THE ANTIBACTERIAL PROPERTIES

OF HONEY

A thesis
submitted in partial fulfilment
of the requirements for the Degree
of
Master of Science in Biological Sciences
at the
University of Waikato
by

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University of Waikato
1983

ABSTRACT

Honey has been used medicinally by man for thousands of years. With the apparent misuse of many antibiotics there has recently been renewed interest in such natural antibacterial substances.

Honey is known to contain several natural antibiotic systems but research so far has been largely confined to the glucose oxidase (inhibine) area. Some past research, however, has indicated the possible existence of anti-bacterial substances other than hydrogen peroxide (inhibine) and the osmotic potential of honey. The aim of this project was to confirm the existence of these substances and to isolate and characterise them if possible.

The antibacterial assay used to detect activity was an agar diffusion technique incorporating Staphylococcus aureus.

Various honey samples were screened for non-peroxide antibacterial activity and the most active honey (manuka) selected for further investigation.

The activity was found to be completely heat-stable (1 hr, 95°C) at acidic pH but less stable at neutral pH, and the honey was less active at neutral pH.

Solvent extraction of the honey was carried out with ethanol, and ether was added to precipitate most of the sugar. All activity was recovered from the honey into the extract in this way. Further isolation of the active fractions was carried out using preparative thin-layer chromatography.

Information on the fractions with activity that were isolated was obtained by ultraviolet spectroscopy, infrared spectroscopy, nuclear magnetic resonance and mass spectrometry.

The compounds identified in the active fractions were:

methyl 3,4,5-trimethoxybenzoate

(o-methyl syringic acid methyl ester)

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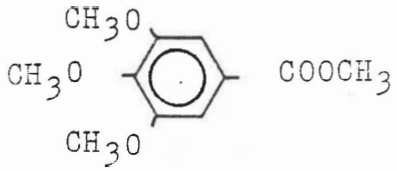

methyl 4-hydroxy-3,5-dimethoxybenzoate

(syringic acid methyl ester)

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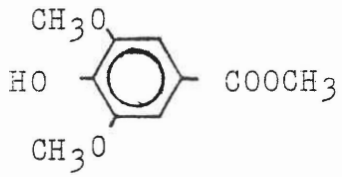

3,4,5-trimethoxybenzoic acid

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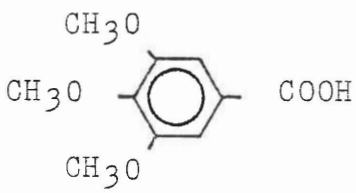


ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr P.C. Molan, for his guidance and constructive criticism during both the practical work and the writing of this thesis.

I would like also to thank Dr A.L. Wilkins for his valuable advice during the practical work, interpretation of spectrographic data and guidance during the writing of Chapter 6.

I am also grateful to:

Murray Reid for obtaining the honey samples.
Ralph Thomson for the n.m.r. spectras.
Dr P. Holland and Dean McGaveston for the mass spectral analysis of fractions.
Rex Julian for the photographs.
Ken Stewart for advice on illustrations.
Jacques Boubée for the translation of French articles.
B.M. Moss for the typing.

Finally I would like to thank my family and friends for their support, advice and encouragement throughout.

CONTENTS

CHAPTER ONE:

INTRODUCTION

Apiary products have been used to treat diverse ailments for thousands of years. With the apparent misuse of many antibiotics and discovery of harmful side-effects from other laboratory-developed drugs there has been renewed interest in recent times in such natural therapeutic substances. Natural antibacterial compounds have possibly been utilised by bees themselves for 10-20 million years as evidenced by the relatively sterile environment of the hive. The active compounds present in hive products can only have altered in as much as the bee itself, or plant from which the materials were obtained, has evolved. These hive products include propolis, bee venom, honey and royal jelly.

Royal jelly is reported to be only weakly antibacterial (Lavie, 1960) but does contain 10-hydroxy-ΔZ-decenoic acid which can prevent both bacterial and fungal growth. (Blum, cited by Harman, 1983).

The pharmacologically active components of propolis have been well researched. Propolis is a resinous, sticky gum collected from the boughs, leaves and buds of various trees, particularly poplar, birch, elm and alder. It is used by bees to attach the comb to the roof of the hive, to seal cracks and to surround the entrance to the hive through which they must pass. There is a lower incidence of bacteria and moulds inside the hive than out and it is thought that the volatile constituents of propolis reduce

the aeroflora within the apiary (Ghisalberti, 1979). An invader in the hive, such as a mouse, is usually killed and then embalmed in propolis and may remain preserved for as long as five years (Donald, 1982). It has been used by man largely in dermatology: to treat bacterial and mycotic infections, eczema and to aid tissue regeneration where it is claimed to speed the remaking of granule cells in wounds (Cizmarik, 1979). It has also been used in the treatment of rheumatism, gout, hyperlipidemia, ulcers, tooth and gingival diseases, ear and respiratory tract infections, some viral infections and to staunch blood loss, for protection against radiation and as an analgesic.

Many of the pharmacologically active compounds in propolis have already been identified. There is evidence that some have come from plants unaltered and some have been transformed by enzymes in the bees saliva (Ghisalberti, 1979). Flavonoids exist in rich variety and highly concentrated form in propolis and exhibit a large number of therapeutic effects. According to Donald (1982) their major known effect is beneficial action on the capillary system, on the fragility and permeability of blood vessels and upon the circulatory system in general as a vasodilator. They also act as diuretics and increase bile production, influence production of compounds from several endocrine glands, including the thymus, thyroid, pancreas and adrenal glands, and have antiviral, antiparasitic, anti-bacterial and anticoagulant effects. In the review on propolis by Ghisalberti (1979) the constituents shown to contribute towards its bacteriostatic or antibacterial activity are galangin (a flavone), pinocembrin (a flavone)

and the cinnamic acid derivatives: caffeic acid and ferulic acid, which are active against both gram positive and gram negative bacteria and show fungistatic activity. An antimycotic effect is also shown by pinobanksin-3-acetate, pinocembrin, p-coumaric acid benzyl ester and a caffeic acid ester. Ferulic acid has a marked astringent effect and a number of the flavones isolated have papaverine-like spasmolytic activity. Stilbenes have also been isolated from propolis and are known to possess fungicidal and fungistatic properties toward wood-rotting fungi and therefore may be responsible for some of the success with mycotic infections. Flavonoid pigments also isolated have antibiotic effects - some also have anti-inflammatory effects on joints, skin and mucous membranes. The many isolated compounds are only a proportion of the total present and their effects require more clinical research (Ghisalberti, 1973).

Bee venom also has various clinical effects owing to its components. It has been used to treat the peripheral nervous system (radiculitis, neuritis, neuralgia), joints (arthritis), rheumatic and allergenic problems and to decrease blood pressure in arteries. It is antibacterial and reported to increase the radiation resistance of mice (though it is not known what fraction achieves this), (Orlov, 1979; Fennell et al., 1968). Approximately 50% of venom consists of mellitin, a polypetide which has arrhythmic effects in non-toxic doses. This fraction is also responsible for venom's antibacterial activity (Fennell et al., 1968) (although Orlov (1979) claims venom solutions are unstable, destroyed by digesting and

oxidising enzymes and liable to bacterial infection and alteration) and has a slightly higher (but insignificant) effect than the venom itself. Various gram positive and gram negative organisms are sensitive to it, including penicillin-resistant S.aureus strain 80, although gram positive organisms are more sensitive. - It was calculated by one group of researchers that one sting had the anti-bacterial potency of about 9 units of penicillin for various gram positive organisms and a range of 9-170 units when measured against a selected group of gram negative organisms (Fennell et al., 1968). The analgesic effect of venom is also attributed to mellitin and the impulse inhibition in the central nervous system, vegetative ganglions and peripheral nervous system. Other known components of bee venom are: the polypeptide apamine which has a specific excitation effect on spine marrow; MSD peptide, which degranulates basophil cells; minimine; phospholipose A, which will split lecithin to lysolecithin and which degrades cell membranes; hyaluronidase, which splits hyaluronic acid, a constituent of conjunctive tissue and therefore favours the spread of active factors of venom throughout the body. A polypeptide, newly identified in bee venom also has one hundred times higher anti-inflammatory effects than hydrocortisone. Venom also stimulates the activity of the hypophysis and suprarenal glands (shown by increased cortisone levels in the blood and increased 17-keto-steroid in urine).

Honey may be one of the worlds oldest medicines. It provided a wide variety of services to ancient and mediaeval man, and is still widely used to treat colds,

burns and other skin problems.. The oldest known written reference to the medicinal use of honey is a Sumerian clay tablet of several prescriptions incorporating it; found in Iraq and dated at about 2,000 B.C. Thirty percent of the 966 prescriptions in the ancient Egyptian Papyrus Ebers (approximately 1500 B.C.) included honey. Hindu, Greek and Roman medicine employed it and reference is made to it in the Koran and the Talmud.

The value of different types of honey was recognised also. Aristotle, in about 350 B.C. specified particular types of honey for eye problems. "The Greek Herbal of Dioscorides", written in the 1st century A.D. and on which herbalists of many nations have drawn their inspiration for 15 centuries, prescribed honey for sores, ulcers, nits and lice, throat and tonsil infections, eye infections and even rabies. Dioscorides also described the best kind of honey to use: honey from Attica was the best and then Sicilian. He said it should be sweet and sharp, fragrant, pale yellow, glutinous and firm and which "in drawing does leap back to the finger." (This may refer to either the property of thixotropy which is due to a relatively high content of certain proteins and is an isothermal gel-sol-gel transformation induced by shearing and subsequent rest, or to dilatancy, increased viscosity with increased rate of shear). Dioscorides also specified that spring honey was the best, and then summer honey, and that Sardinian honey was very good for acne.

Many modern reports can be found in European literature describing the value of honey in treating wounds, burns, infections and urinary disorders. A report in 1982 in the

American Bee Journal (vol. 122, no. 4, pg. 247) said that doctors at several Israeli hospitals are now using it on open wounds after surgery. An Israeli microbiologist claims it prevents infection, speeds healing and that tests show it contains components very similar to antibiotics (no details of these substances were given, however). It was thought to work more quickly than many antibiotics because of easy absorption into the blood stream. The article also claimed it was good for such internal maladies as ulcers by absorbing acids and lubricating the digestive tract. Another reported use of honey on the public is by an English physician (Blomfield, cited by Goulart, 1979) who recommends it for both cleansing and healing. He was using pure natural honey in an accident and emergency department under dry dressings and claimed that it promotes healing of ulcers and burns better than any other local application. (Some of these properties may be partly due to honey providing a good barrier).

In 1970 twelve patients with wound breakdown after surgery for carcinoma of the vulva were treated with honey, and bacteriological wound sterility was attained in 3-6 days and within 8 weeks there was complete healing without the need for any skin grafting. Bacteria cultured from the wounds were killed in vitro by undiluted honey.

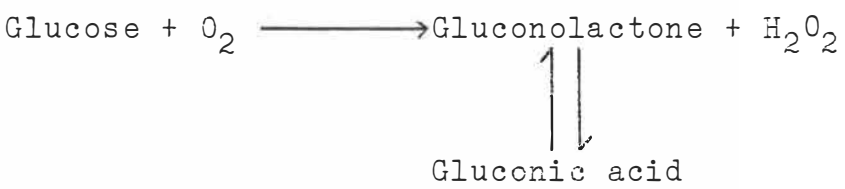
Other therapeutic uses of honey include: raising the haemoglobin content in blood of anaemic childern, cardiac therapy, treatment of stomach ulcers, speeding up alcohol metabolism in drunks and use against sickness resulting from radiation treatment (probably due to its high fructose content), and treatment of respiratory infections and various digestive diseases.

Some of the effects of honey may be ascribed to known components which come from either plant or bee. It seems likely that honey may inherit any curative qualities of plants from which it is gathered because of the discrepancies found between different researchers - one species of honey bee is generally used domestically. However, all honey appears to contain several natural antibiotic systems:

The osmotic pressure in undiluted honey will be important. Honey is basically a super saturated solution of sugars. In one report the osmotic pressure can be more than 2,000 milliosmols. During the ripening stage, honey increases in density, energy and stability due to water evaporation and an increase in simple sugars by the work of inverting enzymes on sucrose.

The acidity of honey also prevents the growth of many bacteria. The general pH range is between 3.2 and 4.5 (average 3.9). The predominant acid is gluconic acid which exists in equilibrium with gluconolactone. pH is affected mainly by the mineral content, i.e. a higher ash content will give a high pH.

The antibacterial property which has probably been studied the most is the glucose oxidase system which produces hydrogen peroxide.



H_2O_2 accumulates during storage of honey, although there are minor components in the honey which will destroy it,

such as vitamin C, catalase and other reducing substances. The glucose oxidase system is reported by several investigators to be active only in diluted honey.

has been recognised as an effective antibiotic for many years and was a major antibacterial component of some of the early penicillin drugs such as "Notatin" or Penicillin A (found to be a glucose oxidase).

Some previous work has also indicated varying levels of non-peroxide antibacterial activity due to other materials in some honeys. Little research so far has been done into these factors alone though, or even to confirm their existence.

Pioneer work was done on honey by the Dutch scientist Van Ketel (1892) who demonstrated the bactericidal effects of honey and by Sackett (1919) who thought it was due to the osmotic potential, but could not explain the phenomenon in diluted honey. In 1937 Dold and co-workers suggested the term "inhibine" for the antibacterial activity observed in diluted honey and found it to be heat and light labile, being inactivated by heating at 100°C for 5 minutes, 80°C for 10 minutes or 56°C for 30 minutes. The absence of inhibine was an indication that the honey had been heated and in 1955 Dold and Witzenhausen reported an assay to standardise inhibine assessment (Table 1). The assay involved the innoculation of S.aureus into agar containing known amounts of honey. After incubation for 24 hours the plates were inspected visually for bacterial colony development.

White and co-workers (1962, 1963) concluded that the liberation and accumulation of hydrogen peroxide by way

TABLE 1: Criteria for an inhibine value.

Dold and Witzenhausen, modified after Adcock (1962).

Honey concentration (per cent) in medium					Inhibine Value
25	20	15	10	5	
+	+	+	+	+	0
-	+	+	+	+	1
-	-	+	+	+	2
-	-	-	+	+	3
-	-	-	-	+	4
-	-	-	-	-	5

\+ growth of S.aureus

\- no growth

of a honey glucose oxidase enzyme system could account for all the known properties of honey inhibine. (Previous work into the enzymic production of gluconic acid in honey led to the discovery of the honey glucose oxidase system). They showed that inhibine was heat sensitive, but with a wide variation in sensitivity to heat, depending on floral source. Of 29 samples heated at 70°C for 10 minutes about two-thirds of the samples lost more than 80% of their activity, five lost 60-80%, two lost 40-60%and two were more heat resistant. The heat resistance of their peroxide accumulation systems varied 70-fold or more. They also concluded that between 55° and 70°C the half-life of the peroxide accumulation is a logarithmic function of temperature. (Different researchers give conflicting results for heat effect though e.g. Gubanski (1962) found that honey boiled for an hour did not lose its anti-bacterial properties especially in so far as intestinal bacteria were concerned). No reasons were proposed for the floral source influencing peroxide accumulation except that the inhibine value is affected not only by glucose oxidase content (which comes from the hypopharyngeal gland of the bee and should therefore be reasonably constant) but also by various minor peroxide-destroying components in the honey from sources such as nectar, pollen, yeasts or by enzymes such as catalase. They proposed that it may also be affected by handling, storage and processing of the honey, and in some cases (again depending on floral source), exposure to light. No explanation has been proposed for effect of light exposure being related to floral source.

White et al, also proposed that light-sensitive honeys contained a sensitiser which when they were mixed with unsensitive honey also rendered it light-sensitive (particularly to ultra-violet radiation). (Table 2).

When White and Subers (1963) actually carried out a chemical assay to relate inhibine number and peroxide accumulation in various honeys they concluded that H_2O_2 accounted for the major portion of the non-osmotic anti-bacterial effect in diluted honey. Inhibine was measured by the usual bioassay method of Dold and Witzenhausen, and H_2O_2 accumulation by a colourimetric method incorporating o-dianisidine. The table they formed is shown in Table 3. Of the 45 samples tested, however, 14 were anomalous - seven gave a higher peroxide accumulation than would be expected for the inhibine numbers obtained and seven gave lower readings than expected for peroxide accumulation. Insufficient material was available to determine the cause of the apparent anomalies.

Adcock (1962), working independently, also showed a connection between inhibine value and peroxide value, which he measured iodometrically (Table 4). He could not explain the lack of correlation between the two sets of values - peroxide was eradicated with catalase and the inhibine value was often only reduced. Adcock claimed that in later tests increasing the amount of catalase could completely destroy the inhibine values of the samples least affected by catalase originally. He did not indicate, however, whether he had increased the concentration of catalase added or whether he just added a greater

TABLE 2: Inhibine assays of irradiated honeys (by the method of Dold and Witzenhausen, 1955). (White and Subers, 1964).

Sensitive Honey	Inhibine No.
Control, unexposed	4
3 min. sun, original density	3
5 min. sun, original density	2
3 min. sun, and filter*, orig. density	4
1 hr. lab. lights, original density	2
5 min. sun, pH 3.9, dilute	2
5 min. sun, pH 6.5, dilute	5
Resistant Honey	
Control, unexposed	5
5 min. sun, original density	5
5 min. sun, pH 6.5, diluted	5
Mixture	
Control, unexposed	4
5 min. sun, original density	1
5 min. sun, pH 6.5, diluted	5

* Glass filter removed blue-green light

TABLE 3: Relation between inhibine number and peroxide accumulation.
(White and Subers, 1963)

Inhibine No.	Peroxide accumulation
0	< 3.4
1	3.4 - 8.7
2	8.8 - 20.5
3	20.6 - 54.5
4	54.6 - 174
5	> 174

Peroxide accumulation given in micrograms per gram of honey in 1 hr under assay conditions.

TABLE 4: Comparison of inhibine and peroxide values of honeys before and after treatment with catalase. (Adcock, 1962)

Country of Origin	Inhibine value		Peroxide value	
	Before catalase treatment	After catalase treatment	Before catalase treatment	After catalase treatment
Australia	5.0	1.5	2.48	0
	4.0	1.0	2.08	0
	2.0	0.5	3.52	0
	2.0	0.5	3.76	0
	2.0	1.0	2.96	0
	1.5	0	3.92	0
	1.5	0.5	4.24	0
	1.5	0.5	2.56	0
	1.0	0	4.48	0
	1.0	0	4.48	0
	1.0	0	4.16	0
	1.0	0	4.64	0
	1.0	0.5	11.52	0
	1.0	0.5	2.40	0
	0.5	0	2.72	0
Argentina	4.0	1.5	9.92	0
	4.0	2.0	4.00	0
	4.0	2.5	3.76	0
	3.0	0.5	10.48	0
Belgium	4.0	0.5	2.80	0
	2.5	1.0	2.90	0
	0.5	0	2.32	0
Germany	3.5	1.0	4.00	0
	2.0	0.5	2.24	0
Mexico	4.5	2.5	5.76	0

quantity of the original concentration - in which case he may have merely diluted away any non-peroxide activity which existed.

It does not appear that the above-mentioned workers have carried out any further research on the antibacterial properties of honey. Some others have suggested the possibility of non-peroxide antibacterial factors existing in diluted honey. These workers have attempted to extract active fractions from honey with various solvents.

In 1951 Vergé extracted antibacterially active fractions of honey with water, alcohol, ether or acetone. Elow? Schuler and Vogel (1956) extracted an active fraction of honey in ether.

Lavie (1960) working with a mixed floral honey found the acetone extract active but the ether extract inactive. He also found that extracting honeydew would give an active substance and that the degree of activity of the extracts varied with sources. The activity was photolabile and reduced by heat but some activity remained after 30 mins at 80°C. Feeding sugar to bees, feeding the product (honey) repeatedly to bees and testing the honey produced each time showed that the activity was increased with each bee "passage". Lavie concluded that the major portion of the activity came from the bee - but not all however as honeydew itself was active. (Plachy, 1944, also tested honeydew honey and floral source honey, finding honeydew honey more active. It might be possible that there are antibacterial substances coming from the plant which are more concentrated in form in the sap of the plant than in the nectar). He also tested several non-nectar solutions

from honeydew (of pine aphids), buds and needles and all possessed activity.

Gonnet and Lavie (1960) working with a multifloral honey containing lavender found an alcohol extract more active than an acetone extract. The acetone extract of this honey was unaffected by light or by neutralising the honey. Heat reduced the activity (although it was still active after autoclaving at 120°C for 15 minutes) but heating and neutralising together eliminated it. The activity returned after reacidifying however. Lavie (1963) also found he was able to obtain a cold ether extract of a honey sample which contained antibacterial substances volatile at 95°C (a fraction collected at a higher temperature than 95°C was inactive). Gonnet and Lavie concluded that besides antibacterial activity which originated from the bee (H_2O_2 produced by glucose oxidase) honey contained another group of antibacterial substances (from the forage plants) which were volatile, photolabile, extractable by solvents and more heat stable than inhibine. Owing to administrative reasons no further work on honey was done in that laboratory and therefore no attempt was made to isolate and characterise the non-peroxide antibacterial compounds. Dustmann (1978) also reported the existance of an antibacterial effect from honey other than the enzyme activity or sugar content but claimed it was only a minor portion.

Papers have been published in recent years which still ignore the possibility of non-osmotic non-peroxide antibacterial effects, (James et al, 1972). Research continues in Europe and Russia but no comprehensive papers appear to

be available at present, i.e. papers containing experimental details and characterisation of the responsible compounds. For instance Mladenov (1974) concludes there are anti-bacterial substances with volatile, heavy volatile and non-volatile character with bacteriostatic and bactericidal effect, but no more details of these compounds were given. There is also much conflict in the literature about which organisms are actually sensitive to honey's activity. Possibly the influence of floral source on a honey's anti-bacterial activity and mode of action is far more important than has previously been reported. This may explain some of the discrepancies in the literature about solvent extraction, heat- and photo- lability, degree of activity and the range of sensitive organisms.

