A Chemical Investigation of Some New Zealand Honeys

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of
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by

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Dedicated to

my mother

Abstract

Ether extracts were made from aqueous solutions of various floral types of New Zealand honeys with the use of a continuous liquid/liquid extractor. The components of the extracts were methylated before being separated and identified by gas chromatography and mass spectrometry.

When a component could not be identified in this way, bulk extraction of representative unifloral honey samples was carried out and the extractives separated by preparative layer chromatography. The structures of the major components were then elucidated by techniques such as high resolution probe mass spectrometry and one and two dimensional ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy, and on one occasion also by X-ray crystallography.

Classes of compounds identified include hydrocarbons, straight chain mono- and dibasic acids, aromatic substances, monoterpenes and degraded carotenoid-like extractives. Compounds reported for the first time in honey include 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoic acid, 3,5,5-trimethylcyclohex-2-en-1-one, 3,5,5-trimethylcyclohex-2-ene-1,4-dione, 4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one, 4-(3-oxo-1-butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one, *trans-cis* and *trans-trans*-abscisic acid, 1,4-dihydroxybenzene and 2,6,6-trimethyl-1-(3-oxo-*trans*-1-butenyl)-cyclohexane-*trans-cis*-1,2,4-triol. The triol appears to be a new compound.

Quantitative analyses of extractable organic substances from a series of white clover type, manuka, ling/heather, thyme, nodding thistle, vipers bugloss, kamahi and willow honeys afforded chromatograms which serve to uniquely characterise the floral sources of the honeys.

Correlation was sought between the occurrence of extractable organic substances and the non-peroxide antibacterial activity of honey. Various potential antibacterial components were identified in this way. Quantitative testing of the pure compounds showed that 1,4-dihydroxybenzene probably accounts for all of the non-peroxide antibacterial activity of vipers bugloss honey, and that 4-hydroxy-3,5-dimethoxybenzoic acid and 2-hydroxy-3-phenylpropionic acid account for 0.2-0.35% and 1.6-3.2% respectively of the non-peroxide antibacterial activity in manuka honey.

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Contents

List of Figures

List of Tables

Abbreviations

The following abbreviation have been used throughout this thesis.

amu	atomic mass unit
$CDCl_3$	deuterochloroform
CI	chemical ionisation
d	doublet
EI	electron impact ionisation
FID	flame ionisation detector
GC	gas chromatography (or gas liquid chromatography)
GC/MS	combined gas chromatography/mass spectrometry
ITDS	Finnigan Ion Trap Detector system
M^+	the ionised molecule; the peak representing the ionised molecule which contains only the isotopes of greatest natural abundance
MS	mass spectrometry
m/z	the mass of the ion divided by its charge (usually unity)
NMR	nuclear magnetic resonance spectroscopy
PLC	preparative layer chromatography
q	quartet
s	singlet
t	triplet
T_1	NMR spin-lattice relaxation time
T_2	NMR transverse relaxation time
UV	ultraviolet

Chapter 1

Introduction and Review

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Introduction and Review

1.1 Introduction

One of the intrinsic features of honey is its natural antibiotic properties. It is known that honey can be kept for long periods of time without becoming spoiled.

Studies at the University of Waikato have shown that some New Zealand native honeys exhibit an additional antibacterial activity, substantially greater than that which could be ascribed solely to the high osmolarity and/or the presence of hydrogen peroxide (Russell, 1983; Molan and Russell, 1988). A part of this additional activity appeared to be correlated with the presence of extractable organic substances other than carbohydrates. A study by Molan *et al.* (1988) showed that the additional activity was nectar-source dependent. Manuka (*Leptospermum scoparium*) honey for example, possessed substantial additional activity whereas honey from white clover (*Trifolium repens*) type was essentially devoid of this activity.

Total extractable organic substances from honey have been little studied. Some work has been reported on the volatile constituents of various unifloral honeys. An account of the extractable organic substances present in four unifloral New Zealand honeys [clover, manuka, rewarewa (*Knightia excelsa*) and heather (*Calluna vulgaris*)] has been reported by Tan (1985). This study showed that the capillary column gas

chromatography (GC) profile of the extractable organic substances was dependent on the nectar source, raising the possibility that the floral source of honeys could be identified from the GC profile. Hitherto, floral source identification has only been possible by pollen analysis (Maurizio, 1975).

The present study reports further advances in the application of combined gas chromatography/mass spectrometry (GC/MS) and other techniques, including one and two dimensional ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and X-ray crystallography in the characterisation and quantification of trace organic substances in honey, with a view to using the information to identify the floral sources of honeys and identifying the antibacterial components.

1.2 Antibacterial Properties of Honey

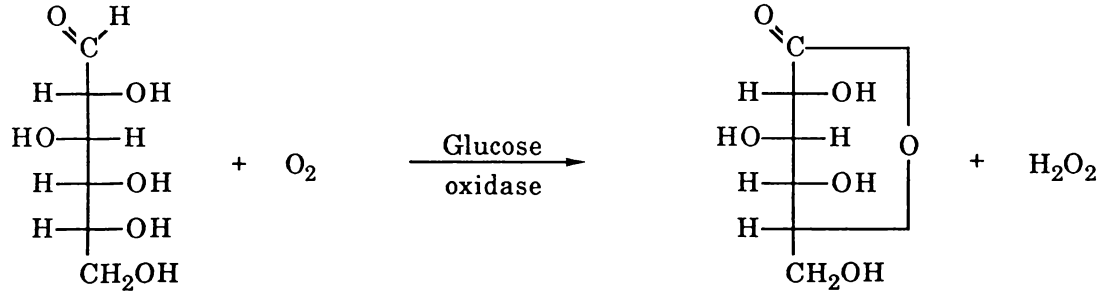
Honey has been used as a medicine to treat bacterial infections from early in recorded history (Aristotle *c.* 350 B.C.). In modern times, honey as medication has been replaced by antibiotics. However, there are reports of the medical profession turning back to honey for the treatment of ulcers, burns and surgical wounds (Cavanagh *et al.,* 1970; Blomfield, 1973; Goulart, 1979 and a report in the *American Bee Journal 122*(4), 247, 1982).

The effectiveness of honey when used in this way is not surprising. The antibacterial effects of honey have long been recognised from testing *in vitro* (Cavanagh *et al.,* 1970; James *et al.,* 1972; Dustmann, 1979; Tóth *et al.,* 1987). Nevertheless, the constituents responsible for this intrinsic property have been little explored.

Sakett in 1919 thought that the antibacterial effect of honey was due to the high sugar content or high osmolarity. He was, however, unable to explain the persistent activity in diluted honey; this activity was greater than that observed for the corresponding undiluted honey. Dold and co-workers (1937) were the first to examine the antibacterial property of honey in detail. They found a substance in honey which appeared to inhibit bacterial growth, and therefore called it 'inhibine'. This was found to be labile to both heat and light.

Subsequent investigations (Prica, 1938; Khristov and Mladenov, 1961; Lindner, 1962) demonstrated that honeys of various origins were active against both gram-positive and gram-negative bacteria. The antibacterial effect was due neither to the normal acidity of honey (average pH of 3.9, White, 1978) nor to its high osmolarity, enzymes, or nitrogenous components, but to a substance which was labile to heat and light and susceptible to low pH. This was believed to be associated with the "inhibine" of Dold.

During the 1960's, White and his co-workers established that the antibacterial property shown in the inhibine assay of honey was the result of the accumulation of hydrogen peroxide (H_2O_2) in honey. Hydrogen peroxide is generated by a natural glucose oxidase system in honey, from glucose, as follows:-



D-(+)-Glucose

Glucono-lactone

This work was published in a series of papers entitled "Studies on honey inhibine" (White *et al.,* 1962; 1963a; 1963b; 1964a; 1964b). Hydrogen peroxide is known to be an effective antibacterial compound which can inhibit bacterial growth (Coulthard *et al.,* 1945).

Glucose oxidase is virtually inactive in full-density honey, but becomes active once honey is diluted. This explains why the antibacterial activity of diluted honey is higher than that of honey that is untreated. Working independently, Adcock (1962) found that the addition of catalase to dissipate hydrogen peroxide also destroyed the inhibitory effect of honey. He therefore suggested a possible connection between the two.

While Dold's inhibine is enzyme-produced hydrogen peroxide and is heat sensitive, other investigators have suggested the possibility of additional antibacterial substances in honey, which could originate from the floral source. Extracts of aqueous solutions of honey with ethanol, acetone and ether were found to possess a strong antibacterial activity (Vergé, 1951; Schuler and Vogal 1956; Lavie 1960). Gonnet and Lavie (1960) reported that the inhibitory extractives were stable to light and relatively stable to heat. Lavie in 1963 also reported that some of the antibacterial substances recovered from an ether extract were volatile at 95°C. More recent reports have demonstrated that honey contains heavy-volatile, non-volatile and volatile antibacterial substances (Mladenov, 1974). Also, Dustmann (1979) reported the existence of antibacterial activity in honey that was not due to peroxide or high osmolarity, while Tóth *et al.* (1987) demonstrated the inhibitory effects of the volatile components of some Hungarian honeys on various gram-negative bacteria.

In an extensive survey of unifloral New Zealand honeys, Molan *et al.* (1988) have noted a marked variation in the inhibitory activity amongst the honey samples. In addition, a definite relationship was found to exist between the antibacterial activity and the floral source of the honey, manuka being the most active group. This finding suggests that the antibacterial activity of honey may originate from the floral source rather than be a by-product of bee metabolism. It is perhaps of passing interest that Aristotle (*c.* 350 B.C.) and Dioscorides (512 A.D.) both specified particular types of honey in the treatment of infections.

Russell *et al.* (1988) in a later study attempted to isolate and characterise some of the components responsible for the additional antibacterial activity observed in manuka honey. They found 4-hydroxy-3,5-dimethoxybenzoic acid, 3,4,5-trimethoxybenzoic acid and their esters in one of the active fractions of the ethanol/ether extracts and suggested that the origin of these compounds could be from the floral source. Structural elucidations were based on the combination of MS, IR (infrared spectroscopy) and ^{1}H NMR data. Although both acids possessed significant antibacterial activity, it was not known whether they accounted for all of the additional activity observed in manuka honey. Gel filtration chromatography, however, demonstrated that the antibacterial substances in manuka honey have molecular weights of less than 1 000 amu (Sealey, 1988).

1.3 Extractable Organic Substances in Honey

The composition of honey has been extensively investigated. The interest in the subject is apparent from the abundant literature and reviews that have been published. However, most of the research has been

directed toward quality control and establishing standards for the honey industry. These investigations have generally been confined to the analysis of the major non-volatile constituents such as glucose, fructose, water, and enzyme activity. A comprehensive review has been published by White (1975) which includes some analyses of minor constituents. A more recent literature survey by Amor (1978) updates the review of White. However, only one later report was added to the list.

Almost all studies concerned with the minor constituents of honey have been limited to either the analysis of flavour and aroma or the hydrocarbon fractions. The characterisation of other minor constituents including phenolic and acidic compounds is scanty. In assessing the significance of extractable organic components in relation to their contribution to additional antibacterial activity in honey, a knowledge of the total extractable organic substances in a substantial variety of honeys is required.

1.3.1 Volatile Components

Maeda *et al.* (1962) ascribed the flavour of honey to fructose, glucose, gluconic acid and proline. It is, however, obvious to anyone who inspects a variety of honeys that an infinite number of aroma and flavour variations can exist.

The journal *Language* reported of wine experts: 'Some said "light" when others said "full-bodied"; some said "withered" when others thought a wine "full of character". The only thing they agreed upon was that the wines they liked were "dry", and wines they didn't like tended to be "sweet". But this was irrespective of sugar content'. Some parallel

comments could be made about honey. The subtle flavour of honey is one of its most unique characteristics, perhaps the most significant reason for the popularity of table honey. However, little work has been directed towards this aspect of honey composition in comparison with other areas of honey research. Technological limitations were the major reason for this restriction, especially prior to the development of GC in the 1960s.

Schmalfuss and Bartmeyer (1929) demonstrated the presence of diacetyl in German heather honey. Methyl anthranilate was identified by Nelson (1930) in orange honey. Two years later, Lothrop (1932) suggested that the presence of methyl anthranilate provides a specific test for orange honey. Later, Deshusses and Gabbai (1962) used thin-layer chromatography (TLC) for this purpose.

Advances in analytical techniques during the last two decades or so have enabled the separation and quantification of minute amounts of complex organic substances. Thus, the development of GC has resulted in the upsurge of interest in research on volatile components from various sources. Dörrscheidt and Friedrich (1962) were the first to use GC to analyse honey volatiles. Out of 31 components separated, only four were common to the six samples being examined. In addition, only two were tentatively identified– methyl acetate and methyl butyrate. They also suggested that the chromatograms might serve as 'fingerprints' for the identification of honey type.

Merz (1963) conducted a qualitative gas chromatographic investigation of ether extracts of honey, and attempted to correlate the quality of the honey to the level of 5-hydroxymethyl-2-furfural. His report also included an organoleptic assessment of GC-eluting components, which showed that compounds eluting after 5-hydroxymethyl-2-furfural

had the distinctive characteristic honey aroma. Vacuum distillation was used by ten Hoopen (1963) to isolate the volatile carbonyl components of honey as their 2,4-dinitrophenylhydrazones. Formaldehyde, acetaldehyde, acetone, isobutyraldehyde and diacetyl were identified following column, TLC and GC separations.

Cremer and Riedmann (1964; 1965a; 1965b), using an early capillary column (Golay 100 m), were able to separate about 120 components, of which more than half were identified. Aliphatic alcohols, their oxidation products and esters dominated the components identified. However, none of the late eluting components reported by Merz were detected. Cremer and Riedmann also noted the level of alcohols increased with storage and suggested they could originate from the corresponding free amino acids by enzymatic degradation. Thus phenylalanine was implicated as a precursor of 2-phenylethanol, phenylacetic acid and its ester.

Two more recent studies have demonstrated the presence of compounds which probably originate from the plant source. Chogovadze *et al.* (1973) used GC to analyse 20 honeys from different parts of Georgia, U.S.S.R. Various terpenoids and esters were detected, of which 17 were identified. Tsuyena *et al.* (1974) demonstrated the presence of 8-*p*-menthene-1,2-diol (major component) and 26 other terpenoids in Linden (*Tilia japonica Simk*) honey by IR, GC, MS and NMR.

Chandler *et al.* in 1974 presented analytical results from a large number of Australian honeys, while Wootton *et al.* (1978) reported the effect of storage on the volatile components of some Australian honeys. Graddon *et al.* (1979) subsequently extended this work; dichloromethane extracts were made from 50 g samples of honeys and analysed by GC/MS.

Almost 100 compounds have been identified with varying degrees of certainty from about 160 components separated. Recently, Bicchi *et al.* (1983) presented analytical results from some unifloral and multifloral Italian honeys using 4 different extraction techniques. A total of 52 components were identified by GC/MS but, as in previous reports, about half of the components detected could not be identified.

Beeswax hydrocarbons have also received some attention. Downing *et al.* (1961), Callow (1963), Streibl *et al.* (1966), Tulloch and Hoffman (1972), Ferber and Nursten (1977) and Tulloch (1971; 1980) have investigated the hydrocarbon and fatty acid fractions of beeswax, while the composition of royal jelly was characterised by Lercker *et al.* (1981; 1982). Recently, the composition of the hydrocarbon fraction of chestnut honey was characterised extensively by Bonaga *et al.* (1986). Tables 1.I, 1.II, 1.III, 1.IV and 1.V summarise the components of honey volatiles that have been identified.

1.4 Floral Source Identification

Identification of honey type is usually based upon the more conventional organoleptic assessment, *i.e.*, flavour, colour, aroma and also the season and location of its production. At the present time the only means of objectively determining the geographical and floral origin of honey for certification is by pollen analysis (Maurizio, 1951; Sawyer, 1975). The technique is known as melissopalynology, the beginning of which goes back as far as the turn of the century. Over the years, research by various workers (Maurizio, 1951; Demianowicz, 1964 and Louveaux *et al.*, 1978) has helped to improve its precision. Pollen analysis can be reliable, especially in the hands of an expert with the necessary experience. The

technique is, however, tedious and very dependent on expert ability and judgement (Howells, 1969).

A detailed discussion of the microscopical determination of the origin of honey was given by Maurizio (1975). However, for honey from which all solid components (including pollen and other plant constituents) have been eliminated by excessive filtration, or by pressure-filtering through diatomaceous earth or similar material, microscopical analysis is of little use.

A recent report on the pollen analysis of New Zealand honeys by Moar (1985) suggests the complications which could arise from the conventional technique. His report includes the general requirements outlined by the International Commission for Bee Botany regarding pollen analysis. For most unifloral honey, absolute pollen content in the range of 20 000 to 100 000 per 10 g of honey is regarded as "normal". Some unifloral honey may contain less than 20 000 pollen grains and others more than 100 000. Honey in the lower range is considered as "under-represented" in terms of "normal" absolute pollen content, and in the higher range "over-represented".

New Zealand annual honey production has exceeded 10 000 tonnes for the past three years, of which about 2 000 tonnes are exported. The native flora yields some most interesting honeys which are exclusive to New Zealand. A number of these have under-represented absolute pollen content; rewarewa, thyme (*Thymus vulgaris*) and ling/heather are examples in this category. The validity of pollen analysis becomes especially important when importing countries question the identity of a honey that is primarily from a floral source which gives a low pollen count.

The proportions of pollen present may be different from the proportions of nectar from which a honey has been produced. Moreover, there are cases where the plant only produces the pollen without any nectar as in the case of kiwifruit (*Actinidia chinensis*). Pollen production varies greatly between species; consequently, pollen analysis can only act as a guide in determining the floral source of honey. Alternative methods that could be more widely and accurately used have been sought for many years. To be viable the components on which the assessment is based would have to originate from the nectar itself.

Chemical means for characterising honeys have been suggested by various groups of investigators since early 1927 (Tillmans and Kiesgen, 1927; Lothrop, 1932; Dörrscheidt and Friedrich, 1962; Curti and Riganti, 1966). In more recent reports (Davies 1975, 1976) GC has been used to study the free amino acids and this seems promising. Although these investigations showed that some nectar and honeydews had a typical amino acid spectrum, which persisted in the honey, the results did not allow any simple evaluation of the contributions of the honeybee and of the plant.

The work of Davies was extended by Bosi and Battaglini (1978) and later by Gilbert *et al.* (1981). Using canonical variates analysis, they were able to distinguish the geographical origin of honey on the basis of free amino acids composition. No attempts were made to correlate the floral origin with the free amino acids composition. In a recent report, Bonaga and Guimanini (1986) also raise the possibility of correlating chemical composition (either derived from the nectar or in some biochemical modification carried out by the bees) with the floral origin of honey.

Unifloral honeys possess distinctive aromas, some of which probably originate from the nectar source; some will be derivatives of the bees' metabolism, while others arise in the processing during harvest. Accordingly, it can be anticipated that the analysis of the aroma and flavour constituents of a unifloral honey, using an advanced analytical technique such as high resolution capillary GC, would give a 'fingerprint' which would be dependent on the floral source.

Studies at the University of Waikato have suggested that the floral source of some New Zealand unifloral honeys can be inferred from the chromatograms of the extractable organic constituents. Inspection of a series of chromatograms revealed that similar patterns were obtained for chromatograms of different samples from the same floral source. On the other hand, different patterns were obtained from chromatograms of samples of honey from different floral sources. Such chromatograms have been reported for clover, manuka, rewarewa and heather honeys (Tan, 1985).

Some of the extractable organic substances present in these honeys have been defined, particularly in the case of clover and manuka honeys (Tan *et al.,* 1988). However a substantial array of other unidentified substances occur in a number of other New Zealand unifloral honeys.

1.5 Aims of Present Study

The present study centres on the accumulation of a detailed knowledge of the extractable trace organic components of New Zealand honeys, in particular, the possibility of the general utilisation of organic extractive profiles for characterising floral sources of honeys. Reports

suggest that GC has been able to dramatically differentiate honey types using a peak or peaks in the chromatograms that are associated with particular characteristic floral source. However, different compounds can have similar GC retention times under standard experimental conditions. Therefore, before GC can be employed to supplement or perhaps even to replace organoleptic judgements, structural identification of these compounds is required.

In order to validate the accuracy and reliability of the chemical data, a large number of samples of each floral type from different seasons and geographical locations will need to be examined. Also it is necessary to extend the technique to honeys of a large number of floral types in order to determine how distinctive is the chemical profile or GC pattern from each floral type.

In addition, the identities and concentrations of extractable substances in honeys also need to be known in order to provide the necessary background information and the possibility of finding a correlation between the level of some organic constituents and the antibacterial activity of various honeys.

Table 1.I Hydrocarbons previously reported in honey.

mol wt	formula	compound	references
92	C_7H_8	toluene	6
104	C_8H_8	styrene	1, 6
106	C_8H_{10}	ethyl benzene	6
106	C_8H_{10}	*p*-xylene	6
106	C_8H_{10}	*m*-xylene	6
106	C_8H_{10}	*o*-xylene	6
120	C_9H_{12}	propylbenzene	6
120	C_9H_{12}	*iso*propylbenzene	6
120	C_9H_{12}	1,2,3-trimethylbenzene	1, 6
132	$C_{10}H_{12}$	tetralin	6
132	$C_{10}H_{12}$	methyl-4-*iso*propylbenzene	1, 4
134	$C_{10}H_{14}$	*p*-cymene	1
134	$C_{10}H_{14}$	tetramethylbenzene	3
136	$C_{10}H_{16}$	β-pinene	1
136	$C_{10}H_{16}$	γ-terpinene	4
136	$C_{10}H_{16}$	camphene	13
136	$C_{10}H_{16}$	limonene	13
138	$C_{10}H_{18}$	decaline	6
142	$C_{10}H_{22}$	*n*-decane	2, 6
156	$C_{11}H_{24}$	*n*-undecane	2, 6
170	$C_{12}H_{26}$	*n*-dodecane	2, 6
184	$C_{13}H_{28}$	*n*-tridecane	2
198	$C_{14}H_{30}$	*n*-tetradecane	2, 6
212	$C_{15}H_{32}$	*n*-pentadecane	2
226	$C_{16}H_{34}$	*n*-hexadecane	1, 2, 6
240	$C_{17}H_{36}$	*n*-heptadecane	1, 2
254	$C_{18}H_{38}$	*n*-octadecane	1, 2
268	$C_{19}H_{40}$	*n*-nonadecane	2
282	$C_{20}H_{42}$	*n*-eicosane	1, 2, 13
296	$C_{21}H_{44}$	*n*-heneicosane	2, 6, 11
310	$C_{22}H_{46}$	*n*-docosane	2, 6, 11
322	$C_{23}H_{46}$	*n*-tricosene	2
324	$C_{23}H_{48}$	*n*-tricosane	1, 2, 6, 11
338	$C_{24}H_{50}$	*n*-tetracosane	2, 6, 11

Table 1.I continued

350	$C_{25}H_{50}$	*n*-pentacosene	2
352	$C_{25}H_{52}$	*n*-pentacosane	1, 2, 6, 11
366	$C_{26}H_{54}$	*n*-hexacosane	2, 6, 11
378	$C_{27}H_{54}$	*n*-heptacosene	2
380	$C_{27}H_{56}$	*n*-heptacosane	1, 2, 6, 11
394	$C_{28}H_{58}$	*n*-octacosane	2, 6, 11
406	$C_{29}H_{58}$	*n*-nonacosene	2
408	$C_{29}H_{60}$	*n*-nonacosane	2, 6, 11
420	$C_{30}H_{60}$	*n*-triacontene	2
422	$C_{30}H_{62}$	*n*-triacontane	2, 6, 11
434	$C_{31}H_{62}$	*n*-hentriacontene	2, 11
436	$C_{31}H_{64}$	*n*-hentriacontane	2, 6
448	$C_{32}H_{64}$	*n*-dotriacontene	2
450	$C_{32}H_{66}$	*n*-dotriacontane	2
462	$C_{33}H_{66}$	*n*-tritriacontene	2, 11
464	$C_{33}H_{68}$	*n*-tritriacontane	2
476	$C_{35}H_{70}$	*n*-pentatriacontene	2
478	$C_{35}H_{72}$	*n*-pentatriacontane	2
518	$C_{37}H_{74}$	*n*-heptatriacontene	2
520	$C_{37}H_{76}$	*n*-heptatriacontane	2

Table 1.II Alcohols previously reported in honey.

mol wt	formula	compound	references
32	C_1H_4O	methanol	5
46	C_2H_6O	ethanol	5
58	C_3H_6O	2-propen-1-ol	5
60	C_3H_8O	1-propanol	5
60	C_3H_8O	2-propanol	5
72	C_4H_8O	2-methylprop-2-en-1-ol	5
72	C_4H_8O	2-buten-1-ol	5
74	$C_4H_{10}O$	2-methylpropan-1-ol	5
74	$C_4H_{10}O$	1-butanol	5
74	$C_4H_{10}O$	2-butanol	5
86	$C_5H_{10}O$	3-methyl-3-buten-1-ol	3

Table 1.II continued

86	$C_5H_{10}O$	3-methyl-2-buten-1-ol	3
88	$C_5H_{12}O$	2-methylbutan-1-ol	5
88	$C_5H_{12}O$	3-methylbutan-1-ol	5
88	$C_5H_{12}O$	3-methylbutan-2-ol	5
88	$C_5H_{12}O$	1-pentanol	5
88	$C_5H_{12}O$	2-pentanol	5
88	$C_5H_{12}O$	3-pentanol	5
90	$C_4H_{10}O_2$	2,3-butanediol	6
94	C_6H_6O	phenol	3, 6
108	C_7H_8O	benzyl alcohol	1, 3, 5, 6, 13,15
116	$C_7H_{16}O$	2-heptanol	5
122	$C_8H_{10}O$	α-methyl-benzyl alcohol	3
122	$C_8H_{10}O$	2-phenylethanol	1, 3, 4, 5, 6, 15
130	$C_8H_{18}O$	1-octanol	4
130	$C_8H_{18}O$	3-octanol	1
134	$C_9H_{10}O$	3-phenylprop-2-en-1-ol	3
136	$C_9H_{12}O$	2,3,5-trimethylphenol	1
136	$C_9H_{12}O$	2,4,6-trimethylphenol	15
136	$C_9H_{12}O$	3,4,5-trimethylphenol	1
136	$C_9H_{12}O$	3-phenylpropan-1-ol	3, 5
138	$C_8H_{10}O_2$	2-methoxybenzyl alcohol	6
138	$C_8H_{10}O_2$	4-methoxybenzyl alcohol	6
150	$C_{10}H_{14}O$	4-phenylbutan-1-ol	5
150	$C_{10}H_{14}O$	*p*-cymol	4
154	$C_{10}H_{18}O$	*p*-menth-1-en-9-ol	1
154	$C_{10}H_{18}O$	1,8-cineole	1
154	$C_{10}H_{18}O$	geraniol	4
154	$C_{10}H_{18}O$	nerol	4
154	$C_{10}H_{18}O$	linalool	4, 6, 13
154	$C_{10}H_{18}O$	α-terpineol	4
154	$C_{10}H_{18}O$	terpinen-4-ol	4
156	$C_{10}H_{20}O$	citronellol	4
156	$C_{10}H_{20}O$	menthol	4
164	$C_{10}H_{12}O_2$	eugenol	1
170	$C_{10}H_{18}O_2$	8-*p*-menthen-1,2-diol	14
222	$C_{15}H_{26}O$	farnesol	13

Table 1.III Aldehydes and ketones previously reported in honey.

mol wt	formula	compound	references
Aldehydes:			
30	C_1H_2O	formaldehyde	5, 12
44	C_2H_4O	acetaldehyde	5, 12
58	C_3H_6O	propanal	5
70	C_4H_6O	2-methylprop-2-enal	5
70	C_3H_6O	2-butenal	5
72	C_4H_8O	2-methylpropanal	5, 12
72	C_4H_8O	butanal	5
86	$C_5H_{10}O$	3-methylbutanal	5
86	$C_5H_{10}O$	pentanal	5
88	$C_4H_8O_2$	3-hydroxybutanal	5
100	$C_6H_{12}O$	hexanal	5
106	C_7H_6O	benzaldehyde	3, 5, 6
107	C_6H_5NO	isonicotinaldehyde	3
118	$C_6H_{14}O_2$	acetal	3
120	C_8H_8O	phenylacetaldehyde	1, 6
132	C_9H_8O	3-phenylprop-2-enal	1, 3
136	$C_8H_8O_2$	methoxybenzaldehyde	6
152	$C_{10}H_{16}O$	1-*p*-menthen-9-al	1
156	$C_{10}H_{20}O$	decanal	3
166	$C_9H_{10}O_3$	3,5-dimethoxybenzaldehyde	6
Ketones:			
58	C_3H_6O	2-propanone	1, 5
72	C_4H_8O	2-butanone	5
74	$C_3H_6O_2$	1-hydroxypropan-2-one	6
84	C_5H_8O	3-penten-2-one	1
86	$C_5H_{10}O$	3-methylbutan-2-one	5
86	$C_4H_6O_2$	2,3-butanedione	5, 6, 12
86	$C_4H_6O_2$	γ-butyrolactone	3, 6
86	$C_5H_{10}O$	2-pentanone	5
86	$C_5H_{10}O$	3-pentanone	5
88	$C_4H_8O_2$	1-hydroxybutan-2-one	6
88	$C_4H_8O_2$	3-hydroxybutan-2-one	5, 6
96	$C_5H_4O_2$	2*H*-pyran-2-one	1

Table 1.III continued

98	$C_6H_{10}O$	4-methyl-3-penten-2-one	1
98	$C_6H_{10}O$	3-hexen-2-one	1
100	$C_5H_8O_2$	2,3-pentanedione	1
100	$C_5H_8O_2$	γ-valerolactone	3, 6
102	$C_5H_{10}O_2$	3-hydroxypentan-2-one	6
102	$C_5H_{10}O_2$	2-hydroxypentan-3-one	6
114	$C_7H_{14}O$	2-heptanone	5
120	C_8H_8O	acetophenone	3
134	$C_9H_{10}O$	2'-methylacetophenone	3, 11
135	C_8H_9NO	3'-aminoacetophenone	3
146	$C_{10}H_{10}O$	cinnamylmethylketone	3
146	$C_6H_{10}O_4$	3,5-dihydroxy-2-methyl-2,6-dihydropyran-4-one	8
150	$C_9H_{10}O_2$	4'-methoxyacetophenone	3
150	$C_{10}H_{14}O$	carvone	4

Table 1.IV Acids and esters previously reported in honey.

mol wt	formula	compound	references
Acids:			
46	CH_2O_2	formic acid	5
60	$C_2H_4O_2$	acetic acid	5
74	$C_3H_6O_2$	propionic acid	5
86	$C_4H_6O_2$	crotonic acid	1
88	$C_4H_8O_2$	2-methylpropionic acid	5, 6
88	$C_4H_8O_2$	butanoic acid	5
102	$C_5H_{10}O_2$	3-methylbutanoic acid	3, 5, 15
102	$C_5H_{10}O_2$	pentanoic acid	3, 5
116	$C_6H_{12}O_2$	hexanoic acid	3, 5
118	$C_4H_6O_4$	butanedioic	11
122	$C_7H_6O_2$	benzoic acid	5, 10, 11
136	$C_8H_8O_2$	2-phenylacetic acid	5, 10, 11
144	$C_8H_{16}O_2$	octanoic acid	2, 3, 11
148	$C_9H_8O_2$	*trans*-3-phenylprop-2-enoic acid	10, 11
150	$C_9H_{10}O_2$	3-phenylpropionic acid	10

Table 1.IV continued

152	$C_8H_8O_3$	α-hydroxyphenylacetic acid	10
152	$C_8H_8O_3$	2-hydroxybenzoic acid	10, 11
152	$C_8H_8O_3$	2-(3-hydroxyphenyl)-acetic acid	10
152	$C_8H_8O_3$	4-hydroxybenzoic acid	10
152	$C_8H_8O_3$	2-(4-hydroxyphenyl)-acetic acid	10
154	$C_7H_6O_4$	2,5-dihydroxybenzoic acid acid	10
154	$C_7H_6O_4$	3,5-dihydroxybenzoic acid	11
154	$C_7H_6O_4$	3,4-dihydroxybenzoic acid	10, 11
158	$C_9H_{18}O_2$	nonanoic acid	2
164	$C_9H_8O_3$	phenylpyruvic acid	10
164	$C_9H_8O_3$	3-(2-hydroxyphenyl)-*trans*-prop-2-enoic acid	10
164	$C_9H_8O_3$	3-(4-hydroxyphenyl)-*trans*-prop-2-enoic acid	10
164	$C_{10}H_{12}O_2$	α-ethyl-2-phenylacetic acid	3
166	$C_9H_{10}O_3$	(+)2-hydroxy-3-phenyl-propionic acid	7, 10, 11
166	$C_9H_{10}O_3$	3-(4-hydroxyphenyl)-propionic acid	10
168	$C_8H_8O_4$	3-hydroxy-4-methoxybenzoic acid	10
170	$C_7H_6O_5$	3,4,5-trihydroxybenzoic acid	11
172	$C_{10}H_{20}O_2$	decanoic acid	2
174	$C_8H_{14}O_4$	octanedioic acid	11
180	$C_9H_8O_4$	4-hydroxyphenylpyruvic acid	10
180	$C_9H_8O_4$	3-(3,4-dihydroxyphenyl)-*trans*-prop-2-enoic acid	10
182	$C_9H_{10}O_4$	2-hydroxy-3-(4-hydroxyphenyl)-propionic acid	11
182	$C_9H_{10}O_4$	2,5-dimethoxybenzoic acid	10
186	$C_{11}H_{22}O_2$	undecanoic acid	2
188	$C_9H_{16}O_4$	nonanedioic acid	11
194	$C_9H_8O_3$	3-(4-hydroxy-3-methoxyphenyl)-*trans*-prop-2-enoic acid	10
198	$C_9H_{10}O_5$	4-hydroxy-3,5-dimethoxy-benzoic acid	10
200	$C_{12}H_{24}O_2$	lauric acid (12:0)	2, 9, 11
200	$C_{10}H_{16}O_4$	*trans*-2-decenedioic acid	11
212	$C_{10}H_{18}O_4$	decanedioic acid	11

Table 1.IV continued

214	$C_{13}H_{26}O_2$	tridecanioc acid	2
224	$C_{11}H_{12}O_5$	3-(4-hydroxy-3,5-dimethoxyphenyl)-*trans*-prop-2-enoic acid	10
228	$C_{14}H_{28}O_2$	myristic acid (14:0)	2, 9, 11
242	$C_{15}H_{30}O_2$	pentadecanoic acid	2
256	$C_{16}H_{32}O_2$	palmitic acid (16:0)	2, 6, 9, 11
270	$C_{17}H_{34}O_2$	heptadecanoic acid	2
278	$C_{18}H_{30}O_2$	α-linolenic acid (18:2)	11
280	$C_{18}H_{32}O_2$	linoleic acid (18:2)	9, 11
282	$C_{18}H_{34}O_2$	oleic acid (18:1)	9, 11
284	$C_{18}H_{36}O_2$	stearic acid (18:0)	2, 9, 11
298	$C_{19}H_{38}O_2$	nonadecanoic acid	2
312	$C_{20}H_{40}O_2$	eicosanoic acid	2, 11
326	$C_{21}H_{42}O_2$	heneicosanoic acid	2
340	$C_{22}H_{44}O_2$	docosanoic acid	2, 11
354	$C_{23}H_{46}O_2$	tricosanoic acid	2
368	$C_{24}H_{48}O_2$	tetracosanoic acid	2, 11
382	$C_{25}H_{50}O_2$	pentacosanoic acid	2
396	$C_{26}H_{52}O_2$	hexacosanoic acid	2, 11
410	$C_{27}H_{54}O_2$	heptacosanoic acid	2
424	$C_{28}H_{56}O_2$	octacosanoic acid	2, 11
Esters:			
60	$C_2H_4O_2$	methyl formate	5
74	$C_3H_6O_2$	ethyl formate	5
74	$C_3H_6O_2$	methyl acetate	5
88	$C_4H_8O_2$	ethyl acetate	5
88	$C_4H_8O_2$	methyl propionate	5
102	$C_5H_{10}O_2$	ethyl propionate	5
102	$C_5H_{10}O_2$	propyl acetate	5
102	$C_5H_{10}O_2$	*iso*propyl acetate	5
102	$C_4H_6O_3$	methyl 2-oxopropionate	5
102	$C_5H_{10}O_2$	methyl butanoate	5
116	$C_6H_{12}O_2$	ethyl butanoate	5
116	$C_6H_{12}O_2$	*iso*amyl formate	5
116	$C_6H_{12}O_2$	butyl acetate	3, 5
116	$C_6H_{12}O_2$	ethyl 2-methylpropionate	5
116	$C_6H_{12}O_2$	methyl 3-methylbutanoate	5

Table 1.IV continued

116	$C_6H_{12}O_2$	methyl pentanoate	5
130	$C_7H_{14}O_2$	ethyl pentanoate	5
130	$C_7H_{14}O_2$	*iso*amyl acetate	5
130	$C_7H_{14}O_2$	propyl 2-methylpropionate	5
130	$C_7H_{14}O_2$	methyl hexanoate (caproate)	5
132	$C_6H_{12}O_3$	2-ethoxyethyl acetate	1
136	$C_8H_8O_2$	methyl benzoate	5, 11
144	$C_8H_{16}O_2$	ethyl hexanoate (caproate)	5
144	$C_8H_{16}O_2$	hexyl acetate	4
144	$C_8H_{16}O_2$	butyl 2-methylpropionate	5
144	$C_8H_{16}O_2$	butyl butanoate	5
144	$C_8H_{16}O_2$	*iso*butyl butanoate	5
150	$C_9H_{10}O_2$	ethyl benzoate	5
150	$C_9H_{10}O_2$	methyl 2-phenylacetate	5
158	$C_9H_{18}O_2$	*iso*amyl 2-methylpropionate	5
158	$C_9H_{18}O_2$	*iso*amyl butanoate	5
164	$C_{10}H_{12}O_2$	ethyl 2-phenylacetate	5
166	$C_9H_{10}O_3$	ethyl 2-hydroxybenzoate	15
170	$C_{10}H_{18}O_2$	*cis*-3-hexenylbutanoate	1, 3, 6
172	$C_{10}H_{20}O_2$	propyl acetate	4
196	$C_{12}H_{20}O_2$	linalyl acetate	4
212	$C_{10}H_{12}O_5$	methyl 4-hyrdoxy-3,5-dimethoxy-benzoate	6, 11
214	$C_{13}H_{26}O_2$	3-methylbutyl octanoate	1
228	$C_{14}H_{28}O_2$	ethyl laurate (12:0)	1
284	$C_{18}H_{36}O_2$	ethyl palmitate (16:0)	1
312	$C_{20}H_{40}O_2$	ethyl stearate (18:0)	1

Table 1.V Furans and other substances previously reported in honey.

mol wt	formula	compound	references
Furans:			
82	C_5H_6O	2-methylfuran	1
84	$C_4H_4O_2$	2-(3H)-furanone	3

Table 1.V continued

96	$C_5H_4O_2$	furfuryl	3, 5, 6
98	$C_5H_6O_2$	furfuryl alcohol	1, 3, 6
110	$C_6H_6O_2$	2-acetylfuran	3, 6
110	$C_6H_6O_2$	5-methyl-2-furfural	1, 6
124	$C_6H_4O_3$	2,5-furandialdehyde	15
126	$C_6H_6O_3$	5-(hydroxymethyl)-2-furfural	3, 6, 8, 15
126	$C_6H_6O_3$	methyl 2-furancarboxylate	1, 6
132	C_9H_8O	2-methylbenzofuran	1
Others:			
74	$C_4H_{10}O_2$	diethyl ether	5
184	$C_{12}H_{12}N_2$	1,1-diphenylhydrazine	3

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1 Bicchi *et al.* (1983)
2 Bonaga *et al.* (1986)
3 Bonaga and Giumanini. (1986)
4 Chogovadze *et al.* (1973)
5 Cremer and Riedmann (1964; 1965a and 1965b)
6 Graddon *et al.* (1979)
7 Hodges and White (1966)
8 Merz (1963)
9 Smith (1963; 1966)
10 Steeg and Montag (1987)
11 Tan *et al.* (1988)
12 ten Hoopen (1963)
13 Tóth *et al.* (1987)
14 Tsuneya *et al.* (1974)
15 Wootton *et al.* (1978)

Chapter 2

Materials and Extraction Procedure

Chapter 2

Materials and Extraction Procedure

2.1 Honey Samples

Honey samples were obtained from apiarists throughout New Zealand, by Mr G. M. Reid, National Apicultural Advisory Officer, Ministry of Agriculture and Fisheries, Hamilton. All honey samples were collected during the 1982-1987 flowering seasons. The majority were considered by the apiarists to be unifloral specimens. Identification of the floral source of each honey was based on the taste, aroma and colour; also the season of collection and location of the hives by the supplying apiarist. A number of honey samples was supplied by Wilson Neill-Hororata Honey Exports Limited, of which floral source identification was based on pollen analysis. Honey samples were stored in the dark in airtight glass or plastic containers at 5°C.

Table 2.I Honey samples analysed.

sample no.	assumed plant of origin[b]	geographical origin	season	form[a]
C1	white clover type	Rocklands Station, Mosgiel	1986/87	e
C2	white clover type	Rocklands Station, Mosgiel	1986/87	e
C16	white clover type	Owaka, Southland	1985/86	s
H408	white clover type	Wilson Neill-Hororata	1986/87	e
H410	white clover type	Wilson Neill-Hororata	1986/87	e
H411	white clover type	Wilson Neill-Hororata	1986/87	e
H412	white clover type	Wilson Neill-Hororata	1986/87	e
H413	white clover type	Wilson Neill-Hororata	1986/87	e

H414	white clover type	Wilson Neill-Hororata	1986/87	e
H415	white clover type	Wilson Neill-Hororata	1986/87	e
M3	manuka	Waikato	1985/86	s
M5	manuka	Te Kauwhata	1985/86	s
M6	manuka	Waikato	1985/86	s
M7	manuka	Te Kauwhata	1985/86	s
M8	manuka	Waikato	1982	c
M9	manuka	Waiotemarama, Hokianga	1985/86	e
M19	manuka	Te Araroa, Gisborne	1986/87	s
M22	manuka	Opotiki	1986/87	s
M24	manuka	Tairua, Coromandel	1987	s
M27	manuka	Tairua, Coromandel	1987	e
M30	manuka	Tairua, Coromandel	1987	s
H416	manuka	Wilson Neill-Hororata	1986/87	e
H361	ling/heather	Wilson Neill-Hororata	1985/86	e
H2	ling/heather	National Park	1986	s
H3	ling/heather	National Park	1986	s
H4	ling/heather	National Park	1986	s
H460	ling/heather	Wilson Neill-Hororata	1987/88	e
H461	ling/heather	Wilson Neill-Hororata	1987/88	e
H462	ling/heather	Wilson Neill-Hororata	1987/88	e
H463	ling/heather	Wilson Neill-Hororata	1987/88	e
H384	thyme	Wilson Neill-Hororata	1985/86	e
H404	thyme	Wilson Neill-Hororata	1986/87	e
H405	thyme	Wilson Neill-Hororata	1986/87	e
H406	thyme	Wilson Neill-Hororata	1986/87	e
H407	thyme	Wilson Neill-Hororata	1986/87	e
T111	thyme	Cromwell, Central Otago	1987	e
T139	thyme	Wedderburn, Central Otago	1987	e
T1	thyme	Central Otago	1986	e
T2	thyme	Central Otago	1986	e
T3	thyme	Central Otago	1986	e
T4	thyme	Central Otago	1986	e
N1	nodding thistle	Waikato	1984/85	e
N3	nodding thistle	Oamaru	1986	e
TH1	nodding thistle	Oamaru	1986	e
8N	nodding thistle	Tauranga	1987	s
38N	nodding thistle	Te Puna	1987	e

68N	nodding thistle	Ohaupo, Waikato	1987	e
77N	nodding thistle	Whakamaru	1987	s
205N	nodding thistle	Hawkes Bay	1987	s
206N	nodding thistle	Hawkes Bay	1987	s
H403	nodding thistle	Wilson Neill-Hororata	1986/87	e
VB102	vipers bugloss	Oamaru	1987	s
VB103	vipers bugloss	Oamaru	1987	s
VB104	vipers bugloss	Oamaru	1987	s
VB105	vipers bugloss	Oamaru	1987	s
VB106	vipers bugloss	Oamaru	1987	s
VB107	vipers bugloss	Oamaru	1987	s
VB124	vipers bugloss	Oamaru	1987	s
VB125	vipers bugloss	Oamaru	1987	s
VB126	vipers bugloss	Oamaru	1987	s
VB1	vipers bugloss	Molesworth	1986	s
M1	kamahi	Kumara	1986	s
H363	kamahi	Wilson Neill-Hororata	1985/86	e
H402	kamahi	Wilson Neill-Hororata	1986/87	e
KA2	kamahi	Tikau	1985/86	e
KA3	kamahi	Westport	1986	e
KA4	kamahi	Westport	1986	e
KA5	kamahi	Karamea	1986	e
KA6	kamahi	Owaka, Southland	1985/86	s
KA7	kamahi	Owaka, Southland	1985/86	s
KA8	kamahi	Owaka, Southland	1985/86	s
KA9	kamahi	Owaka, Southland	1985/86	s
W207	willow	Takapau, Hawkes Bay	1987	s

[a]Honey supplied in the form of:-
- c: comb honey
- e: extracted honey
- s: scraped from comb

[b]Botanical name:-

clover	*Trifolium repens*
manuka	*Leptospermum scoparium*
ling/heather	*Calluna vulgaris*
thyme	*Thymus vulgaris*
nodding thistle	*Carduus nutans*
vipers bugloss	*Echium vulgare*
kamahi	*Weinmannia racemosa*
willow	*Salix* sp.

2.2 Extraction Procedure

2.2.1 Reagents

All solvents were bulk or commercial grade and were carefully redistilled and checked by gas chromatography prior to use.

2.2.2 Honey Extraction

Preliminary studies (Russell 1983) revealed the presence in some honeys of acidic and/or phenolic substances (*e.g.* methyl 4-hydroxy-3,5-dimethoxybenzoate and methyl 3,4,5-trimethoxybenzoate) of comparatively low volatility and appreciable water solubility. Consequently, it was considered (Tan *et al.*, 1988) that simple separating funnel extraction procedures, purging and/or resin absorption techniques (van Rossum and Webb, 1978) would result in a poor recovery of these classes of compounds. Accordingly, liquid/liquid extraction procedures were routinely employed.

Whilst liquid/liquid extraction is tedious (~ 12 hours) and is subject to the limitation that very volatile compounds may be lost during the extraction and concentration steps, it has the major advantage of its ability to quantitatively recover small quantities of organic materials with high water solubilities (*e.g.* polar species such as polyhydroxylated phenols and acidic components).

2.2.3 Extractors and Choices of Solvent

A variety of extraction systems and choices of solvent were extensively investigated in earlier work (Tan 1985). In general similar extraction procedures were adopted in the present study. However,

certain modifications were necessary to increase the efficiency of the extraction when smaller samples were extracted; an increased rate of solvent turn over was achieved by utilising a 250 ml extractor and 10 g honey samples instead of the 700 ml extractor and 50 g samples. The volume of solvent (ether) used in each extraction was about 200 ml.

2.2.4 Choice of Extraction Conditions

With the smaller extractor, examination of the influence of extraction time revealed that 12 hours was needed to recover over 95 percent of the extractable organic substances. Longer extraction times (up to 36 hours) did not give any significant increase in recovery. Furthermore, there were no observable quantitative differences between extractions using 10 g, 20 g, 25 g, 40 g and 50 g of honey. In fact the larger sample size complicated the extraction as higher concentrations favour the formation of an emulsion which can result in significant amounts (up to 100 ml) of the aqueous fraction being transferred to the solvent flask. Consequently, 12 hours was considered to be an appropriate extraction time with 10 g of honey.

2.2.5 Typical Extraction

In the preparation of honey extracts, two procedures were adopted, depending on the nature of the honey sample:

(a) Extracted honey without comb: 10 g of honey was dissolved in a beaker with 200 ml of distilled water. The resultant solution was stirred at room temperature for 5 minutes by means of a magnetic stirrer. The

honey solution was then introduced into the continuous liquid/liquid extractor. The beaker itself was washed three times with the extracting solvent (ether), to avoid possible losses of organic species that were adsorbed onto the beaker surface, and the washings introduced to the extractor. Two aliquots of internal standards, undecane (C_{11}) and methyl heptadecanoate (17:0 fatty acid methyl ester) in dichloromethane (1 mg/ml) were added directly into the extractor at the concentration of 10 μg/g of honey. As comparatively higher levels of extractable organic substances were present in ling/heather, kamahi, thyme, willow and manuka honey, internal standards were added at two times the usual concentration for the first four honeys and four times the usual concentration in manuka honey respectively.

(b) Comb honey or scraped honey with comb: the presence of honey comb rendered the extraction procedures somewhat more complicated, since the honey comb is comprised of wax esters and contains a series of hydrocarbons (from C_{21} to C_{33}, Tulloch, 1980; Gojmerac, 1980), as well as triglycerides and a series of fatty acids (from C_{12} to C_{34}, Tulloch, 1980; Gojmerac, 1980). These classes of compounds are also extractable by ether or other organic solvents. However, some of these substances are not GC-volatile under the conditions used in this study (*e.g.* waxes, triglycerides and higher hydrocarbons); thus their presence in the final extract would contaminate the GC column. Therefore the comb was removed prior to extraction. This was achieved by coarse-filtering the honey solution (10 g of honey/200 ml of water) through a cotton or glass wool plug. The glass funnel was not washed with the solvent, but was left to air dry before being reweighed in order to determine the actual amount of honey used.

2.2.6 Extraction and Treatments of Extractives

All *Quick-Fit* joints were carefully sealed with PTFE tape (John Cropper Ltd.) prior to extraction of the honey samples. After 12 hours of extraction, the ether extract was dried over anhydrous $MgSO_4$ (10 minutes with stirring). The extract was then concentrated under reduced pressure in an all-glass rotary evaporator at 25°C.

When the volume was suitably reduced (2-3 ml), the extractive solution was transferred to a 10 ml vial, and methylated with diazomethane for 1.5 minutes. Longer derivatisation times resulted in the progressive methylation of some of the phenolic hydroxyl groups. Excess diazomethane was blown off with a stream of nitrogen. After final concentration (air drying) to about 1 ml, the vial was sealed and stored in the dark at 5°C until required.

Chapter 3

Quantification and Identification of Components

Chapter 3

Quantification and Identification of Components

3.1 Introduction

As discussed in Chapter 1, the general utilisation of organic extractive profiles in determining the floral source of honeys requires the accumulation of detailed chemical data from a large number of samples of each floral type of honey, including samples from different flowering seasons and geographical locations. However, different compounds can have similar GC retention times under standard experimental conditions. Therefore, before chemical fingerprinting can be employed to characterise the floral origin of honey, the structural identity and the concentration of these components must be known. This can be achieved by using GC/MS in addition to GC to quantitatively characterise the total organic extractives from honeys.

3.2 Chromatography

Gas chromatography was performed on a Pye Unicam Model PU4500 chromatograph equipped with a flame ionisation detector (FID), modified for a capillary column with a split injection system (SGE Unijector, Melbourne). Injections were split, 1 part to the column and 200

parts to waste. The retention times (in minutes) and peak areas were measured using a Shimadzu CR-3A reporting integrator.

Analyses were performed on a fused silica open tubular (FSOT) column (12 m or 16 m x 0.22 mm i.d.), coated with non-polar dimethyl-silicone stationary phase (SGE Ltd. Melbourne), using hydrogen as a carrier.

In general, analyses were temperature-programmed commencing at 40°C (3 minutes initial hold) and increasing to 250°C (30 minutes final hold for the 12 m column and 60 minutes final hold for the 16 m column) at 4°C per minute with a carrier gas linear velocity of 45 cm/sec. Between 2 to 5 µl of each concentrated extract was injected depending on the floral type of the honey. Carbon numbers were determined by interpolation of GC retention times to those of a series of *n*-alkanes run under identical conditions, using third order polynomial fitting on an Apple Macintosh Computer running the Cricket Graph software package. Examination of blanks (from methylation and from ether) under the conditions employed did not show any interfering GC peaks.

3.3 Quantification

Quantification was performed relative to an internal standard of heptadecanoic acid (17:0) methyl ester. Response factors were determined for benzaldehyde, butanedioic acid, benzoic acid, 2-hydroxybenzoic acid, 1,4-dihydroxybenzene, 2'-methoxyacetophenone, 2-methoxybenzoic acid, 2-hydroxy-3-phenylpropionic acid, *trans*-3-phenylprop-2-enoic acid, octanedioic acid, 3,4-dimethoxybenzaldehyde, 2,6-di-*tert*-butyl-4-methylphenol, 3,4-dimethoxybenzoic acid, 3,4,5-trimethoxybenzoic acid, methyl 2-

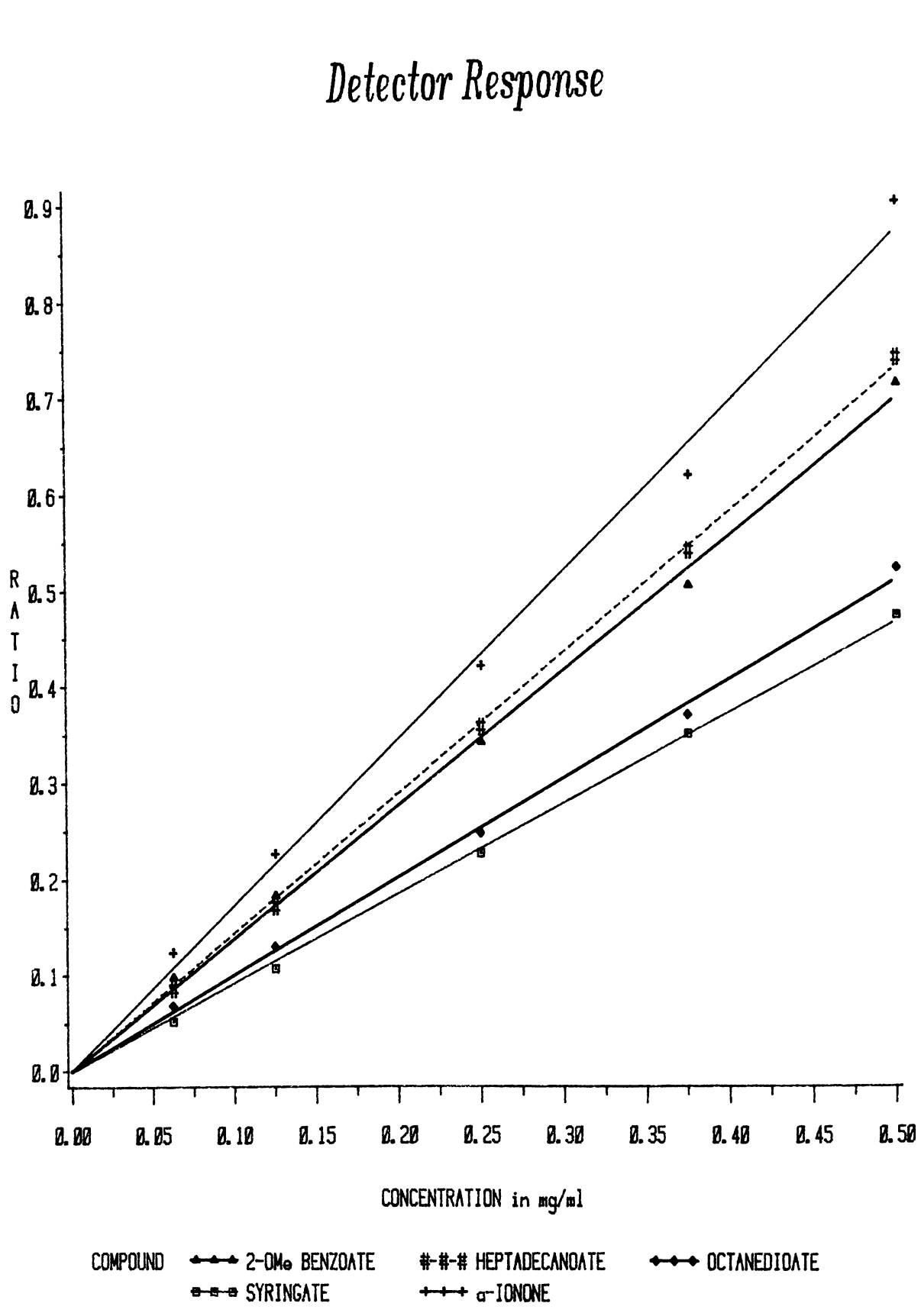


Figure 3.1 Detector response expressed as the ratios of standard peak area over C_{18} hydrocarbon peak area versus concentration of a 0.5 mg/ml added standard. In all cases, C_{18} was added at the rate of 1 mg/ml.

hydroxy-3,5-dimethoxybenzoate and tetracosane (C_{24} alkane), using 1 to 1 mixtures of the named compounds and the internal standard. Typical response factors are given in Table 3.I. In general, response factors varied by less than ± 20% in repetitive runs. Reasonable linearity in detector response relative to a C_{18} hydrocarbon internal standard was demonstrated over the range of concentrations encountered in the study (see Figure 3.1). Class response factors were applied to the series of obviously related compounds. For example, all fatty acids 6:0 to 20:0 were assigned the same response factor as fatty acid 17:0.

Table 3.I FID response factors relative to fatty acid 17:0.

compound	response factor
methyl heptadecanoate (17:0)	1.00
benzaldehyde	1.04
butanedioic acid	0.54
benzoic acid	1.16
2-hydroxybenzoic acid	0.80
1,4-dihydroxybenzene	1.47
2'-methoxyacetophenone	0.94
2-methoxybenzoic acid	1.02
2-hydroxy-3-phenylpropionic acid	0.89
trans-3-phenylprop-2-enoic acid	0.63
octanedioic acid	0.76
3,4-dimethoxybenzaldehyde	0.69
2,6-di-*tert*-butyl-4-methylphenol	1.38
3,4-dimethoxybenzoic acid	0.74
3,4,5-trimethoxybenzoic acid	0.75
methyl 2-hydroxy-3,5-dimethoxybenzoate	0.42
tetracosane (C_{24} alkane)	1.15

The unknown organic components were quantified as "butanedioic acid equivalents" for peaks with retention times up to 2-hydroxybenzoic acid, "2-hydroxybenzoic acid equivalents" for peaks with retention times up to 2-hydroxy-3-phenylpropionic acids, "2-phenylprop-2-enoic acid equivalents" for peaks with retention times up to fatty acid 17:0 and "fatty acid 17:0 equivalents" for peaks with retention times of fatty acid 17:0 and above. The results are reported as μg/g of honey used. No allowance was made for variation in water content of the samples of honey.

3.4 Combined GC/MS Analyses

Combined gas chromatographic/mass spectroscopic (GC/MS) analyses were performed on a Hewlett Packard 5890/5970 GC/MSD system interfaced to a 12 m x 0.22 mm i.d. HP-1 dimethylsilicone column. Alternatively, a Varian 3700 GC instrument was used coupled to a Finnigan Mat 700 ion trap detector system interfaced to a 12 m x 0.22 mm i.d. BP-1 column (via an open split interface) using helium as a carrier and with the temperature programmed from 40 to 240°C at 6°C per minutes (3 minutes initial hold, 10 minutes final hold). The scan repetition rate was one second over a mass range of 40 amu to 480 amu.

Chemical ionisation (CI) (*iso*butane) and high resolution GC/MS or high resolution probe MS were carried out on a Kratos MS80RFA mass spectrometer coupled with a Carlo Erba Mega gas chromatograph. This system was operated by Ruakura Agricultural Centre, Ministry of Agriculture and Fisheries, Hamilton.

3.4.1 Identification of Compounds

Mass spectra were first examined by computer search (*PBM* search software) against the *NBS Mass Spectrometry Data Base* library of approximately 42 000 spectra. The identification of individual components was based on a combination of the GC retention times and the published mass spectra of the compounds found. Identifications were confirmed by peak matching with authentic samples where possible. When a component could not be identified in this way, an attempt was made to elucidate the structure from its fragmentation pattern. Table 3.II summarises the compounds present in the honeys examined, with their retention carbon numbers and prominent ions. Representative mass spectra are depicted in Appendix A.

Unless otherwise stated, GC/FID and GC/MS total ion current chromatograms were similar despite some differences in temperature programming, carrier gases (hydrogen and helium) and linear velocity. It was assumed that the elution order was the same on both systems.

3.5 Preparative Layer Chromatography

A number of unifloral honeys possessed relatively high levels of extractable substances, the structure of which could not be identified by GC/MS alone. Sufficient quantities were isolated by multiple preparative layer chromatography (PLC) on 1 mm silica gel (Merck $P_{254+366}$) coated glass plates. Plates were developed using hexane-ether (1:4) or toluene-dioxane-acetic acid (90:25:3) solvent systems and the bands were located by viewing under UV illumination at λ = 254 and/or 350 nm.

3.6 Nuclear Magnetic Resonance Spectroscopy

^1H and ^{13}C NMR spectra were determined in $CDCl_3$ on a Jeol FX90Q, Bruker AM-400 or Varian XL-300 instrument using chloroform as the internal standard. 90 MHz ^1H NMR spectra were acquired across an 1 200 Hz spectral window with 8k data points resulting in J values accurate to 0.3 Hz. 400 MHz spectra were recorded with a 4 000 Hz spectral window and 32k data points, resulting in J values accurate to 0.25 Hz.

22.5 MHz ^{13}C NMR spectra were obtained with broadband decoupling, while 100.6 MHz spectra were obtained with broad band WALTZ-16 (Shaka *et al.* 1983) decoupling. Assignment ambiguities were resolved where possible by either the SEFT (Le Cocq and Lallemand, 1981) pulse sequence using $\tau = 1/J$ to invert methylene and quarternary carbon signals relative to methyl and methine carbon signals; or the INEPT (Doddrell and Pegg, 1980) pulse sequence using $\tau = 1/4J$ and a delay $\Delta = 3/4J$ to suppress quarternary resonances and invert methylene resonances relative to methine and methyl resonances. The DEPT (Doddrell *et al.* 1982) sequence was employed in a like manner using $\tau = 1/2J$ and $\theta = 135°$. Quarternary resonances were identified where necessary in noise modulated off resonance decoupled (NMORD) experiments in which the decoupling field was located *c.* 5 KHz to lowfield of the usual position.

The two dimensional double quantum filtered COSY (DQFCOSY) spectra were determined on the AM-400 spectrometer across spectral windows of the order 2 000-3 000 Hz as appropriate, using 2k data points in the F_2 domain and 512 increments zero-filled to 1k in the F_1 domain. Normally 16 repetitions per increment were acquired, with a pulse

repetition rate of 2 sec. Data sets were transformed using shifted sinebell window functions (SSB1 and SSB2 = 5), and symmetrised after transformation.

Table 3.II Summary of GC/MS data for honeys extracts: components are listed in order of elution time and carbon numbers, and prominent MS ions are given

peak	C no.	prominent MS ions	compound
1	8.11		unknown[a]
2	8.13	43, 59, 73, 74, 85, 104 M^+	methyl 3-hydroxypropionate[b]
3	8.22	43, 59, 74, 83, 101	unknown[b]
4	8.39	43, 45, 59, 71, 74, 87, 103	methyl 3-hydroxybutanoate[b]
5	8.48	44, 58, 74, 75, 88, 103	unknown[b] (kamahi)
6	8.57	43, 59, 74, 85, 101, 117	methyl 3-hydroxy-3-methylbutanoate[b]
7	8.58		unknown[a] (manuka)
8	8.71	55, 54, 80, 82, 108 M^+	2,5-cyclohexadiene-1,4-dione
9	8.75	43, 55, 73, 90, 103	methyl 2-hydroxy-3-methylbutyroate
10	8.83	45, 60, 72, 89, 118	manuka (unknown)
11	9.04	43, 55, 59, 71, 74, 87, 101	unknown (kamahi)
12	9.09	43, 59, 74, 87, 99, 130 M^+	methyl caproate (6:0)
13	9.13	41, 59, 68, 74, 113, 128 M^+	methyl 3-hexenoate
14	9.18	43, 59, 71, 74, 87, 99, 102 M^+	unknown (kamahi)
15	9.23	51, 77, 105, 106 M^+	benzaldehyde
16	9.27	45, 57, 70, 87, 110	manuka (unknown)
17	9.28	41, 59, 68, 69, 96, 97	unknown (kamahi)
18	9.43	41, 53, 68, 95, 126 M^+	methyl 3-furancarboxylate
19	9.44	41, 57, 85, 95, 126, 144 M^+	methyl 3-methyl-2-oxopentanoate
20	9.47	43, 55, 71, 87, 99, 115	unknown (kamahi)
21	9.48	43, 55, 88, 99, 115, 130 M^+	methyl 4-oxopentanoate
22	9.56	41, 55, 69, 84, 112, 128 M^+?	unknown (thyme)
23	9.59		unknown[a] (manuka)
24	9.66	55, 65, 66, 77, 94 M^+	phenol
25	9.67	43, 45, 57, 69, 87, 90, 113	methyl 2-hydroxy-4-methylpentanoate
26	9.72	45, 57, 69, 87, 90, 113	methyl 2-hydroxy-3-methylpentanoate
27	9.72	41, 67, 68, 95, 96, 138 M^+	unknown (kamahi)

28	9.82	41, 55, 68, 69, 83, 98 M^+	unknown (kamahi)
29	9.84	42, 55, 70, 79, 125, 140 M^+	unknown (kamahi)
30	9.85	43, 59, 87, 113, 129, 144 M^+	unknown (kamahi)
31	10.01	55, 59, 87, 114, 115	dimethyl butanedioate
32	10.02	51, 77, 79, 107, 108 M^+	benzyl alcohol
33	10.03	51, 65, 91, 92, 120 M^+	phenylacetaldehyde
34	10.20	43, 69, 73, 101, 126, 158 M^+	unknown (ling/heather)
35	10.36	41, 55, 59, 69, 74, 101, 129	methyl 2-methylsuccinaldehydate
36	10.38		unknown[a] (manuka)
37	10.39	41, 53, 67, 95, 126 M^+	methyl 2-furancarboxylate
38	10.41		unknown[a] (nodding thistle)
39	10.64	55, 59, 73, 101, 115, 143	dimethyl 2,2-dimethylbutanedioate
40	10.65	51, 77, 105, 136 M^+	methyl benzoate
41	10.78	65, 78, 91, 92, 104, 122 M^+	2-phenylethanol
42	10.82	54, 67, 82, 95, 123, 138 M^+	3,5,5-trimethylcyclohex-2-en-1-one
43	10.82	43, 58, 66, 94, 125 M^+?	unknown (clover)
44	10.83	43, 67, 71, 82, 109, 125	unknown
45	10.83		unknown[a] (manuka)
46	10.96	51, 53, 65, 80, 108, 109	unknown (willow)
47	10.99	43, 68, 96, 109, 137, 152 M^+	3,5,5-trimethylcyclohex-2-ene-1,4-dione
48	10.98	51, 78, 106, 137 M^+	methyl pyridinecarboxylate
49	11.00	43, 57, 71, 85, 127, 156 M^+	*n*-undecane (C_{11}, internal standard)
50	11.02	55, 59, 74, 87, 100, 129	dimethyl pentanedioate
51	11.06	43, 55, 74, 87, 127, 158 M^+	methyl caprylate (8:0)
52	11.09		unknown[a] (manuka)
53	11.13	55, 59, 75, 85, 117, 146 M^+	unknown (rewarewa?)
54	11.13	42, 56, 69, 70, 139, 154 M^+	2,6,6-trimethylcyclohexane-1,4-dione
55	11.14	43, 55, 67, 71, 93, 111, 153	unknown (nodding thistle)
56	11.16	43, 55, 59, 83, 98, 110	unknown (clover)
57	11.18	56, 70, 98, 112, 139, 154 M^+	unknown (ling/heather)
58	11.22	43, 55, 67, 71, 93, 111, 153	unknown (nodding thistle)
59	11.23	43, 59, 71, 129, 141, 172 M^+	unknown (kamahi)
60	11.30	43, 55, 83, 97, 98, 126	unknown (clover)
61	11.36	43, 55, 67, 71, 93, 111, 153	unknown (nodding thistle)
62	11.48	51, 65, 77, 91, 150 M^+	methyl 2-phenylethanoate
63	11.55	43, 59, 83, 101, 116, 141, 159	unknown
64	11.61	43, 67, 71, 82, 109, 125, 137	unknown
65	11.62	65, 92, 120, 152 M^+	methyl 2-hydroxybenzoate

66	11.85	41, 53, 69, 97, 109, 126 M^+	5-hydroxymethyl-2-furfural
67	11.86	53, 81, 95, 109, 124 M^+	4-methoxyphenol
68	11.87	43, 55, 67, 71, 93, 111, 155	unknown (nodding thistle)
69	11.87		unknown[a] (manuka)
70	11.94	43, 57, 71, 83, 109, 152, 155	unknown[b] (kamahi)
71	12.01	43, 55, 67, 71, 93, 111, 155	unknown (nodding thistle)
72	12.02	43, 70, 81, 101, 109, 152, 170	unknown[b] (kamahi)
73	12.02	55, 79, 94, 108, 136, 168 M^+	unknown (thyme)
74	12.06	43, 55, 74, 87, 141, 172 M^+	methyl nonanoate (9:0)
75	12.06	42, 57, 72, 83, 128, 152, 155	unknown[b] (kamahi)
76	12.07	65, 77, 92, 107, 135, 136 M^+	methoxybenzaldehyde
77	12.08	55, 59, 74, 101, 114, 143	dimethyl hexanedioate
78	12.09	65, 77, 91, 149, 164 M^+	ethyl phenylethanoate
79	12.14	43, 55, 71, 82, 95, 103, 198	unknown[b] (kamahi)
80	12.25	43, 61, 71, 103, 123, 141, 154	unknown (thyme)
81	12.29	43, 55, 93, 111, 137, 155	unknown (nodding thistle)
82	12.32	43, 55, 85, 98, 111, 151, 183	unknown (thyme)
83	12.39	51, 65, 77, 91, 104, 164 M^+	methyl 3-phenylpropionate
84	12.43	53, 55, 69, 81, 82, 110 M^+	1,4-dihydroxybenzene
85	12.45	77, 79, 91, 107, 166 M^+	methyl 2-hydroxy-2-phenylethanoate
86	12.48	43, 77, 92, 135, 150 M^+	2'-methoxyacetophenone
87	12.49	43, 55, 81, 95, 109, 151, 169	unknown (thyme)
88	12.50	43, 55, 57, 93, 111, 137, 155	unknown (nodding thistle)
89	12.52	55, 83, 140, 154, 167, 182 M^+	2-methoxy-3,5,5-trimethyl-cyclohex-2-ene-1,4-dione
90	12.54	43, 65, 92, 120, 135 M^+	3'-aminoacetophenone
91	12.54	41, 69, 70, 98, 112, 154, 171	unknown[b] (kamahi)
92	12.56	41, 69, 83, 100, 125, 128, 152	unknown (ling/heather)
93	12.65	43, 55, 67, 75, 93, 111, 155	unknown (nodding thistle)
94	12.65	51, 78, 92, 105, 115, 134 M^+	3-phenylprop-2-en-1-ol
95	12.66	51, 77, 103, 131, 162 M^+	methyl *cis*-3-phenylprop-2-enoate
96	12.66	43, 55, 111, 139, 183, 198 M^+	unknown[b] (kamahi)
97	12.72	43, 55, 71, 85, 109, 140, 169	unknown (thyme)
98	12.73	43, 85, 109, 127, 141, 169	unknown (ling/heather)
99	12.74	43, 55, 85, 98, 108, 127, 140	unknown (clover)
100	12.77	43, 70, 95, 111, 140, 168, 180	unknown[b] (kamahi)
101	12.79	43, 55, 67, 75, 93, 137, 171	unknown (nodding thistle)
102	12.83	43, 67, 70, 95, 140, 168, 182	unknown[b] (kamahi)

103	12.84	77, 91, 103, 121, 136 M^+	trimethylphenol
104	12.91	43, 59, 60, 87, 118, 129, 159	unknown[b]
105	12.91	41, 59, 69, 97, 129, 156 M^+	unknown[b] (kamahi)
106	12.93	43, 55, 85, 116, 130, 153, 166	unknown (ling/heather)
107	12.94	77, 92, 105, 133, 135, 166 M^+	methyl 2-methoxybenzoate
108	13.00	41, 69, 97, 125, 139, 156	unknown (clover)
109	13.03	43, 55, 71, 95, 135, 139, 170	unknown (willow)
110	13.03	43, 59, 60, 69, 118, 153, 171	unknown[b] (kamahi)
111	13.07	43, 69, 71, 97, 124, 152, 170	unknown (nodding thistle)
112	13.07	43, 55, 71, 96, 109, 135, 171	unknown (ling/heather)
113	13.10	43, 77, 95, 107, 124 M^+	hydroxybenzyl alcohol
114	13.12	43, 55, 71, 96, 109, 139, 157	unknown (nodding thistle)
115	13.15	43, 79, 97, 121, 136, 151, 184	unknown
116	13.16	43, 67, 71, 84, 119, 137, 152	unknown
117	13.21	50, 65, 93, 121, 122 M^+	hydroxybenzaldehyde
118	13.28	43, 55, 67, 71, 119, 135, 166	unknown[b] (kamahi)
119	13.28	65, 91, 121, 162, 180 M^+	methyl 2-hydroxy-3-phenylpropionate
120	13.31	77, 92, 107, 135, 166 M^+	methyl 3-methoxybenzoate
121	13.36	43, 67, 71, 93, 110, 119, 137	unknown (nodding thistle)
122	13.40	55, 69, 85, 117, 121, 166, 199	unknown (thyme)
123	13.43	51, 77, 103, 131, 162 M^+	methyl *trans*-3-phenylprop-2-enoate
124	13.43	43, 65, 75, 91, 135, 162 M^+	unknown (manuka)
125	13.44	43, 59, 68, 71, 111, 137, 155	unknown (thyme)
126	13.49	43, 59, 68, 71, 111, 137, 155	unknown[b] (kamahi)
127	13.59	43, 97, 139, 181, 196 M^+	unknown (ling/heather)
128	13.63	41, 83, 123, 151, 180 M^+	unknown (ling/heather)
129	13.72	59, 75, 95, 99, 127, 154, 187	unknown (manuka)
130	13.73	43, 55, 69, 97, 109, 129, 155	unknown
131	13.80	43, 69, 85, 117, 121, 178, 199	unknown (thyme)
132	13.81	43, 69, 71, 85, 139, 154, 189	unknown[b] (kamahi)
133	13.82	43, 77, 79, 107, 120 180 M^+	ethyl 2-hydroxy-2-phenylethanoate
134	13.85	51, 77, 91, 107, 138	unknown (willow)
135	13.86	51, 63, 77, 106, 121, 180 M^+	methyl 2-(methoxyphenyl)-ethanoate
136	14.03	65, 91, 103, 121, 148, 176	ethyl 2-hydroxy-3-phenylpropionate
137	14.06	55, 59, 69, 74, 97, 138, 171	dimethyl octanedioate
138	14.11	43, 109, 123, 191, 224 M^+	1-(3-oxo-1-butenyl)-2,6,6-trimethyl-1,2-epoxycyclohexan-4-ol
139	14.15	65, 93, 121, 152 M^+	methyl 3-hydroxybenzoate

140	14.18	43, 59, 67, 71, 117, 151, 180	methyl 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoate
141	14.23	65, 77, 95, 151, 165, 166 M^+	3,4-dimethoxybenzaldehyde
142	14.33	43, 59, 79, 109, 121, 152, 177	unknown (willow)
143	14.40	55, 70, 95, 107, 127, 196 M^+	unknown (ling/heather)
144	14.41	43, 55, 83, 97, 111, 143, 158	unknown (thyme)
145	14.42	55, 67, 79, 93, 107, 151, 166	unknown (willow)
146	14.45	43, 77, 91, 107, 121, 166 M^+	methyl 2-(hydroxyphenyl)-ethanoate
147	14.52	77, 91, 120, 148, 204 M^+	4-(3-oxo-1-butynyl)-3,5,5-trimethyl-cyclohex-2-en-1-one
148	14.54	55, 59, 81, 108, 136, 140 168	unknown
149	14.61	43, 65, 92, 120, 135, 163 M^+	unknown (thyme)
150	14.66	77, 91, 103, 131, 162, 190 M^+	unknown (manuka)
151	14.70	65, 91, 107, 151, 182 M^+	methyl 4-hydroxy-3-methoxybenzoate
152	14.77	43, 69, 85, 109, 127, 169	unknown (ling/heather)
153	14.78	43, 77, 109, 123, 191, 224 M^+	1-(3-oxo-1-butenyl)-2,6,6-trimethyl-1,2-epoxycyclohexan-4-ol
154	14.85	77, 91, 145, 177, 205, 220 M^+	2,6-di-*tert*-butyl-4-methylphenol
155	14.90	55, 59, 74, 87, 97, 125, 157	unknown, fatty acid?
156	15.06	43, 67, 71, 83, 98, 113, 153	unknown[b] (kamahi)
157	15.07	55, 74, 87, 143, 171, 214 M^+	methyl laurate (12:0)
158	15.10	55, 59, 74, 83, 111, 152, 185	dimethyl nonanedioate
159	15.38	45, 55, 67, 81, 100, 113, 156	unknown (nodding thistle)
160	15.41	77, 91, 107, 138, 165, 196 M^+	methyl 3,5-dimethoxybenzoate
161	15.42	77, 89, 133, 161, 192 M^+	methyl 3-(methoxyphenyl)-prop-2-enoate
162	15.42	41, 55, 81, 95, 113, 138, 166	unknown
163	15.47	79, 91, 107, 137, 165, 196 M^+	methyl 3,4-dimethoxybenzoate
164	15.48	78, 91, 102, 120, 148, 180 M^+	unknown (manuka)
165	15.62	55, 74, 87, 111, 129, 152, 172	unknown, fatty acid?
166	15.73	41, 69, 121, 175, 193, 208 M^+	unknown (willow)
167	15.78	77, 91, 107, 137, 150, 210 M^+	methyl 3-hydroxy-3-(methoxyphenyl)-propanoate
168	15.78	77, 91, 121, 151, 179, 210 M^+	methyl 2-hydroxy-3-(4-methoxy-phenyl)-propionate
169	15.97	51, 65, 91, 119, 147, 178 M^+	methyl 3-(4-hydroxyphenyl)-*cis*-prop-2-enoate
170	15.99	43, 77, 95, 108, 135, 150	unknown (clover)

171	16.00	43, 77, 119, 147, 162, 204 M^+	4-(3-oxo-1-butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one
172	16.01	43, 77, 91, 108, 152, 208 M^+	4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one
173	16.08	55, 67, 81, 95, 127, 168, 198	unknown (ling/heather)
174	16.10	55, 59, 74, 98, 125, 166, 199	dimethyl decanedioate
175	16.19	43, 55, 81, 95, 152, 180 M^+?	unknown[b] (kamahi)
176	16.20	45, 77, 91, 106, 147, 162 M^+?	unknown (ling/heather)
177	16.23	77, 118, 133, 161, 192 M^+	methyl 3-(4-methoxyphenyl)-*trans*-prop-2-enoate
178	16.25	43, 81, 95, 113, 137, 152	unknown (vipers bugloss)
179	16.29		unknown[a] (manuka)
180	16.30	77, 91, 107, 137, 150, 210 M^+	methyl 3-hydroxy-3-(methoxyphenyl)-propanoate
181	16.49	43, 93, 125, 136, 164, 220 M^+	4-hydroxy-4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one
182	16.53	45, 77, 93, 137, 180, 224 M^+	4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one
183	16.60	55, 69, 95, 81, 136, 164, 196	dimethyl *trans*-2-decenedioate
184	16.71	93, 109, 155, 195, 211, 226 M^+	methyl 3,4,5-trimethoxybenzoate
185	16.84	43, 77, 91, 107, 125, 208 M^+	unknown (willow)
186	16.91	51, 65, 91, 119, 147, 178 M^+	methyl 3-(4-hydroxyphenyl)-*trans*-prop-2-enoate
187	17.07	43, 74, 87, 143, 199, 242 M^+	methyl myristate (14:0)
188	17.12	67, 93, 123, 153, 181, 212 M^+	methyl 4-hydroxy-3,5-dimethoxy-benzoate
189	17.18	45, 77, 93, 137, 180, 224 M^+	4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one
190	17.20	77, 91, 147, 191, 207, 222 M^+	methyl 3-(3,4-dimethoxyphenyl)-*cis*-prop-2-enoate
191	17.25	43, 77, 95, 124, 163, 180, 209	unknown (willow)
192	17.28		unknown[a] (kamahi)
193	17.29	43, 69, 95, 124, 166, 222 M^+	4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one [**9**]
194	17.35	43, 69, 95, 124, 166, 222 M^+	isomer of [**9**] (peak 193)
195	17.40		unknown[a] (kamahi)
196	17.45		unknown[a] (manuka)
197	17.58	77, 92, 135, 179, 199	unknown[b] (manuka)

198	17.96	81, 106, 160, 188, 215, 216 M^+	unknown (vipers bugloss)
199	18.08	43, 74, 87, 143, 213, 256 M^+	methyl pentadecanoate (15:0)
200	18.25	77, 91, 147, 191, 207, 222 M^+	methyl 3-(3,4-dimethoxyphenyl)-*trans*-prop-2-enoate
201	18.29	41, 69, 109, 124, 152, 165, 222	unknown[b] (kamahi)
202	18.50	43, 83, 98, 125, 181, 239, 268	unknown[b] (kamahi)
203	18.53	81, 106, 160, 118, 215, 216 M^+	unknown (vipers bugloss)
204	18.44	43, 91, 134, 162, 278 M^+	unknown (ling/heather)
205	18.66	43, 97, 123, 125, 166, 224	isomer of **[22]** (peak 206)
206	18.77	43, 97, 123, 125, 166, 224	1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans-cis*-1,2,4-triol **[22]**
207	18.81	41, 55, 74, 97, 236, 268 M^+	methyl palmitioleate (16:1)
208	18.88	69, 109, 124, 152, 222, 250	unknown[b] (kamahi)
209	19.09	43, 74, 87, 143, 239, 270 M^+	methyl palmitate (16:0)
210	19.66	55, 71, 83, 109, 125, 152, 170	unknown[b] (kamahi)
211	19.80	41, 83, 127, 155, 179, 225, 252	unknown[b] (kamahi)
212	20.06	66, 94, 108, 135, 163, 220	unknown (manuka)
213	20.10	43, 74, 87, 143, 241, 284 M^+	methyl margarate (17:0, internal standard)
214	20.30	91, 125, 134, 162, 190, 278 M^+	methyl abscisate
215	20.39	89, 133, 177, 194, 208, 276 M^+	unknown (clover)
216	20.70	55, 67, 81, 109, 263, 294 M^+	methyl linoleate (18:2)
217	20.73	55, 67, 79, 108, 236, 292 M^+	methyl α-linolenate (18:3)
218	20.79	55, 69, 74, 110, 264, 296 M^+	methyl oleate (18:1)
219	20.86	55, 69, 74, 110, 264, 296 M^+	methyl oleate isomer (18:1)
220	21.00	43, 57, 71, 85, 113, 296 M^+	*n*-heneicosane (C_{21})
221	21.09	43, 74, 87, 143, 255, 298 M^+	methyl stearate (18:0)
222	21.12	91, 125, 134, 162, 190, 278 M^+	methyl abscisate
223	21.72	41, 83, 127, 155, 179, 266 M^+?	unknown[b] (kamahi)
224	22.00	43, 57, 71, 85, 127, 310 M^+	*n*-docosane (C_{22})
225	23.00	43, 57, 71, 85, 113, 324 M^+	*n*-triacosane (C_{23})
226	23.18	43, 74, 87, 143, 283, 326 M^+	methyl arachidate (20:0)
227	24.00	43, 57, 71, 85, 127, 338 M^+	*n*-tetracosane (C_{24})
228	25.00	43, 57, 71, 85, 127, 352 M^+	*n*-pentacosane (C_{25})
229	25.16	43, 74, 87, 143, 311, 354 M^+	methyl behenate (22:0)
230	26.00	43, 57, 71, 85, 127, 366 M^+	*n*-hexacosane (C_{26})
231	27.00	43, 57, 71, 85, 127, 380 M^+	*n*-heptacosane (C_{27})
232	27.15	43, 74, 87, 143, 339, 382 M^+	methyl lignocerate (24:0)

233	28.00	43, 57, 71, 85, 113, 394 M^+	*n*-nonacosane (C_{28})
234	29.00	43, 57, 71, 85, 113, 408 M^+	*n*-nonacosane (C_{29})
235	29.16	43, 74, 87, 143, 367, 410 M^+	methyl cerotate (26:0)
236	30.70	43, 57, 69, 97, 139, 434 M^+	*n*-hentriacontene (C_{31})
237	30.78	43, 57, 69, 97, 139, 434 M^+	*n*-hentriacontene (C_{31})
238	31.00	43, 57, 71, 85, 141, 436 M^+	*n*-hentriacontane (C_{31})
239	31.20	43, 74, 87, 143, 395, 438 M^+	methyl montanate (28:0)
240	32.70	43, 57, 83, 97, 153, 462 M^+	*n*-tritriacontene (C_{33})
241	33.10	43, 74, 87, 143, 423, 466 M^+	methyl triacontanoate (30:0)

[a] detected in GC/FID but not in GC/MS.

[b] peak assignment is ambiguous due to different GC/FID and GC/MS response.

Chapter 4

Identification of the Components of Extracts of Various Types of Honey

Chapter 4

Identification of the Components of Extracts of Various Types of Honey

4.1 Introduction

In the work on the quantification and identification of the components of honey described in Chapter 3, it was noted that a number of unifloral honeys possessed a variety of compounds at relatively high levels, the mass spectra of which did not correspond with those of any of the compounds reported in the honey literature or recorded in the *EPA/NIH Mass Spectral Data Base.* Table 3.II includes some of the new components identified in the present study, *e.g.* Peaks 42, 140, 193 and 206.

As discussed in Chapter 1, the present study centres on the accumulation of a detailed knowledge of the extractable trace organic components of New Zealand honeys and the possibility of the general utilisation of organic extractive profiles for characterising the floral sources of honeys. Although one is able to locate in the chromatogram the peak or peaks that are associated with a particular characteristic floral source, different compounds can have similar GC retention times under standard experimental conditions. Therefore, structural identification of these compounds is required before GC can be employed to supplement, or perhaps even to replace organoleptic judgements.

It is apparent that GC/MS alone could not be employed to successfully elucidate the structures of these components. For charac-

terisation and structural elucidation by other techniques such as probe high resolution MS and one and two dimensional NMR, isolation of the unknowns in larger quantities was necessary. Consequently, bulk extraction of representative unifloral honey samples was carried out and the extracts subjected to PLC separation followed by GC analysis to determine the unknowns. Attempts to obtain crystals of the major isolated components by vapour diffusion techniques (toluene/hexane) for X-ray crystallography were unsuccessful in all but one case.

Unifloral honeys contained components which were unique for each floral type. The characterisation of these components was pursued as they were discovered.

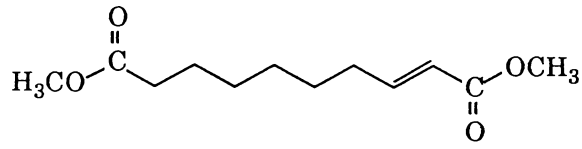
4.2 White Clover Type Honey

Overall, white clover type honey was found to be relatively low in extractives (< 50 μg/g). The major extractable components of this honey proved to be aliphatic diacids with an unsaturated C_{10} diacid predominating. The geometry and the position of the double bond in the unsaturated diacid were elucidated by NMR spectroscopy.

4.2.1 ^{1}H and ^{13}C NMR of Dimethyl *trans*-2-decenedioate [1]

A bulk extraction (90 g) of clover honey (sample 42 described in an earlier study; Tan, 1985) afforded a mixture of extractives which were separated by multiple PLC on silica gel (Merck $PF_{245+366}$) with hexane-ether (4:1) (3 developments). One band was recovered (*c.* 1 mg) which exhibited a purple colouration when the PLC plate was viewed under UV

illumination at λ = 350 nm. Methylation of this band afforded a compound identified as dimethyl *trans*-2-decenedioate [**1**].



[**1**]

The identification of dimethyl *trans*-2-decenedioate was initially based on a match of retention index and mass spectrum of that reported for a urinary acid (Spiteller *et al.*, 1979) and a component of royal jelly (Lercker *et al.*, 1981; 1982). It is the only decenedioic acid isomer to display a higher carbon number than decanedioic acid in GC as methyl esters on a non-polar phase.

The configuration and position of the double bond was confirmed as *trans*-2 by NMR analysis of the methylated diacid. There appeared in the 200 MHz ^{1}H NMR spectrum of the diester two methoxy signals (δ 3.73 and 3.67 ppm) together with two olefinic methine signals centred at δ 5.81 [doublet (J = 15.6 Hz) of triplets (J = 1.6 Hz)] and 6.96 ppm [doublet (J = 15.6 Hz) of triplets (J = 7.0 Hz)] respectively. The large ^3J coupling (15.6 Hz) establishes the π bond geometry to be *trans* rather than *cis*, whilst the marked downfield shift (to δ 6.96 ppm) experienced by one of the olefinic methine protons is indicative of conjugation with an ester group.

Consistent with this conclusion, there appeared in the ^{13}C NMR spectrum of the diester carbonyl signals at δ 174.2 (isolated $COOCH_3$) and 167.2 ppm (conjugated $COOCH_3$), together with the olefinic signals at δ 121.0 (C-3) and 149.5 ppm (C-2) and the aliphatic signals at δ 32.1, 28.9, 28.7, 27.8, 24.8 and 34.0 ppm (C-4 to C-9). The structure of the methylated diacids was further substantiated by a comparison of the ^{1}H and ^{13}C NMR

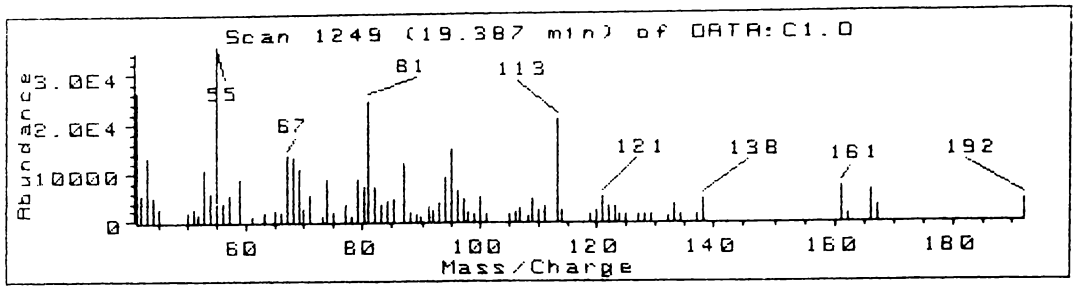
spectral data determined for a methylated specimen of *trans*-2-dodecene-dioic acid (Sigma Chemical Co., St. Louis) which displayed similar coupling constants and chemical shifts.

Typically the level of 2-decenedioic acid was in the range of 1.2 to 7.5 μg/g. However some large variations were found, for example 181 μg/g in sample 42 of the earlier study (Tan, 1985) and 0.8 μg/g in sample C2 of the present study (See Table 5.I). The saturated and *trans*-2 unsaturated C_{10} diacids, but not the *cis*-2 unsaturated diacid (*trans* is the only active form; Blum *et al.,* 1971), are known to be part of the pheromone system of the honeybee *Apis mellifera* (Pain, 1979) and are therefore not considered as part of the contribution from the floral source. The C_{10} acids are also the constituents of the fatty acid fraction of royal jelly (Lercker *et al.,* 1981, 1982).

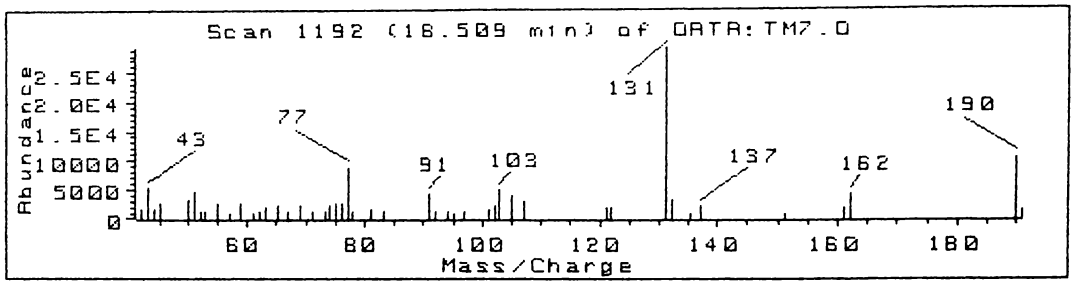
Although aliphatic diacids were found in most of the other unifloral honeys investigated in the present study, they were absent from some unifloral ling/heather honey samples.

4.2.2 Unidentified Components

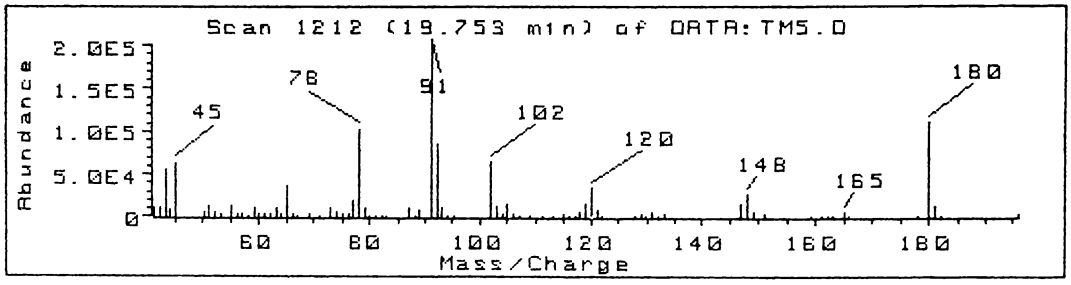
In addition to the identified compounds, a number of components could not be identified, the majority of which were trace or co-eluting components which gave rise to poor quality mass spectra. For example, peak 162 (carbon number 15.42) of clover honey possessed a mass spectrum [see Figure 4.1(a)] which suggested it to be a mixture of two components, one of which had a molecular ion of m/z 192, while the highest occurring ion from the second component appeared at m/z 166. Loss of a methoxy radical from the ion of m/z 192 would give rise to the ion



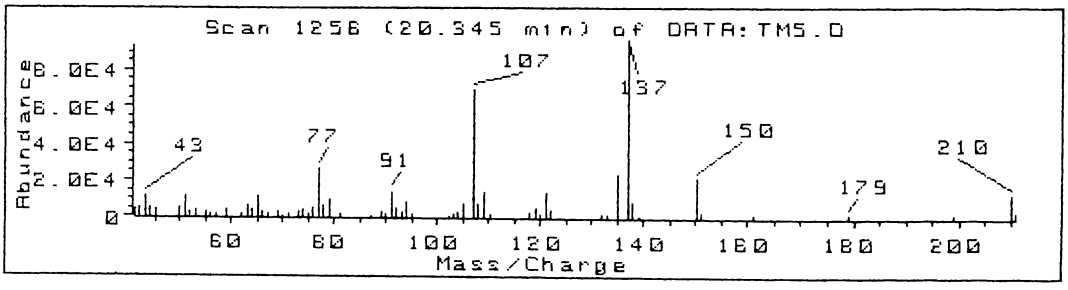
(a) peak 162



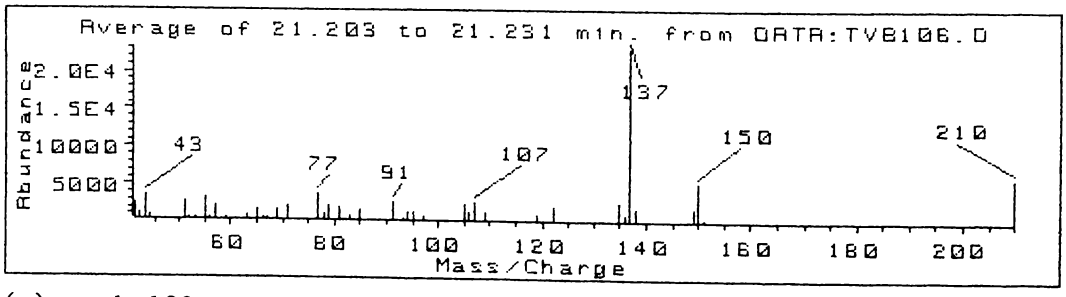
(b) peak 150



(c) peak 164



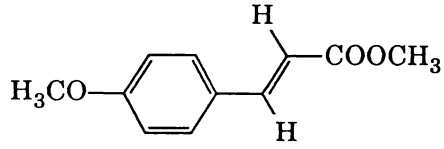
(d) peak 167



(e) peak 180

Figure 4.1 Mass spectra of tentatively identified components in white clover type (peaks 162) and manuka (peaks 150, 164, 167 and 180) honeys.

of m/z 161 [see Figure 4.1(a)]. Methyl *trans*-3-(4-methoxyphenyl)-prop-2-enoate [**2**] appeared to exhibit a similar fragmentation pattern to one of the components of peak 162, but this compound eluted at carbon number 16.23. It is possible that peak 162 is the corresponding *cis* isomer, or the *ortho*- or *meta*- analogue of the *trans* isomer.



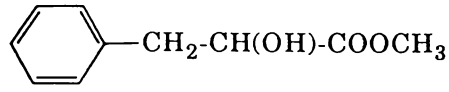
[**2**]

4.3 Manuka Honey

In the earlier study (Tan, 1985) identification of 2-hydroxy-3-phenylpropionic acid, the dominant component in manuka honey, was based only on mass spectral data. It was therefore considered desirable that the structure of this compound be confirmed by its isolation and spectroscopic comparison.

4.3.1 ^{1}H and ^{13}C NMR of Methyl 2-hydroxy-3-phenylpropionate [3]

A bulk extraction (50 g) of manuka honey afforded a mixture of extractives which were separated by multiple PLC on silica gel (Merck $PF_{245+366}$) with hexane-ether (4:1) (3 developments). One band was recovered (*c.* 2 mg) which exhibited a purple colouration when the PLC plate was viewed under UV illumination at $\lambda = 350$ nm. Methylation of this band afforded methyl 2-hydroxy-3-phenylpropionate [**3**], identical with an authentic specimen (Sigma Chemical Co., St. Louis).

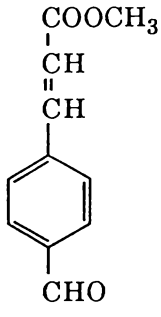


[3]

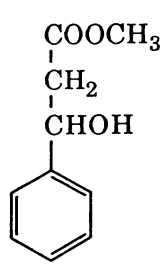
In the ^{1}H NMR spectrum of 2-hydroxy-3-phenylpropionate [**3**] there appeared an ester methoxy signal (δ 3.72 ppm) together with a two proton ABX like aliphatic multiplet, [δ 2.94, doublet (^{2}J = 13.9 Hz) of doublet (^{3}J = 6.5 Hz) and 3.16 ppm, doublet (^{2}J = 13.9 Hz) of doublet (^{3}J = 4.7 Hz)], a broad hydroxyl and hydroxyl methine signal at δ 4.45 ppm, ($W_{1/2}$ = 10.0 Hz) and an aromatic signal at δ 7.25 ppm. In the ^{13}C NMR spectrum of the ester there appeared carbonyl signal at δ 174.5 ppm, together with aliphatic and oxygenated aliphatic signals at δ 40.6 and 71.3 ppm and aromatic signals at δ 129.5, 128.4 and 126.9 ppm in the ratio of 2:2:1.

4.3.2 Unidentified Components

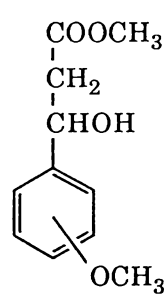
Mass spectra of four tentatively identified components (peaks 150, 164, 167 and 180) from manuka honey are depicted in Figure 4.1(b)-(e). It is believed that peaks 150 and 164 possess structures [**4**] and [**5**] respectively, and that peaks 167 and 180 are isomers of structure [**6**]. The mass spectra of these compounds are similar to those recorded for methyl 3-phenylprop-2-enoate [**7**], methyl 2-hydroxy-3-phenylpropionate [**3**] and methyl 2-hydroxy-3-(4-methoxyphenyl)-propionate [**8**] respectively.



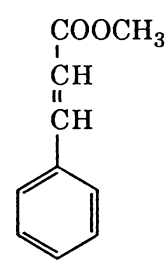
[4]



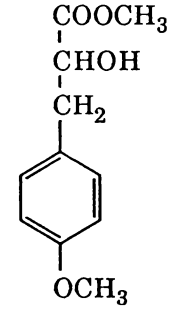
[5]



[6]



[7]



[8]

4.4 Ling/Heather Honey

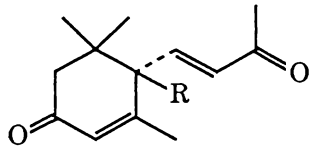
Gas chromatographic analysis of ling/heather honey extracts revealed an array of early eluting substances of established structure, methyl benzoate, methyl phenylethanoate and methyl 2-hydroxy-3-phenylpropionate being the dominant components. In addition to the known components, a variety of unidentified components were also present (see Table 5.V). PLC separations of these unknown substances have resulted in the characterisation in ling/heather honey, a family of 3,5,5-trimethylcyclohex-2-ene-1-one derivatives possessing fifteen, thirteen, nine or eight carbon atoms, often described as degraded carotenoids. Concentrations of these substances and other extractives in heather honeys are presented in Section 5.4.

4.4.1 Isolation of Degraded Carotenoids

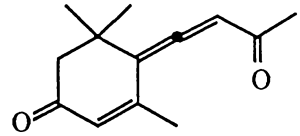
A bulk extraction of a mixture of samples H2, H3 and H4 (100 g combined weight) afforded a mixture of extractives which were separated by multiple PLC on silica gel (Merck $PF_{254+366}$) with toluene-dioxane-acetic acid (90:25:3) (first and second development) and hexane-ether (4:1) (third and fourth development). Twenty bands were recovered from the PLC plate; the two major degraded carotenoid bands (peaks 171 and 193, *c.* 1 and 2 mg respectively) exhibited a deep purple colouration when the plate was viewed under UV illumination at $\lambda = 350$ nm.

The quantities of the two major components (peaks 171 and 193) were sufficient for their structures to be elucidated (structures [**9**] and [**10**]), principally by a combination of high resolution MS and high field one and two dimensional NMR spectroscopy. Thereafter, a consideration of

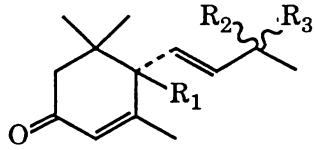
the mass spectral fragmentation patterns of these substances, and of those reported in the literature for related compounds, enabled the structures of the other heather honey compounds to be determined with a high degree of assurance (structures [**11-20**]).



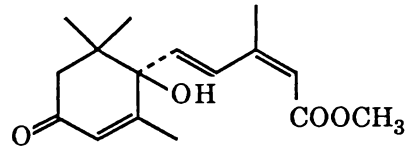
[**9**] R = OH
[**21**] R = H



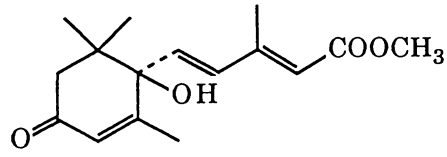
[**10**]



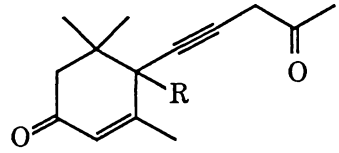
[**11**] $R_1 = R_2 = OH, R_3 = H$
[**12**] $R_1 = R_3 = OH, R_2 = H$
[**17**] $R_1 = R_2 = H, R_3 = OH$



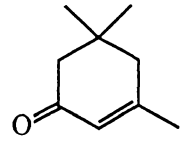
[**13**]



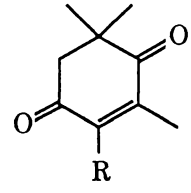
[**14**]



[**15**] R = OH
[**16**] R = H



[**18**]



[**19**] R = H
[**20**] $R = OCH_3$

4.4.2 Peak 193 [9]

^{13}C NMR spectroscopy revealed the presence of thirteen carbon signals (see Table 4.I), assignable to four methyl carbons, two conjugated carbonyl groups, and the carbons of two olefinic double bonds (one trisubstituted, the other disubstituted), while high resolution mass spectroscopy established the molecular formula $C_{13}H_{18}O_3$ (found m/z = 222.1257 M^+, required 222.1256). Since this molecular formula requires a total of five rings, and/or double bonds, it follows that peak 193, which the ^{13}C NMR data requires to be a diketo-diene, must be monocyclic. High resolution mass spectroscopy established that the ion of m/z 205 arises by loss of a hydroxyl radical. This suggested the presence of a tertiary hydroxyl group, an observation that is in keeping with the occurrence at δ 79.3 ppm in the ^{13}C NMR spectrum of a signal assignable to an oxygenated quaternary carbon. ^1H NMR spectroscopy indicated the presence of two aliphatic tertiary methyl groups, an olefinic methyl group, a methyl ketone, an isolated two proton multiplet (AB_q, ^2J = 17.5 Hz) and three conjugated olefinic protons, two of which were mutually *trans* coupled (AB_q, ^3J = 15.8 Hz). While this data establishes the functional groups present and demonstrates that peak 193 possesses a six membered ring, it does not define the stereochemical disposition of substituent groups.

Even at 90 MHz, the half peak width and height of the two tertiary methyl group signals differed by *c.* 15%. This was despite the observation that the methyl group protons possess similar T_1 values (5α-CH_3, 0.88 sec; 5β-CH_3, 1.01 sec), and therefore by implication T_2 values. Hence it can be concluded that one of the tertiary methyl group signals is more extensively long-range coupled than the other. This observation prompted the determination of the two dimensional double quantum filtered COSY

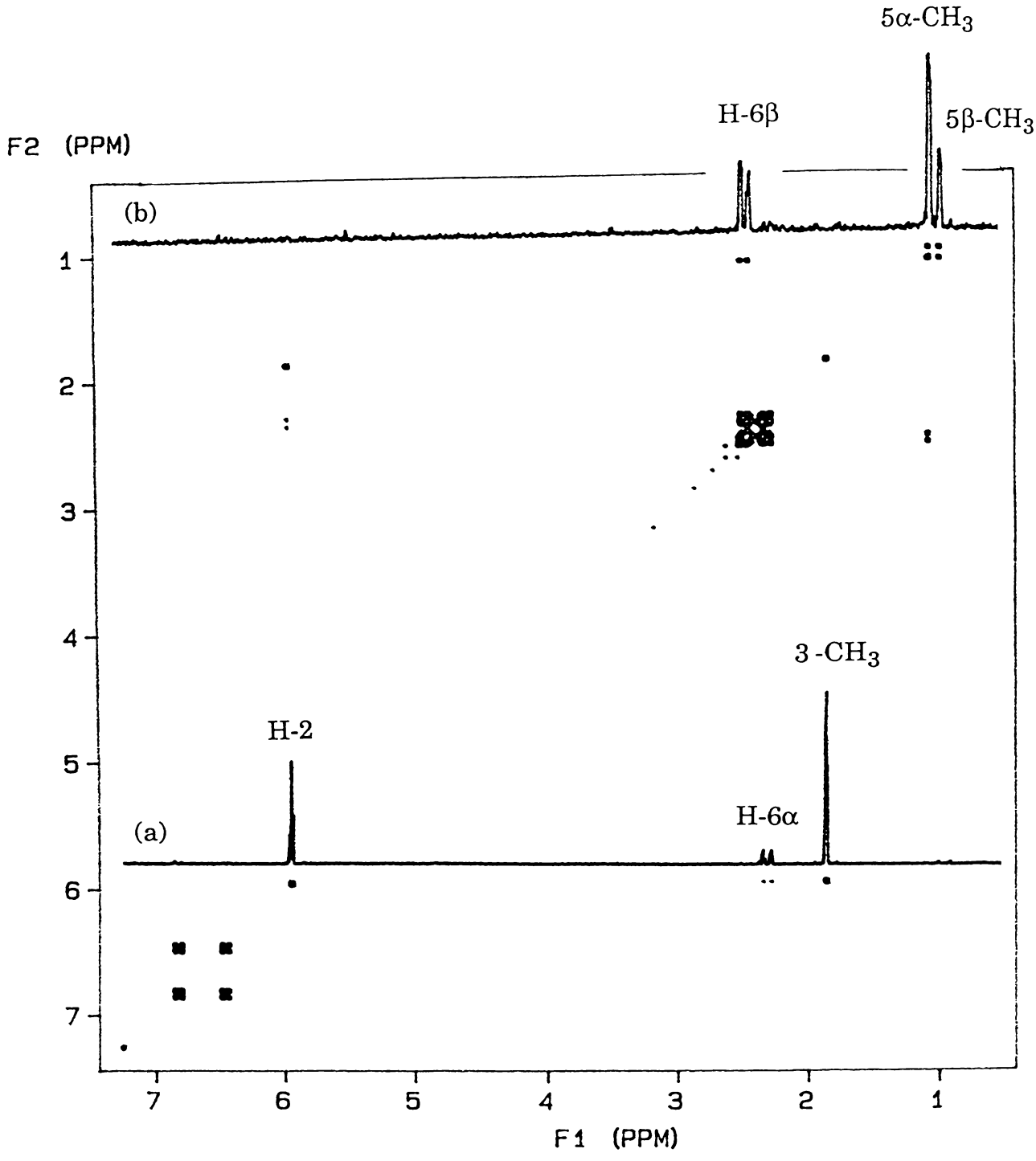
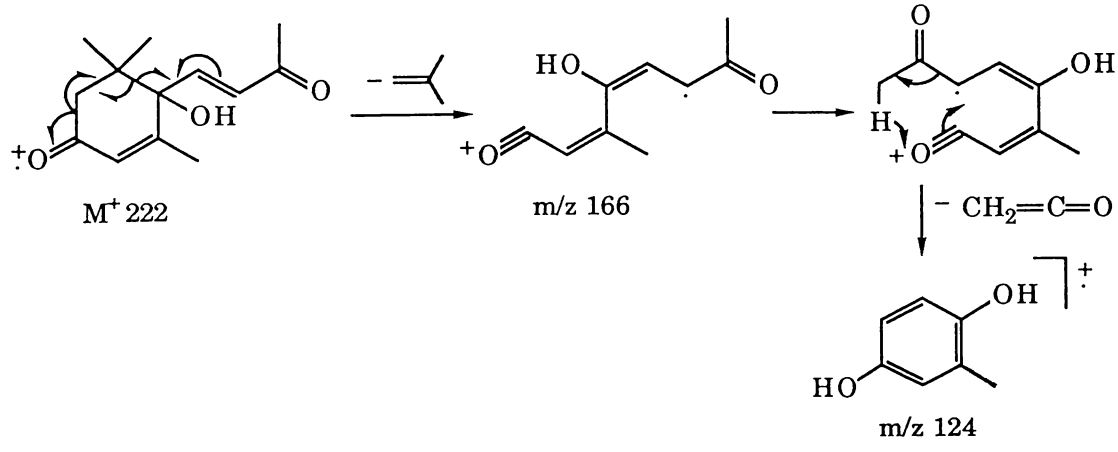


Figure 4.2 300 MHz double quantum filtered COSY NMR spectrum of 4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one [9]; with cross section at the resonance frequency of (a) H-2 and (b) 5α-CH_3.

(DQFCOSY) spectrum of peak 193 at 300 MHz under conditions designed to detect ^4J couplings. While ^4J couplings are usually not resolved in conventional ^{1}H NMR spectroscopy, they can be detected as cross peaks in two dimensional COSY and DQFCOSY spectra. Thus it emerged (Figure 4.2) that the tertiary methyl groups were mutually ^4J coupled and that one of the methyl groups (that with the lesser peak height) was also ^4J coupled to one of the adjacent methylene protons. Such a ^4J coupling between an axial methylene proton and the protons of a 1,2-*trans*- oriented methyl group is typical of that occurring in steroids (Marat *et al.*, 1987) and triterpenes (Wilkins *et al.*, 1989). These protons can be aligned such that they exhibit a planar W type interaction. The other (equatorial) methylene proton exhibited a ^4J coupling (0.9 Hz) with the conjugated olefinic proton which resonated at δ 5.95 ppm. Such a coupling is typical of that which occurs between protons which flank a carbonyl group. The olefinic proton also displayed a resolvable ^4J coupling (1.4 Hz) with the olefinic methyl group. The foregoing four bond connectivities require that peak 193 is 4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one [**9**]. In accordance with this conclusion there appeared in the mass spectrum of this compound strong losses of ketene (from the oxobutenyl side chain) and butene (from the six membered ring).



Levels of this component in honeys from the 1985-1986 season ranged from 100-180 μg/g. A minor component (peak 194) exhibited an identical mass spectrum; since ^1H NMR established that peak 193 possesses a *trans* double bond it is possible that peak 194 is the *cis* analogue.

Table 4.I ^{13}C and ^1H NMR chemical shifts of 4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one [**9**] and 4-(3-oxo-1-butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one [**10**] in $CDCl_3$.

Peak no	193 [**9**]		171 [**10**]	
Site	δ ^{13}C ppm	δ ^1H ppm	δ ^{13}C ppm	δ ^1H ppm
1	197.5[a]		197.4	
2	127.8	5.96	127.3	6.20
3	160.5		148.4	
4	79.3		117.1	
5	41.5		37.1	
6	49.6	2.48, 2.35[b]	50.9	2.45
7	24.4	1.02 (5α-CH_3)	28.8	1.24
8	18.7	1.10 (5β-CH_3)	21.5	1.28
9	23.0	1.88 (3-CH_3)	27.2	2.02
1'	145.1	6.82[c]	214.2	
2'	130.5	6.45[c]	102.5	6.00
3'	197.1[a]		196.9	
4'	28.4	2.30 ($COCH_3$)	28.8	2.27

[a] Assignments interchangeable, [b] AB_q, J = 17.5 Hz, [c] AB_q, J = 15.8 Hz.

4.4.3 Peak 171 [10]

High resolution mass spectroscopy established the molecular formula $C_{13}H_{16}O_2$ ((found m/z = 204.1165 M^+, required 204.1150), while ^{13}C

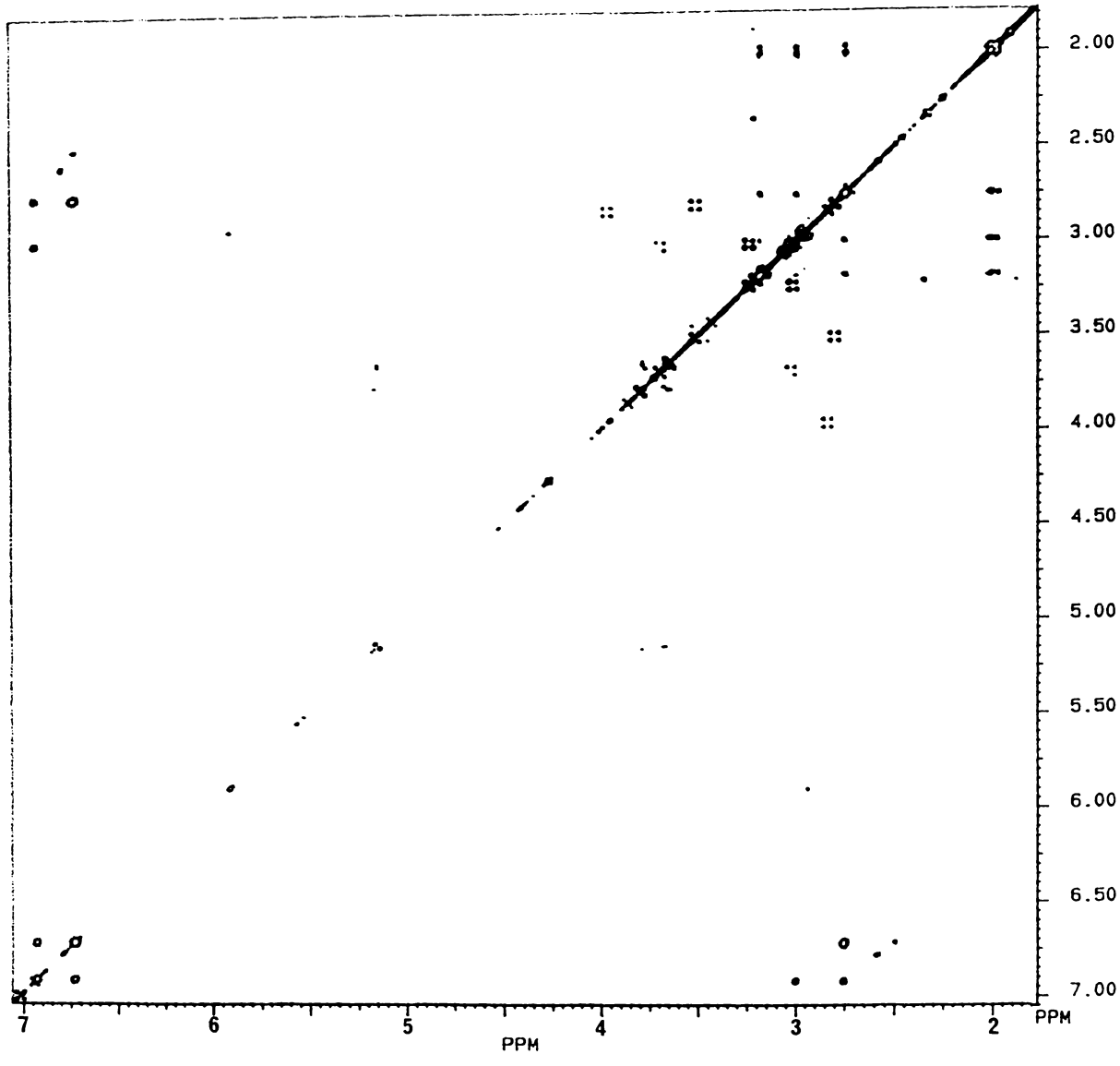


Figure 4.3 400 MHz double quantum filtered COSY NMR spectrum of 4-(3-oxo-1-butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one [**10**].

and ^1H NMR spectroscopy established the presence an array of functional groups similar to that found for [**9**] save that the tertiary hydroxyl group and the disubstituted double bond had been replaced by an allenic system. Structure [**10**] was thus assigned to peak 171. Consistent with this conclusion, a DQFCOSY NMR experiment established that within the six membered ring system there existed a network of ^4J couplings identical to those established for [**9**], while the allenic proton proved to be long range coupled with the olefinic proton, the olefinic methyl group and the methyl ketone protons (see Figure 4.3). The mass spectrum of this compound exhibited a strong loss of ketene, but lacked a butene loss. Levels of this component in honeys from the 1985-86 season ranged from 27-36 μg/g.

4.4.4 Peaks 182 and 189

The mass spectra of peaks 182 and 189 were indistinguishable; both displayed molecular ions at m/z 224. Accurate mass measurement established the molecular formula $C_{13}H_{20}O_3$ (found m/z = 224.1371 M^+, required 224.1412). Reduction of one of the carbonyl groups in structure [**9**] ($C_{13}H_{18}O_3$) was indicated. Since the strong ketene loss of [**9**] was in both of peaks 182 and 189, replaced by a 44 amu loss (C_2H_4O by accurate mass measurement), it can be concluded that these compounds possess structures [**11**] and [**12**]. These alcohols are a pair of diastereoisomers as C-4 is asymmetric, and are therefore resolvable. However, the absolute configuration of the alcohols was not determined. Compound [**12**] (the later eluting isomer) predominated in the 1985-86 season honey with levels of 30-60 μg/g.

4.4.5 Abscisic Acid Isomers

The presence of [**9-12**], all of which are related to abscisic acid, a well known plant growth hormone, prompted a search for this substance. Methylation of authentic specimens of *trans-cis* and *trans-trans* abscisic acid afforded the methyl esters [**13**] and [**14**] respectively. GC/MS analysis on two capillary columns of differing polarity, together with high resolution mass measurements and selected ion chromatograms revealed the presence in the extracts of low levels of the *trans-cis* and *trans-trans* esters (peaks 214 and 222 respectively).

4.4.6 Other Extractives

High resolution mass spectroscopy established the presence of another two compounds, each possessing thirteen carbon atoms. These substances exhibited molecular ions at m/z 204, $C_{13}H_{16}O_2$, peak 147, (found m/z = 204.1150 M^+, required 204.1165) and at m/z 220, $C_{13}H_{16}O_3$, peak 181, (found m/z = 220.1102 M^+, required 220.1099). Since peak 181 possessed two fewer hydrogen atoms than [**9**], another ring or double bond must be present. The mass spectra of both compounds included a distinctive loss of a C_4H_8 (butene) fragment. Mass spectral studies (Enzell and Wahlberg, 1986) have demonstrated that a butene loss is characteristic of degraded carotenoids which possess a 3,5,5-trimethylcyclohex-2-en-1-one ring system. Thus a six membered ring system similar to that established for [**9-12**] was present and it follows that the additional double bond must be located in the side chain. This proposition lead to the conclusion that peak 181 was the acetylenic analogue of [**9**] and possessed structure [**15**].

The presence of an acetylenic bond in [**15**] appeared to inhibit the loss of the side chain methyl ketone as ketene. A weak CH_3CO^+ ion loss and a strong CO loss also occurred in the mass spectrum of this compound. Peak 147 also lacked a ketene loss, but displayed strong butene and CO losses and a weak CH_3CO^+ ion loss. Since this compound possesses two oxygen atoms it follows that it has structure [**16**]. Selected ion GC/MS analysis demonstrated the presence of a shoulder peak (not resolved in GC/FID traces) corresponding to a substance of molecular weight 208 amu with an identical mass spectrum to the recorded library mass spectrum of 4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one [**17**].

Several of the early eluting compounds also gave mass spectra which showed strong losses of butene and CO. Two of these compounds were characterised. Peak 42 (m/z 138 M^+, $C_9H_{14}O$) proved to be identical with an authentic specimen (Sigma Chemical Co., St. Louis) of 3,5,5-trimethylcyclohex-2-en-1-one [**18**] (isophorone), while peak 47 (m/z 152 M^+, $C_9H_{12}O_2$) was considered to be the diketo analogue [**19**]. Another substance, peak 89 (m/z 182 M^+), gave a mass spectrum identical to the *NBS Mass Spectrometry Data Base* library spectrum of 2-methoxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione [**20**].

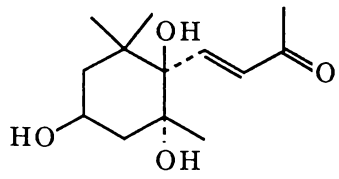
The four ling/heather samples collected during the 1985-86 season (*i.e.* H361, H2, H3 and H4) all possessed similar levels of 3,5,5-trimethyl-cyclohex-2-en-1-one derivatives (see Table 5.V). Substances possessing structures of this type are frequently referred to as degraded carotenoids and have hitherto been reported from a number of plant sources. For example, an array of closely related compounds possessing allenic, olefinic, or acetylenic side chains were isolated from tobacco by Enzell and Wahlberg (1986). Compounds of uncertain structure were detected by

Graddon *et al.* (1979) in several Australian honeys. The mass spectra they published of compounds suggested to be 4-butylcyclopenten-3-one and 4-butylcyclohexen-3-one corresponded exactly with those which were determined in the present study for compounds [**18**] and [**19**]. Additionally it should be noted that their published mass spectrum of a compound of uncertain structure and molecular weight 206, corresponds exactly with that recently presented by Enzell and Wahlberg for 3-oxo-α-ionone [**21**].

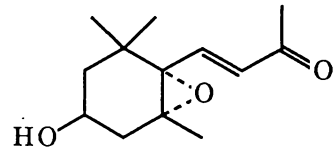
The presence of the foregoing extractives prompted a search for carotenoids in the honey samples. However, none were detected using an established non-aqueous reverse phase high pressure liquid chromatography procedure (Lauren *et al.*, 1986). Detection limits were below 1 μg/g for β-carotene and a range of hydroxy- or carboxy- carotenes.

4.5 Thyme Honey

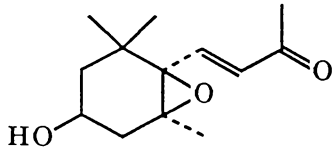
As for ling/heather honey, GC/MS analysis of thyme honey revealed an array of extractives of established structure, in addition to a variety of unidentified components. This section reports the isolation and characterisation of the dominant trihydroxyketone isolated from the ether extracts of a representative New Zealand thyme honey. The trihydroxyketone was shown by X-ray crystallographic analysis to be 1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans-cis*-1,2,4-triol [**22**]. Since this compound appears to occur only in thyme honeys, its presence is considered to afford a chemical procedure for the verification of floral source. Peaks 138 and 153 were characterised as related structures.



[22]



[23]



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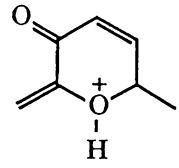

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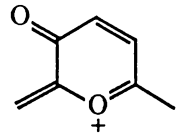

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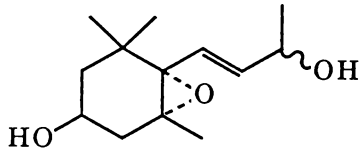
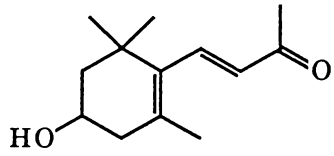
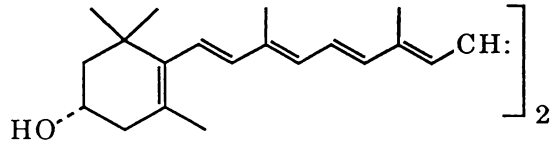


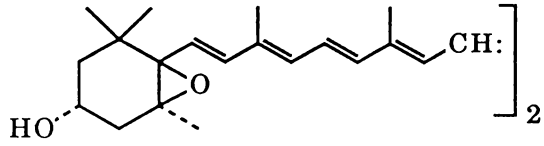
[27]



[28]



[29]



[30]

4.5.1 Experimental

Isolation of 1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans*-*cis*-1,2,4-triol [22]. Gas chromatographic analysis of the ether extracts of a number of thyme honeys revealed the consistent presence of a dominant peak 206 (see Figure 5.6) of uncertain structure. A bulk extraction of a mixture of thyme honeys T1, T2, T3, T4 and T111 (200 g combined weight) afforded a mixture of extractives. Separation was achieved by multiple PLC on silica gel (Merck $PF_{254+366}$) developed with

ether-hexane 1:1 mixture (4 developments). The major band (5 mg) displayed a deep purple colouration when the plate was viewed under UV illumination at λ = 350 nm.

^{1}H and ^{13}C NMR spectra were determined in $CDCl_3$ on a Bruker AM-400 spectrometer using chloroform as an internal reference. The DQFCOSY spectrum of [**22**] was determined on the AM-400 spectrometer across a 2 823 Hz window using 2k data points and 512 increments (zero-filled to 1k).

Colourless rectangular crystals of [**22**], melting point 68-69°C, suitable for crystallographic study were obtained by vapour diffusion techniques from toluene/hexane.

^{1}H NMR. (400 MHz, $CDCl_3$) δ: 0.85, s, 6β-Me; 1.16, s, 2-Me; 1.27, s, 6α-Me; 1.62, m, 5-H_2 and 3 x OH; 1.78, dd, ^{2}J 13.1 and ^{3}J 11.2 Hz, 3β-H; 1.88, ddd, ^{2}J 13.1, ^{3}J 4.6 and 1.5 Hz, 3α-H; 2.31, s, $COCH_3$; 4.17, m, $W_{1/2}$ 21 Hz, 4α-H; 6.36 and 7.25, 2 x d, ^{3}J 15.8 Hz, *trans*-CH=CH.

^{13}C NMR. (100.6 MHz, $CDCl_3$) δ: 25.6 (q), 26.6 (q), 27.4 (q), 27.8 (q), 40.2 (t), 45.2 (s), 45.5 (t), 64.2 (d), 77.1 (s), 78.7 (s), 131.1 (d), 148.8 (d) and 198.2 (s).

Mass spectrum. (GC/MS, EI, 70 eV) m/z: 224 (6%), 141 (9), 140 (8), 125 (43), 124 (10), 123 (17), 109 (8), 99 (7), 97 (23), 95 (6), 83 (9), 71 (13), 69 (8), 55 (17) and 43 (100). High resolution GC/MS, EI, 24 eV, found m/z 224.1426, calculated for $C_{13}H_{20}O_3$ (M^+ - H_2O) m/z 224.1412; found 125.0575 $C_7H_9O_2$, requires m/z 125.0602; found m/z 123.0834 $C_8H_{11}O$, requires m/z 123.0810.

Crystal data. $C_{13}H_{22}O_4.H_2O$, M_r = 242.32 (260.33 with H_2O); orthorhombic, space group $P2_12_12_1$ (No. 19), a = 7.887, b = 8.957, c =

40.994 Å, V = 2 896.0 Å^3, D_c = 1.19 g cm^{-3} for Z = 8, $F(000)$ = 1 136, monochromatic Mo–Kα X-radiation, λ = 0.7107 Å, specimen size *c.* 0.25 by 0.1 by 0.1 mm.

Data collection. Nonius CAD4 diffractometer, $\theta/2\theta$ scan, $2\theta_{max}$ 40° yielding 1861 independent reflections, 1094 with $I > 2\sigma(I)$ being considered observed and used in the structure refinement.

Structure solution. SHELX86 (Sheldrick, 1986); Refinement: SHELX76 (Sheldrick, 1976) with cycles of least-squares full-matrix refinement, and hydrogen atoms included in calculated positions with C-H distance set at 1.08 Å. Thermal parameters: ring atoms isotropic; water molecules and selected non ring atoms anisotropic (6 in molecule A, 9 in molecule B) depending on U_{11} parameter values after the final cycle of isotropic refinement (which had R = 0.12), hydrogen atoms isotropic with common U_H assigned to the *CH* and *CH*$_2$, *CH*$_3$ and *OH* hydrogens (0.036, 0.093 and 0.062 respectively). Because of the limited number of reflections it was not considered appropriate to increase the number of anisotropic atoms. Residuals on *F*: R, R' 0.078, 0.079. Final maximum Δ/e.s.d = 0.4 involving a rotation parameter of the C-10 methyl group. Largest feature in final difference map = 0.29 e Å^{-3}. Reflection weights: $1.0972[\sigma^2(F_O) + 0.0037(F_O^2)]^{-1}$. Thermal parameters, calculated hydrogen atom positions and tables of structure factor amplitudes are displayed in the Appendix B.

Peak 151 (GC/MS, EI, 70 eV) m/z: 224 (M^+, 12%), 191 (1), 166 (1), 151 (1), 149 (2), 125 (3), 124 (10), 123 (100), 109 (5), 95 (4), 77 (4), 55 (4), 43 (45). High resolution GC/MS, EI, 24 eV, molecular formula $C_{13}H_{20}O_3$; found m/z = 224.1426 M^+, required 224.1412; found m/z = 123.0817 $C_8H_{11}O$, requires m/z 123.0810.

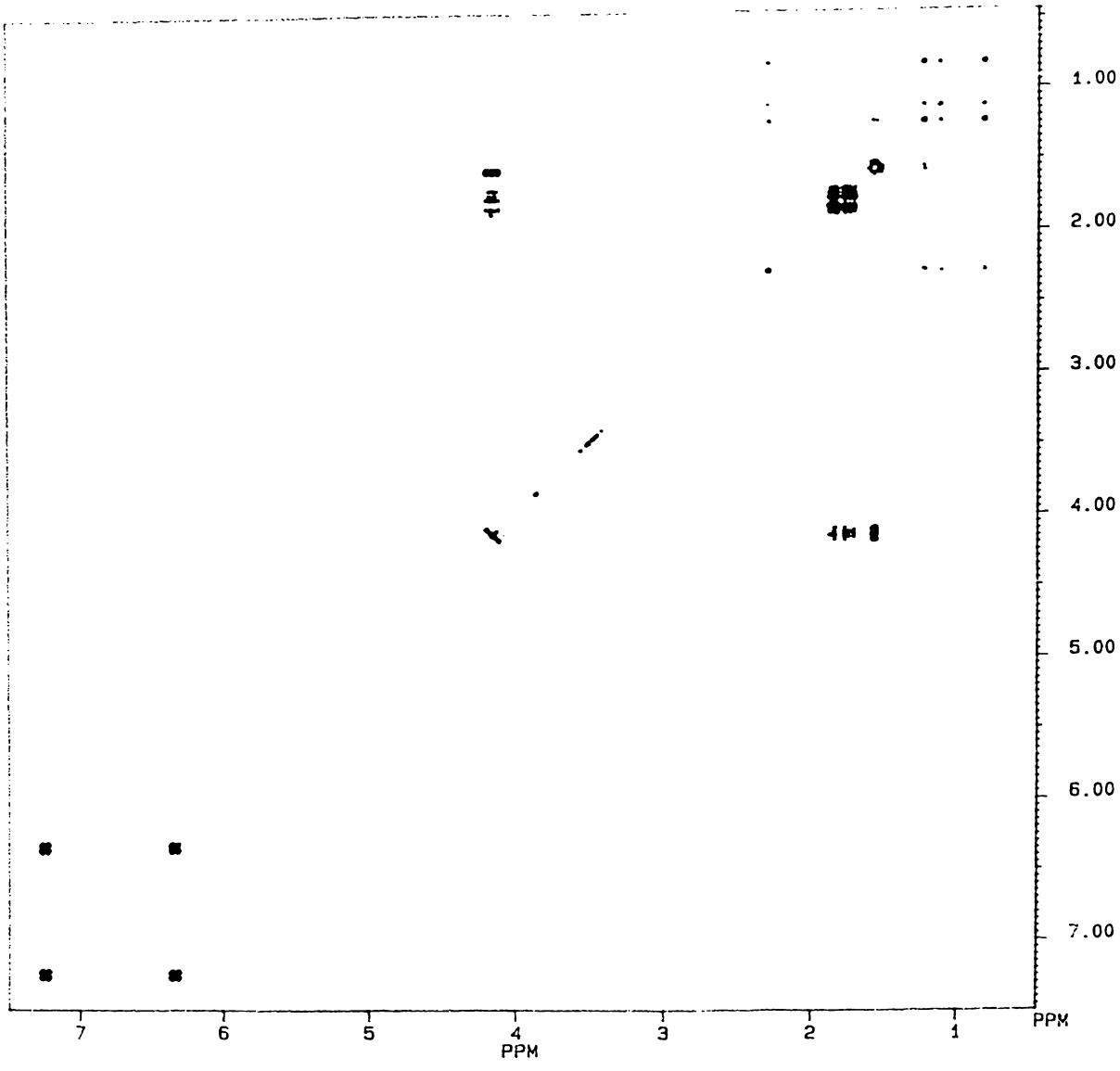


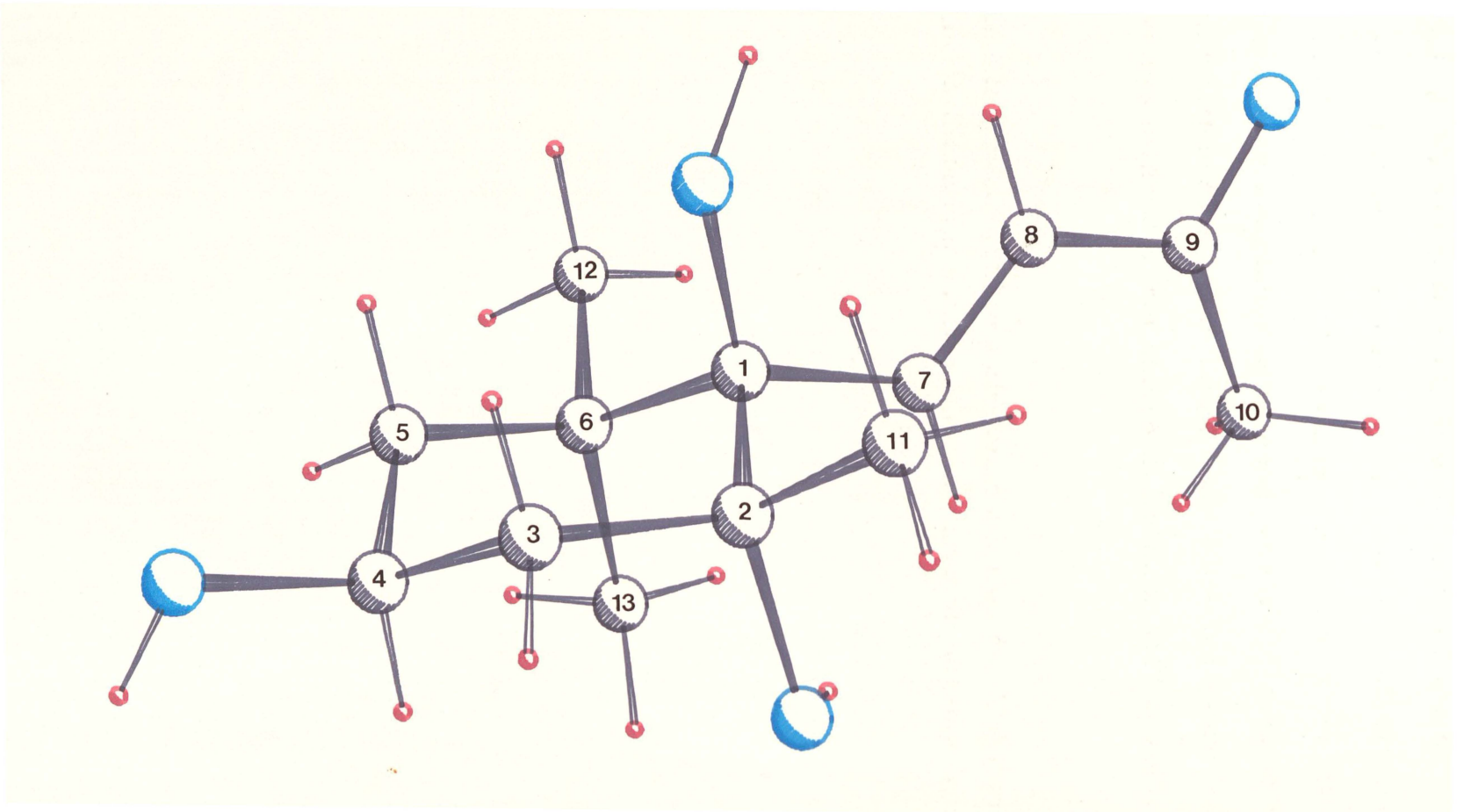
Figure 4.4 400 MHz double quantum filtered COSY NMR spectrum of 1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans-cis*-1,2,4-triol [**22**].

4.5.2 Peak 206 [22]

The highest observed ion in the mass spectrum of 1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans-cis*-1,2,4-triol [**22**] occurred at m/z 224 ($C_{13}H_{20}O_3$ by high resolution measurement). ^1H NMR (see Experimental section) revealed the presence of signals attributable to three tertiary methyl groups, a *trans* disubstituted double bond, a methylketone and a lowfield methine multiplet, possibly arising from a secondary hydroxyl group. A two dimensional DQFCOSY NMR experiment (see Figure 4.4) established that the lowfield methine proton was strongly coupled to four methylene protons, while two of the tertiary methyl groups exhibited a cross peak attributable to a mutual ^4J coupling. Of these two methyl groups, that at higher field also exhibited a ^4J coupling with one of the foregoing methylene protons. These ^1H NMR characteristics are, in part, reminiscent of those determined for the hydroxydiketone [**9**] which was isolated from heather honey. The NMR and mass spectral evidence thus far described lead to the conclusion that the thyme honey compound might be one of the diastereoisomeric epoxides [**23**] or [**24**].

^{13}C NMR data (see Experimental section), while consistent with the presence of a 1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane skeleton, was not consistent with the presence of an epoxide functionality. In particular the downfield shifts exhibited by two of the quarternary carbons (δ 77.1 and 78.7 ppm) suggested that the thyme honey compound should be formulated as the trihydroxyketone [**22**], and that the highest observed ion in EI/MS (m/z 224) arose by loss of water from an absent molecular ion of m/z 242 ($C_{13}H_{22}O_4$). A molecular weight of 242 could not be confirmed by CI(isobutane)/MS (no significant MH^+ ion at m/z 243). The uncertainty regarding the thyme honey compound's structure led to examination by X-ray crystallography.

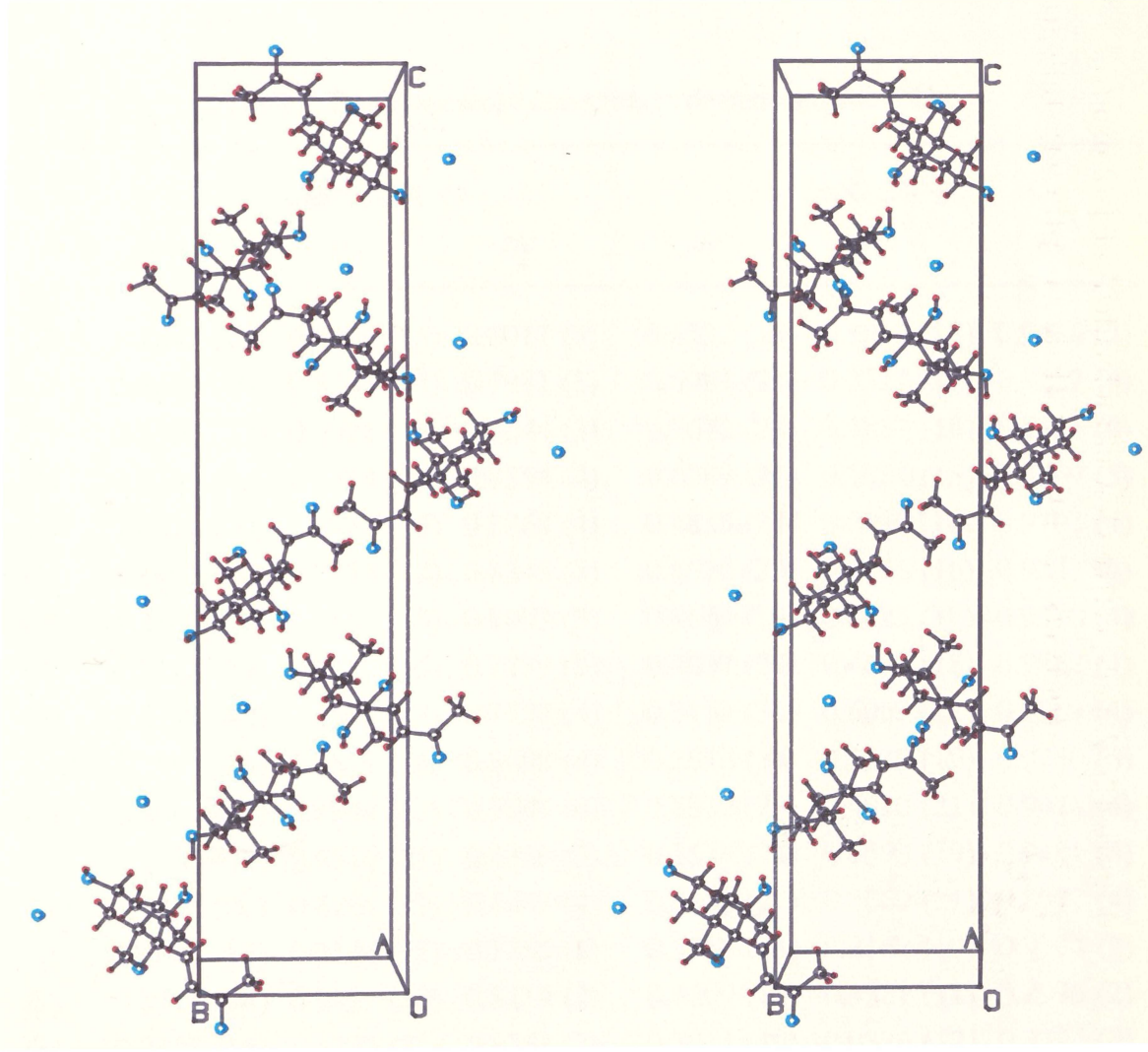
Figure 4.5 A perspective view of molecule A of 1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans*-*cis*-1,2,4-triol [**22**] showing the crystallographic numbering scheme.