Therefore, because of conflicting reports in the literature about the existence and properties of non-peroxide antibacterial activity in honey it was decided that a valuable area of research would be to confirm its apparent existence - to commence by screening various honey samples for this activity; to extract and purify all potentially antibacterial (non-peroxide) compounds from the most active honey sample available and to characterise these compounds if possible.

CHAPTER TWO:

MATERIALS AND METHODS

a. Honey

Honey samples were obtained from various beekeepers by Mr M. Reed, Apiary Advisory Officer, Ministry of Agriculture and Fisheries, Hamilton. The age, origin and previous treatment of each honey sample is given in Chapter 3.

The honey sample used for all experiments, other than screening a range of samples for activity, was unextracted manuka honey, 18 months old.

The honey was stored in air-tight plastic containers at 4°C until required for use.

b. Antibacterial Assay

Antibacterial activity was tested using an agar well diffusion technique.

Plates were prepared using a 24-48 hour bacterial culture in nutrient broth (0.08 g in 10 ml distilled water). The culture (10 ml) was added to 150 ml of sterile liquid nutrient agar (3.45 g in 150 ml distilled water) at about 45°C. The plates were poured immediately in a Laminar Flow Cabinet and stored at 4°C for a maximum of 4 weeks.

Holes, 8 mm in diameter, were cut in the agar and the wells filled with test solution. The plates were incubated at 37°C for 18 hours (Figure 1).

The relative extent of inhibition of growth was measured with a ruler as the distance between the edge of the well and the edge of the clearing. (The extent of

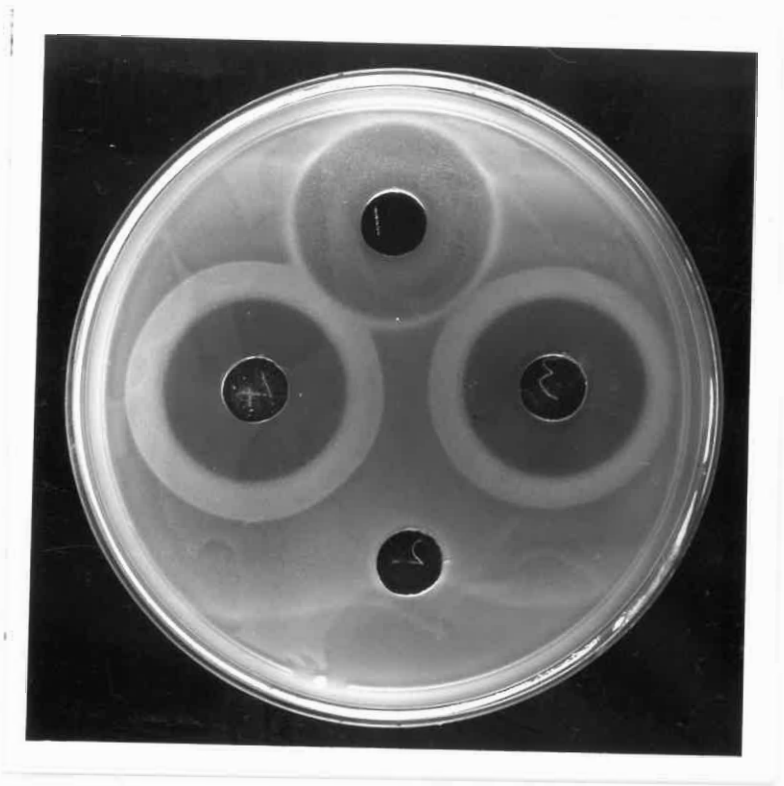


FIGURE 1: Antibacterial assay by technique of agar well diffusion.

1. 1.01 mol/l H_2O_2

4. honey + catalase (6255 units/ml) 3. 50% v/v honey solution

2. 1.01 mol/l H_2O_2 + catalase (6255 units/ml)

clearing was checked under a dissecting microscope).

This assay method is semi-quantitative: the extent of bacterial inhibition is not linearly related to the amount of antibacterial activity present.

In order to find the most sensitive method of detecting antibacterial compounds a method of detecting activity with sensitivity discs was also tried. Discs of 8 mm diameter were cut from ashless filter paper. They were soaked in water, ethanol, honey or honey extract for one minute and dried with a hairdryer. Following this they were laid on a seeded agar plate for 2 hours at 4°C, to allow infiltration of the activity into the agar before bacterial growth could occur and "mask" its presence i.e. so that the bacteria would not grow before the inhibitory compound could potentially create an area of inhibition. The plates were incubated for 18 hours at 37°C but no inhibition of growth was seen around any of the discs. Using a bacterial lawn instead of a seeded agar plate also showed no inhibition of growth.

Hence the agar diffusion technique was selected as the most sensitive technique of antibiotic detection.

c. Buffers

Phosphate buffer (0.1 mol/l) was prepared by dissolving 3.12 g of sodium dihydrogen phosphate in 200 ml distilled water and adjusting to pH 7 with NaOH.

Citric acid buffer (0.1 mol/l) was prepared by dissolving 5.2535 g citric acid in 250 ml distilled water.

d. Staining Reagents

Lactate dehydrogenase detection reagent contained 10 mg NAD^+, 6 mg nitroblue tetrazolium, 15 mg lithium lactate and 0.3 mg phenazine methosulphate dissolved in 2 ml distilled water. This was made up immediately before use, the agar plate flooded with it and re-incubated for a further 30 minutes. Living bacteria are stained purple and other areas remain clear.

Triphenyl tetrazolium chloride (Kirchner, 1978) is a biological detection reagent. Innoculated nutrient agar was mixed with 0.7 ml of a 5% solution of triphenyltetrazolium chloride in 50% methanol per 50 ml of medium. This was poured gently onto the surface of the developed chromatogram and when cool, a layer of sterile agar was poured on top of this. Prior to incubation the plate was placed in the refrigerator (4°C) for 1 hour in order to allow any inhibitory activity to diffuse into the agar from the silica.

p-Aminophenol (Dawson et al., 1969), the staining reagent for sugars, was prepared fresh with 0.5 g p-aminophenol and 2 g H_3PO_4 in 50 ml 96% ethanol. This was sprayed (with a Shandon spray unit) onto the commercially prepared TLC plate which was then heated for 5 minutes at 105-110°C. This stains:

glucose	⟶ dark brown
fructose	⟶ lemon yellow
sucrose and maltose	⟶ brownish yellow
raffinose	⟶ light brown

Sulphuric acid stain contained 25 ml 98% sulphuric acid in 220 ml methanol i.e. a 10% solution. TLC plates were sprayed with this and then incubated at 110°C for up to 20 minutes. A 10% solution of aqueous sulphuric acid was also used for staining commercial aluminium - foil-backed TLC plates and heated in the same way.

Molybdophosphoric acid general spray reagent (Dawson et al., 1969) consisted of a 10% solution of molybdophosphoric acid in 95% ethanol. Following spraying with this the TLC plates were heated at 110°C for 10 minutes.

Ferric chloride stain (Kirchner, 1978) was a saturated solution of anhydrous ferric chloride in methanol, for detection of phenols and terpene phenols.

Anisaldehyde-sulphuric acid staining reagent (Stahl, 1969) was prepared fresh before use. Concentrated H_2SO_4 (1 ml) was added to 0.5 ml anisaldehyde in 50 ml acetic acid. Following spraying with this solution the TLC plate was heated at 100°C for 10 minutes to detect phenols, terpenes, sugars or steroids.

Ferric chloride-potassium ferricyanide stain (Dawson et al., 1969) was prepared with aqueous 1% solutions of each salt (A.R. quality) mixed equally. The solution had to be orange-brown with no trace of blue. Reactive phenols stained blue immediately without heating. (A 0.1N HCl solution was sprayed on top to give a permanent record).

Vanillin-sulphuric acid stain (Randerath, 1966) contained 1 g vanillin in 100 ml conc. H_2SO_4. The TLC plate was sprayed with this and then heated at 120°C for 10 minutes.

e. Artificial honey

An artificial honey solution, containing only sugars was used to test the resistance of bacteria to the sugar content of honey. The solution consisted of sugars and water in the average proportions Thomson (1936) found in 21 New Zealand honey samples (Table 5), i.e. 17.5 ml water, 36.2 g glucose, 40 g fructose and 2.8 g sucrose.

f. Catalase

Catalase solution was made up fresh when used and consisted of 0.0278 g catalase powder (from horseradish origin, Sigma) in 10 ml distilled water, giving 6,255 units/ml activity.

g. H_2O_2 determination

An attempt to determine the H_2O_2 production of manuka honey was carried out using the enzymic reaction:

$$H_2O_2 + DH_2 \xrightarrow{\text{peroxidase}} 2H_2O + D$$

In the course of the peroxidase reaction the hydrogen donor o-dianisidine (DH_2) is oxidised to give a brown-coloured product (D). Optical density was measured at 436 nm. The assay mixture consisted of:

2.47 ml o-dianisidine solution (6.6 mg dissolved in 100 ml 0.1M phosphate buffer and neutralised with NaOH).

0.01 ml peroxidase suspension (4.93 mg peroxidase with 73 units/mg activity in 1 ml distilled water).

0.52 ml sample (honey solution or H_2O_2 standard).

TABLE 5: The chemical composition of manuka honey and the average for 21 various other New Zealand samples (Thomson, 1936).

	Manuka	Average
water %	18.4	17.5
glucose %	34.5	36.2
fructose %	42	40
sucrose %	2.8	2.8
ash %	.15	.18
nitrogen %	.038	.040
dextrins and c; (undetermined) %	2.1	-
titrable acidity C.c	2.8	3.3
pH value	4.05	3.81
formol titration C.c	1.2	1.3
specific gravity	1.407	1.423
glucose:fructose	1:1.22	1:1.11

The spectrophotometer was zeroed on water, and the optical density of the reagents alone, with no sample, was read to compensate for their contribution to the coloured products.

The background colour of the honey interfered too much with the brown-coloured indicator. Hence o-tolidine (0.04 g in 1 ml absolute ethanol plus 2 ml 30% acetic acid [200 µl in 100 ml phosphate buffer]) was substituted for o-dianisidine in the assay. This also produced a brown-coloured product, however.

h. Ultrafiltration

Ultrafiltration of honey solution was attempted with a Chemlab. Ultrafiltration Cell (Model C 50) with an Amicon Diaflow UM-05 membrane (nominal retentivity 500 molecular weight). Pressure was applied with oxygen-free nitrogen.

i. Thin-layer Chromatography

Commercial chromatograms used for preliminary work were Eastman Chromagram Sheet 6060 with a plastic backing. The silica contained a fluorescent indicator. Chromatogram strips were developed in screw-top jars which had been equilibrated with solvent for 1 hour beforehand. Developed strips were allowed to air-dry in a fume cupboard.

Preparative TLC plates were prepared using Merck Kieselgel 60 $PF_{254+366}$ (silica gel with a plaster of Paris binder and two fluorescent indicators).

Powder (100 g) was suspended in 240 ml distilled water, swirled to mix and left to stand for 1 hour.

Glass plates were cleaned with an abrasive detergent, rinsed, soaked in 3% Decon overnight, rinsed and dried. They were then spread with the gel, using a Shandon Unoplan Spreader (to a thickness of approximately 2 mm). The plates were left to dry overnight in a draughtless room or cupboard and activated at 120°C for 2 hours.

Plates were developed in a metal chamber with a glass plate across the top and the atmosphere saturated with the aid of solvent-soaked filter papers down the sides. Saturation of the atmosphere gives faster separation and a straighter solvent front. It was found that developing the plate a few centimetres, allowing it to dry standing upright, and then continuing development, did not produce sharper bands. Also application size did not increase linearly with the amount of material it contained. After the preliminary experiments using preparative plates, further plates were "cleared" of contaminants, such as phthalate, before use by development (to the top of the gel) in a slightly more polar solvent than the one to be used.

Solvent systems used to obtain final fractions:

I - ethanol:diethylether (60:40)

II - toluene:chloroform:acetone (40:25:35)

III - toluene:chloroform:acetone (40:20:40)

j. Ultra-violet absorption spectroscopy

A quartz cell was used with distilled water as a reference. Analysis was carried out with a Pye Unicam SP8 - 500 u.v./vis. spectrophotometer in the range 500 nm - 200 nm.

k. Infra-red spectroscopy

This was carried out using a Shimadzu IR-27G recording infra-red spectrophotometer in the range 4000-400 nm.

Potassium bromide discs were made of each freeze-dried isolated sample. The anhydrous KBr and sample were finely ground together with a pestle and mortar and a 13 mm disc formed under 154 x 10^3 kP pressure under vacuum for 1 minute.

l. Nuclear magnetic resonance

The samples to be analysed were dissolved in deuterated chloroform and analysed using a Jeol FX 60 Q n.m.r. machine.

m. Mass spectrometry

This analysis of samples was carried out on a Varian Mat CH5 mass spectrophotometer, which assigned masses. It had an associated data system, a 90° magnetic sector instrument and samples were run at 24 ev.

CHAPTER THREE:

INTRINSIC PROPERTIES OF NATURAL HONEY

3.1 Developing on Assay for Antibacterial Activity

An assay was developed in order to detect in honey antibacterial activity other than that due to hydrogen peroxide, osmolarity or acidity. The assay required a test organism which was both insensitive to the high osmolarity and acidity of honey and the growth of which was inhibited by the non-peroxide antibacterial factors present. It also required a means of eliminating hydrogen peroxide from honey.

Previous work in this laboratory had demonstrated that manuka honey possessed antibacterial activity in addition to that due to hydrogen peroxide production (Molan, personal communication). Therefore this floral type of honey was used during development of the bioassay.

a. Estimating Hydrogen Peroxide Production

In order to devise a means of eliminating hydrogen peroxide it was necessary to estimate how much might be produced in honey during the incubation period of a bioassay (approximately 18 hours).

An attempt was made to do this using the reaction:

$$H_2O_2 + DH_2 \xrightarrow{\text{peroxidase}} 2H_2O + D$$

where D represents the hydrogen -donor o-dianisidine, which is oxidised to a brown-coloured product. The colour produced from honey was compared with that produced from a range of H_2O_2 standards, by measuring absorption at 436 nm.

The reaction mixture consisted of:

2.47 ml o-dianisidine solution (6.6 mg in 100 ml → 0.1 mol/l phosphate buffer, neutralised with NaOH)

0.01 ml peroxidase suspension (4.93 mg of enzyme, activity 73 units/mg, in 1 ml water)

0.52 ml honey solution (incubated 18 hours), or standard H_2O_2 solution.

Dilutions of honey were used since the glucose oxidase system is active only in diluted honey (White and Subers, 1963). This method, however, was unsuccessful as the background colour of the honey solutions interfered with the absorbance readings. White and Subers (1963) used o-dianisidine in their assay of peroxide accumulation in various American honeys but their samples may have contained fewer coloured compounds.

Therefore, peroxide was estimated by comparing the relative antibacterial activity of a range of H_2O_2 standards with a range of honey dilutions. Activity was measured using an agar well diffusion technique. The agar was seeded with Staphylococcus aureus, shown by Dustmann (1979) to be highly sensitive to hydrogen peroxide and used by Dold and Witzenhausen (1955) to determine honey inhibine numbers.

A preliminary assay showed that a peroxide concentration as low as 0.032 mol/l would inhibit growth of S.aureus.

The results are shown in Table 6.

Some of the inhibition by honey would have been due

TABLE 6: Inhibition of growth of S.aureus on agar plates by H_2O_2 standards and manuka honey.

HYDROGEN PEROXIDE STANDARDS

H_2O_2 concentration (mol/l)	Diameter of zone of Inhibition (mm)
2.020	13
1.010	7.3
0.510	5.3
0.250	3.5
0.130	4.5
0.063	2.5
0.032	0.5

HONEY

Honey concentration (% v/v)	Diameter of zone of Inhibition (mm)
100	6.0
50	5.0
25	3.5
10	2.5
1	0.3

to non-peroxide antibacterial action. Thus the relative peroxide production of the honey was overestimated. During the incubation period the honey produced a clearance approximating that of an H_2O_2 concentration between the range 0.51 mol/l and 1.01 mol/l and it was therefore taken that the H_2O_2 content could not be greater than 1.01 mol/l.

b. Elimination of Hydrogen Peroxide with Catalase

To find the concentration of catalase required to remove the H_2O_2 produced by honey, a range was tested with 1.01 mol/l H_2O_2 against growth of S.aureus.

0.5 ml catalase suspension (in the strength range 6,255 sigma units/ml to 100,080 sigma units/ml) was shaken with 0.5 ml 1.01 mol/l H_2O_2 and the mixture assayed for antibacterial activity as above. H_2O_2 was also assayed alone as a control.

It was found that the lowest concentration of catalase used, 6,255 sigma units/ml (2.78 g/l), totally eliminated the antibacterial activity of 1.01 mol/l H_2O_2. This concentration of catalase was therefore used during antibacterial assays to eliminate hydrogen peroxide when studying the other antibacterial factors in honey.

c. Selecting a Test Organism

A variety of bacterial species was screened in an agar diffusion test for resistance to the sugar content of honey and for sensitivity to non-peroxide antibacterial factors. It was then demonstrated by neutralising the honey in a bioassay that the non-peroxide activity was not due to the

acid pH of honey.

In order to test the osmotic effect alone, a solution of sugars was mixed in similar proportions to the constitution of honey (Thomson, 1936. Table 7). The sugar solution contained:

17.5 ml water
36.2 g glucose
40.0 g fructose
2.8 g sucrose

All organisms were tested with the following solutions:

1.01 mol/l H_2O_2 (control)

0.5 ml 1.01 mol/l H_2O_2 + 0.5 ml catalase (control)

100% (v/v) honey solution
50% (v/v) honey solution
25% (v/v) honey solution
10% (v/v) honey solution
1% (v/v) honey solution

100% (v/v) sugar solution
50% (v/v) sugar solution
25% (v/v) sugar solution
10% (v/v) sugar solution
1% (v/v) sugar solution

0.5 ml 100% (v/v) honey solution + 0.5 ml catalase
0.5 ml 50% (v/v) honey solution + 0.5 ml catalase
0.5 ml 25% (v/v) honey solution + 0.5 ml catalase
0.5 ml 10% (v/v) honey solution + 0.5 ml catalase
0.5 ml 1% (v/v) honey solution + 0.5 ml catalase

The results are shown in Table 8.

TABLE 7: Extract from "Chemical Composition of New Zealand Honey", R.H.K. Thomson.

		Clover (Trifolium sp.) unknown origin	Penny Royal (mentha pulegium) Auckland	Manuka (Leptospermum sp.) Auckland	Average (of 21 samples)
water	%	16.6	18.0	18.4	17.5
glucose	%	38.4	37.1	34.5	36.2
fructose	%	39.7	38.9	42.0	40.0
sucrose	%	1.9	2.3	2.8	2.8
ash	%	0.050	0.370	0.150	0.180
nitrogen	%	0.029	0.056	0.038	0.040
dextrins, and c, (undetermined)	%	3.3	3.2	2.1	...
titrable acidity	C.c	2.8	4.6	2.8	3.3
pH value		3.42	3.70	4.05	3.81
formol titration	C.c	1.2	1.8	1.2	1.3
specific gravity		1.421	1.416	1.407	1.423
glucose:fructose		1:1.03	1:1.05	1:1.22	1:1.11

... uncalculated

TABLE 8: Inhibition of growth of various bacteria by manuka honey and sugar solutions

x no inhibition
/ slight inhibition
// inhibition > 1 mm
... not tested

Test Organism	Sugar solution % (v/v)					Honey solution % (v/v)					Honey + Catalase % (v/v)					H_2O_2	H_2O_2 + catalase
	100	50	25	10	1	100	50	25	10	1	100	50	25	10	1		
Alcaligenes viscolactis*	-	-	-	-	-	-	-	-	-	-	...	...	...	...	...	...	...
Proteus vulgaris	/	/	/	/	x	//	//	//	//	x	...	...	...	...	...	...	...
Citrobacter frendii	/	/	/	/	x	//	//	//	//	x	...	...	...	...	...	...	...
Shigella flexneri	//	//	//	//	x	//	//	//	//	x	...	...	...	...	...	...	...
Salmonella typhimurium	/	/	/	/	x	//	//	//	/	x	//	//	/	x	x	//	x
Pseudomonas aeruginosa	x	x	x	x	x	x	x	x	x	x	...	...	...	...	...	...	...
Serratia marcescens	x	x	x	x	x	/	/	/	x	x	/	/	x	x	x	//	x
Escherichia coli K12 F-	x	x	x	x	x	//	//	//	/	x	//	x	x	x	x	//	x
Escherichia coli K12 HFr	/	/	/	/	x	//	//	//	//	x	...	...	...	...	...	...	...
Escherichia coli K12 λ	//	//	//	//	x	//	//	//	//	x	...	...	...	...	...	...	...
Escherichia coli CSH34	//	//	//	x	x	/	/	/	x	x	...	...	...	...	...	...	...
Staphylococcus aureus	x	x	x	x	x	//	//	//	//	x	//	//	//	x	x	//	x
Micrococcus luteus	x	x	x	x	x	x	x	x	x	x	...	...	...	...	...	...	...
Streptococcus faccilis	x	x	x	x	x	//	//	/	x	x	/	x	x	x	x	//	x
Sarcina lutea	...	...	...	...	...	/	/	x	x	x	x	x	x	x	x	//	x
Bacillus circulans	x	x	x	x	x	x	x	x	x	x	...	...	...	...	...	...	...

* Culture did not grow

Alcaligenes viscolactis did not grow during the 18 hour incubation period and was therefore not considered a possible test organism. It was found that Citrobacter frendii grew during storage at 4°C and hence it also was not considered.