4.5.3 X-Ray Crystallographic Analysis

Solution of the X-ray data set as described in the Experimental section revealed the thyme honey compound to be 1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans*-*cis*-1,2,4-triol [**22**]. Two crystallographically distinct thyme honey compound molecules and two hydrogen bonded water molecules were found in the asymmetric unit. One of the molecules of [**22**] exhibited three intermolecular hydrogen bonds, whereas

Figure 4.6 A stereographic projection of [**22**] showing the arrangement of molecules in the unit cells including water of crystallisation.



the other molecule of [**22**] exhibited only two such interactions. Figure 4.5 is a perspective view of molecule A of [**22**] showing the crystallographic numbering scheme, while Figure 4.6 depicts a stereographic projection of [**22**] showing the arrangement of molecules in the unit cells including water of crystallisation.

Heavy atom coordinates and bond lengths and bond angles with their standard deviations are given in Tables 4.II and 4.III respectively. Tables of other crystallographic data, including calculated hydrogen atom positions, thermal parameters and observed and calculated F_o and F_c values are given in the Appendix B as supplementary material.

Table 4.II Heavy atom coordinates determined for [**22**].

	molecule A			molecule B		
	x/a	y/b	z/c	x/a	y/b	z/c
C1	1.1245 (20)	0.8094 (15)	0.8017 (3)	0.5332 (19)	0.3012 (15)	0.9446 (3)
C2	0.9288 (16)	0.8351 (14)	0.7981 (3)	0.6991 (21)	0.3322 (17)	0.9257 (4)
C3	0.8300 (20)	0.6921 (14)	0.8044 (3)	0.7672 (23)	0.1887 (18)	0.9105 (4)
C4	0.8765 (22)	0.6114 (16)	0.8354 (4)	0.6399 (21)	0.1084 (15)	0.8909 (3)
C5	1.0617 (20)	0.5865 (16)	0.8368 (4)	0.4835 (21)	0.0750 (16)	0.9103 (4)
C6	1.1697 (20)	0.7255 (17)	0.8345 (3)	0.4020 (20)	0.2143 (16)	0.9247 (4)
C7	1.2140 (19)	0.9567 (15)	0.8003 (3)	0.4564 (19)	0.4423 (17)	0.9570 (4)
C8	1.3288 (19)	0.9930 (15)	0.7779 (3)	0.4037 (25)	0.4678 (18)	0.9862 (4)
C9	1.4197 (20)	1.1341 (17)	0.7758 (4)	0.3137 (31)	0.6069 (22)	0.9969 (4)
C10	1.3991 (25)	1.2519 (16)	0.8008 (4)	0.2816 (34)	0.7343 (19)	0.9750 (6)
C11	0.8814 (24)	0.8944 (17)	0.7647 (4)	0.8330 (23)	0.4010 (21)	0.9474 (4)
C12	1.3585 (22)	0.6816 (21)	0.8329 (5)	0.2534 (24)	0.1590 (19)	0.9484 (4)
C13	1.1434 (26)	0.8221 (19)	0.8647 (4)	0.3130 (27)	0.3069 (21)	0.8977 (4)
O1	1.1613 (14)	0.7166 (11)	0.7755 (2)	0.5758 (13)	0.2162 (10)	0.9734 (2)
O2	0.8714 (14)	0.9437 (10)	0.8219 (2)	0.6699 (16)	0.4381 (11)	0.8996 (2)
O4	0.7803 (15)	0.4727 (11)	0.8362 (2)	0.7014 (19)	-0.0336 (12)	0.8794 (3)

Table 4.II continued

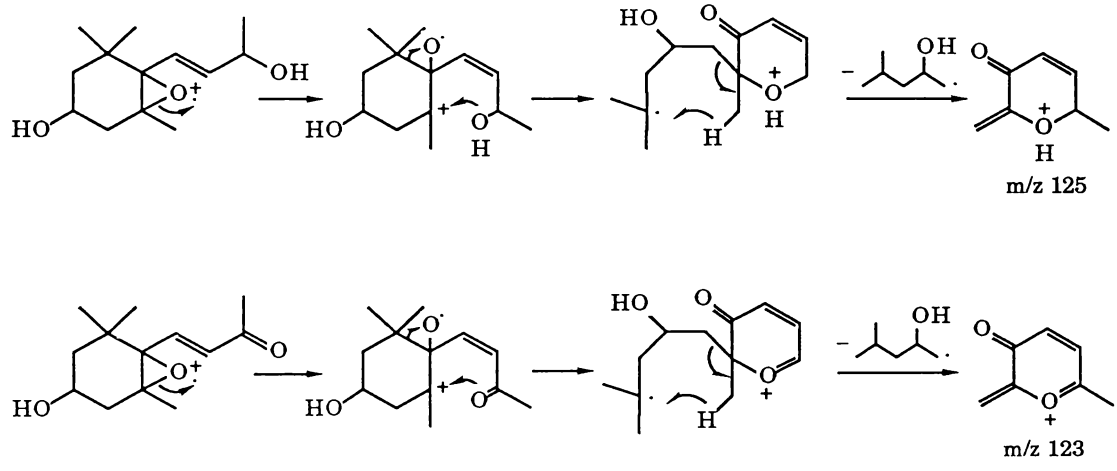
O9	1.5220 (15)	1.1519 (12)	0.7532 (3)	0.2478 (24)	0.6078 (16)	1.0239 (3)
O(W)	0.8767 (19)	0.2301 (11)	0.7996 (3)	0.6640 (21)	-0.2755 (11)	0.9182 (3)

Table 4.III Bond lengths (Å) and bond angles (degrees) determined for [**22**].

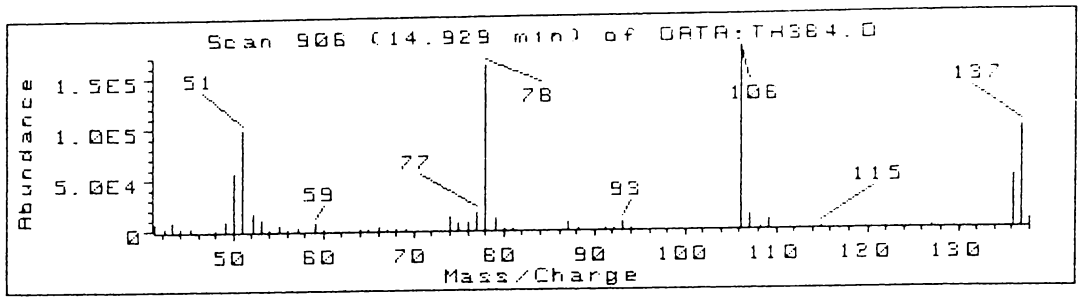
	molecule A	molecule B		molecule A	molecule B
Bonds			Bonds		
C1-C2	1.567 (19)	1.546 (20)	C4-O4	1.456 (17)	1.441 (17)
C1-C6	1.580 (19)	1.531 (19)	C5-C6	1.511 (19)	1.524 (20)
C1-C7	1.498 (19)	1.490 (19)	C6-C12	1.542 (23)	1.551 (21)
C1-O1	1.390 (15)	1.444 (16)	C6-C13	1.524 (21)	1.600 (22)
C2-C3	1.521 (18)	1.525 (21)	C7-C8	1.329 (18)	1.288 (18)
C2-C11	1.517 (17)	1.512 (21)	C8-C9	1.456 (20)	1.500 (25)
C2-O2	1.450 (15)	1.448 (17)	C9-C10	1.482 (21)	1.475 (24)
C3-C4	1.506 (19)	1.475 (20)	C9-O9	1.237 (17)	1.221 (18)
C4-C5	1.478 (22)	1.498 (21)			
Angles			Angles		
C2-C1-C6	111.9 (1.2)	113.3 (1.2)	C3-C4-O4	107.5 (1.2)	112.4 (1.4)
C2-C1-C7	109.3 (1.1)	111.3 (1.2)	C5-C4-O4	112.7 (1.3)	105.9 (1.1)
C2-C1-O1	102.7 (1.1)	107.9 (1.1)	C4-C5-C6	115.5 (1.3)	113.0 (1.0)
C6-C1-C7	110.2 (1.2)	109.7 (1.2)	C1-C6-C5	108.5 (1.2)	109.8 (1.3)
C6-C1-O1	109.1 (1.1)	108.9 (1.1)	C1-C6-C12	107.6 (1.3)	109.2 (1.2)
C7-C1-O1	113.4 (1.2)	105.3 (1.1)	C1-C6-C13	113.0 (1.2)	114.5 (1.3)
C1-C2-C3	111.4 (1.1)	110.6 (1.3)	C5-C6-C12	109.7 (1.4)	106.9 (1.2)
C1-C2-C11	112.3 (1.1)	111.6 (1.2)	C5-C6-C13	109.9 (1.3)	110.6 (1.2)
C1-C2-O2	110.0 (1.1)	110.7 (1.2)	C12-C6-C13	108.1 (1.4)	105.4 (1.4)
C3-C2-C11	108.8 (1.1)	109.7 (1.4)	C1-C7-C8	124.3 (1.3)	126.7 (1.5)
C3-C2-O2	106.9 (1.0)	107.9 (1.2)	C7-C8-C9	126.1 (1.4)	125.0 (1.6)
C11-C2-O2	107.2 (1.0)	106.2 (1.3)	C8-C9-C10	121.5 (1.4)	123.0 (1.5)
C2-C3-C4	115.0 (1.2)	113.2 (1.4)	C8-C9-O9	118.6 (1.4)	118.2 (1.7)
C3-C4-C5	110.2 (1.4)	111.6 (1.2)	C10-C9-O9	119.8 (1.5)	118.3 (1.9)

4.5.4 Peaks 138 and 153

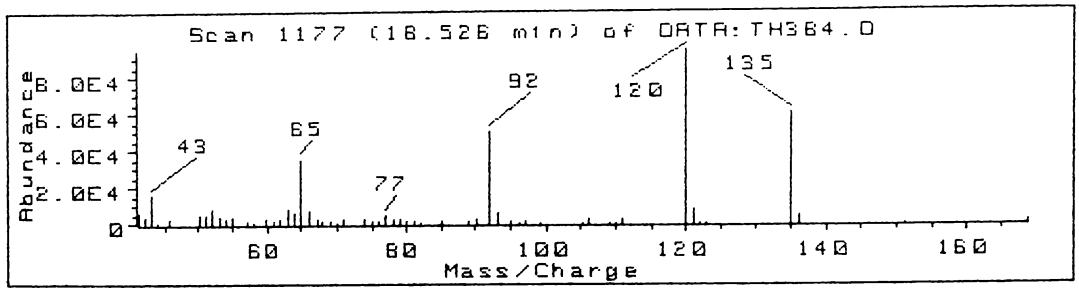
Selected ion GC/MS analysis (m/z 224 and 123 ion profiles) established the location in the thyme honey extracts of two earlier eluting substances; peaks 138 and 153 (138 in trace quantities only). High resolution GC/MS demonstrated the molecular formula $C_{13}H_{20}O_3$ (Found m/z = 224.1425 M^+, requires m/z 224.1412) and m/z 123 $C_8H_{11}O$ (found m/z = 123.0817, requires m/z 123.0810).



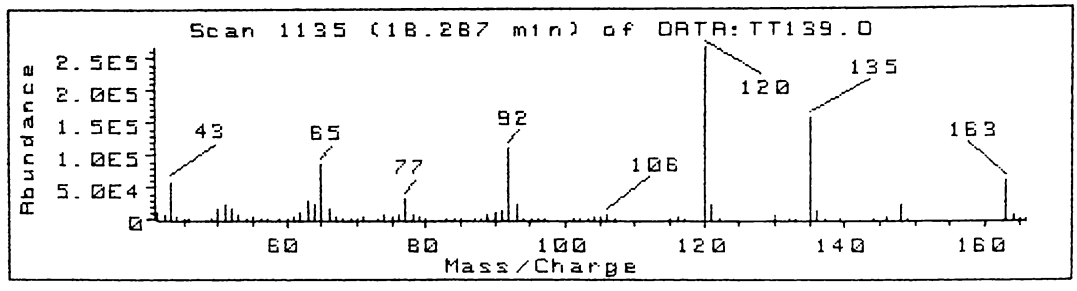
The mass spectral characteristics of peaks 138 and 153 appeared to correspond with those reported by Enzell and Wahlberg (1986) for the epoxides [**23**] and [**24**]. These authors proposed structure [**25**] for the ions of m/z 123 ($C_7H_7O_2$) displayed by these epoxides, while structure [**26**] was proposed for the ion of m/z 125 ($C_7H_9O_2$) displayed by the dihydroxyepoxide [**27**]. However, high resolution measurements established that the ions of m/z 123 which appeared in the mass spectra of the thyme honey compound and the more dominant of the thyme epoxides, (43 and 100% respectively) arose from $C_8H_{11}O$ (rather than $C_7H_7O_2$) fragments. On the other hand the ion of m/z 125 (100%) displayed by the thyme honey compound exhibited a $C_7H_9O_2$ formulation (found m/z = 125.0575, required



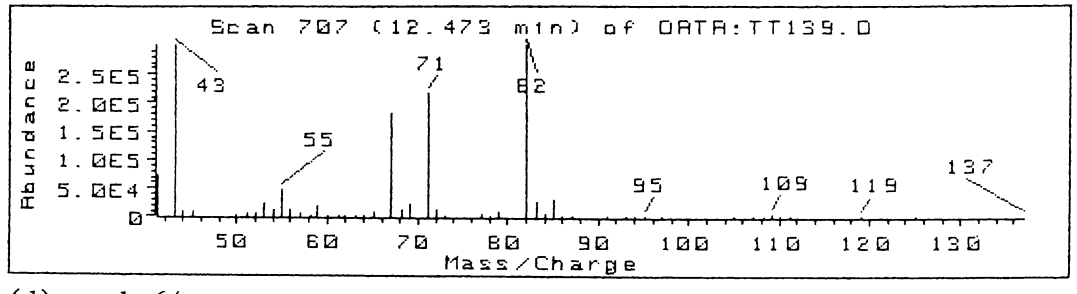
(a) peak 48



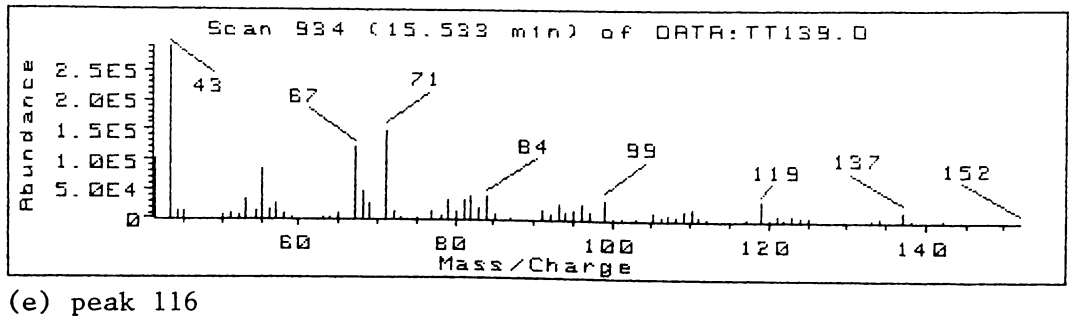
(b) peak 90



(c) peak 149



(d) peak 64



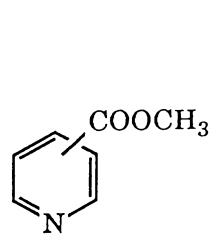
(e) peak 116

Figure 4.7 Mass spectra of tentatively identified (peaks 48, 90, 149) and unidentified components (peaks 64, 116) in thyme honey.

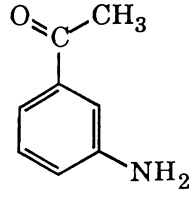
125.0602). Using the rationale of Enzell and Wahlberg (1986), the latter observation can be interpreted as implying the presence in the thyme honey compound of a 3-hydroxy group, rather than a 3-keto group.

4.5.5 Other Extractives

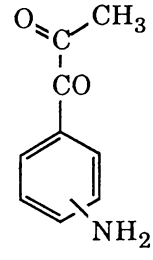
Mass spectra of three tentatively identified components (peaks 48, 90 and 149) from thyme honey are depicted in Figure 4.7(a)-(c). High resolution mass spectroscopy established the presence of a nitrogen atom in each of these compounds which possessed seven, eight and nine carbon atoms respectively. These organic extractives exhibited molecular ions at m/z 137, $C_7H_7NO_2$, peak 48, (found m/z = 137.0449 M^+, required 137.0477); m/z 135, C_8H_9NO, peak 90, (found m/z = 135.0718 M^+, required 135.0684) and m/z 163, $C_9H_9NO_2$, peak 149, (found m/z = 163.0672 M^+, required 163.0633). It is believed that peaks 48, 90 and 149 possess structures [**31**], [**32**] and [**33**]. The mass spectra of the first two compounds are similar to those recorded for methyl benzoate [**34**] and 3'-aminoacetophenone [**32**] (Bonaga and Giumanini, 1986).



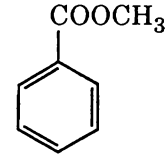
[**31**]



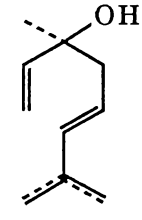
[**32**]



[**33**]



[**34**]



[**35**]

In addition to the tentatively identified compounds, a number of components could not be identified, the majority of which were trace or co-

eluting components. For example, peak 64 possessed the mass spectrum depicted in Figure 4.7(d) with a base peak at m/z 82 and a highest observable ion at m/z 137. Chemical ionisation (CI), with *iso*butane as the ionising gas displayed the appropriate MH^+ (M+1) quasi-molecular ion at m/z 153. Loss of a water molecule from the ion of m/z 153 gave rise to an ion of m/z 135 in the CI mass spectrum. High resolution GC/MS, EI at 24 eV, established the molecular formula $C_{10}H_{16}O$, (found m/z = 152.1188 M^+, required 152.1201; found m/z = 134.1076 $M^+ - H_2O$, required m/z 134.1045). The strong ion of m/z 71 is reminiscent of that displayed by methyl 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoate (see Section 4.7). It is possible that peaks 44 and 64 are the *cis* and *trans* diene isomers of **[35]**.

Among the more significant unknown compounds in thyme honey is peak 116, the mass spectrum of which is shown in Figure 4.7(e). This compound also appears to be a monoterpene of formula $C_{10}H_{16}O$.

4.5.6 Discussion

Although a wide variety of degraded carotenoid-like substances have been isolated, including some with a similar 2,6,6-trimethyl-*trans*-cyclohexane-1,2-diol skeleton, this appears to be the first occasion the trihydroxyketone **[22]** has been reported. The biosynthesis of this compound can be envisaged as proceeding via the alkene **[28]** and one or both of the epoxides **[23]** and **[24]**. The carotenoids such as zeaxanthin **[29]** and violaxanthin **[30]**, from which these compounds are probably derived, are widely distributed in plants (Nakanishi *et al.* 1974).

Examination of the extractives of more than two hundred New Zealand honey samples in the present study revealed the occurrence of **[22]**

to be confined to samples which include a thyme component. This is despite the occurrence of a number of related degraded carotenoid-like substances in other unifloral honeys.

The presence of the foregoing extractives prompted a search for carotenoids in thyme honey samples. However, as in the case for heather honey, none were detected using an established non-aqueous reverse phase high pressure liquid chromatography procedure (Lauren *et al.*, 1986). Detection limits were below 1 μg/g for β-carotene and a range of hydroxy- or carboxy- carotenes.

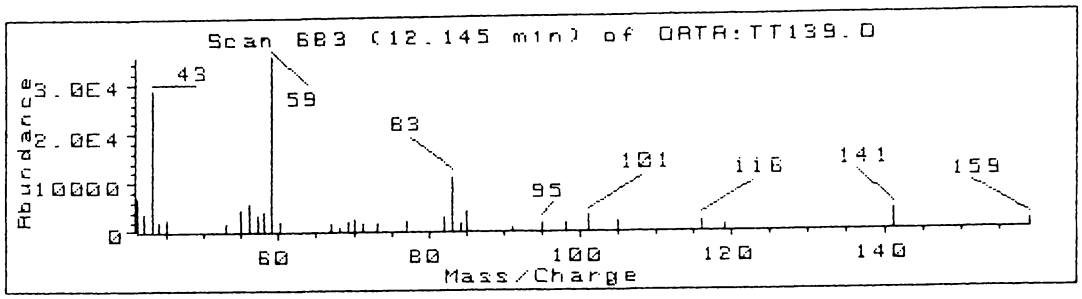
4.6 Vipers Bugloss Honey

The identification of the major extractable component of vipers bugloss honey is described in this section. It was shown by MS to be a dihydroxybenzene. The position of the hydroxyl groups on the benzene ring were elucidated as being 1,4- by NMR.

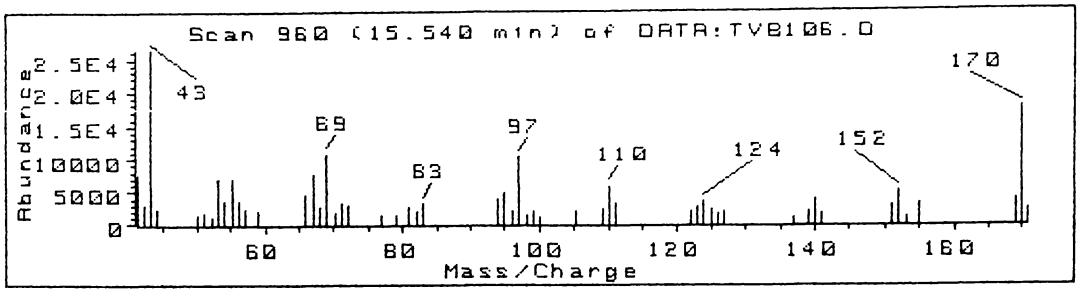
4.6.1 ^{1}H and ^{13}C NMR of 1,4-Dihydroxybenzene [36]

A bulk extraction of vipers bugloss sample VB1 (100 g) afforded a mixture of extractives which were separated by multiple PLC on silica gel (Merck $PF_{245+366}$) with hexane-ether (4:1) (first, second and third development). One band was recovered (*c.* 1 mg) from a PLC plate which exhibited a purple colouration when the plate was viewed under UV illumination at λ = 350 nm.

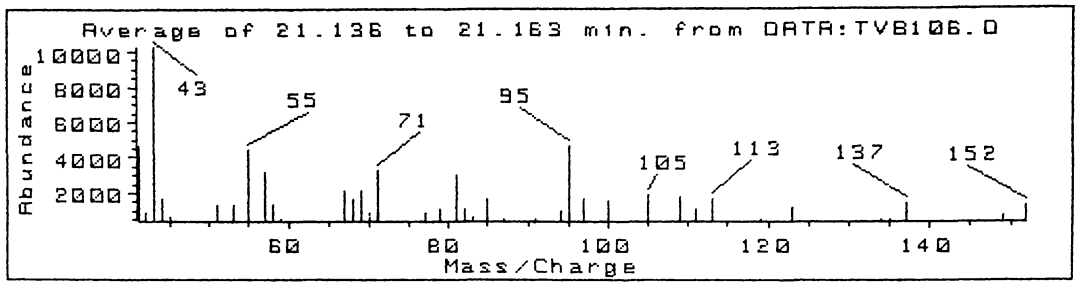
The highest observable ion of the recovered substances occurred at m/z = 110. Rearrangement ions at m/z 82 and 81, resulting from the loss



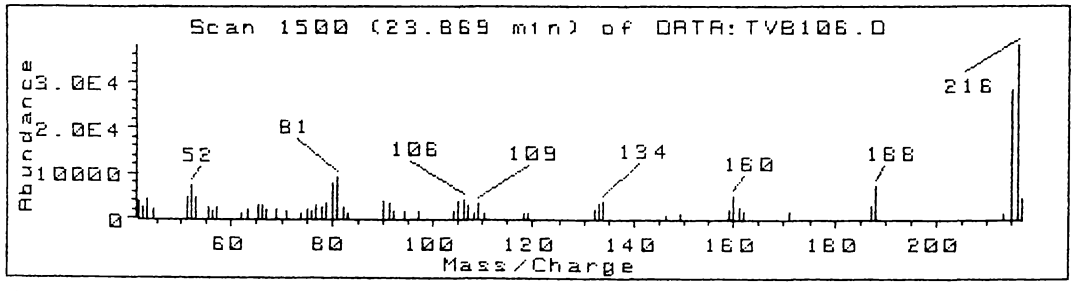
(a) peak 63



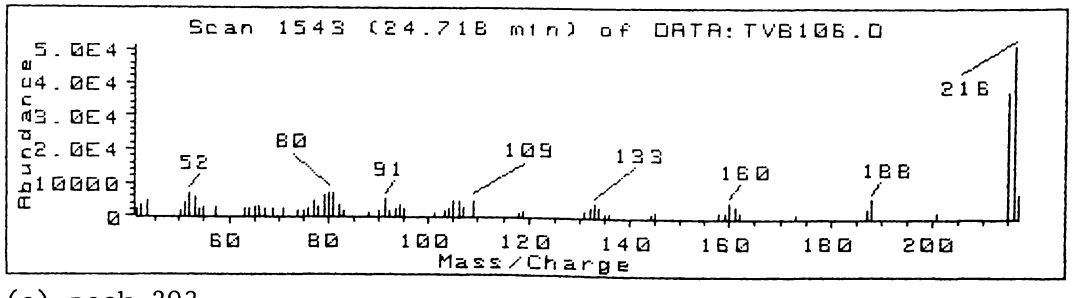
(b) peak 112



(c) peak 178



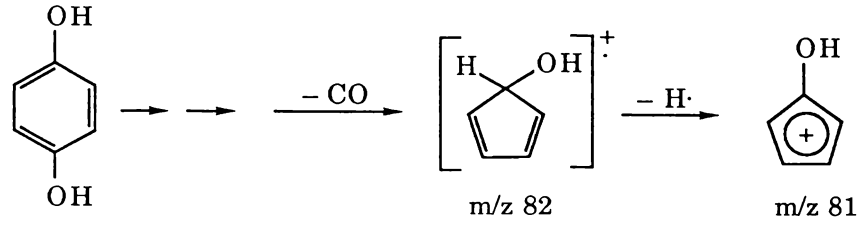
(d) peak 198



(e) peak 203

Figure 4.8 Mass spectra of some of the unidentified components in vipers bugloss honey.

of CO (M^+ - 28) and CHO (M^+ - 29), were typical of those found in phenols (Silverstein *et al.* 1981). The identification of the recovered band as a dihydroxybenzene was initially based on a match of library mass spectra against the recorded mass spectrum.



[36]

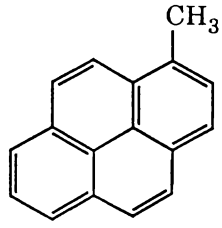
The position of the two hydroxyl groups was confirmed as 1,4- by NMR analysis of the isolated component. Thus there appeared in the 90 MHz ^1H NMR spectrum an aryl proton signal at δ 6.73 ppm together with a broad hydroxyl signal at δ 7.06 ppm in the ratio of 2:1 or 4:2 respectively. The presence of only a single aryl signal established *para-* rather than *ortho-* or *meta-* substitution. Consistent with this conclusion there appeared in the ^{13}C NMR spectrum a single protonated aryl carbon signal at δ 116.2 ppm.

As 1,4-dihydroxybenzene was detected only in vipers bugloss honey, the implication is that it originates from the plant source, hence this determination is a valid chemical procedure for the verification of this floral source. Levels of the said component in vipers bugloss honeys ranged from 16-28 μg/g.

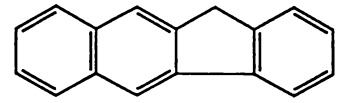
4.6.2 Unidentified Components

As in previous honey types, a number of components in vipers bugloss honey could not be identified. Figure 4.8 shows the mass spectra

of some of the unknowns detected in the present study. A library search suggested that peaks 198 and 203 (MW 216) might be structural isomers of either 1-methylpyrene [**37**] or 11*H*-benzo[b]fluorene [**38**].



[37]



[38]

4.7 Nodding Thistle Honey

Examination of mass spectra recorded for nodding thistle honeys revealed the presence of an array of components dominated by ions, among others, of m/z 67 and 71. Another feature of this class of compounds is the low intensity molecular ions which suggests they may be aliphatic rather than cyclic or aromatic substances. Bulk extraction was undertaken in order that the more dominant of these extractives could be isolated in sufficient quantity for structural characterisation by other techniques such as one and two dimensional NMR.

4.7.1 Isolation of Monoterpene Acid

A bulk extraction of sample N3 (900 g) afforded a mixture of extractives which were separated by multiple (x 3) PLC on silica gel (Merck $PF_{254+366}$) with hexane-ether (4:1). About twenty bands were recovered from the PLC plate; the three major bands (peaks 66, 140 and 183, *c.* 2 mg each) exhibited a purple colouration when the plate was

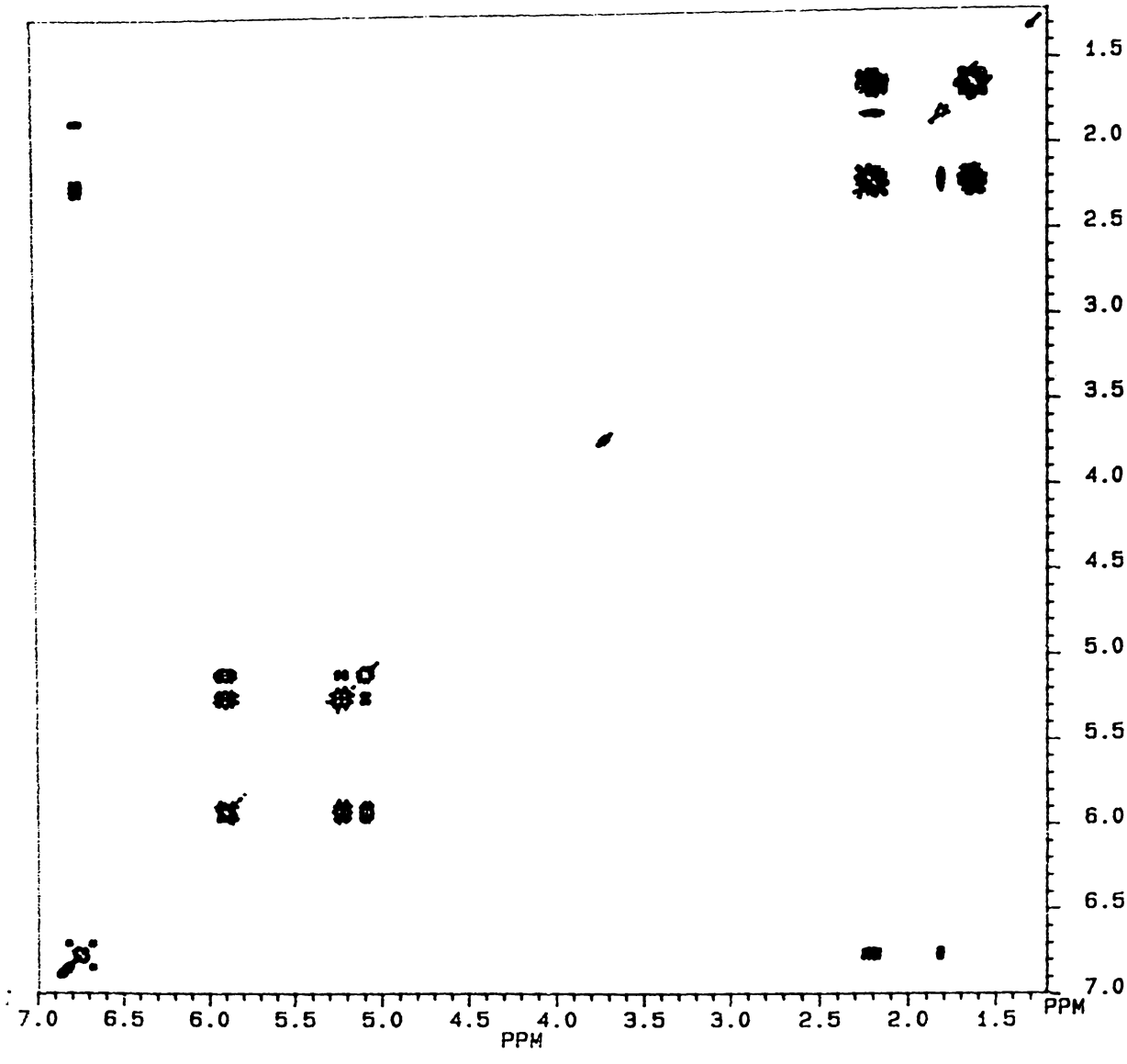


Figure 4.9 400 MHz double quantum filtered COSY NMR spectrum of methyl 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoate.

viewed under UV illumination at λ = 350 nm. Methylation of these bands followed by NMR analysis revealed that peak 183 was dimethyl *trans*-2-decenedioate (see Section 4.2). NMR analysis established that peak 140 was not pure and the band was therefore replated.

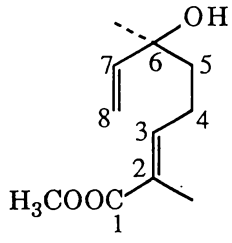
4.7.2 Peak 140 [39]

^{13}C NMR spectroscopy demonstrated the presence of eleven carbon signals, assignable to two methyl carbons, one conjugated carbonyl group, and the carbons of two olefinic double bonds (one trisubstituted, the other disubstituted; see Table 4.IV). High resolution mass spectroscopy established the molecular formula $C_{11}H_{18}O_3$ (found m/z = 198.1250 M^+; required 198.1256). Since this molecular formula requires a total of three rings, and/or double bonds it follows that peak 140, which ^{13}C NMR data indicates to be a keto-diene, must be acyclic. High resolution mass spectroscopy further established that a fragment ion of m/z 180 arose by loss of a water molecule (found m/z = 180.1170 $M^+ - H_2O$, required 180.1150). The ^{13}C NMR spectrum of peak 140 included an oxygenated quaternary carbon signal at δ 73.1 ppm consistent with the presence of a tertiary hydroxyl group. ^1H NMR spectroscopy indicated the presence of an aliphatic tertiary methyl group, an olefinic methyl group, a carboxymethyl group, four methylene protons and four olefinic protons, three of which were mutually coupled (see Table 4.IV). While this data establishes the functional groups present and demonstrates that peak 140 is an acyclic compound, it does not define the stereochemical disposition of the substituent groups.

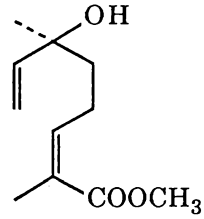
The 400 MHz two dimensional DQFCOSY spectrum of peak 140 is depicted in Figure 4.9. Cross peaks revealed the presence of an isolated

vinyl group (CH_2=CH–), and that the other olefinic proton (δ 6.75 ppm) was coupled to a pair of equivalent methylene protons (δ 2.21 ppm) and to the olefinic methyl group (δ 1.82 ppm). Additionally the olefinic methyl group was found to be long range coupled to the foregoing pair of methylene protons, which were in turn coupled to the other pair of methylene protons (δ 1.65 ppm). These observations established peak 140 to be either **[39]** or **[40]**.

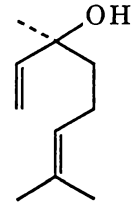
With the exception of the signals attributable to the carbomethoxy group, the ^{13}C NMR signals of linalool **[41]** were similar to those observed for peak 140. Okada *et al.* (1980) and Konoshima and Sawada (1982) have reported the isolation of a number of saponins from the fruits of *Gleditsia japonica* Miquel. Hydrolysis of one of the saponins afforded a compound identified as (+)-2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoic acid. Methylation of this compound yielded the corresponding methyl ester **[39]**. The ^{13}C NMR signal assignments reported for the ester corresponded with those reported in Table 4.IV for peak 140. On the other hand the ^{1}H NMR chemicals shifts observed in this study (determined relative to internal $CHCl_3$) differed by *c.* 0.05 ppm from those reported relative to internal tetramethylsilane).



[39]



[40]



[41]

The presence of 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoic acid only in nodding thistle honey highlights its application in the

chemical verification of the floral source. Levels of this compound detected in nodding thistle honey ranged from 8.6-30 μg/g.

Table 4.IV ^{13}C and ^{1}H NMR chemical shifts of linalool and methyl 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoate in $CDCl_3$.

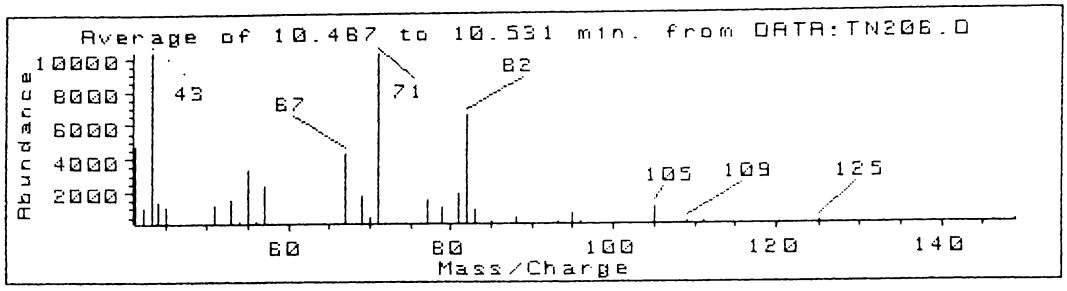
compound	linalool [41]	[39] (reported[a])		peak 140	
site	δ ^{13}C	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H (ppm)
C-1	25.7	168.6		168.7	
C-2	131.9	127.5		127.7	
C-3	124.4	144.5	6.96	144.6[b]	6.75 (t)[c]
C-4	22.9	23.5	1.95-2.35	23.5	2.19-2.24 (m)
C-5	42.2	40.6	1.45-1.71	40.7	1.61-1.68 (m)
C-6	73.5	72.9		73.1	
C-7	145.2	142.3	5.84	142.4[b]	5.91 (d of d)[d]
C-8	111.7	112.1	5.00, 5.14	112.2	5.09, 5.23 (d of t)[e, f]
$C_{(2)}$-CH_3	17.7	12.3	1.77	12.4	1.82 (s)
$C_{(6)}$-CH_3	27.9	27.9	1.26	28.0	1.31 (s)
$-COOCH_3$		51.6	3.65	51.7	3.72 (s)

[a] Okada *et al.* (1980) and Konoshima and Sawada (1982)

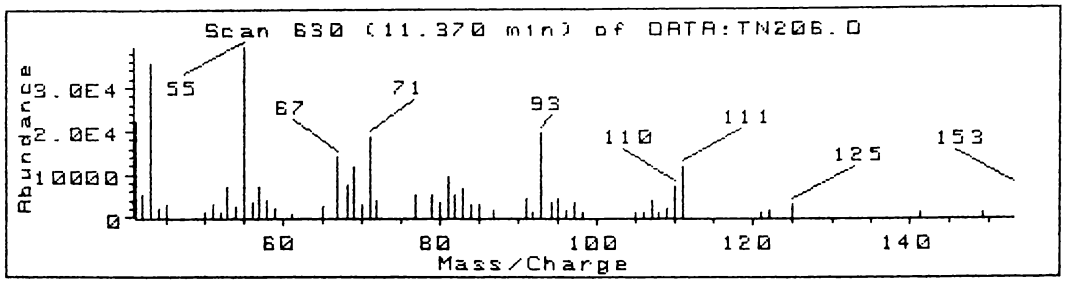
[b] Assignments interchangeable, [c] J = 7.5 Hz, [d] J = 10.8 Hz, [e] J = 10.8 and 1.1 Hz, [f] J = 17.4 and 1.1 Hz

4.7.3 Peak 66 [42]

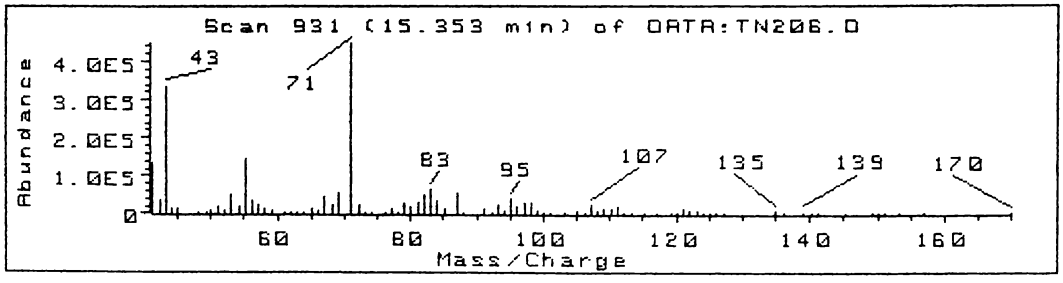
A substance of molecular weight 126 was variably present in many honey samples examined in this work. On occasions it was the dominant extractable compound, whilst in other honey samples from the same floral source it was completely absent. The mass spectrum of this compound, while similar to that of 5-hydroxymethyl-2-furfural(HMF) (50% library match), was not sufficiently convincing to demonstrate its identity.



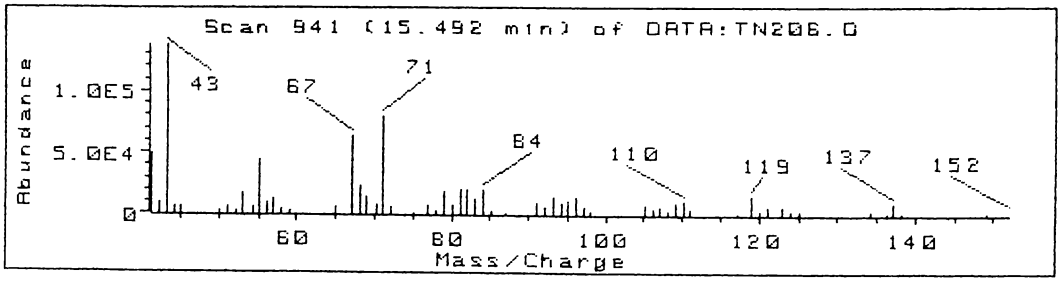
(a) peak 44



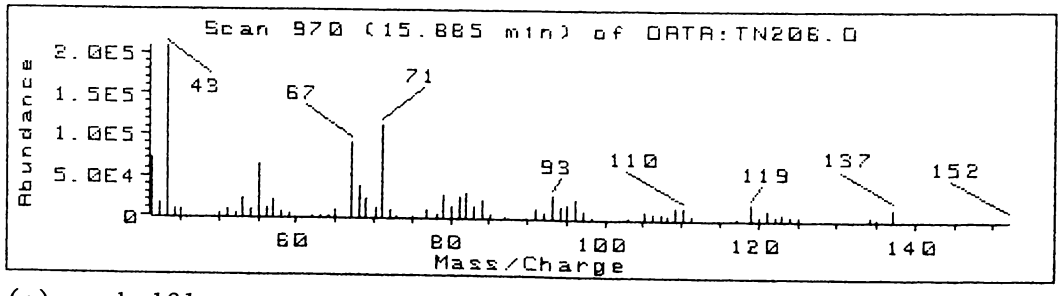
(b) peak 58



(c) peak 111



(d) peak 116

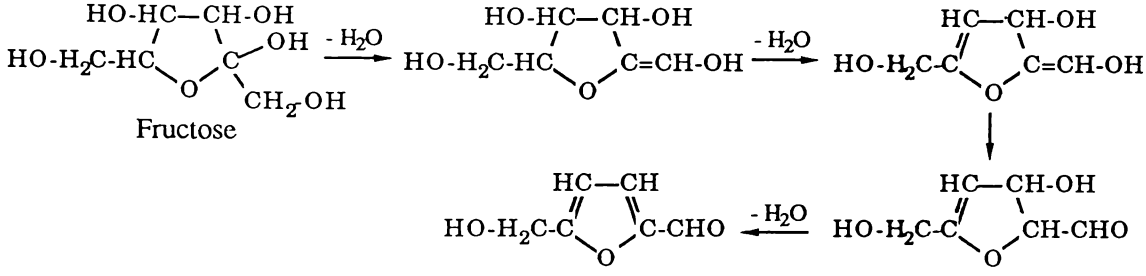


(e) peak 121

Figure 4.10 Mass spectra of some of the unidentified components in nodding thistle honey.

However, the ^{1}H and ^{13}C NMR spectral data determined for peak 66 [^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.72 (s, CH_2OH), 6.52 (d, *J* 3.6 Hz, =CH), 7.22 (d, *J* 3.6 Hz, =CH, 9.60 (s, CHO); ^{13}C NMR (22.5 MHz, $CDCl_3$) δ: 57.7 (t), 110.0 (d), 122.6 (d), 152,5 (s) 160.7 (s) and 177.7 (d)] proved to be identical with an authentic specimen of HMF [**42**].

It is believed that HMF is formed from the thermal elimination of water molecules from glucose and fructose in the presence of acids according to the following scheme:-



Fructose

HMF [**42**]

The rate of formation is exponentially dependent on temperature. Therefore the level of HMF is higher in honey which has been over-heated in processing or storage. Crane (1980) reported that every extra 10°C increases HMF production by a factor of 4.5.

4.7.4 Other Extractives

A number of components could not be identified. Several of the compounds exhibited strong fragment ions at m/z 61, 71, 82, 93 and/or 111 [see Figure 4.10(a)-(e)]. These compounds may be oxygenated mono-terpenes. While the majority of the unidentified compounds occurred only in nodding thistle honey, one of the compounds, the mass spectrum of

which appears as Figure 4.10(a), also occurred in thyme and kamahi (but not heather) honeys.

4.8 Kamahi Honey

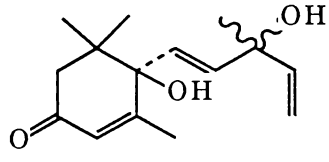
An array of unidentified substances was detected in kamahi honeys. The mass spectra of these compounds suggested that many of them were related; amongst others, ions of m/z = 83, 98, 125, 152 and 170 were displayed by most of the compounds. These mass spectral characteristics were reminiscent of those for the degraded carotenoid-like substances isolated from heather and thyme honeys. A bulk extraction was therefore undertaken so that the more dominant of these extractives could be isolated in sufficient quantities for structural elucidation by other techniques (*e.g.* NMR).

A bulk extraction of a mixture of samples KA6, KA7, KA8 and KA9 (200 g combined weight) afforded a mixture of extractives which were separated by multiple (x 3) PLC on silica gel (Merck $PF_{254+366}$) with hexane-ether (4:1). About twenty bands were recovered from the PLC plate; the two major bands (peaks 208 and 223, *c.* 1 mg each) exhibited purple colouration when the plate was viewed under UV illumination at $\lambda = 350$ nm. Methylation of these bands, followed by GC analysis, revealed that peak 223 was not pure; consequently this band was replated.

4.8.1 Peak 208

^{1}H and ^{13}C NMR analysis suggested peak 208 to be a mixture of at least three isomers of a compound of formula $C_{15}H_{22}O_3$. GS/MSD analysis

displayed weak ions of m/z 250 and 223 (M^+ - OH), together with strong ions at 170, 152 and 124. The latter two ions appear to arise from the ion of m/z 170 by the sequential loss of H_2O and CO. A significant ion of m/z 251 (probably an MH^+ ion) appeared in the GC/ITDS mass spectrum of the this peak. ^{13}C NMR data, whilst complicated by some of the carbon atoms appearing as clusters of peaks of similar chemical shift [*e.g.* three signals (attributable to the presence of a conjugated carbonyl group, see Section 4.4.2) occurred at δ 195.5, 195.2 and 195.0 ppm], pointed to the presence of a 4-hydroxy-3,5,5-trimethylcyclohex-2-en-1-one skeleton similar to that established for the series of degraded carotenoid-like substances detected in heather honey samples. ^1H NMR proton data revealed the presence of a collection of signals attributable to olefinic protons; a comparatively sharp cluster of signals at *c.* δ 5.9 ppm was typical of the conjugated H-2 signal of a 4-hydroxycyclohex-2-en-1-one derivatives (see Table 4.1), while a more extensively coupled cluster of signals in the region *c.* δ 4.6 to 5.6 ppm indicated the presence of a number of olefinic (non-conjugated) protons. The foregoing spectral data leads to the conclusion that peak 208 may be *cis*/*trans*-R/S isomers of structure [**43**].

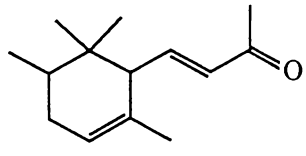


[**43**]

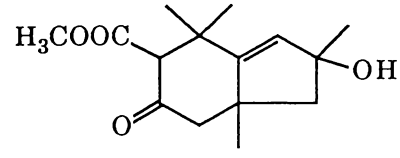
4.8.2 Peak 223

Peak 223 appeared to be a compound of formula $C_{15}H_{22}O_4$. The ^1H NMR spectrum indicated the presence of four tertiary methyl group signals (δ 1.29, 1.20, 0.98 and 0.90 ppm), two pairs of isolated methylene

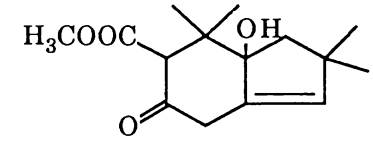
protons (δ 2.62 and 2.25, ppm, AB_q, J = 14.4 Hz; 2.30 and 2.17 ppm, AB_q, J = 14.2 Hz), a one proton doublet (δ 3.62 ppm J = 0.7 Hz), a carbomethoxy group (δ 3.68 ppm) and an olefinic proton signal (δ 5.50 ppm). A 2D COSY NMR spectrum showed that the singlet like signal at δ 3.62 ppm was long ranged coupled with a pair of methylene protons, and two of the methyl groups. The ^{13}C NMR spectrum included signals assignable to four aliphatic methyl carbons (δ 16.8, 18.8, 23.6 and 24.9 ppm), a methoxy methyl carbon (δ 51.8 ppm), two methylene carbons (δ 37.5 and 37.6 ppm), two methine carbons (δ 84.7 and 105.3 ppm) and six quaternary carbons (δ 43.1, 47.2, 52.3, 101.8, 171.7 and 216.8 ppm). In part, the spectral data recorded for this compound suggests it may be a degraded carotenoid, while the molecular formula requires the presence of 5 rings or double bonds. Since ^{13}C NMR data indicates the presence of 3 double bonds (two carbonyl groups and an olefinic double bond), it follows that peak 223 is dicyclic. The presence of a carboxymethyl group in addition to four quaternary methyl groups suggests that peak 223 may be an analogue of a compound such as β-irone [**44**]. While the structure of peak 223 remains uncertain, it appears that it possesses a dicyclic structure similar to [**45**] or [**46**]. Enzell and Wahlberg have characterised the presence of a variety of dicyclic degraded carotenoids in tobacco, however, none of the compounds appear to correspond with peak 223.



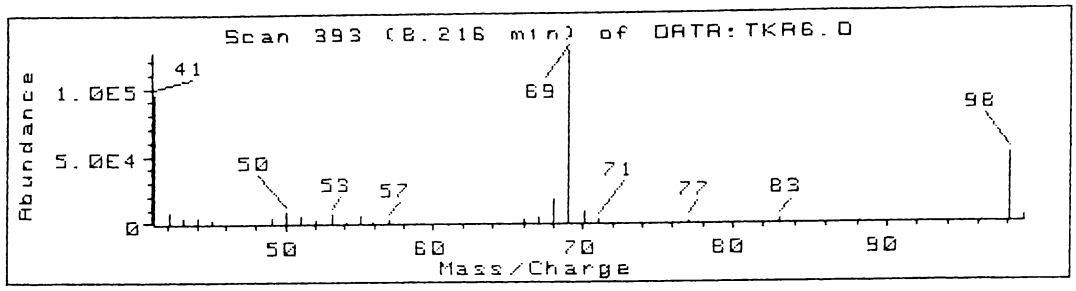
[**44**]



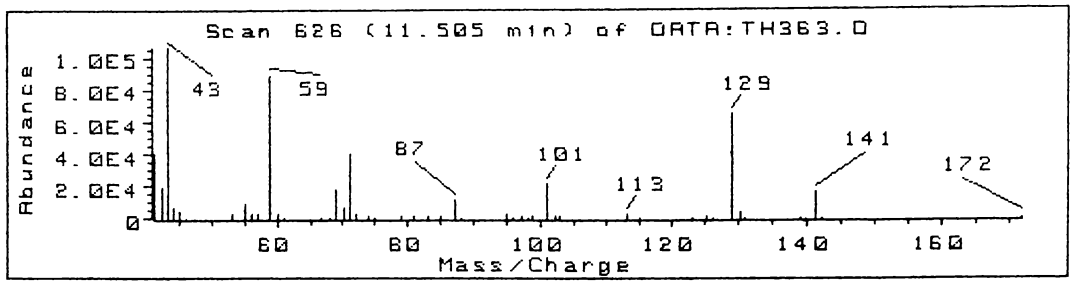
[**45**]



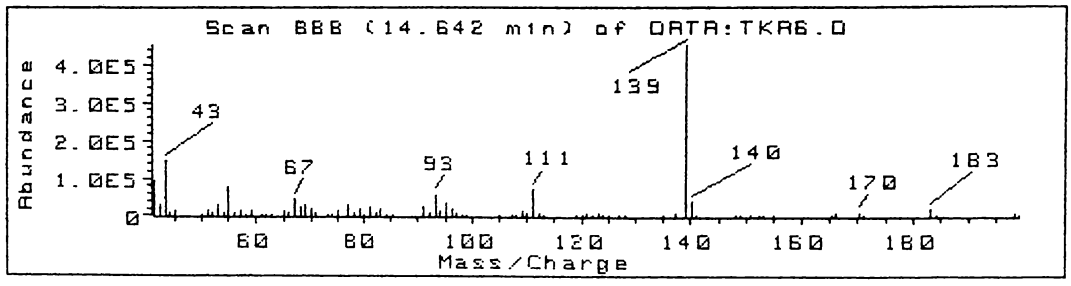
[**46**]



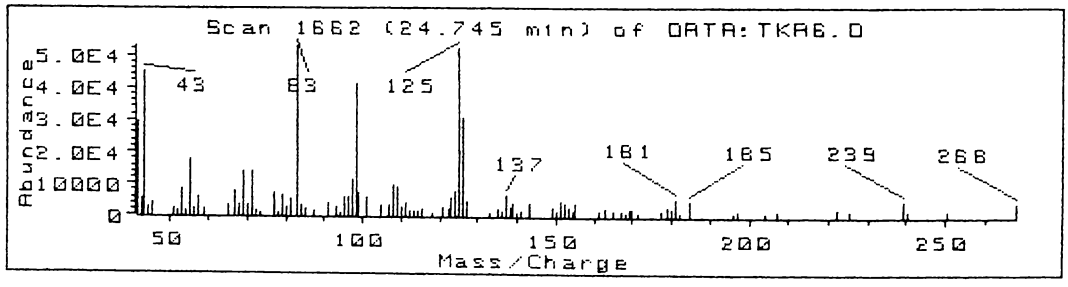
(a) peak 28



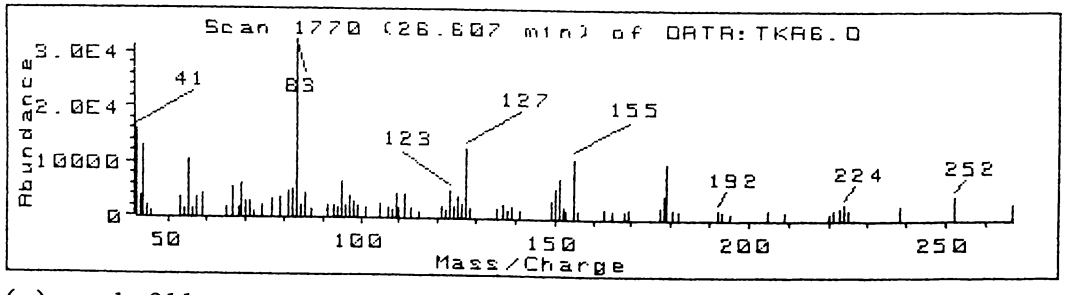
(b) peak 59



(c) peak 96



(d) peak 202



(e) peak 211

Figure 4.11 Mass spectra of some of the unidentified components in kamahi honey.

4.8.3 Other Unidentified Extractives

In addition to peaks 208 and 223, a number of peaks could not be identified. The mass spectra of some of these unknown are depicted in Figure 4.11(a)-(e). Although their MS characteristics suggested that they could be related to degraded carotenoid-like compounds, their identities remain uncertain.

4.9 Willow Honey

GC analysis of willow honey extractive revealed two dominant peaks 214 and 222. GC/MS on the other hand revealed that the mass spectra characteristics were reminiscent of those of the degraded carotenoid-like substances isolated from heather and thyme honeys. A bulk extraction was undertaken to allow the more dominant of these extractives to be isolated in sufficient quantity for structural elucidation by other techniques (*e.g.* NMR and probe high resolution MS).

A bulk extraction of sample W207 (120 g) afforded a mixture of extractives which were separated by multiple (x 5) PLC on silica gel (Merck $PF_{254+366}$) with hexane-ether (4:1). Ten bands were recovered from the PLC plate; the major band (*c.* 0.5 mg) exhibited purple colouration when the plate was viewed under UV illumination at λ = 350 nm. Methylation of these bands followed by GC analysis demonstrated that the single band from PLC indeed consisted of two peaks, 214 and 222. The GC/ITDS and TLC characteristics of the band were identical to those of an authentic sample of abscisic acid (mixed isomers) (Sigma Chemical Co., St. Louis). While the present data is limited, thus far only willow honey has been found to exhibit high levels of abscisic acids.

Chapter 5

Determination of the Floral Source of Honeys

Chapter 5

Determination of the Floral Source of Honeys

5.1 Introduction

In Chapter 1, the principal objective means of determining the geographical and floral origin of honey was discussed. Melissopalynology or pollen analysis, can be reliable where the necessary experience with the relevant pollens has been obtained. It is, however, tedious and very dependent on expert ability and judgement (Howells, 1969). Alternative methods that could be more widely and accurately used for characterising honeys have been sought for many years. To anyone who examines a variety of honeys it is evident that an infinite variety of aromas, flavours and colours can exist. Consequently, it can be anticipated that the chemical analyses of the aroma and flavour components of a unifloral honey could give a fingerprint which would be dependent on the floral source.

In the course of a study to identify the nectar-derived antibacterial components of honey it was observed that different types of honey afforded qualitatively distinct gas chromatograms of the trace extractable organic constituents. Inspection of a series of chromatograms revealed that similar patterns were observed for honeys derived from the same floral source. On the other hand, different patterns were observed for honeys derived from different floral sources.

The method appears to be promising because most of the components on which the assessment of the floral origin is based seem to originate from the nectar itself. Bonaga and Giumanini (1986) have suggested that "the next step in this type of research will be an attempt to correlate floral source with the presence of certain compounds originating either in the nectar or in some biochemical modification carried out by the bee".

In the preceding chapter, the chemical identities of the extractable organic components in white clover type, manuka, ling/heather, thyme, vipers bugloss, nodding thistle, kamahi and willow honeys were presented. The present chapter describes aspects of the chemical investigations which lead to a plausible alternative method to pollen analysis in characterising the floral origin of honey. The approach is based on the recognition of substances dependent on the floral source which can be quantitatively identified amongst the total extractable trace organic constituents recovered from honey samples using diethyl ether liquid/liquid extraction. A large number of samples had to be examined to establish the reliability of this method.

5.2 White Clover Type Honey

In a preliminary study (Tan, 1985), the chemical identities of the majority of the extractable organic components in clover, heather, rewarewa (*Knightia excelsa*), manuka and kanuka honeys collected during the 1982-84 flowering seasons from various geographical locations around the North Island of New Zealand were reported.

Table 5.I Concentration (μg/g) of components in clover honey (acids as methyl esters).

Peak	Compound (prominent MS ions)	C16	C1	C2	H415	H414	H413	H412	H411	H410	H408
5.Ia.	Individual components:										
1	unknown[a]	5.2	18	20	16	6.4	10.7	1.9	1.1	3.3	2.9
3	43, 59, 74, 83, 101	13	0.8	0.5	1.2	-	-	-	-		
7	unknown[a]	-	-	-	3.4	-	-	-	-		-
15	benzaldehyde	-	-	-	-	0.2	0.7	-	-		-
18	methyl 3-furancarboxylate	-	-	-	-	-	0.1	0.1	0.1	trace	
23	unknown[a]	-	-	-	103	-	44	-	-	-	-
25	methyl 2-hydroxy-4-methylpentanoate	-	-	-	-	-	-	-	-	trace	-
26	methyl 2-hydroxy-3-methylpentanoate	-	-	-	-	-	-	-	-	0.1	-
31	dimethyl butanedioate	0.1	0.1	0.1	0.3	0.2	0.3	0.5	0.6	0.1	1.2
32	benzyl alcohol	-	-	-	-	-	trace	trace	trace	-	-
35	methyl 2-methylsuccinaldehydate	0.2	-	-	0.5	0.2	0.3	0.1	0.1	0.1	0.1
40	methyl benzoate	0.2	0.1	0.1	0.6	0.4	0.9	0.1	0.1	0.1	0.1
41	2-phenylethanol	-	-	-	0.1	0.3	0.4	-	-	-	-
43	43, 58, 66, 94, 125 M^+	trace	trace	trace	trace	trace	trace	0.1	0.2	trace	trace
44	43, 67, 71, 82, 109, 125	-	-	-	-	0.2	0.6	trace	trace	-	-
49	*n*-undecane (C_{11}, internal standard)										
50	dimethyl pentanedioate	-	-	-	-	-	-	trace	trace	-	trace
51	methyl caprylate (8:0)	trace	trace	trace	trace	0.1	0.1	0.1	trace	0.1	0.1
56	43, 55, 59, 83, 98, 110	-	-	-	-	0.2	1.3	-	-	0.1	-
60	43, 55, 83, 97, 98, 126	-	-	-	-	-	-	0.1	0.1	-	-
62	methyl 2-phenylethanoate	0.1	trace	trace	0.6	0.6	0.8	0.3	0.1	trace	0.1
63	43, 59, 83, 101, 116, 141, 159	0.9	0.1	0.1	0.6	0.6	0.1	0.2	0.1	0.2	0.2
64	43, 67, 71, 82, 109, 125, 137	-	-	-	-	*	*	*	*	-	-
65	methyl 2-hydroxybenzoate	0.2	0.1	0.1	0.5	0.9*	0.9*	0.3*	0.1*	trace	0.1
66	5-hydroxymethyl-2-furfural	0.8	0.2	0.1	1.0	-	-	0.1	trace	0.3	0.4
74	methyl nonanoate (9:0)	0.1	0.1	0.1	0.2	-	-	-	-	0.1	-
77	dimethyl hexanedioate	-	-	-	-	0.1	0.5	0.1	0.3	-	trace
83	methyl 3-phenylpropionate	-	trace	-	0.2	0.1	0.1	trace	trace	trace	0.1
86	2'-methoxyacetophenone	-	-	-	3.5	0.4	1.3	-	-	-	-
95	methyl *cis*-3-phenylprop-2-enoate	-	0.1	-	0.1	trace	0.2	-	-	-	-
99	43, 55, 85, 98, 108, 127, 140	-	0.1	-	-	-	-	0.2	0.7	0.1	0.1
107	methyl 2-methoxybenzoate	-	-	-	5.0	0.8	2.7	-	-	-	-
108	41, 69, 97, 125, 139, 156	-	-	-	-	-	-	-	-	-	0.4
109	43, 69, 71, 97, 124, 152, 170	-	-	-	-	0.3	0.5	0.1	0.1		0.1
115	43, 67, 79, 97, 121, 136, 151, 184	-	-	-	0.6	-	-	-	-	-	-
119	methyl 2-hydroxy-3-phenylpropionate	trace	trace	-	9.9	1.4	4.6	0.4	0.4	0.1	0.2
120	methyl 3-methoxybenzoate	1.9	0.8	0.7	trace	1.0	1.1	0.2	0.5	trace	0.2
123	methyl *trans*-3-phenylprop-2-enoate	-	1.2	0.3	1.2	1.4	1.2	0.3	0.6	0.8	0.3
130	43, 55, 69, 97, 109, 129, 155	-	0.1	-	-	0.1	0.4	trace*	trace*		
133	ethyl 2-hydroxy-2-phenylethanoate	-	-	-	0.3	-	-	-	-	-	-
135	2-(methoxyphenyl)-ethanoate	-	-	-	-	0.1	0.1	trace*	trace*	-	-
137	dimethyl octanedioate	0.1	0.2	trace	0.5	trace	0.4	0.3	0.4	0.2	0.3
139	methyl 3-hydroxybenzoate	0.2	0.4	0.2	0.5	-	0.3	0.2	*	0.1	*
140	methyl 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoate	-	-	-	0.8	1.9	0.5	0.3	0.7*	0.3	0.3*
141	3,4-dimethoxybenzaldehyde	-	-	-	trace	-	-	-	trace	-	-
146	methyl 2-(hydroxyphenyl)-ethanoate	-	-	-	-	0.3	-	0.1	trace	-	-
148	55, 59, 81, 108, 136, 140 168	-	0.1	-	-	-	0.3	0.9	0.1	0.1	-
154	2,6-di-*tert*-butyl-4-methylphenol	0.1	0.1	trace	-	-	0.1	trace	0.1	-	-
155	55, 59, 74, 87, 97, 125, 157 fatty acid ?	0.3	0.5	0.1	-	-	-	-	-		

157	methyl laurate (12:0)	0.1	0.1	0.1	-	0.1	0.2	0.1	0.2	trace	0.6
158	dimethyl nonanedioate	0.2	0.1	trace	-	0.2	0.3	0.1	0.1	trace	0.3
160	methyl 3,5-dimethoxybenzoate	1.8	-	-	1.9	0.2	0.6	0.2	trace	trace	-
162	55, 67, 81, 113, 138, 161, 192	0.6	1.7*	0.3	1.1	0.6	0.2	0.3	0.2	-	0.2
163	methyl 3,4-dimethoxybenzoate	0.2	*	-	0.9	0.6	1.4	0.4	0.6	-	0.1
165	55, 74, 87, 111, 129, 152, 172	-	-	-	-	-	0.1	0.1	-		-
167	methyl 3-hydroxy-3-(methoxyphenyl)-propionate	-	-	-	2.0*	*	*	-	-		-
168	methyl 2-hydroxy-3-(4-methoxyphenyl)-propionate	-	-	-	*	0.2*	0.6*	trace	0.1	-	
170	43, 77, 95, 108, 135, 150	-	-	-	-	0.5	0.3	-	-	-	-
174	dimethyl decanedioate	1.2	2.3	0.3	2.1	1.0	2.8	1.5	1.4	1.7	1.7
177	methyl 3-(4-methoxyphenyl)-*trans*-prop-2-enoate	0.3	0.1	-	0.2	0.2	0.6	0.1	0.2	trace	-
180	methyl 3-hydroxy-3-(methoxyphenyl)-propionate	-	0.2	-	trace	0.2	0.1	0.1	0.2	0.1	0.1
183	dimethyl *trans*-2-decenedioate	4.6	7.5	0.8	4.0	1.2	2.8	4.9	4.4	4.7	5.1
184	methyl 3,4,5-trimethoxybenzoate	-	-	0.1	13	3.6	7.8	1.0	0.5	0.1	0.4
187	methyl myristate (14:0)	0.1	0.4	0.4	0.2	0.2	0.1	0.3	0.3	0.1	0.2
188	methyl 4-hydroxy-3,5-dimethoxybenzoate	-	-	-	5.4	0.3	0.6	-	-	-	-
190	methyl 3-(3,4-dimethoxyphenyl)-*cis*-prop-2-enoate	0.2	0.3	0.3	-	1.4	3.1	0.8	1.9	0.8	0.4
193	4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	-	-	-	-	0.2	0.8	0.4	0.8	-	-
199	methyl pentadecanoate (15:0)	0.1	0.1	0.3	0.3	0.1	0.1	0.3	0.5	0.2	0.1
200	methyl 3-(3,4-dimethoxyphenyl)-*trans*-prop-2-enoate	-	-	-	0.9	1.3	2.3	0.4	1.0	0.4	0.1
207	methyl palmitioleate (16:1)	0.1	0.3	0.7	0.2	0.2	0.3	0.2	0.2	0.2	0.2
209	methyl palmitate(16:0)	1.8	2.7	3.2	3.9	3.6	5.3	4.7	3.7	4.3	3.4
212	43, 66, 94, 108, 120, 135, 163, 220	-	-	-	2.1	-	-	-	-	-	-
213	methyl margarate (17:0, internal standard)										
214	methyl abscisate	trace	trace	0.1	5.5	0.6	4.3	1.0	1.4	0.1	0.1
215	89, 117, 133, 177, 194, 208, 276 M^+	-	-	-	-	0.5	0.5	0.1	0.3	0.2	-
216	methyl linoleate (18:2)	0.4	*	*	*	*	*	*	*	*	*
217	methyl α-linolenate (18:3)	0.7	2.7*	2.6*	5.3*	5.3*	7.3*	6.5*	3.5*	6.2*	4.0*
218	methyl oleate (18:1)	1.8	2.8	0.5	3.4	2.3	3.3	3.6	3.9	3.0	2.2
220	*n*-heneicosane (C_{21})	0.1	0.4	0.1	0.4	0.4	0.4	0.4	0.5	0.3	0.6
221	methyl stearate (18:0)	0.5	0.7	0.9	2.1	0.7	2.4	1.0	1.2	0.6	0.5
222	methyl abscisate	-	-	-	trace	-	-	-	-	-	-
225	*n*-triacosane (C_{23})	0.4	0.8	0.7	1.3	0.8	1.1	2.0	2.3	1.1	2.6
Ib.	Component plus classes:										
	Carotenoids-like substances	trace	trace	0.1	5.5	0.8	5.1	1.4	2.2	0.1	0.1
	Aromatics and acids[b]	5.1	3.4	2.0	46	16	33	5.0	6.4	2.6	2.2
	Diacids	6.0	10.1	1.2	6.9	2.7	7.1	7.4	7.2	6.7	8.6
	Unknown	20	20	20	130	20	59	4.3	3.5	4.2	4.3

[a] detected in GC/FID but not in GC/MS.

[b] excluding aliphatic fatty acids and beeswax hydrocarbons.

* unresolved GC/FID peak.

To extend that data, further samples of white clover type honey samples from the 1985-87 flowering seasons were analysed in the present study. Moar (1985) found that only 4 out of 28 North Island clover honeys exhibited clover pollen contributions of more than 80%. Conversely, of the 27 South Island clover honeys investigated, 20 displayed clover pollen contributions in excess of 80%. Consequently, to minimise extraneous floral source contributions to white clover type honeys, ten South Island clover honeys were selected for the present study. The quantities of extractable organic substances recovered from these honeys are shown in Table 5.I. Six of the samples were commercial samples, the floral source integrity of which had been ascertained by pollen analysis in addition to the more traditional organoleptic characteristics of flavour, colour and aroma. The results of the pollen analysis are shown in Table 5.II

Table 5.II Pollen composition of clover and manuka (H416) honeys expressed as a percentage of total pollen of nectar plants counted.

pollen type	H408	H410	H411	H412	H413	H414	H415	H416
white clover type (*Trifolium*)	92	90	42	35	87	90	85	12
cruciferae (*Brassica* type)	7	-	-	37	-	-	-	-
rosaceae (blackberry type)	-	7	35	-	-	-	-	-
lotus	-	-	16	23	7	-	5	7
manuka (*Leptospermum*)	-	-	-	-	-	-	6	74
remainder	1	3	7	5	6	10	4	7

Figure 5.1 is a typical GC profile of the extractable organic substances recovered from one of these white clover type honeys of high purity. Peaks 226-241 were shown to be hydrocarbons or fatty acids (see Table 3.II). Since high molecular weight hydrocarbons (C_{21-33} or higher)

and fatty acids (C_{18-28} or higher) are part of the beeswax composition, the concentrations and distribution of which have been well documented (Graddon *et al.*, 1979; Tulloch, 1980; Bonaga and Giumanini, 1986; Tan *et al.*, 1988), details of their characterisation are not repeated here.

It is apparent from Table 5.I that white clover type honeys possess only low levels of extractable organic substances; in none of the samples did the levels of individual substances exceed 10 μg/g. Amongst the more dominant extractives of white clover type honeys is *trans*-2-decenedioic acid (peak 183). It is typically present in white clover type honey in the concentration ranges 1.2 to 7.5 μg/g; average level 4.0 μg/g. It is noteworthy that *trans*-2-decenedioic acid is detected in all honey samples investigated in the present study except heather honey samples H361, H2, H3 and H4. Its level in all the honeys ranged from 0.2 to 49 μg/g, with a mean level of 7.0 μg/g. The implication of *trans*-2-decenedioic acid as a part of the pheromone system of the honeybee was discussed earlier in Section 4.2.1.