The results show that four of the organisms tested were both resistant to the high osmolarity of honey and sensitive to the non-peroxide factors. These were Serratia marsescens, Escherichia coli K12 F -, Streptococcus faecilis and Staphylococcus aureus. Serratia marsescens was discarded because inhibition of growth by all concentrations of honey was only slight and therefore difficult to see. Staphylococcus aureus was chosen as the test organism for all antibacterial assays because of its greater sensitivity to the non-peroxide activity and its sensitivity to lower concentrations of this factor. (E. coli K12 F- and Streptococcus faecilis were inhibited by non-peroxide activity only in undiluted honey).

d. Effect of Honey Acidity on S.aureus

The effect of the low pH of manuka honey on growth of S.aureus was assayed. Honey solutions were buffered with 0.1 mol/l citric acid and the pH altered with sodium hydroxide solution. Artificial sugar solution (as used to screen bacteria, 3.1c) was treated in the same way and used as a control.

The following solutions were assayed against S.aureus:

1.01 mol/l H_2O_2

0.5 ml 1.01 mol/l H_2O_2 + 0.5 ml catalase

0.5 ml honey + 1.5 ml water, pH 3.53

0.5 ml honey + 0.5 ml water, pH 3.53 + 1 ml catalase

0.5 ml honey + 0.5 ml citric acid, pH 4 + 1 ml water

0.5 ml honey + 0.5 ml citric acid, pH 4 + 1 ml catalase

0.5 ml honey + 0.5 ml citric acid, pH 7 + 1 ml water

0.5 ml honey + 0.5 ml citric acid, pH 7 + 1 ml catalase

0.5 ml sugar + 0.5 ml citric acid, pH 4 + 1 ml water

0.5 ml sugar + 0.5 ml citric acid, pH 7 + 1 ml water

The results are given in Table 9.

The acidic pH of manuka honey had a slight effect on the growth of S.aureus. A 25% (v/v) neutralised honey solution produced a clear zone of inhibition however.

3.2 Screening Honeys for Activity

Honeys of different floral origin and age were compared for non-peroxide antibacterial activity. Samples diluted to 50% (v/v) and 25% (v/v) were assayed, both with and without catalase. The types assayed were:

TABLE 9: The effect of pH on growth of S.aureus in an agar diffusion test.
(Concentration of all sugar and honey solutions: 25% v/v)

Solution assaved	Diameter of zone of inhibition (mm)
H_2O_2	11
H_2O_2 + catalase	0
Natural honey (pH 3.53)	3.0
Natural honey (pH 3.53) + catalase	2.0
Honey (pH 4)	3.0
Honey (pH 4) + catalase	3.0
Honey (pH 7)	2.0
Honey (pH 7) + catalase	2.0
Sugar (pH 4)	0.3
Sugar (pH 7)	0

Floral Type	Origin	State	Age
Ling Heather (Calluna vulgaris)	Urewera National Park	(a) comb	2 mths
		(b) extracted	2 mths
Manuka (Leptospermum sp.)	-	(a) comb	18 mths
	-	(b) comb	18 mths
	-	(c) comb	6 mths
	-	(d) extracted and boiled	11 years
Thyme (Thymus vulgaris)	Central Otago	extracted	2 years
Penny Royal (Mentha pulegium)	Waikato	extracted	1 year
Thistle-Nodding or California	-	extracted	4 years
Astelia (banksii or nervosa)	-	extracted	4 years
Cabbage tree (Cordyline indivisa)	Putaruru	extracted	1 year
Clover (Trifolium sp.)	(a) -	extracted	1 year
	(b) Kihikihi	extracted	1 year
Rewarewa (Knightia excelsa)	-	(a) comb	6 mths
	-	extracted: (b) clear top layer (c) granular lower layer	4 years
Towai (Weimannia silvicola)	-	extracted: (a) clear top layer (b) granular lower layer	4 years
Various	Wairoa	extracted	1 year

(Sample pollen count: White clover 43%, lotus 31%, salix 9%, reworewa 4%, briar 3%, dandelion 1%, gum 1%, grass 1%, cabbage tree 1%, manuka 1%).

Extracted honeys had been heated at approximately 45°C during the extraction process.

H_2O_2 and a solution of H_2O_2 and catalase were assayed on each agar plate to check that the catalase was

active. The results are shown in Table 10.

Four types assayed had antibacterial activity in addition to that from hydrogen peroxide. This activity was strong in all manuka honey samples tested. It was also present in rewarewa honey, and penny royal honey possessed slight activity. The activity in the honey labelled "various" may indicate that the factor in rewarewa or manuka honey is very active even in low concentrations, or it may be due to one of the other floral components not tested in isolation.

It was also concluded from these results that the non-peroxide activity being investigated originated in the plant and not the bee. These honey samples were all collected from small commercial producers and it can be assumed that all were produced by the honeybee Apis mellifera. Therefore existence of non-peroxide activity appears to depend on the floral source of the honey.

The manuka honey which was 11 years old and boiled had reduced activity in comparison to the three other manuka samples. It was not possible to tell whether this was due to prolonged heating at high temperatures or its age.

Manuka honey produced in 1980 was selected for further experimental work and extraction because it had the strongest antibacterial activity and of the active samples was available in the largest quantity. After 1 year of storage, in the dark at 4°C, it did not appear to have any reduction in its activity against S.aureus.

3.3 Heat Stability

The heat stability of the non-peroxide antibacterial factor was determined by subjecting raw manuka honey to a

TABLE 10: Activity of various New Zealand honeys, with and without catalase, against growth of S.aureus.

✓, inhibition of growth

x, no inhibition

Floral Type		Honey Concentration (v/v)			
		Alone		Including Catalase	
		50%	25%	50%	25%
Heather	(a)	x	x	x	x
	(b)	✓	x	x	x
Manuka	(a)	✓	✓	✓	✓
	(b)	✓	✓	✓	✓
	(c)	✓	✓	✓	✓
	(d)	✓	✓	✓	✓
Thyme		x	x	x	x
Penny Royal		✓	x	sl.	sl.
Thistle		✓	x	x	x
Astelia		✓	x	x	x
Cabbage Tree		x	x	x	x
Clover	(a)	x	x	x	x
	(b)	x	x	x	x
Rewarewa	(a)	✓	x	✓	x
	(b)	✓	x	✓	x
	(c)	✓	x	sl.	x
Towai	(a)	x	x	x	x
	(b)	✓	x	x	x
Various		✓	✓	✓	✓

range of temperatures for up to 1 hour and then assaying its activity.

A 50% (v/v) solution of honey was divided into thirty 1 ml aliquots and heated at temperatures of 45°, 55°, 65°, 75°, 85° or 95°C in waterbaths for 10, 15, 30, 45 or 60 minutes. The solutions were then allowed to cool to room temperature, and 1 ml catalase solution was added to each becore assaying for antibacterial activity. H_2O_2 (1.01 mol/l) and H_2O_2 + catalase were also tested to check that the catalase was effective. The results are given in Table 11.

The factor retained all its activity after heating at 95°C for 1 hour. All test solutions (25% v/v) gave approximately 4 mm diameter zones of clearance.

3.4 Effect of pH on the Non-peroxide Activity

The effect of pH was determined in order to see if the factor required an acidic environment to be active.

A 50% (v/v) solution of honey in 0.1 mol/l citric acid was divided into eight 1 ml aliquots and the pH of pairs of aliquots brought to 4, 5, 6 or 7 with sodium hydroxide solution. 1 ml of catalase or distilled water was then added to each and the solutions assayed. The experimental sugar solution was treated in the same way to gauge the effect of pH alone on S.aureus. The zones of clearing measured are shown in Table 12.

Increasing the pH of the honey solution decreased the activity of the factor but this was partially due to a direct effect of low pH on bacterial growth.

3.5 Combined effect of Heating and Neutralising Honey

The combined effect of pH and heat on the non-peroxide

TABLE 11: Inhibition of growth of S.aureus, as an indication of heat stability of the non-peroxide antibacterial factor.

Temperature (°C)	Time (minutes)				
	10	15	30	45	60
45	✓	✓	✓	✓	✓
55	✓	✓	✓	✓	✓
65	✓	✓	✓	✓	✓
75	✓	✓	✓	✓	✓
85	✓	✓	✓	✓	✓
95	✓	✓	✓	✓	✓

✓ , inhibition of growth S.aureus

TABLE 12: The effect of pH on the antibacterial factor, shown as inhibition of S.aureus.

Solutions	Diameter of Zone of Inhibition (mm)			
	pH 4	pH 5	pH 6	pH 7
Buffered honey + water	3.0	3.0	2.0	2.0
buffered honey + catalase	3.5	3.0	2.0	2.0
Buffered sugar + water	0.3	0	0	0

antibacterial factor was investigated and compared with the individual effects of heat and neutralising. Gonnet and Lavie (1960) experimented with multifloral honeys and found that neutralising their honey extracts gave no decrease in antibacterial activity, and heating for 30 mins at 80°C produced only a 50% decrease in activity. However when they combined the treatments (heating at 80°C for 30 mins at pH 8.4) the activity was lost, regardless of which treatment was carried out first. They found that activity returned as the pH was returned to the natural pH of the honey. At pH 7 it was still very slight.

50% solutions of honey in water or citric acid were neutralised or heated at 90°C for 30 minutes or both. Catalase was added to half of them and the solutions were assayed for antibacterial activity. Untreated honey was also assayed for comparison.

The test solutions contained:

1.01 mol/l H_2O_2 (control)

1 ml 1.01 mol/l H_2O_2 + 1 ml catalase (control)

Unheated

0.5 ml honey + 1.5 ml water
0.5 ml honey + 0.5 ml water + 1 ml catalase

0.5 ml honey + 0.5 ml citric acid, pH 7 + 1 ml water
0.5 ml honey + 0.5 ml citric acid, pH 7 + 1 ml catalase

Heated at 90°C for 30 mins

0.5 ml honey + 1.5 ml water
0.5 ml honey + 0.5 ml water + 1 ml catalase

0.5 ml honey + 0.5 ml citric acid, pH 7 + 1 ml water
0.5 ml honey + 0.5 ml citric acid, pH 7 + 1 ml catalase

The zones of clearance measured are shown in Table 13.

TABLE 13: Inhibition of growth of S.aureus by manuka honey that has been heated, neutralised or both.
(All solutions 25% v/v honey)

Solution	Diameter of zone of inhibition (mm)
Honey	4.5
Honey + catalase	6.0
Neutralised honey	3.5
Neutralised honey + catalase	3.5
Heated honey	4.5
Heated honey + catalase	4.5
Heated, neut. honey	2.5
Heated, neut. honey + catalase	1.5

The results demonstrated that heating at low pH did not decrease the antibacterial activity in manuka honey; neutralising produced a slight decrease in activity, and combining the treatments reduced the activity by about half. No explanation could be found for the apparent increase in activity of untreated honey when catalase was added or the decrease when catalase was added to heated, neutralised honey.

3.6 Ultrafiltration

Ultrafiltration with an Amicon UM 05 membrane was attempted to obtain an idea of the size of the anti-bacterial molecule(s) involved. The membrane had a nominal retentivity of 500 daltons.

A 25% v/v honey solution was used and pressure was applied for two hours with continuous stirring. At the end of this time however nothing had passed through the membrane. It was concluded that the honey solution was too viscous for ultrafiltration due to the high concentration of sugar present.

3.7 Summary

Non-peroxide antibacterial activity was found in manuka, rewarewa and penny royal honey. The activity in manuka honey was further studied. S.aureus was the most sensitive osmotolerant organism and was used to assay the activity. The active compound(s) was heat-stable at an acidic pH but less stable at neutral pH, and was less active at neutral pH.

CHAPTER FOUR:

SOLVENT EXTRACTION OF THE ANTIBACTERIAL ACTIVITY FROM HONEY

Gonnet and Lavie (1960) obtained an antibacterial fraction from several multifloral honeys by extraction with cold acetone. This extract contained only a portion of the activity of the whole honey, whereas alcohol extracted the activity totally. None of the activity would pass into ether. In further research Lavie (1963) obtained an ether extract of a hot alcohol extract. He was also able to extract this honey (unspecified type) in cold ether and when the extract was redissolved in water and distilled he found the active fraction was volatile at 95°C.

A solvent or solvent mixture was required for manuka honey which would extract all the non-peroxide antibacterial activity, and would leave behind most of the sugar present. It was necessary to eliminate most of the sugar from the extract because it would become more concentrated when dried on a chromatogram and may appear as an area of inhibition during a bioassay for activity.

4.1 Solvent Extraction

Honey (10 ml) was homogenised with absolute ethanol (20 ml) in a Waring blender for about 2 minutes. The solution was allowed to cool to room temperature (about 15 minutes) and then centrifuged at 8,000 r.p.m. for 15 minutes. The supernatant was rotary evaporated

and freeze-dried to remove all the alcohol. The yellow viscous produce was redissolved in 10 ml distilled water and assayed for antibacterial activity.

The test solutions were:

0.5 ml extract + 0.5 ml water + 1 ml catalase

0.5 ml extract + 1.5 ml water + 2 ml catalase

0.5 ml honey + 0.5 ml water + 1 ml catalase

0.5 ml honey + 1.5 ml water + 2 ml catalase

1.01 mol/l H_2O_2 (control)

1 ml 1.01 mol/l H_2O_2 + 1 ml catalase (control)

The results are given in Table 14.

All non-peroxide antibacterial activity could be extracted in absolute ethanol.

The above procedure was repeated using diethyl ether instead of ethanol but no activity was extracted. After the rotary evaporation stage a waxy white precipitate was left around the flask. This would not dissolve into water and it was difficult to redissolve into ether. An attempt to extract the dried alcohol extract with diethyl ether gave the same result.

4.2 Effect of Acid and Alkali on Factor Stability

An attempt was made to extract the active factor into ether from an acidic and an alkaline solution of the alcohol extract in water.

An alcohol extract of 10 ml honey redissolved in 10 ml water was divided into two 5 ml aliquots; one aliquot was acidified to pH 3 with HCl and the other taken to pH 10 with NaOH. They were then each vigorously shaken with

TABLE 14.: Comparison of inhibition of growth of *S.aureus* by raw manuka honey and an ethanol extract of it.

Test Solution (containing 50% v/v catalase)	Diameter of zone of inhibition (mm)
Extract (25% v/v)	5
Extract (12.25% v/v)	2
Honey (25% v/v)	5
Honey (12.25% v/v)	2
H_2O_2 (no catalase)	16
H_2O_2 + catalase	0

15 ml ether in separating funnels and left to settle. The ether fractions were dried by rotary evaporation, 1 ml distilled water was added to each and the solutions assayed. The remaining aqueous phase from each separating funnel was neutralised and assayed. The results are shown in Table 15.

Lowering the pH for the extraction did not make the factor more soluble in ether. The high pH inactivated the factor completely. It was therefore not possible to see which fraction it had entered. Hence the above procedure for extracting from alkaline solution was repeated but both phases were acidified before neutralising and assaying to see if the activity would return. When the ether fraction had been dried by rotary evaporation acidified water was added to the flask, shaken, then neutralised and assayed. As a control the second 5 ml aliquot of extract was checked for antibacterial activity with no further treatment.

The results obtained were the same as above. The alcohol extract itself was active however. Alkali again destroyed the antibacterial activity and this was not restored by reacidifying before neutralising. The same results were also obtained when the procedure was repeated in the cold room (at 4°C) with chilled ether.

The effect of taking the aqueous solution to pH 8 was then investigated.

An aqueous solution of the alcohol extract was adjusted to pH 8 with NaOH. The solution was left to stand for approximately the same time (about 15 minutes) as the factor had been exposed to pH 10 during the ether extraction attempt in the separating funnel. The solution

TABLE 15: Activity of ether extracts of manuka honey measured against growth of S.aureus.

TREATMENT	Acid		Alkali	
PHASE	water	ether	water	ether
INHIBITION	inhibition	no inhibition	no inhibition	no inhibition

All test solutions 50% v/v honey with catalase.

Control

Test Solution	Inhibition
H_2O_2	inhibition
H_2O_2 + catalase	no inhibition

was then neutralised and assayed. The results are given in Table 16.

The extract retained only slight antibacterial activity. It was concluded that increasing the pH of the active solution brought a concomittant decrease in antibacterial activity.

4.3 Extracts of Inactive Honeys

The alcohol extracts of two honey types, which were previously thought to have no non-peroxide antibacterial activity, were assayed to test if any components were lost during extraction which inhibited their activity while raw.

The extracts of heather and clover honeys were obtained by the same procedure as for manuka honey and assayed by an agar diffusion test against S.aureus. The results are shown in Table 17.

It was concluded that these contained no non-peroxide antibacterial activity.

4.4 Thin-layer Chromatography on commercial plates

Some preliminary chromatography was carried out with the extract of manuka honey using commercial thin-layer chromatograms to obtain information on how the non-peroxide activity migrated in various solvent mixtures of ethanol and diethyl ether. A bioassay was carried out to find its approximate position, by laying the used chromatogram on seeded agar plates for 2 hours, then removing it and incubating the plates overnight. Areas of clearing of S.aureus served to indicate the position to which the activity had migrated during chromatography. It was thought to be unnecessary to incorporate catalase into this assay because any glucose oxidase not precipitated during extraction (White et al. 1962) would be denatured and

TABLE 16: Effect of pH 8 on antibacterial activity of an alcohol extract of manuka honey, measured as inhibition of growth of S.aureus.

Test Solution (50% v/v)	Inhibition of growth
Extract	inhibition
Extract + catalase	inhibition
Extract - neutralised	inhibition
Extract - neut. + catalase	inhibition
Treated extract - neut.	slight inhibition
Treated extract - neut. + catalase	slight inhibition
H_2O_2	inhibition
H_2O_2 + catalase	no inhibition

sl. inhibition = < 1 mm diameter zone of inhibition.

TABLE 17: Activity of extracts of two inactive raw honeys, against growth of S.aureus.

Honey samples: Heather (b)
Clover (b)

Solution (50% v/v)	Activity	
	Heather	Clover
Honey	slight inhibition	inhibition
Honey + catalase	no inhibition	no inhibition
Extract + catalase	no inhibition	no inhibition
H_2O_2	inhibition	
H_2O_2 + catalase	no inhibition	

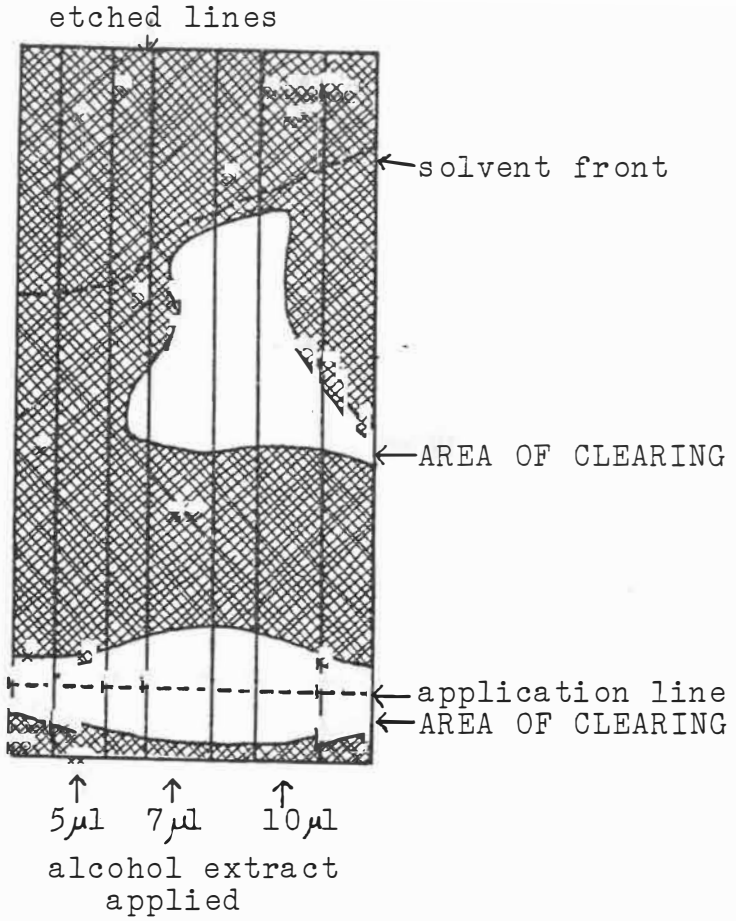
slight inhibition = < 1mm diameter zone of clearance

therefore inactivated by the high temperatures attained by the hairdryer used to dry applications on the chromatogram.

In order to find the loading necessary for this assay method a solution of honey extract in alcohol or twice its equivalent concentration in raw honey was applied to a thin layer chromatogram in 5, 7 and 10 µl amounts. The assay procedure was carried out without developing the chromatogram in solvent. The agar plate was incubated overnight and then flooded with a freshly-made solution of lactate dehydragenase detection reagent and incubated for another 30 minutes in order to make clearings more visible in the disturbed agar. Clearings were observed at the three points where material from the extract had diffused into the agar from the chromatogram.

As the loadings appeared to be appropriate it was thus repeated with the same quantities of extract applied and run in a solvent mixture of 80% ethanol - 20% diethyl ether. Lines were etched along the chromatograms to ensure the extract applications migrated in straight lines. The appearance of the seeded agar plate is illustrated in Figure 2.

This indicated that to some extent the areas of no growth were artefacts due to the thickness of the agar and surface effects. No explanation could be found for the shape of the clearing in the centre of the plate or for the 10 µl point of application producing a smaller clearing than the 7 µl point of application. The experiment was repeated. The results of the second assay showed little bacterial growth on the top half of the plate (the agar was also thinner here) and on the lower half there was a



stained purple i.e. normal growth of S.aureus

no staining i.e. inhibition of growth of S.aureus

Figure 2: Appearance of seeded agar plate after superimposing thin-layer chromatogram, incubation and staining with lactate dehydrogenase detection reagent.

clearing over the position of the application line again. This time the area of clearing was larger with increasing volume of extract applied.

To test whether this consistent clearing over the application area might be due to a high concentration of sugar, a mixture of sugars was made up in the same proportions as honey (as in Chapter 3) and extracted with alcohol as was the honey. The sugar extract was applied to a chromatogram in 5, 7 and 10μl quantities, developed in alcohol:ether (80:20) solvent and assayed as above. A clearing corresponding to each application was observed on the agar plate with the largest clearing where 10μl of sugar extract was applied.