Other diacids detected include decanedioic acid (peak 174), octanedioic acid (peak 137) and butanedioic acid (peak 31). Traces of nonanedioic acid (peak 158), hexanedioic acid (peak 77) and pentanedioic acid (peak 50) were also characterised. In addition to the diacids, other components detected include phenolic (*e.g.* peaks 65, 154 and 188) and aromatic acids (*e.g.* peaks 40, 62, 83, 119, 120, 123 and 190) as well as a number of unknowns (*e.g.* peaks 43, 63, 148, 162 and 215). Some furan derivatives were also detected, *e.g.* 3-furancarboxylic acid (peak 18) and 5-hydroxymethyl-2-furfural (peak 66). The variety of components detected was wide ranging: however, none appear to be specific to white clover type honey.

It is interesting to note that the levels of extractable organic substances obtained in the present study are much lower than those obtained in the earlier investigation. Noticeably, the levels of 2-hydroxy-3-phenylpropionic acid (peak 119) (levels between 2.5 μg/g to 67 μg/g in the earlier study, 0.1 μg/g to 10 μg/g in the present study) and methyl 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoate (peak 140) corresponding to the unknown peak 83 in the earlier study (1.3 μg/g to 30 μg/g in the earlier study, 0.3 μg/g to 1.9 μg/g in the present study) were much higher than those detected here.

At the time of the earlier study it was suggested (Tan, 1985) that even modest contamination of the presumed unifloral white clover type honey, by for example manuka honey, would dramatically increase the level of 2-hydroxy-3-phenylpropionic acid. Levels of 2-hydroxy-3-phenyl-propionic acid akin to those reported in the initial study were found in honey samples which apiarists described as white clover type honey, but pollen analysis revealed that these contained some manuka contribution. Such an example is found in sample H415 (6% manuka pollen, with the level of 2-hydroxy-3-phenylpropionic acid elevated to 10 μg/g).

It is now clear that the white clover type honey samples utilised in the initial study were of lower purity than those collected during the 1985-87 flowering seasons. Similarly, the level of peak 83 in the initial study was probably due to a contribution from nodding thistle honey (see Section 4.7).

It appears that white clover type honey does not possess unique extractable substances, and that unifloral clover type honeys of high purity have low levels of extractable organic substances (less than 50 μg/g excluding fatty acids and beeswax hydrocarbons). Thus for a sample to be

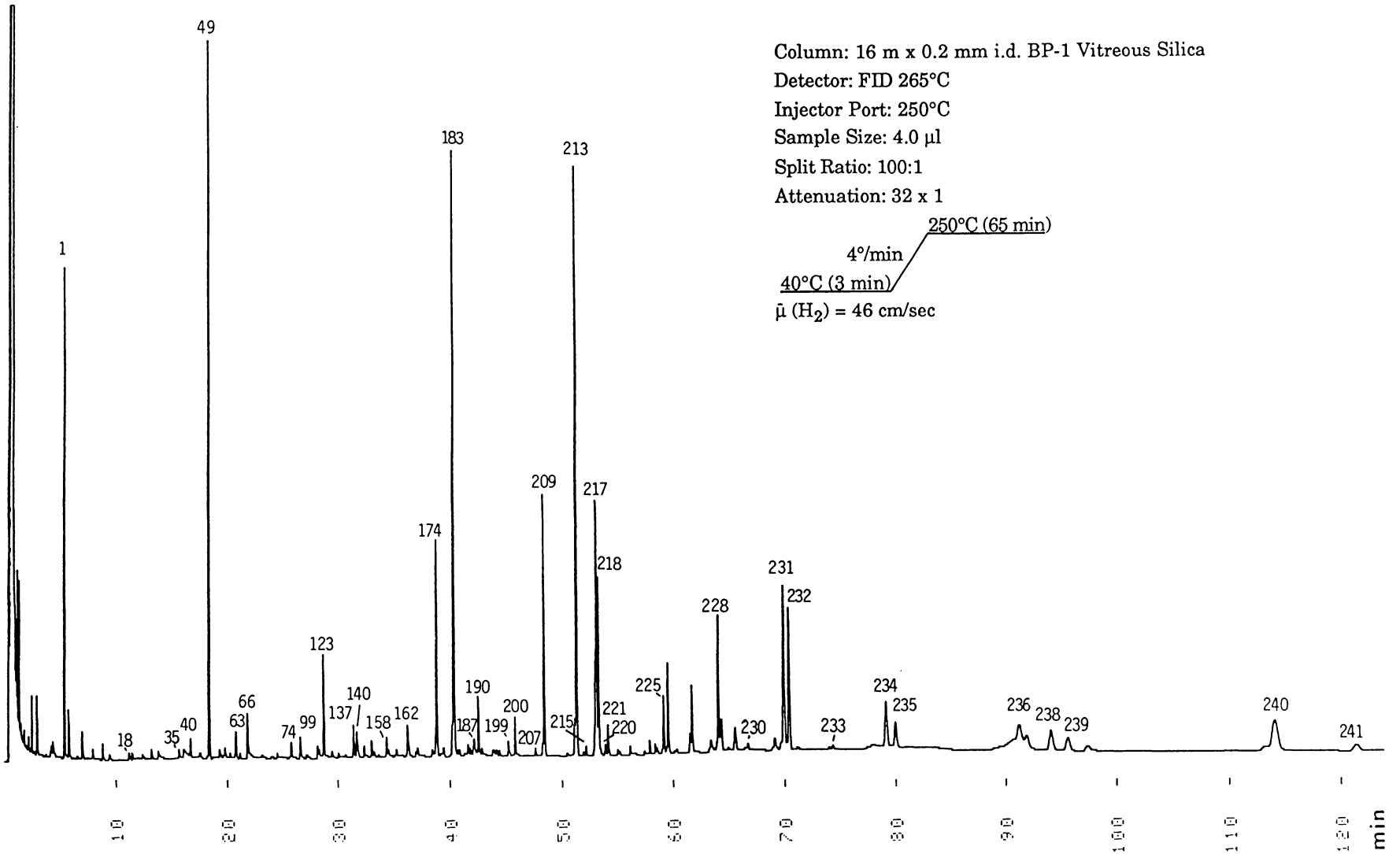


Figure 5.1 Gas chromatographic profile of a representative white clover type honey (sample H410). For peak identification see Tables 3.II and 5.I.

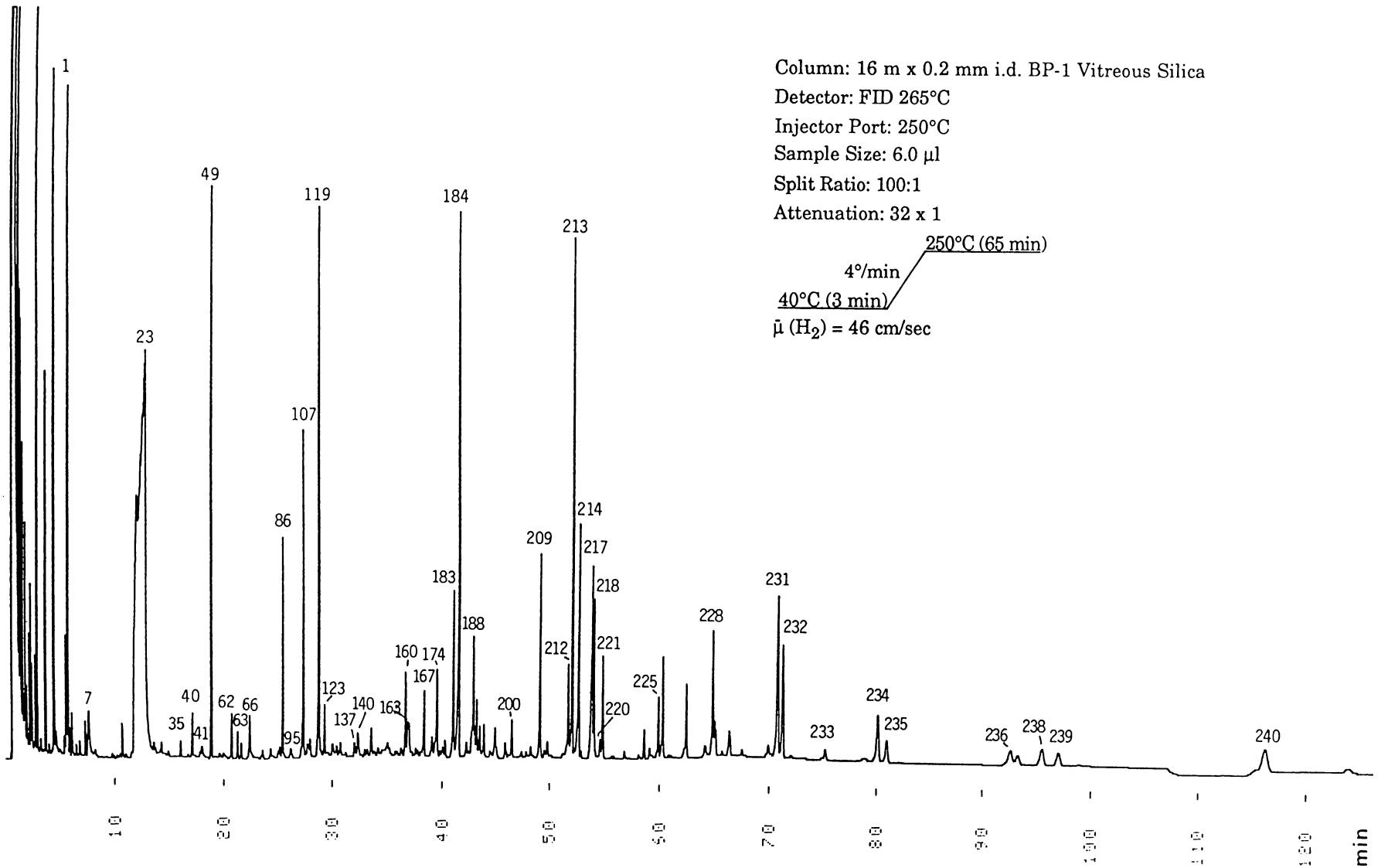


Figure 5.2 Gas chromatographic profile of a low purity white clover type honey (sample H415). For peak identification see Tables 3.II and 5.I.

considered as truly unifloral white clover type honey, *i.e.* greater than 80% clover contribution from pollen analysis, it should afford a GC trace akin to Figure 5.1 and levels of extractable substances similar to those found for samples H408, H410 and C1. Those which contain less than 50% clover contribution by pollen count tend to give GC traces typical of those shown in Figure 5.2. Considerable care must, however, be exercised in the interpretation of the chemical data versus pollen composition; the reason for this is seen in the next section, where comparison can be made between high and low extractable organic components.

It is possible that white clover type honey could be contaminated with other floral sources which would not be detected by GC analysis if these other floral sources also had low extractable organic substances and no unique components. Although such floral types were not encountered in the present study, it could possibly apply to others.

White clover type honey is the major honey sold on the domestic market. Substantial quantities are also exported. For the year June 1985-1986 export earnings from all honeys (excluding honeydew and beeswax) exceeded 5.5 million dollars, with more than half attributable to white clover type. This figure reflects the role white clover plays in New Zealand's pastoral economy, and hence its value in honey production (Godley, 1979).

5.3 Manuka Honey

In the present investigation manuka honey samples from the 1985-87 (including one sample from 1982) flowering seasons were analysed, and compared with data obtained for eight samples from the 1982-84 seasons

Table 5.III Concentration (μg/g) of components in manuka honey (acids as methyl esters).

Peak	Compound (prominent MS ions)	M8	M3	M5	M6	M7	M9	H416	19M	22M	24M	27M	30M
1	unknown[a]	365	206	117	138	193	83	9.7	25	5.9	5.4	16	4.2
2	methyl 3-hydroxypropionate	2.6	4.2	6.7	5.8	6.3	4.4	0.5	2.7	4.3	4.5	5.6	3.4
3	43, 59, 74, 83, 101	-	-	-	-	-	-	4.0	61	21	23	81	27
4	methyl 3-hydroxybutanoate	2.1	1.4	1.6	2.2	1.7	1.5	1.2	36	3.0	9.4	15	3.7
7	unknown[a]	40	43	40	44	50	25	24	160	26	23	78	18
9	methyl 2-hydroxy-3-methylbutyroate	4.2	2.7	13	2.4	3.0	1.1	0.5	1.0	0.3	-	0.4	0.1
10	45, 60, 72, 89, 118	1.8	2.1	2.4	2.0	2.3	2.3	0.4	1.2	0.4	1.4	1.6	0.6
15	benzaldehyde	-	-	-	-	-	-	trace	0.4	-	-	-	-
16	45, 57, 70, 87, 110	0.3	1.5	2.4	1.0	1.1	2.3	1.1	4.8	1.2	3.7	9.5	2.1
18	methyl 3-furancarboxylate	1.6*	0.9*	1.1*	1.2*	1.0*	*	-	-	-	-	1.9	-
19	methyl 3-methyl-2-oxo-pentanoate	*	*	*	*	*	6.5*	trace	-	14	1.8	-	1.1
23	unknown[a]	10.5	4.4	17	7.2	10.9	6.7	58	36	8.4	17	36	12
25	methyl 2-hydroxy-4-methylpentanoate	5.5	2.5	14	6.8	4.8	2.4	trace	3.5	0.9	0.3	2.4	0.7
26	methyl 2-hydroxy-3-methylpentanoate	5.4	2.2	13	3.9	3.1	2.0	trace	3.8	1.5	1.2	1.9	1.1
31	dimethyl butanedioate	3.1	4.6	2.8	10.1	4.5	37	0.9	2.0	37	40	31	26
32	benzyl alcohol	0.2*	0.5*	0.6*	0.7*	0.4*	0.1*	-	1.7*	-	-	trace	-
33	phenylacetaldehyde	*	*	*	*	*	*	-	*	-	-	-	-
35	methyl 2-methylsuccinaldehydoate	-	-	-	-	-	1.4	0.2	0.7	1.0	0.6	2.1	0.2
36	unknown[a]	4.6	3.7	2.9	3	3.6	1.6	-	-	-	-	-	-
40	methyl benzoate	1.9	1.2	1.1	1.1	1.0	0.9	0.2	1.5	1.1	1.0	1.3	0.5
44	43, 55, 67, 71, 82, 109, 125	-	-	-	-	-	-	-	0.8	-	-	1.2	-
45	unknown[a]	9.0	6.0	2.2	4.8	5.5	2.2	-	-	-	-	-	-
49	*n*-undecane (C_{11}, internal standard)												
50	dimethyl pentanedioate	-	-	-	-	-	-	-	-	0.4	1.0	0.3	0.7
51	methyl caprylate (8:0)	trace	trace	trace	trace	trace	trace	trace	0.9	0.6	0.7	0.8	0.9
52	unknown[a]	7.8	2.7	2.1	1.9	2.3	1.5	-	-	-	-	-	-
62	methyl 2-phenylethanoate	1.7	1.2	1.9	1.1	1.4	2.1	0.6	1.5	3.1	0.9	1.0	0.5
63	43, 59, 83, 101, 129, 141, 159	-	-	-	-	-	1.2	0.2	1.5	1.3	1.3	1.5	0.8
64	43, 67, 71, 82, 109, 125,137	-	-	-	-	-	0.3	-	*	-	-	-	-
65	methyl 2-hydroxybenzoate	1.5	1.5	1.2	1.1	1.5	4.7	0.5	2.7*	3.4	1.3	1.2	0.9
66	5-hydroxymethyl-2-furfural	5.3	-	-	-	1.5	0.7	-	-	0.9	-	0.6	0.4
69	unknown[a]	18	9.5	9	11	7.6	1.5	-	0.6	-	-	1.8	-
83	methyl 3-phenylpropionate	-	0.2	0.1	-	0.1	0.1	-	-	0.1	trace	-	trace
86	2'-methoxyacetophenone	7.5	15	13	8.5	15	5.6	14	18	2.4	3.8	21	3.8
95	methyl *cis*-3-phenylprop-2-enoate	-	-	-	-	-	-	0.1	0.7	0.3	-	0.4	0.1
107	methyl 2-methoxybenzoate	36	32	31	23	35	11	1.9	16	1.2	2.2	7.0	1.9
119	methyl 2-hydroxy-3-phenylpropionate	1058	856	570	754	813	1143	234	591	87	106	383	79
123	methyl *trans*-3-phenylprop-2-enoate	13	0.4	5.3	1.4	0.5	7.0*	0.8	8.5	0.6	1.2	3.1	0.5
124	65, 75, 91, 105, 135, 162 M^+	5.8	3.6	3.0	2.1	2.0	*	0.1	3.7	0.5	0.9	4.0	0.9
129	59, 75, 95, 99, 127, 154, 187	0.6	1.1	0.7	0.7	1.1	-	-	1.8	-	-	0.8	-
133	ethyl 2-hydroxy-2-phenylethanoate	2.6	1.8	1.1	1.4	1.7	1.3	0.6	2.9	0.5	0.5	2.4	1.2
135	methyl 2-(methoxyphenyl)-ethanoate	-	-	-	-	-	0.3	-	-	-	-	-	-
136	ethyl 2-hydroxy-3-phenylpropionate	1.4	1.7	0.7	1.5	1.1	1.4	0.2	0.6	0.2	-	0.7	0.1
137	dimethyl octanedioate	-	0.3	-	0.9	0.5	1.3	0.2	-	0.8	1.1	0.5	0.8
139	methyl 3-hydroxybenzoate	-	0.4	*	-	-	2.2*	0.1	-	1.0	0.5	0.5	0.9
141	3,4-dimethoxybenzaldehyde	1.0	0.5	1.1*	1.3	1.2	*	0.2	0.7	-	-	-	-
146	methyl 2-(hydroxyphenyl)-ethanoate	-	-	0.3	-	-	-	0.3	0.4	0.4	0.5	0.9	0.4
148	55, 59, 81, 108, 136, 140, 168	0.2	0.9	0.3	0.6	0.9	4.1	0.6	0.4	0.8	trace	0.9	trace
150	77, 91, 103, 131, 162, 190 M^+	0.8	-	0.7	-	0.5	1.9	1.1	0.9*	-	0.2*	0.5	*
151	methyl 4-hydroxy-3-methoxybenzoate	0.5	0.8	0.6	0.7	0.4	2.6	-	*	0.1	*	-	0.3*
157	methyl laurate (12:0)	trace	trace	trace	trace	trace	trace	-	trace	trace	trace	trace	trace

158	dimethyl nonanedioate	0.8	0.3	0.3	0.9	1.0	0.8	trace	0.1	0.5	0.5	0.8	0.5
160	methyl 3,5-dimethoxybenzoate	9.5	4.9	4.6	4.2	4.9	2.4	3.4	4.1	0.6	1.5	5.0	1.7
162	55, 67, 81, 113, 138, 161, 192	*	*	trace*	*	*	*	0.9	-	*	2.9	1.6	*
163	methyl 3,4-dimethoxybenzoate	3.5*	5.2*	trace*	5.8*	6.8*	10.5*	1.3	3.5	1.5*	1.4	2.8	1.8*
164	65, 78, 91, 102, 120, 148, 180 M^+	0.7	-	5.9*	-	-	-	-	-	5.8	-	-	3.6
167	methyl 3-hydroxy-3-(methoxy-phenyl)-propanoate	11*	7.9*	6.7*	*	8.5*	*	*	5.7	*	2.8	5.5*	*
168	methyl 2-hydroxy-3-(4-methoxyphenyl)-propionate	*	*	*	71*	*	114	9.7*	-	4.3*	-	*	3.3
174	dimethyl decanedioate	1.9	4.8	2.8	8.2	8.2	11	1.0	0.3	7.5	5.2	3.5	5.4
177	methyl 3-(4-methoxyphenyl)-*trans*-prop-2-enoate	-	-	-	-	-	-	-	-	0.3	0.5	-	0.3
179	unknown[a]	6.9	5.2	4.8	6.4	5.3	4.6	1.9	-	-	-	-	-
180	methyl 3-hydroxy-3-(methoxy-phenyl)-propanoate	-	-	-	-	-	-	-	1.0	0.6	-	1.0	0.4
183	dimethyl *trans*-2-decenedioate	2.7	12	5.7	24	19	49	3.1	1.8	24	14	9.7	13
184	methyl 3,4,5-trimethoxybenzoate	6.1	9.4	13	12	13	8.2	7.8	0.9	0.6	0.9	4.7	1.6
187	methyl myristate (14:0)	-	0.7	0.2	0.5	-	trace	0.6	trace	0.5	0.4	1.3	0.7
188	methyl 4-hydroxy-3,5-dimethoxy-benzoate	150	213	132	171	230	74	38	20	12	7.7	41	18
190	methyl 3-(3,4-dimethoxyphenyl)-*cis*-prop-2-enoate	1.5	-	1.4	-	0.7	trace	0.5	-	0.3	0.5	5.3	1.1
196	unknown[a]	6.4	9.7	5.4	6.1	10.3	6.7	4.3	3.6	1.5	0.7	3.7	2.2
197	77, 92, 135, 179, 199	6.9	6.7	7.1	8.3	6.4	3.4	2.2	3.0	0.5	0.5	1.8	1.6
207	methyl palmitioleate (16:1)	0.2	0.2	0.3	0.2	0.3	0.4	0.3	0.3	0.2	0.4	0.3	0.3
209	methyl palmitate (16:0)	1.0	0.8	1.0	1.0	1.1	1.4	1.2	2.1	1.3	1.6	1.6	1.7
210	55, 71, 83, 109, 125, 152, 170	-	-	-	-	-	-	2.3	1.1	0.2	-	1.4	1.1
211	41, 83, 127, 155, 179, 225, 252	1.5	2.5	1.2	1.4	0.8	0.5	0.5	4.3	0.4	0.3	3.0	1.2
212	43, 108, 120, 135, 163, 220 M^+	4.2	6.1	8.8	4.2	8.2	4.2	3.2	1.4	trace	trace	4.9	1.1
213	methyl margarate (17:0, internal standard)												
214	methyl abscisate	-	0.5	-	0.6	0.8	trace	0.2	-	0.2	trace	5.7	0.9
216	methyl linoleate (18:2)	*	trace*	trace*	*	*	0.2	*	0.3	0.3	*	0.3	0.1
217	methyl α-linolenate (18:3)	0.6*	trace*	trace*	0.5*	0.4*	0.2	0.3*	0.5	0.7	0.1*	0.5	0.2
218	methyl oleate (18:1)	1.4	1.0	1.0	1.4	1.5	1.1	0.5	0.9	1.3	0.8	0.9	0.9
220	*n*-heneicosane (C_{21})	trace	trace	trace	trace	trace	trace	0.3	0.6	0.3	0.3	0.2	0.7
221	methyl stearate (18:0)	0.4	0.3	0.3	trace	0.4	0.1	0.2	0.4	0.3	0.3	0.3	0.5
225	*n*-triacosane (C_{23})	0.5	0.3	0.2	0.3	0.2	0.6	1.2	1.6	0.9	1.1	1.2	2.1

[a] detected in GC/FID but not in GC/MS.

* unresolved GC/FID peak.

(Tan, 1985). Table 5.III list the components and the concentrations found in the twelve honey samples studied in this work. The GC profiles of 1985-87 manuka honeys proved to be similar to those obtained in the original study. A representative chromatogram of manuka honey extractives obtained from the 1985-87 flowering seasons is depicted in Figure 5.3.

Manuka honey is notable for its high levels of honey extractives, particularly 2-hydroxy-3-phenylpropionic acid (peak 119), 2'-methoxy-acetophenone (peak 86), 2-hydroxybenzoic acid (peak 107) and methyl 4-hydroxy-3,5-dimethoxybenzoate (peak 188). In the twelve samples examined the average levels of these four compounds are 550, 10, 16 and 92 μg/g respectively. In addition to the aromatic extractives, high levels of *trans*-2-decenedioic acid (peak 183) were also detected (average level 15 μg/g for manuka honey, 4.0 μg/g in white clover type). Other extractives include 2-hydroxy-4-methylpentanoic acid (peak 25) and 2-hydroxy-3-methylpentanoic acid (peak 26) (average levels 3.7 μg/g and 3.3 μg/g respectively).

Some seasonal variations appear to exist. For example, the levels of 2-hydroxy-3-phenylpropionic acid (peak 119) in the 1986-87 samples (average level 247 μg/g) are much lower than those obtained in the earlier seasons (average levels 1 769 μg/g for 1982-84 and 866 μg/g for 1985-86 flowering seasons respectively). The 1986-87 manuka honey samples contained a modest amount of clover contribution, *e.g.* pollen analysis revealed 12% clover pollen and 74% manuka pollen in sample H416. The result of the pollen analysis carried out on the manuka honey is shown in Table 5.II.

As discussed in Chapter 1, the absolute pollen content of most unifloral honeys is in the range of 20 000 to 100 000 per 10 g sample. Such

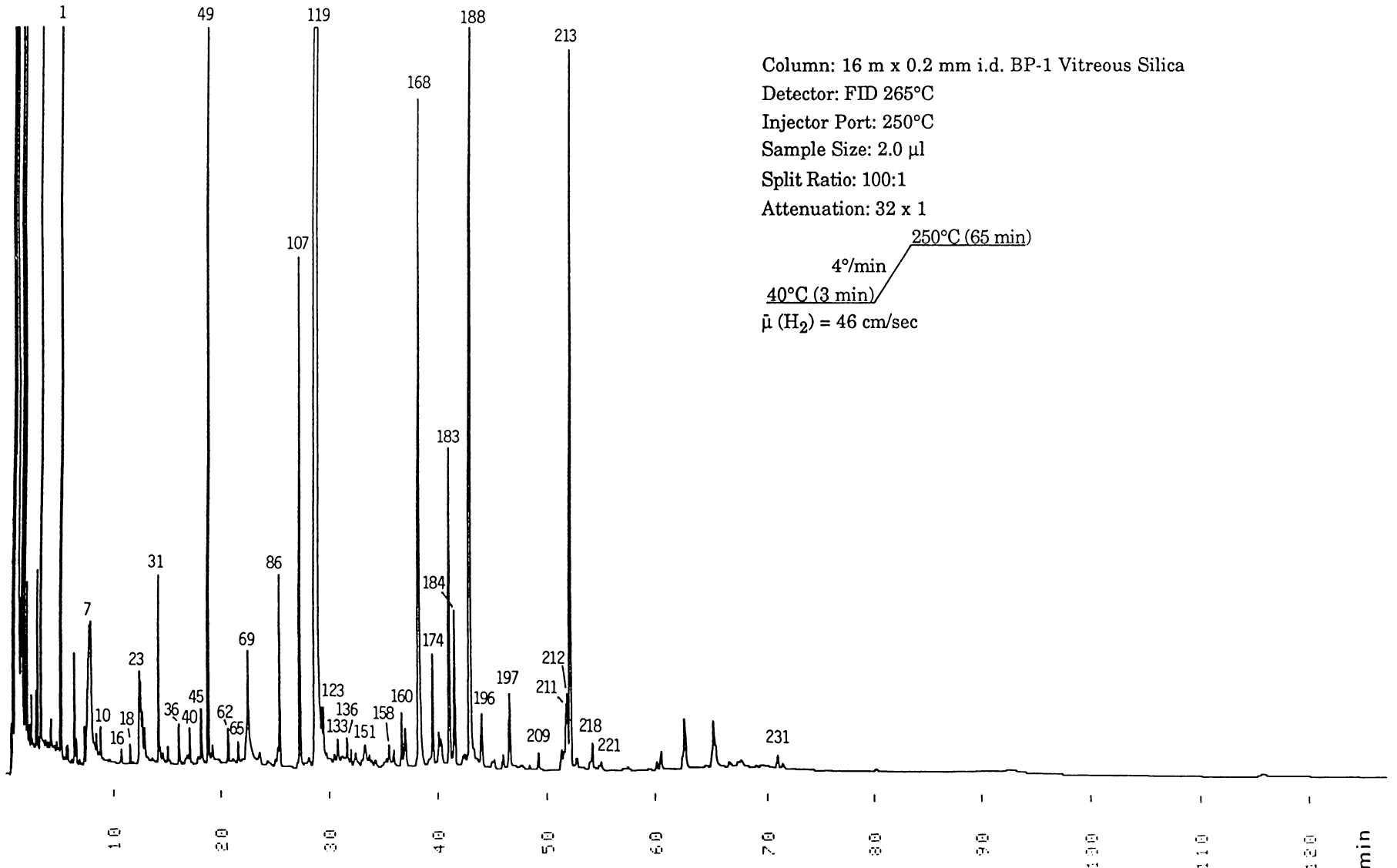


Figure 5.3 Gas chromatographic profile of a representative manuka honey (sample M6). For peak identification see Tables 3.II and 5.III.

values are regarded as "normal" (Crane 1975), and in this category, the principal pollen type is predominant and present in frequencies of 45% or more. However, pollen production differs vastly between species: some unifloral honey may contain less than 20 000 pollen grains and others more than 100 000 pollen grain. Honey in the lower range is "under-represented" in terms of "normal" absolute pollen content, and that in the higher range is "over-represented".

Moar (1985) has shown that manuka is in the "over-represented" category where a minimum of 70% manuka pollen is necessary to classify a sample as unifloral, while clover in the "normal" category needs 45% contribution to be considered unifloral. On the other hand, thyme in the "under-represented" category only needs 20%. Therefore, according to Moar's calculation, the amount of manuka pollen in sample H416 corrected to the "normal" category should be considered as:

$$= (74\% \times 45\%) \div 70\% = 47.6\%$$

As noted previously the levels of extractives recovered from manuka honey are substantially greater than those obtained from clover honey; typically the level of aromatic and acid extractives in clover samples does not exceed 50 μg/g (see Table 5.I and 5.III). It follows that the levels and nature of chemical components from extractives of manuka honey can be utilised to differentiate white clover type honey from manuka and vice versa, *i.e.* samples with higher levels of the four major compounds should be the purest manuka honey, while those with low levels of extractives should contain a high proportion of clover contribution. Manuka honey samples 22M and 30M (2-hydroxy-3-phenylpropionic acid levels of 87 and 79 μg/g) appeared to be

predominantly white clover type (*cf.* clover sample H415; 6% manuka pollen, with a 2-hydroxy-3-phenylpropionic acid level of 10 μg/g).

Thus far the available evidence indicates that 2-hydroxy-3-phenyl-propionic acid (average level over all samples and seasons of 556 μg/g), 2'-methoxyacetophenone (average level over all samples and seasons of 11 μg/g), 2-hydroxybenzoic acid (average level over all samples and seasons of 17 μg/g), 3,4,5-trimethoxybenzoic acid (average level over all samples and seasons of 6.5 μg/g) and methyl 4-hydroxy-3,5-dimethoxybenzoate (average level over all samples and seasons of 92 μg/g) are diagnostic of manuka honey.

5.4 Ling/Heather Honey

In an extension of the ling/heather honey study, eight samples from the 1985-87 flowering seasons were analysed. As in the case of clover honeys, judgement was exercised in the regional selection of samples in order to minimise extraneous floral source contributions. Ling/heather honey samples (unheated) were obtained from beekeepers with hives kept in the central North Island of New Zealand (National Park region) and commercial honey samples from the same region were obtained from Wilson Neill-Hororata Honey Exports Limited. Honey purity was ascertained by pollen analysis. The results of the pollen analyses are given in Table 5.IV. Ling/heather pollen is poorly represented (Moar, 1985). Maurizio and Louveaux (1964) note that an almost pure honey can be produced from ling/heather despite that pollen exhibits only a secondary contribution.

Table 5.IV Pollen composition of ling/heather honey expressed as a percentage of total pollen of nectar plants counted.

pollen type	H361	H460	H461	H462	H463
five-finger (*Pseudopanax*)	47	-	-	-	-
ling (*Calluna*)	31	3	3	2	5
hebe-type (*Hebe*)	6	-	-	-	-
white clover type (*Trifolium repens type*)	6	80	81	98*	95*
remainder	10	17	16	*	*

* white clover type and others.

Figure 5.4 is a typical GC trace of the extractable organic substances recovered from a heather honey sample. Peaks 226-241 were shown to be hydrocarbons or fatty acids; a similar collection of fatty acids and hydrocarbons were found in other unifloral honeys (Tan *et al.*, 1988). As in the case for white clover type and manuka honeys, details of their characterisation are not repeated here.

Table 5.V lists the quantitative composition of chemical components detected in eight ling/heather honeys collected over two flowering seasons (*i.e.* 1985-86, sample H361, H2, H3 and H4; 1986-87, sample H460, H461, H462 and H463). It is clear from pollen data that ling/heather honeys from the 1985-86 season were of higher purity than those collected in 1986-87 (see Table 5.IV). The heather extractives included a number of compounds previously detected in other types of unifloral honeys, among others a range of aliphatic and aromatic acids (*cf.* Table 5.V, 5.I and 5.III). It is noteworthy that the diacids which were a prominent component of clover and manuka honey extractives were detected only in the 1986-87 season heather honeys and not in the purer 1985-86 season heather honey.

Table 5.V Concentration (μg/g) of components in heather honey (acids as methyl esters).

peak	compound (prominent MS ions)	H361	H2	H3	H4	H460	H461	H462	H463
1	unknown[a]	45	4.4	5.6	4.2	23	4.4	5.0	4.9
2	methyl 3-hydroxypropionate	-	-	-	-	trace	trace	trace	trace
4	methyl 3-hydroxybutanoate	-	-	-	-	trace	trace	trace	trace
6	methyl 3-hydroxy-3-methylbutanoate	-	-	-	-	1.1	1.1	1.2	1.4
12	methyl caproate (6:0)	1.1	1.6	1.5	1.3	0.3	0.1	0.1	0.1
15	benzaldehyde	1.0	0.3	0.3	0.3	0.3	0.2	0.2	0.2
18	methyl 3-furancarboxylate	-	-	-	-	0.7	0.6	0.6	0.6
21	methyl 4-oxo-pentanoate	-	-	-	-	0.2	0.1	0.2	0.2
44	phenol	0.5	0.7	0.7	0.5	0.1	trace	trace	trace
25	methyl 2-hydroxy-4-methylpentanoate	-	-	-	-	1.2	0.9	1.1	1.3
26	methyl 2-hydroxy-3-methylpentanoate	-	-	-	-	1.2	0.7	0.8	1.0
31	dimethyl butanedioate	-	-	-	-	7.5	6.2	6.2	5.8
32	benzyl alcohol	2.0	1.7	1.8	1.2	0.7*	0.3*	0.4*	0.5*
33	phenylacetaldehyde	0.2	0.4	0.3	0.2	*	*	*	*
34	43, 69, 73, 101, 126, 143, 158 M^+	3.0	1.7	2.2	1.4	0.9	0.5	0.6	0.8
35	methyl 2-methylsuccinaldehydate	-	-	-	-	0.4	0.3	0.3	0.3
40	methyl benzoate	114	68	83	63	20	12	15	18
41	2-phenylethanol	0.9	0.8	0.9	0.6	0.2	0.1	0.1	0.1
42	3,5,5-trimethylcyclohex-2-en-1-one	5.0	2.5	2.9	2.1	1.4	0.8	1.0	1.1
47	3,5,5-trimethylcyclohex-2-ene-1,4-dione	trace*	trace*	trace*	trace*	trace*	trace*	trace*	trace*
49	*n*-undecane (C_{11}, internal standard)	*	*	*	*	*	*	*	*
51	methyl caprylate (8:0)	0.9	2.5	2.7	1.6	0.1	0.2	0.1	trace
57	56, 70, 98, 112, 139, 154 M^+	1.5	1.3	1.1	0.8	0.4	0.2	0.3	0.4
62	methyl 2-phenylethanoate	213	173	214	153	57	34	45	49
65	methyl 2-hydroxybenzoate	0.3	0.3	0.4	0.2	0.6	0.4	0.4	0.5
66	5-hydroxymethyl-2-furfural	-	-	-	-	1.0	0.4	0.3	0.4
76	methoxybenzaldehyde	0.9	2.9	2.6	2.0	-	-	-	-
77	dimethyl hexanedoiate	-	-	-	-	1.1	0.7	0.7	0.8
78	ethyl 2-phenylethanoate	0.2	0.2	0.2	0.2	-	-	-	-
83	methyl 3-phenylpropionate	0.2	0.2	0.3	0.1	0.2	0.1	0.1	0.1
85	methyl 2-hydroxy-2-phenylethanoate	-	-	-	-	2.0	1.4	1.3	1.6
89	2-methoxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione	1.6	1.4	1.6	1.2	0.4	0.2	0.3	0.2
92	41, 69, 83, 100, 125, 128, 152	1.1	0.8	1.0	0.7	0.4	0.2	0.3	0.3
94	3-phenylprop-2-en-1-ol	2.4	3.2	3.7	2.5	-	-	-	-
95	methyl *cis*-3-phenylprop-2-enoate	-	-	-	-	1.1	0.5	0.7	0.1
98	43, 85, 109, 127, 141, 169	1.1	1.3	1.5	1.0	0.9	0.7	0.4	0.3
103	trimethylphenol	0.3	0.2	0.3	0.2	-	-	-	-
106	43, 55, 85, 116, 130, 153, 166	-	-	-	-	1.2	0.7	0.8	0.4
112	43, 55, 71, 83, 95, 109, 135, 170	7.1	5.4	5.8	4.3	-	-	-	-
119	methyl 2-hydroxy-3-phenylpropionate	2.2	5.1	6.8	3.2	262*	135*	160*	195*
120	methyl 3-methoxybenzoate	0.7	0.6	0.7	0.7	*	*	*	*
123	methyl *trans*-3-phenylprop-2-enoate	1.8	5.2	5.2	4.2	4.8	2.8	2.9	3.2
127	43, 97, 139, 181, 196 M^+	3.1	0.9	0.6	0.8	0.2	0.3	0.4	0.5
128	41, 83, 123, 151, 180 M^+	1.5	1.4	1.8	1.2	0.4	0.3	0.4	0.3
133	ethyl 2-hydroxy-2-phenylethanoate	-	-	-	-	1.7	1.1	1.0	1.1
136	ethyl 2-hydroxy-3-phenylpropionate	-	-	-	-	0.4	0.2	0.2	0.3
137	dimethyl octanedioate	-	-	-	-	1.5	0.9	0.9	1.0
139	methyl 3-hydroxybenzoate	2.1	2.1	2.7	2.4	5.5	3.0	3.5	3.6

143	55, 70, 95, 107, 127, 196 M^+	3.8	3.6	4.2	3.3	1.0	0.6	0.8	1.2
146	methyl 2-(hydroxyphenyl)-ethanoate	-	-	-	-	1.8	1.0	1.0	0.8
147	4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one	1.2	1.2	1.1	1.4	5.3*	2.6*	3.1*	3.8*
148	55, 59, 81, 108, 136, 140, 168	-	-	-	-	*	*	*	*
157	methyl laurate (12:0)	0.9	0.7	0.7	0.7	0.5	0.4	0.5	0.3
158	dimethyl nonanedioate	-	-	-	-	0.4	0.2	0.1	0.2
160	methyl 3,5-dimethoxybenzoate	1.8	1.7	1.7	1.2	1.3	1.0	1.3	1.4
163	methyl 3,4-dimethoxybenzoate	1.1	1.5	1.3	0.6	0.8	0.4	0.6	0.6
165	55, 74, 87, 111, 129, 152, 172 fatty acid?	1.3	0.3	0.5	0.5	-	-	-	-
168	methyl 2-hydroxy-3-(4-methoxyphenyl)-propionate	-	-	-	-	0.1	0.1	0.2	0.1
171	4-(3-oxo-1-butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one	27	35	36	32	7.3	3.5	4.4	5.1
172	4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	trace	trace	trace	trace	trace	trace	trace	trace
173	55, 67, 81, 95, 127, 168, 198	4.1	3.4	4.3	3.8	-	-	-	-
174	dimethyl decanedioate	-	-	-	-	5.2	2.6	3.3	3.3
176	45, 77, 93, 119, 147, 162 M^+	1.5	2.4	1.9	2.2	0.9	0.4	0.6	0.7
177	methyl 3-(4-methoxyphenyl)-*trans*-prop-2-enoate	-	-	-	-	1.1	0.2	0.3	0.4
180	methyl 3-hydroxy-3-(methoxyphenyl)-propanoate	-	-	-	-	0.4	0.4	0.4	0.3
181	4-hydroxy-4-(3-oxo-1-butynyl)-3,5,5-trimethyl-cyclohex-2-en-1-one	1.6	1.7	1.4	1.5	-	-	-	-
183	dimethyl *trans*-2-decenedioate	-	-	-	-	15	7.7*	9.0*	9.3*
182	4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	4.4	0.5	0.3	0.3	0.2	*	*	*
184	methyl 3,4,5-trimethylbenzoate	1.9	1.6	1.9	1.8	0.6	2.8	2.8	2.9
187	methyl myristate (14:0)	16.3	3.5	4.2	3.8	0.6	0.4	0.4	0.5
188	methyl 4-hydroxy-3,5-dimethoxybenzoate	-	-	-	-	45	23	19	28
189	4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	30	36	60	39	8.9	15	14	19
193	4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one [9]	107	158	148	185	42	13	17	13
194	isomer of [9]	9.0	7.0	8.1	8.0	2.6	1.1	1.4	0.1
199	methyl pentadecanoate (15:0)	0.5	0.7	0.3	0.6	0.5	0.3	0.2	0.4
200	methyl 3-(3,4-dimethoxyphenyl)-*trans*-prop-2-enoate	0.5	0.5	0.4	0.8	1.7	0.9	1.0	1.0
204	43, 91, 134, 162, 278 M^+	0.4	0.6	0.8	0.9	0.3	0.2	0.2	0.2
207	methyl palmitioleate (16:1)	0.6	1.6	1.1	0.8	0.2	0.2	0.2	0.2
209	methyl palmitate(16:0)	6.0	6.9	4.5	5.2	15	6.0	8.0	10.3
213	methyl margarate (17:0, internal standard)								
214	methyl abscisate	1.6	4.2	5.0	5.7	1.4	0.6	0.7	0.5
216	methyl linoleate (18:2)	1.1	1.1	0.8	1.5	*	*	*	*
217	methyl α-linolenate (18:3)	1.1	6.4	2.6	3.3	25*	9.8*	14*	18*
218	methyl oleate (18:1)	6.9	16	0.8	7.3	5.7	2.3	2.6	2.6
219	isomer of methyl oleate (18:1)	1.7	1.0	-	-	-	-	-	-
220	*n*-heneicosane (C_{21})	0.9	1.6	0.4	1.2	1.2	0.7	0.6	0.7
221	methyl stearate (18:0)	2.7	5.5	4.7	5.5	2.1	1.2	1.2	1.2
222	methyl abscisate	trace	trace	trace	trace	trace	trace	trace	trace
225	*n*-triacosane (C_{23})	3.1	3.0	1.2	2.1	3.9	1.9	2.0	1.9

[a] detected in GC/FID but not in GC/MS.

* unresolved GC/FID peak.

Samples collected during the 1985-86 season (*i.e.* H361, H2, H3 and H4) possessed similar levels of the 3,5,5-trimethylcyclohex-2-en-1-one derivatives (*e.g.* peaks 42, 47, 89, 171, 182, 189 and 193) which were much higher than the samples collected during the 1986-87 season (see Table 5.V). Substances possessing structures of this type are frequently referred to as degraded carotenoids. Chief amongst the degraded carotenoids are 4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one (peak 193), 4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one (peak 189) and 4-(3-oxo-1-butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one (peak 171). Concentrations of these components in the 1985-86 season honeys ranged from 107 to 185 μg/g, 30 to 60 μg/g and 27 to 36 μg/g; whilst in the 1986-87 season honeys, the concentration ranged from 13 to 42 μg/g, 9 to 19 μg/g and 3.5 to 7.3 μg/g respectively.

Other trimethylcyclohex-2-en-1-one derivatives detected included 3,5,5-trimethylcyclohex-2-en-1-one (peak 42, average level of 2.1 μg/g), 3,5,5-trimethylcyclohex-2-ene-1,4-dione (peak 47), 2-methoxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione (peak 89, average level of 0.9 μg/g), 4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one (peak 147, average level of 1.2 μg/g), 4-hydroxy-4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one (peak 181, average level of 1.6 μg/g) and abscisic acid (peak 214, average level of 2.5 μg/g).

Degraded carotenoids have previously been reported from a number of plant sources (see Section 4.4). In addition to the degraded carotenoids, heather honeys are also dominated by benzoic acid (peak 40), 2-phenylethanoic acid (peak 62) and 2-hydroxy-3-phenylpropionic acid (peak 119). Levels of the former two acids are much higher in the 1985-86 season samples (average levels of 82 μg/g and 188 μg/g) than the 1986-87 season samples (average levels of 16 μg/g and 46 μg/g). Conversely, the

levels of the latter acid in the 1985-86 season samples (average levels of 4.3 μg/g) are much lower than in the 1986-87 season samples (average levels of 188 μg/g). On the other hand, the diacids (peaks 31, 77, 137, 158, 174 and 183) which were common to both clover and manuka honeys were only detected in the 1986-87 season ling/heather samples. Moreover, 2-hydroxy-4-methylpentanoic acid (peak 25) and 2-hydroxy-3-methylpentanoic acid (peak 26) which were detected in manuka honeys were only found in the later season samples.

There appears to be some seasonal variations in the total extractable organic substances recovered from ling/heather honey. One possible explanation is that such variations are due to the different levels of contributing floral sources. Pollen data in Table 5.IV clearly supports this presumption. A substantial quantity of 2-hydroxy-3-phenylpropionic acid occurred in the 1986-87 samples (H460, H461, H462 and H463) which were predominantly white clover type. Pollen analysis (Table IV) revealed that these samples include a significant contribution (up to 17%) from unstated species at least one of which appears to contribute some 2-hydroxy-3-phenylpropionic acid. It is perhaps of passing interest that both manuka and ling/heather honeys possess abnormal (non-Newtonian) flow properties due to their relatively high content of certain proteins (Crane, 1980) and are therefore often extracted together in New Zealand honeyhouses.

In a recent report, Steeg and Montag (1987) described the composition of both free and bound acids from a variety of European honeys including heather. The levels of benzoic acid and 2-phenylethanoic acid found in ling/heather honeys in the present study were similar to those found in European heather honeys. However, the levels of 2-hydroxy-3-phenylpropionic acid recorded for the 1985-86 season

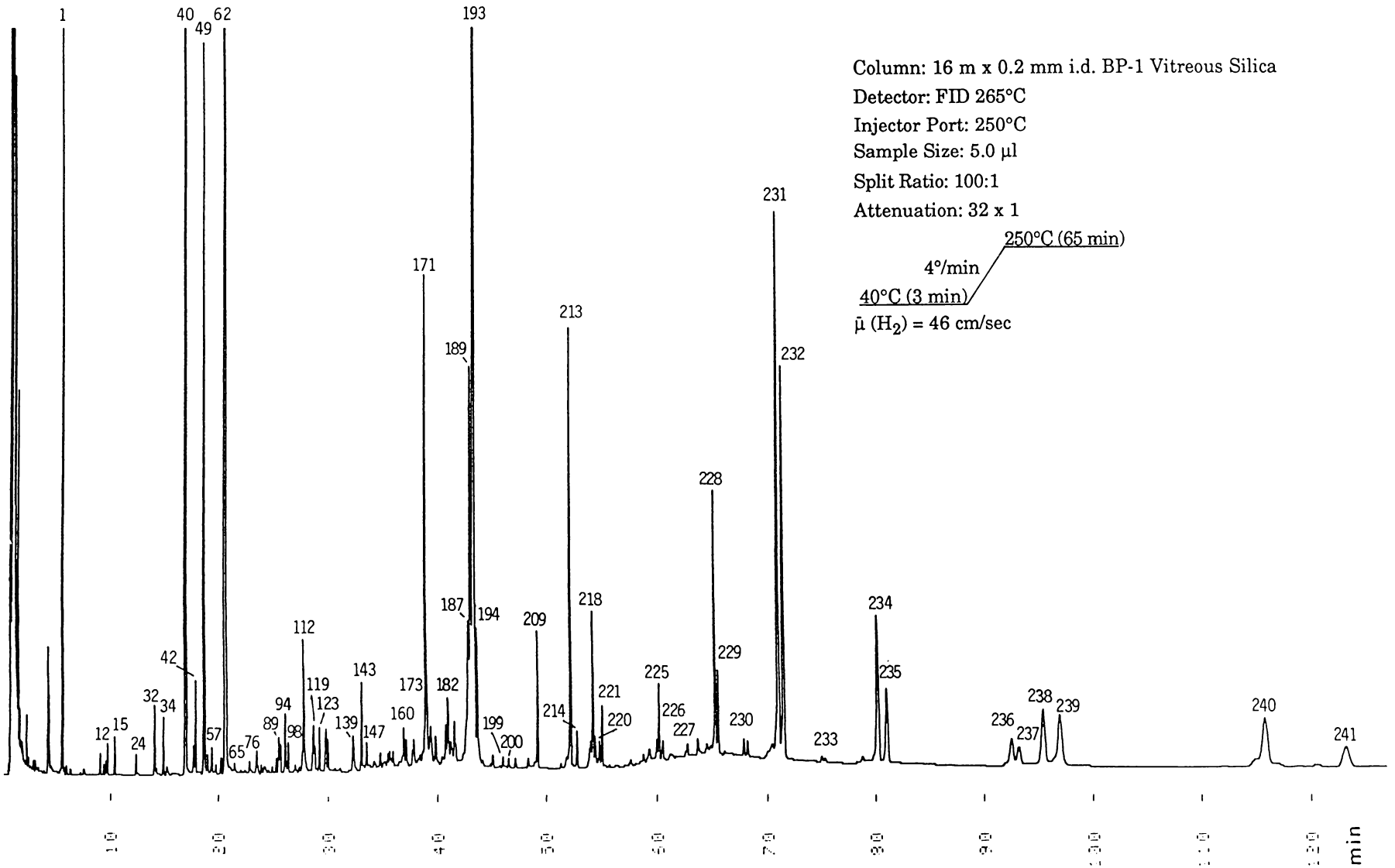


Figure 5.4 Gas chromatographic profile of a representative heather honey (sample H361). For peak identification see Tables 3.II and 5.V.

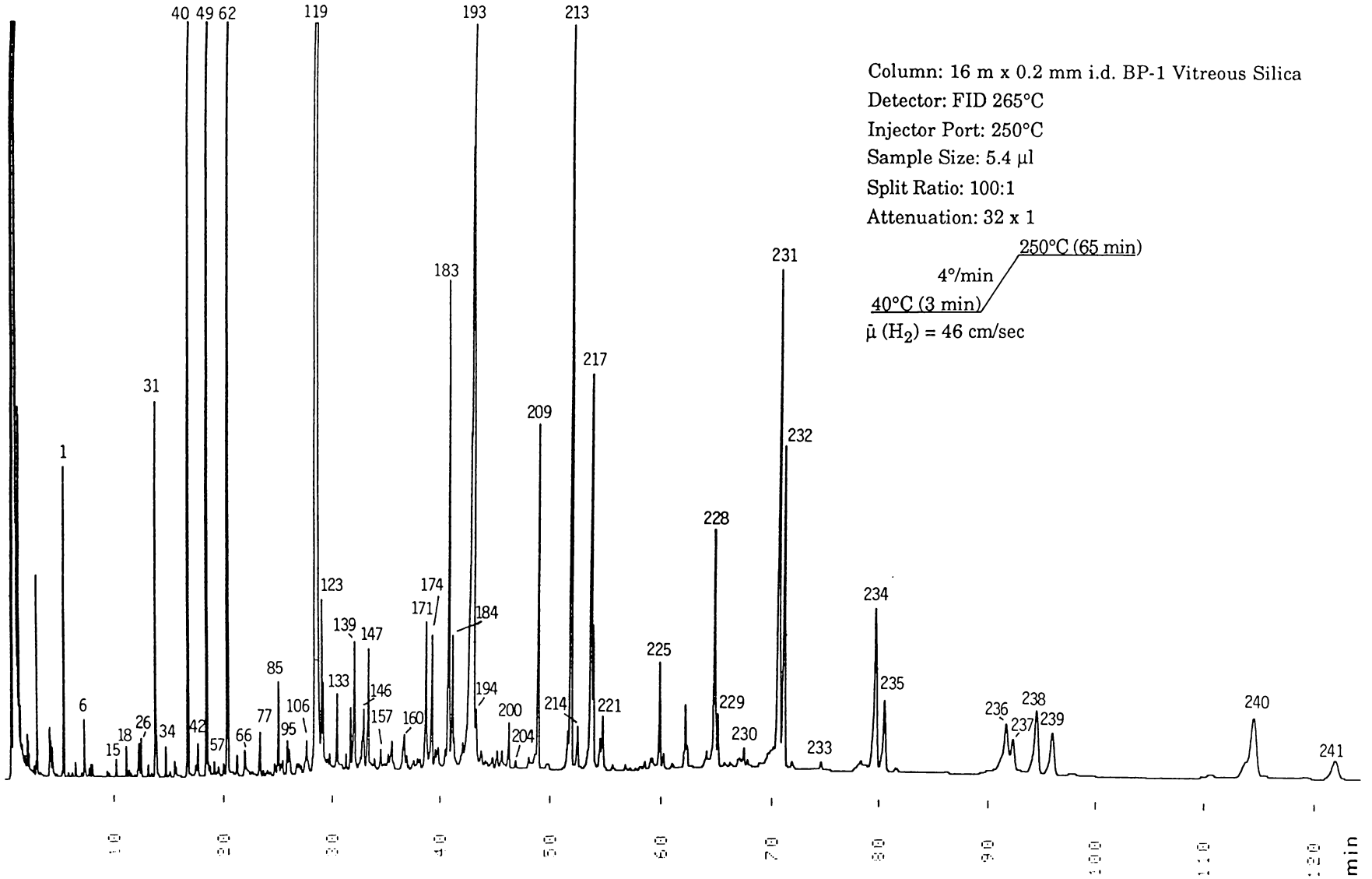


Figure 5.5 Gas chromatographic profile of a low purity heather honey (sample H462). For peak identification see Tables 3.II and 5.V.

ling/heather honey were lower. Levels of 2-hydroxy-3-phenylpropionic acid akin to those reported by Steeg and Montag were found only in 1986-87 season honey samples. Although apiarists described these as heather honeys, pollen analysis revealed that samples with elevated 2-hydroxy-3-phenylpropionic acid levels exhibited low heather pollen counts (less than 5%, see Table 5.IV). The present results indicate that high quality heather honey is essentially devoid of 2-hydroxy-3-phenylpropionic acid. In addition, none of the diacids identified in the present study were reported by Steeg and Montag.

Since degraded carotenoids are absent in New Zealand white clover type and manuka unifloral honeys, the implication of the present findings is that they originate specifically from the heather plant source. The degraded carotenoids are probably of specific biosynthetic origin. However, no intact carotenoids were detected in the honeys (see Section 4.4.6). Two commonly encountered C_{13} plant substances of related structure are α- and β-ionone; both are described as possessing sweet floral fragrances reminiscent of violets (Bauer and Garbe 1985). These compounds were not detected in the heather honey. New Zealand heather honey is described by Walsh (1967) as being "reddish in colour and of mild but pronounced flavour". Possibly the degraded carotenoids detected in this study contribute to the heather honey flavour.

Although there are insufficient data to completely define New Zealand heather honey using chemical criteria, the apparent characteristic distribution of degraded carotenoid-like compounds points to their utility in distinguishing heather honey from less valuable honeys derived from other floral sources. Thus, for a sample to be considered as unifloral ling/heather honey, it should afford a GC trace akin to Figure 5.4 and levels of extractable degraded carotenoid-like substances similar to those

found for samples H361, H2, H3 and H4. Those which contain less than 10% ling/heather contribution by pollen count tend to give GC traces similar to that of Figure 5.5.

5.5 Thyme and Willow Honeys

A representative GC trace of thyme honey is displayed in Figure 5.6. Table 5.VI lists the components and their concentrations found for the 11 thyme honey samples studied including five commercial honey samples. Thyme honey is under-represented in terms of pollen analysis (Moar, 1985; Maurizio, 1975); Moar concluded that a minimum of 20% thyme pollen is required for classification as a unifloral honey. Table 5.VII lists the results of the pollen analysis of some of the thyme honeys.

Thyme honey is characterised by the presence of 3-hexenoic acid (peak 13), 3'-aminoacetophenone (peak 90), an unknown peak 149, and 1-(3-oxo-1-butenyl)-2,6,6-trimethyl-1,2-epoxycyclohexan-4-ol [**24**] (peak 153) with 1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans-cis*-1,2,4-triol [**22**] (peak 206) dominating the GC trace. In the 11 honey samples examined, the concentration of these compounds ranged from 3.5 to 8.1 μg/g (average level 4.8 μg/g), 0.7 to 5.1 μg/g (average level 2.5 μg/g), 1.4 to 5.6 μg/g (average level 3.8 μg/g), 0.6 to 4.8 μg/g (average level 1.8 μg/g) and 25 to 110 μg/g (average level 65 μg/g) respectively.

Other components detected include the diacids (peaks 31, 50, 77, 137, 158, 174 and 183), pyridinecarboxylic acid (peak 48), and abscisic acids (peaks 214 and 222 respectively). In addition to the components unique to thyme honey, other components were detected including some substances associated with other unifloral honeys. For example, unknown peaks 44

Table 5.VI Concentration (μg/g) of components in thyme and willow honeys (acids as methyl esters).

peak	compound (prominent MS ions)	W207	H384	H404	H405	H406	H407	111T	139T	T1	T2	T3	T4
1	unknown[a]	7.4	7.5	11	8.0	7.0	4.7	4.5	7.6	72	2.1	0.9	3.9
3	43, 59, 74, 83, 101	1.1	1.4	0.5	2.0	1.5	0.5	0.3	1.5	-	-	-	-
4	methyl 3-hydroxybutanoate	-	0.7	-	-	-	-	0.1	0.1	0.6	0.7	0.5	0.5
6	methyl 3-hydroxy-3-methylbutanoate	-	0.5	0.2	0.2	0.2	0.1	0.1	0.1	0.4	0.4	0.4	0.3
8	2,5-cyclohexadiene-1,4-dione	-	0.3	-	-	-	-	0.2	0.4	-	-	-	-
12	methyl caproate (6:0)	-	0.8	0.8	1.0	0.9	1.1	1.6	1.8	0.7	0.8	0.9	0.7
13	methyl 3-hexenoate	-	4.6	3.8	4.7	4.3	5.7	6.5	8.1	3.9	3.7	4.3	3.5
18	methyl 3-furancarboxylate	-	1.5	1.6	0.6	0.4	0.1	0.2	0.3	1.1	1.0	1.1	0.8
22	41, 55, 69, 84, 112, 128 M^+	-	1.0	-	-	-	-	-	-	0.6	0.7	0.6	0.5
31	dimethyl butanedioate	0.1	7.0	0.3	0.4	0.4	0.1	0.3	0.3	8.0	9.1	8.4	2.3
32	benzyl alcohol	0.1	-	-	-	-	-	-	-	-	-	-	-
33	phenylacetaldehyde	-	-	-	-	-	-	trace	0.1	trace	trace	trace	0.4
35	methyl 2-methylsuccinaldehydate	-	0.2	-	-	-	-	-	-	0.2	0.2	0.2	0.1
40	methyl benzoate	0.2	1.5	1.3	1.5	1.4	0.6	0.8	1.1	2.6	2.8	3.0	2.4
41	2-phenylethanol	0.1	0.1	-	0.1	0.1	-	-	-	-	-	-	-
44	43, 55, 67, 71, 82, 109, 125	0.1	3.3	2.0	1.7	1.3	0.5	0.8	1.4	2.7	3.1	3.6	3.3
46	51, 53, 65, 80, 108, 109	2.5	-	-	-	-	-	-	-	-	-	-	-
48	methyl pyridinecarboxylate	-	2.3	0.8	trace	0.1	0.1	0.2	0.2	*	*	*	*
49	*n*-undecane (C_{11}, internal standard)												
50	dimethyl pentanedioate	-	-	-	-	-	-	-	-	0.4*	0.5*	0.6*	0.4*
51	methyl caprylate (8:0)	-	-	0.3	-	-	-	trace	trace	0.9	1.1	0.8	0.9
62	methyl 2-phenylethanoate	0.5	1.4	2.7	1.9	1.5	0.3	0.8	0.6	1.8	2.7	1.9	1.7
63	43, 59, 83, 101, 116, 141, 159	0.4	0.4	0.7	0.8	0.8	0.3	0.3	0.1	2.2	0.9	1.1	1.2
64	43, 67, 71, 82, 109, 125, 137	0.5	17	13	14	12	5.8	8.0	8.3	21	22	22	20
66	5-hydroxymethyl-2-furfural	-	0.2	-	-	-	-	-	0.1	-	-	-	-
73	43, 55, 67, 71, 79, 94, 108, 136, 168 M^+	-	0.6	0.6	0.8	0.7	0.4	0.7	0.6	1.0	1.0	1.1	0.9
74	methyl nonanoate (9:0)	0.2*	-	-	-	-	-	-	-	-		-	
76	methoxybenzaldehyde	*	-	-	-	-	-	-	-	-	-	-	
77	dimethyl hexanedioate	-	0.5	-	-	-	-	0.1	0.1	-	-	-	-
80	43, 55, 61, 71, 94, 103, 123, 141, 154	-	2.0	0.9	0.9	0.8	0.4	0.5	0.6	2.0	2.3	2.5	1.9
82	43, 55, 85, 98, 111, 115, 130, 151, 183	-	0.2	0.7	0.7	0.6	0.2	0.3	0.2	1.3	2.1	1.6	1.9
83	methyl 3-phenylpropionate	-	-	-	0.1	0.1	trace	0.1	0.3	0.3	0.2	0.2	0.8
84	1,4-dihydroxybenzene	-	5.5	-	-	-	-	-	-	-	-	-	-
86	2'-methoxyacetophenone	0.2	-	-	-	-	-	-	-	-	-	-	-
87	43, 55, 71, 81, 95, 109, 151, 169	-	2.9	0.1	*	*	0.5	0.3	0.3	1.9	2.5	1.3	2.7
89	2-methoxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione	1.4	-	-	-	-	-	-	-	-	-	-	-
90	3'-aminoacetophenone	-	2.6	2.5	2.8	2.0	0.7	1.4	0.9	4.8	5.1	4.8	4.9
95	methyl *cis* 3-phenylprop-2-enoate	-	-	-	-	-	-	trace	0.1	-	-	-	-
97	43, 55, 71, 85, 109, 127, 140, 169	0.1	1.1	0.4	0.4	0.2	0.2	0.3	0.2	1.5	2.3	1.2	1.5
104	43, 59, 69, 74, 87, 110, 118, 130, 159	0.1	2.9	0.7	0.7	0.7	0.3	0.2	0.5	0.7	0.6	1.1	0.6
109	43, 55, 71, 83, 95, 102, 109, 135, 139, 170	0.6	-	-	-	-	-	-	-	-	-	-	-
116	43, 55, 67, 71, 84, 119, 137, 152	0.6	5.6	7.7	6.5	5.1	2.4	3.7	3.0	10.2	10.2	12	10.7
119	methyl 2-hydroxy-3-phenylpropionate	-	-	-	0.4	0.6	trace	-	-	1.7*	1.7*	1.8*	1.7*
120	methyl 3-methoxybenzoate	0.1	-	0.7	0.4	0.5	0.3	0.3	0.3	*	*	*	*
122	43, 55, 69, 80, 85, 117, 121, 166, 199	-	0.5	0.4	0.3	0.2	0.1	0.1	-	0.9	0.9	1.1	0.9
123	methyl *trans* 3-phenylprop-2-enoate	-	0.6	0.4	*	*	*	0.1	0.7	*	*	*	*
125	43, 55, 59, 68, 71, 94, 111, 125, 137, 155	0.3	2.2	1.7	1.6*	1.0*	0.5*	0.3	2.0	0.8*	0.8*	1.0*	0.9*
131	43, 55, 69, 85, 117, 121, 178, 199	-	5.4	6.7	7.0	6.9	2.8	2.9	-	0.6	0.8	0.8	0.6
134	51, 77, 91, 107, 138	5.2	-	-	-	-	-	-	-	-	-	-	-
137	dimethyl octanedioate	trace	0.9	0.2	0.3	0.3	0.1	0.1	0.2	0.7	0.7	0.7	0.8

138	1-(3-oxo-1-butenyl)-2,6,6-trimethyl-1,2-epoxycyclohexan-4-ol	-	-	trace	-	-	-	*	0.3				
139	methyl 3-hydroxybenzoate	*	*	-	-	-	-	*	*	-	-		
140	methyl 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoate	1.6*	4.7*	3.9	3.1	2.9	1.1	1.3*	1.3*	4.8	6.1	5.1	5.0
142	43, 59, 79, 109, 121, 137, 152, 177	4.6	-	-	-	-	-	-	-	-	-	-	-
144	43, 55, 69, 83, 97, 111, 126, 143, 158	-	-	0.2	0.8	0.8	0.1	0.1	0.2	1.6	1.8	1.9	1.7
145	41, 55, 67, 79, 93, 107, 121, 151, 166	7.6	-	-	-	-	-	-	-	-	-	-	-
148	55, 81, 108, 136, 140, 168 M^+	-	1.5	0.8	-	0.4	0.1	0.5	0.2	1.1	1.5	1.5	1.8
149	43, 65, 92, 120, 135, 163 M^+	-	1.4	5.0	5.6	4.1	2.6	4.4	2.7	4.4	4.6	4.1	3.1
151	methyl 4-hydroxy-3-methoxybenzoate	-	1.0	2.1	1.7	1.2	1.3	1.1	0.9	1.1	1.4	1.3	1.2
153	1-(3-oxo-1-butenyl)-2,6,6-trimethyl-1,2-epoxycyclohexan-4-ol	-	0.6	0.8	1.1	0.8	0.4	1.7	0.6	3.5	1.4	4.8	4.1
157	methyl laurate (12:0)	-	0.1	0.7	-	-	-	trace	0.8	0.2	0.2	0.2	0.1
158	dimethyl nonanadioate	-	0.3	0.1	0.1	0.2	trace	0.1	0.1	0.3	0.3	0.3	0.2
162	41, 55, 81, 95, 113, 138, 161, 166, 192	3.0	0.9	-	-	-	-	0.2	-	-	-	-	-
163	methyl 3,4-dimethoxybenzoate	-	0.4	0.9	1.6	1.5	0.6	0.7	0.3	0.5	0.9	0.6	0.7
166	41, 55, 69, 105, 121, 175, 193, 208 M^+	0.4	-	-	-	-	-	-	-	-	-	-	-
168	methyl 2-hydroxy-3-(4-methoxyphenyl)-propionate	-	-	-	-	-	-	-	-	0.5	0.6	0.2	0.2
171	4-(3-oxo-1-butenylidene)-3,5,5-trimethyl cyclohex-2-en-1-one	0.9	-	-	-	-	-	-	-	-	-	-	-
174	dimethyl decanedioate	0.4	4.4	1.3	1.8	1.5	0.4	0.6	1.1	2.0	2.3	2.9	2.5
177	methyl 3-(4-methoxyphenyl)-*trans*-prop-2-enoate	0.7	0.2	0.1	0.2	0.2	trace	0.1	0.1	0.3	0.3	0.2	0.3
180	methyl 3-hydroxy-3-(methoxyphenyl)-propionate	0.1	0.3	trace	-	0.1	trace	0.1	0.2	0.6	0.6	0.6	0.2
183	dimethyl *trans* 2-decenedioate	0.7	14	3.3	3.5	2.5	0.7	1.1	2.3	8.2	7.6	9.8	8.5
184	methyl 3,4,5-trimethoxybenzoate	0.5	0.3	4.1	4.4	3.6	2.4	3.2	0.5	0.4	0.3	0.5	0.5
185	43, 77, 91, 107, 125, 147, 167, 208 M^+	1.8	-	-	-	-	-	-	-	-	-	-	-
186	methyl 3-(4-hydroxyphenyl)-*trans*-prop-2-enoate	1.5	0.7	0.2	-	0.4	0.1	0.1	0.2	0.7	-	0.3	0.4
187	methyl myristate (14:0)	0.2	0.4	0.5	0.1	0.3	0.2	0.2	0.2	0.5	0.4	-	0.4
188	methyl 4-hydroxy-3,5-dimethoxybenzoate	-	4.2	0.7	2.4	3.1	0.7	0.4	2.8	11	14	12	8.4
190	methyl 3-(3,4-dimethoxyphenyl)-*cis*-prop-2-enoate	-	0.9	-	0.2	0.3	0.2	0.3	0.4	0.6	0.3	0.3	0.5
191	43, 77, 95, 124, 163, 180, 209	5.2	-	-	-	-	-	-	-	-	-	-	-
193	4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	0.6	1.8	1.3	-	-	-	-	-	-	-	-	-
199	methyl pentadecanoate (15:0)	0.2	trace	0.2	0.1	0.2	trace	0.1	0.1	0.1	0.1	0.1	0.1
200	methyl 3-(3,4-dimethoxyphenyl)-*trans*-prop-2-enoate	0.1	-	-	-	-	-	0.3	0.2	-	-	-	-
205	isomer of [22]	-	4.4	15	17	10.2	9.4	7.4	2.0	1.2	43	1.8	23
206	1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethyl-cyclohexane-*trans*-*cis*-1,2,4-triol [22]	-	82	72	61	52	40	25	27	72	82	110	87
209	methyl palmitate (16:0)	1.5	1.9	4.3	4.5	4.9	1.7	2.5	2.7	7.6	10.5	9.5	8.9
213	methyl margarate (17:0, internal standard)												
214	methyl *trans*-*cis*-abscisate	106	10.1	8.1	7.7	10.6	0.5	1.6	5.3	1.3	0.8	0.9	1.5
216	methyl linoleate (18:2)	*	*	*	*	*	*	1.0	*	*	*	*	*
217	methyl α-linolenate (18:3)	2.3*	2.4*	4.7*	7.8*	6.9*	1.2*	1.9	3.5*	9.9*	15*	13*	14*
218	methyl oleate (18:1)	1.0	2.1	3.5	2.7	3.4	1.8	1.8	1.7	3.0	4.5	5.2	3.9
220	*n*-heneicosane (C_{21})	0.2	0.1	0.7	0.5	0.5	0.2	0.3	0.5	0.2	0.8	0.5	0.4
221	methyl stearate (18:0)	trace	*	5.3	4.0*	5.6*	0.6	1.2	*	1.0	0.5	0.5	0.4
222	methyl *trans*-*trans*-abscisate	42	7.4	*	*	*	-	-	3.1*	-	-	-	-
225	*n*-triacosane (C_{23})	0.4	0.9	1.3	1.1	1.3	1.0	1.4	1.4	1.7	2.6	3.4	1.5

[a] detected in GC/FID but not in GC/MS.

* unresolved GC/FID peak.

and 64 (average levels 2.0 μg/g and 14 μg/g for thyme honey, 0.6 μg/g and 2.4 μg/g; 1.3 μg/g and 22 μg/g were found in nodding thistle and kamahi honeys respectively), unknown peak 63 (average levels 0.8 μg/g for thyme honey, 0.3 μg/g were found in white clover type honey), 4-hydroxy-3,5-dimethoxybenzoic acid (peak 188, average level 5.4 μg/g for thyme honey, 92 μg/g were found in manuka honey) and 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoic acid (peak 138, average level 3.4 μg/g for thyme honey, 16 μg/g were found in nodding thistle honey).

Table 5.VII Pollen composition of thyme honeys expressed as a percentage of total pollen of nectar plants counted.

pollen type	W207	H384	H404	H405	H406	H407
thyme (*Thymus*)	-	26	28	28	18	27
willow (*Salix*)	90	41	33	24	28	5
white clover type (*Trifolium*)	-	13	16	22	26	7
matagouri (*Discaria*)	-	-	13	12	10	27
rosaceae	-	5	-	7	13	8
gorse type (*Ulex* type)	-	-	-	-	-	20
vipers bugloss (*Echium vulgare*)	-	9	-	-	-	-
remainder	10	6	10	7	5	6

Thus for a sample to be considered as unifloral thyme honey in terms of chemical criteria, it should afford levels of extractives similar to those found for thyme honeys in Table 5.VI and a gas chromatogram similar to Figure 5.6.