A second sugar chromatogram was run in the same way and stained for sugar with p-Aminophencl instead of assaying for antibacterial activity. The appearance of the stained chromatogram is illustrated in Figure 3.

It was concluded that there was still a very high percentage of sugar in the extract and that this was probably producing the clearing corresponding to the application point on the thin-layer chromatogram.

4.5 Further Extraction

The activity due to the sugar could have been masking another antibacterial component. It was therefore necessary to modify the extraction process to eliminate the sugar. A solvent mixture was required which was sufficiently non-polar to dissolve only a little of the sugar but polar enough for the antibacterial acitvity to be soluble in it. Solvent mixtures of absolute ethanol and diethyl ether were investigated.

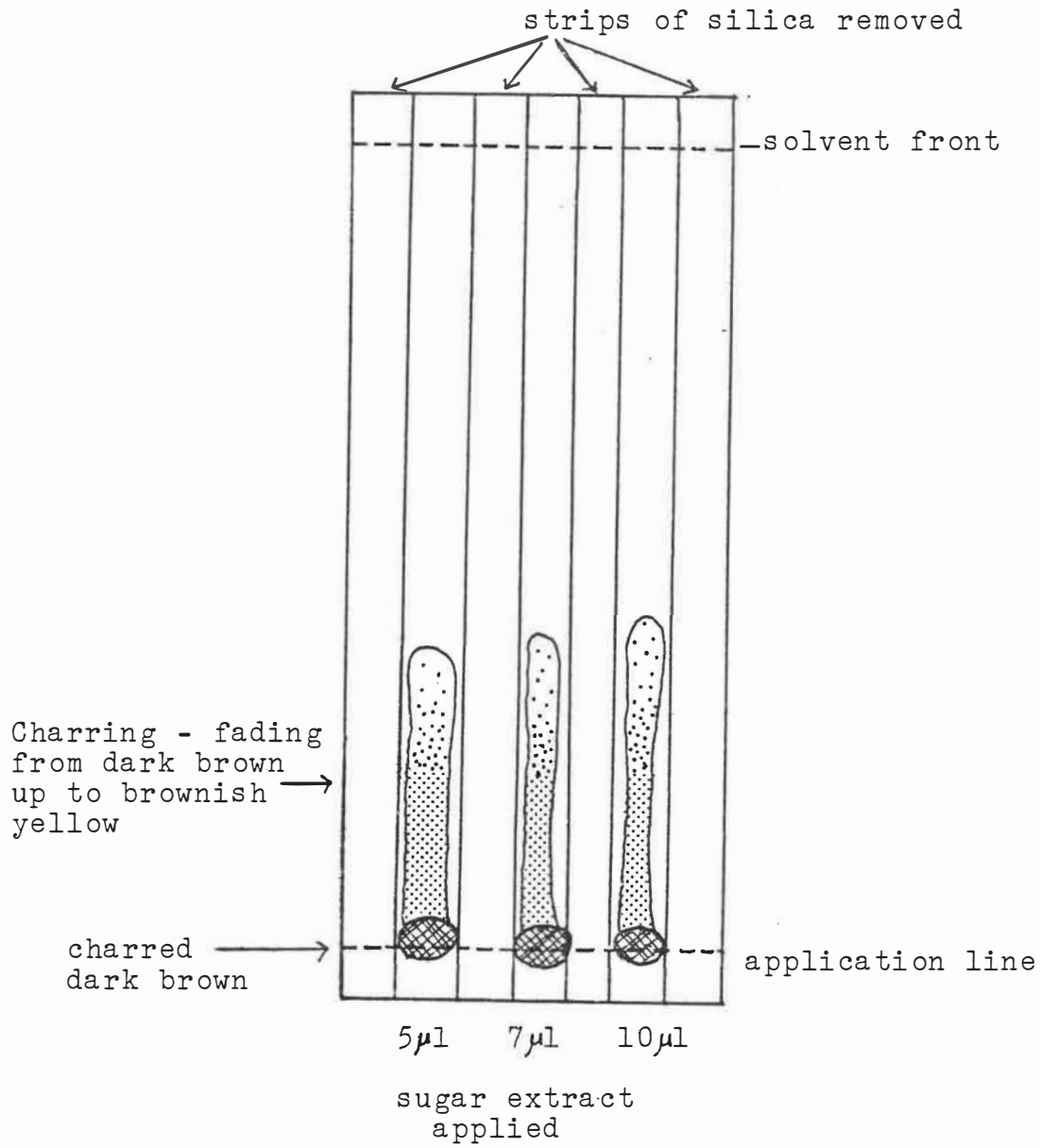


Figure 3: Appearance of thin-layer chromatogram with sugar extract applied. It was run in ethanol: ether (80:20), dried, sprayed with p-Aminophenol and charred at 110°C, 5 mins.

An alcohol extract of 10 ml of honey was divided into five aliquots and ethanol and ether added in the quantities: 4 ml + 1 ml, 3 ml + 2 ml, 2 ml + 2 ml, 2 ml + 3 ml and 1 ml + 4 ml.

Each mixture was shaken on a vortex mixer for 2 minutes to aid sugar precipitation and centrifuged at 8000 r.p.m. for 10 minutes. The supernatants were removed and dried by rotary evaporation. Distilled water (1 ml) was added to all dried fractions and precipitates and the resulting solutions assayed for antibacterial activity. The results are shown in Table 18.

The activity was soluble in all solvent mixtures tested. The precipitate from the 80% ether solution may have caused inhibition of growth of S.aureus by its high sugar concentration only but a 60% ether solution was selected for use in further extraction because some of the inhibition may have been due to the presence of a little of the antibacterial factor. The loss of this from the supernatant may have been so small as to fall within the marginal error for measuring activity by zones of clearance.

The modified extraction procedure subsequently adopted was thus:

(1) - extract 10 ml honey in 20 ml ethanol and centrifuge
(2) - rotary evaporate supernatant to remove alcohol
(3) - freeze dry to eliminate as much water as possible
(4) redissolve in 20 ml ethanol and add 30 ml diethyl ether
(5) - agitate on vortex mixer until sugar (white) precipitate forms
(6) - centrifuge to remove sugar
(7) - concentrate supernatant by rotary evaporation

TABLE 18: Solubility of antibacterial activity in solvent mixtures of different polarity, shown as inhibition of growth of S.aureus.

Solvent mixture (ethanol:ether)	Fraction	
	precipitate	supernatant
80:20	no inhibition	slight inhibition
60:40	no inhibition	slight inhibition
50:50	no inhibition	inhibition
40:60	no inhibition	inhibition
20:80	inhibition	inhibition

slight inhibition = < 1 mm diameter zone of inhibition

An extract obtained by the modified procedure was applied in 10, 20 and 30µl quantities to two thin-layer chromatograms and developed in solvent as before. One was stained with p-Aminophenol and the other was assayed for antibacterial activity. Application and drying of extract was found to be quicker and easier with less sugar present.

The agar plate on which one chromatograph was superimposed showed a clearing which extended across the tracks from the 10µl and 20µl applications, R_f = 0.68. This may have been an artefact as it was continuous across the area between the two tracks where no extract had been applied. There was a clearing again over the area corresponding to the application points of the chromatogram, which was continuous and increased in size and intensity with the larger extract applications. p-Aminophenol stain, however, indicated little sugar present.

4.6 Summary

All the non-peroxide antibacterial activity of manuka honey could be extracted into absolute ethanol, but none would dissolve in ether.

Ether was added (60%) to the ethanol extract to precipitate most of the sugar from the honey but maintained the solubility of the activity.

The solubility and behaviour on thin-layer chromatography indicated that the activity was fairly polar.

Acidifying the extracted activity did not make it more soluble in ether. Adding alkali to pH 10 eliminated the activity, making it undetectable.

Extracts of inactive raw honey were also inactive when assayed for activity.

CHAPTER FIVE:

ISOLATION OF THE NON-PEROXIDE ANTIBACTERIAL ACTIVITY

Thin-layer chromatography (TLC) is a useful technique for separating the compounds within a mixture because it is rapid, convenient, sensitive and gives good resolution. With preparative thin-layer chromatography heavier loading is possible and the various fractions can subsequently be eluted and evaluated. Hence preparative TLC was selected to aid isolation of the non-peroxide antibacterial factor or factors.

Migration of the different substances in the honey extract was followed by various visual methods after each chromatographic run, as R_f values vary in preparative TLC. The R_f of a compound can vary with size of application, length of chromatogram, activity of adsorbant, sealing and dimensions of the chamber, atmospheric conditions (temperature and humidity), differences in the solvent mixture and with thickness of the layer.

Simple solvent mixtures were used to begin with as they give better reproducibility.

The ascending method was used.

5.1 Preliminary Preparative TLC

Some preliminary TLC was carried out in order to find a solvent mixture which would separate the activity from the other components of the extract, particularly the sugars. This was necessary as, for reasons stated above, migration on a preparative plate would not be the same as that on the Eastman Chromagram Sheet used previously. Therefore the

location of the active components had to be retraced.

Plate preparation is described in Chapter 2. After activation and cooling of the plates an alcohol-ether extract (5 ml) of 10 ml honey was applied to a line marked 15 mm from the adsorbant edge, and dried with a hairdryer. The high temperatures reached by the hairdryer would also inactivate any glucose oxidase enzyme (and any other proteins) present should any have remained soluble in the ethanol. During these preliminary investigations, plates of size 50 x 200 mm were used and development was carried out in a glass chamber sealed with a silicone-greased glass plate. Bioassays, to localise activity, were carried out after chromatography by superimposing the air-dried TLC plates on agar plates, of the same size, for two hours in a sealed plastic container (lined with damp strips of filter paper). The agar plates were then incubated at 37°C overnight and later stained for lactate dehydragenase, as described previously.

Agar plates were prepared in the cold room (about 4°C), the warm, seeded agar being poured onto a tray of glass plates which had been chilled (4°C) for several hours beforehand, to obtain a thinner agar. It was necessary for the agar plates to be thin so that any activity would diffuse through the whole depth of agar and would not be masked, after staining, by bacterial growth beyond the limiting dilution of activity.

The general detection reagents sulphuric acid and molybdophosphoric acid were used to visualise the position of the separated compounds on the TLC plates which were not bioassayed.

a. Testing General Reagents and Bioassay

Initially three chromatograms were run in the same solvent in order to check the method of detecting the activity described above and to see if the sulphuric acid and molybdophosphoric acid stains detected the active factor(s) and other compounds present. These preliminary runs were also used to check that a heavier loading of extract did not significantly alter the migration pattern of compounds and that sugar was not affecting the bioassay.

Three preparative TLC plates were activated, cooled and each given an application of 50 µl honey extract, 50 µl sugar solution extract and 75 µl honey extract. The plates were developed at the same time in 80% ethanol - 20% ether in a sealed glass tank until the solvent front was 10-20 mm below the top edge of the silica. After removal from the chamber the plates were allowed to air dry and then one was used for the bioassay, as described above. One plate was sprayed with sulphuric acid and the other with molybdophosphoric acid and then both were incubated at 100-110°C for 30 minutes to complete staining.

After staining with lactate dehydrogenase detection reagent, the agar plate showed two green "spots" of different size (corresponding to applied honey extract) which represented zones of growth inhibition of S.aureus. These were approximately just below the position of the solvent front. An area of clearing (green) was also present about 30 mm above the origin in the line of migration of the 75 µl of honey extract. Unlike the clearings near the solvent front, this one did not correspond in position to any compounds stained with the

general reagents on the other two plates. The stained plates are represented in Figure 4.

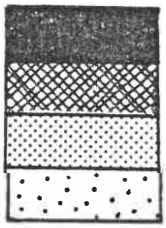
The time taken for stained spots and streaks to appear with heating was about 2-10 minutes. They became darker and more distinct with further heating. Comparing all three plates as closely as possible it was concluded that the upper areas of bacterial inhibition corresponded to areas somewhere within the upper 20 mm of development. The purple colour of compounds stained with sulphuric acid in this region indicated that they were possibly phenolic derivatives.

Sugar from the honey extract was located by comparison with the migration of the extract of the sugar solution, but the honey had produced a larger and more darkly charred spot. This indicated that other compounds in the honey extract had moved to the same position as the sugar. As there was no inhibition on the bioassay plate corresponding to these areas it was concluded that there was no need to attempt further separation and isolation of these compounds.

Comparing the migration patterns of the 50μl and 75μl honey extract spots showed that a heavier loading did not significantly alter the chromatographic behaviour and hence the position of separated fractions.

Further preliminary TLC work was necessary to elucidate certain possible artefacts before attempting to isolate active factor(s). The clearing produced 30 mm above the application line could have been an artefact as with previous TLC, or a compound with strong antibacterial activity which was in such a small quantity as to be undetectable with a spray reagent. This could be confirmed

KEY TO FIGURES:



very dark

very faint

x = no inhibition of growth

sl = < 1 mm inhibition

✓ = ⩾ 1 mm inhibition

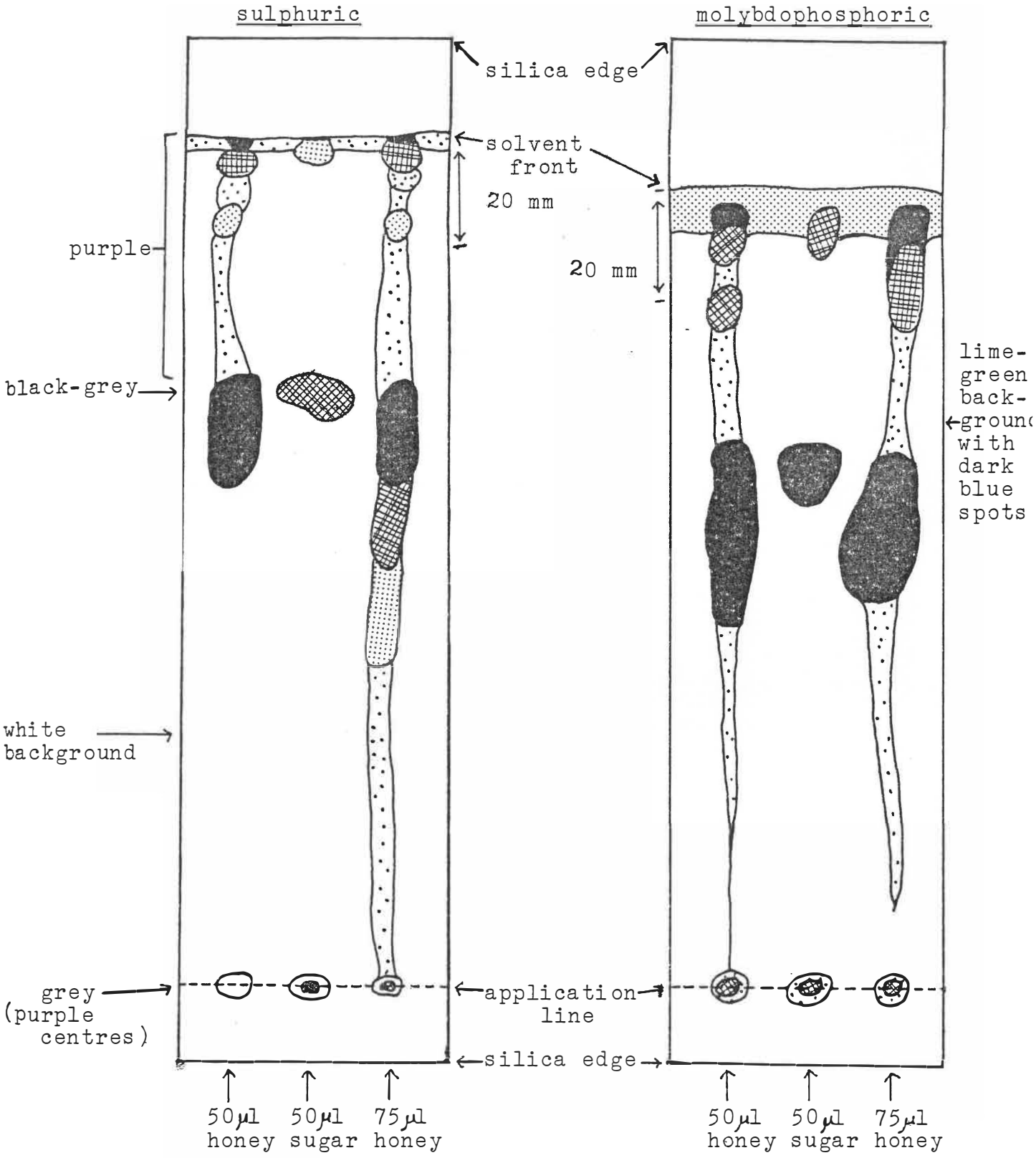


FIGURE 4: Staining of preparative TLC plates, following honey and sugar extract application and development in 80% ethanol:20% ether.

by repeating chromatography with the same solvent mixture and others, less polar and more polar, in order to see if the position of the area of clearing was changed in the path of migration. Also both stained TLC plates indicated an artefactual staining was produced along the solvent front, which was also visibly yellow before spraying and charring. Hence the darker stained compounds on the solvent front, which occurred in line with both sugar and honey extract origins, may also have been artefacts due to the use of bulk solvents (less than 95% pure) during these preliminary investigations.

b. Effect of Different Solvent Mixtures on Compound Separation

A solvent mixture for chromatogram development was required which would give a good separation distance between components, particularly between sugar and antibacterial factor(s), and sharp migration patterns, i.e. limited tailing and spreading of spot size.

Two solvent mixtures were investigated other than the 80% ethanol-20% ether previously used; a more polar solvent, 100% ethanol and a less polar mixture 60% ethanol-40% ether. Three chromatograms were developed in each solvent and treated as previously with either sulphuric acid spray, molybdophosphoric acid spray or bioassayed for antibacterial activity.

Honey extract (75 μl) was applied to each plate and the migration pattern compared with that from 75 μl sugar extract and a control of 75 μl of 40% alcohol-60% ether solution, in order to see which stained spots consisted of sugar and which were artefacts due to the solvents used. The results are shown in Figures 5, 6 and 7.