Pollen data in Table 5.VII revealed that the thyme honey samples H384, H404, H405, H406 and H407 possessed a significant contribution from willow pollens (5 to 41%). Only a single willow honey sample (90%

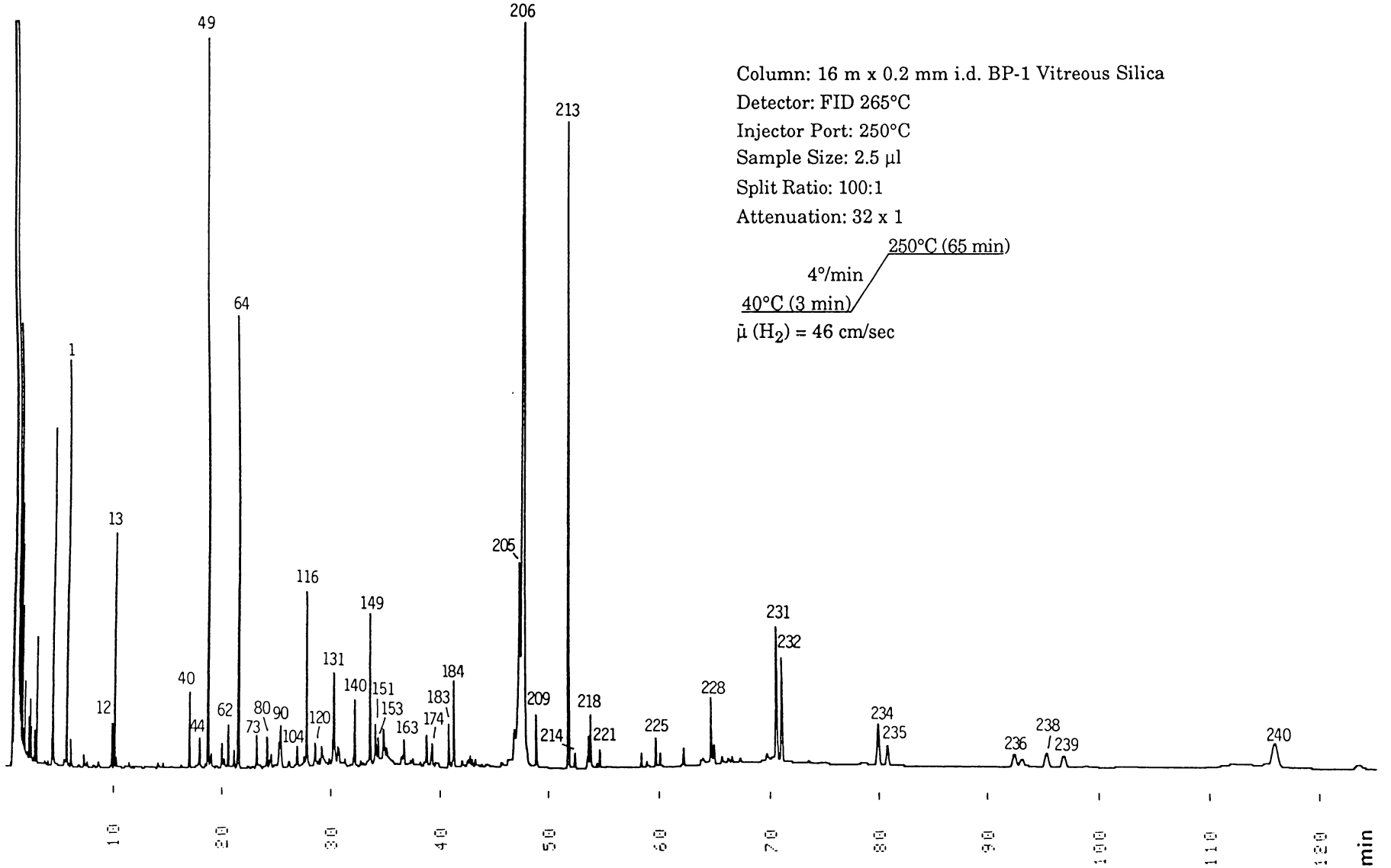


Figure 5.6 Gas chromatographic profile of a representative thyme honey (sample H407). For peak identification see Tables 3.II and VI.

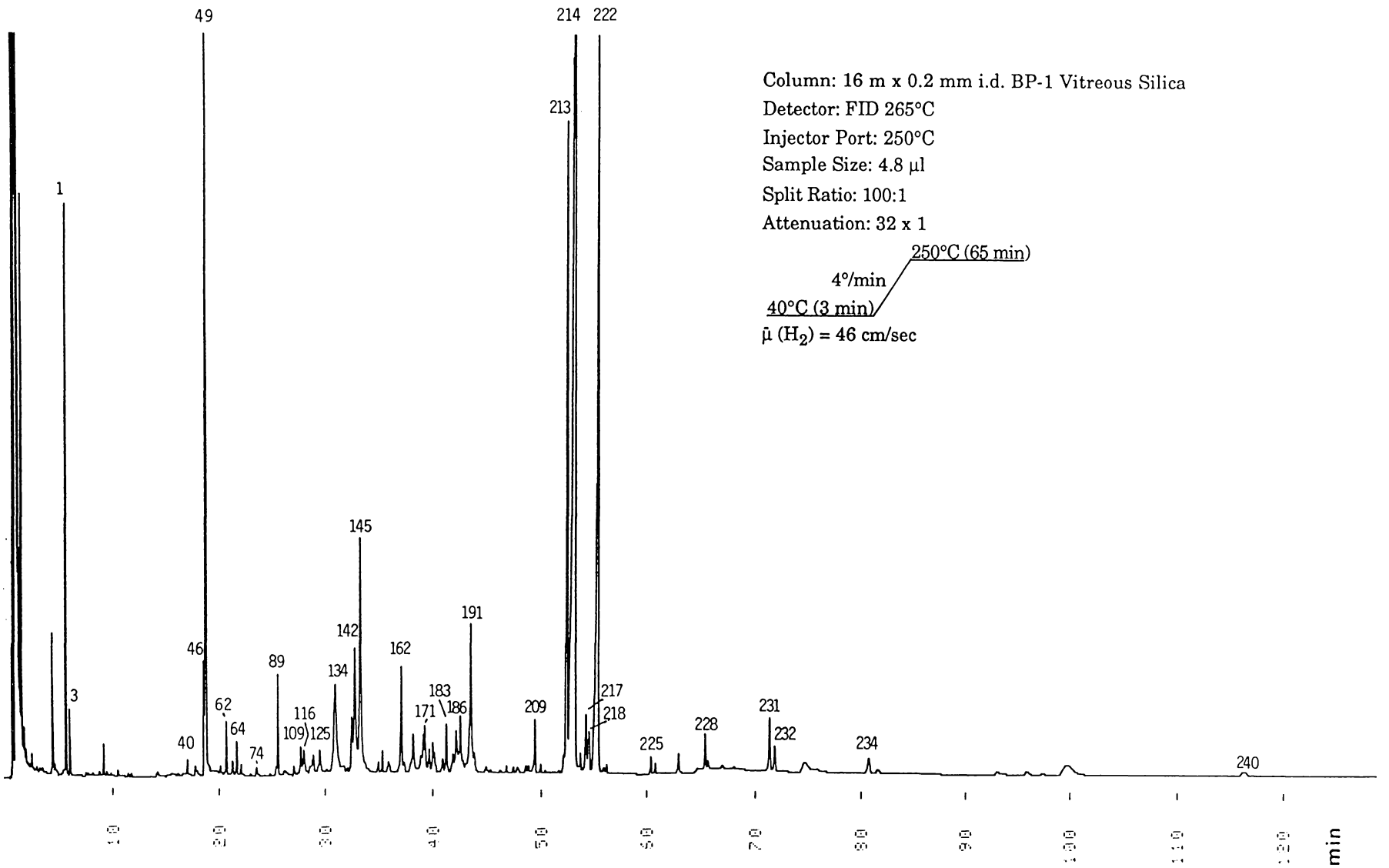


Figure 5.7 Gas chromatographic profile of willow honey (sample W207). For peak identification see Tables 3.II and 5.VI.

willow pollen) was available, hence data for this sample is included in Table 5.VI and VII.

The GC trace of the willow honey extractives is shown in Figure 5.7. While thyme honey extractives were dominated by peak 206, the willow extractives were dominated by *trans-cis* and *trans-trans*-abscisic acids (peaks 214 and 222, see Section 4.9). Other extractives include 2-methoxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione (peak 89, 1.4 μg/g *cf.* average level 1.5 μg/g in the 1985-86 season heather honeys), 4-(3-oxo-1-butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one (peak 171, 0.9 μg/g *cf.* average level 33 μg/g in the 1985-86 season heather honeys) and the unknown peaks 46, 134, 142, 145 and 191.

Because data for only a single willow honey is available, levels of components in willow honey cannot be regarded as typical. The available data indicates that willow honey should possess high levels of *trans-cis* and *trans-trans*-abscisic acids and exhibit a gas chromatogram similar to Figure 5.7.

Given Moar's requirement (see above) that a 20% thyme pollen contribution is necessary for unifloral classification, it can be concluded that samples H384, H404, H405 and H407 are unifloral. The 18% thyme pollen contribution of sample H406 implies that this sample is also substantially thyme honey.

The level of *trans-cis*-abscisic acid (peak 214) was 106 μg/g in the willow honey sample (90% willow pollen contribution as revealed by pollen analysis). Although significant willow pollen were found in samples H384, H404, H405 and H406, much lower levels of *trans-cis*-abscisic acid were found (10.1, 8.1, 7.7 and 6.9 μg/g respectively). These levels suggest willow contributions of 10 to 7% in the thyme honey samples, as opposed to

41 to 24% contributions by pollen analysis. It is apparent that in circumstances where the pollen counts of the contributing species differ substantially (thyme honey is known to exhibit under-represented pollen counts), chemical data can afford a more reliable assessment of the contributing nectar sources. It is likely that the pollen data and abscisic acid levels are related in a non-linear fashion.

Sample H384 also possessed a 9% pollen contribution from vipers bugloss. Examination of the chemical data in Table 5.VI revealed that this sample contained 5.5 μg/g of 1,4-dihydroxybenzene which was not detected in any other honeys except samples with vipers bugloss contribution (average level 21 μg/g, see Section 5.6).

5.6 Vipers Bugloss honey

Figure 5.8 is a representative chromatogram of vipers bugloss honey. Table 5.VIII lists the components and the concentrations found for the ten honey samples examined, all of which displayed similar GC profiles. Absolute pollen counts of vipers bugloss honeys demonstrate that it falls into the "normal" category where a minimum of 45% vipers bugloss pollen is required to be reasonably considered a sample as unifloral (Moar, 1985).

Vipers bugloss honey is characterised by the presence of 2,5-cyclohexadiene-1,4-dione (peak 8), 4-methoxyphenol (peak 67), hydroxybenzyl alcohol (peak 113), hydroxybenzaldehyde (peak 117) and a high concentration of 1,4-dihydroxybenzene (hydroquinone, peak 84). Concentrations of these compounds were in the range of 0.2 to 3.0 μg/g, average 0.9 μg/g; 0.3 to 1.7 μg/g, average 0.8 μg/g; 0.8 to 3.4 μg/g, average 1.8 μg/g;

Table 5.VIII Concentration (μg/g) of components in vipers bugloss honeys (acids as methyl esters).

Peak	Compound (prominent MS ions)	VB102	VB103	VB104	VB105	VB106	VB107	VB124	VB125	VB126	VB1
1	unknown[a]	5.0	14	5.2	12	1.9	4.0	1.9	1.3	3.0	0.7
3	43, 59, 74, 83, 101	2.0	5.8	0.1	5.5	0.5	0.3	0.5	0.2	1.6	-
8	2,5-cyclohexadiene-1,4-dione	1.0	1.8	0.6	3.0	0.7	1.1	0.3	0.2	0.5	0.2
15	benzaldehyde	0.1	0.2	0.1	0.4	trace	0.2	trace	trace	0.1	-
31	dimethyl butanedioate	-	0.1	0.1	0.2	trace	trace	-	trace	0.1	trace
33	phenylacetaldehyde	0.1	0.7	0.2	0.4	0.1	0.3	0.3	0.2	0.6	0.4
40	methyl benzoate	0.1	0.3	0.1	0.3	trace	0.1	0.1	trace	0.2	0.1
43	43, 58, 66, 94, 125 M^+	trace	0.1	0.1	-	-	-	0.1	trace	trace	0.1
49	*n*-undecane (C_{11}, internal standard)										
62	methyl 2-phenylethanoate	0.1	0.3	0.1	0.3	0.1	0.2	0.2	0.1	0.3	0.1
63	43, 59, 83, 101, 116, 141, 159	0.5	0.5	0.2	0.5	0.1	0.3	0.4	0.2	0.3	-
66	5-hydroxymethyl-2-furfural	0.3	0.4	0.2	0.2	-	0.4	0.5	0.2	0.3	0.4
67	4-methoxyphenol	1.4	0.6	0.5	1.7	0.5	0.5	0.7	0.3	1.0	1.1
76	methoxybenzaldehyde	1.5	0.5	0.9	3.8	1.4	0.3	0.2	0.1	0.2	0.1
84	1,4-dihydroxybenzene (hydroquinone)	18	19	22	28	21	22	22	25	16	21
104	43, 59, 74, 87, 118, 130, 150 fatty acid?	0.2	0.2	0.2	0.3	0.1	0.4	trace	-	-	-
112	43, 69, 71, 97, 124, 152, 170 M^+?	1.9	0.5	0.5	1.2	0.8	0.2	0.6	0.1	0.2	-
113	hydroxybenzyl alcohol	3.4	2.7	2.0	1.8	0.8	1.6	2.2	0.6	0.9	1.6
117	hydroxybenzaldehyde	1.0	2.2	2.1	1.8	0.3	4.1	1.1	1.1	1.4	-
120	methyl 3-methoxybenzoate	0.2	0.2	0.1	0.3	0.1	0.1	0.1	trace	0.1	trace
123	methyl *trans*-3-phenylprop-2-enoate	0.2	0.4	0.3	0.4	0.1	0.3	1.2	0.2	1.8	0.3
137	dimethyl octanedioate	0.1	-	-	0.1	0.1	0.1	trace	trace	-	trace
139	methyl 3-hydroxybenzoate	1.2	1.4	1.0	1.5	0.6	1.2	1.1	0.5	0.9	0.8
155	55, 59, 74, 87, 97, 125, 157	1.2	0.8	1.1	0.9	0.3	1.2	0.8	0.4	0.4	-
162	41, 55, 81, 95, 113, 138, 166	2.6	1.6	2.7	1.6	0.6	3.2	2.0	0.8	0.6	-
169	methyl 3-(4-hydroxyphenyl)-*cis*-prop-2-enoate	0.1	trace	0.1	trace	0.1	-	0.4	0.1	0.3	-
174	dimethyl decanedioate	0.8	0.2	0.2	0.4	0.4	0.3	0.2	0.1	0.1	0.1
178	43, 81, 95, 113, 137, 152	0.7	-	0.2	0.2	0.2	0.1	0.1	0.1	trace	-
180	methyl 3-hydroxy-3-(methoxyphenyl)-propanoate	0.2	0.1	0.3	0.3	0.2	0.3	0.2	0.1	0.1	-
183	dimethyl *trans* 2-decenedioate	2.3	0.8	0.8	1.4	1.8	1.2	0.6	0.4	0.5	0.2
186	methyl 3-(4-hydroxyphenyl)-*trans*-prop-2-enoate	trace	trace	trace	-	trace	0.1	0.5	0.1	0.3	-
187	methyl myristate (14:0)	0.2	0.2	trace	trace	trace	0.1	0.1	trace	0.1	0.2
190	methyl 3-(3,4-dimethoxyphenly)-*cis*-prop-2-enoate	-	-	-	-	trace	-	0.2	trace	0.1	0.1
198	81, 106, 160, 188, 215, 216 M^+	0.2	-	-	0.2	0.5	-	-	-	-	0.4
199	methyl pentadecanoate (15:0)	0.1	-	trace	-	-	-	-	trace	0.1	-
200	methyl 3-(3,4-dimethoxyphenly)-*trans*-prop-2-enoate	-	-	-	-	trace	-	0.1	-	0.1	-
203	81, 106, 160, 118, 215, 216 M^+	0.3	-	-	0.2	0.6	-	-	-	-	0.6
207	methyl palmitioleate (16:1)	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.2
209	methyl palmitate (16:0)	1.0	0.9	1.3	0.5	0.6	0.8	0.7	0.3	0.8	1.6
213	methyl margarate (17:0, internal standard)										
214	methyl abscisate	0.2	0.1	0.2	0.1	0.2	0.2	0.4	0.3	0.2	0.3
216	methyl linoleate (18:2)	0.1	0.2*	0.5*	0.1*	0.2	0.2*	0.1	0.1*	0.3*	0.2
217	methyl α-linolenate (18:3)	0.3	*	*	*	0.2	*	0.2	*	*	0.6
218	methyl oleate (18:1)	0.4	0.5	0.6	0.3	0.4	0.5	0.5	0.2	0.4	2.1
220	*n*-heneicosane (C_{21})	0.1	0.1	0.3	0.1	0.1	0.2	0.1	trace	0.1	0.1
221	methyl stearate (18:0)	0.3	0.3	0.3	0.2	0.3	0.2	0.3	0.1	0.2	0.8
225	*n*-triacosane (C_{23})	0.5	0.7	1.7	0.5	0.5	0.7	0.5	0.2	0.4	0.5

[a] detected in GC/FID but not in GC/MS.

* unresolved GC/FID peak.

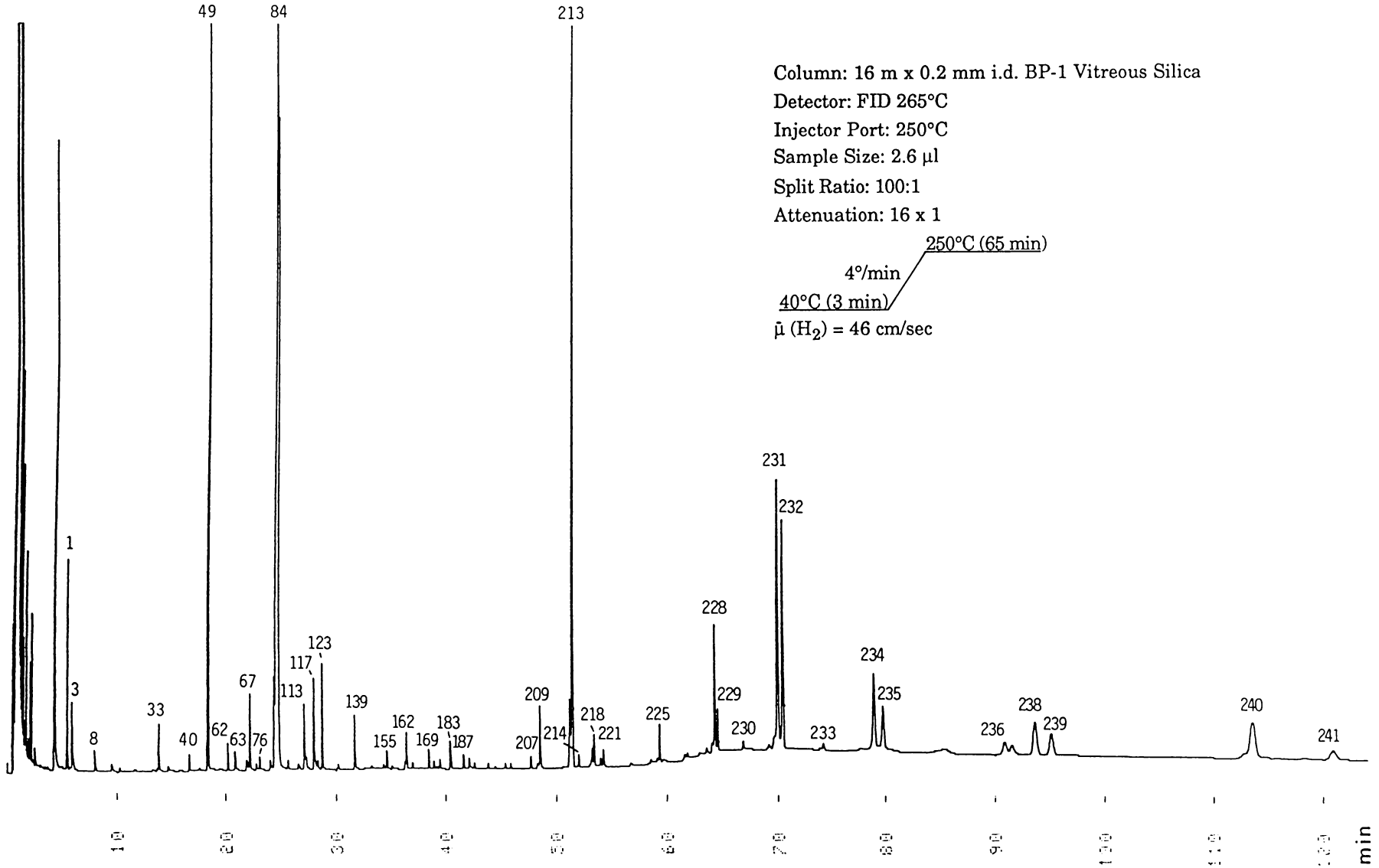


Figure 5.8 Gas chromatographic profile of a representative vipers bugloss honey (sample VB126). For peak identification see Tables 3.II and 5.VIII.

trace to 2.2 μg/g, average 1.5 μg/g and 16 to 28 μg/g, average 21 μg/g respectively.

In addition to the above compounds, which appear to be specific to vipers bugloss honey, other substances detected included unknown peak 43 (average level 0.1 μg/g), unknown peak 63 (average level 0.3 μg/g *cf.* 0.3 μg/g in white clover type honey), *trans*-3-phenylprop-2-enoate (peak 123, average level 0.5 μg/g *cf.* 0.7 μg/g in white clover type honey) and 3-hydroxybenzoic acid (peak 139, average 1.0 μg/g cf. 0.2 μg/g in white clover type honey).

For a sample to be considered as a unifloral vipers bugloss honey, it should possess a level of 1,4-dihydroxybenzene similar to those found in Table 5.VIII and a gas chromatogram similar to that shown in Figure 5.8. Since 1,4-dihydroxybenzene was only detected in honeys having a vipers bugloss contribution, its presence in the extracts of other unifloral honeys is indicative of a minor vipers bugloss honey contribution to these honeys. An example of this is found in thyme honey sample H384 with 5.5 μg/g (see Table 5.VI); the pollen analysis of this sample includes a 9% vipers bugloss contribution.

5.7 Nodding Thistle Honey

Ten nodding thistle honeys were analysed. A representative GC trace of nodding thistle honey is displayed in Figure 5.9. Table 5.IX lists the components and the concentrations found in the ten honeys examined, all of which gave similar GC profiles. Pollen analysis results for only one nodding thistle honey (sample H403) were available. It is clear from the data included in Table 5.X that sample H403 possess a significant white

Table 5.IX Concentration (μg/g) of components in nodding thistle honeys (acids as methyl esters).

Peak	Compound (prominent MS ions)	N1	N3	TH1	8N	38N	68N	77N	205N	206N	H403
1	unknown[a]	1.9	3.7	2.6	4.2	4.0	4.6	1.7	2.8	1.7	3.7
3	43, 59, 74, 83, 101	2.1	2.7	1.6	0.8	0.6	1.1	0.3	1.0	0.6	0.8
18	methyl 3-furancarboxylate	0.2	0.3	-	-	0.2	0.2	-	0.1	-	0.1
23	unknown[a]	-	-	-	-	-	-	-	-	-	0.8
31	dimethyl butanedioate	2.4	1.2	0.1	0.2	4.9	3.2	0.1	0.6	0.2	0.8
32	benzyl alcohol	0.3	trace	-	trace	trace	0.1	trace	trace	trace	0.1
37	methyl 2-furancarboxylate	0.2	0.1	-	-	0.5	0.2	0.1	0.1	-	0.4
38	unknown[a]	-	-	-	0.2	-	-	0.2	-	0.2	-
40	methyl benzoate	1.0	0.4	0.3	0.5	0.4	0.3	0.2	0.2	0.1	0.4
41	2-phenylethanol	trace	trace	0.3	0.1	0.2	0.1	trace	0.1	trace	0.1
44	43, 67, 71, 82, 109, 125	1.4	1.4	1.0	0.2	0.5	0.2	0.2	0.4	0.3	0.4
48	51, 78, 106, 137 M^+	0.1	trace	-	trace	0.2	0.3	0.1	trace	trace	0.1
49	*n*-undecane (C_{11}, internal standard)										
55	43, 55, 67, 71, 93, 111, 125, 153	1.3	2.6	1.1	0.4	0.7	0.5	0.3	2.0	0.7	-
58	43, 55, 67, 71, 93, 111, 125, 153	2.6	5.0	2.1	0.8	1.3	1.7	0.4	3.7	1.2	-
61	43, 55, 67, 71, 93, 111, 125, 153	1.5	2.7	1.2	0.4	0.7	0.5	0.2	1.9	0.5	-
62	methyl 2-phenylethanoate	3.6	5.5	3.7	1.5	3.0	1.3	1.0	3.0	1.7	1.9
63	43, 59, 83, 116, 141, 159	1.0	1.4	1.3	0.4	0.2	0.2	0.2	0.3	0.3	0.5
64	43, 67, 71, 82, 109, 125, 137	3.5	6.0	2.8	1.9	2.5	1.0	0.8	2.9	1.8	1.0
66	5-hydroxymethyl-2-furfural	2.1	0.2	0.2	0.2	1.4	1.9	1.0	0.5	0.4	2.1
68	43, 55, 67, 71, 93, 111, 125, 155	2.4	2.3	0.8	0.6	0.7	0.5	0.2	0.9	0.6	0.6
71	43, 55, 67, 71, 93, 111, 125, 155	-	-	-	0.1	-	-	-	-	0.1	-
76	methoxybenzaldehyde	-	1.1	0.7	-	-	-	-	-	0.3	1.0
77	dimethyl hexanedioate	0.6	-	-	0.1	0.4	0.1	-	0.2	-	-
81	43, 55, 93, 111, 137, 155	-	-	-	-	-	-	trace	0.2	0.2	-
83	methyl 3-phenylpropionate	-	-	-	-	-	0.1	0.1	-	-	-
88	43, 55, 57, 75, 93, 111, 137, 155	-	-	-	-	-	-	-	-	0.2	-
93	43, 55, 67, 75, 93, 111, 137, 155	-	-	-	-	-	-	0.1	0.4	0.4	0.4
95	methyl *cis*-3-phenylprop-2-enoate	-	-	-	0.1	0.5	trace	0.1	-	-	-
101	43, 55, 67, 75, 93, 111, 137, 155, 171	-	-	-	-	-	-	0.1	0.2	0.3	-
104	43, 59, 74, 87, 118, 129, 130, 156	0.2	0.3	0.2	0.3	0.6	0.2	0.1	0.2	0.1	0.7
111	43, 55, 69, 71, 97, 109, 135, 152, 170	5.8	12	6.0	3.0	4.9	2.1	1.6	8.7	6.5	2.1
114	43, 55, 71, 96, 109, 139, 157	2.2	13*	3.7	1.7	2.7	1.1	0.8	2.8	2.2	1.6
116	43, 67, 71, 84, 119, 137, 152	5.3	*	3.5	1.8	2.3	1.3	0.9	3.0	2.0	1.6
119	methyl 2-hydroxy-3-phenylpropionate	-	-	0.7	-	0.4	0.2	-	-	-	-
121	43, 67, 71, 93, 110, 119, 137	9.1	11	8.3	3.1	6.1	3.0	1.9	4.6	4.2	3.4
123	methyl *trans*-3-phenylprop-2-enoate	4.7	3.3	4.2	1.5	1.6	3.7	6.5	0.7	0.3	2.5
130	43, 55, 69, 97, 109, 129, 155	-	-	0.2	0.5	1.2	0.6	0.5	1.0	1.7	1.3
137	dimethyl octanedioate	0.7	0.3	0.2	0.4	0.4	0.3	0.1	0.1	0.1	0.3
140	methyl 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoate	30	29	28	9.9	14	6.5	6.4	11	8.6	13
148	55, 59, 81, 108, 136, 140, 168 M^+	-	-	-	0.2	0.7	0.3	-	-	-	-
155	55, 59, 74, 87, 125, 150, 157	0.7	1.2	0.9	0.2	0.5	0.1	0.4	1.0	1.0	0.7
157	methyl laurate (12:0)	0.1	trace	-	-	0.2	0.1	-	0.2	0.3	-
158	dimethyl nonanedioate	0.3	0.2	0.1	0.1	0.4	0.2	0.1	0.3	0.3	0.1
159	45, 55, 67, 81, 100, 113, 156	*	*	*	0.6	0.6	0.3	0.4	0.3	-	0.5
160	methyl 3,5-dimethoxybenzoate	*	*	*	*	*	*	*	*	*	*
162	41, 55, 81, 95, 113, 138, 166	2.3	4.6*	1.8*	1.7*	1.3*	0.5*	0.6*	2.4*	1.9*	1.3*
165	55, 74, 87, 98, 110, 123, 172	-	-	-	-	-	0.1	0.2	-	-	-
174	dimethyl decanedioate	4.3	1.6	0.3	1.5	2.3	1.4	0.1	0.9	0.8	2.2
177	methyl 3-(4-methoxyphenyl)-*trans*-prop-2-enoate	1.0	0.5	0.4	0.4	0.3	0.1	0.1	0.3	0.5	0.9

180	methyl 3-hydroxy-3-(methoxyphenyl)-propanoate	0.1	0.6	0.6	0.2	0.4	0.3	0.1	0.2	0.1	0.3
183	dimethyl *trans*-2-decenedioate	15	6.8	6.1	6.7	7.6	5.4	4.4	2.9	2.4	8.8
184	methyl 3,4,5-trimethoxybenzoate	0.1	0.2	0.2	-	0.1	trace	0.1	-	0.1	0.5
186	methyl 3-(4-hydroxyphenyl)-*trans*-prop-2-enoate	1.6	0.2	0.5	0.2	0.4	0.2	0.3	0.4	0.3	0.9
187	methyl myristate (14:0)	0.4	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.1	0.2
188	methyl 4-hydroxy-3,5-dimethoxybenzoate	1.5	0.6	0.6	-	0.6	0.5	-	-	0.4	0.9
190	methyl 3-(3,4-dimethoxyphenyl)-*cis*-prop-2-enoate	1.2	0.3	0.8	0.3	0.4	0.7	1.1	0.2	0.2	1.2
199	methyl pentadecanoate (15:0)	0.4	0.1	0.3	0.1	0.2	trace	0.3	trace	trace	0.1
200	methyl 3-(3,4-dimethoxyphenyl)-*trans*-prop-2-enoate	1.1	0.4	0.6	0.1	0.4	1.7	2.2	0.1	0.1	1.2
207	methyl palmitioleate (16:1)	0.2	0.3	0.2	0.1	0.5	0.1	0.2	0.1	0.1	0.1
209	methyl palmitate(16:0)	2.4	1.8	1.5	1.0	1.9	1.2	1.1	0.8	0.7	1.1
213	methyl margarate (17:0, internal standard)										
214	methyl abscisate	0.3	0.5	0.7	0.2	0.2	0.2	0.2	0.2	0.2	0.2
215	unknown (77, 91, 105, 134, 181, 208, 276 M^+)	-	-	0.2	-	-	0.1	0.2	-	-	0.3
216	methyl linoleate (18:2)	0.8	0.4	0.4	0.2	0.7	*	0.5	0.2	0.3	0.5
217	methyl α-linolenate (18:3)	1.4	0.7	0.8	0.4	1.5	1.5*	0.5	0.2	0.3	0.2
218	methyl oleate (18:1)	2.7	2.1	1.7	1.0	1.8	1.3	1.1	0.7	0.8	1.3
220	*n*-heneicosane (C_{21})	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.2
221	methyl stearate (18:0)	0.7	0.5	0.5	0.2	0.3	0.2	0.3	0.2	0.2	0.3
225	*n*-triacosane (C_{23})	0.9	0.4	0.8	0.6	0.6	0.4	0.6	0.5	0.4	0.7

[a] detected in GC/FID but not in GC/MS.

* unresolved GC/FID peak.

clover type contribution; even so, the relatively low thistle component (7%) indicates a considerable input of nodding thistle nectar (Moar, personal communication).

Table 5.X Pollen composition of nodding thistle and kamahi honeys expressed as a percentage of total pollen of nectar plants counted.

pollen type	H403	H363	H402	M1
white clover type (*Trifolium repens type*)	77	47	58	8
kamahi (*Weinmannia*)	-	32	26	79
nodding thistle (*Cirsium* type)	7	-	-	-
rewarewa (*Knightia excelsa*)	-	7	-	-
pohutukawa (*Metrosideros*)	6	-	-	-
lotus	5	-	-	-
remainder	5	4	16	13

Nodding thistle honey is dominated by 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoic acid (peak 140). Levels of this compound range from 6.4 to 30 µg/g, average 16 µg/g. In addition to peak 140, a number of components could not be identified which appeared to be unique to nodding thistle honey. For example, peak 55 (average level 1.1 µg/g), peak 58 (average level 2.1 µg/g), peak 61 (average level 1.1 µg/g), peak 68 (average level 0.9 µg/g), peak 111 (average level 5.3 µg/g), peak 114 (average level 3.2 µg/g), peak 116 (average level 2.4 µg/g) and peak 121 (average level 5.5 µg/g).

Other components detected include peak 44 (average level 0.6 µg/g *cf.* 2.0 and 1.3 µg/g in thyme and kamahi honeys respectively), peak 64 (average level 2.4 µg/g *cf.* 14 and 22 µg/g in thyme and kamahi honeys respectively), peak 63 (average level 0.6 *cf.* 0.3 µg/g in both white clover type

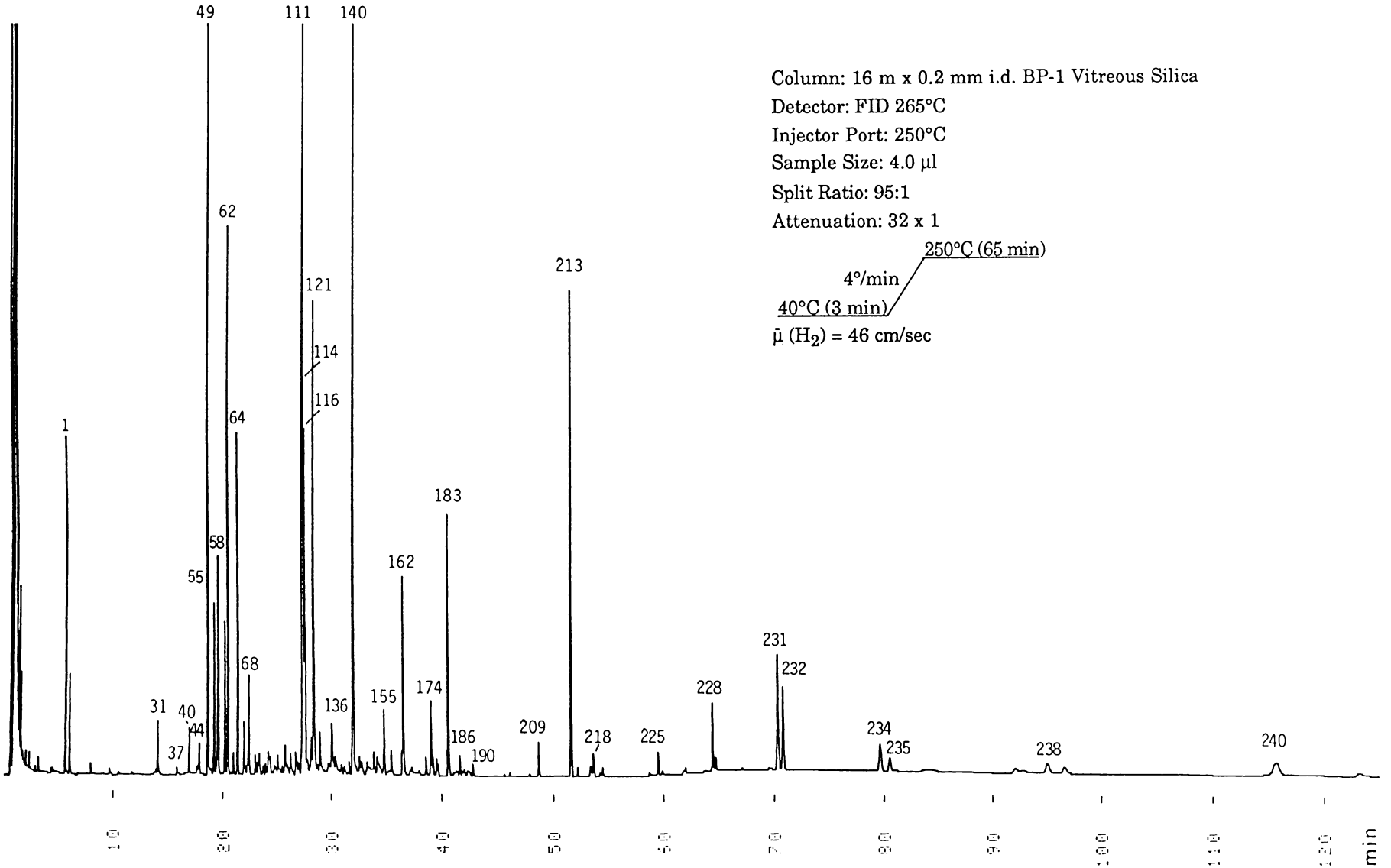


Figure 5.9 Gas chromatographic profile of a representative nodding thistle honey (sample N205). For peak identification see Tables 3.II and 5.IX.

and vipers bugloss honeys), phenylethanoic acid (peak 62, average level 2.9 μg/g *cf.* 0.2 and 117 μg/g in white clover type and heather honeys respectively), 5-hydroxymethyl-2-furfural (peak 66 average level 1.0 μg/g which arise from the dehydration of glucose and fructose), *trans*-3-phenylprop-2-enoic acid (peak 123, average level 2.9 μg/g *cf.* 0.8 and 0.5 μg/g in white clover type and vipers bugloss honeys respectively) and *cis*- and *trans*-3-(3,4-dimethoxyphenyl)-prop-2-enoic acid (peaks 190 and 200, average levels 0.6 and 1.8 μg/g respectively) as well as the diacids (peaks 31, 77, 137, 158, 174 and 183).

3-(3,4-Dimethoxyphenyl)-prop-2-enoic acid is most certain to arise from 3-(3,4-dihydroxyphenyl)-prop-2-enoic acid, 3-(4-hydroxy-3-methoxy-phenyl)-prop-2-enoic acid or 3-(3-hydroxy-4-methoxyphenyl)-prop-2-enoic acid. This should also be true for 3-(4-methoxyphenyl)-prop-2-enoic acid (peak 177) which could be considered to arise from 3-(4-hydroxyphenyl)-prop-2-enoic acid (peak 186). It is perhaps of passing interest that 3-(4-hydroxy-3-methoxyphenyl)-*trans*-prop-2-enoic acid is considered to be a softwood lignin precursor. The existence of this component in honey, however, remains uncertain.

Although some of the nodding thistle honeys examined contained significant white clover type contributions (as revealed by pollen analysis, see Table 5.X), the range of extractives found and their concentrations was greater than that which could be attributed solely to white clover type. This points to at least one other predominant contributing floral source (compare Figure 5.9 and 5.1).

The available information indicates that high quality nodding thistle honey should possess high levels of 2,6-dimethyl-6(S)-hydroxy-2-

trans-2,7-octadienoic acid (peak 140) and exhibit a GC profile similar to Figure 5.9.

5.8 Kamahi honey

A representative GC profile of kamahi honey is depicted in Figure 5.10. Table 5.XI lists the components and the concentrations found for the 11 kamahi honeys examined. The results of pollen analysis obtained for three of the kamahi honey samples are shown in Table 5.X above.

The most abundant component in kamahi honey is peak 208. Its concentration ranged from 10.8 to 156 μg/g, average 85 μg/g. A structure **[43]** has been proposed for peak 208 (see Section 4.8.1). Besides peak 208, other substances which appear to be unique to kamahi honey are unknown peak 28 (average level 5.2 μg/g), 2,6,6-trimethylcyclohexane-1,4-dione (peak 54, average level 0.7 μg/g), peak 59 (average level 4.2 μg/g), peak 201 (average level 3.6 μg/g), peak 202 (average level 6.9 μg/g), peak 210 (average level 6.5 μg/g) and peak 211 (average level 6.7 μg/g). Efforts to elucidate the structures of these and other unknown peaks are continuing.

Apart from the unique components, others substances detected include peaks 44 and 64 (average levels 1.3 and 22 μg/g *cf.* 0.6 and 2.4 μg/g and 2.0 and 14 μg/g in nodding thistle and thyme honeys respectively), peak 62 (average level 0.5 μg/g *cf.* 117 μg/g in ling/heather honey), peak 63 (average level 0.8 μg/g *cf.* 0.3 μg/g in white clover type honey), *trans-cis*-abscisic acid (peak 214, average level 1.0 μg/g cf. 106 μg/g in willow honey).

One kamahi honey sample (M1) was labeled in the field as manuka. The GC profile (compare Figure 5.11, 5.10 and 5.3) and the

Table 5 XI Concentration (μg/g) of components in kamahi honey (acids as methyl esters).

peak	compound (prominent MS ions)	M1	H363	H402	KA2	KA3	KA4	KA5	KA6	KA7	KA8	KA9
1	unknown[a]	16	11	5.6	18	30	9.4	18	59	35	13	27
3	43, 59, 74, 83, 101	0.2	0.2	0.8	2.9	1.3	0.1	0.2	1.1	0.7	0.7	0.5
5	44, 58, 74, 75, 88, 103	-	-	-	-	-	1.0	-	1.3	1.3	1.1	-
7	unknown[a] (manuka)	-	-	-	-	1.5	8.2	-	-	-	-	-
11	43, 55, 59, 71, 74, 87, 101	-	0.9	0.3	-	-	-	1.8	1.0	1.3	1.0	1.5
14	43, 59, 71, 74, 87, 99, 102 M^+	5.1	0.6	0.3	3.3	6.7	6.1	6.1	1.2	2.1	0.8	2.4
17	41, 59, 68, 69, 96, 97	0.1	-	-	0.8	0.1	2.7	0.5	0.6	0.9	0.3	-
18	methyl 3-furancarboxylate	-	1.1	-	1.2	-	0.5	-	-	-	-	-
20	43, 55, 71, 87, 99, 115	-	-	-	0.4	0.5	0.3	-	0.3	0.3	0.3	-
24	phenol	-	2.8	1.2	-	-	-	-	-	-	-	-
25	methyl 2-hydroxy-4-methylpentanoate	0.2	-	-	0.1	0.6	2.3	-	-	-	-	-
26	methyl 2-hydroxy-3-methylpentanoate	0.3	-	-	*	0.7*	1.9	-	-	-	-	-
27	41, 67, 68, 95, 96, 109, 110, 123, 138 M^+	trace	0.7	-	1.0*	*	-	0.4	0.8	0.6	0.4	0.4
28	41, 55, 68, 69, 83, 98 M^+	3.1	2.0	1.2	3.8	7.0	6.5	7.7	3.4	9.5	2.8	10.5
29	42, 55, 70, 79, 97, 125, 140 M^+	1.3	1.7	-	1.4	1.4	1.6	1.2	2.1	1.4	1.3	1.0
30	43, 59, 69, 87, 102, 112, 113, 129, 144 M^+	0.9	-	0.3	-	-	-	1.8	0.7	1.0	0.3	3.6
31	dimethyl butanedioate	0.4	20	0.2	19	2.0	10.8	0.3	5.0	5.2	3.9	0.3
32	benzyl alcohol	0.1	0.4	0.2	0.6	0.3	trace	-	-	-	0.2	-
35	methyl 2-methylsuccinaldehydate	1.0	3.3	0.1	3.9	5.8	12	0.6	6.6	8.8	5.3	1.1
39	dimethyl 2,2-dimethylbutanedioate	0.3	-	-	0.1	0.3	0.2	0.6	0.9	1.1	0.4	1.4
40	methyl benzoate	1.9	1.2	0.6	0.8	0.9	1.0	2.1	0.3	0.8	0.4	0.9
44	43, 55, 67, 71, 82, 109, 125	0.6	1.7	0.1	1.5	1.2	2.8	1.2	2.3	1.3	1.1	0.7
47	3,5,5-trimethylcyclohex-2-ene-1,4-dione	*	0.8	0.3	*	*	*	*	*	*	*	*
49	*n*-undecane (C_{11}, internal standard)	*			*	*	*	*	*	*	*	*
53	43, 59, 75, 85, 101, 117, 146 M^+	-	0.8	-	-	-	-	-	-	-	-	-
54	2,6,6-trimethylcyclohexane-1,4-dione	0.4	trace	0.7	2.0	0.5	0.3	1.2	0.9	0.9	0.3	0.5
59	43, 59, 71, 87, 101, 129, 141, 172 M^+	5.4	1.0	1.0	2.3	6.7	4.3	11	0.8	4.3	1.9	7.4
62	methyl 2-phenylethanoate	0.4	1.1	0.3	0.6	0.8	1.0	0.7	0.1	0.1	0.1	0.1
63	43, 59, 83, 101, 116, 141, 159	0.1	0.3	0.5	1.1	0.4	0.3	0.4	0.8	1.3	1.2	1.0
64	43, 55, 67, 71, 82, 109, 125	21	7.8	5.7	13	33	32	47	15	19	7.0	41
65	5-hydroxymethyl-2-furfural	-	2.0	-	-	-	-	-	0.4	-	0.8	-
70	43, 57, 71, 83, 101, 109, 123, 152, 155	1.2	-	0.5	1.2	1.9	2.2	3.4	0.6	1.9	0.7	4.2
72	43, 55, 70, 81, 101, 109, 124, 152, 170	2.6	-	0.3	0.7	2.0	1.7	2.9	0.4	1.3	0.5	2.2
75	42, 57, 72, 83, 109, 128, 152, 155	2.4	-	0.8	2.0	3.0	3.2	5.0	1.0	2.2	1.1	3.6
79	43, 55, 71, 82, 95. 103, 198	2.0	-	-	-	1.0	-	1.3	-	1.5	1.1	2.2
86	2'-methoxyacetophenone	trace	-	-	-	trace	5.1	*	-	-	-	-
89	2-methoxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione	-	0.4	0.4	-	-	trace	1.6*	-	-	-	-
91	41, 69, 70, 98, 112, 130, 154, 171	-	-	-	-	1.0	0.7	2.1	0.6	1.0	0.4	2.0
96	43, 55, 67, 93, 111, 139, 170, 183, 198 M^+	0.5	1.5	0.3	2.1	1.6	6.0	0.2	3.4	2.6	2.0	1.1
100	43, 55, 70, 95, 111, 125, 140, 157, 168, 180	0.8	-	-	-	1.0	0.8	-	0.5	-	-	-
102	43, 67, 70, 95, 125, 140, 154, 168, 182	3.2	0.3	-	1.0	1.9	2.2	-	0.8	1.2	0.6	1.1
104	43, 59, 60, 69, 87, 97, 118, 129, 156	-	-	0.9	1.9	5.3*	-	3.6*	1.6	2.4	1.3	2.0
105	41, 59, 69, 97, 101, 129, 156 M^+	-	9.9	-	-	-	-	-	1.5	2.5	1.3	3.7
107	methyl 2-methoxybenzoate	trace	-	-	-	*	8.3	*	-	-	-	-
110	43, 59, 60, 69, 83, 99, 118, 139, 153, 171	1.9	-	0.7	-	2.8	1.9	40	1.7	2.9	0.9	1.8
118	43, 55, 67, 71, 82, 93, 119, 135, 166	*	1.9	3.4	10.5*	34	*	23*	3.3	9.4	4.8	18
119	methyl 2-hydroxy-3-phenylpropionate	17*	6.8	-	*	8.6	186*	*	-	-	1.0	-
123	methyl *trans*-3-phenylprop-2-enoate	trace	1.7	1.7	1.8	-	0.5	*	0.2	trace	trace	-
126	43, 55, 59, 68, 71, 83, 94, 111, 125, 137, 155	1.6	2.5	0.8	-	11	8.6	30*	6.6	27	8	34
132	43, 69, 71, 85, 98, 139, 154, 189	-	4.9	1.2	-	6.1	15	16	9.1	18	11	15
137	dimethyl octanedioate	1.1	0.8	0.2	0.8	trace	0.3	0.2	0.2	0.5	0.3	0.5

139	methyl 3-hydroxybenzoate	-	*	*	-	-	-	*	*	*	*	*
140	2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoate	1.2	10.6*	2.7*	5.2	-	-	0.6*	2.9*	3.9*	2.4*	4.9*
148	55, 59, 81, 108, 136, 140, 168 M^+	-	0.7	0.3	-	-	-	-	-	-	-	-
155	55, 59, 74, 87, 97, 125, 143, 157	-	0.3	1.4	-	1.5	-	-	0.5	trace	0.2	trace
156	43, 55, 67, 71, 83, 98, 113, 137, 153	-	-	-	-	-	-	-	-	9.8	0.7	18
157	methyl laurate (12:0)	0.1	0.1	-	0.1	-	-	-	0.2	-	1.1	-
158	dimethyl nonanedioate	0.4	0.4	0.3	0.3	0.4	0.5	1.0	0.4	0.1	0.2	0.1
160	methyl 3,5-dimethoxybenzoate	trace	-	-	-	-	0.7	-	-	-	-	-
162	55, 67, 81, 113, 138, 161, 192	4.8	0.9	7.0*	1.7	6.9	*	8.3	1.6*	1.9*	0.2*	1.6*
163	methyl 3,4-dimethoxybenzoate	0.4	0.5	*	0.3	-	4.5*	-	*	*	*	*
165	55, 74, 87, 111, 129, 152, 172	-	-	1.8	0.4	1.1	-	1.6	-	-	-	-
168	methyl 2-hydroxy-3-(4-methoxyphenyl)-propionate	4.5	-	-	-	10.1	1.0	2.1	-	0.4	0.5	-
174	dimethyl decanedioate	2.1	4.3	4.1	4.4	4.3	2.8	7.0	1.5	1.7	1.4	0.9
175	43, 55, 71, 81, 95, 100, 135, 152, 180 M^+	-	-	2.2	-	-	-	*	0.4	0.8	0.3	1.1
180	methyl 3-hydroxy-3-(methoxyphenyl)-propanoate	0.2	0.4	-	0.7	1.8	0.5	2.5*	-	-	-	-
183	dimethyl *trans*-2-decenedioate	6.2	13	5.5	13	9.8	9.7	8.7	6.1	6.4	5.2	0.4
184	methyl 3,4,5-trimethoxybenzoate	1.5	-	trace	trace	0.2	0.3	1.0	-	-	0.1	-
187	methyl myristate (14:0)	1.5	0.3	0.3	0.5	1.5	0.5	1.4	0.4	0.2	0.2	0.3
188	methyl 4-hydroxy-3,5-dimethoxybenzoate	4.7	0.2	-	1.0	6.5	25	trace	-	-	0.4	-
190	methyl 3-(3,4-dimethoxyphenyl)-*cis*-prop-2-enoate	-	-	0.4	1.1	-	-	-	-	-	-	-
192	unknown[a]	12	0.5	0.9	0.7	4.4	5.0	17	1.6	1.9	0.6	2.2
195	unknown[a]	2.8	0.3	0.2	0.7	3.8	4.9	5.6	1	1.3	0.3	1.9
201	41, 69, 95, 109, 124, 137, 152, 165, 174, 222	4.9	0.6	0.7	1.5	10.0	5.4	15	0.3	0.3	0.1	0.4
202	43, 83, 98, 125, 137, 151, 181, 239, 250, 268	8.9	1.7	1.1	3.8	10.4	13	14	4.9	6.6	2.8	9.2
208	69, 83, 99, 109, 124, 137, 152, 170, 222, 250	63	26	10.8	45	107*	156*	127*	118	106	40	140
209	methyl palmitate(16:0)	13	2.6	2.1	7.8	*	*	*	6.9	-	3.4	-
210	55, 71, 83, 109, 125, 152, 170	6.6	1.5	0.7	5.7	10.5	16	11	5.8	5.8	2.0	5.7
211	41, 55, 67, 83, 95, 109, 127, 155, 179, 225, 252	8.3	0.8	1.3	2.6	11	9.2	18	3.3	5.7	2.3	11
213	methyl margarate (17:0, internal standard)											
214	methyl abscisate	1.4	0.2	0.2	0.4	1.4	2.5	3.2	0.3	0.7	0.1	0.9
216	methyl linoleate (18:2)	0.3	0.3	*	*	0.3	*	*	0.4	0.3	0.4	0.3
217	methyl α-linolenate (18:3)	0.4	0.9	0.9*	2.3*	0.4	0.2*	0.5*	0.8	0.4	0.6	0.4
218	methyl oleate (18:1)	2.3	1.1	1.2	4.0	1.4	0.8	2.4	1.7	1.2	1.6	1.1
220	*n*-heneicosane (C_{21})	1.4	0.1	0.3	0.2	1.1	1.7	1.7	0.4	0.4	0.3	0.3
221	methyl stearate (18:0)	1.3	0.3	0.5	1.1	1.4	1.6	1.3	0.7	1.0	0.3	1.5
223	41, 55, 83, 95, 127, 155, 179, 252, 266 M^+	0.7	0.6	-	1.7	1.4	5.8	-	3.0	1.0	0.9	0.8
225	*n*-triacosane (C_{23})	0.4	0.3	0.2	0.6	0.3	0.4	0.5	0.5	0.5	0.5	0.2

[a] detected in GC/FID but not in GC/MS.

* unresolved GC/FID peak.

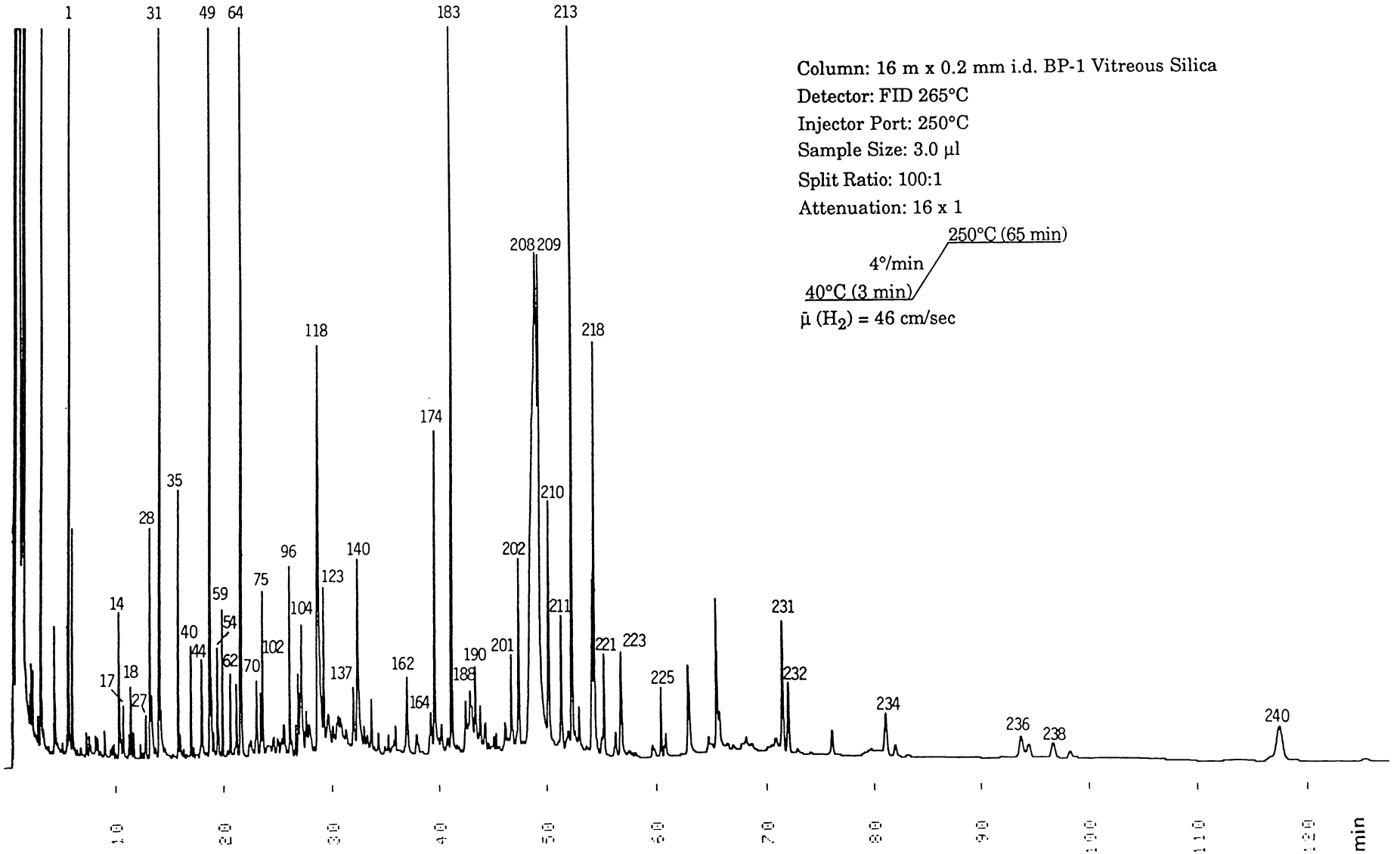


Figure 5.10 Gas chromatographic profile of a representative kamahi honey (sample KA2). For peak identification see Tables 3.II and 5.XI.

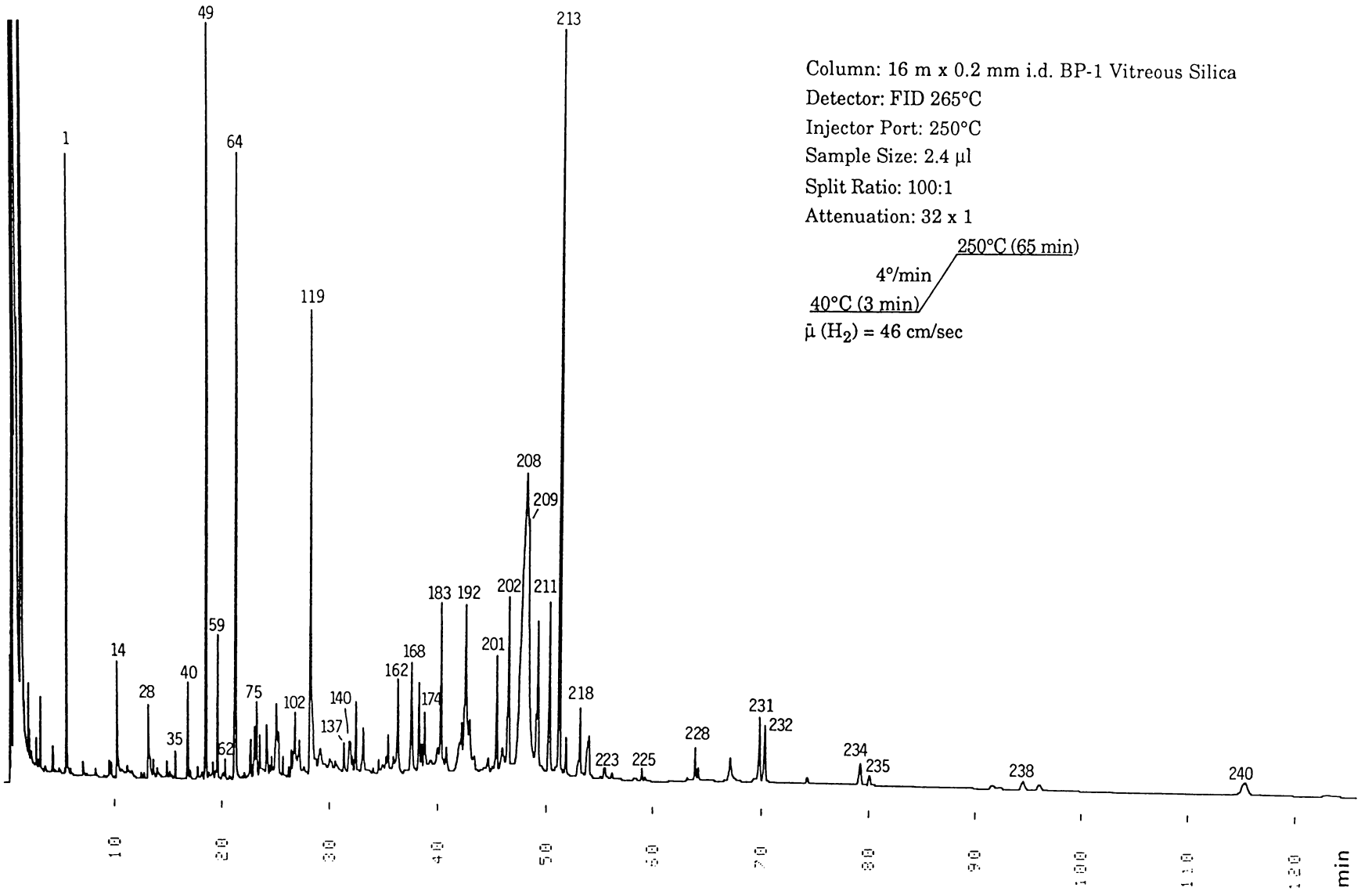


Figure 5.11 Gas chromatographic profile of the kamahi honey sample M1. For peak identification see Tables 3.II and 5.XI.

chemical composition clearly demonstrated that sample M1 possessed the characteristics of kamahi honey more so than those of manuka honey. Thus components characteristic of manuka honey such as 2'-methoxy-acetophenone (peak 86), 2-hydroxybenzoic acid (peak 107), 2-hydroxy-3-phenylpropionic acid (peak 119), methyl 4-hydroxy-3,5-dimethoxybenzoate (peak 188), 2-hydroxy-4-methylpentanoic acid (peak 25) and 2-hydroxy-3-methylpentanoic acid (peak 26) were detected in much lower concentrations than necessary for the sample to be classified as manuka honey. Subsequent pollen analysis supported this observation (see Table 5.X).

5.9 Discussion

In order to confirm the reliability of the chemical technique in the identification of floral source, it is necessary to analyse quantitatively a large number of samples of each floral type so that the variation in concentration of the extractable organic substances unique to a particular floral type can be assessed. In addition, it is important to characterise structurally particular marker compounds and establish that they are indeed characteristic for each floral source. Such information requires long-term studies and guaranteed sample purity. The present investigation covering four flowering seasons and 8 honey types provides some of the data required to validate the proposal that floral source can be reliably inferred from chemical analysis.

Most of the samples received for chemical investigation (except samples from Wilson Neill-Hororata Honey Exports Limited) were named in the field on the basis of their organoleptic characteristics and the floral source(s) available to the bees in the immediate vicinity of the hives, and

were as pure as the collector could obtain. Such a method of identification is, however, not foolproof. Mis-identification can occur through mistaking the honey flavour or through an inadequate knowledge of a hive's working history (*e.g.* manuka honey M1). Fortunately, in the present investigation, pollen analysis showed that mis-identification had occurred only rarely, and was in the main confined to honeys from pastoral floral plants and certain bush honeys. These problem samples probably reflect the combination of plant species available; *i.e.* the area covered by particular plants was not large enough for the bees to produce truly unifloral honeys.

Moar (1985) in an extensive investigation of pollen composition of 119 honey samples from various parts of New Zealand demonstrated that some unifloral honeys are easier to obtain than others. For example, honey samples named in the field as barberry (*Berberis vulgaris*), buttercup (*Ranunculus*), penny royal (*Mentha pulegium*) and rewarewa frequently displayed predominantly white clover type pollen. Conversely, honey named in the field as manuka and kamahi were invariably confirmed by pollen analysis.

Adams and Smith (1981) compared the proportion of different pollen types in freshly collected nectar with that in extracted honey (see Table 5.XII). The experiment involved weighing three colonies twice weekly on Monday and Thursday, and taking samples of freshly collected nectar. From 9th to 22nd July 1979 lime was predominant in the pollen and the colonies gained considerable weight. From 22nd July to 16th August birdfoot trefoil (*Lotus*) and loosestrife (*Lythrum*) pollens gained in frequency while lime pollens decreased. However, during this period the colonies gain in weight was modest in comparison to that during the lime flow. Table 5.XII displays the percentage of pollen detected in the nectar over the period 9th to 16th August compared to that found in the honey

extracted on 16th August. Because the colonies weight gain was substantial while lime was the predominant pollen, the presumption was that the honey was predominantly lime honey and indeed it possessed the flavour and colour of lime. However, the honey contained only 6.7% lime pollen, well below the limit set (10 to 20%, Vorwohl, 1973; Maurizio, 1975) for legitimate labeling as lime honey.

Table 5.XII Percentage of pollen detected in the nectar.

pollen type	nectar 9 July to 16 August	honey 16 August
lime	20.9	6.7
bird's foot trefoil	23.2	29.7
loosestrife	14.5	23.0

Taken from Adams and Smith, 1981.

Another consideration is that where, due to the nature of the floral structure of the plants, pollen analysis was unable to confirm the dominant floral contribution. Thyme (Moar, 1985) and heather (Maurizio and Louveaux, 1964; Maurizio, 1979) honeys are in this category. For example, thyme is a gynodioecious plant (*i.e.* hermaphrodite flowers are produced by some plants, female flowers are produced by other plants), and there is evidence that environmental factors play an important and complex role in determining sexual expression in thyme (Dommee *et al.*, 1978). Both types of flower produce abundant nectar and are equally available to foraging bees, although pollen is only available in a proportion of flowers (*i.e.* hermaphrodite). Conversely, manuka is andromonoecius (*i.e.* male and hermaphrodite flowers occur on the same plant), and

nectar is produced in small quantities by both types of flower (Primack and Lloyd, 1980). The small manuka pollen grains are therefore available whenever bees visit the flowers. Demianowicz (1964) reported that highest absolute pollen counts were often recorded for plants with the smallest pollen grains.

Recent experiments with willows (*Salix caprea*) suggest that honeybees exhibit a degree of faithfulness to either the male or female plants of dioecious species (van der Werf, 1983). Crack willow (*Salix fragilis*) and weeping willow (*S. babylonica*), the two most common species in New Zealand, are dioecious. The apparent preference of bees for either male or female plants of dioecious plants such as willow would result in producing honeys from the same plants with different pollen frequencies. The chemical results for willow contribution in thyme honeys in Section 5.5 appeared to support this observation. It is possible that the single sample of willow honey (W207) may or may not have been predominantly willow nectar because of the sex preferences of bees. Such preference may also be relevant for other species as well.

Despite the limitations of obtaining unifloral honey samples, the results of the present chemical investigation provide a basis for the identification of the floral source of New Zealand honeys, and also contributes to a better understanding of some of the organoleptic nature of local honeys.

Thus honey with low extractable organic substances (< 50 μg/g) is characteristic of white clover type, while manuka honey is characterised by high levels of extractable aromatic substances like 2-hydroxy-3-phenylpropionic acid (peak 119), 2'-methoxyacetophenone (peak 86) and methyl 4-hydroxy-3,5-dimethoxybenzoate (peak 188). On the other hand,

heather honey is characterised by the presence of high levels of degraded carotenoid-like substances such as 4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one (peak 193) and 4-(3-oxo-1-butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one (peak 171), thyme honey is characterised by 3'-aminoacetophenone (peak 90), 1-(3-oxo-1-butenyl)-2,6,6-trimethyl-1,2-epoxycyclohexan-4-ol (peak 153) and 1-(3-oxo-1-butenyl)-2,6,6-trimethyl-cyclohexane-*trans-cis*-1,2,4-triol (peak 206) and willow honey is characterised by high levels of *trans-cis* and *trans-trans*-abscisic acid (peaks 214 and 222). The presence of 1,4-dihydroybenzene (peak 84) and 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoic acid (peak 140) is diagnostic of vipers bugloss and nodding thistle honeys respectively. Finally, the unknown peaks 201, 202, 208, 210 and 211 are unique to kamahi honey. Table 5.XIII summarises the characteristics of each honey type investigated in the present study.

Table 5.XIII Summary of honey characteristics.

honey type	honey characteristics or unique compound(s)	common range µg/g	mean µg/g
white clover type	low extractable organic substances	total < 50	
manuka	2-hydroxy-3-phenylpropionic acid	240 - 1200	750
	methyl 4-hydroxy-3,5-dimethoxybenzoate	20 - 230	130
	2'-methoxyacetophenone	6 - 20	10
	2-hydroxybenzoic acid	2 - 40	20
ling/heather	4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	10 - 200	150
	4-(3-oxo-1-butenylidene)-3,5,5-trimethyl-cyclohex-2-en-1-one	30 - 40	30
thyme	1-(3-oxo-1-butenyl)-2,6,6-trimethylcyclo-hexane-1,2,4-triol	25 - 110	65

	1-(3-oxo-1-butenyl)-2,6,6-trimethyl-1,2-epoxycyclohexan-4-ol	1 - 5	2
	3'-aminoacetophenone	1 - 5	3
	unknown peak 149	2 - 6	4
	3-hexenoic acid	4 - 8	5
willow	*trans-cis*-abscisic acid	100	100
	trans-trans-abscisic acid	40	40
vipers bugloss	1,4-dihydroxybenzene	15 - 30	20
nodding thistle	2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoate	10 - 30	15
	unknown peak 111	2 - 9	5
	unknown peak 114	1 - 6	3
	unknown peak 116	1 - 6	3
	unknown peak 121	2 - 9	6
kamahi	unknown peak 208	10 - 150	80
	unknown peak 201	0.5 - 10	4
	unknown peak 202	1 - 10	7
	unknown peak 210	2 - 10	7

Pollen analysis is usually directed towards the identification of the dominant contributing floral sources and may fail to identify a contributing source which exhibits low pollen counts because of the morphological nature of the flower (*e.g.* thyme). A major advantage in the chemical technique is that it affords a fingerprint which appears to be nectar source dependent, hence this leads to a more complete identification of the floral contribution. In addition, the level of 5-hydroxymethyl-2-furfural in a honey reflects treatment during processing and storage. Studies have shown that excessive heating in processing and storing of honey produces high levels of 5-hydroxymethyl-2-furfural which are recovered in the total honey extractives (see Section 4.7.3); while the

presence of ethyl ester components reflects the state of the honey with respect to fermentation (Tan, 1985).

Pollen composition is dependent upon the method of extraction (Moar, 1985) as well as the distance of the hive from the floral source (Maurizio, 1975). A comprehensive account of such variation and its significance in floral sources identification of honey was given by Maurizio (1975), while a concise summary was given by Cowan (1988). Thus comparison of results can only be made from samples extracted by the same method. For example, the absolute pollen content of a sample obtained by centrifugal extraction may be greatly reduced from that of a sample obtained by pressing.

On the other hand, chemical studies of samples acquired from these two methods should give similar chemical composition and GC profiles despite the different pollen frequencies. A study (Tan unpublished) of the chemical composition of kiwifruit and pastoral pollen revealed that apart from fatty acids, little other extractives are present in the pollen. As yet no evidence is available to show that the chemical composition of the nectar remains invariant during the transformation of the nectar into honey.

The chemical approach also gives a better indication of the contributing nectar as demonstrated in the case for nodding thistle (see section 5.7) and willow and thyme (see Section 5.5). Because the present chemical data are limited, it is not possible to show the percentage contribution from individual floral sources. Nevertheless, since one is able to locate in the chromatogram the peak or peaks that are associated with particular floral sources, correction factors can be determined

experimentally once more data becomes available. However, such information requires long term studies and guaranteed sample purity.

The present study indicates that certain components are unique to a particular floral type and therefore points to their general utilisation as marker compounds for floral source identification. Such observations are only based on a limited number of floral types (eight). As yet there is no evidence for the marker compounds of a floral source being truly unique; therefore future work is needed to investigate a full range of floral types to establish "uniqueness". In addition, multiple samples of more floral types are needed to establish characteristic patterns for identifying honey types.