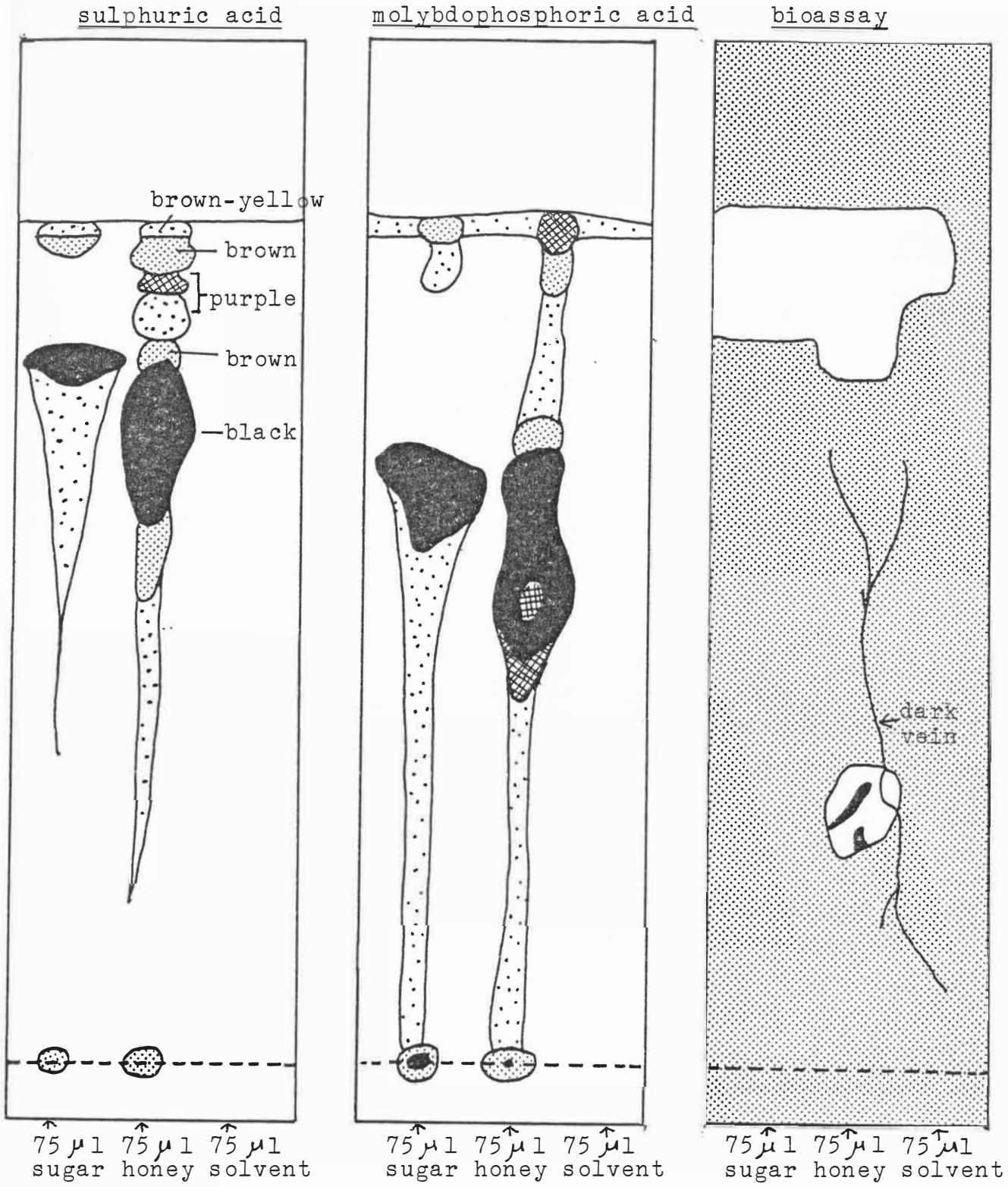


FIGURE 5: Chromatogram developed in 100% ethanol

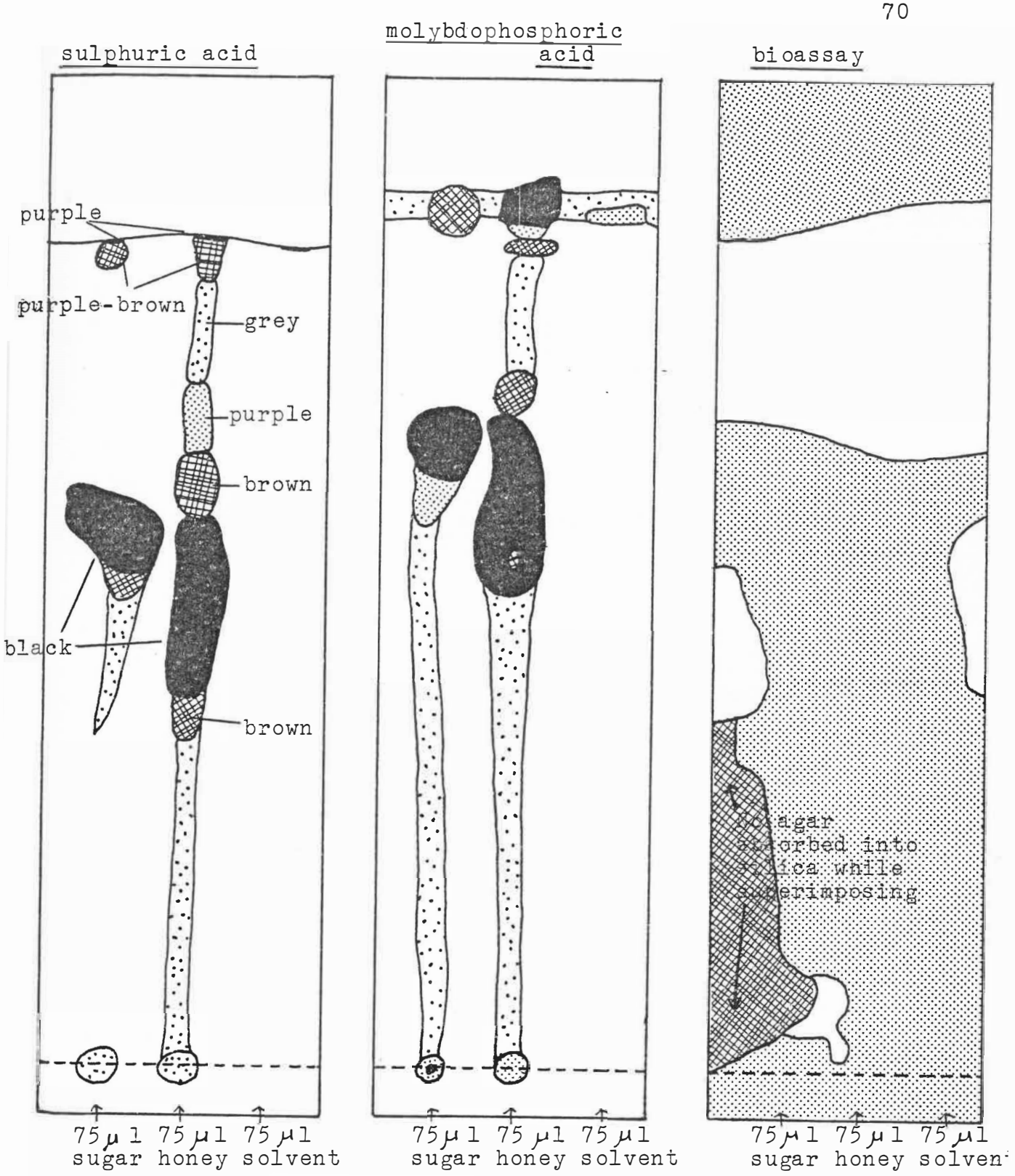


FIGURE 6: Chromatogram developed in 80% ethanol:20% ether

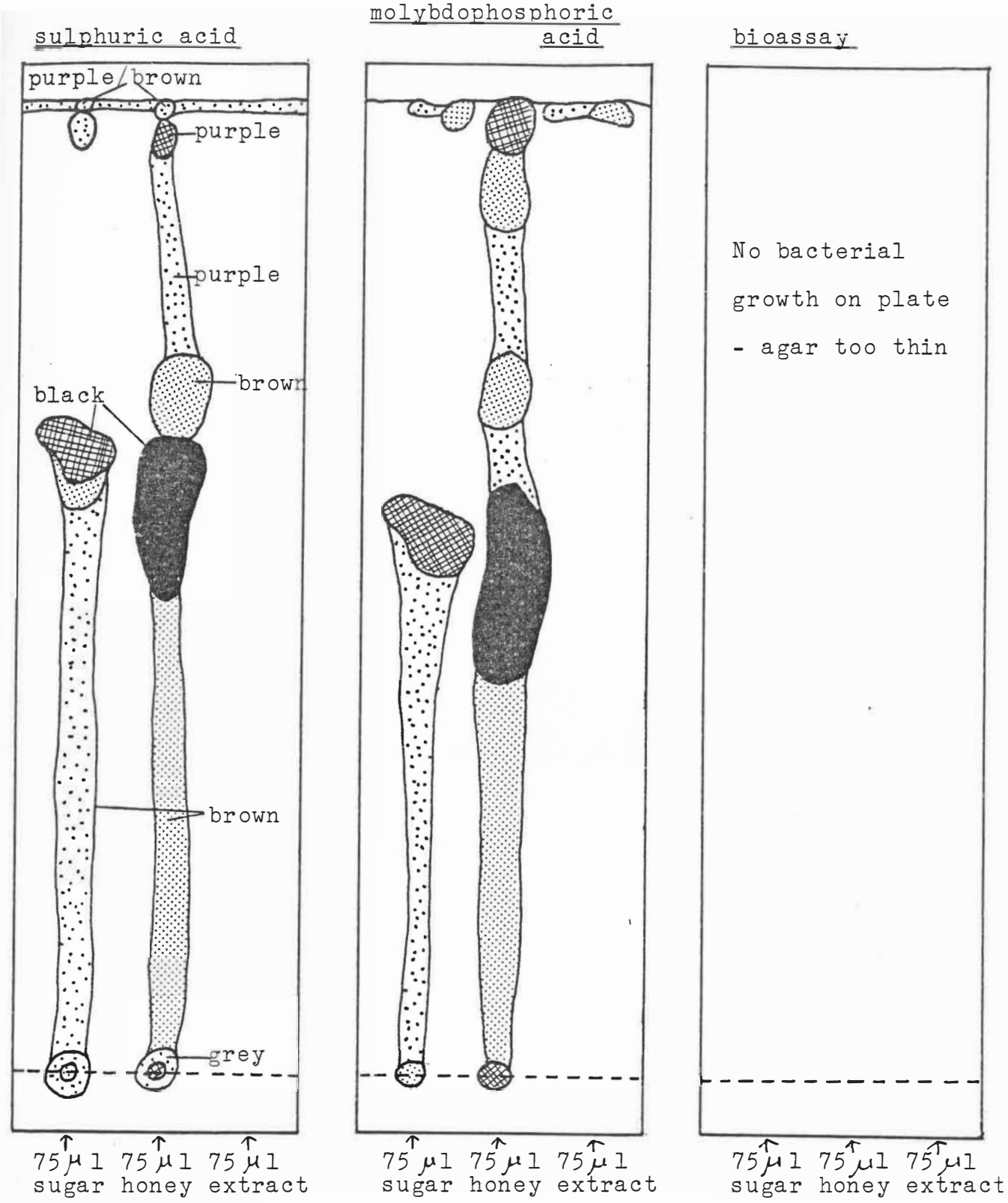


FIGURE 7: Chromatogram developed in 60% ethanol:40% ether

The stained and charred plates indicated that 60% ethanol-40% ether solvent produced the greatest separation of fractions. Ethanol, alone, carried most of the honey extract into the upper half of the chromatogram, with little distance between different compounds. These plates also confirmed that artefactual staining was produced at the front from the solvent alone. Therefore it was concluded that, prior to loading and developing chromatograms for qualitative or quantitative analysis, they would have to be cleared of impurities, such as phthalate, by development in a more polar solvent to the end of the adsorbant and then reactivated prior to development with extracts. This would have to be halted several centimetres before the end of the silica to avoid the band of impurities.

Variability in R_f values was also clearly demonstrated.

Two of the bioassay plates gave results. In both, large clearings were produced just behind the solvent front. The antibacterial activity here appeared to be quite potent, as in both cases it extended across the possible path of sugar extract, where no compounds were detected with either spray reagent. It was concluded that an active fraction did migrate to near the solvent front as it was visualised in all three bioassays which had worked, but that this method of bioassay was not precise enough to indicate the exact position of the fraction. The second clearing which appeared in all cases on the lower half of the agar plates was concluded to be an artefact of the bioassay procedure. Its "migration" only approximated that of honey extract components and did not

correlate with the polarity of solvent mixture used i.e. with development in 80% ethanol it did not migrate to the same position both times. Each time, too, it was associated with a "fault" in the agar.

It was decided to discard this method of bioassay because of its inaccuracy due to the ease of diffusion of the antibacterial factor(s) and the difficulty in making agar plates of adequate and constant depth. Pouring plates in the cold to allow quicker setting and therefore thinner layers caused uneven layers as overlapping of agar occurred, without mixing. Hence while superimposing chromatograms small areas were not in contact with the silica and very thin areas of agar were often completely absorbed into it, or would not support growth of S.aureus.

An attempt was made to improve the plates by pouring them in individual metal dishes. It was difficult to obtain metal dishes with a flat bottom however and this did not eliminate the production of dark veins and spots throughout the agar after staining. These were covered by silica adsorbing to the agar during superimposing. They could not be washed off until after incubation and growth of S.aureus by which time they were completely embedded in the agar.

In order to avoid the artefacts mentioned above, a biological detection method (Kirchner, 1978) was tried which involved coating the thin-lay chromatogram itself with seeded agar. After air-drying the developed chromatogram,inoculated agar, containing 0.7 ml of a 5% solution of triphenyltetrazolium chloride in 50% methanol per 50 ml of media was poured gently onto its surface.

After cooling this layer was protected by pouring a thin coating of sterile agar on top. When this was set the plate was kept in a closed container in the refrigerator ($0^{o}C$) for one hour to allow the antibacterial compounds to diffuse into the agar. After incubation ($37^{o}C$ overnight) bright yellow spots of inhibition should have appeared in a red-brown background. However, when warm agar was poured onto the TLC plate areas of silica bubbled up and became pitted and after incubation the area of agar in between remained colourless.

5.2 Locating the Antibacterial Activity

Several methods of detection were attempted in order to find out more accurately which compounds were antibacterial and to discover, if possible, to which chemical group they belonged. Also a detection method was required which was non-destructive to enable qualitative chemical analysis of the antibacterial factor(s) once located.

a. Biological Detection of Antibacterial Factor(s)

Antibacterial activity was assayed using an agar well diffusion technique.

Honey extract (1 ml) was applied as a band to each of two activated TLC plates. The chromatograms were developed in 60% ethanol-40% ether (Solvent I) and air-dried. One chromatogram was then divided into thirty horizontal strips (approximately 5 mm in width) across the path of migration of the honey extract. Each strip of silica was removed from the glass plate, mixed with 200-300μl distilled water (to form a wet sludge) and added to a well in a seeded agar plate, which was then incubated. The second plate was sprayed with sulphuric acid to indicate the location of

extract fractions. The results are given in Figure 8. Repeating this with sugar extract, instead of honey extract, produced no bands of inhibitory activity.

Following this a 300 mm preparative TLC plate was loaded with a band of 6 ml honey extract and developed in Solvent I. Ascending development was carried out in a metal chamber with filter papers lining the sides to maintain a solvent-saturated atmosphere. A glass plate was laid on top to seal the chamber. The plate was air-dried and vertical strips of 50 mm width were used in the following investigations. This enabled a more consistent comparison of the different detection methods because the antibacterial fraction(s) would be located at the same migration distance in each test.

b. Detection Under Ultra-Violet Illumination

Ultra-violet illumination of the developed chromatogram was investigated as a non-destructive method of locating components of the honey. A section of the chromatogram was visualised under U.V. illumination of 254 nm and 350 nm wave length and the patterns recorded. The same section was then assayed for antibacterial activity as described above. The degree of inhibition of bacterial growth was given a relative visual assessment only - the zone of clearing was not measured. The results are shown in Figure 9.

The inhibition of growth shown by the strips numbered 11 and 29 was only slight and therefore assumed to be artefacts. This was confirmed when this procedure was repeated with a 50 mm TLC plate, where only fractions corresponding to strips 32-36 produced inhibition of growth.

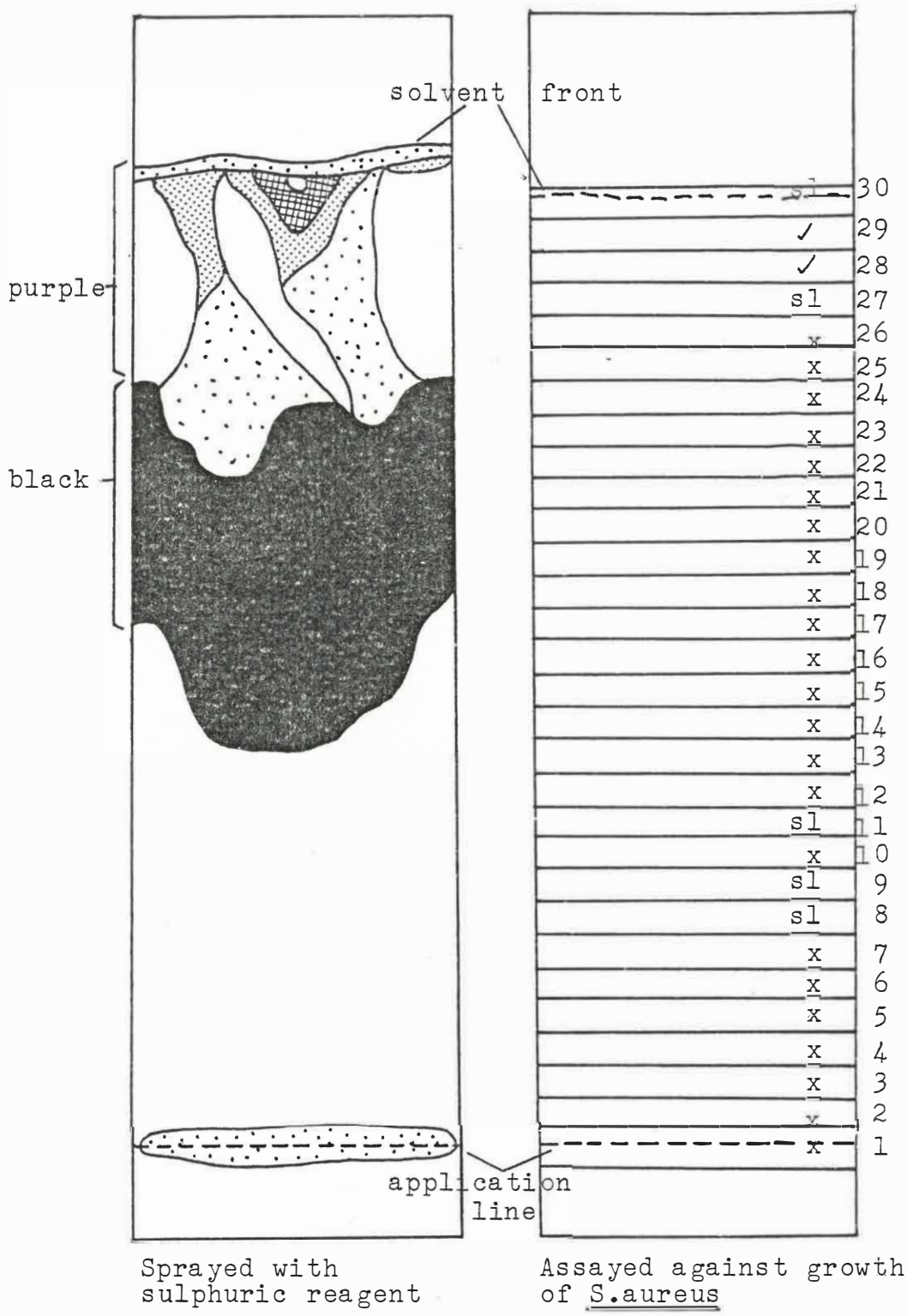
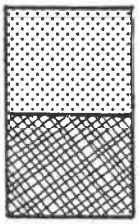


FIGURE 8: Comparison of 2 chromatograms of 1 ml honey extract, developed in Solvent I.

One plate was stained with sulphuric acid and the other assayed by agar well diffusion for antibacterial activity in the strips removed from the plate.

Key to Figure 9



Absorbance

Strong absorbance

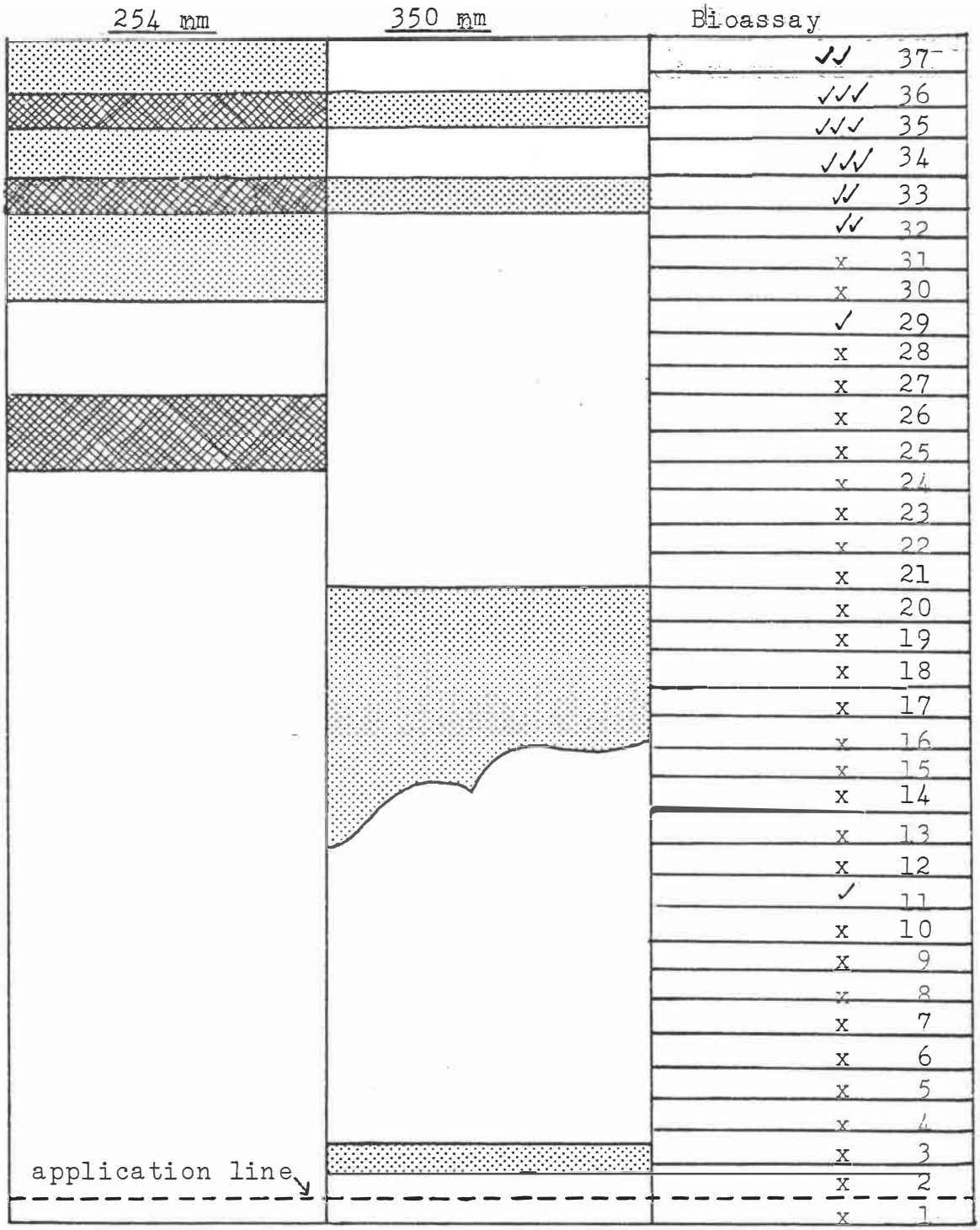


FIGURE 9: Location of antibacterial fraction(s) by comparing appearance under u.v. illumination with assay against S.aureus.

Strip 37 (the front) was also concluded to be an artefact. Inhibition by No. 37 was probably due to a high concentration of phthalate and other impurities in the Keiselgel. The solvent in which the 300 mm TLC plate was developed had been allowed to ascend to the top of the adsorbant and the plate had not been "cleared" prior to this development.

A chromatogram, applied with sugar extract and developed in SolventI, when viewed under ultra-violet illumination of 350 nm wavelength showed that sugar had move approximately to the first band of absorbance (assay bands numbered 14-20, Figure 9).

c. Sprav Reagents

Spray reagents can be used to give an indication of what type of compounds are in the separated fractions on a chromatogram. The sulphuric acid reagent used to locate compounds gave a purple colour in the region where anti-bacterial activity was detected. This reagent was similar in composition to a spray of concentrated H_2SO_4 - water - 95% ethanol (1:1:8) which colours phenolic derivatives purple or blue (Elliot et al.,1969). All artefacts of solvent development, on the solvent front, also coloured purple after staining and heating. Therefore, an attempt was made to confirm whether the active fraction(s) was/were phenolic by using other spray reagents.

Five spray reagents were used to stain 50 mm sections side by side on the 300 mm preparative plate. Aluminium foil was used to protect other sections during spraying.

Sulphuric Acid

The general spray reagent used previously appeared to stain purple only the solvent front i.e. kieselgel and solvent impurities. The bands of activity just behind this were not stained. An area half-way up the plate was charred black and it was concluded that this was sugar.

Ferric Chloride

A section was sprayed with a saturated solution of anhydrous ferric chloride in methanol. Kirchner (1978) lists this spray for terpene phenols. Elliot et al,,(1969) lists a similar reagent:- a saturated solution in anhydrous dioxan or chloroform to stain most phenolics red-violet. They also claim that phenols having two or more vicinal hydroxy groups, or ortho (or peri) hydroxycarbonyl groups, give coloured chelates with ferric salts.

In this case however, none of the antibacterial bands were stained.

Anisaldehyde-Sulphuric Acid

Concentrated H_2SO_4 (1 ml) was added to 0.5 ml anisaldehyde in 50 ml acetic acid. The chromatogram was sprayed immediately after the solution was mixed, and heated to 100°C for 5-10 minutes (Stahl, 1969). Lichen constituents, phenols, terpenes, sugars and steroids yield violet, blue, red, grey or green products (Stahl, 1969).

This was found to stain all compounds which had been visualised previously with a general reagent. The area of antibacterial activity was stained bright pink-mauve. This was repeated with a small TLC plate spot-applied with honey extract and the active area was stained pink-orange when sprayed.

Ferric Chloride-Potassium Ferricyanide

This stain is fairly specific for phenolic compounds. Aqueous 1% solutions of each salt (A.R. quality) were mixed in equal volumes to form an orange-brown solution, with no trace of blue (Elliott et al., 1969). Reactive phenols should give blue spots on a pale blue background immediately, without heating,

The two active bands corresponding to strong absorbance under 254 nm u.v. illumination appeared dark blue immediately after staining. The two bands of less strong absorbance above each of these also appeared blue, but with less intensity. This result was confirmed with a chromatogram run from a spot application. However only one elongated blue spot appeared on staining. This plate was treated with ferric stain immediately after air-drying whereas the larger plate had been left a few days before staining. Partial oxidation of the activity may have occurred on standing and only then have given the appearance of two separate bands of u.v. absorbance (Figure 9).

Vanillin-Sulphuric Acid

Owing to the inconclusive result obtained with the anisaldehyde stain and the possible plant origin of the antibacterial activity, a reagent which detects terpenes and essential oils was tested. A section of chromatogram was sprayed with a solution of 1 g vanillin in 100 ml 95% H_2SO_4 and heated at 120°C. The solvent front and sugar fraction only became stained.

Conclusions

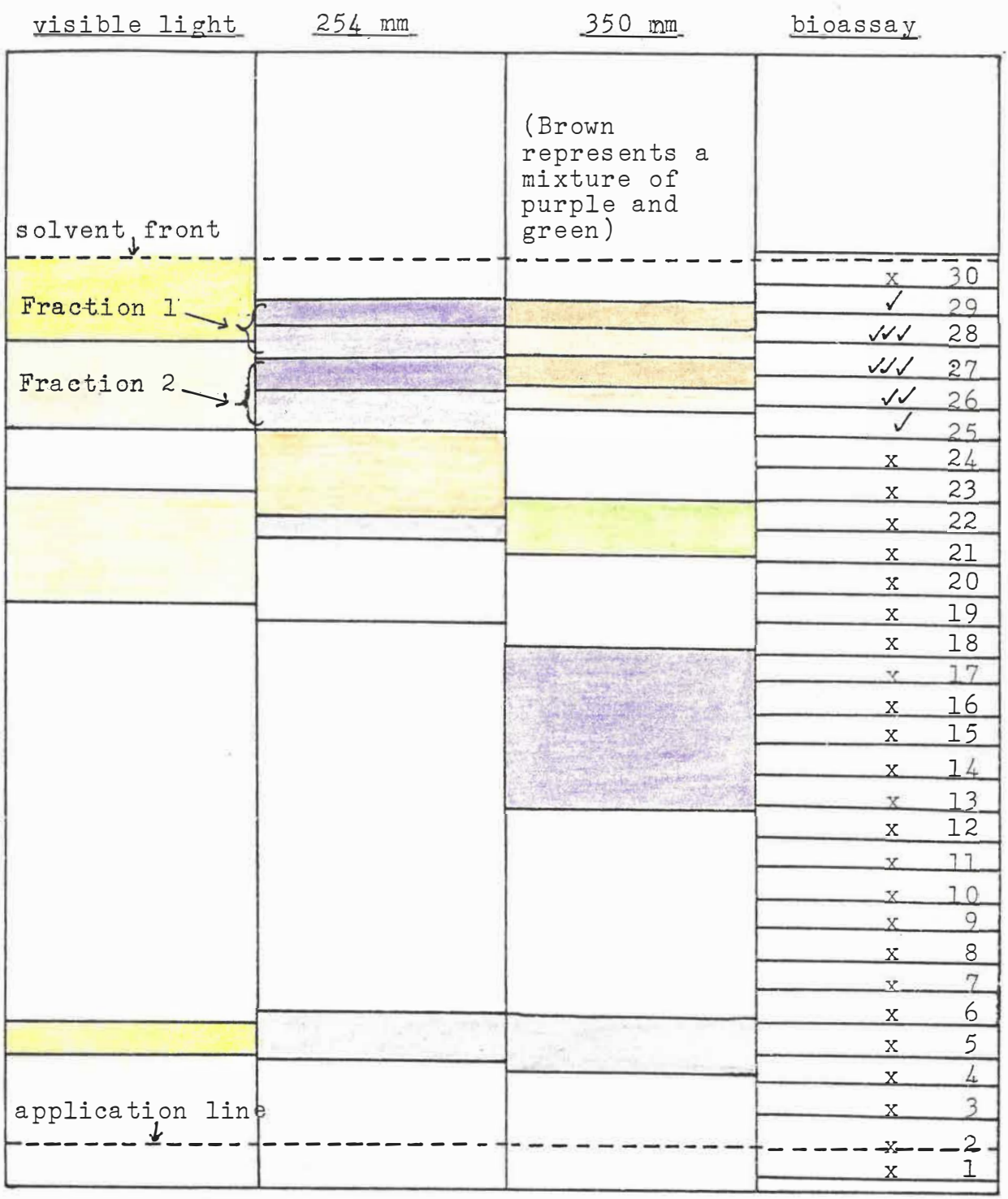
Although staining the chromatogram with sulphuric acid or anisaldehyde gave inconclusive results about the chemical nature of the antibacterial activity, the ferric chloride-potassium ferricyanide spray indicated that it was probably phenolic. The negative results obtained with vanillin or ferric chloride stains tend to eliminate the possibility of it being a terpene. The lack of detection with ferric chloride also limits the possible structure somewhat, i.e. it does not have two or more vicinal hydroxy groups.

5.3 Further Separation with Preparative TLC

On primary TLC of honey extract two antibacterial bands appeared to migrate just behind the solvent front and were visualised as dark absorbing bands under ultra-violet illumination. Two lighter absorbing antibacterial bands were immediately behind each one of these (Figure 9). Further separation of the active fractions was necessary in order to see if the lighter bands were different compounds or tailings of the darker bands. Sharper separation of all active bands was desirable before qualitative analysis, to avoid overlap of fractions. Secondary development on a chromatogram would also give an indication of how pure these bands were.

A 400 mm preparative plate was loaded with the alcohol extract of 40 ml of honey and developed in Solvent I. It was air-dried for two days, the u.v. patterns recorded and a 50 mm vertical strip was divided into thirty 5 mm strips, cross-wise. These were removed and assayed for anti-bacterial activity and the results are shown in Figure 10.

Under ultra-violet illumination of 254 nm wavelength



✓ relative inhibition of S.aureus growth

x no inhibition

FIGURE 10: Preliminary TLC of honey extract with Solvent I viewed under u.v. illumination and assayed for antibacterial activity.

the remaining active area (labelled Fractions 1 and 2 in Figure 10) was scraped from the plate. It was added to 30 ml absolute ethanol and ground up in a pestle and mortar to allow all compounds adsorbed to the silica to re-dissolve into the alcohol. The alcohol and sludge were centrifuged at 8000 r.p.m. for 15 minutes and the supernatant reduced to 14 ml by rotary evaporation. Seven 50 mm preparative TLC plates were loaded with 2 ml each of the concentrated supernatant and developed in one of the following solvents:

chloroform

n-hexane-ethylacetate (95:5)

n-hexane-ethylacetate (72:29)

ethyl acetate - petroleum ether (3:1)

10% acetic acid in chloroform

diethyl ether - petroleum ether (1:1)

toluene-chloroform-acetone (40:25:35)

Each chromatogram was then air-dried for two days and the band patterns viewed under u.v. illumination (254 nm and 350 nm wavelength) and recorded. They were then sub-divided into 5 mm strips across their width and assayed for activity.

All plates which had been developed in solvents containing ethyl acetate or acetic acid indicated varying degrees of inhibition in all strips assayed from them. It was concluded that these plates still contained acetic acid bound to the silica (although it was not detectable by smell) and that these would not be convenient solvents to use for secondary development.

The chromatograms which had been developed in the other solvent mixtures demonstrated no bands of bacterial inhibition, although under u.v. illumination they appeared

to have bands of varying absorbance and fluorescence. It was considered that either the solvents used were reacting with the antibacterial compounds and rendering them inactive or the activity was not being removed from the silica of the first plate.

In order to test this, 5 ml of honey extract was applied to a 100 mm preparative plate and chromatographed in Solvent I. After drying, one half of the plate was assayed for antibacterial activity as a positive control. The active bands were scraped from the second half and ground up in 10 ml absolute ethanol with a pestle and mortar. After centrifuging, the supernatant was concentrated and applied to a 50 mm TLC plate and developed again in Solvent I. The plate was then air-dried, viewed under u.v. illumination and the bands seen assayed for activity. The results are shown in Figure 11.

It can be seen that the loss of activity was not due to the use of a second solvent mixture. It appeared from the band patterns, seen under u.v. illumination, that the compounds were redissolving into the alcohol and being applied to the second chromatogram, although they did not show up as strongly and their migration patterns seemed to altered slightly. It was concluded that grinding up the active compounds with silica in alcohol was rendering them inactive, or that the active component(s) was not being eluted.

A method of removing the active fractions from the silica of the primary TLC plate by elution was investigated. Alcohol extract of 40 ml of honey was applied to a 400 mm preparative plate and chromatographed with Solvent I.

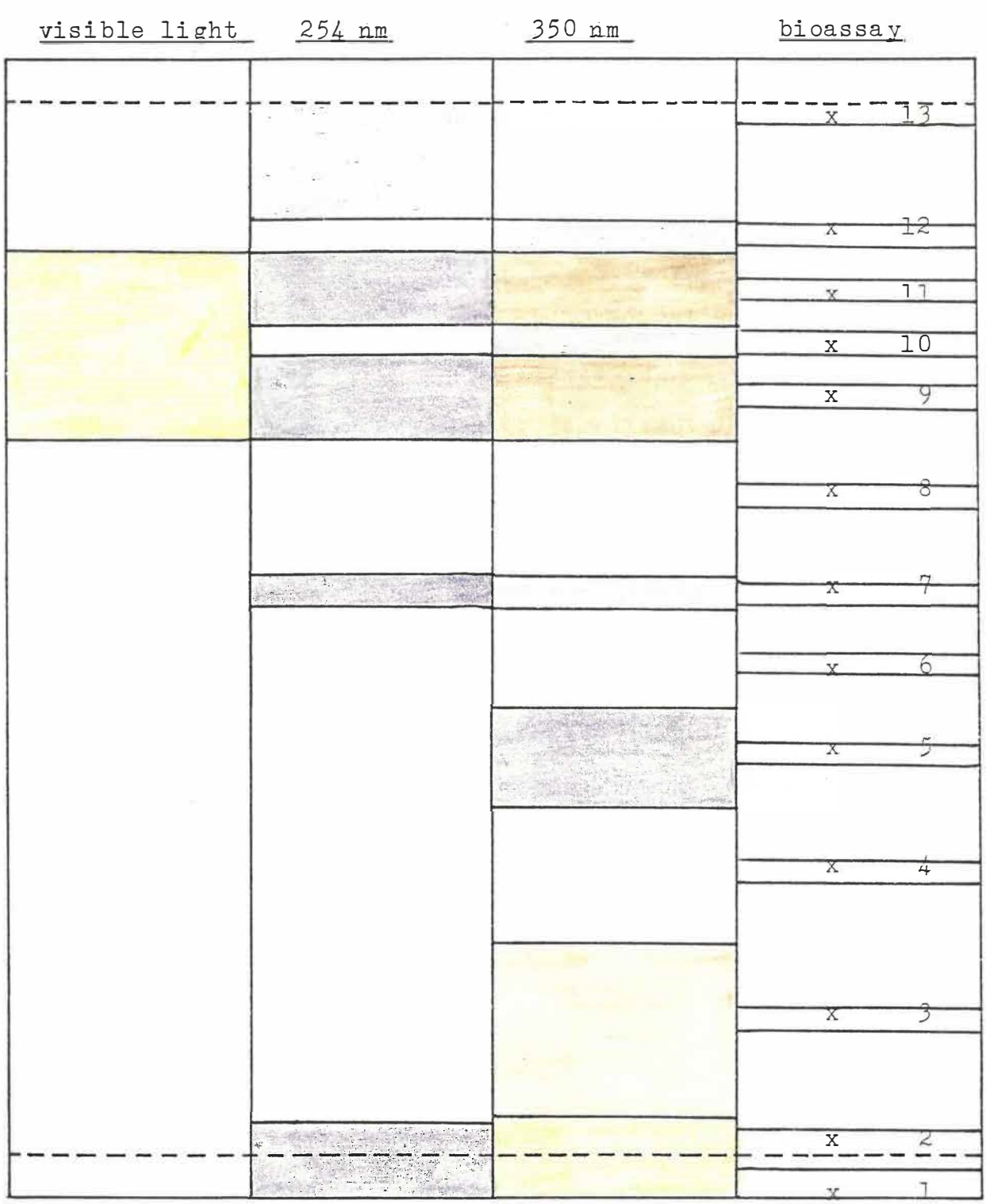


FIGURE 11: Re-chromatograph of honey extract with Solvent I.

When dry the two fractions of antibacterial activity (Figure 10) were scraped from the plate separately. They were put in separate glass columns with packed cottonwool in the bottom and eluted with twice their volume of Solvent I. The eluted extracts were then concentrated to 20 ml. One millilitre of each was rotary evaporated to dryness and 200 μl distilled water added and shaken vigorously. The brown coating inside each flask was only partially soluble in water but the aqueous solutions of fractions 1 and 2 were both assayed for antibacterial activity.

Fraction 1 produced a 1.5 mm diameter zone of inhibition and Fraction 2 4 mm. This indicated that the elution method of removing active fractions from a TLC plate was successful and activity was being recovered. The antibacterial activity of the two fractions appeared to be relatively less than the estimation of what it should have been. This, and the lack of solubility of the eluted material, indicated that activity may have been trapped in water-insoluble material. It also indicated that the two fractions were still very impure and needed further separation. This was confirmed when pooled samples of Fraction 2 were analysed by nuclear magnetic resonance. Many small peaks were obtained, some possibly indicating aromatic substances, and a very large peak indicating aliphatic substance.

A secondary solvent system was investigated for separating the compounds in Fractions 1 and 2, and to give an indication of how many compounds were in the mixtures. A solvent mixture commonly used for chromatography of

phenols was used. A 50 µl sample of Fraction 2, dissolved in deuterated chloroform, was spot-applied to a commercial aluminium foil-backed silica plate. The plate was developed in toluene:dioxan:acetic acid (180:50:8) in a sealed jar, dried, sprayed with aqueous 10% sulphuric acid and charred at 100°C for 30 minutes. When viewed under ultra-violet illumination of 254 nm wavelength the chromatogram appeared to have approximately eight separated compounds, with a large spot of hydrocarbon material (identified by colour and position) just behind the solvent front.

Because of the difficulty, found previously, in removing acetic acid from thin-layer chromatograms a solvent mixture of similar elutive power, but containing no acetic acid was tested. This solvent contained toluene:chloroform: acetone (40:25:35) and produced a resolution at least as good. The appearance of both stained and charred chromatograms is represented in Figure 12. This solvent mixture was subsequently used for all secondary chromatography and designated Solvent II.

5.4 Locating Antibacterial Compounds after Secondary Chromatography

In order to find the position of antibacterial bands after secondary chromatography two 400 mm preparative plates were cleared in Solvent I and re-activated. One was loaded with alcohol extract of 40 ml of honey and developed in Solvent I. A 300 mm width of the active area (Fractions 1 and 2, together) was then removed and the material eluted and applied to 300 mm of the second plate. This was developed in Solvent II. The bands were then located by viewing under u.v. illumination and

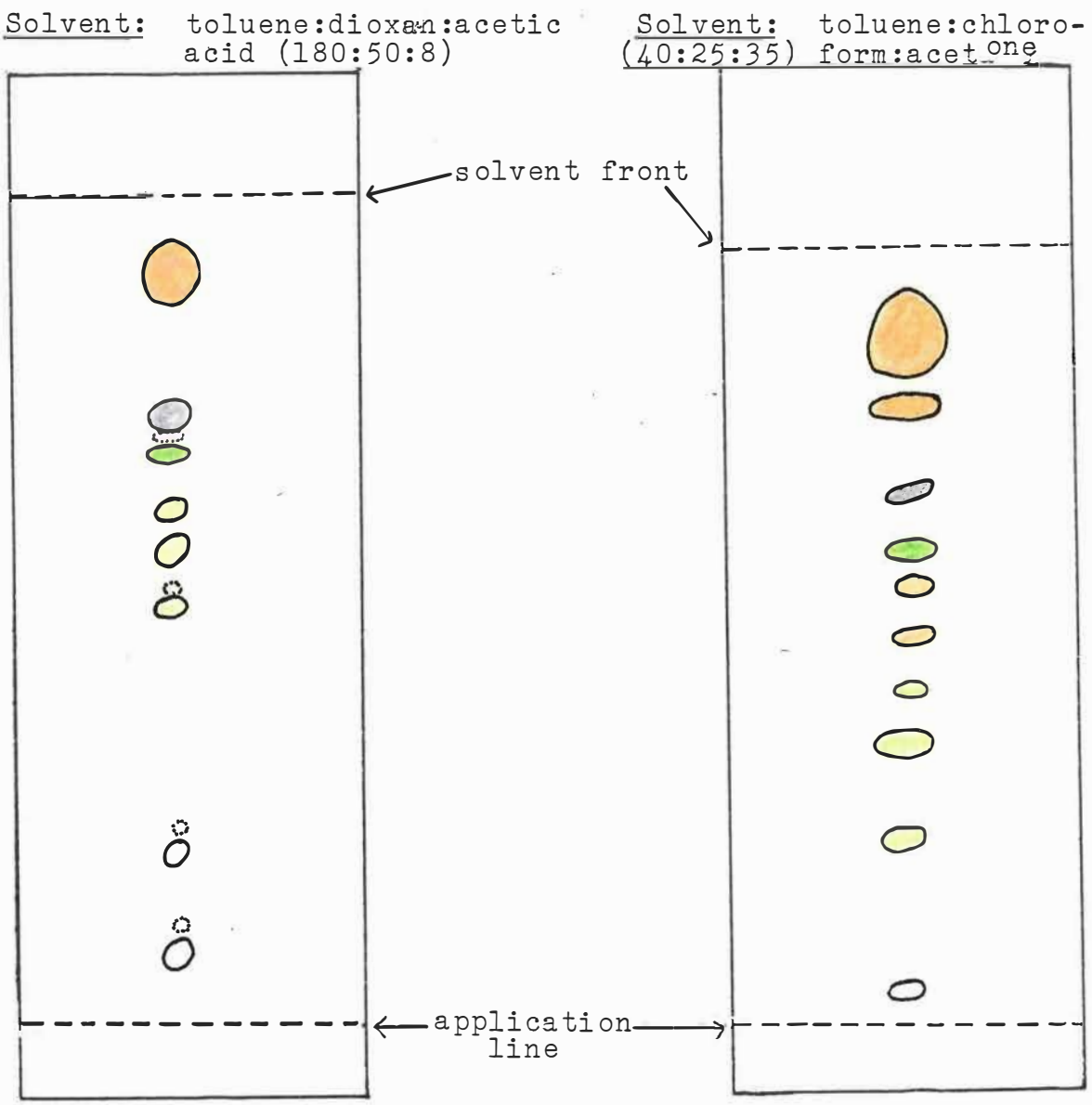


FIGURE 12: Appearance of TLC plates loaded with Fraction 2, developed with the solvents shown and stained with aqueous 10% sulphuric acid.

a 50 x 5 mm strip of each one removed, eluted in Solvent II, dried and assayed (in 200μl water) for antibacterial activity. A 50 mm wide strip of the active area from the first plate was eluted in Solvent I and assayed to see if any activity was lost during secondary development (any insoluble material was also scraped into the agar well).

The active area from the first plate produced a 4-5 mm diameter zone of inhibition. The results for the second plate are given in Figure 13.

Zones of inhibition cannot be added to give total inhibition but it was clear from the assay of the second plate that either activity was being lost between plates or the compounds work synergistically. Therefore elution and assaying was carried out with 50 mm strips of bands designated 6, 8, 9 and 11 (Figure 13) from the second plate, a pooled mixture of 6, 8, 9 and 11, a 50 mm wide strip from the total length of the second plate (i.e. including all separated compounds), and a 50 mm wide strip of active area from the first plate for comparison. The results are given in Table 19.

It was concluded that the loss of activity was not due to a synergistic requirement of the active compounds and that activity was being lost after application to the second plate. The results also indicated that not all activity was located in bands 6, 8, 9 and 11. Therefore assaying of bands in total, rather than 5 mm strips from in them, was carried out and the area from which strip no. 12 had been taken was found to contain slight antibacterial activity.