Chapter 6

Antibacterial Activity

Chapter 6

Antibacterial Activity

6.1 Introduction

In Chapter 1, the antibacterial properties of honey were discussed. While the antibacterial activity attributable to the hydrogen peroxide component has been well documented, little is known about the additional activity which was demonstrated to be nectar-dependent, manuka honey being the most active (Molan *et al.,* 1988). Russell (1983) in a later study attempted to isolate and characterise the components responsible for the additional antibacterial activity. They found 4-hydroxy-3,5-dimethoxybenzoic acid and 3,4,5-trimethoxybenzoic acid and their esters in some of the active fractions recovered from the PLC separation of the ethanol/ether extracts of manuka honey samples. Although both of the foregoing acids possessed significant antibacterial activity (Russell 1983), the concentrations at which these compounds were present in the honey were not known, hence it could not be determined if these compounds could account for all of the additional activity observed in manuka honey.

Since the concentrations of 4-hydroxy-3,5-dimethoxybenzoic acid, 3,4,5-trimethoxybenzoic acid and a number of other potentially antibacterial phenols in honey were determined in the present study (see Chapter 5), quantitative assessment of their contribution to the antibacterial activity of the honeys could be made. All samples of honey analysed were also assayed for antibacterial activity. Correlations were

then sought between activity and the occurrence of any of the constituents with the hope of identifying any other antibacterial substances present.

6.2 Assessment of Antibacterial Activity

The antibacterial activity of all honeys was assayed by an agar well diffusion method. Large square plates (243 mm x 243 mm x 18 mm) seeded with *Staphylococcus aureus* (ATCC 25923) were prepared by adding 100 µl of a 24-hour culture of the bacteria in nutrient broth (BBL, 8 g/l) to 150 ml of sterilised nutrient agar [made with nutrient broth (BBL, 8 g/l), plus Difco agar, 15 g/l] cooled to 45°C. The plates were poured to a depth of 4 mm immediately after mixing, and stored at 4°C for 24 hours before use.

The micro-organism, *S. aureus* was chosen because it was demonstrated (Molan and Russell, 1988) to be sensitive to the non-peroxide antibacterial component(s) of honey and not affected by the acidity or osmolarity of the honey samples.

Sixty-four wells were made in the agar using a cooled flamed 8 mm cork borer, and 100 µl of 25 percent w/w honey sample in water was added to each well (4 replicates per sample in a randomised pattern). Catalase (Sigma Chemical Co., St. Louis) was added to a concentration of 3 000 units/ml in all of the diluted honey samples to remove hydrogen peroxide (Molan and Russell, 1988). Standards of phenol in the concentration range of zero to 10% were included in each plate. The plates were incubated for 18 hours at 37°C.

The diameter of the zone of inhibition was recorded using a Vernier gauge. The zone of inhibition and the antibacterial activity are related in a non-linear manner (Kavanagh, 1972). A plot of the square of the diameter of the zone of inhibition versus the concentration of the

sample gave the best fit straight line. (Figure 6.1 shows the linearity obtained with this type of plot of the results obtained with a range of phenol standards and a range of concentrations of a manuka honey.) The mean diameter of the clearings obtained with each sample of honey was compared with the plot obtained from the phenol standards, and the result was expressed as the equivalent concentration of phenol. The results are shown in Table 6.I.

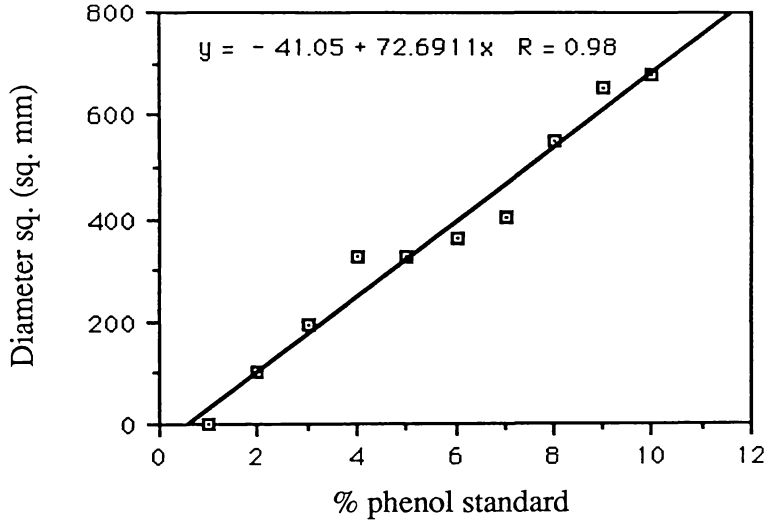


Figure 6.1(a) Plot of square of diameter of inhibition zone versus concentration of phenol standard.

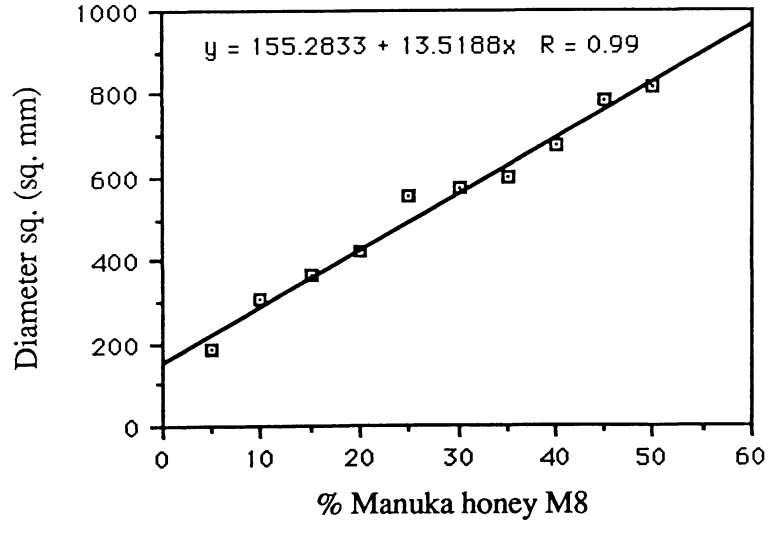


Figure 6.1(b) Plot of square of diameter of inhibition zone versus concentration of manuka honey.

Table 6.I Antibacterial activity of honeys. Results are expressed as the equivalent concentration of phenol per 25% honey.

sample no.	assumed plant of origin	form[a]	season	zone size in mm 1	2	3	4	ave.	equiv.% phenol
C1	white clover type	e	1986/87	-	-	-	-	-	-
C2	white clover type	e	1986/87	-	-	-	-	-	-
C16	white clover type	s	1985/86	-	-	-	-	-	-
H408	white clover type	e	1986/87	-	-	-	-	-	-
H410	white clover type	e	1986/87	-	-	-	-	-	-
H411	white clover type	e	1986/87	-	-	-	-	-	-
H412	white clover type	e	1986/87	-	-	-	-	-	-
H413	white clover type	e	1986/87	-	-	-	-	-	-
H414	white clover type	e	1986/87	-	-	-	-	-	-
H415	white clover type	e	1986/87	-	-	-	-	-	-
M3	manuka	s	1985/86	23.0	21.0	21.0	21.1	21.5	6.9
M5	manuka	s	1985/86	20.0	19.5	19.0	19.2	19.4	5.7
M6	manuka	s	1985/86	19.5	19.0	20.0	19.5	19.5	5.8
M7	manuka	s	1985/86	22.0	22.0	22.2	21.0	21.8	7.1
M8	manuka	c	1982	23.5	24.0	22.5	23.5	23.4	8.1
M9	manuka	e	1985/86	16.0	14.8	15.0	16.0	15.5	3.8
M19	manuka	s	1986/87	14.0	11.5	13.0	13.0	12.9	2.7
M22	manuka	s	1986/87	-	-	-	-	-	-
M24	manuka	s	1987	-	-	-	-	-	-
M27	manuka	e	1987	12.8	12.0	13.0	12.8	12.7	2.6
M30	manuka	s	1987	17.8	17.9	17.5	17.5	17.7	4.0
H416	manuka	e	1986/87	-	-	-	-	-	-
H361	ling/heather	e	1985/86	-	-	-	-	-	-
H2	ling/heather	s	1986	-	-	-	-	-	-
H3	ling/heather	s	1986	-	-	-	-	-	-
H4	ling/heather	s	1986	-	-	-	-	-	-
H460	ling/heather	e	1987/88	-	-	-	-	-	-
H461	ling/heather	e	1987/88	-	-	-	-	-	-
H462	ling/heather	e	1987/88	-	-	-	-	-	-
H463	ling/heather	e	1987/88	-	-	-	-	-	-
H384	thyme	e	1985/86	-	-	-	-	-	-
H404	thyme	e	1986/87	-	-	-	-	-	-

H405	thyme	e	1986/87	-	-	-	-	-	-
H406	thyme	e	1986/87	-	-	-	-	-	-
H407	thyme	e	1986/87	-	-	-	-	-	-
T111	thyme	e	1987	-	-	-	-	-	-
T139	thyme	e	1987	-	-	-	-	-	-
T1	thyme	e	1986	-	-	-	-	-	-
T2	thyme	e	1986	-	-	-	-	-	-
T3	thyme	e	1986	-	-	-	-	-	-
T4	thyme	e	1986	-	-	-	-	-	-
N1	nodding thistle	e	1984/85	-	-	-	-	-	-
N3	nodding thistle	e	1986	-	-	-	-	-	-
TH1	nodding thistle	e	1986	-	-	-	-	-	-
8N	nodding thistle	s	1987	-	-	-	-	-	-
38N	nodding thistle	e	1987	-	-	-	-	-	-
68N	nodding thistle	e	1987	-	-	-	-	-	-
77N	nodding thistle	s	1987	-	-	-	-	-	-
205N	nodding thistle	s	1987	-	-	-	-	-	-
206N	nodding thistle	s	1987	-	-	-	-	-	-
H403	nodding thistle	e	1986/87	-	-	-	-	-	-
VB102	vipers bugloss	s	1987	8.5	9.0	9.0	9.0	8.9	1.1
VB103	vipers bugloss	s	1987	9.0	9.0	9.0	9.0	9.0	1.1
VB104	vipers bugloss	s	1987	9.0	9.0	9.0	9.0	9.0	1.1
VB105	vipers bugloss	s	1987	-	-	-	-	-	-
VB106	vipers bugloss	s	1987	8.5	9.0	9.0	8.8	8.8	1.1
VB107	vipers bugloss	s	1987	-	-	-	-	-	-
VB124	vipers bugloss	s	1987	-	-	-	-	-	-
VB125	vipers bugloss	s	1987	-	-	-	-	-	-
VB126	vipers bugloss	s	1987	-	-	-	-	-	-
VB1	vipers bugloss	s	1986	-	-	-	-	-	-
M1	kamahi	s	1986	-	-	-	-	-	-
H363	kamahi	e	1985/86	-	-	-	-	-	-
H402	kamahi	e	1986/87	-	-	-	-	-	-
KA2	kamahi	e	1985/86	-	-	-	-	-	-
KA3	kamahi	e	1986	-	-	-	-	-	-
KA4	kamahi	e	1986	-	-	-	-	-	-
KA5	kamahi	e	1986	-	-	-	-	-	-
KA6	kamahi	s	1985/86	-	-	-	-	-	-
KA7	kamahi	s	1985/86	-	-	-	-	-	-

KA8	kamahi	s	1985/86	-	-	-	-	-	-
KA9	kamahi	s	1985/86	-	-	-	-	-	-
W207	willow	s	1987	-	-	-	-	-	-

- no detectable inhibition

[a] Honey supplied in the form of:- c: comb honey
e: extracted honey
s: scraped from comb

6.3 Correlation of Antibacterial Activity with Composition

Amongst the 73 honey samples tested only 4 vipers bugloss and 9 manuka samples were found to possess additional antibacterial activities, equivalent to levels of 1.1 to 8.1 % phenol (see Table 6.I). From the results in Table 6.I, the 73 honey samples can be divided into three principal groups:

(a) honey samples with no detectable activity,

(b) active manuka honey and

(c) active vipers bugloss honey.

The mean concentration of each component in each of these three groups was calculated, and the means were compared to find any compounds which occurred at substantially higher levels in the two active groups. These are shown in Table 6.II.

Table 6.II Components with mean concentrations higher in honeys with antibacterial activity.

		mean conc. in honey (μg/g)		
peak	component	grp (a)	grp (b)	grp (c)
1	unknown (manuka)	10.3	127	6.5
7	unknown (manuka)	1.4	55	0

23	unknown (manuka)	3.8	16	0
31	butanedioic acid	3.7	13.5	0.1
45	unknown (manuka)	0	3.3	0
52	unknown (manuka)	0	2.0	0
69	unknown (manuka)	0	6.6	0
84	1,4-dihydroxybenzene	0	0	20
86	2'-methoxyacetophenone	0.5	12	0
107	2-hydroxybenzoic acid	0.4	21	0
119	2-hydroxy-3-phenylpropionic acid	24	583	0
124	unknown (manuka)	0	2.8	0
160	3,5-dihydroxybenzoic acid	0.4	4.6	0
163	3,4-dihydroxybenzoic acid	0.5	4.4	0
167	3-hydroxy-3-(hydroxyphenyl)-propionic acid	0.1	5.0	0
168	2-hydroxy-3-(4-hydroxyphenyl)-propionic acid	0.6	21	0
179	unknown (manuka)	0	3.7	0
184	3,4,5-trimethoxybenzoic acid	1.3	7.7	0
188	4-hydroxy-3,5-dimethoxy-benzoic acid	4.6	117	0
196	unknown (manuka)	0.1	6.0	0
197	unknown (manuka)	0.1	5.0	0
212	unknown (manuka)	0.1	4.8	0

It is clear from Table 6.II that the high concentration of some substances is associated with antibacterial activity. Some appeared to be more obviously associated than others, for example unknown peak 1, 1,4-dihydroxybenzene (peak 84), 2-hydroxybenzoic acid (peak 86), 2-hydroxybenzoic acid (peak 107), 2-hydroxy-3-phenylpropionic acid (peak 119) and 4-hydroxy-3,5-dimethoxybenzoic acid (peak 188). While peak 1 was detected in GC/FID analysis, it did not produce a recognisable GC/MS peak, and hence its identity remains uncertain. In addition to peak 1, other unknown substances with significant association with activity were

peaks 7, 23, 45, 52, 69, 124, 179, 196, 197 and 212. The antibacterial activity of these unknowns could not be assessed since pure standards of these compounds could not be obtained. Owing to time constraints, only peaks 84, 86, 107, 119 and 188 from the list of identified compounds were available for antibacterial activity assessment.

6.4 Testing of Potentially Antibacterial Compounds

The compounds of established structure found to be correlated with antibacterial activity were tested for their relative activity. The assays of these compounds were carried out quantitatively so that their contribution to the total additional antibacterial activity could be determined. Each was tested at a range of concentrations by dissolving the finely ground compound in a 50% solution of an inactive honey (N39, previously determined to be inactive) at 100°C.

6.4.1 1,4-Dihydroxybenzene (hydroquinone)

Solutions of a range from 0.001 to 0.01% w/v of 1,4-dihydroxybenzene in a 50% solution of an inactive honey (N39) were prepared. The antibacterial activities of these solutions were tested and the results compared with those obtained for vipers bugloss honeys tested at 50% strength (with catalase added). The square of the diameter of the zone of inhibitions were plotted against concentrations of 1,4-dihydroxybenzene (see Figure 6.2). The results obtained from the 50% honey are shown in Table 6.III.

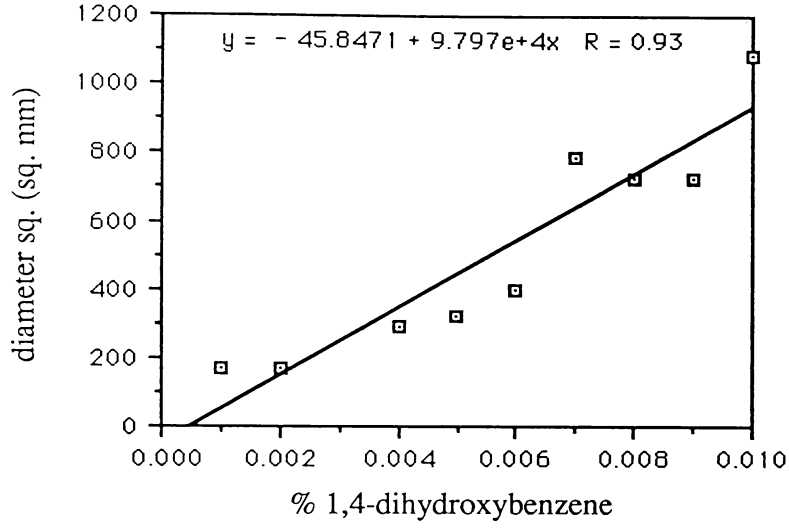


Figure 6.2 Plot of square of diameter of inhibition zone versus concentration of 1,4-dihydroxybenzene.

Table 6.III Antibacterial activity of vipers bugloss honeys tested at 50% concentration.

sample	mean diameter of clearing (mm)	mm^2
VB102	15.8 ± 1.9	250
VB103	17.3 ± 1.4	299
VB104	16.9 ± 0.5	286
VB106	17.1 ± 1.4	292

From the gradient in Figure 6.2 the activity due to the 1,4-dihydroxybenzene present in the 50% solutions of VB102, VB103, VB104 and VB106 (50% of the values in Table 5.VIII) was calculated. The equivalent inhibition zones were estimated to be 88, 93, 108 and 103 mm^2 respectively. From these results 1,4-dihydroxybenzene appeared to account for 35, 31, 38 and 35% respectively of the activity found in the viper bugloss honey.

As the results for the honey appeared high compared with the results in Table 6.I, the determination was repeated. On this occasion,

lower values were obtained with the 1,4-dihydroxybenzene while no activity was detected in the vipers bugloss honeys. However, at 10 μg/g, 1,4-dihydroxybenzene remained active.

Since the activity of 1,4-dihydroxybenzene was found to be higher than necessary in one case and lower in the other, the likelihood is that 1,4-dihydroxybenzene is responsible for the activity found in vipers bugloss honey.

There also appears to be a degree of inconsistency in the results from vipers bugloss honeys with respect to the antibacterial activity of 1,4-dihydroxybenzene concentration in Table 6.I. One possible explanation is that at 25% dilution, the level of 1,4-dihydroxybenzene in the honey samples is towards the limit of detection, hence inactive samples can be found among active ones even though the levels of 1,4-dihydroxybenzene were similar in both cases.

6.4.2 2-Hydroxy-3-phenylpropionic acid

Solutions of 0.1-2.0% w/w of 2-hydroxy-3-phenylpropionic acid in a 50% solution of an inactive honey (N39) were prepared. The antibacterial activity of these solutions were tested and the results compared with the results from several manuka honeys tested at the same time at 50% concentration (with catalase added). The square of the diameter of clearings were plotted against concentrations of 2-hydroxy-3-phenylpropionic acid (see Figure 6.3). The results obtained from the honey samples are shown in Table 6.IV. From the gradient in Figure 6.3 the activity due to 2-hydroxy-3-phenylpropionic acid present in the 50% solutions of M3, M7, M8 and M9 (50% of the values in Table 5.III) was

calculated. The activity was estimated to be 13, 13, 16 and 17 mm^2 respectively. Although the level at which 2-hydroxy-3-phenylpropionic acid occurs in manuka honey is substantial, the results demonstrate that it only accounts for 1.6-3.2% of the activity.

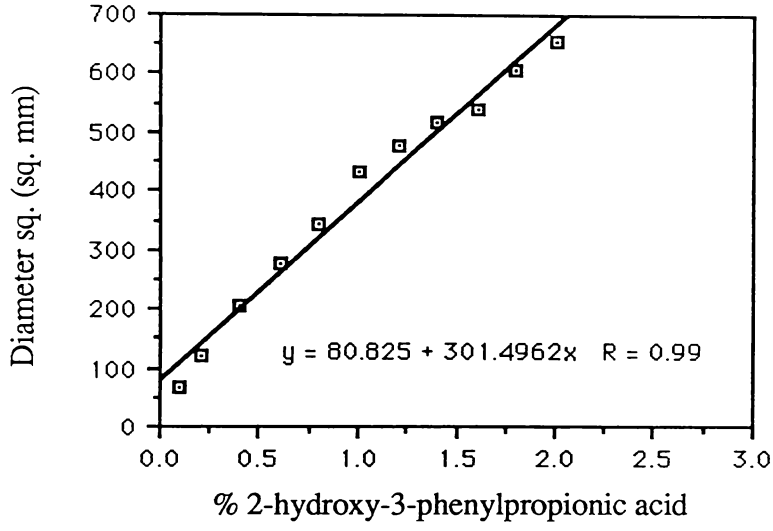


Figure 6.3 Plot of square of diameter of inhibition zone versus concentration of 2-hydroxy-3-phenylpropionic acid.

Table 6.IV Antibacterial activity of manuka honeys tested at 50% concentration.

sample	mean diameter of clearing (mm)	mm^2
M3	28.2 ± 1.1	795
M7	28.4 ± 2.0	805
M8	30.1 ± 1.6	904
M9	23.0 ± 2.3	529

6.4.3 4-Hydroxy-3,5-dimethoxybenzoic acid

4-Hydroxy-3,5-dimethoxybenzoic acid was one of the active compounds in manuka honey identified by Russell *et al.* (1988) in an

earlier study. Solutions of 0.1-1.0% w/w of 4-hydroxy-3,5-dimethoxybenzoic acid in a 50% solution of an inactive honey (N39) were prepared. The antibacterial activity of these solutions was tested and the results compared with several manuka honeys tested at the same time at 50% strength (with catalase added). The square of the diameter of the zones of inhibition were plotted against concentrations of 4-hydroxy-3,5-dimethoxybenzoic acid (see Figure 6.4). The results obtained from the honey samples are shown in Table 6.V. From the gradient in Figure 6.4 the activity due to 4-hydroxy-3,5-dimethoxybenzoic acid present in the 50% solutions of M3, M6, M7 and M9 (50% of the values in Table 5.III) was calculated. The activity was estimated to be equivalent to 2.8, 2.3, 3.1 and 1.0 mm^2 respectively. Although 4-hydroxy-3,5-dimethoxybenzoic acid was the principal antibacterial component isolated by Russell *et al.* (1988), the results here demonstrate that it only accounts for 0.2-0.35% of the activity in manuka honey.

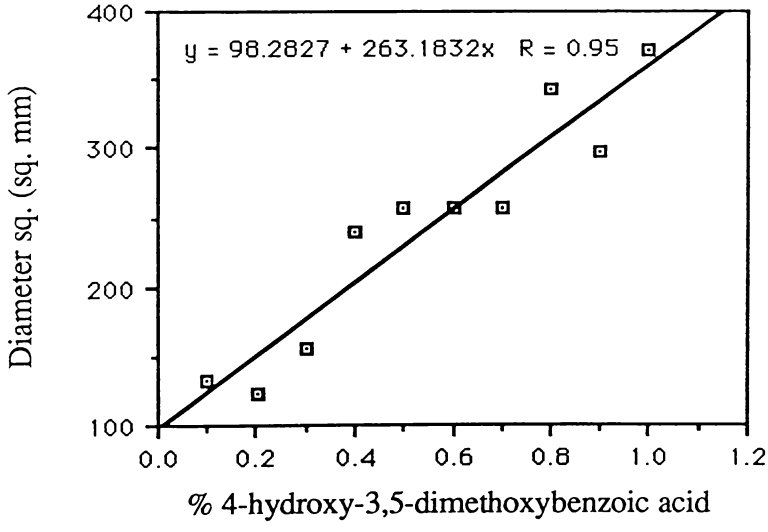


Figure 6.4 Plot of square of diameter of inhibition zone versus concentration of 4-hydroxy-3,5-dimethoxybenzoic acid.

Table 6.V Antibacterial activity of manuka honeys tested at 50% concentration.

sample	mean diameter of clearing (mm)	mm^2
M3	29.5 ± 1.1	871
M6	27.8 ± 1.7	775
M7	29.0 ± 1.7	841
M9	22.4 ± 0.8	502

6.4.4 3,4,5-Trimethoxybenzoic acid

3,4,5-Trimethoxybenzoic acid was another of the active compounds in manuka honey identified by Russell *et al.* (1988) in an earlier study. The minimum concentration at which activity could be detected in the present study with 3,4,5-trimethoxybenzoic acid in 50% inactive honey (N39) was 0.02%. The level of 3,4,5-trimethoxybenzoic acid in manuka honey ranges 0.000 09 - 0.001 3% (average level 0.000 65%), which is too low for it to make a noticeable contribution to the antibacterial activity.

6.4.5 2'-Methoxyacetophenone

Solutions of 0.2-10.0% 2'-methoxyacetophenone in a 50% solution of inactive honey (N39) were prepared. The antibacterial activity of these solutions were tested and the results compared with several manuka honeys tested at the same time at 50% strength (with catalase added). The square of the diameter of the zones of inhibition were plotted against concentrations of 2'-methoxyacetophenone (see Figure 6.5). The results obtained from the honey samples are shown in Table 6.IV. From the gradient in Figure 6.5 the activity due to the 2'-methoxyacetophenone

present in the 50% solutions of M3, M7, M8 and M9 (50% of the values in Table 5.III) was calculated. The activity was estimated to be equivalent to 0.02, 0.02, 0.01 and 0.005 mm^2 respectively. The results obtained therefore showed that 2'-methoxyacetophenone contributes only a negligible proportion of the activity.

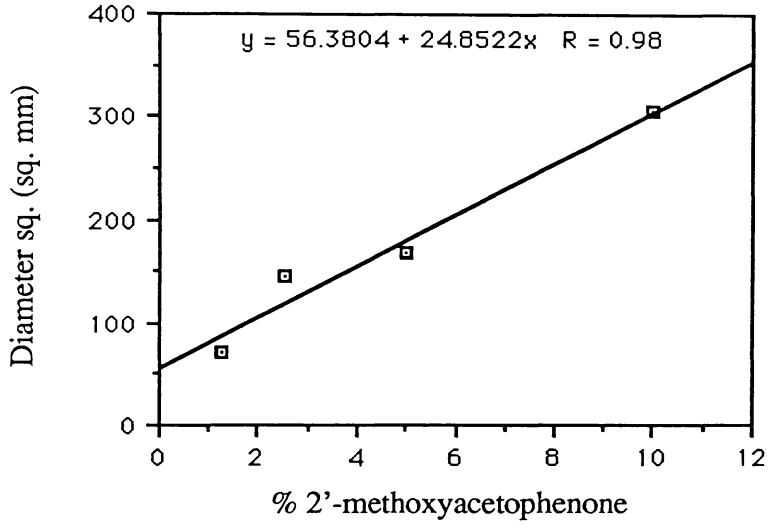


Figure 6.5 Plot of square of diameter of inhibition zone versus concentration of 2'-methoxyacetophenone.

6.4.6 2-Hydroxybenzoic acid

Solutions of 0.01-0.1% 2-hydroxybenzoic acid in a 50% solution of inactive honey (N39) were prepared. The antibacterial activity of these solutions was tested and the results compared with several manuka honeys tested at the same time at 50% strength (with catalase added). The square of the diameter of the zones of inhibition were plotted against concentrations of 2-hydroxybenzoic acid (see Figure 6.6). The results obtained from the honey samples are shown in Table 6.IV. From the gradient in Figure 6.5 the activity due to the 2-hydroxybenzoic acid present

in the 50% solutions of M3, M7, M8 and M9 (50% of the values in Table 5.III) was calculated (peak 107, methyl 2-methoxybenzoate, being assumed to have been 2-hydroxybenzoic acid in the honeys prior to methylation). The activity was estimated to be equivalent to 2.5, 2.7, 2.8 and 1.1 mm^2 respectively. The results obtained thus showed that 2-hydroxybenzoic acid contributes only 0.2-0.3% of the activity in manuka honey.

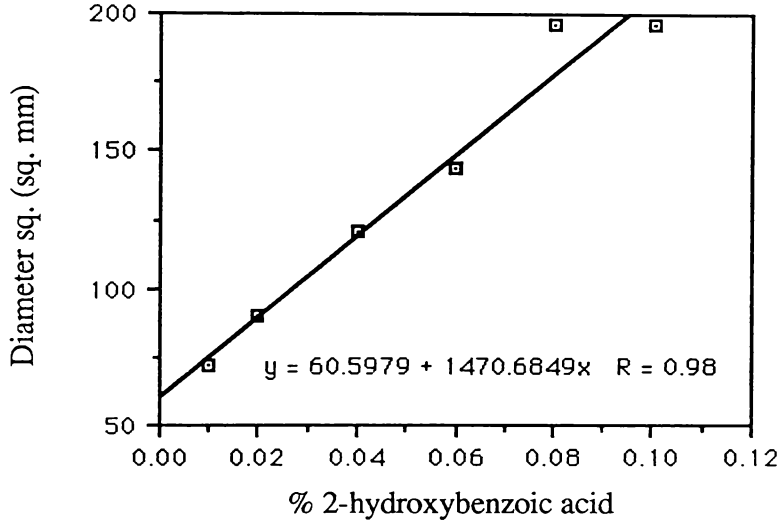


Figure 6.6 Plot of square of diameter of inhibition zone versus concentration of 2-hydroxybenzoic acid.

6.4.7 Antibacterial Activity of Manuka Honey

Examination of manuka extractives revealed that other than 2-hydroxy-3-phenylpropionic acid and 4-hydroxy-3,5-dimethoxybenzoic acid, there were no observable unique components or any other components which occur at high concentration. However, the present study shows that the level at which both acids occur in manuka honeys can only account for a very small proportion of the total additional activity observed.

The foregoing observations lead to the conclusion that the antibacterial substances responsible for the additional activity in manuka honeys are either the unidentified peaks or were not detected in GC analysis either because they were not volatile under the analysis condition or because the antibacterial substances were not being extracted. In order to determine which was the case, an experiment was conducted to measure how much of the activity was extracted.

A honey sample (50 g) was dissolved in 200 ml of distilled water by means of a magnetic stirrer at room temperature. A 10 ml fraction (sample A) of the resultant honey solution was kept at 4°C while the rest was transferred into liquid/liquid extractor and was extracted for 24 hours as described in Section 2.2.4.

After 24 hours extraction, the aqueous and the ethereal fractions were divided into two equal portions. One half of each aqueous and ethereal portion was recombined (sample B) while the other aqueous portion was denoted sample C. In another part of the experiment, 25 g of inactive clover honey was dissolved in 100 ml of distilled water. A portion (5 ml) of the resultant solution was kept aside as sample E while the remaining portion (95 ml) was combined with the ether fraction of the manuka honey extract to form sample D.

Ether was distilled from the aqueous samples B, C and D, in an all-glass rotory evaporator under reduced pressure at 25°C. Samples A to E were assayed for antibacterial activity as described above (see Section 6.2), and the results expressed as the equivalent concentration of phenol are shown in Table 6.VI.

Table 6.VI Antibacterial activity expressed as the equivalent concentration of phenol.

sample A (original honey)	5.7
sample B (re-combined phases)	5.7
sample C (aqueous phase)	3.9
sample D (ether phase in inactive honey)	2.5
sample E (inactive honey)	not detectable

This experiment demonstrated that most of the antibacterial activity is retained (*i.e.* it is not destroyed by the extraction procedure), and that a somewhat greater level of activity is associated with the aqueous fraction (sample C) compared with the ether fraction (sample D).

6.5 Discussion

Although the results obtained for vipers bugloss honeys were inconclusive due to the variability of the antibacterial assay, the low antibacterial activity in vipers bugloss honey could well be accounted for by 1,4-dihydroxybenzene.

The study of Russell *et al.* (1988) and the present study identified several antibacterial components in manuka honey. Although some of these acids possessed significant antibacterial activity, the concentrations at which these compounds were present could not account for all the additional activity observed in manuka honeys. The one that occurred at very high levels (2-hydroxy-3-phenylpropionic acid) was relatively low in activity. Russell *et al.* found the methyl esters of the acids to be present as well as the free acids. These were not assayed for activity in the present

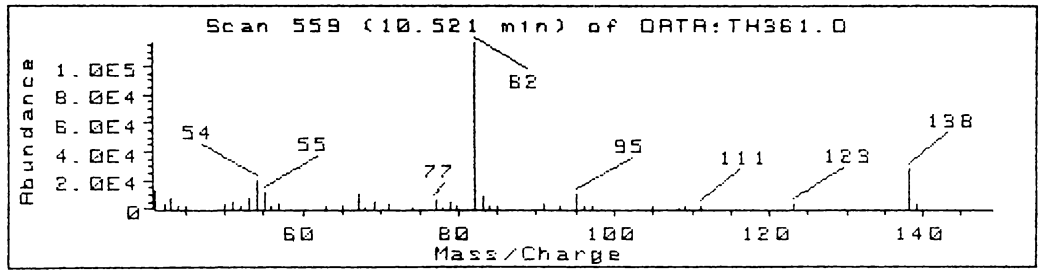
study, but preliminary tests and the work of Russell *et al.* (1988) found these to be less active than the free acids. Thus the components responsible for the major part of the high additional activity in manuka honey remain to be identified.

Earlier studies (Tan, 1985) demonstrated that the liquid/liquid extraction technique afforded an exhaustive recovery of the trace organic substances including phenolic and acidic components (see section 2.2.4). Therefore it is unlikely, but is possible, that additional quantities of 4-hydroxy-3,5-dimethoxybenzoic acid and other acids could be retained in the aqueous fraction after extraction.

The ether extraction experiment and the assessment of the various fractions demonstrated that about one-third of the activity was extracted (see Table 6.VI). However, only a small proportion of the activity of this fraction is accounted for by the identified compounds. The foregoing observations lead to the conclusion that the larger part of the active fraction from manuka honey is retained in the aqueous fraction. It is possible that the active compounds are highly hydrophilic, maybe glycosides of glucose and/or fructose, and therefore not extracted. This proposal is consistent with the results obtained by Sealey (1988) who found that the antibacterial substances in manuka honey have molecular weights of less than 1 000 amu and were not retained on reverse phase HPLC. Further work is needed to identify the unknowns detected in the ether extractives in this study, while the non-volatiles retained in the aqueous fraction need to be investigated by other techniques.

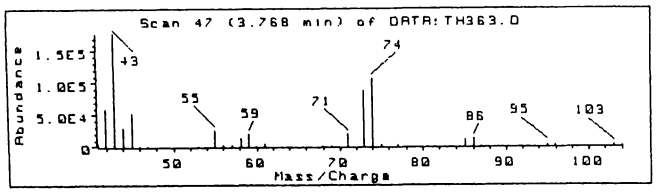
Appendix A

Mass Spectra of Honey Components

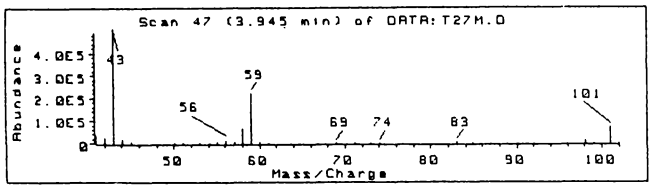


42 10.82 3,5,5-trimethylcyclohex-2-en-1-one

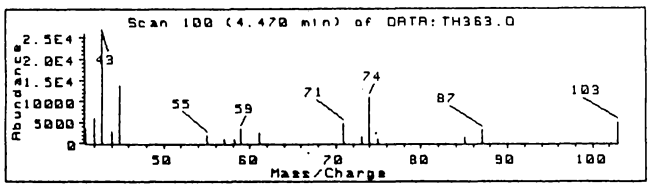
Peak number Carbon number Name if known



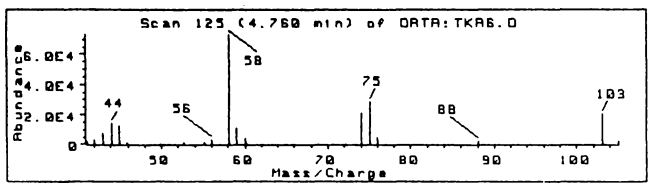
2 8.13 methyl 3-hydroxypropionate



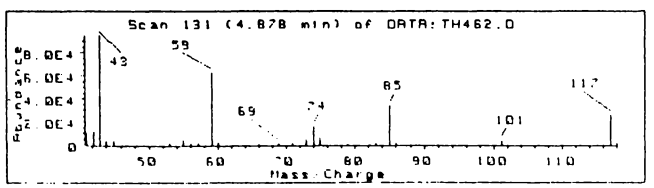
3 8.22 unknown[b]



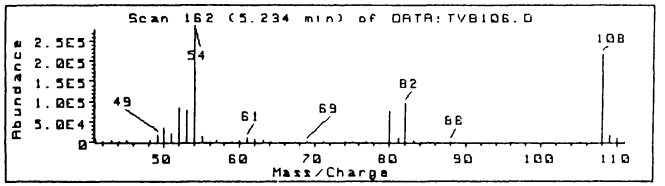
4 8.39 methyl 3-hydroxybutanoate[b]



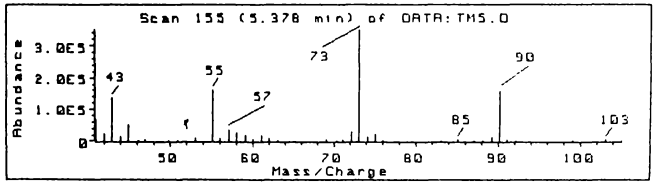
5 8.48 unknown[b] (kamahi)



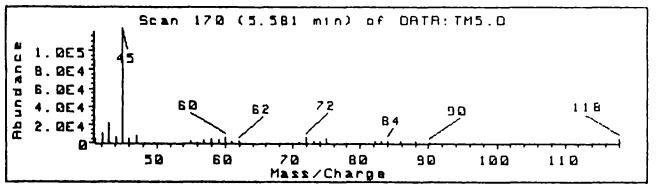
6 8.57 methyl 3-hydroxy-3-methylbutanoate[b]



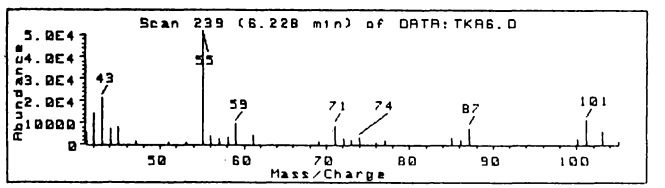
8 8.71 2,5-cyclohexadiene-1,4-dione



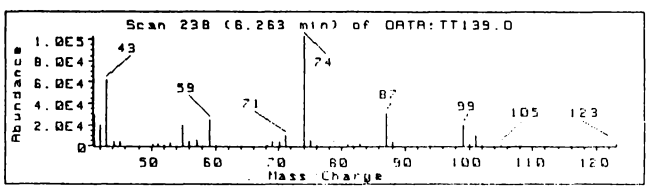
9 8.75 methyl 2-hydroxy-3-methylbutyroate



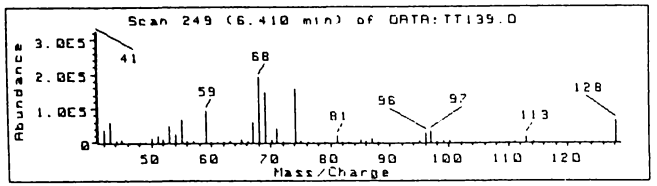
10 8.83 manuka (unknown)



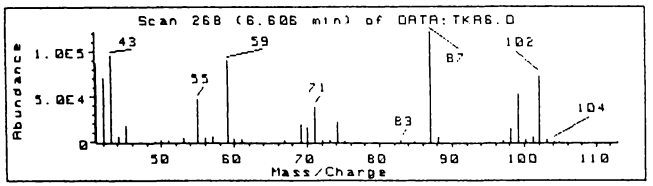
11 9.04 unknown (kamahi)



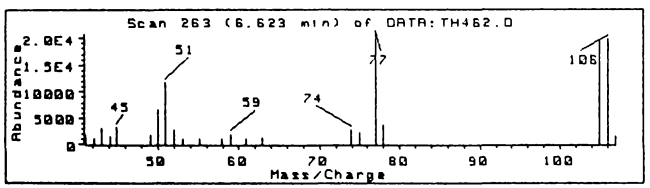
12 9.09 methyl caproate (6:0)



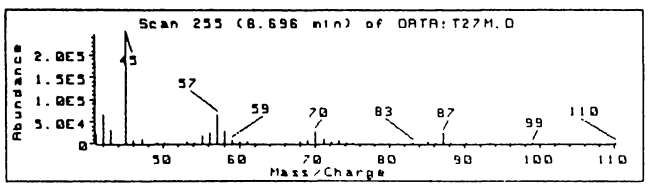
13 9.13 methyl 3-hexenoate



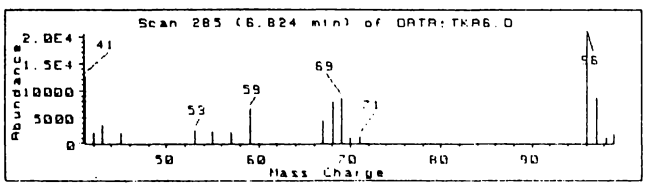
14 9.18 unknown (kamahi)



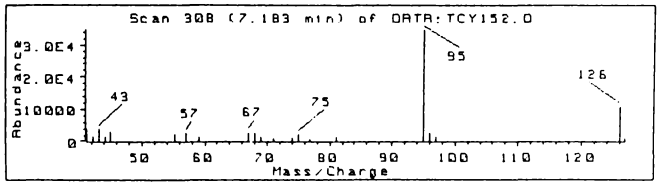
15 9.23 benzaldehyde



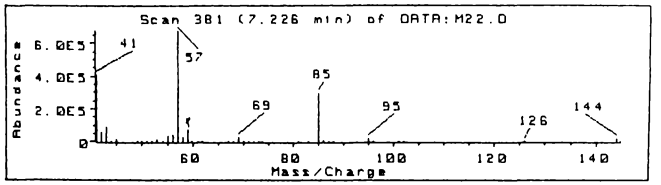
16 9.27 manuka (unknown)



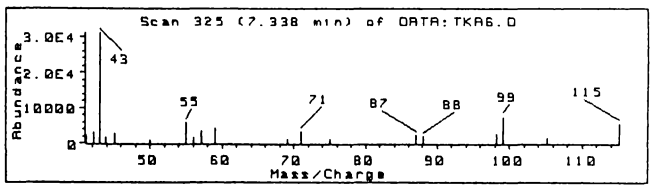
17 9.28 unknown (kamahi)



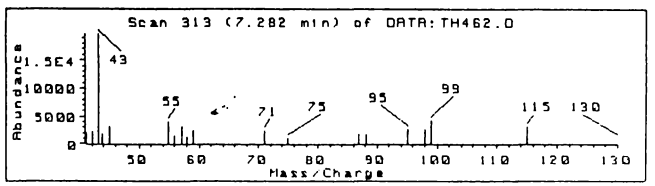
18 9.43 methyl 3-furancarboxylate



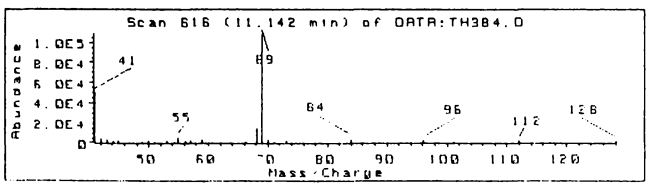
19 9.44 methyl 3-methyl-2-oxo-pentanoate



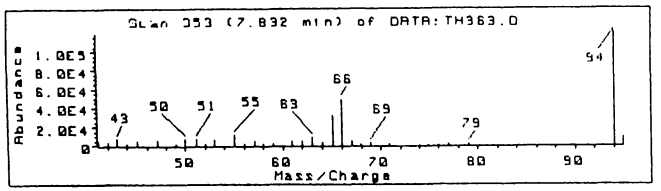
20 9.47 unknown (kamahi)



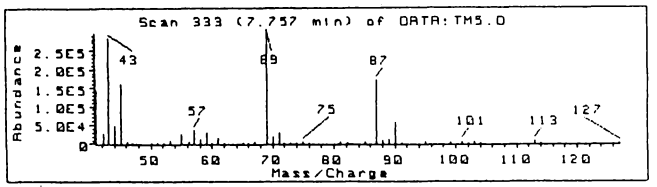
21 9.48 methyl 4-oxo-pentanoate



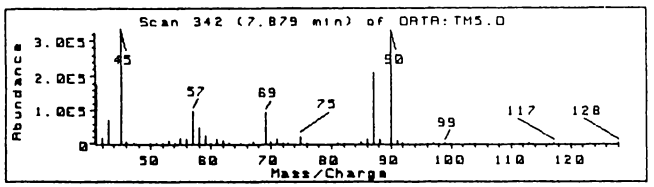
22 9.56 unknown (thyme)



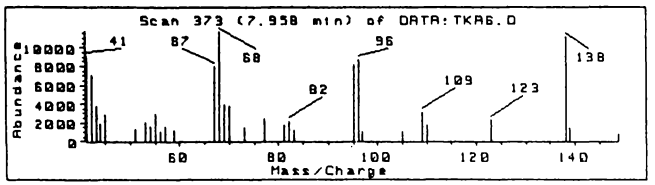
24 9.66 phenol



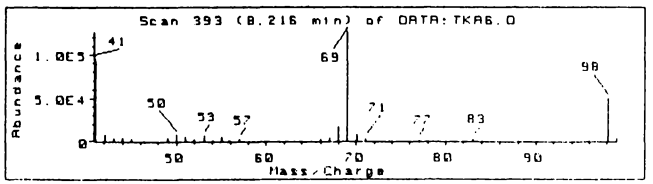
25 9.67 methyl 2-hydroxy-4-methylpentanoate



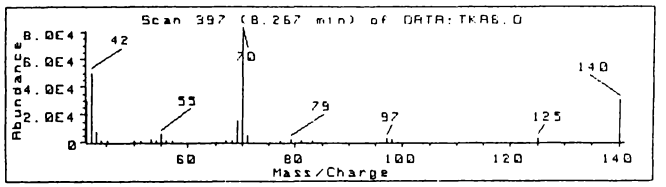
26 9.72 methyl 2-hydroxy-3-methylpentanoate



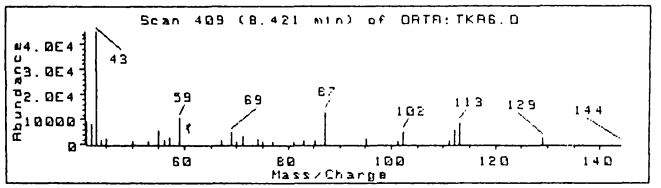
27 9.72 unknown (kamahi)



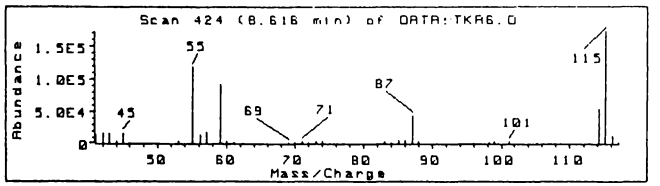
28 9.82 unknown (kamahi)



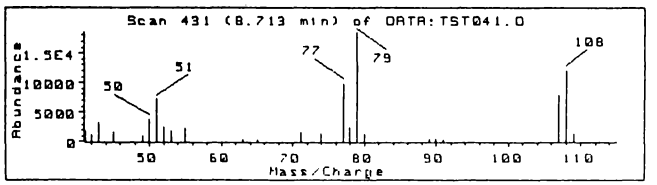
29 9.84 unknown (kamahi)



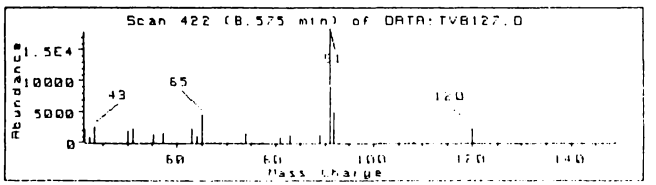
30 9.85 unknown (kamahi)



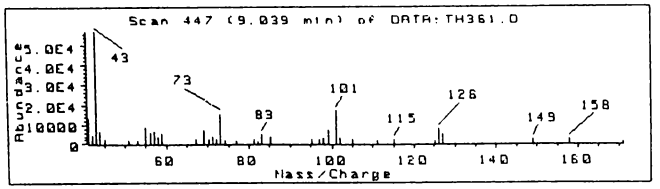
31 10.01 dimethyl butanedioate



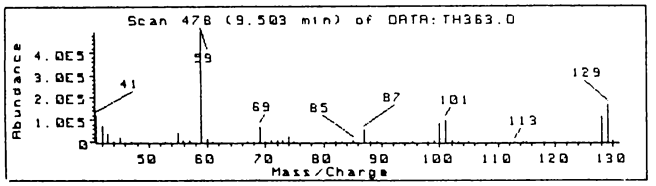
32 10.02 benzyl alcohol



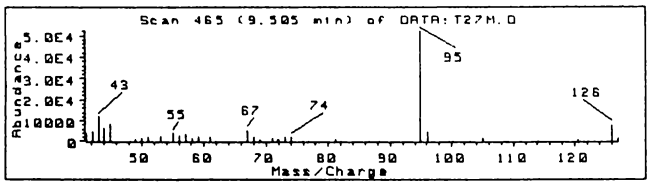
33 10.03 phenylacetaldehyde



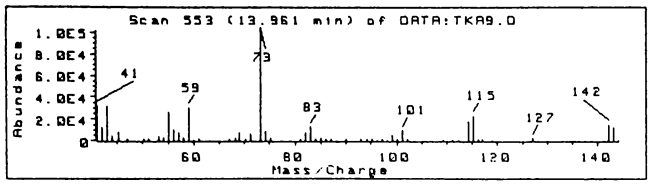
34 10.20 unknown (ling/heather)



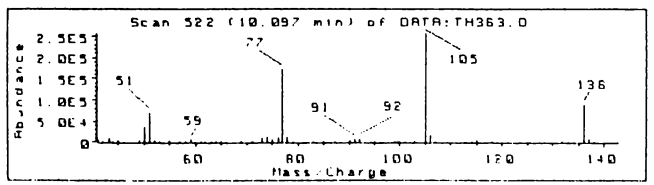
35 10.36 methyl 2-methylsuccinaldehydate



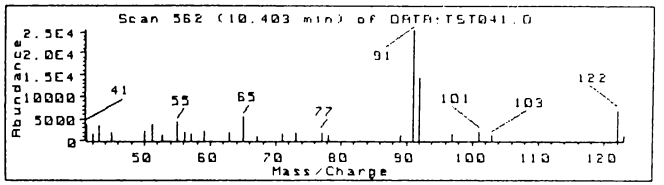
37 10.39 methyl 2-furancarboxylate



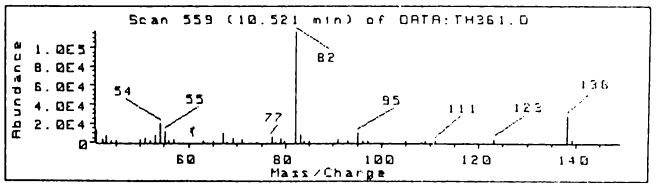
39 10.64 dimethyl 2,2-dimethylbutanedioate



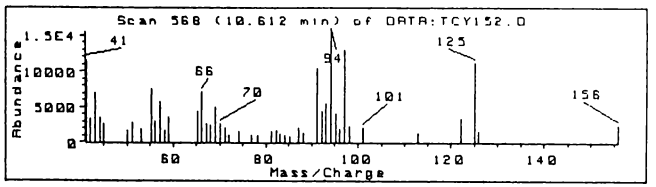
40 10.65 methyl benzoate



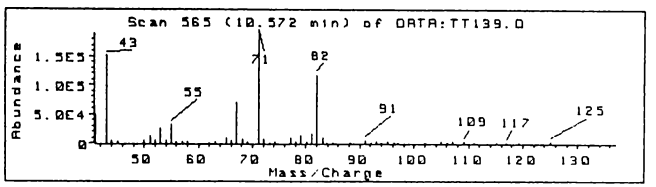
41 10.78 2-phenylethanol



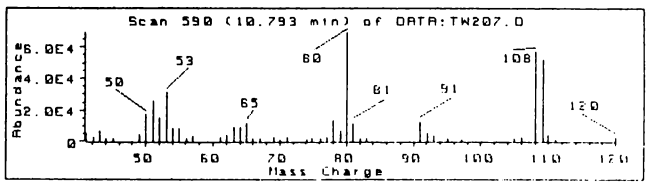
42 10.82 3,5,5-trimethylcyclohex-2-en-1-one



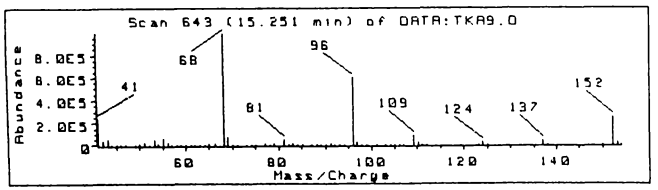
43 10.82 unknown (clover)



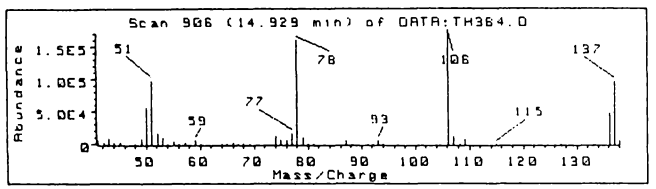
44 10.83 unknown



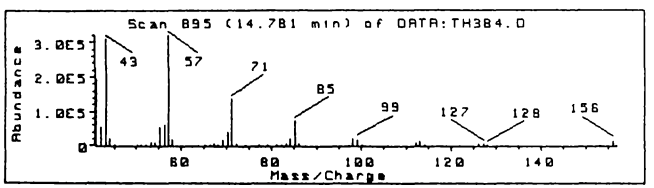
46 10.96 unknown (willow)



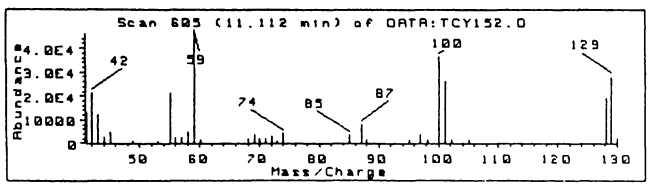
47 10.99 3,5,5-trimethylcyclohex-2-ene-1,4-dione



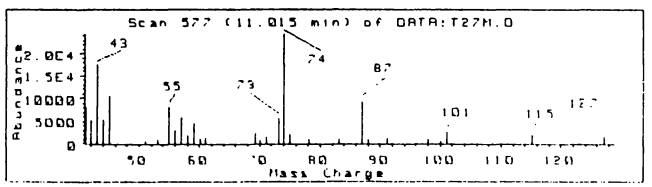
48 10.98 methyl pyridinecarboxylate



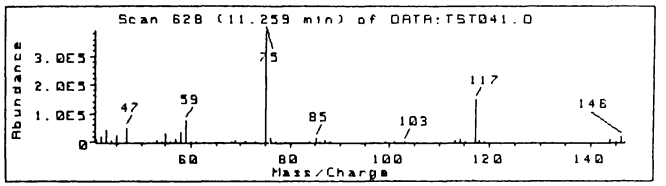
49 11.00 *n*-undecane (C_{11}, internal standard)



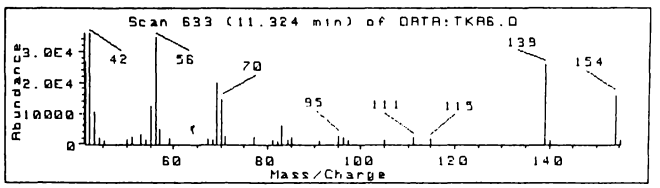
50 11.02 dimethyl pentanedioate



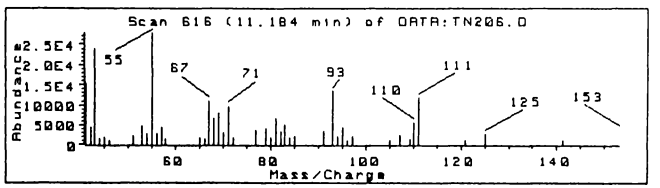
51 11.06 methyl caprylate (8:0)



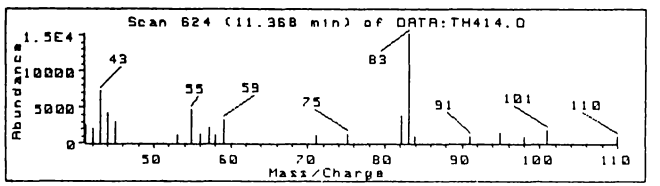
53 11.13 unknown (rewarewa?)



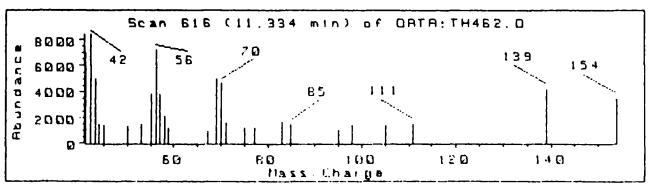
54 11.13 2,6,6-trimethylcyclohexane-1,4-dione



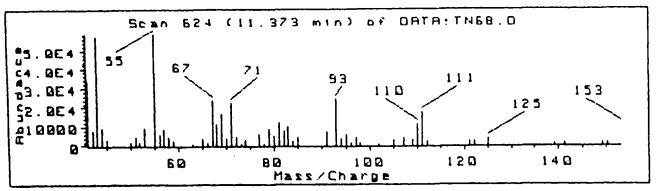
55 11.14 unknown (nodding thistle)



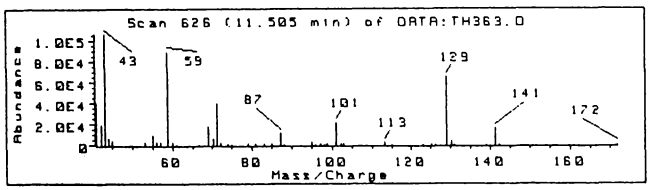
56 11.16 unknown (clover)



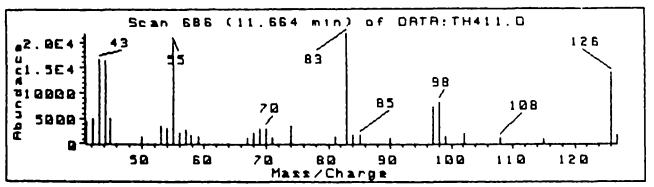
57 11.18 unknown (ling/heather)



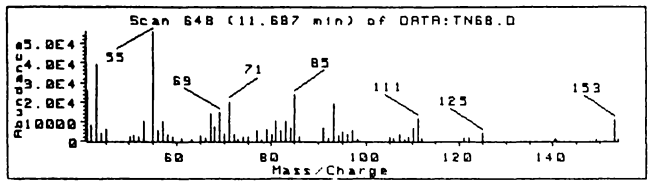
58 11.22 unknown (nodding thistle)



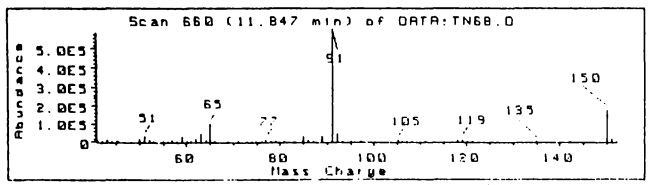
59 11.23 unknown (kamahi)



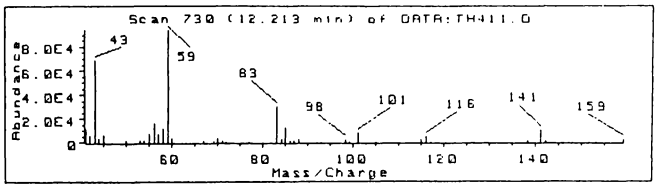
60 11.30 unknown (clover)



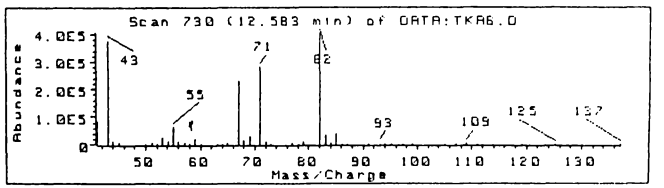
61 11.36 unknown (nodding thistle)



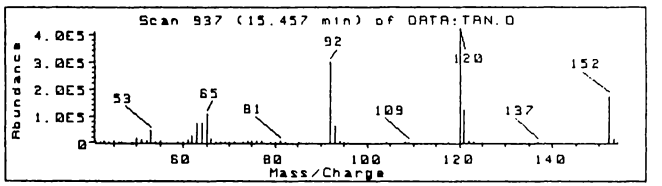
62 11.48 methyl 2-phenylethanoate



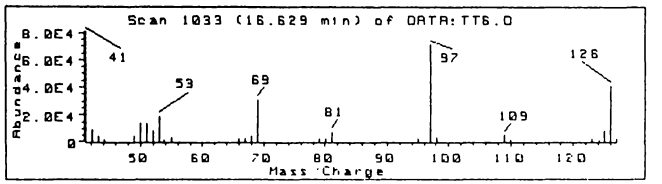
63 11.55 unknown



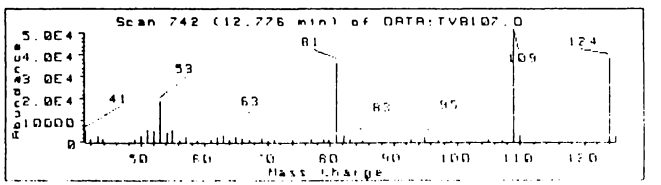
64 11.61 unknown



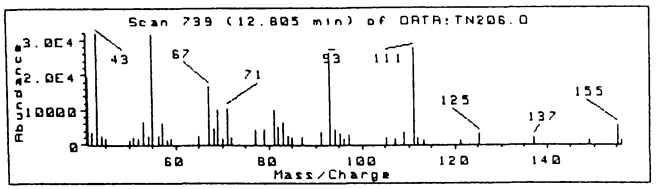
65 11.62 methyl 2-hydroxybenzoate



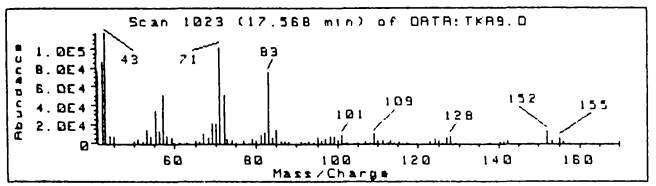
66 11.85 5-hydroxymethyl-2-furfural



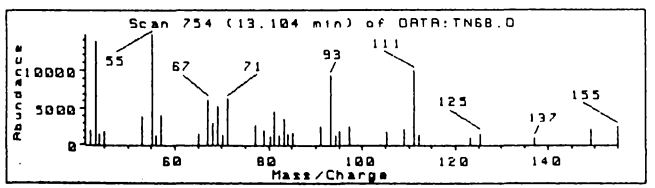
67 11.86 4-methoxyphenol



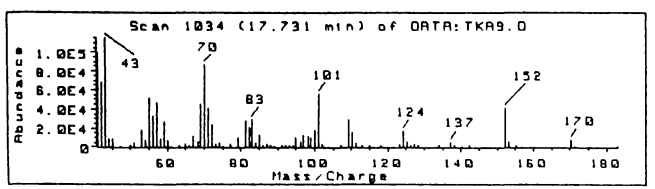
68 11.87 unknown (nodding thistle)



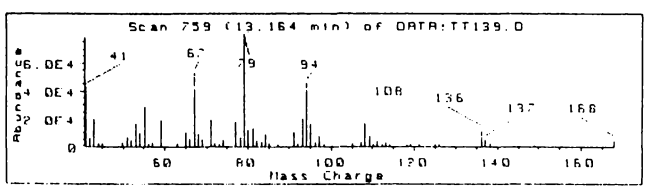
70 11.94 unknown[b] (kamahi)



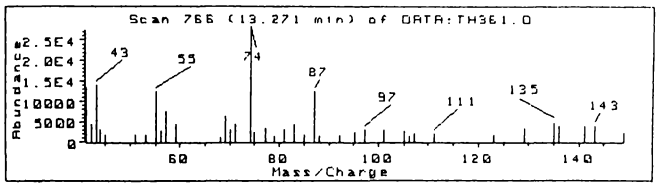
71 12.01 unknown (nodding thistle)



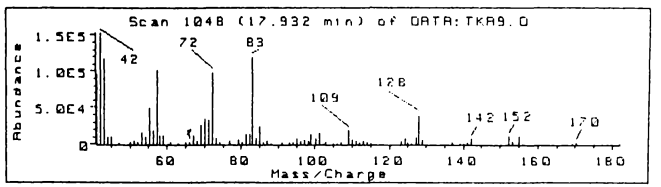
72 12.02 unknown[b] (kamahi)



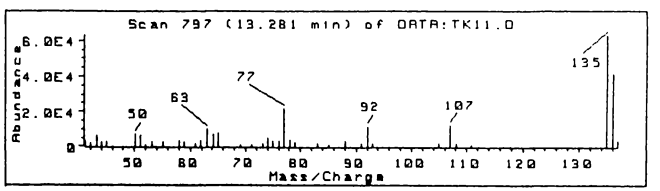
73 12.02 unknown (thyme)



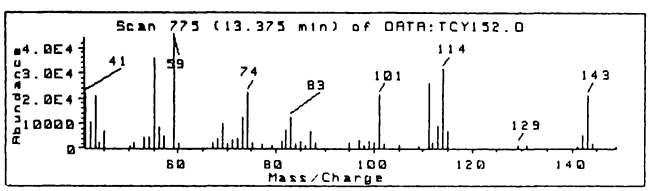
74 12.06 methyl nonanoate (9:0)



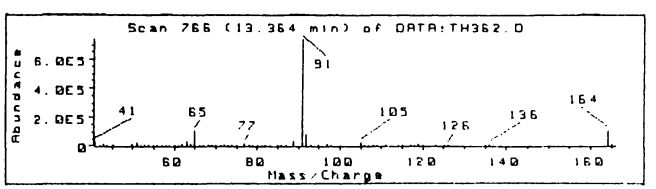
75 12.06 unknown[b] (kamahi)



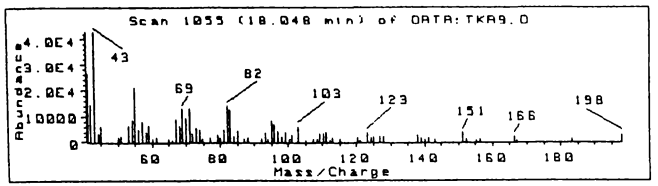
76 12.07 methoxybenzaldehyde



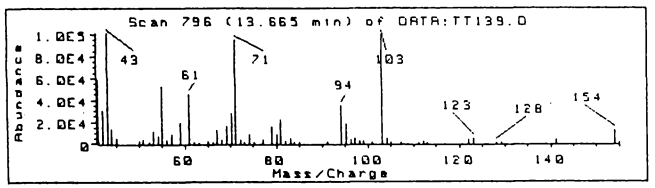
77 12.08 dimethyl hexanedioate



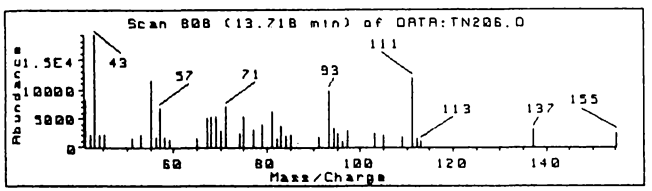
78 12.09 ethyl phenylethanoate



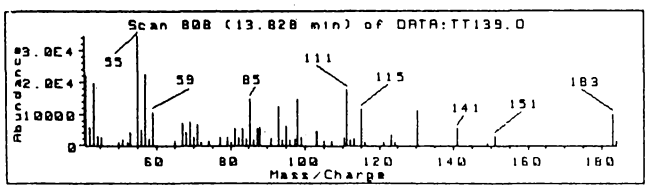
79 12.14 unknown[b] (kamahi)



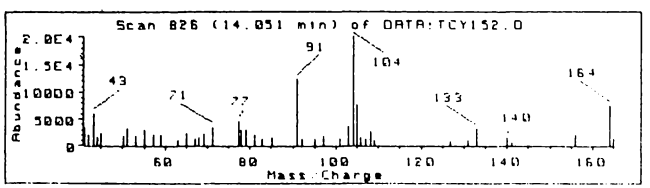
80 12.25 unknown (thyme)



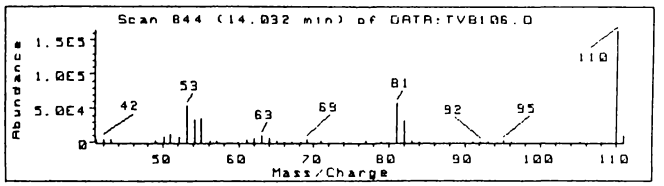
81 12.29 unknown (nodding thistle)



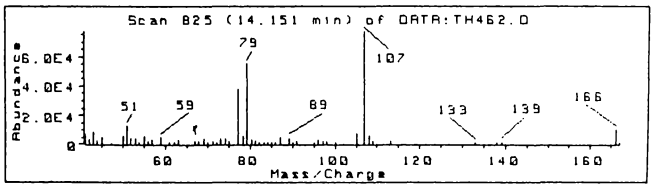
82 12.32 unknown (thyme)



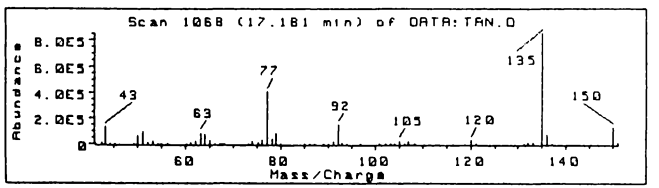
83 12.39 methyl 3-phenylpropionate



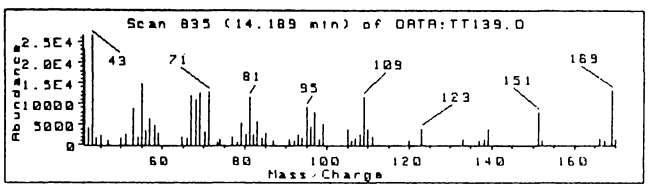
84 12.43 1,4-dihydroxybenzene



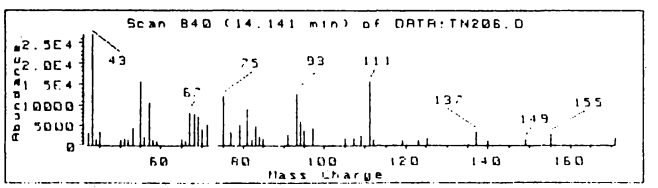
85 12.45 methyl 2-hydroxy-2-phenylethanoate



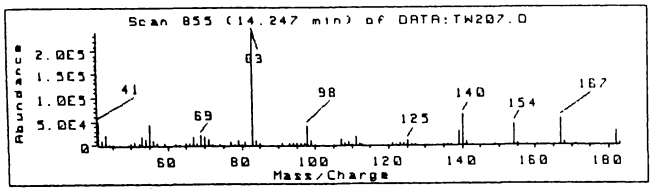
86 12.48 2'-methoxyacetophenone



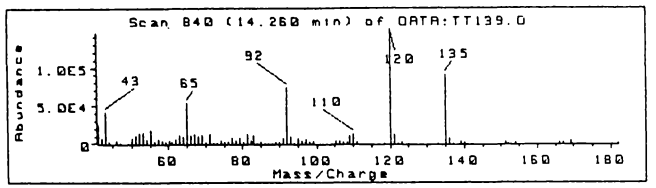
87 12.49 unknown (thyme)



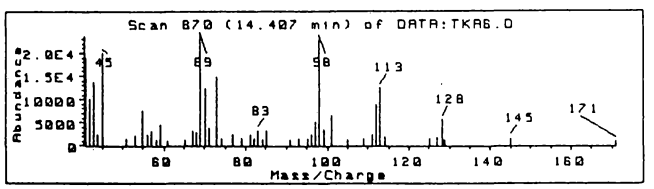
88 12.50 unknown (nodding thistle)



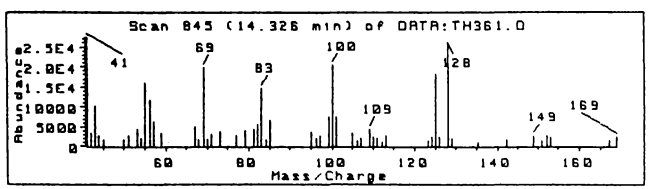
89 12.52 2-methoxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione



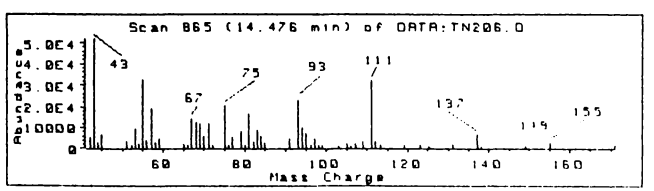
90 12.54 3'-aminoacetophenone



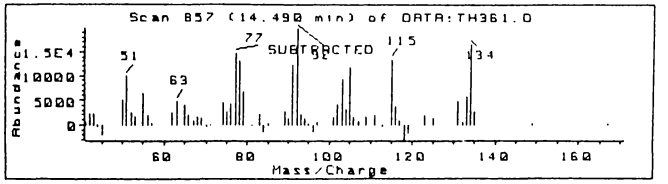
91 12.54 unknown[b] (kamahi)



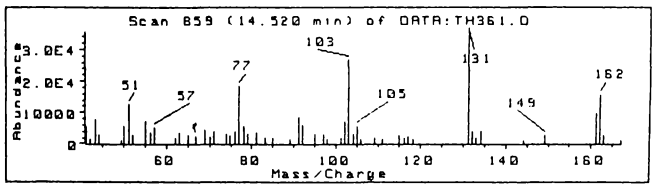
92 12.56 unknown (ling/heather)



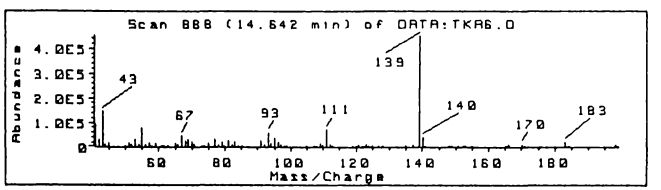
93 12.65 unknown (nodding thistle)



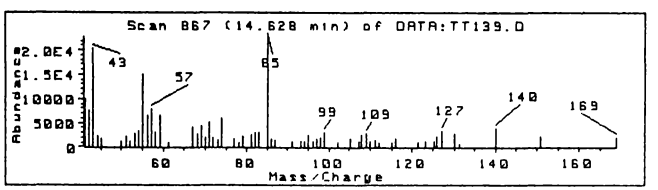
94 12.65 3-phenylprop-2-en-1-ol



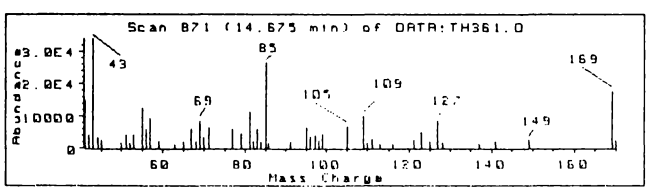
95 12.66 methyl *cis*-3-phenylprop-2-enoate



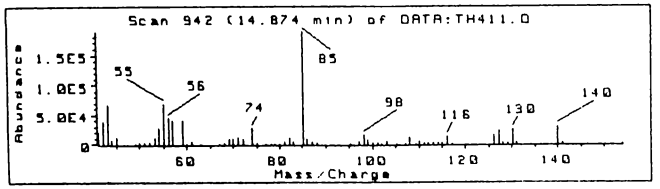
96 12.66 unknown[b] (kamahi)



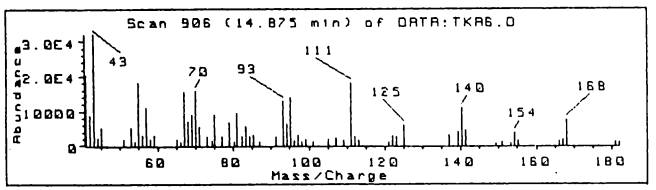
97 12.72 unknown (thyme)



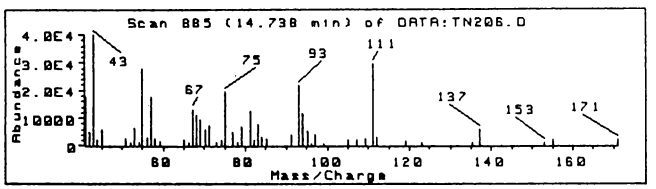
98 12.73 unknown (ling/heather)



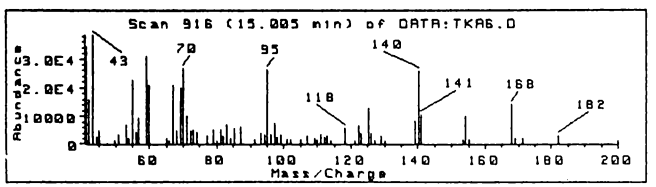
99 12.74 unknown (clover)



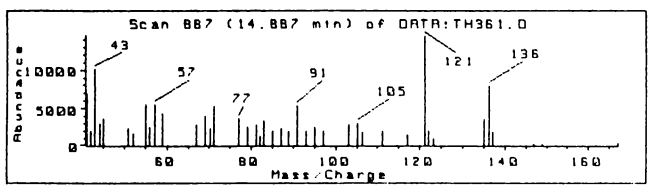
100 12.77 unknown[b] (kamahi)



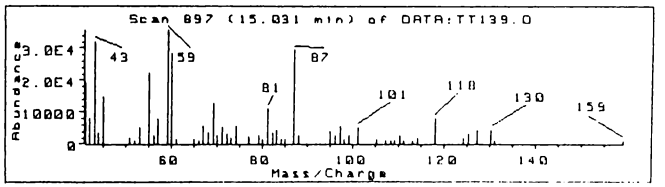
101 12.79 unknown (nodding thistle)



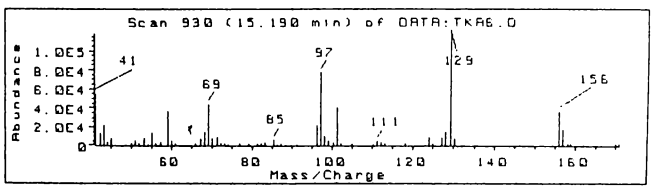
102 12.83 unknown[b] (kamahi)



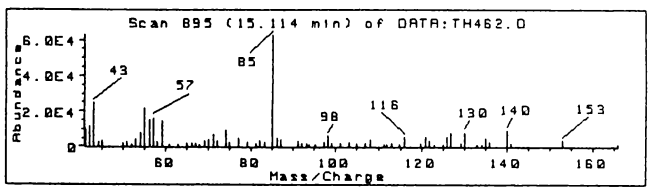
103 12.84 trimethylphenol



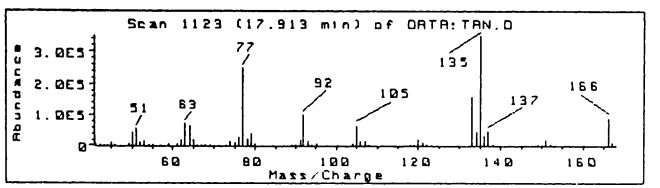
104 12.91 unknown[b]



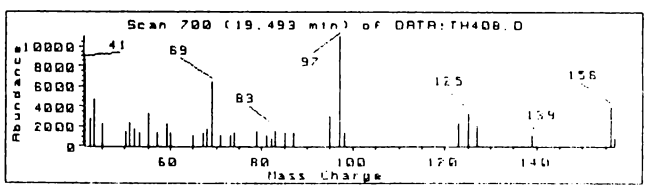
105 12.91 unknown[b] (kamahi)



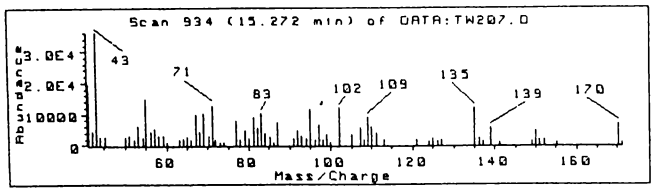
106 12.93 unknown (ling/heather)



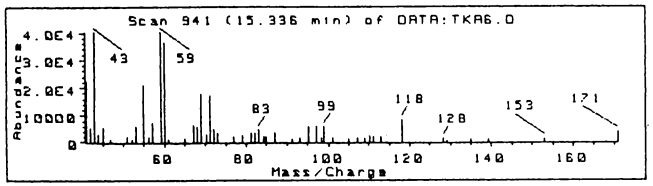
107 12.94 methyl 2-methoxybenzoate



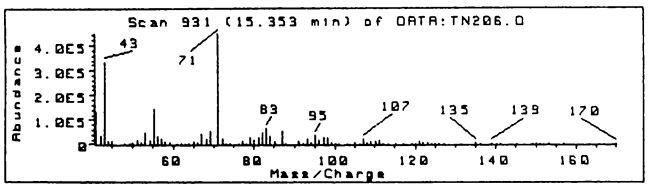
108 13.00 unknown (clover)



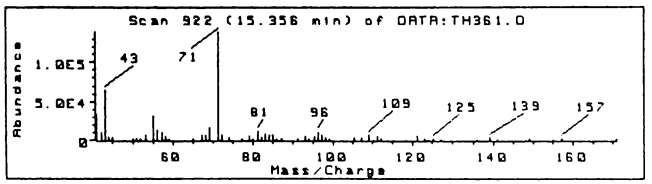
109 13.03 unknown (willow)



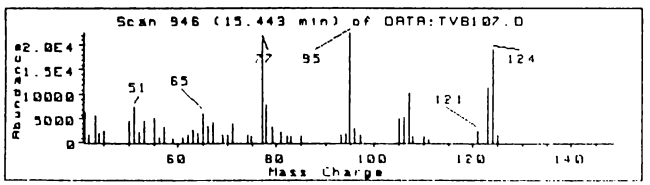
110 13.03 unknown[b] (kamahi)



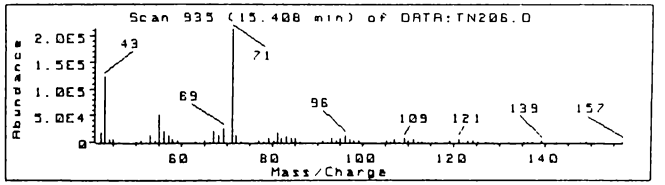
111 13.07 unknown (nodding thistle)



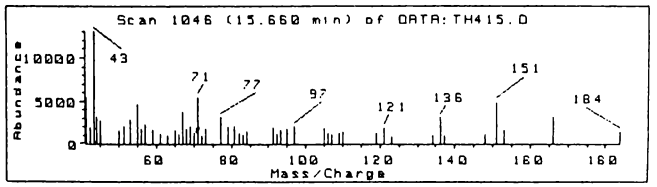
112 13.07 unknown (ling/heather)



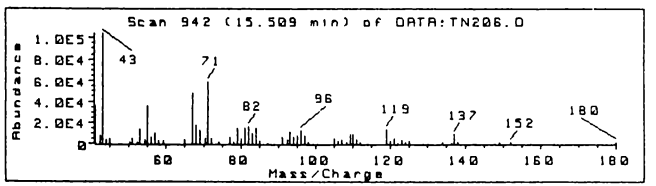
113 13.10 hydroxybenzyl alcohol



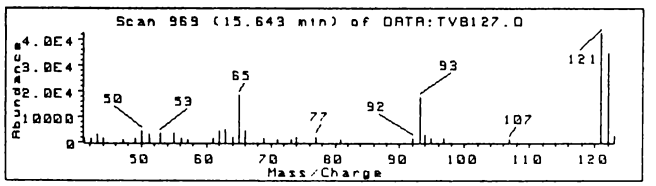
114 13.12 unknown (nodding thistle)



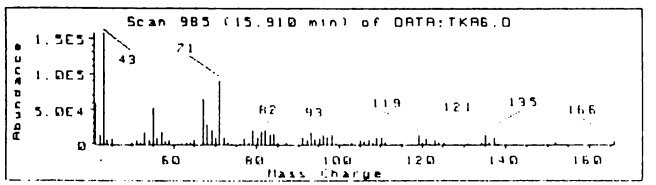
115 13.15 unknown



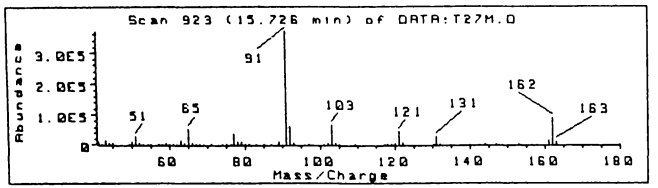
116 13.16 unknown



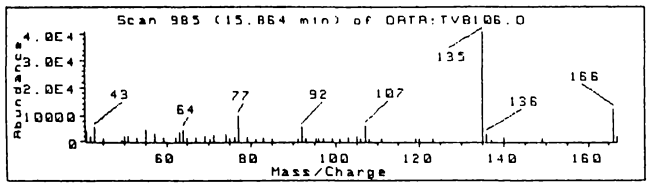
117 13.21 hydroxybenzaldehyde



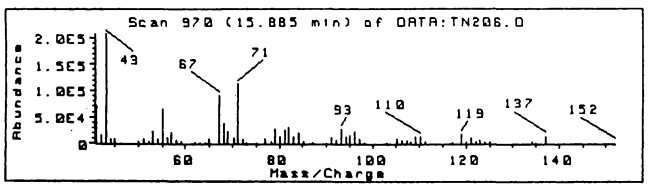
118 13.28 unknown[b] (kamahi)



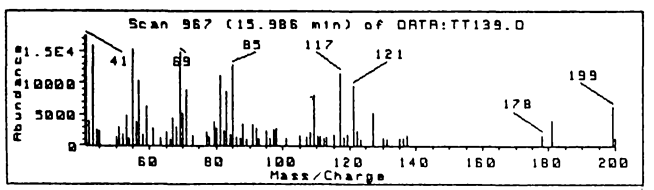
119 13.28 methyl 2-hydroxy-3-phenylpropionate



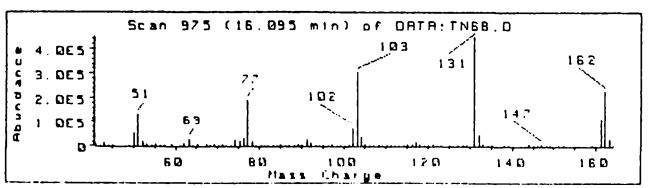
120 13.31 methyl 3-methoxybenzoate



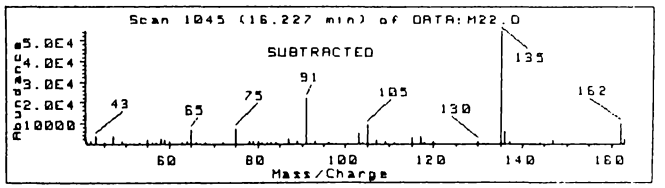
121 13.36 unknown (nodding thistle)



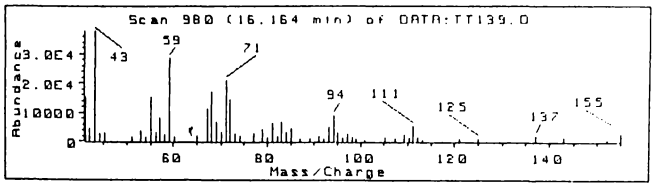
122 13.40 unknown (thyme)



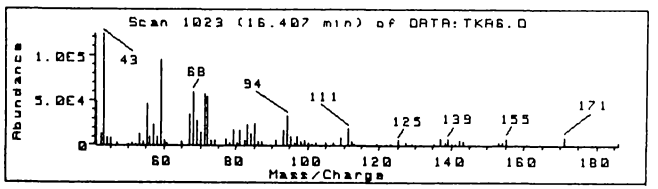
123 13.43 methyl *trans*-3-phenylprop-2-enoate



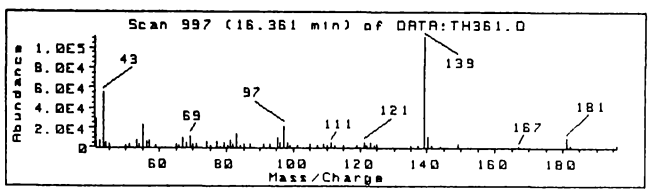
124 13.43 unknown (manuka)



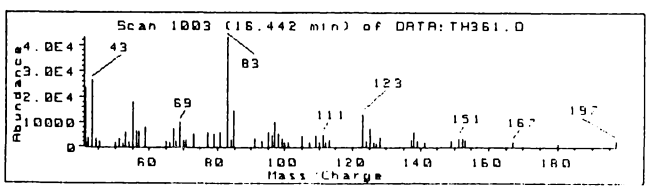
125 13.44 unknown (thyme)



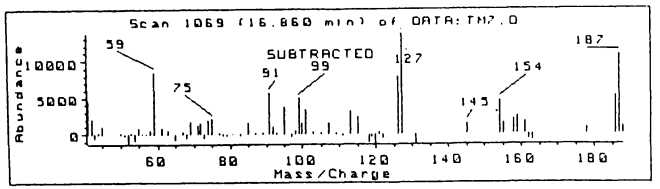
126 13.49 unknown[b] (kamahi)



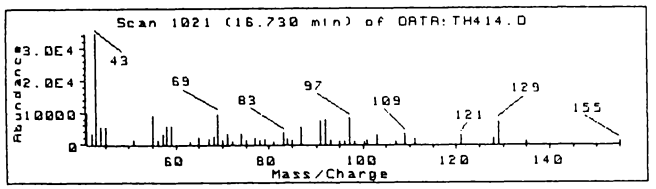
127 13.59 unknown (ling/heather)



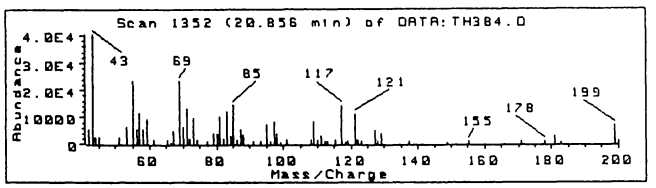
128 13.63 unknown (ling/heather)



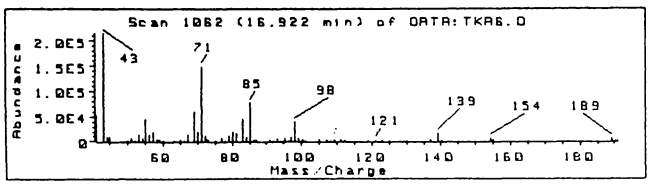
129 13.72 unknown (manuka)



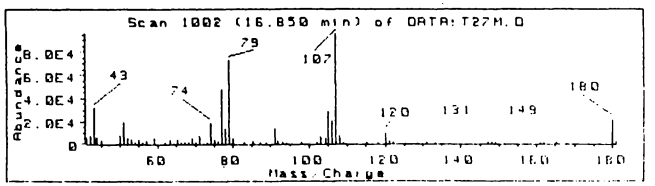
130 13.73 unknown



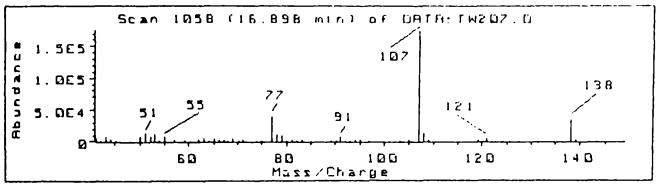
131 13.80 unknown (thyme)



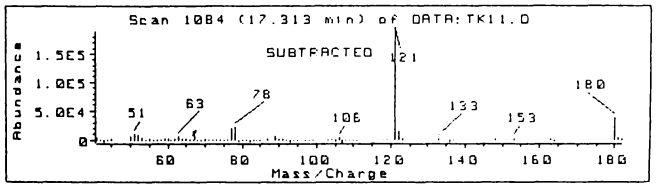
132 13.81 unknown[b] (kamahi)



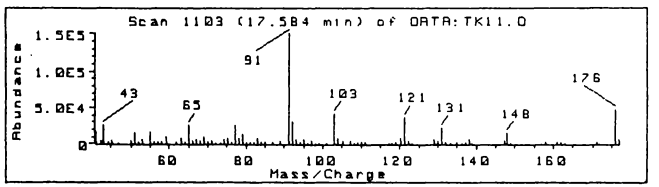
133 13.82 ethyl 2-hydroxy-2-phenylethanoate



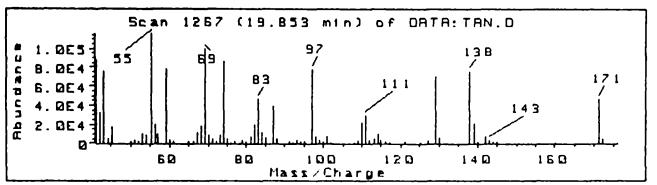
134 13.85 unknown (willow)



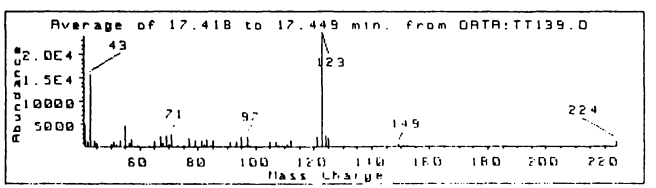
135 13.86 methyl 2-(methoxyphenyl)-ethanoate



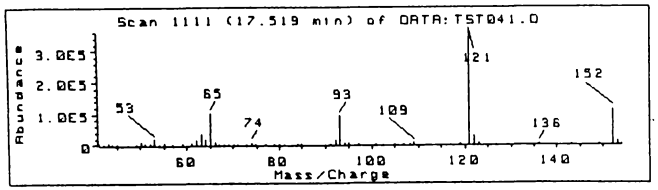
136 14.03 ethyl 2-hydroxy-3-phenylpropionate



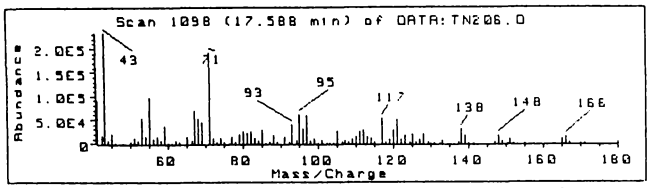
137 14.06 dimethyl octanedioate



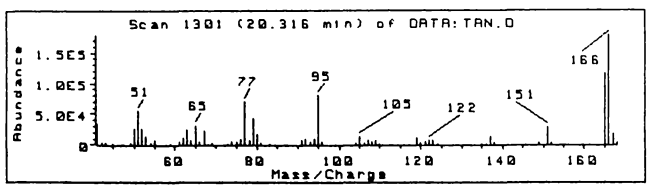
138 14.11 1-(3-oxo-1-butenyl)-2,6,6-trimethyl-1,2-epoxycyclohexan-4-ol



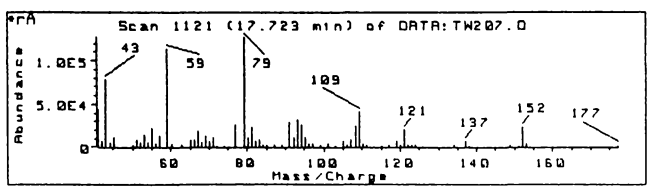
139 14.15 methyl 3-hydroxybenzoate



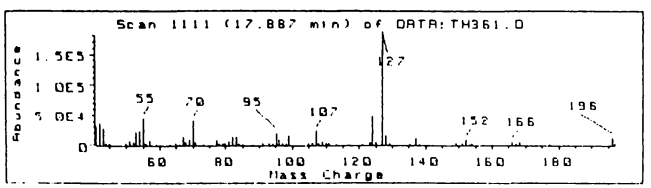
140 14.18 methyl 2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octadienoate



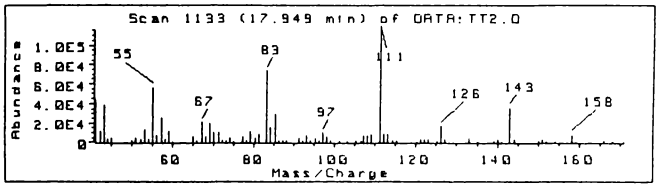
141 14.23 3,4-dimethoxybenzaldehyde



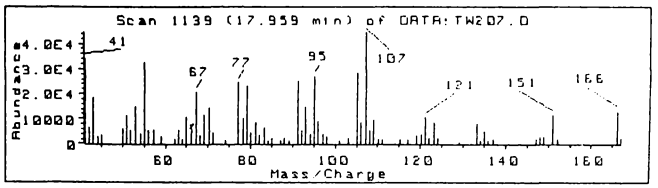
142 14.33 unknown (willow)



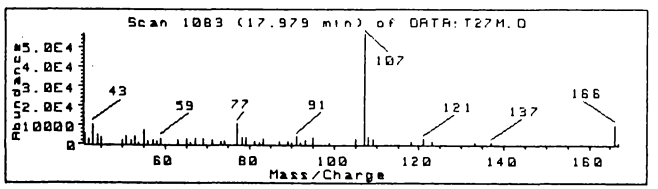
143 14.40 unknown (ling/heather)



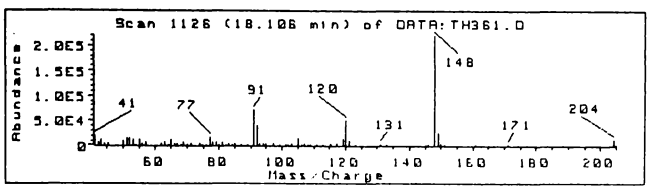
144 14.41 unknown (thyme)



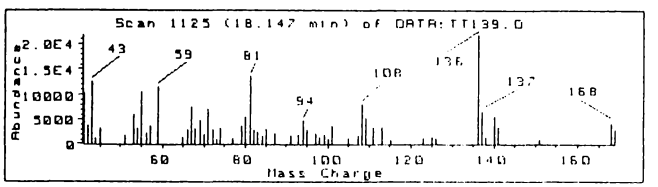
145 14.42 unknown (willow)



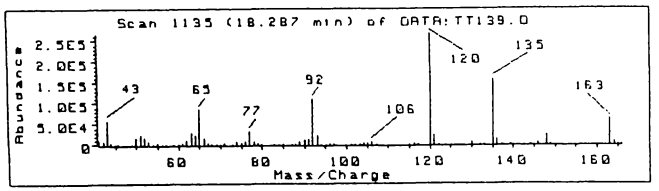
146 14.45 methyl 2-(hydroxyphenyl)-ethanoate



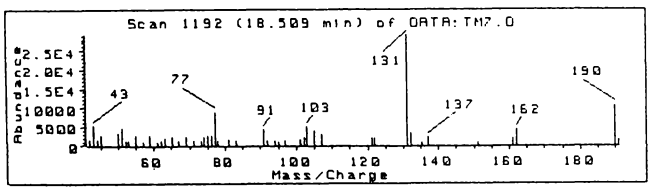
147 14.52 4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one



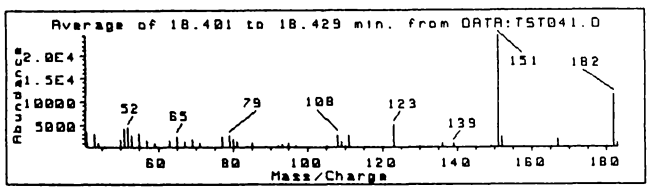
148 14.54 unknown



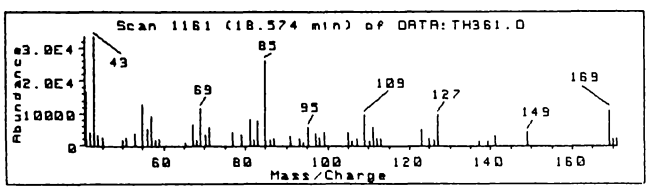
149 14.61 unknown (thyme)



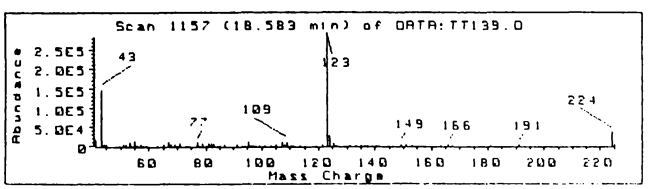
150 14.66 unknown (manuka)



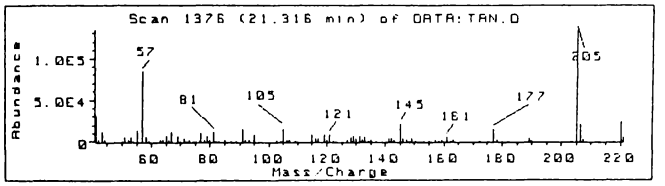
151 14.70 methyl 4-hydroxy-3-methoxybenzoate



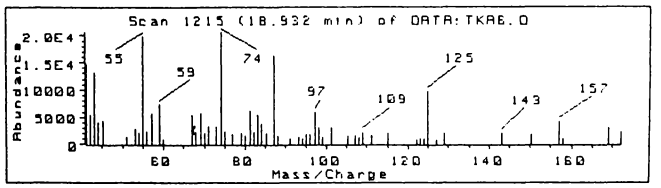
152 14.77 unknown (ling/heather)



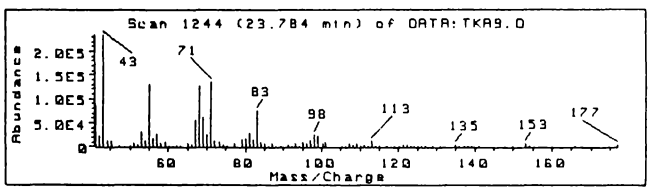
153 14.78 1-(3-oxo-1-butenyl)-2,6,6-trimethyl-1,2-epoxycyclohexan-4-ol



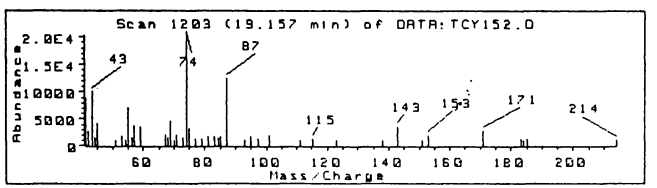
154 14.85 2,6-di-*tert*-butyl-4-methylphenol



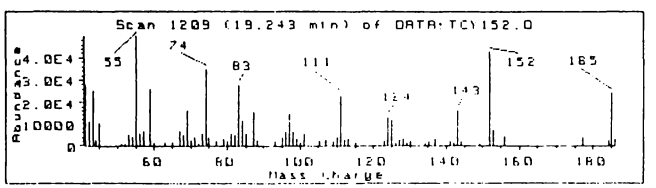
155 14.90 unknown, fatty acid?



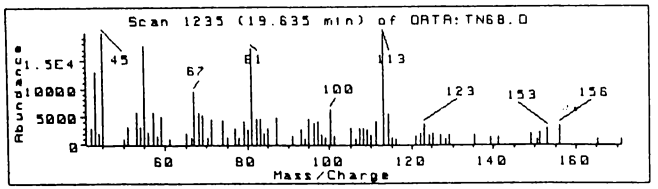
156 15.06 unknown[b] (kamahi)



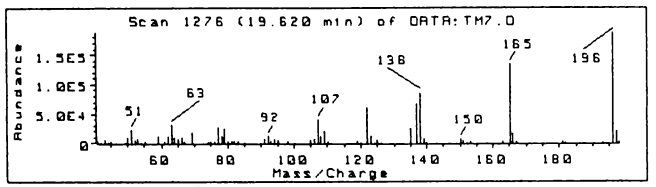
157 15.07 methyl laurate (12:0)



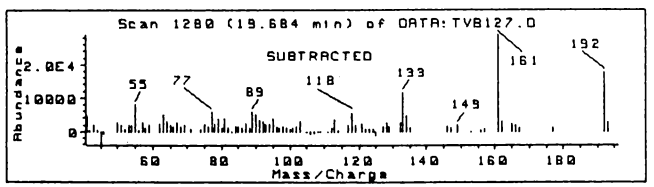
158 15.10 dimethyl nonanedioate



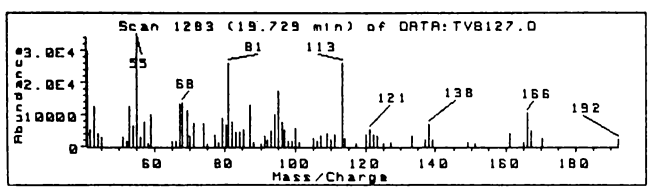
159 15.38 unknown (nodding thistle)



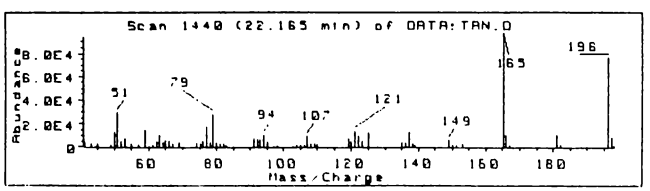
160 15.41 methyl 3,5-dimethoxybenzoate



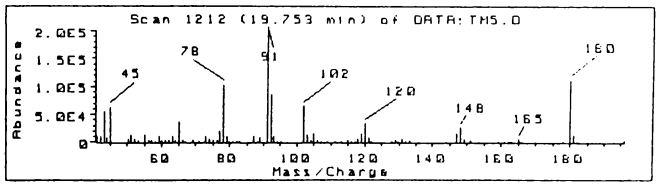
161 15.42 methyl 3-(methoxyphenyl)-prop-2-enoate



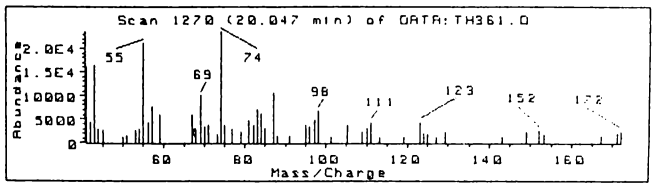
162 15.42 unknown



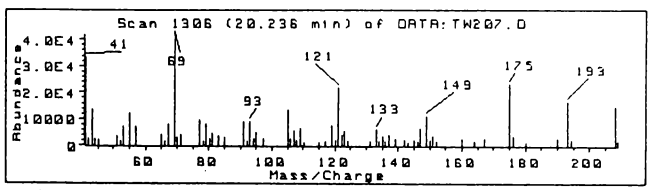
163 15.47 methyl 3,4-dimethoxybenzoate



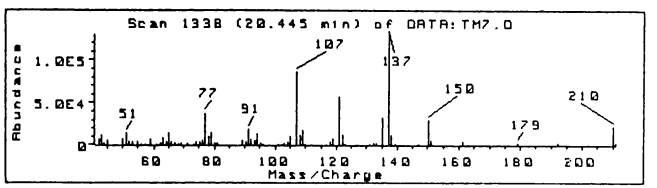
164 15.48 unknown (manuka)



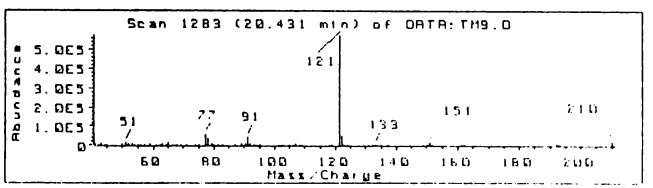
165 15.62 unknown, fatty acid?



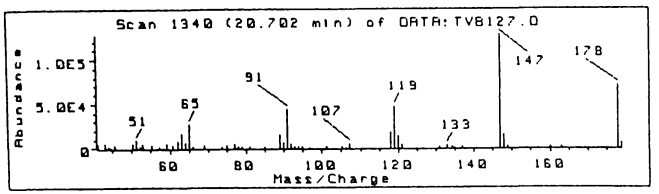
166 15.73 unknown (willow)



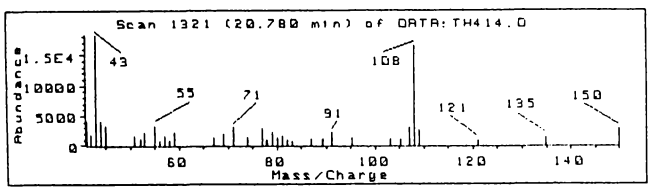
167 15.78 methyl 3-hydroxy-3-(methoxyphenyl)-propanoate



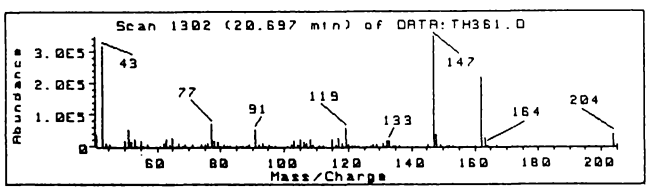
168 15.78 methyl 2-hydroxy-3-(4-methoxyphenyl)-propionate



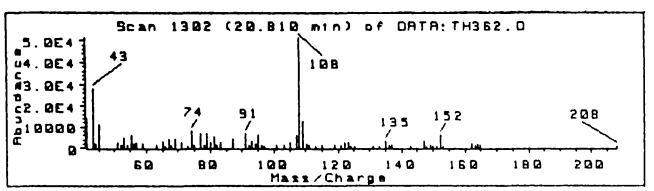
169 15.97 methyl 3-(4-hydroxyphenyl)-*cis*-prop-2-enoate



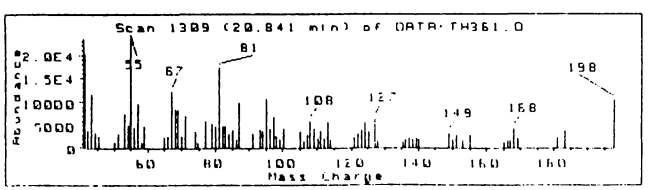
170 15.99 unknown (clover)



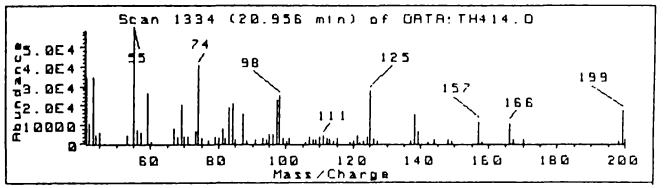
171 16.00 4-(3-oxo-1-butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one



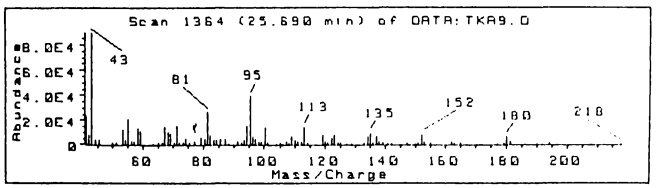
172 16.01 4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one



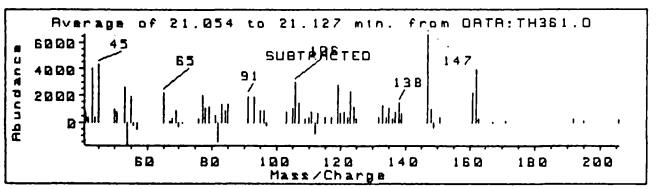
173 16.08 unknown (ling/heather)



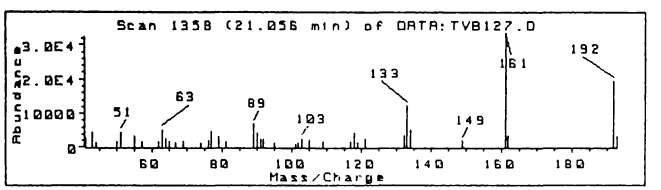
174 16.10 dimethyl decanedioate



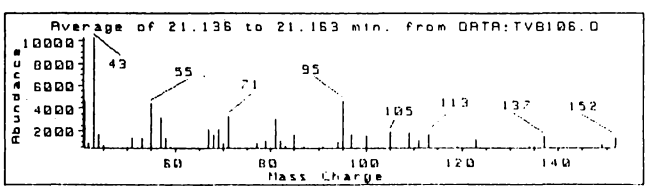
175 16.19 unknown[b] (kamahi)



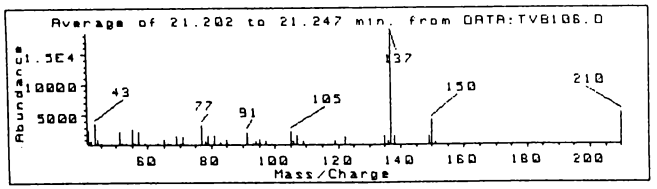
176 16.20 unknown (ling/heather)



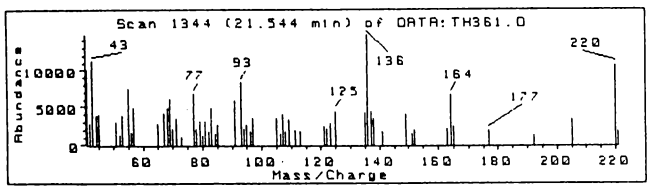
177 16.23 methyl 3-(4-methoxyphenyl)-*trans*-prop-2-enoate



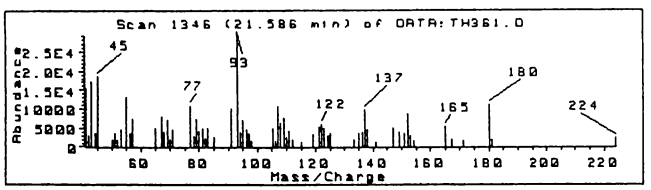
178 16.25 unknown (vipers bugloss)



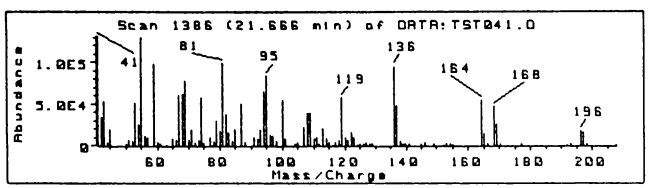
180 16.30 methyl 3-hydroxy-3-(methoxyphenyl)-propanoate



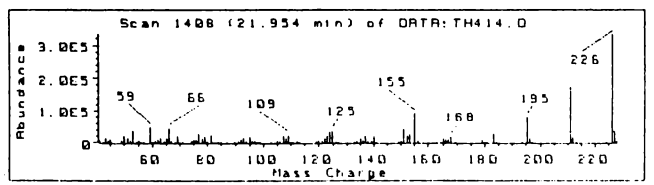
181 16.49 4-hydroxy-4-(3-oxo-1-butynyl)-3,5,5-trimethyl-cyclohex-2-en-1-one



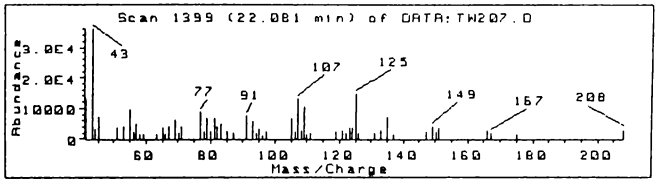
182 16.53 4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one



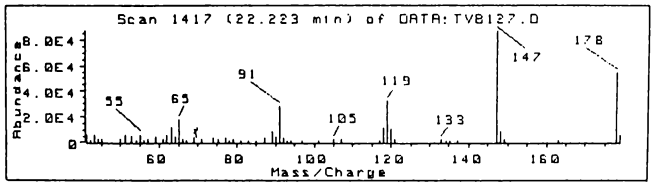
183 16.60 dimethyl *trans*-2-decenedioate



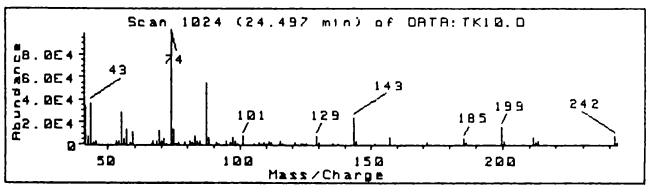
184 16.71 methyl 3,4,5-trimethoxybenzoate



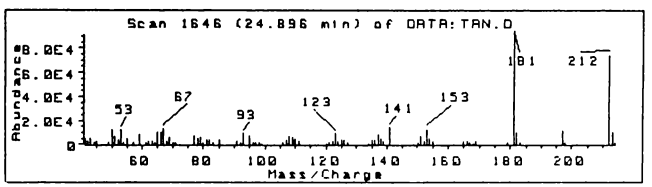
185 16.84 unknown (willow)



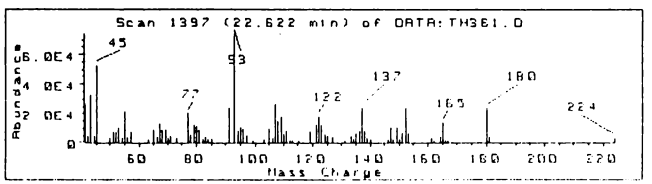
186 16.91 methyl 3-(4-hydroxyphenyl)-*trans*-prop-2-enoate



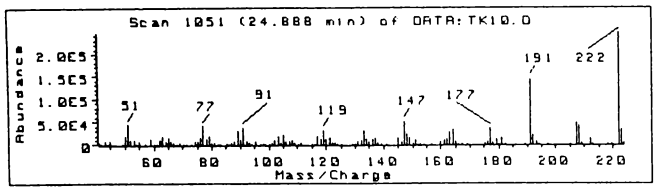
187 17.07 methyl myristate (14:0)



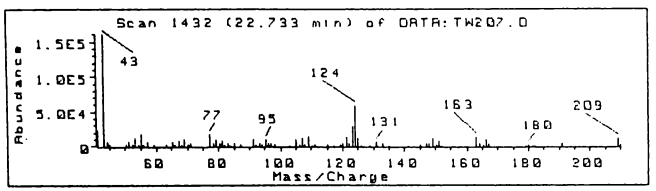
188 17.12 methyl 4-hydroxy-3,5-dimethoxybenzoate



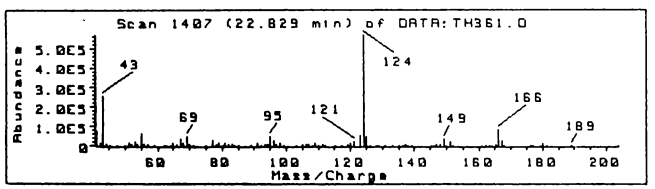
189 17.18 4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one



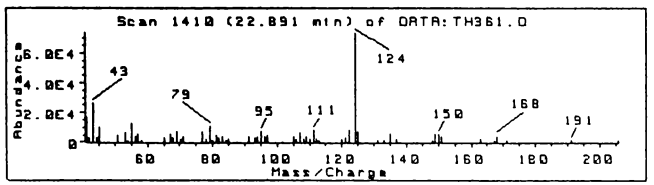
190 17.20 methyl 3-(3,4-dimethoxyphenyl)-*cis*-prop-2-enoate



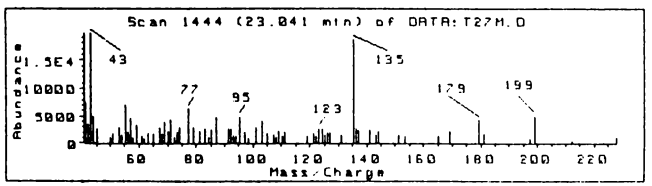
191 17.25 unknown (willow)



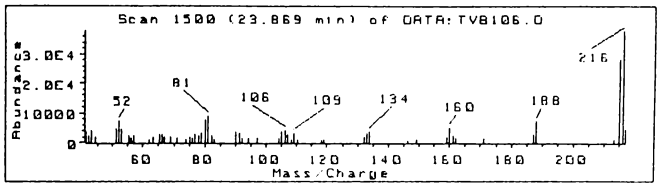
193 17.29 4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one [9]



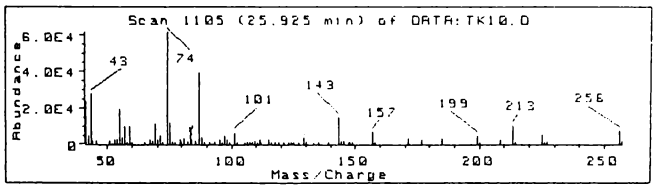
194 17.35 isomer of [9] (peak 193)



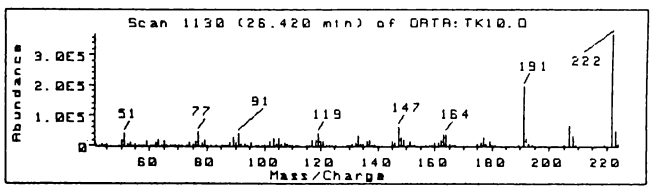
197 17.58 unknown (manuka)



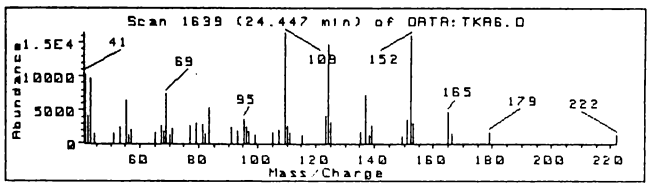
198 17.96 unknown (vipers bugloss)



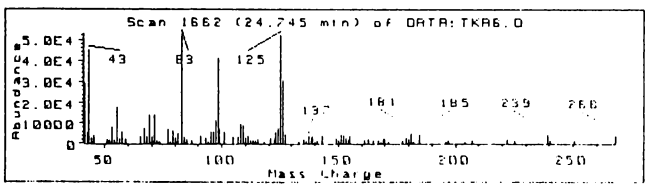
199 18.08 methyl pentadecanoate (15:0)



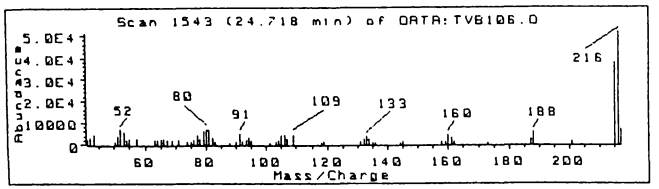
200 18.25 methyl 3-(3,4-dimethoxyphenyl)-*trans*-prop-2-enoate



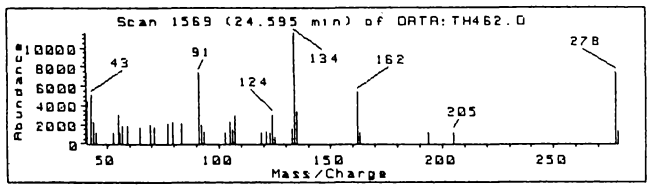
201 18.29 unknown[b] (kamahi)



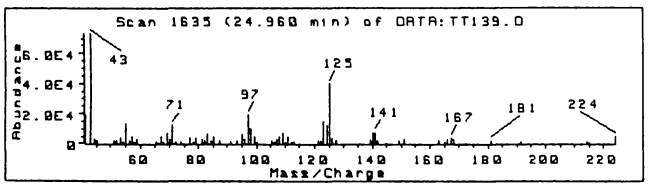
202 18.50 unknown[b] (kamahi)



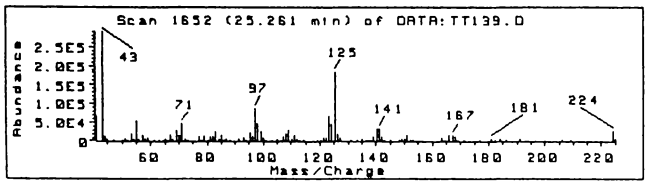
203 18.53 unknown (vipers bugloss)



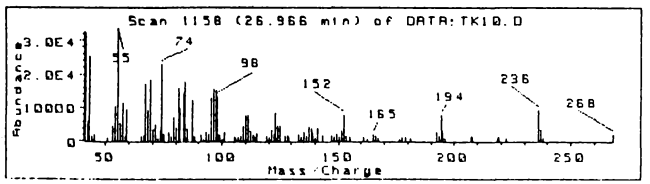
204 18.44 unknown (ling/heather)



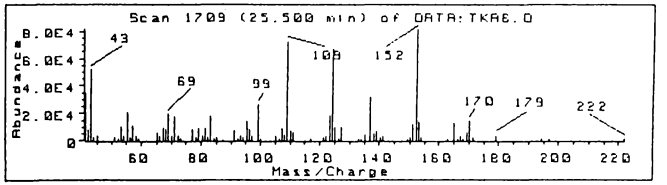
205 18.66 isomer of [22] (peak 206)



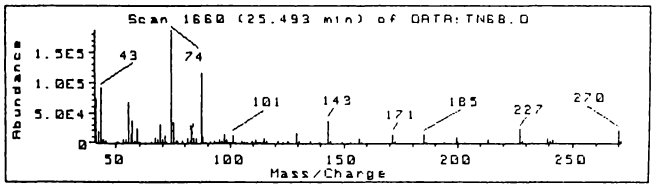
206 18.77 1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethyl-cyclohexane-*trans*-*cis*-1,2,4-triol [22]



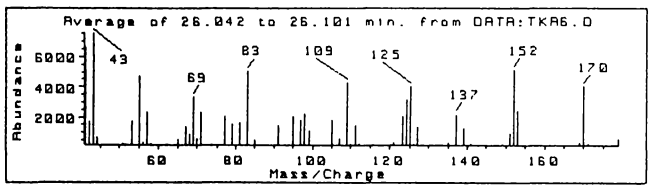
207 18.81 methyl palmitioleate (16:1)



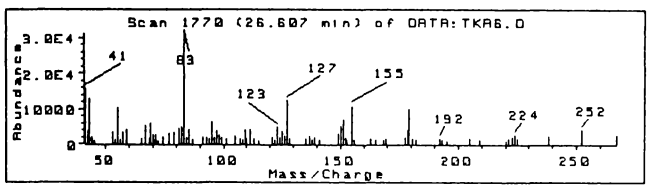
208 18.88 unknown[b] (kamahi)



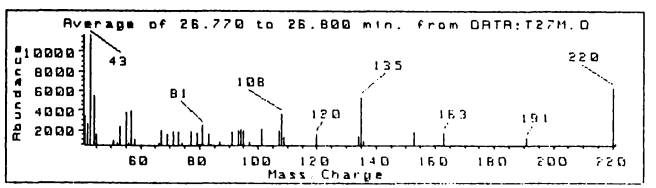
209 19.09 methyl palmitate (16:0)



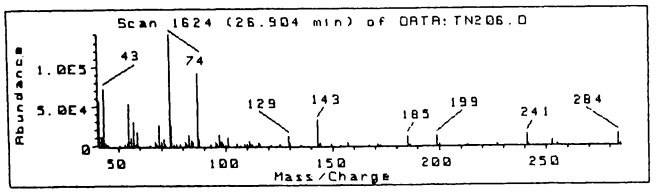
210 19.66 unknown[b] (kamahi)



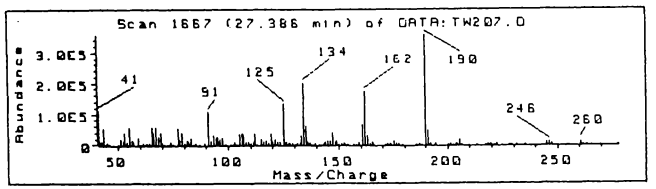
211 19.80 unknown[b] (kamahi)



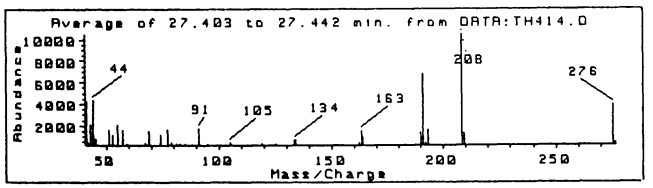
212 20.06 unknown (manuka)



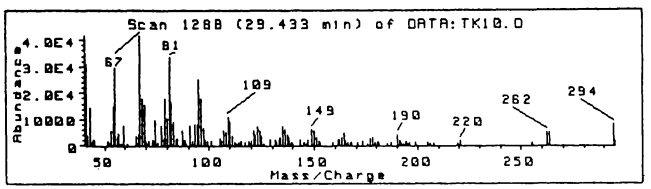
213 20.10 methyl margarate (17:0, internal standard)



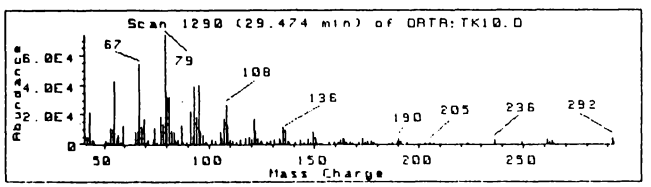
214 20.30 methyl abscisate



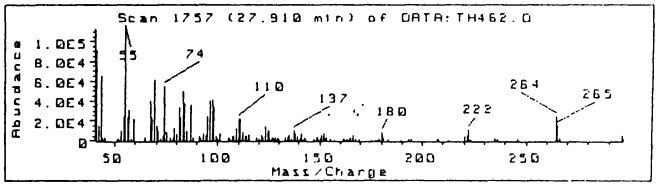
215 20.39 unknown (clover)



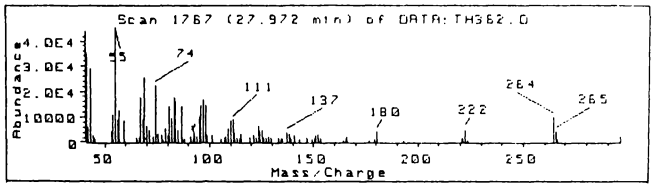
216 20.70 methyl linoleate (18:2)



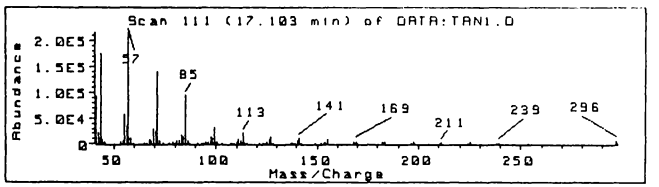
217 20.73 methyl α-linolenate (18:3)



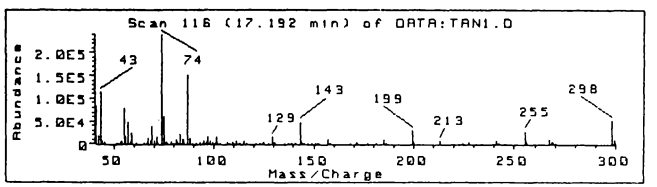
218 20.79 methyl oleate (18:1)



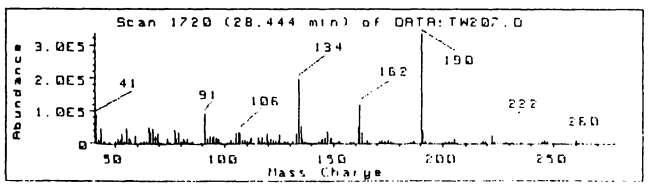
219 20.86 methyl oleate isomer (18:1)



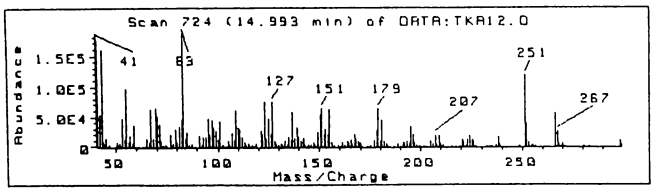
220 21.00 *n*-heneicosane (C_{21})



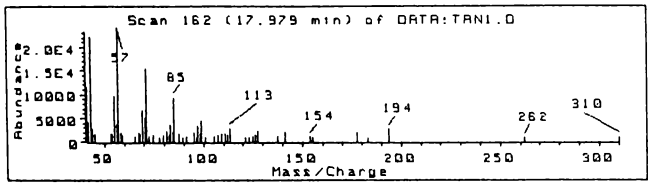
221 21.09 methyl stearate (18:0)



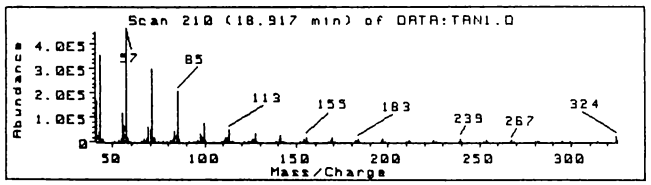
222 21.12 methyl abscisate



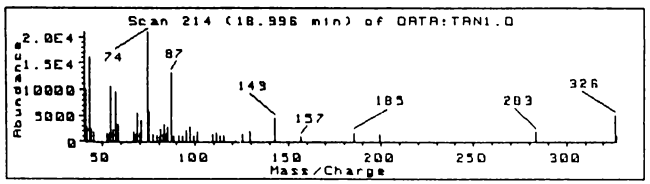
223 21.72 unknown[b] (kamahi)



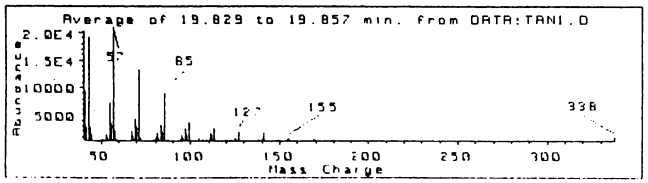
224 22.00 *n*-docosane (C_{22})



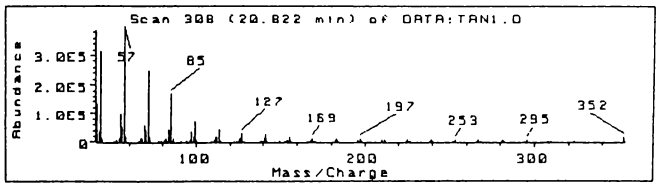
225 23.00 *n*-triacosane (C_{23})



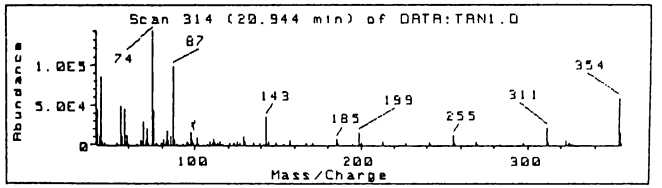
226 23.18 methyl arachidate (20:0)



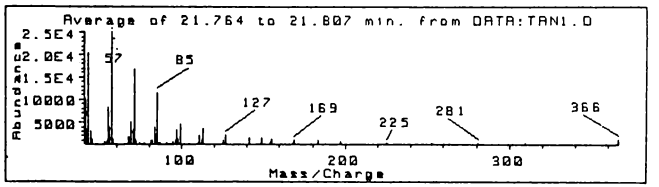
227 24.00 *n*-tetracosane (C_{24})



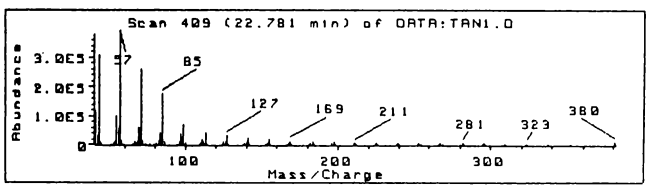
228 25.00 *n*-pentacosane (C_{25})



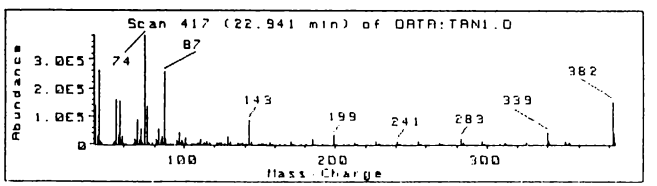
229 25.16 methyl behenate (22:0)



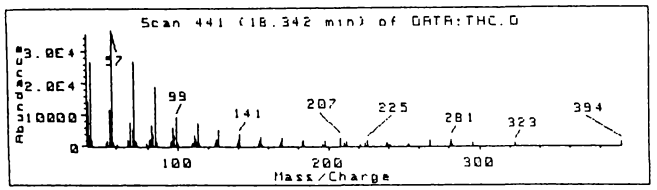
230 26.00 *n*-hexacosane (C_{26})



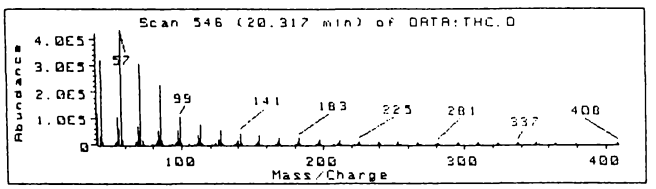
231 27.00 *n*-heptacosane (C_{27})



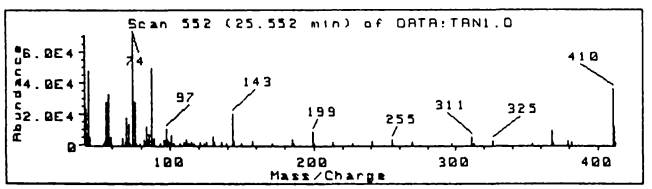
232 27.15 methyl lignocerate (24:0)



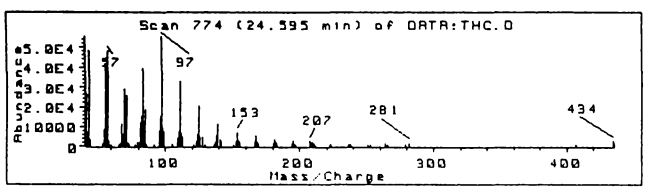
233 28.00 *n*-nonacosane (C_{28})



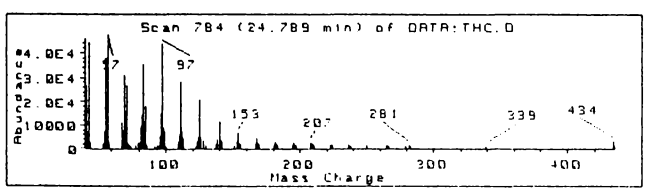
234 29.00 *n*-nonacosane (C_{29})



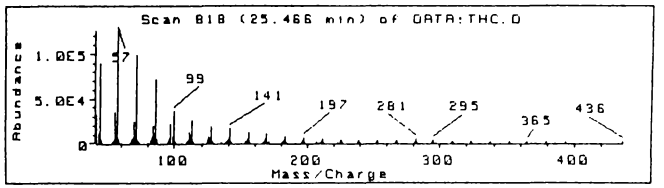
235 29.16 methyl cerotate (26:0)



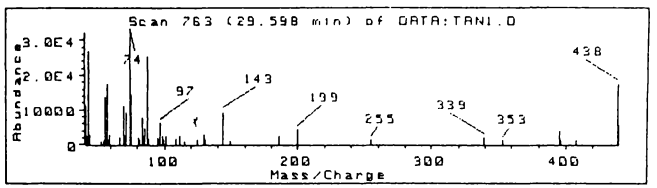
236 30.70 *n*-hentriacontene (C_{31})



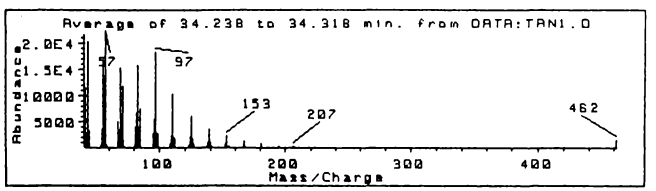
237 30.78 *n*-hentriacontene (C_{31})



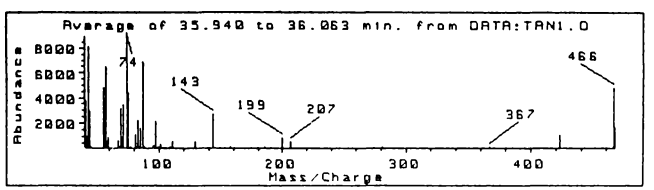
238 31.00 *n*-hentriacontane (C_{31})



239 31.20 methyl montanate (28:0)



240 32.70 *n*-tritriacontene (C_{33})



241 33.10 methyl triacontanoate (30:0)

Appendix B

X-Ray Crystallographic Data

for

1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethyl-cyclohexane-*trans-cis*-1,2,4-triol