Antibacterial activity after secondary development was

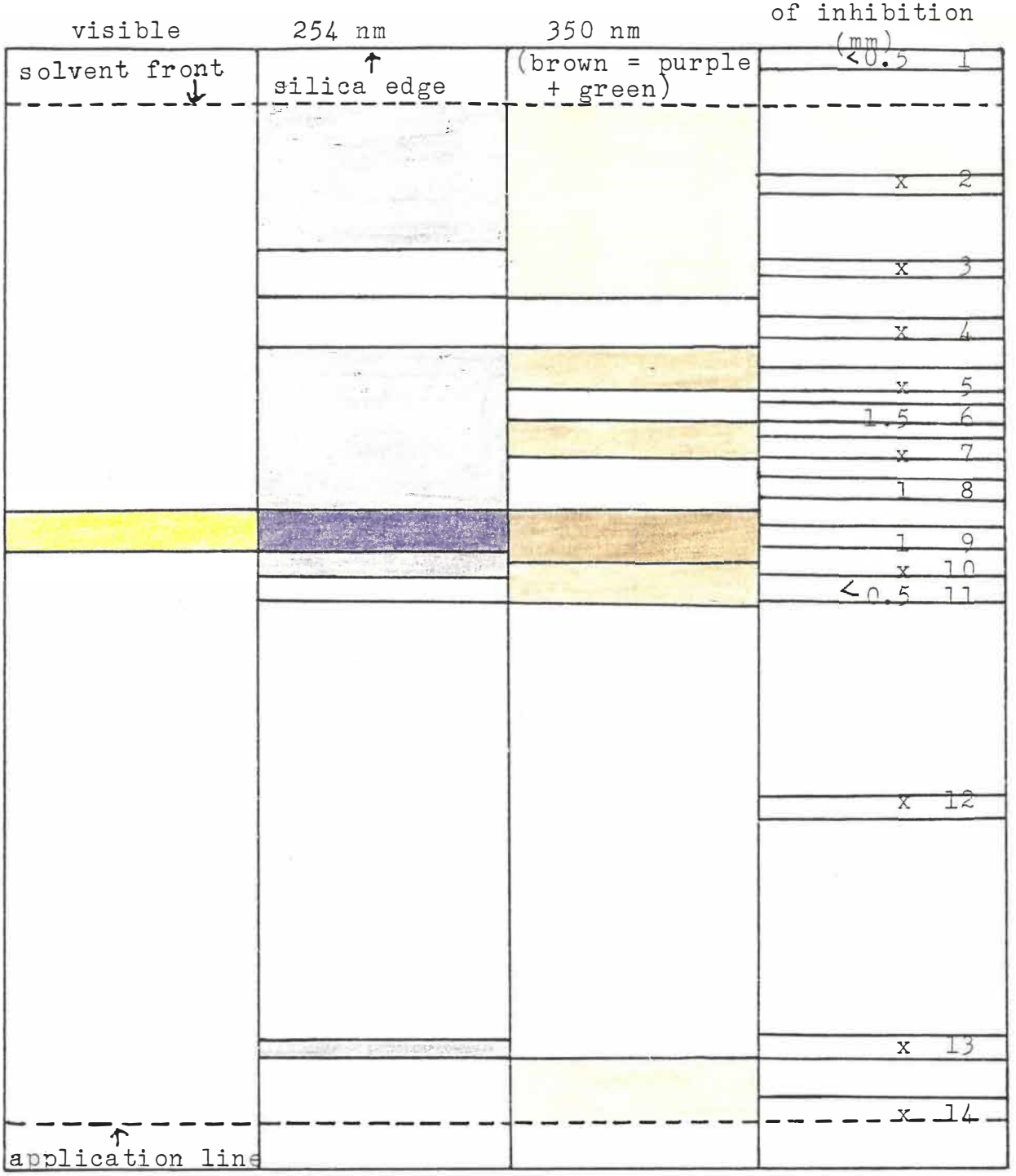


FIGURE 13: Secondary chromatography of honey extract in Solvent II. Viewed under u.v. illumination and assayed for antibacterial activity.

TABLE 19: Inhibition of growth of S.aureus by various eluted chromatographic fractions.

Test Fraction	Diameter of Zone of Inhibition (mm)
Active bands of first run (with Solvent I) (eluted with Solvent I)	4-5
50 mm-wide strip of total length of re-run active bands (eluted in Solvent II)	2
The four active bands of re-run (pooled, eluted in Solvent II)	1
Band 6 from re-run (eluted in Solvent II)	< 0.5
Band 8 from re-run (eluted in Solvent II)	1
Band 9 from re-run (eluted in Solvent II)	1.5
Band 11 from re-run (eluted in Solvent II)	1

not being totally recovered, either because of chemical instability on exposure to oxygen and the secondard solvent, or inefficient removal from silica by Solvent II during elution. To test these possibilities the following solutions from 50 mm wide strips of preparative plates were assayed for activity:

(1) active area of first run (with Solvent I) eluted with Solvent I.

(2) active area of first run, (with Solvent I) eluted with Solvent I, dried by rotary evaporation, 20 ml Solvent II added, poured into 90 mm diameter petri dish and left uncovered in fume cupboard for 2 hours, dried and assayed.

(3) total length of a second run (with Solvent II) plate, eluted in Solvent I.

(4) the four most active bands of the secondary plate, i.e. 6, 8, 9 and 11 (Figure 13), eluted with Solvent I.

The diameters of zones of inhibition of S.aureus, for these test solutions are given in Table 20.

The results obtained (Tables 19 and 20) showed that loss of activity was not due to inefficient elution by Solvent II but was due to inactivation by exposure to oxygen and Solvent II.

In order to find out which component of Solvent II was oxidising the antibacterial activity a 250 mm width of the active area of a first run (with Solvent I) was eluted with Solvent I and divided evenly between five 50 ml flasks. The solutions were then dried by rotary evaporation and treated by one of the following methods:

TABLE 20: Inhibition of S.aureus by antibacterial fractions treated in various ways.

Test Solution	Diameter of Zone of Inhibition (mm)
Plate 1 activity	3
Plate 1 activity - Solvent II treated	<0.5
Plate 2 in total - eluted in Solvent I	0.5-1
Plate 2 - 4 major active bands, eluted in Solvent I	1

Flask No.		Treatment
(1)	control	200 µl distilled water added, shaken vigorously at 30 minute intervals over 3 hours and the water-soluble fraction (plus insoluble material) assayed for activity.
(2)		15 ml toluene added, shaken, left one hour; dried and treated as flask (1).
(3)		15 ml chloroform added, shaken, left one hour; dried and treated as flask (1).
(4)		15 ml acetone added, shaken, left one hour; dried and treated as flask (1).
(5)		15 ml Solvent II added, shaken, left one hour; dried and treated as flask (1).

The results of the bioassay are shown in Table 21. Acetone was found to be the solvent component which was reducing the antibacterial activity. Exposure to oxygen was also found to be an important factor: when treated with Solvent II and exposed to a small amount of oxygen there was no inactivation of the antibacterial activity.

Bulk acetone had been used in Solvent II up to this point and it was thought likely that iron from the metal storage drums could have been catalysing the oxidation of the active compounds. In order to test this, the effect of reagent grade acetone on activity from primary TLC was compared with that of bulk acetone. A 200 mm width of primary plate activity was eluted with Solvent I and divided equally between four 50 ml flasks. The solutions were dried by rotary evaporation and the residues treated in one of the following ways:

TABLE 21: Inhibition of S.aureus by active fraction from primary TLC, treated with various solvents.

Solvent treatment	Diameter of Inhibition Zone (mm)
control	5
toluene	5
chloroform	6
acetone	<1
Solvent II	5

Flask No.	Treatment
(1) - control -	200 μl distilled water added, shaken at 30 minute intervals over 3 hours and the water-soluble fraction assayed for activity.
(2)	15 ml Solvent II (containing reagent grade acetone) added, poured into a 90 mm petri dish and left one hour, dried and treated as flask (1).
(3)	15 ml reagent grade acetone added, dried after one hour and treated as flask (1).
(4)	15 ml bulk acetone added, dried after one hour and treated as flask (1).

The results of the bioassay are given in Table 22. It was concluded that loss of activity was due to the use of bulk acetone in Solvent II, used for developing the secondary chromatogram. It was also concluded that it was not necessary to exclude oxygen from the system when reagent grade acetone was used.

Location of antibacterial bands was attempted again but with a chromatogram developed in Solvent II containing reagent grade acetone. The results are shown in Figure 14. (The full extent of the bands in the direction of migration was taken for assay). This secondary chromatographic development produced three areas containing antibacterial activity. The first, marked A, occurred in the upper third of the plate and had two bands of slight activity. The second occurred just above the middle of the plate (B) and contained three antibacterial bands which appeared under u.v. illumination of 350 nm wavelength as a purple band of strong absorbance, a band of medium absorbance and a band of green fluorescence. The medium

TABLE 22: Inhibition of S.aureus by activity from primary TLC subsequently treated with bulk acetone or reagent grade acetone.

Test Solution	Diameter of Inhibition Zone (mm)
Control	6
Solvent II (with reagent grade acetone)	6
Reagent grade acetone	6
Bulk acetone	1

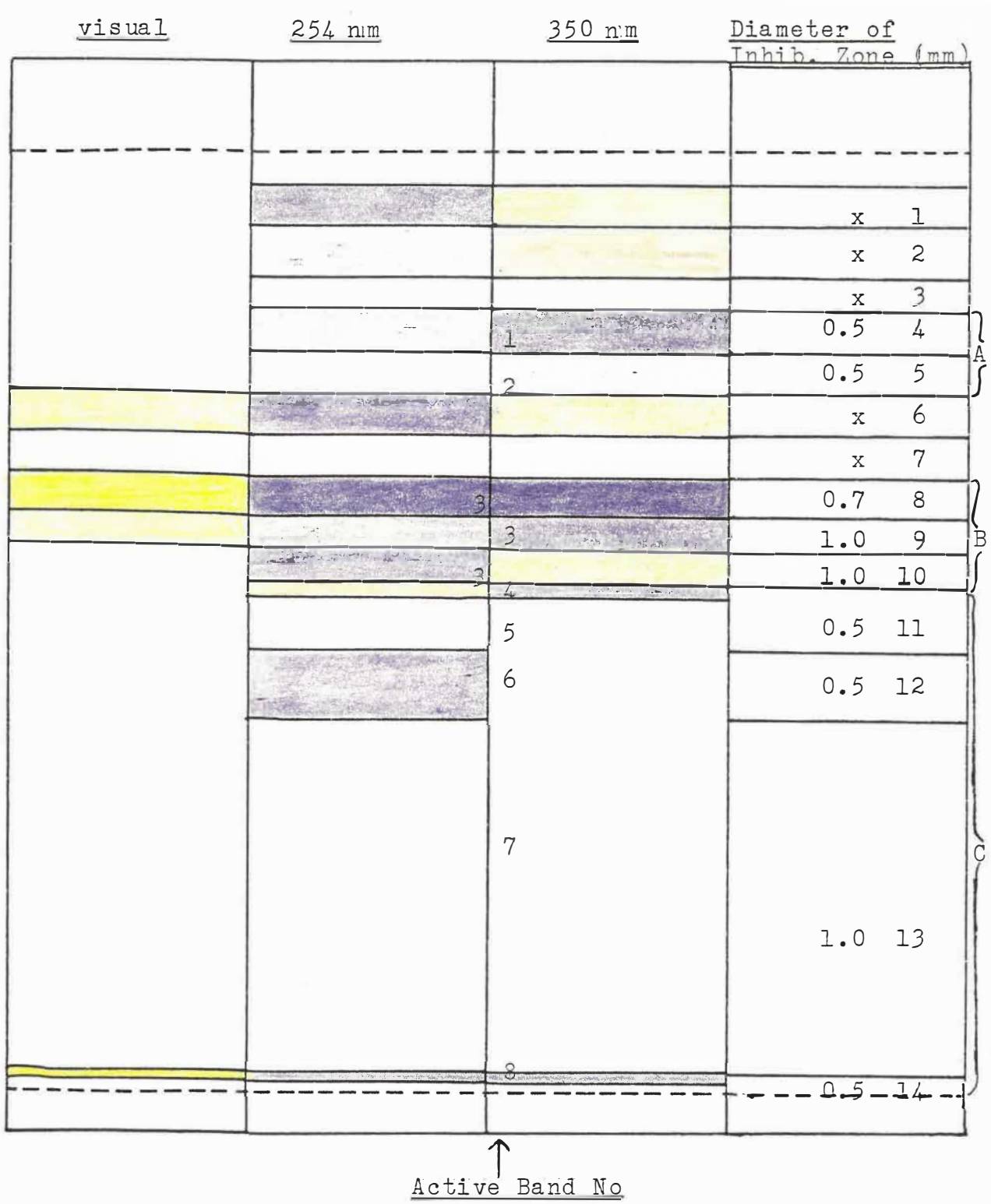


FIGURE 14: Secondary TLC of honey extract in Solvent II. Viewed under u.v. illumination and assayed for antibacterial activity.

purple band and the green band appeared to overlap slightly and the division between the two purple bands was indistinct. It was decided therefore that the bands in area B required further separation before their analysis was attempted.

The third area, C, occupied the lower half of the plate. This antibacterial area was mainly colourless, with some absorbing material corresponding to activity on the origin and a band (No. 6) which was antibacterial and appeared only on some secondary plates, and showed slight absorbance under u.v. illumination of 254 nm. The activity of the colourless area could have been due to a honey component, or components present without u.v. absorbance or fluorescence, or an antibacterial residue from the silica gel or solvent which is not concentrated enough to show up in other assayed bands (with which shorter lengths of the plate were taken). In order to test this the following 60 x 50 mm preparative chromatogram strips were scraped and eluted with Solvent I and arranged for activity:

(1) A blank strip run with Solvent I and reactivated, to check if activity was due to something in the silica.

(2) A blank strip run with Solvent I, reactivated and developed in Solvent II (no extract also), to see if inhibition was due to a residue from Solvent II.

Neither of these inhibited the growth of S.aureus and it was concluded that antibacterial material from the honey extract was causing inhibition from the colourless area. The next step was to see if this activity could be located in any particular position in the colourless area.

A 150 mm secondary plate was developed and dried. This time no coloured band corresponding to No. 6 of

Figure 14 was present. A 100 mm wide strip of the plate was used instead of 50 mm in order that any activity might be more easily detected. The length of the colourless area between the application line and orange band (unavoidably transferred from the primary plate) was 85 mm. The area in the 100 mm width corresponding to region C was divided into three equal lengths of approximately 28 mm each. The remaining 50 mm width of the TLC plate was divided into eight lengths of 10 mm and one of 5 mm which was removed from just below region B. All fractions were individually eluted in Solvent I, dried, 200μl water added and assayed for antibacterial activity. The results are given in Table 23.

It appeared that the colourless area may have contained some antibacterial material which was dissolving slowly off the origin and was not concentrated enough to be detected in sections of 10 mm. The material may have been carried slowly by the solvent, only migrating to higher areas when lower ones were saturated with it, in which case the blank area would be chemically homogenous, or it was being oxidised as it was migrating and therefore would not be chemically homogeneous. It is possible that several unrelated compounds (not visible under u.v. illumination), were present, the most active being coincidentally closest to the origin but this is less likely. It was not possible to find the correct explanation until various sections of the colourless area had been chemically analysed.

5.5 Tertiary Chromatography of Section B

Better separation of the three antibacterially active bands of section B was necessary before characterisation of the bands could be attempted. It would also serve to

TABLE 23: Antibacterial activity of sections of area C from secondary development of honey extract.

Section from 50 mm width of plate	Diameter of Zone of Inhibition (mm)
1 - top 5 mm of area C (next to B)	0
2 - next 10 mm	0
3 - next 10 mm	0
4 - next 10 mm	0
5 - next 10 mm	0
6 - next 10 mm	0.15
7 - next 10 mm	0.3
8 - next 10 mm	0.3
9 - next 10 mm	0.5 - 1.0
Section from 100 mm width of plate	
1 - top 28 mm of area C	1.3
2 - middle 28 mm of area C	1.3
3 - bottom 28 mm of area C	1.5

indicate whether the medium purple band was a tailing of the dark purple band (as it was always directly behind it with no intervening space) or a different compound altogether. An indication of how much activity was due to overlap with area C compounds might also be given.

A 100 mm-wide strip of area B was eluted in Solvent I and then applied to a cleared 50 mm preparative plate and developed in a different toluene:chloroform;acetone mixture (40:20:40), designated Solvent III. Bands were viewed under u.v. illumination, and eluted and assayed against S.aureus. The results are shown in Figure 15. They indicated that the band of medium absorbance is a different compound to that contained in the band of dark absorbance, which is only very slightly active. Some of the inhibition previously attributed to these two bands was concluded to be due to overlap with activity in area C. This showed up as a light coloured mixture of purple and green under u.v. illumination of 350 mm wavelength on the tertiary TLC plate, as did most of area C on the secondary plate. Both the absorbing bands (3a and 3d in Figure 15) must possess some activity of their own, other than that due to the possible overlap mentioned, as they both extended about 10 mm, which was too little to display activity when compared with strips of 10 mm extent from region C (Table 23).

5.6 Assessment of Recovery of Activity from Primary TLC

Recovery of activity from the second TLC plate was estimated in order to see if all antibacterial activity had been located.

A 50 mm wide strip from a primary plate was eluted and assayed and the diameter of the zone of inhibition

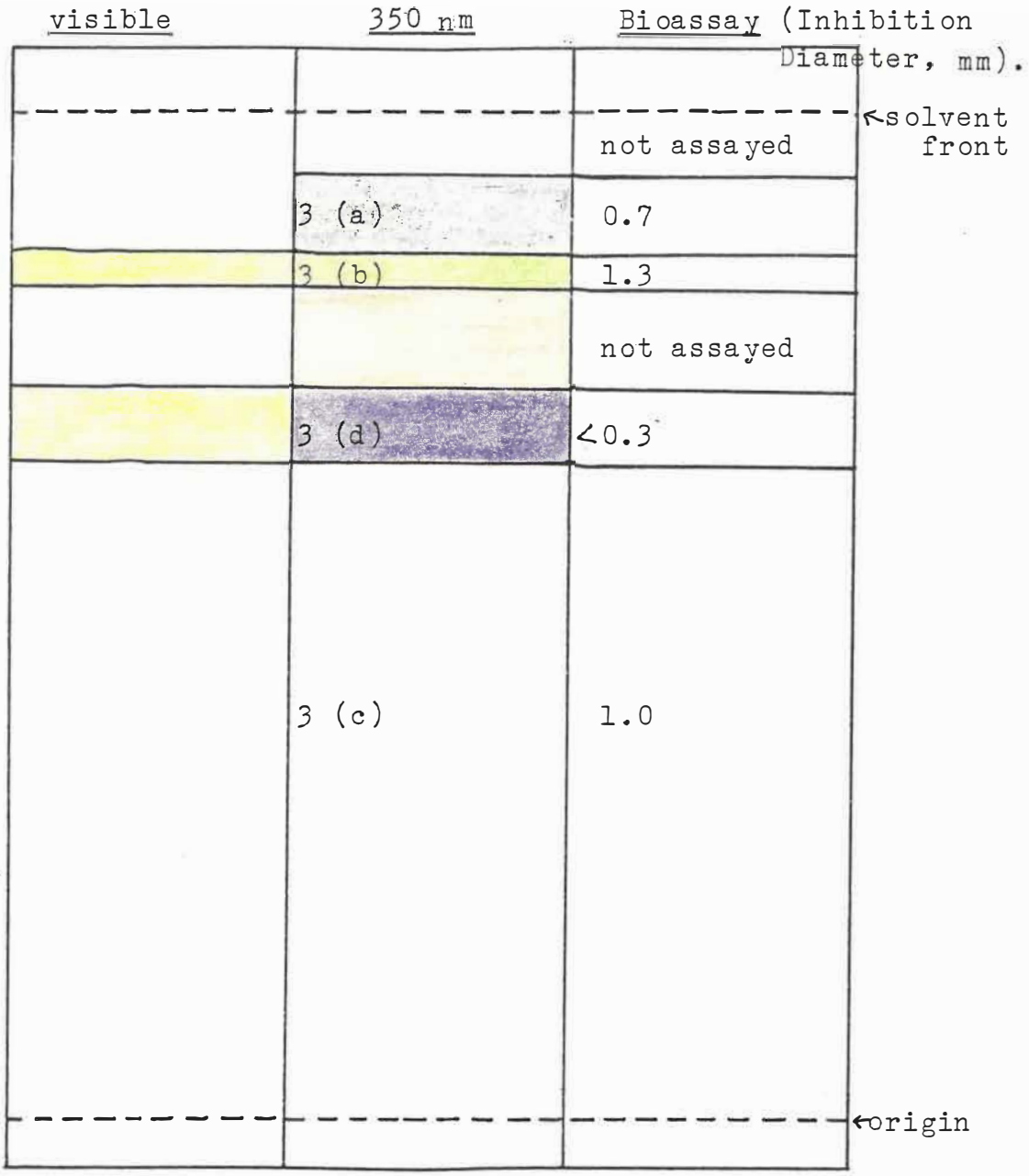


FIGURE 15: Chromatography with toluene:chloroform: acetone (40:20:40) of area B from secondary TLC, i.e. tertiary chromatography. Viewed under 350 mm wavelength u.v. illumination and assayed for antibacterial activity.

compared with that of the pooled areas A, B and C eluted from a 50 mm secondary plate. Both plates were developed with the same set of extracts (i.e. half of material eluted from primary plate was used to load secondary plate). The results showed that the pooled fractions were nearly 0.5 mm less active. This was concluded to be within experimental error after development on two chromatograms and two elutions.

5.7 Summary

Separation of honey extract fractions required three chromatographic developments before characterisation of the compounds was attempted. Primary development was with 60% ethanol-40% ether (Solvent I). All activity was localised just behind the solvent front. It was eluted from the silica in columns with the same solvent and developed on a second chromatogram with toluene:chloroform: acetone (40:25:35, Solvent II). The areas of activity were separated, one of which required further separation (toluene:chloroform:acetone, 40:20:40; Solvent III).

Reagent grade solvents were used as oxidation of the antibacterial activity occurred with bulk acetone.

CHAPTER SIX:

CHARACTERISATION OF ANTIBACTERIAL COMPOUNDS

Characterisation of the various antibacterial substances was attempted by obtaining structural information from ultra-violet and infra-red analysis, nuclear magnetic resonance and mass spectroscopy.