Table I Observed and calculated structure factors

Table II Calculated hydrogen atom positions

Table III Thermal parameters

OBSERVED AND CALCULATED STRUCTURE FACTORS

H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC
2	0	0	131	128	3	0	1	81	77	4	8	1	17	9	2	7	2	13	17	2	5	3	14	15
4	0	0	76	72	5	0	1	16	20	1	9	1	13	14	5	7	2	17	18	3	5	3	13	10
2	1	0	53	47	0	1	1	51	59	3	9	1	17	13	1	8	2	31	32	5	5	3	23	24
3	1	0	61	61	1	1	1	45	47	1	0	2	74	69	2	8	2	16	12	1	6	3	38	39
4	1	0	25	25	2	1	1	49	48	2	0	2	38	40	3	8	2	13	14	2	6	3	12	10
5	1	0	16	13	3	1	1	13	13	3	0	2	32	30	1	9	2	15	13	6	6	3	18	21
0	2	0	104	103	4	1	1	33	31	4	0	2	62	63	1	0	3	137	145	0	7	3	40	42
1	2	0	76	72	5	1	1	19	18	8	0	2	14	7	2	0	3	30	30	2	7	3	16	11
4	2	0	37	38	6	1	1	18	18	0	1	2	10	10	3	0	3	80	74	3	7	3	15	11
5	2	0	23	21	7	1	1	16	14	1	1	2	115	131	4	0	3	31	29	4	7	3	13	11
1	3	0	28	31	0	2	1	20	20	2	1	2	42	38	6	0	3	13	17	0	8	3	18	16
2	3	0	82	80	1	2	1	65	63	3	1	2	37	38	0	1	3	108	124	1	8	3	23	25
3	3	0	27	24	2	2	1	48	47	1	2	2	90	93	1	1	3	33	33	0	9	3	17	15
4	3	0	37	40	3	2	1	48	48	2	2	2	41	39	2	1	3	55	57	0	0	4	31	31
6	3	0	41	46	4	2	1	21	23	3	2	2	33	34	3	1	3	18	18	1	0	4	70	75
7	3	0	13	8	7	2	1	18	15	4	2	2	31	30	4	1	3	33	36	2	0	4	63	58
0	4	0	48	46	0	3	1	19	16	5	2	2	27	29	6	1	3	13	15	5	0	4	17	18
1	4	0	29	29	1	3	1	24	24	6	2	2	17	15	0	2	3	32	33	0	1	4	44	43
2	4	0	33	31	2	3	1	20	21	1	3	2	58	58	1	2	3	48	45	1	1	4	34	37
3	4	0	23	24	3	3	1	30	31	2	3	2	79	76	2	2	3	42	40	2	1	4	28	27
5	4	0	33	35	5	3	1	27	31	5	3	2	13	12	3	2	3	63	63	3	1	4	54	57
1	5	0	11	12	6	3	1	31	33	6	3	2	28	29	4	2	3	38	40	4	1	4	27	27
2	5	0	47	46	8	3	1	16	19	0	4	2	12	12	6	2	3	22	25	6	1	4	20	21
6	5	0	16	19	0	4	1	92	96	1	4	2	12	11	8	2	3	16	15	0	2	4	50	53
0	6	0	15	13	1	4	1	48	49	2	4	2	70	67	0	3	3	37	37	1	2	4	48	48
1	6	0	19	19	2	4	1	54	52	3	4	2	38	38	1	3	3	57	56	2	2	4	21	19
2	6	0	13	9	5	4	1	20	21	5	4	2	20	19	2	3	3	20	20	3	2	4	31	33
3	6	0	12	12	1	5	1	25	23	6	4	2	16	21	3	3	3	22	23	6	2	4	19	18
4	6	0	22	22	2	5	1	37	36	1	5	2	12	9	4	3	3	13	16	0	3	4	35	33
5	6	0	16	16	0	6	1	22	21	2	5	2	20	20	5	3	3	40	43	1	3	4	31	28
1	7	0	21	22	3	6	1	13	8	3	5	2	15	13	6	3	3	19	18	2	3	4	43	41
4	7	0	16	12	0	7	1	20	19	6	5	2	15	16	1	4	3	57	56	3	3	4	11	9
0	8	0	21	19	2	7	1	20	18	0	6	2	19	20	2	4	3	25	26	4	3	4	12	14
1	9	0	15	13	4	7	1	14	15	1	6	2	13	13	3	4	3	14	12	5	3	4	24	27
1	0	1	14	14	0	8	1	40	41	5	6	2	27	28	6	4	3	21	21	7	3	4	14	10
2	0	1	34	31	3	8	1	13	11	1	7	2	15	15	1	5	3	37	35	0	4	4	15	14
1	4	4	19	14	4	3	5	20	22	3	1	6	47	47	2	0	7	48	49	0	8	7	16	15
2	4	4	36	36	5	3	5	38	39	5	1	6	16	16	3	0	7	81	79	0	0	8	102	96
3	4	4	33	33	7	3	5	13	8	6	1	6	17	18	4	0	7	42	43	1	0	8	19	17
4	4	4	23	23	0	4	5	41	42	7	1	6	14	17	5	0	7	26	26	4	0	8	15	15
6	4	4	22	22	1	4	5	28	28	0	2	6	49	48	0	1	7	115	124	0	1	8	116	123
1	5	4	16	16	2	4	5	17	16	1	2	6	66	67	1	1	7	19	18	1	1	8	94	96
2	5	4	22	19	3	4	5	32	34	2	2	6	39	37	2	1	7	41	45	2	1	8	50	46
3	5	4	32	29	6	4	5	12	12	3	2	6	53	52	3	1	7	28	29	3	1	8	43	45
4	5	4	12	7	0	5	5	23	22	4	2	6	15	15	4	1	7	19	18	5	1	8	12	7
1	6	4	20	19	1	5	5	13	15	5	2	6	12	18	7	1	7	13	12	0	2	8	79	79
2	6	4	27	25	2	5	5	22	21	6	2	6	16	13	0	2	7	18	18	1	2	8	65	63
5	6	4	13	12	3	5	5	20	21	7	2	6	19	20	1	2	7	44	44	2	2	8	21	22
1	7	4	18	18	4	5	5	18	17	0	3	6	15	19	2	2	7	40	42	3	2	8	61	60
0	8	4	18	20	5	5	5	24	25	1	3	6	21	22	3	2	7	52	51	4	2	8	20	22
0	9	4	20	17	0	6	5	34	32	2	3	6	45	44	4	2	7	27	27	5	2	8	15	15
1	0	5	146	158	1	6	5	23	23	3	3	6	22	23	7	2	7	13	5	6	2	8	14	19
2	0	5	149	148	2	6	5	30	29	5	3	6	19	17	0	3	7	30	30	0	3	8	35	34
3	0	5	24	24	3	6	5	13	18	6	3	6	31	33	1	3	7	50	52	1	3	8	33	32
4	0	5	22	22	4	6	5	16	17	0	4	6	24	27	3	3	7	30	31	2	3	8	43	45
6	0	5	16	17	5	6	5	13	9	1	4	6	42	43	4	3	7	11	7	3	3	8	22	19
0	1	5	132	158	0	7	5	15	17	2	4	6	30	29	5	3	7	13	13	4	3	8	22	24
1	1	5	46	44	4	7	5	14	8	3	4	6	20	22	6	3	7	27	26	5	3	8	15	13
2	1	5	79	82	0	8	5	15	13	4	4	6	13	11	0	4	7	29	28	6	3	8	21	20
3	1	5	12	12	1	8	5	18	18	7	4	6	14	8	1	4	7	45	42	0	4	8	47	45
4	1	5	38	39	3	8	5	20	18	2	5	6	16	16	2	4	7	16	14	1	4	8	28	28
5	1	5	13	14	4	8	5	15	8	3	5	6	29	28	3	4	7	20	22	2	4	8	32	36
6	1	5	15	20	0	9	5	28	32	4	5	6	15	13	4	4	7	12	8	3	4	8	15	16
0	2	5	12	10	1	0	6	47	56	0	6	6	13	12	5	4	7	19	21	4	4	8	13	13
1	2	5	38	37	2	0	6	16	18	1	6	6	23	20	1	5	7	23	25	5	4	8	24	25
2	2	5	31	29	3	0	6	39	38	3	6	6	17	16	2	5	7	28	29	1	5	8	26	28
4	2	5	31	29	4	0	6	41	40	1	7	6	24	24	3	5	7	21	24	2	5	8	30	30
7	2	5	13	9	6	0	6	17	19	3	7	6	17	19	5	5	7	22	22	5	5	8	20	23
0	3	5	90	85	7	0	6	14	8	2	8	6	13	9	7	5	7	17	9	1	6	8	21	23
1	3	5	28	28	0	1	6	24	26	3	8	6	15	20	1	6	7	26	24	2	6	8	32	33
2	3	5	55	48	1	1	6	74	79	4	8	6	19	16	3	6	7	15	15	3	6	8	21	23
3	3	5	15	15	2	1	6	52	50	1	0	7	87	88	0	7	7	19	17	0	7	8	14	14
2	7	8	33	35	3	6	9	15	15	5	5	10	24	27	3	5	11	15	16	3	4	12	11	9
0	8	8	18	20	0	7	9	24	22	6	5	10	13	11	1	6	11	33	34	5	4	12	14	15
1	8	8	13	12	1	7	9	13	12	0	6	10	17	19	2	6	11	17	15	6	4	12	18	22
4	8	8	17	17	2	7	9	13	15	3	6	10	25	25	3	6	11	22	20	1	5	12	24	26
1	0	9	38	36	3	7	9	12	14	4	6	10	19	23	1	7	11	19	22	2	5	12	14	12
2	0	9	34	31	0	8	9	15	18	6	6	10	14	8	2	7	11	24	26	0	6	12	14	15
3	0	9	12	11	0	0	10	75	74	0	7	10	15	19	3	7	11	21	21	1	6	12	24	22
4	0	9	39	39	1	0	10	64	64	1	7	10	14	14	1	8	11	15	16	3	6	12	18	16
6	0	9	17	18	2	0	10	58	55	5	7	10	13	5	2	8	11	18	14	4	6	12	18	24
1	1	9	41	41	3	0	10	27	27	2	8	10	17	16	3	8	11	14	10	0	7	12	26	27
2	1	9	55	53	4	0	10	38	41	1	0	11	20	21	0	0	12	22	22	2	8	12	15	9
3	1	9	50	50	5	0	10	20	22	2	0	11	33	32	1	0	12	63	63	1	0	13	56	54
4	1	9	29	29	0	1	10	103	101	3	0	11	44	43	3	0	12	22	22	2	0	13	13	14
7	1	9	16	12	1	1	10	52	52	1	1	11	25	22	5	0	12	16	16	3	0	13	28	27
1	2	9	41	37	2	1	10	63	63	2	1	11	38	38	0	1	12	25	24	8	0	13	15	15
2	2	9	33	31	3	1	10	32	30	3	1	11	76	76	1	1	12	11	11	0	1	13	62	61

OBSERVED AND CALCULATED STRUCTURE FACTORS

H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC
3	2	9	28	27	4	1	10	14	14	5	1	11	13	15	2	1	12	27	29	1	1	13	40	40
4	2	9	17	17	5	1	10	18	15	7	1	11	21	23	3	1	12	17	15	2	1	13	23	21
6	2	9	13	16	0	2	10	35	33	0	2	11	68	71	4	1	12	15	16	3	1	13	12	14
1	3	9	71	68	1	2	10	21	22	1	2	11	73	71	6	1	12	18	16	4	1	13	30	32
2	3	9	49	46	2	2	10	27	26	2	2	11	44	44	0	2	12	29	27	5	1	13	20	23
3	3	9	12	13	3	2	10	55	56	3	2	11	40	40	1	2	12	11	10	0	2	13	40	39
4	3	9	27	26	4	2	10	16	18	4	2	11	12	11	2	2	12	28	28	1	2	13	50	51
6	3	9	13	17	5	2	10	16	18	7	2	11	13	11	3	2	12	28	27	2	2	13	80	77
0	4	9	20	19	0	3	10	45	44	0	3	11	11	15	4	2	12	27	27	3	2	13	37	34
1	4	9	25	24	1	3	10	33	34	1	3	11	28	26	5	2	12	13	17	4	2	13	49	51
2	4	9	35	34	2	3	10	70	69	2	3	11	23	21	6	2	12	14	12	5	2	13	13	13
3	4	9	14	15	3	3	10	14	10	4	3	11	21	20	7	2	12	15	16	0	3	13	28	29
4	4	9	20	19	4	3	10	23	25	5	3	11	17	21	0	3	12	21	21	1	3	13	22	20
1	5	9	17	19	0	4	10	42	43	1	4	11	42	41	1	3	12	34	36	2	3	13	31	27
2	5	9	22	25	1	4	10	34	34	2	4	11	20	22	2	3	12	37	34	3	3	13	22	21
3	5	9	15	11	2	4	10	42	40	4	4	11	17	16	3	3	12	40	42	4	3	13	16	19
4	5	9	18	13	3	4	10	17	16	7	4	11	14	12	4	3	12	17	18	5	3	13	26	25
0	6	9	15	12	4	4	10	14	13	0	5	11	16	20	7	3	12	14	16	6	3	13	16	11
1	6	9	24	25	5	4	10	15	13	1	5	11	19	21	0	4	12	21	20	0	4	13	45	48
2	6	9	17	16	1	5	10	16	15	2	5	11	19	19	2	4	12	18	19	1	4	13	30	30
2	4	13	27	27	4	3	14	25	23	2	3	15	33	33	5	2	16	15	13	0	2	17	31	30
4	4	13	16	14	6	3	14	17	12	5	3	15	14	16	0	3	16	31	31	1	2	17	55	54
5	4	13	36	37	0	4	14	14	12	1	4	15	25	24	1	3	16	81	78	2	2	17	35	31
0	5	13	26	25	1	4	14	24	23	2	4	15	25	26	2	3	16	24	23	3	2	17	16	14
2	5	13	24	27	2	4	14	20	23	4	4	15	14	14	3	3	16	12	17	4	2	17	22	24
3	5	13	17	21	5	4	14	26	25	5	4	15	33	34	4	3	16	46	47	1	3	17	10	12
4	5	13	17	16	6	4	14	15	13	6	4	15	16	19	5	3	16	29	32	2	3	17	11	13
3	6	13	13	11	0	5	14	37	35	1	5	15	19	17	0	4	16	19	18	3	3	17	15	9
4	6	13	15	14	1	5	14	27	26	2	5	15	23	20	1	4	16	14	11	4	3	17	14	14
5	6	13	15	16	2	5	14	29	30	3	5	15	15	15	2	4	16	15	14	5	3	17	16	13
0	7	13	50	52	0	6	14	26	25	4	5	15	31	32	3	4	16	15	14	1	4	17	14	13
2	7	13	20	19	2	6	14	44	44	0	6	15	41	42	4	4	16	23	20	2	4	17	25	26
4	7	13	13	14	3	6	14	14	14	1	6	15	36	35	5	4	16	26	30	4	4	17	16	14
0	8	13	14	11	4	6	14	18	16	2	6	15	26	27	6	4	16	13	15	6	4	17	15	11
3	8	13	19	20	1	7	14	15	19	3	6	15	12	10	0	5	16	26	25	0	5	17	45	48
0	0	14	56	58	3	7	14	16	13	4	6	15	15	17	1	5	16	27	27	1	5	17	38	38
3	0	14	37	37	4	7	14	14	17	5	6	15	20	23	3	5	16	19	20	2	5	17	21	21
5	0	14	21	22	0	8	14	12	11	0	7	15	41	46	6	5	16	17	10	3	5	17	16	15
7	0	14	29	27	2	8	14	15	10	1	7	15	23	25	0	6	16	21	22	4	5	17	18	21
0	1	14	46	41	3	8	14	12	7	4	7	15	13	10	1	6	16	15	16	5	5	17	13	11
1	1	14	41	41	1	0	15	19	17	0	8	15	29	30	2	6	16	15	15	0	6	17	23	22
2	1	14	82	81	3	0	15	36	37	0	0	16	39	41	3	6	16	16	11	1	6	17	38	36
3	1	14	18	18	4	0	15	26	26	2	0	16	31	30	0	7	16	29	28	3	6	17	14	17
4	1	14	14	14	5	0	15	17	21	3	0	16	16	17	1	7	16	24	27	3	7	17	17	14
5	1	14	14	9	1	1	15	38	40	5	0	16	14	15	3	7	16	30	28	1	8	17	17	11
6	1	14	18	18	2	1	15	18	17	7	0	16	14	15	2	8	16	15	13	3	8	17	22	20
0	2	14	44	43	3	1	15	32	31	0	1	16	27	29	3	8	16	18	15	1	0	18	11	10
1	2	14	25	25	4	1	15	18	16	1	1	16	34	34	2	0	17	20	20	2	0	18	25	24
2	2	14	19	18	0	2	15	38	36	3	1	16	13	15	6	0	17	15	20	4	0	18	12	14
3	2	14	12	10	1	2	15	35	34	4	1	16	21	22	0	1	17	10	12	5	0	18	16	14
4	2	14	20	20	2	2	15	49	49	5	1	16	20	17	1	1	17	23	24	0	1	18	62	61
7	2	14	15	16	4	2	15	14	16	6	1	16	17	16	2	1	17	24	24	2	1	18	15	17
0	3	14	13	10	5	2	15	16	17	1	2	16	97	92	3	1	17	15	19	3	1	18	13	7
1	3	14	48	46	7	2	15	15	14	2	2	16	53	50	4	1	17	21	20	4	1	18	19	17
2	3	14	13	10	0	3	15	44	46	3	2	16	24	27	5	1	17	22	22	5	1	18	14	14
3	3	14	12	12	1	3	15	48	47	4	2	16	24	24	7	1	17	16	13	6	1	18	21	24
0	2	18	90	90	2	2	19	32	30	1	4	20	12	10	6	5	21	14	10	4	2	23	27	28
1	2	18	44	45	3	2	19	13	12	2	4	20	12	8	0	6	21	28	30	5	2	23	14	14
2	2	18	12	14	4	2	19	26	28	4	4	20	15	12	1	6	21	29	26	6	2	23	17	15
3	2	18	25	27	5	2	19	18	19	2	5	20	12	13	2	6	21	20	15	0	3	23	25	27
4	2	18	21	17	0	3	19	29	29	4	5	20	22	25	1	7	21	16	17	2	3	23	20	20
6	2	18	19	19	1	3	19	40	41	1	6	20	18	21	0	0	22	37	38	4	3	23	34	30
0	3	18	22	22	2	3	19	18	17	1	7	20	22	20	1	0	22	12	12	6	3	23	16	12
1	3	18	34	31	3	3	19	14	13	0	8	20	16	16	3	0	22	26	28	2	4	23	17	21
2	3	18	21	18	5	3	19	21	21	1	8	20	17	14	6	0	22	16	16	4	4	23	17	16
3	3	18	11	9	0	4	19	14	13	2	0	21	19	20	0	1	22	16	17	5	4	23	18	16
4	3	18	20	22	2	4	19	13	12	6	0	21	15	17	2	1	22	17	18	0	5	23	17	18
5	3	18	46	46	3	4	19	21	21	7	0	21	20	20	4	1	22	14	8	3	5	23	17	16
7	3	18	14	8	4	4	19	26	24	0	1	21	31	29	0	2	22	32	33	4	5	23	21	21
0	4	18	23	21	0	5	19	24	27	1	1	21	36	34	2	2	22	31	29	1	6	23	15	14
1	4	18	31	29	1	5	19	32	31	2	1	21	44	44	3	2	22	21	18	2	6	23	12	11
2	4	18	24	21	2	5	19	17	20	3	1	21	32	31	4	2	22	22	23	0	7	23	16	12
4	4	18	25	28	4	5	19	25	25	4	1	21	27	27	0	3	22	27	27	0	8	23	32	28
0	5	18	20	18	5	5	19	18	18	0	2	21	14	12	3	3	22	21	21	0	0	24	21	21
1	5	18	14	16	0	6	19	12	12	1	2	21	22	20	4	3	22	25	26	1	0	24	41	41
2	5	18	13	10	1	6	19	27	31	3	2	21	16	15	1	4	22	28	27	2	0	24	12	10
4	5	18	17	15	2	6	19	19	16	4	2	21	17	21	3	4	22	13	12	4	0	24	18	18
5	5	18	29	31	3	6	19	18	15	5	2	21	34	32	5	4	22	15	11	0	1	24	20	19
1	6	18	23	23	1	7	19	14	13	0	3	21	22	21	2	5	22	29	32	1	1	24	16	16
0	7	18	13	13	1	0	20	12	11	1	3	21	38	35	3	5	22	13	16	2	1	24	15	14
1	7	18	21	23	2	0	20	20	20	2	3	21	16	16	1	6	22	14	17	5	1	24	14	12
2	0	19	25	27	3	0	20	12	9	4	3	21	16	16	2	6	22	23	26	1	2	24	11	11
3	0	19	34	33	4	0	20	17	21	5	3	21	19	21	1	7	22	12	15	4	2	24	24	28
4	0	19	13	10	0	1	20	53	55	0	4	21	23	23	7	0	23	17	13	5	2	24	20	21
7	0	19	19	17	1	1	20	35	38	1	4	21	24	22	0	1	23	24	23	1	3	24	13	12
0	1	19	35	36	1	2	20	34	35	2	4	21	13	12	2	1	23	25	23	2	3	24	27	25
1	1	19	16	16	3	2	20	20	22	4	4	21	16	20	3	1	23	12	7	4	3	24	19	18
2	1	19	35	36	5	2	20	16	18	5	4	21	28	31	4	1	23	26	28	1	4	24	16	18

OBSERVED AND CALCULATED STRUCTURE FACTORS

H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC
3	1	19	23	23	0	3	20	16	18	0	5	21	18	19	0	2	23	26	24	2	4	24	18	19
7	1	19	16	19	1	3	20	17	16	1	5	21	23	26	1	2	23	23	24	4	4	24	19	14
0	2	19	14	10	5	3	20	22	21	4	5	21	18	17	2	2	23	39	39	1	5	24	17	16
1	2	19	19	18	0	4	20	30	29	5	5	21	14	6	3	2	23	21	21	2	5	24	43	42
3	5	24	13	11	3	4	26	31	31	3	3	28	17	18	2	0	31	13	8	1	3	33	16	13
1	6	24	19	19	1	5	26	21	21	0	4	28	19	20	3	0	31	14	8	2	3	33	18	11
2	6	24	26	23	2	5	26	19	23	2	4	28	16	13	1	1	31	17	17	2	4	33	18	15
0	7	24	14	14	3	5	26	15	12	3	4	28	14	12	0	2	31	23	19	1	5	33	13	9
2	0	25	15	17	1	6	26	13	13	1	5	28	13	7	1	2	31	13	16	0	0	34	19	21
3	0	25	22	22	2	6	26	15	14	2	5	28	25	26	2	2	31	15	9	1	1	34	31	33
5	0	25	14	12	3	0	27	16	12	3	5	28	13	12	1	3	31	17	19	2	1	34	19	21
0	1	25	23	25	0	1	27	13	10	4	5	28	14	14	4	3	31	14	13	3	1	34	14	10
2	1	25	15	15	2	1	27	20	21	1	0	29	34	34	2	4	31	19	17	4	1	34	15	13
3	1	25	12	11	3	1	27	15	17	3	0	29	25	24	3	4	31	14	17	5	1	34	14	13
1	2	25	15	15	4	1	27	24	25	1	1	29	25	28	2	6	31	16	12	1	2	34	16	20
2	2	25	26	23	5	1	27	13	12	3	1	29	29	33	0	0	32	26	27	3	2	34	15	16
3	2	25	12	6	1	2	27	23	22	4	1	29	23	21	1	0	32	33	34	3	3	34	13	14
4	2	25	20	23	2	2	27	21	24	2	2	29	18	21	3	0	32	18	22	1	4	34	23	20
0	3	25	20	18	4	2	27	15	17	3	2	29	16	19	4	0	32	18	22	2	1	35	21	17
3	3	25	20	18	0	3	27	20	14	0	3	29	19	22	0	1	32	37	40	3	1	35	13	10
2	4	25	14	16	1	3	27	18	15	1	3	29	15	16	1	1	32	13	13	0	2	35	16	16
4	4	25	16	17	2	3	27	30	28	3	3	29	12	12	2	1	32	18	15	0	3	35	17	14
2	5	25	13	15	1	4	27	14	15	1	4	29	17	13	3	1	32	37	38	1	3	35	14	14
3	5	25	19	16	2	4	27	16	17	2	4	29	30	30	0	2	32	15	13	0	4	35	14	5
5	5	25	14	8	0	5	27	16	22	0	5	29	16	16	1	2	32	14	13	2	5	35	16	14
0	6	25	20	21	1	5	27	23	24	1	5	29	32	33	2	2	32	22	22	0	0	36	44	44
2	7	25	14	10	4	5	27	18	11	2	5	29	20	23	3	2	32	17	14	0	1	36	26	26
2	0	26	14	10	5	5	27	15	6	3	6	29	13	5	4	2	32	17	15	1	1	36	14	14
4	0	26	15	14	0	0	28	56	54	0	0	30	41	44	2	3	32	22	19	0	2	36	32	33
0	1	26	24	22	3	0	28	27	28	2	0	30	14	13	4	4	32	14	11	3	3	36	14	5
2	1	26	15	16	5	0	28	17	16	4	0	30	26	25	1	5	32	12	10	2	0	37	15	18
3	1	26	26	27	1	1	28	24	21	1	1	30	20	22	2	5	32	17	21	3	0	37	21	23
4	1	26	19	26	2	1	28	25	24	3	1	30	13	11	4	0	33	25	27	4	0	37	28	30
3	2	26	12	13	4	1	28	15	13	0	2	30	19	17	0	1	33	16	15	1	1	37	18	22
4	2	26	27	28	0	2	28	14	16	4	2	30	22	26	1	1	33	12	8	2	1	37	13	13
0	3	26	17	18	1	2	28	19	19	1	3	30	29	27	3	1	33	14	12	0	2	37	29	33
1	3	26	25	21	4	2	28	14	21	2	3	30	25	25	1	2	33	18	17	3	2	37	23	20
2	3	26	16	14	6	2	28	16	11	4	3	30	13	9	3	2	33	12	12	2	3	37	15	18
3	3	26	16	18	0	3	28	27	27	2	4	30	16	15	4	2	33	15	15	2	0	38	13	4
2	4	26	17	12	2	3	28	20	22	5	4	30	17	16	0	3	33	23	24	2	1	38	18	16
2	3	38	15	9	2	1	39	14	7	3	2	39	16	11	0	0	40	33	35	2	1	41	15	14
0	4	38	14	14	3	1	39	14	13	0	3	39	16	17	3	0	40	17	20	1	2	41	16	11
2	4	38	18	14	1	2	39	21	23	0	4	39	18	17	0	2	40	19	13					

II Calculated hydrogen atom positions for [22].

	molecule A			molecule B		
	x/a	y/b	z/c	x/a	y/b	z/c
H31	0.8494 (20)	0.6240 (14)	0.7861 (3)	0.8053 (23)	0.1226 (18)	0.9281 (4)
H32	0.7094 (20)	0.7177 (14)	0.8055 (3)	0.8637 (23)	0.2138 (18)	0.8965 (4)
H4	0.8479 (22)	0.6721 (16)	0.8545 (4)	0.6165 (21)	0.1747 (15)	0.8724 (3)
H51	1.0870 (20)	0.5377 (16)	0.8576 (4)	0.4011 (21)	0.0249 (16)	0.8961 (4)
H52	1.0929 (20)	0.5199 (16)	0.8188 (4)	0.5152 (21)	0.0081 (16)	0.9282 (4)
H7	1.1866 (19)	1.0317 (15)	0.8169 (3)	0.4429 (19)	0.5235 (17)	0.9412 (4)
H8	1.3533 (19)	0.9175 (15)	0.7613 (3)	0.4256 (25)	0.3906 (18)	1.0026 (4)
H101	1.2913 (25)	1.2203 (16)	0.8105 (4)	0.2923 (34)	0.7204 (19)	0.9514 (6)
H102	1.3833 (25)	1.3470 (16)	0.7894 (4)	0.3744 (34)	0.7976 (19)	0.9828 (6)
H103	1.4839 (25)	1.2643 (16)	0.8181 (4)	0.1728 (34)	0.7824 (19)	0.9799 (6)
H111	0.8973 (24)	0.8194 (17)	0.7475 (4)	0.8901 (23)	0.3556 (21)	0.9662 (4)
H112	0.9615 (24)	0.9767 (17)	0.7613 (4)	0.7432 (23)	0.4728 (21)	0.9551 (4)
H113	0.7652 (24)	0.9327 (17)	0.7638 (4)	0.9166 (23)	0.4525 (21)	0.9338 (4)
H121	1.3914 (22)	0.6160 (21)	0.8148 (5)	0.2978 (24)	0.0874 (19)	0.9643 (4)
H122	1.3690 (22)	0.6274 (21)	0.8535 (5)	0.1541 (24)	0.1162 (19)	0.9374 (4)
H123	1.4329 (22)	0.7693 (21)	0.8332 (5)	0.2206 (24)	0.2512 (19)	0.9596 (4)
H131	1.0379 (26)	0.8690 (19)	0.8720 (4)	0.3896 (27)	0.3359 (21)	0.8800 (4)
H132	1.2341 (26)	0.8969 (19)	0.8642 (4)	0.2472 (27)	0.3940 (21)	0.9047 (4)
H133	1.1744 (26)	0.7418 (19)	0.8798 (4)	0.2357 (27)	0.2289 (21)	0.8900 (4)
HO1	1.1613 (14)	0.7166 (11)	0.7755 (2)	0.6640 (13)	0.1518 (10)	0.9641 (2)
HO2	0.8714 (14)	0.9437 (10)	0.8219 (2)	0.5736 (16)	0.4327 (11)	0.8845 (2)
HO4	0.7803 (15)	0.4727 (11)	0.8362 (2)	0.8212 (19)	-0.0504 (12)	0.8743 (3)

III Thermal parameters determined for heavy atoms of [**22**].

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
Molecule A						
C1	0.0319 (39)					
C2	0.0156 (34)					
C3	0.0332 (39)					
C4	0.0397 (44)					
C5	0.0396 (44)					
C6	0.0382 (42)					
C7	0.0307 (38)					
C8	0.0298 (39)					
C9	0.0215 (102)	0.0412 (98)	0.0458 (103)	-0.0007 (88)	0.0090 (94)	-0.0013 (88)
C10	0.0654 (138)	0.0311 (98)	0.0697 (127)	-0.0003 (92)	0.0118 (123)	-0.0334 (98)
C11	0.0466 (46)					
C12	0.0357 (120)	0.0698 (131)	0.0910 (144)	0.0049 (113)	-0.0323 (127)	0.0176 (118)
C13	0.0569 (53)					
O1	0.0457 (78)	0.0301 (52)	0.0382 (64)	-0.0200 (50)	0.0253 (58)	-0.0024 (63)
O2	0.0334 (26)					
O4	0.0770 (93)	0.0307 (59)	0.0433 (63)	-0.0080 (55)	0.0302 (70)	-0.0260 (66)
O9	0.0356 (77)	0.0484 (69)	0.0828 (87)	0.0111 (69)	0.0320 (74)	-0.0098 (68)
OW1	0.1047 (108)	0.0325 (63)	0.0583 (77)	0.0059 (61)	0.0118 (84)	-0.0055 (77)
Molecule B						
C1	0.0280 (39)					
C2	0.0378 (43)					
C3	0.0513 (51)					
C4	0.0339 (41)					
C5	0.0383 (43)					
C6	0.0347 (41)					

III continued

C7	0.0302 (39)					
C8	0.0949 (39)	0.0328 (84)	0.0378 (92)	0.0108 (88)	0.0299 (109)	0.0159 (115)
C9	0.1052 (205)	0.0757 (146)	0.0757 (146)	0.0329 (107)	-0.0063 (102)	-0.0099 (148)
C10	0.1439 (244)	0.0430 (118)	0.1158 (185)	0.0199 (126)	0.0506 (189)	0.0482 (152)
C11	0.0274 (126)	0.0811 (140)	0.0651 (127)	0.0134 (108)	0.0061 (108)	-0.0166 (110)
C12	0.0590 (143)	0.0291 (92)	0.0957 (161)	-0.0015 (102)	-0.0044 (122)	0.0005 (105)
C13	0.0863 (172)	0.0681 (133)	0.0446 (113)	0.0006 (100)	-0.0026 (115)	0.0164 (136)
O1	0.0332 (27)					
O2	0.0648 (95)	0.0393 (59)	0.0252 (53)	0.0128 (53)	-0.0068 (63)	-0.0028 (69)
O4	0.1048 (127)	0.0277 (58)	0.0686 (78)	-0.0181 (59)	0.0516 (84)	0.0132 (83)
O9	0.0356 (77)	0.0484 (69)	0.0828 (87)	0.0111 (69)	0.0320 (74)	-0.0098 (68)
OW2	0.1597 (150)	0.0249 (56)	0.0585 (78)	-0.0133 (55)	0.0065 (103)	-0.0036 (90)

Appendix C

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Extractives from New Zealand Honeys. 1. White Clover, Manuka, and Kanuka Unifloral Honeys

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Ether extracts were made from aqueous solutions of manuka (*Leptospermum scoparium*), kanuka (*Leptospermum ericoides*), and clover (*Trifolium repens*) honeys with use of a continuous liquid/liquid extractor. The components of the extracts were methylated before being separated and identified by gas chromatography and mass spectrometry, and also by preparative thin-layer chromatography followed by ^1H and ^{13}C NMR analyses. A total of 61 different compounds were detected, and 56 of these were identified. Their concentrations ranged from 0.1 to 4000 $\mu g/g$. Classes of compounds detected included hydrocarbons (C_{21}–C_{33}) and straight-chain monobasic (C_8–C_{28}), dibasic, and aromatic acids. The concentration of aromatic acids in manuka and kanuka honeys was much higher than in clover honey. These acids were not present in a chloroform extract of manuka flowers, which contained many terpenes, none of which were present in manuka honey. Compounds reported for the first time in honey include 2-decenedioic, decanedioic, nonanedioic, and octanedioic acids.

One of the intrinsic features of honey is its natural antibiotic properties. It is known that honey can be kept for long periods of time without becoming spoiled.

Honey is of high osmotic pressure (estimated to be about 2000 mosm), which increases the resistance to spoilage by microorganisms (White, 1975). The acids in honey also contribute to its resistance to spoilage by bacteria (White, 1975). Generally, the pH ranges from 3.4 to 6.1, averaging at about 3.9 (White, 1978). The most studied antibacterial property of honey (Gauhe, 1941; Cocker, 1951; White et al., 1958, 1963) is the action of the enzyme glucose oxidase. Glucose oxidase is virtually inactive in full-density honey but becomes active again in diluted honey, producing hydrogen peroxide (H_2O_2) from glucose.

We have however observed that some New Zealand native honeys, particularly manuka, exhibited a level of additional antibacterial activity greater than that which could be ascribed solely to the three systems listed above (Russell, 1983). Manuka honey typically possessed substantial additional activity whereas honey from white clover was essentially devoid of additional activity (Molan et al., 1987).

Preliminary studies revealed that the antibacterial compounds were unaffected by heat and light and were soluble in organic solvents such as ethanol and ether. Ethanol extraction of manuka honey and subsequent analyses established that two of the major antibacterial components were methyl 4-hydroxy-3,5-dimethoxybenzoate and methyl 3,4,5-trimethoxybenzoate (Russell, 1983).

Total extractable organics from honey have been little studied. Some work has been reported on the volatile constituents of various unifloral honeys (Cremer and Riedmann, 1964, 1965; Wootton et al., 1978; Graddon et al., 1979; Bicchi et al., 1983). Recently the composition of the hydrocarbon fraction of chestnut honey was reported in detail (Bonaga et al., 1986).

In the course of a study to identify the plant-derived antibacterial components of honey, a survey of the extractives in honeys was initiated. The techniques used for the isolation of relatively volatile components are not appropriate for the quantitative recovery of polar phenolic and acidic substances. In this paper we report a procedure for the recovery of these groups of compounds and the application of this technique to a number of white clover, manuka, and kanuka honeys. A preliminary study was also made of the contribution of manuka flower components to honey extractives.

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MATERIALS AND METHODS

Samples. A collection of honey samples was obtained from beekeepers throughout New Zealand by the Ministry of Agriculture and Fisheries, Hamilton, New Zealand. All honey samples were collected during the 1982-1986 flowering seasons. The majority were considered to be primarily unifloral specimens. Floral source identification of each honey was based on the flavor, color, and aroma, and also the season and location of its production.

Reagents. All solvents were bulk grade and were redistilled and checked by gas chromatography.

Honey Extraction. In a typical extraction, 20 g of honey was placed in a beaker with 700 mL of distilled water. The resultant mixture was stirred at room temperature for 5 min by means of a magnetic stirrer. Two aliquots of internal standards, undecane (C_{11}) and methyl heptadecanoate (17:0 fatty acid methyl ester) in chloroform (1 mg/mL), were added at the concentration of 10 $\mu g/g$ of honey. As comparatively higher levels of extractable organics were present in manuka and kanuka honey, internal standards were added at 4 times the usual concentration for these honeys. The honey solution (with the internal standards) was then introduced into a standard continuous liquid/liquid extractor. The beaker itself was washed with diethyl ether (3 × 100 mL), and the washings were introduced to the extractor.

All quick-fit joints were carefully sealed with Teflon tape (John Cropper Ltd.). After 24 h of extraction, the ether extract was dried over anhydrous $MgSO_4$. The extract was then concentrated under reduced pressure in an all-glass rotary evaporator at 25 °C. When the volume was suitably reduced (3–5 mL), the extractive solution was transferred to a 10-mL vial and methylated with etheral diazomethane for 2.5 min. Longer derivatization times resulted in the progressive methylation of some of the phenolic hydroxyl groups. Excess diazomethane was blown off with a stream of nitrogen. After final concentration (air drying) to about

0021-8561/88/1436-0453$01.50/0

2 mL, the vial was sealed and stored at 5 °C until required.

Flower Extraction. Fresh whole manuka flowers were soaked in chloroform for 2 h. [Simple soaking may not be the appropriate technique (Erickson et al., 1979).] The filtered solution was concentrated and methylated prior to gas chromatography (GC), preparative layer chromatography (PLC), and gas chromatography/mass spectrometry.

Chromatography. Gas chromatography was performed on a Pye Unicam Model PU4500 chromatograph equipped with a flame ionization detector (FID), modified for capillary column with a split injection system (SGE Unijector, split ratio 200:1). The retention times (in seconds) and peak areas were measured by a Spectra-Physics minigrator. Analyses were performed on a fused silica open tubular (FSOT) column, 0.25 mm (i.d.) × 12 m, coated with methylsilicone (SGE Ltd., Melbourne) with hydrogen as carrier. Injector and FID temperatures were 250 °C.

The column oven was temperature programmed from 40 °C (3-min initial hold) to 250 °C (35-min final hold) at 4 °C/min with a carrier gas linear velocity of 40 cm/s. Between 6 and 10 μL of each concentrated extract was injected. Carbon numbers were determined by interpolation of GC retention times to those of a series of *n*-alkanes run under identical conditions.

Quantification was performed relative to an internal standard of heptadecanoic acid (17:0) methyl ester. Response factors were determined for succinic acid, benzoic acid, caprylic acid (8:0), phenylacetic acid, ethyl phenylacetate, 2′-methoxyacetophenone, 2-methoxybenzoic acid, 2-hydroxy-3-phenylpropionic acid, (*E*)-cinnamic acid, octanedioic acid, 2,6-di-*tert*-butyl-4-methylphenol, 3,5-dimethoxybenzoic acid, 3,4-dimethoxybenzoic acid, 3,4,5-trimethoxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, and tetracosane (C_{24} alkane), with 1:1 mixtures of the named compounds and the internal standard. Reasonable linearity in detector response relative to the C_{18} hydrocarbon internal standard was demonstrated over the range of concentrations encountered in the study. Class response factors were applied to the series of obviously related compounds.

The unknown organic components were quantified as "succinic acid equivalents" for peaks with retention time up to that of phenylacetic acid, "phenylacetic acid equivalents" for peaks with retention time up to that of 2-hydroxy-3-phenylpropionic acids, "cinnamic acid equivalents" for peaks with retention time up to that of fatty acid 16:0, and "fatty acid 17:0 equivalents" for peaks with retention time of those of fatty acid 16:0 and above. The results are reported as micrograms/gram of honey (fresh weight).

Gas Chromatography/Mass Spectrometry. A Varian MAT CH5 mass spectrometer was coupled with a Varian 2700 gas chromatograph via an open-split interface. An OV-1 FSOT column, 0.32 mm × 25 m (diameter), was employed with helium as carrier gas and the temperature programmed from 40 to 280 °C at 8 °C/min (no initial hold, 10-min final hold). The mass spectra were recorded in electron ionization (EI) mode at 70 eV, ion source temperature 200 °C. The scan repetition rate was 4 s over a mass range of 35 amu to 700 amu. Chemical ionization (isobutane) GC/MS was carried out on a Hewlett-Packard HP5985 mass spectrometer.

^{1}H and ^{13}C NMR. Sufficient quantities were recovered where possible, isolated by PLC on silica gel for ^{1}H and ^{13}C NMR to be recorded. ^{1}H NMR spectra were determined in $CDCl_3$ on a Jeol FX90Q (89.6-MHz) FT NMR spectrometer or Bruker AC200 FT NMR spectrometer using tetramethylsilane as an internal reference. ^{13}C NMR spectra were determined in $CDCl_3$ on a Jeol FX90Q (22.5-MHz) FT NMR spectrometer or a Bruker AC200 (50 MHz) FT NMR spectrometer using tetramethylsilane as an internal standard.

RESULTS

Various extracting solvents were investigated in the liquid/liquid extractor: ethyl acetate, chloroform, ether, hexane. The volume of solvent used in each extraction was about 300 mL. Each of the solvents gave extractive mixtures for which generally similar profiles were observed. The higher the extraction temperature, the greater the level of (hydroxymethyl)furfural (HMF). The use of ether substantially reduced the level of HMF and ethyl ester analogues of the parent acids. It is concluded that HMF arises from an acid-catalyzed thermal elimination of water from glucose and fructose. Crane (1980) reported that every extra 10 °C increases HMF production by about 4.5 times.

Additionally, these studies revealed that at the higher extraction temperatures the ethyl ester levels increased at the expense of the parent acids, particularly for aromatic acids. Presumably the higher levels of esters reflect an increased rate of acid-catalyzed esterification of these acids of ethanol, which had probably arisen from fermentation of glucose and fructose by yeast cells in the honey samples supplied.

Although higher temperatures facilitated dissolving of the honey in water prior to extraction, significant losses of more volatile components occurred. Accordingly, dissolution was carried out at room temperature with stirring for 5 min. Adjusting to pH 2 did not increase the quantity of extracted material. Extraction was therefore carried out at natural pH to avoid possible degradation of some compounds. Liquid/liquid extraction was carried out for 24 h as no increase in yields was obtained at longer extraction times.

The identification of individual components was based on a combination of the GC carbon number and published mass spectra. Identification was confirmed by comparison with authentic samples in most cases. In the case of uncertain or absent molecular ions, CI (isobutane) mass spectra were used to deduce molecular weight. Examination of blanks (from methylation and from ether) under the conditions employed did not show any interfering components.

The acidic compounds were identified and quantified as their methyl esters. However, gas chromatography of unmethylated extracts demonstrated that the majority of the acids were present in the free form.

Table I summarizes the compounds present in the clover, manuka, and kanuka honeys examined, together with the prominent ions in the EI mass spectra.

White Clover Honey. A representative GC trace of white clover honey is depicted in Figure 1a. All eight clover honey samples gave remarkably similar GC profiles. Some samples showed extra peaks, which were identified by GC/MS as the ethyl esters of the major parent acids. Presumably these arose by acid-catalyzed esterification with ethanol. Quantitative data for the eight clover honeys examined are given in Table II.

The hydrocarbons (peaks 35, 41, 42, 44, 45, 48, 49, 52, 53, 56, 57, 58, 59, and 61) consisted mainly of *n*-alkanes typically ranging from C_{21} to C_{33}, with C_{27} most abundant. Even-numbered alkanes were in much lower concentrations. Some unsaturated analogues were also detected (see Table II). Despite their variation from sample to sample, the relative concentrations of C_{23} alkane, C_{25} alkane, C_{27}

Table I. GC/MS Summary of Honey Extracts (Components in Order of Elution Time)

peak no.	C no.	prominent ions	compound	basis for ident[a-c]
1	9.74	43, 57, *69*, 87	unknown (manuka)	MS
2	9.76	54, 57, *90*	unknown (manuka)	MS
3	10.04	55, 59, 87, 114, *115*	dimethyl succinate	GC, MS
4	10.64	51, 77, *105*, 136 M^+	methyl benzoate	GC, MS
5	10.70	55, 59, 87, 101, *115*	ethyl methyl succinate	MS
6	11.07	*74*, 87, 158 M^+	methyl caprylate (8:0)	GC, MS
7	11.44	65, *91*, 150 M^+	methyl phenylacetate	GC, MS
8	12.24	65, *91*, 105, 164 M^+	ethyl phenylacetate	GC, MS
9	12.50	77, 91, 105, *135*, 150 M^+	2′-methoxyacetophenone	GC, MS
10	12.95	77, 92, *135*, 151, 166 M^+	methyl 2-methoxybenzoate	GC, MS
11	13.36	65, *91*, 121, 162, 180 M^+	methyl 2-hydroxy-3-phenylpropionate	GC, MS, NMR
12	13.50	77, 103, *131*, 162 M^+	methyl (*E*)-cinnamate	GC, MS
13	14.07	69, *74*, 97, 107, 138, 171	dimethyl octanedionate	GC, MS
14	14.15	55, *71*, 97, 117, 165, 180 M^+	unknown (clover)	MS
15	14.45	*91*, 103, 121, 176, 194 M^+	ethyl 2-hydroxy-3-phenylpropionate	MS
16	14.78	*74*, 87, 119, 214	methyl laurate (12:0)	GC, MS
17	14.85	*55*, 74, 83, 111, 152, 185	dimethyl nonanedioate	MS
18	15.10	107, 122, 138, 165, *196* M^+	methyl 3,5-dimethoxybenzoate	GC, MS
19	15.20	69, *165*, 181, 196 M^+	methyl 3,4-dimethoxybenzoate	GC, MS
20	15.40	91, *121*, 151, 192, 210 M^+	methyl 2-hydroxy-3-(4-methoxyphenyl)propionate	MS
21	16.20	59, *74*, 98, 125, 157, 199	dimethyl decanedioate	MS
22	16.60	87, 95, *136*, 164, 196, 197	dimethyl 2-decenedioate	MS, NMR
23	16.78	59, 195, 211, *226* M^+	methyl 3,4,5-trimethoxybenzoate	GC, MS
24	16.92	74, 98, *125*, 166, 199, 213	ethyl methyl decanedioate	MS
25	17.15	*74*, 87, 107, 121, 242 M^+	methyl myristate (14:0)	GC, MS
26	17.25	153, 181, 197, *212* M^+	methyl 4-hydroxy-3,5-dimethoxybenzoate	GC, MS
27	17.35	81, 108, 119, *136*, 164, 197	ethyl methyl 2-decenedioate	MS
28	17.53	73, 103, 223, *254*, 269, 284 M^+	unknown (manuka)	MS
29	18.30	75, *103*, 167, 227, 242 M^+	unknown (mamuka)	MS
30	19.04	*74*, 87, 143, 239, 270 M^+	methyl palmitate (16:0)	GC, MS
31	19.70	*88*, 101, 157, 284 M^+	ethyl palmitate (16:0)	MS
32	20.61	59, 74, *81*, 95, 109, 294 M^+	methyl linoleate (18:2)	GC, MS
33	20.69	59, 67, *79*, 95, 108, 292 M^+	methyl α-linolenate (18:3)	MS
34	20.76	55, 69, *74*, 83, 97, 264, 296 M^+	methyl oleate (18:1)	GC, MS
35	21.00	*57*, 71, 85, 113, 296 M^+	C_{21} alkane	GC, MS
36	21.16	59, *74*, 87, 225, 298 M^+	methyl stearate (18:0)	GC, MS
37	21.39	50, *74*, 83, 95, 109, 308 M^+	ethyl linoleate (18:2)	MS
38	21.55	59, 67, *79*, 95, 108, 306 M^+	ethyl α-linolenate (18:3)	MS
39	21.60	69, *83*, 97, 101, 264, 310 M^+	ethyl oleate (18:1)	MS
40	22.02	59, *74*, 88, 101, 312 M^+	ethyl stearate (18:0)	MS
41	22.00	*57*, 71, 85, 99, 310 M^+	C_{22} alkane	GC, MS
42	23.00	*57*, 71, 85, 324 M^+	C_{23} alkane	GC, MS
43	23.02	*74*, 87, 354 M^+	methyl arachidate (20:0)	GC, MS
44	24.00	*57*, 71, 85, 338 M^+	C_{24} alkane	GC, MS
45	25.00	*57*, 71, 85, 352 M^+	C_{25} alkane	GC, MS
46	25.10	*74*, 87, 354 M^+	methyl behenate (22:0)	GC, MS
48	26.00	*57*, 71, 85, 99, 366 M^+	C_{26} alkane	GC, MS
49	27.00	*57*, 71, 85, 99, 380 M^+	C_{27} alkane	GC, MS
50	27.10	*74*, 87, 143, 382 M^+	methyl lignocerate (24:0)	GC, MS
52	28.00	*57*, 71, 85, 99, 394 M^+	C_{28} alkane	GC, MS
53	29.00	*57*, 71, 85, 99, 408 M^+	C_{29} alkane	GC, MS
54	29.04	*74*, 87, 143, 410 M^+	methyl cerotate (26:0)	GC, MS
56	30.0	*57*, 71, 85, 422 M^+	C_{30} alkane	GC, MS
57	30.62	55, *57*, 69, 83, 97, 434 M^+	$C_{31:1}$ alkene	MS
58	30.85	55, 57, 69, 83, *97*, 434 M^+	$C_{31:1}$ alkene	MS
59	31.00	*57*, 71, 85, 99, 436 M^+	C_{31} alkane	GC, MS
60	31.25	*74*, 87, 143, 438 M^+	methyl montanate (28:0)	GC, MS
61	32.20	57, 69, 83, *97*, 111, 462 M^+	$C_{33:1}$ alkene	MS

[a] GC, gas chromatographic data. [b] MS, mass spectral data. [c] ^1H and ^{13}C NMR data.

alkane, C_{29} alkane, C_{31} alkene, C_{31} alkene, C_{31} alkane, and C_{33} alkene in each sample remained consistently in the approximate ratios of 1:2:6:3:1:1:2:3.

GC investigation of the ether-extractable lipids revealed the presence of dibasic as well as monobasic acids. The first to elute was succinic acid (peak 3). Other dibasic acids detected were octanedioic (peak 13), decanedioic (peak 21), and 2-decenedioic (peak 22), and a trace of nonanedioic (peak 17) acids. None of the diacids gave molecular ions in EI mass spectra.

The identification of 2-decenedioic acid dimethyl ester was initially based on a match of retention index and mass spectrum to that reported for a urinary acid (Spiteller and Spiteller, 1979) and a component of royal jelly (Lercker et al., 1981, 1982). It is the only decenedioic acid isomer with a carbon number higher than decanedioic acid on a nonpolar phase.

The position and configuration of the double bond were confirmed as 2(*E*) by NMR analysis of a diacid fraction isolated by PLC fractionation of clover honey sample 3 (Table II). The structure of the methylated diacid was further substantiated by a comparison of the ^1H and ^{13}C NMR spectral data determined for a specimen of 2(*E*)-dodecenedioic acid dimethyl ester (Sigma, St. Louis, MO).

The monobasic acids (peaks 6, 16, 25, 30, 32, 33, 34, 36, 43, 46, 50, 54, and 60) revealed a great variation in terms of concentration and carbon chain length. In general, palmitic acid (16:0) (peak 30), lignoceric acid (24:0) (peak

Table II. Concentration (μg/g) of Methylated Components in Clover Honey

peak	compound	sample number 1	2	3	4	5	6	7	8	mean
3	dimethyl succinate	13.1	7.1	36.9	43.8	13.6	62.8	5.1	24.7	25.9
4	methyl benzoate	0.4	1.0	1.1	0.5	0.8	0.9	0.2	24.7	3.7
6	methyl caprylate (8:0)			0.2				0.1		0.2
7	methyl phenylacetate	1.2	1.0	1.1	0.5	0.8	0.9	0.2	0.4	0.8
9	2′-methoxyacetophenone		1.5							1.5
10	methyl 2-methoxybenzoate	1.3	11.3	2.8				0.6	1.1	3.4
11	methyl 2-hydroxy-3-phenylpropionate	2.5	66.9	4.5	4.7	9.3	10.6	33.5	44.6	22.1
12	methyl (*E*)-cinnamate	3.9	1.6	3.3	3.6	2.3	1.8		1.7	2.6
13	dimethyl octanedioate	0.9	1.5	9.3	0.8	0.9	0.7	0.8	1.2	2.0
14	unknown	12.5	30.2		12.0	5.9	9.1		1.3	11.8
16	methyl laurate (12:0)	0.1	0.2	0.4	0.9		0.1	0.2	0.5	0.3
17	dimethyl nonanedioate	0.3	0.5	0.8	0.2	0.1	0.2	0.9	0.2	0.4
18	methyl 3,5-dimethoxybenzoate		0.3				0.1	0.3	0.2	0.2
19	methyl 3,4-dimethoxybenzoate	0.4	0.5	2.9	0.3	0.3	0.3		0.5	0.7
20	methyl 2-hydroxy-3-(4-methoxyphenyl)propionate		0.2			0.2			20.6	7.0
21	dimethyl decanedioate	4.0	8.7	50.9	4.9	5.0	4.7	4.7	7.5	11.3
22	dimethyl 2-decenedioate	14.9	30.3	181.2	17.1	19.8	18.0	14.3	26.9	40.3
23	methyl 3,4,5-trimethoxybenzoate		1.6						1.0	1.3
25	methyl myristate (14:0)	0.3	0.8	1.2	0.1	0.3	0.1	0.6	0.5	0.5
26	methyl 4-hydroxy-3,5-dimethoxybenzoate		2.6						1.8	2.2
30	methyl palmitate (16:0)	3.9	12.2	16.0	3.9	11.4	7.4	6.2	17.5	13.6
32	methyl linoleate (18:2)	0.7	1.5					1.3		
				11.9[a]	4.1[a]	15.0[a]	9.6[a]		12.9[a]	8.4[a]
33	methyl α-linolenate (18:3)	2.6	4.7					2.6		
34	methyl oleate (18:1)	2.0	10.7	11.2	3.2	4.6	3.8	5.0	14.3	6.9
35	heneicosane (C_{21})	0.2	0.3	0.4	0.1	0.1	0.2	1.6	0.4	0.4
36	methyl stearate (18:0)	0.5	2.2	2.7	0.5	0.8	0.7	0.9	2.6	1.4
41	docosane (C_{22})	0.3	0.3	0.2				0.2		0.3
42	tricosane (C_{23})	0.9	1.8	3.1	0.8	1.3	1.1	3.0	1.8	1.7
43	methyl arachidate (20:0)	0.1	0.1	0.4		0.1		0.1	0.1	0.2
44	tetracosane (C_{24})	0.6	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.2
45	pentacosane (C_{25})	1.9	4.1	7.5	1.8	2.9	2.1	5.0	4.3	3.7
46	methyl behenate (22:0)	0.3	0.9	1.2	0.3	0.8	0.5	2.3	1.2	0.9
48	hexacosane (C_{26})	0.1	0.4	0.4	0.1	0.2	0.1	0.3	0.3	0.2
49	heptacosane (C_{27})	4.9	12.8	12.2	4.7	8.1	5.9	13.7	12.8	9.4
50	methyl lignocerate (24:0)	2.6	8.1	7.3	3.3	6.9	4.3	9.8	10.9	6.7
52	octacosane (C_{28})	0.3	0.5	1.9	0.1	0.3	0.1	0.5	0.3	0.5
53	nonacosane (C_{29})	2.8	6.3	7.0	2.4	3.9	3.1	6.3	6.1	4.7
54	methyl cerotate (26:0)	0.8	2.5	2.1	1.0	2.2	1.6	2.8	3.3	2.0
56	triacontene (C_{30})	0.1	0.1	0.3		0.1	0.1	0.1	0.1	0.1
57	hentriacontene ($C_{31:1}$)	1.6	2.4	5.6	0.9	1.6	1.4	1.4	2.0	2.1
58	hentriacontena ($C_{31:1}$)	1.0	1.7	3.4	0.6	1.2	0.9	1.0	1.5	1.4
59	hentriacontane (C_{31})	1.7	3.3	4.3	1.3	2.4	1.8	3.4	3.5	2.7
60	methyl montanate (28:0)	0.8	2.6	2.2	0.8	1.9	1.2	2.8	2.5	1.9
61	tritriacontene ($C_{33:1}$)	3.0	4.6	12.1	2.2	4.4	2.9	3.8	5.8	4.9

[a] Fatty acids 18:2 and 18:3 unresolved.

Table III. Relative Percent Composition of Straight-Chain Fatty Acids in Clover Honeys

chain length	sample number 1	2	3	4	5	6	7	8	mean
8:0	0.1	0.1	0.4				0.5		0.3
12:0	0.5	0.4	0.7	0.5	0.1	0.2	0.7	0.7	0.5
14:0	1.7	1.6	2.1	0.9	0.6	0.5	2.2	0.8	1.3
16:0	26.4	26.3	27.6	22.7	26.8	25.2	21.7	26.3	25.3
18:3	17.5	10.2					9.0		
			20.5[a]	23.6[a]	34.1[a]	32.7[a]		19.5[a]	21.9[a]
18:2	4.7	3.2					4.4		
18:1	13.8	22.9	19.4	18.6	10.5	13.1	17.7	21.6	17.2
18:0	3.8	4.7	4.7	2.7	1.9	2.5	3.3	3.9	3.4
20:0	0.7	0.3	2.5	0.2	0.3	0.1	0.4	0.2	0.6
22:0	2.3	2.0	2.0	1.9	1.8	1.6	8.0	1.8	2.7
24:0	17.6	17.4	12.6	18.9	15.8	14.7	34.5	16.4	18.5
26:0	5.5	5.4	3.7	5.8	4.9	5.4	9.8	5.0	5.7
28:0	5.4	5.5	3.9	4.3	4.2	4.0	9.9	3.8	5.1

[a] Fatty acids 18:2 and 18:3 unresolved.

50), oleic acid (18:1) (peak 34), and α-linolenic acid (18:3) (peak 33) dominated the GC traces. Certain pairs were only partially resolved, e.g. linoleic (18:2) (peak 32) and α-linolenic acids. Table III shows the relative percentage composition of these acids in the eight clover honey samples studied.

A wide variety of aromatic substances were detected. In clover honey, among the more dominant aromatic compounds was 2-hydroxy-3-phenylpropionic acid (peak 11). Its identity was confirmed by direct comparison with an authentic standard. The amount of 2-hydroxy-3-phenylpropionic acid varied greatly in different honey samples,

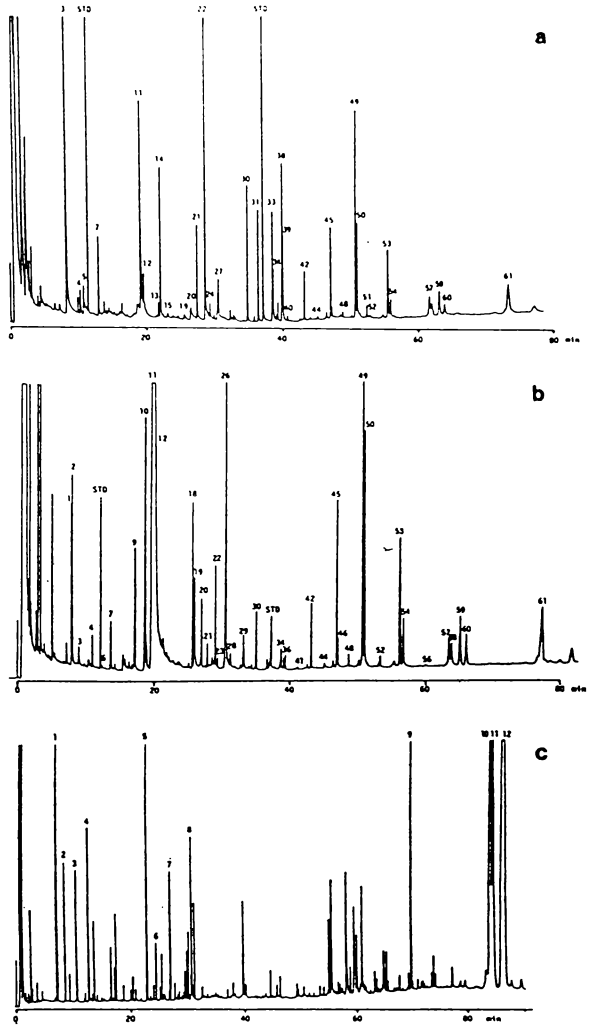

Figure 1. Representative chromatograms: (a) clover honey; (b) manuka and kanuka honeys; (c) chloroform extract of whole manuka flowers. For peak identification, see Table I. GC conditions are as stated in Materials and Methods except for (c) in which the detector and injector temperature was maintained at 270 °C and the column oven was temperature programmed from 35 °C (2-min initial hold) to 270 °C (30-min final hold) at 3 °C/min with carrier gas (H_2) linear velocity of 46 cm/s.

the absolute quantity varying from 2.5 μg/g (clover 1) to 66.9 μg/g (clover 2).

Other aromatic compounds detected were phenylacetic acid (peak 7), (*E*)-cinnamic acid (peak 12), and benzoic acid (peak 4). Their identities were also confirmed by direct comparison with authentic standards. Clover sample 8 revealed the presence of a large amount (20.6 μg/g) of 2-hydroxy-3-(4-methoxyphenyl)propionic acid (peak 20). This compound was not a major component in other clover honey samples (concentration in the range 0–0.2 μg/g). This suggests that another floral source contributed to clover sample 8.

A number of minor components could not be identified from the mass spectra. For example, peak 14 (see Figure 2) possessed a mass spectrum suggesting it to be a mixture of two components with molecular ions *m/e* 166 and 180. Loss of a methyl radical from these two M^+ ions would give rise to the ions at *m/e* 151 and 165, respectively.

Kanuka and Manuka Honeys. Kanuka and manuka

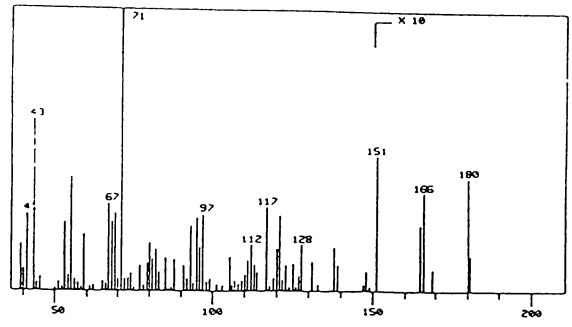

Figure 2. Mass spectrum of an unidentified compound that occurs as a significant component in clover honey extracts.

are of the same family and genus. Consequently it can be anticipated that the two species may give rise to similar types of honeys, and this indeed was observed. Accordingly, they are discussed together under the same heading.

Two kanuka and six manuka honeys were analyzed (sample numbers 9–16); a representative GC trace is shown in Figure 1b. As in the clover honey, the GC analyses were complicated by a series of ethyl ester peaks. Table IV lists the components and their concentrations found in the two kanuka and six manuka honey samples studied. It is evident from Table IV that the manuka honeys possessed much higher concentrations of aromatic acids than the clover honeys. Hydrocarbon and higher fatty acid compositions and concentrations were similar to clover honey and are not reported.

Aromatic acids were invariably dominated by 2-hydroxy-3-phenylpropionic acid (peak 11). Lesser amounts of 2-methoxybenzoic acid (peak 10), 2′-methoxyacetophenone (peak 9), 2-decenedioic acid (peak 22), and 4-hydroxy-3,5-dimethoxybenzoic acid (peak 26) were also detected. The latter compound was consistently detected in all the samples studied. It was also one of the components detected by Russell (1983) in her studies of antibacterial activity in honey. This was one of the few acids found to be in the honey mainly in the methyl ester form.

Manuka Flower Extractives. Because of the high levels of phenolic and acidic compounds recovered from manuka honey, an attempt was made to correlate the extractives from manuka flowers with the components found in the honey.

Figure 1c depicts the GC profile of the methylated extractive. Little similarity exists between this GC trace (Figure 1c) and the representative manuka honey profile shown in Figure 1b.

The principal compounds recovered from manuka flower were demonstrated to be triterpenoids (peaks 10–12). PLC on silica gel was used to isolate fractions for ^1H and ^{13}C NMR. The molecular weights of peaks 10–12, as determined by probe MS, were in each case 470. From a combination of the MS and ^1H and ^{13}C NMR data, peaks 10–12 were shown to be triterpene acid methyl esters: methyl ursolate, methyl oleanolate, and methyl betulinate.

The principal classes of the minor components were monoterpenes and sesquiterpenes. Chief amongst the monoterpenes were α-pinene (peak 1), β-pinene (peak 2), cineole (peak 3), and linalool (peak 4), while the principal sesquiterpenes appeared to be cadinene (peak 6), caryophyllene (peak 7), and unidentified peaks 5 and 8. A collection of other substances having molecular weights in the range 220–262 were also detected. These appeared to be oxygenated sesquiterpenes. Since none of these substances were found in the honey, their structures were not investigated further. Some hydrocarbons were also

Table IV. Concentration (μg/g) of Methylated Components in Kanuka and Manuka Honeys

peak	compound	sample number								mean
		9	10	11	12	13	14	15	16	
1	unknown	6.5	3.2	71.2	1.3	5.4	9.8	0.8	1.5	12.5
2	unknown	2.5	2.0	84.0	1.7	2.5	5.1	1.0	1.6	12.6
3	dimethyl succinate	7.5	23.4	21.1	7.0	19.1	59.2	65.0	27.4	28.7
4	methyl benzoate	2.7	1.0	9.0	2.5	2.1	1.8	3.1	1.5	3.0
6	methyl caprylate (8:0)	0.4	0.3	0.4	0.3	0.2	0.6	1.1	0.2	0.4
7	methyl phenylacetate	2.2	1.8	12.4	2.5	15.3	4.3	34.4	2.6	9.4
9	2'-methoxyacetophenone	15.9	5.0	52.1	32.9	17.8	33.4	21.3	8.1	23.3
10	methyl 2-methoxybenzoate	103	21.5	143	20.5	36.6	19.0	12.0	14.7	46.3
11	methyl 2-hydroxy-3-phenylpropionate	733	816	4474	1440	1668	1466	2348	1209	1769
12	methyl (*E*)-cinnamate	1.6	327	200	8.0	5.1	115	95.7	92.7	106
13	dimethyl octanedioate	0.4	0.8	1.5	0.5	0.2	0.6		1.2	0.7
16	methyl laurate (12:0)	0.2	1.2	0.5	0.2	0.1	0.3		0.3	0.4
17	dimethyl nonanedioate	0.7	1.4	2.6	1.1	0.2	1.4	1.3	1.2	1.2
18	methyl 3,5-dimethoxybenzoate	22.9	0.5	52.2	6.6	8.9	20.4	3.3	3.6	14.8
19	methyl 3,4-dimethoxybenzoate	7.2	2.5	42.0	7.3	6.3	6.9	2.1	10.0	10.5
20	methyl 2-hydroxy-3-(4-methoxyphenyl)propionate	22.4	44.3	29.5	6.2	14.4	6.5	508	12.4	80.5
21	dimethyl decanedioate	5.4	3.4	10.5	3.4	0.3	6.0	8.6	11.5	6.1
22	dimethyl 2-decenedioate	19.6	39.2	42.5	13.1	3.5	16.3	59.5	55.1	31.1
23	methyl 3,4,5-trimethoxybenzoate	21.4	3.7	7.3	28.6	2.5	16.3	8.5	4.8	11.6
25	methyl myristate (14:0)	0.4	0.5	2.3	0.3	0.2	0.3	1.6	0.7	0.8
26	methyl 4-hydroxy-3,5-dimethoxybenzoate	470	26	355	329	306	372	312	105	277
28	unknown	3.3	4.2	5.9	0.5	20.6	26.4	4.6	3.9	8.7
29	unknown	6.0	9.5	10.6	5.4	13.0	18.3		6.4	9.9
30	methyl palmitate (16:0)	3.0	1.5	23.0	2.4	0.5	9.1	13.2	6.4	7.4
32	methyl linoleate (18:2)		0.4		0.3	0.1	0.3	7.0		
		1.1[a]		3.8[a]					2.3[a]	4.8[a]
33	methyl α-linolenate (18:3)	-	0.5		1.0	0.5	0.6	20.5		
34	methyl oleate (18:1)	2.3	1.3	9.6	2.4	0.5	2.2	9.4	4.3	4.0

[a] Fatty acids 18:2 and 18:3 unresolved.

characterized, nonacosane (peak 9) being dominant.

DISCUSSION

The results of GC/MS studies have shown that honey extracts are intricate mixtures containing various classes of components, the concentrations of which vary from 0.1 to 4000 μg/g. The principal constituents recovered were hydrocarbons (C_{21}–C_{33}), fatty acids (C_8–C_{28}), phenolic acids, and diacids. The majority of the substances listed in Table I have been reported previously in honey apart from the diacids.

The presence of high molecular weight hydrocarbons in honey extracts were not unexpected. Undoubtedly they arise from beeswax, which has not been completely separated during harvest and processing. Further characterization of this fraction was not attempted as it was not likely to give significant biological activity. A detailed compositional analysis for chestnut honey has been reported (Bonaga et al., 1986), including structural elucidation of unsaturates. They showed that the abundance order of hydrocarbons was C_{27} alkane and then C_{29} alkane followed by C_{23} alkane and C_{25} alkane. The concentrations of odd-numbered *n*-alkanes were much higher than the even-numbered *n*-alkanes. This is similar to the results found in the present study except that the level of C_{25} alkane is always higher than C_{23} alkane. In fact the relative amounts of alkanes found in this study are more closely related to those reported by Graddon et al. (1979) and Tulloch and Hoffman (1972).

While the absolute amounts of the individual fatty acids varied from sample to sample, the relative amounts (see Table III) did not vary greatly. The major components were palmitic acid (16:0) (peak 30) and lignoceric acid (24:0) (peak 50), followed by oleic acid (18:1) (peak 34) and α-linolenic acid (18:3) (peak 33).

The composition of fatty acids in cotton honey has previously been reported by Smith (1963, 1966) and in beeswax by Tulloch and Hoffman (1972). Smith reported that the highest fatty acid level in cotton honey was oleic acid (18:1). On the other hand, Tulloch's report on the studies of 80 Canadian beeswax samples showed the highest level of fatty acid to be lignoceric acid (24:0). Unsaturated fatty acids of the same chain length were not assessed separately in his study. [Components of the lipid fraction of beeswax were described in an earlier paper (Tulloch, 1971).] In addition, Tulloch's report also includes a range of much higher carbon fatty acids up to C_{52}. These fatty acids were not detected in the present study because the GC conditions used only eluted methyl esters up to C_{30}.

It is interesting to note that, as early as 1911, Ehrlich and Jacobsen (1911) demonstrated that 2-hydroxy-3-phenylpropionic acid could be produced by the action of microorganisms on phenylalanine. Its presence in honey was first reported by Hodges and White (1966), who isolated one optically active isomer, namely the (+)-form from a New Zealand honey.

The concentrations of trace organics in manuka/kanuka honey were 1000 times greater than those in white clover honey (Tables II and IV). Even modest contamination of the presumed unifloral source clover honey samples by (for example) manuka honey would dramatically increase the level of 2-hydroxy-3-phenylpropionic acid detected.

It is accepted that a truly unifloral honey is impossible to obtain. Strong flavored and dark colored honeys such as manuka may also be derived from a significant percentage of clover nectar. Although pollen analysis can give some guide, the proportions of pollen may differ from the proportions of nectars contributing to a honey.

New Zealand white clover honeys were characterized by low overall recoverable trace organics. Only three constituents of the clover honeys were generally present in concentrations greater than 5 μg/g, these being the unknown component (peak 14), succinic acid (peak 3), and 2-decenedioic acid (peak 22). Those honeys with 2-hydroxy-3-phenylpropionic acid as a major component are considered to contain contributions from other floral sources.

Manuka and kanuka honeys are characterized by the presence of 4-hydroxy-3,5-dimethoxybenzoic acid (peak 26), 2′-methoxyacetophenone (peak 9), and 2-methoxybenzoic acid (peak 10), with 2-hydroxy-3-phenylpropionic acid (peak 11) dominating the GC trace. These components serve to differentiate manuka or kanuka honey from clover honey.

The presence of triterpenoids in manuka flowers was not surprising since Cambie (1976) reported the presence of betulic acid, oleanolic acid, and ursolic acid acetate in the bark of manuka. Despite the dominance of these triterpenoids in manuka flower, no traces were found in the honey studied. Similarly, monoterpenes and sesquiterpenes were not detected in manuka honey. The absence of 2-hydroxy-3-phenylpropionic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, 2′-methoxyacetophenone, and 2-methoxybenzoic acid in manuka flowers suggests that these substances may be derived from honeydew.

Manuka plants are generally attacked by an Australian scale insect (*Eriococcus orariensis*), resulting in what is commonly known as manuka blight (Hoy, 1961). This insect lies on the bark and drains the sap, copiously excreting honeydew. Sooty mold fungi grow on the honeydew, giving rise to a blackened appearance of the bark and leaves. Honeydew is known to be collected by foraging bees. Phenylalanine in honeydew could be metabolized to 2-hydroxy-3-phenylpropionic acid by bacteria or fungi in the honeydew or by the bee during honey production.

Behavioral Substances. Of the compounds identified, the diacids are of special interest. Octanedioic, nonanedioic, decanedioic, and 2-decenedioic acids are reported in honey for the first time, despite the fact that they have long been recognized as part of the pheromone system of the honey bee *Apis mellifera*. Octanedioic acid has been reported in the extracts of worker larvae (Gochnauer and Shearer, 1981) while nonanedioic, decanedioic, and 2-decenedioic have been reported in the extracts of queen larvae or royal jelly (Collow et al., 1964; Lercker et al., 1981, 1982).

2-Decenedioic acid was the most prominent diacid in the honeys. Its concentration in clover, kanuka, and manuka honeys ranged from 3.5 to 181.2 μg/g, with a mean level of 32.1 μg/g. The presence of these compounds in honey poses interesting questions as to their possible significance to the bee pheromone system, particularly in relation to behavioral control of worker bees or sexual development of bee larvae.

CONCLUSIONS

Liquid/liquid extraction provides a mild and efficient means of extracting organic compounds from honey. Patterns of extractives, mostly acidic, are produced that are characteristic of the source of the honey. Manuka/kanuka honeys contain much higher concentrations of aromatic acids than those derived from white clover, which serves to differentiate the two types. Their origin does not appear to be directly related to extractives in manuka flowers. Diacids are prominent extractives in both clover and manuka/kanuka honeys. Investigations are continuing to determine the contribution of honeydew to manuka honey, the origin of the diacids, and the general utility of organic extractive profiles for charactering floral sources of honeys.

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Registry No. 8:0, 124-07-2; 12:0, 143-07-7; 14:0, 544-63-8; 16:0, 57-10-3; 16:0 ethyl ester, 628-97-7; 18:0, 57-11-4; 18:0 ethyl ester, 111-61-5; 18:1, 112-80-1; 18:1 ethyl ester, 111-62-6; 18:2, 60-33-3; 18:2 ethyl ester, 544-35-4; 18:3, 463-40-1; 18:3 ethyl ester, 1191-41-9; 20:0, 506-30-9; 22:0, 112-85-6; 24:0, 557-59-5; 26:0, 506-46-7; 28:0, 506-48-9; C_{21}, 629-94-7; C_{22}, 629-97-0; C_{23}, 638-67-5; C_{24}, 646-31-1; C_{25}, 629-99-2; C_{26}, 630-01-3; C_{27}, 593-49-7; C_{28}, 630-02-4; C_{29}, 630-03-5; C_{30}, 36731-14-3; C_{31}, 630-04-6; $C_{31:1}$, 77046-64-1; $C_{33:1}$, 85792-06-9; succinate, 110-15-6; benzoate, 65-85-0; phenylacetate, 103-82-2; 2′-methoxyacetophenone, 579-74-8; 2-methoxybenzoate, 579-75-9; 2-hydroxy-3-phenylpropionate, 156-05-8; (*E*)-cinnamate, 140-10-3; octanedioate, 505-48-6; nonanedioate, 123-99-9; 3,5-dimethoxybenzoate, 1132-21-4; 3,4-dimethoxybenzoate, 93-07-2; 2-hydroxy-3-(4-methoxyphenyl)propionate, 28030-15-1; decanedioate, 111-20-6; 2-decenedioate, 6048-93-7; 3,4,5-trimethoxybenzoate, 118-41-2; 4-hydroxy-3,5-dimethoxybenzoate, 530-57-4; ethyl methyl succinate, 627-73-6; ethyl phenylacetate, 101-97-3; ethyl 2-hydroxy-3-phenylpropionate, 15399-05-0; dimethyl decanedioate, 106-79-6; dimethyl 2-decenedioate, 28598-91-6; ethyl methyl decanedioate, 692-88-6; ethyl methyl 2-decenedioate, 111495-84-2; 2,6-di-*tert*-butyl-4-methylphenol, 128-37-0.

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