Alcohol extracts of 160 ml of honey were obtained and chromatographed in Solvent I. Secondary chromatography was carried out in Solvent II and the active bands, labelled 1, 2, 3, 5, 6, 7 and 8 in Figure 14, were removed from the TLC plates separately. Bands labelled 3 were removed together and further chromatographed in Solvent III and the bands, labelled as in Figure 15, were then removed. A sample (3 e) was also removed from the tertiary TLC plate which had appeared as small aligned spots of medium absorbance under u.v. illumination directly behind 3 d. This may have been tailing of 3 d due to the humid conditions during chromatography. Humid conditions can often give phenols a distorted/streaked migration pattern.

Each sample was removed from the silica by elution with Solvent I, which was subsequently removed from the samples by rotary evaporation. Then, to help obtain pure samples, 1 ml distilled water was added to each and shaken vigorously at intervals of 30 minutes over a three hour period. The

solutions of the fractions were then filtered with Whatman No. 1 filter paper and the filtrate made up to 3 ml with water.

6.1 Ultraviolet Spectroscopy

The ultraviolet absorption spectrum of samples was measured with a Pye Unicam SP8-500 spectrophotometer with quartz sample cells and distilled water as a reference. The scan range was 200 nm-500 nm. The results are shown in Figures 16 and 17 and a summary of the results is given in Table 24.

Carbonyl groups show absorbance in the range 275 nm-295 nm (Dyer, 1965). Assuming a 10 nm margin of error, samples 1, 3 a, 3 c, 3 d, 3 e and 8 showed absorbance within this range. Samples 5 and 7 required better resolution.

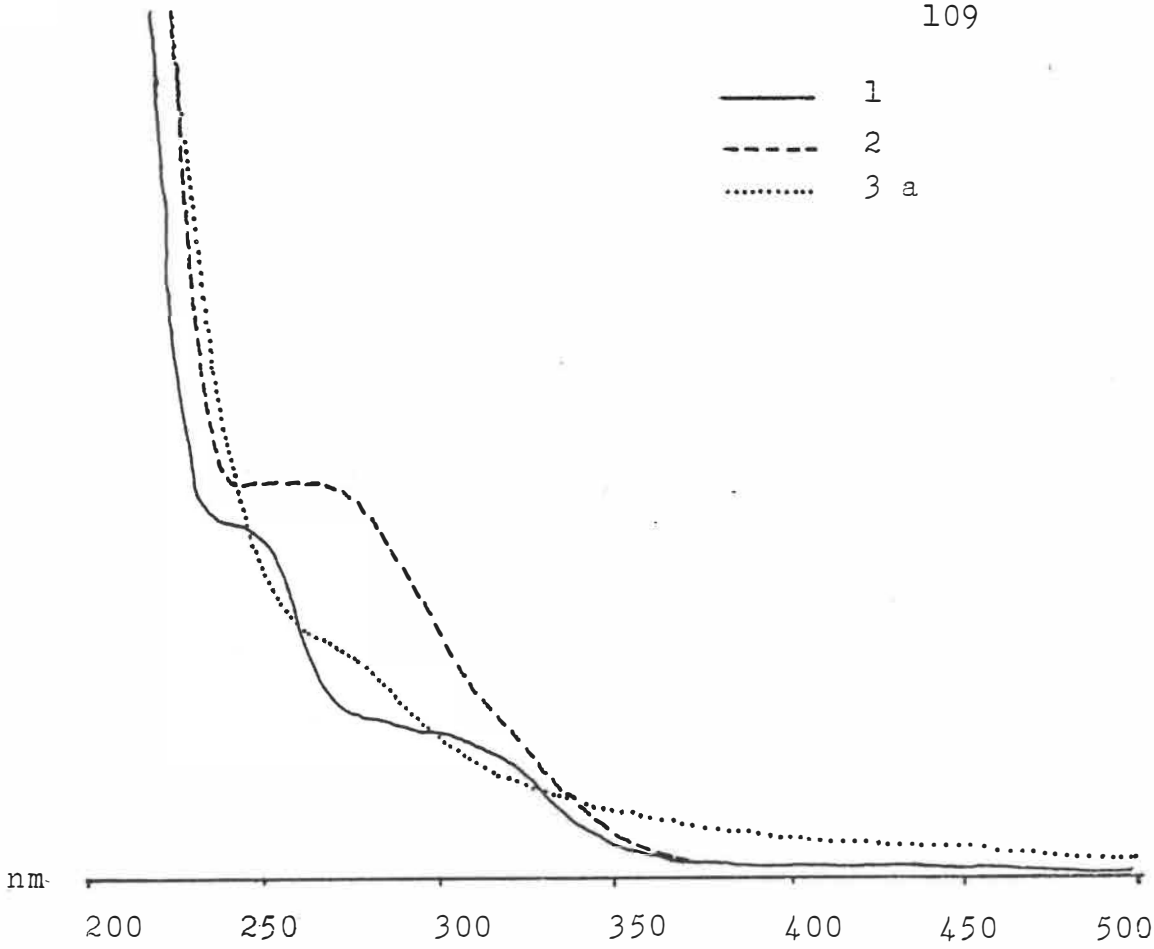
6.2 Infrared Spectroscopy

The samples used for obtaining the u.v. spectra were freeze-dried to remove water prior to infrared analysis.

Samples were ground with potassium bromide and formed into discs under 154 x 10^3 kP pressure under vacuum for 1 minute. Spectroscopy was carried out with a Shimadzu IR-27G spectrophotometer in the range 4000 nm-400 nm.

Most of the fractions produced infrared spectra with only broad absorption peaks which had little diagnostic value and indicated heterogeneity in the samples. Band 3 d, however, displayed relatively sharp absorption peaks at 1695, 1085 and 1100 cm^{-1}. This indicated the presence of a conjugated acid, carbonyl and -c-o- groups respectively. A broad absorption peak at approximately 3400 cm^{-1} also indicated the presence of one or more hydroxyl groups. It was therefore decided to carry out further analysis on band 3 d.

Figure 16 and 17: Ultra violet absorption spectra of antibacterial compounds from manuka honey.


1
2
3 a
nm
200
250
300
350
400
450
500

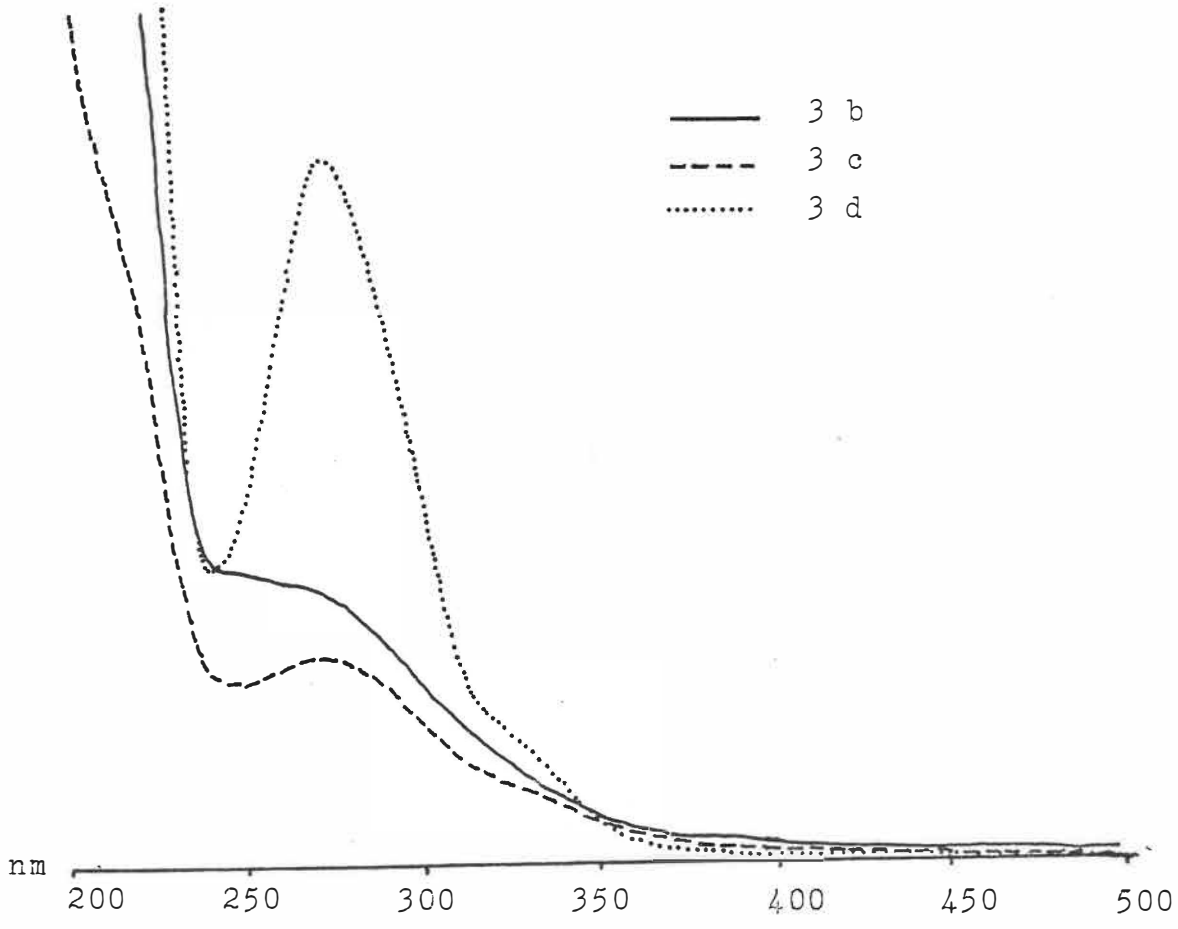
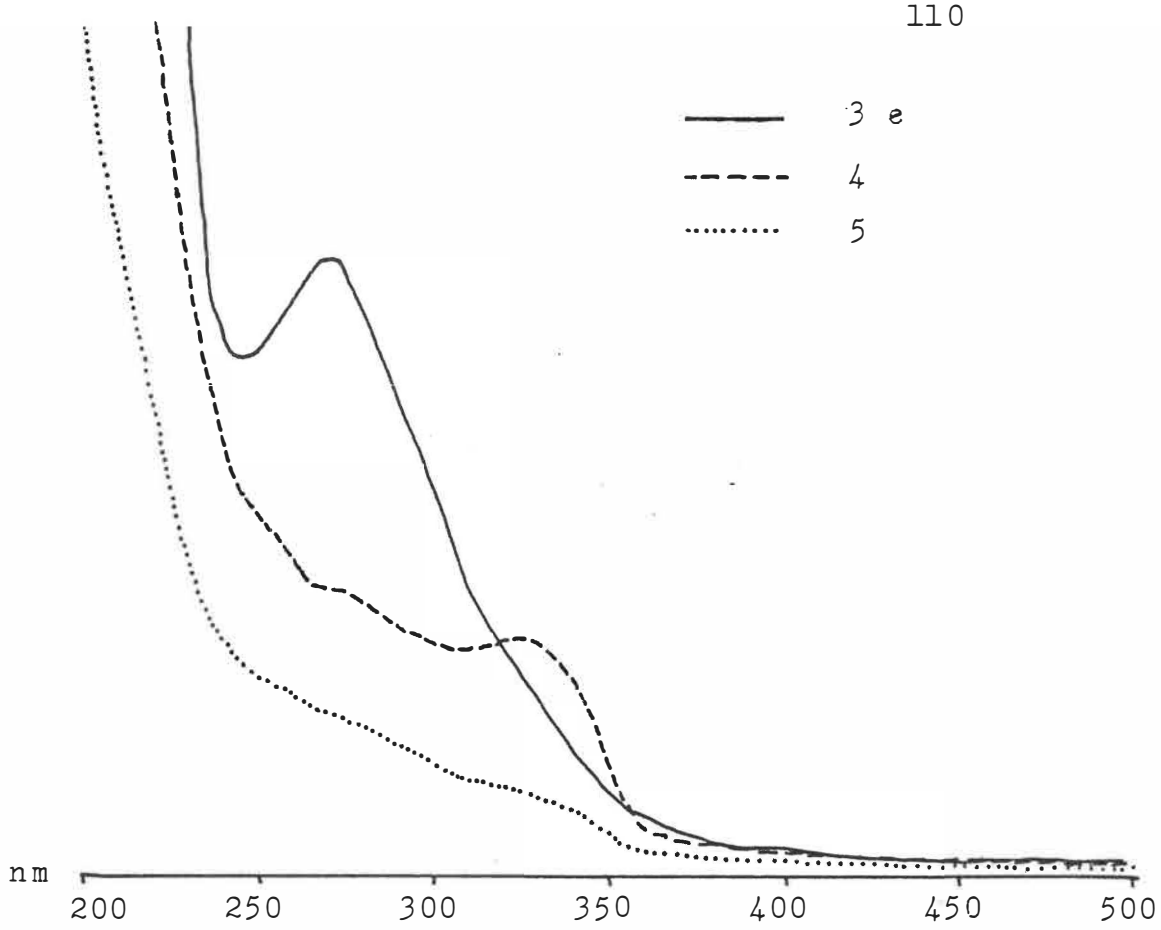

3 b
3 c
3 d
nm
200
250
300
350
400
450
500

Figure 16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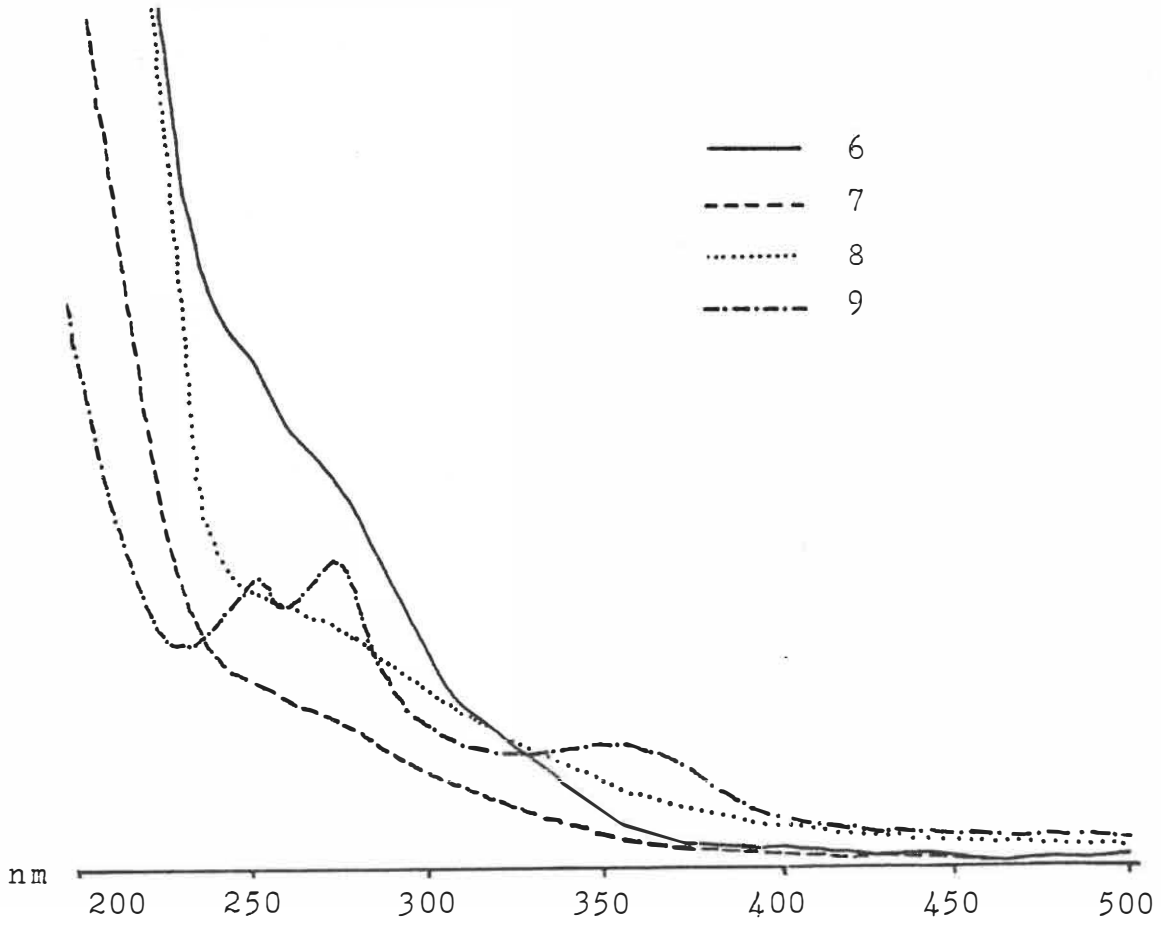

6
7
8
9
nm
200
250
300
350
400
450
500

Figure 17

TABLE 24: Peaks/plateaux of ultraviolet absorbance of antibacterial substances from manuka honey.

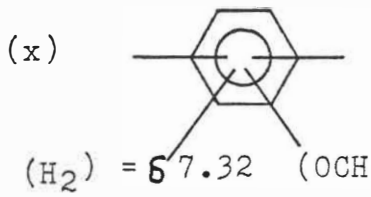
	Wavelength (nm)		
Sample no.	Peak(s)	Plateau(x)	Notes
1	-	243,289	
2	-	256	
3 a	-	268	
3 b	-	249	
3 c	271	-	
3 d	272.5	-	
3 e	270	-	
5	-	323	Possibly three plateaux on further dilution
6	-	239,258,311	
7	-	-	Possibly three plateaux on further dilution
8	-	265	

The infrared spectrum of sample 3 d is shown in Figure 18.

6.3 Nuclear Magnetic Resonance (n.m.r)

In order to obtain further information on the structure of 3 d, proton n.m.r analysis was carried out using a Jeol FX 60 Q instrument.

A sample of 3 d was dissolved in deuterated chloroform and its proton n.m.r spectrum measured. Absorption signals were present at δ 7.32, 3.94 and 3.89 ppm in the approximate ratios of 2:6:3. From the combination of I.R. and n.m.r data it was therefore concluded that 3 d was possibly a tri-substituted aromatic compound of the type:



δ 3.89 is consistent with the possibility of a $COOCH_3$ group here but does not exclude other possibilities.

The ratios of peak intensity of the above chemical shifts indicated one aromatic hydrogen environment and one methoxyl group environment, which further suggested that the molecule possessed a plane of symmetry.

The proton n.m.r spectrum of fraction 3 d is given in Figure 19.

Figure 18: IR spectrum of fraction 3 d

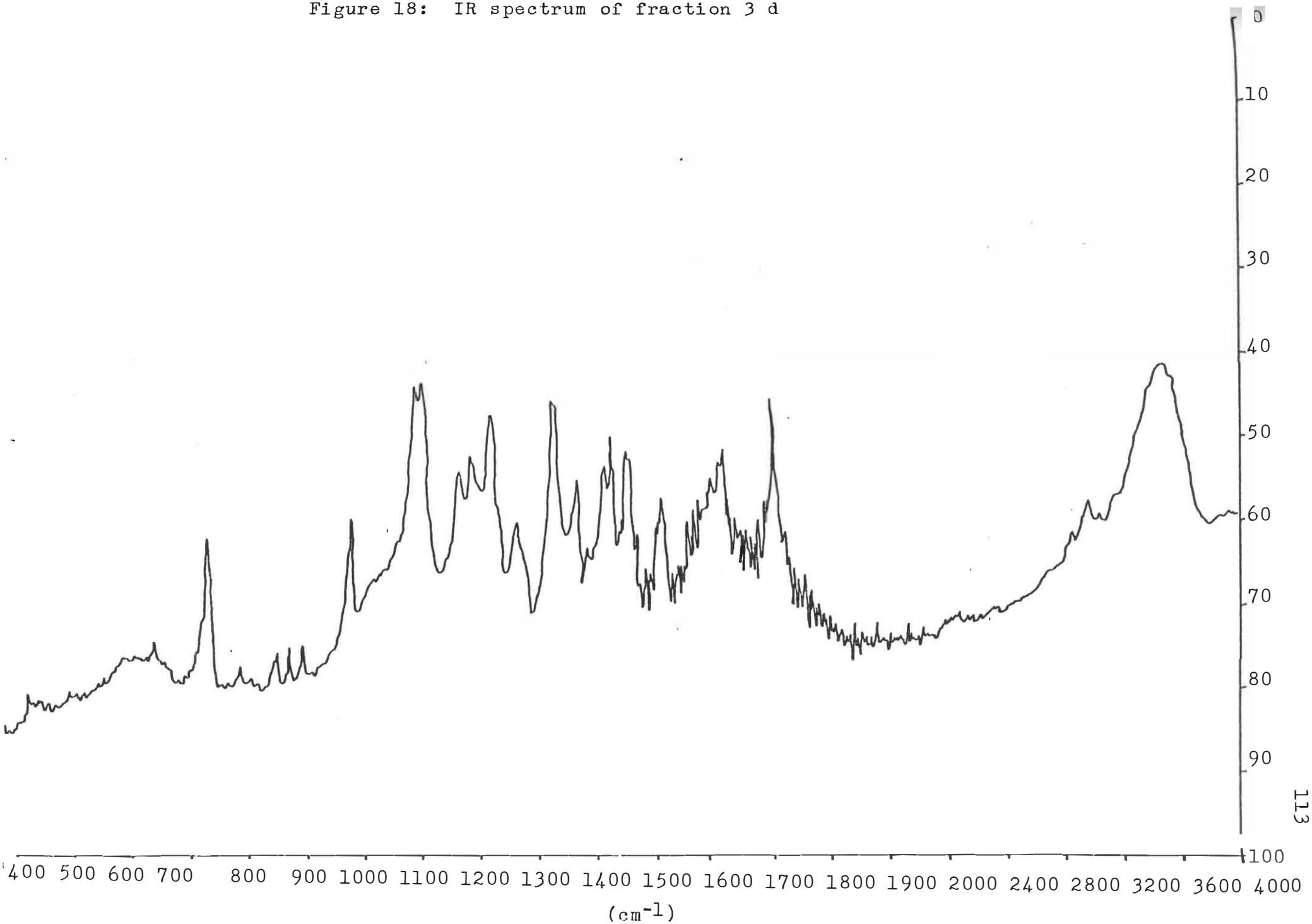
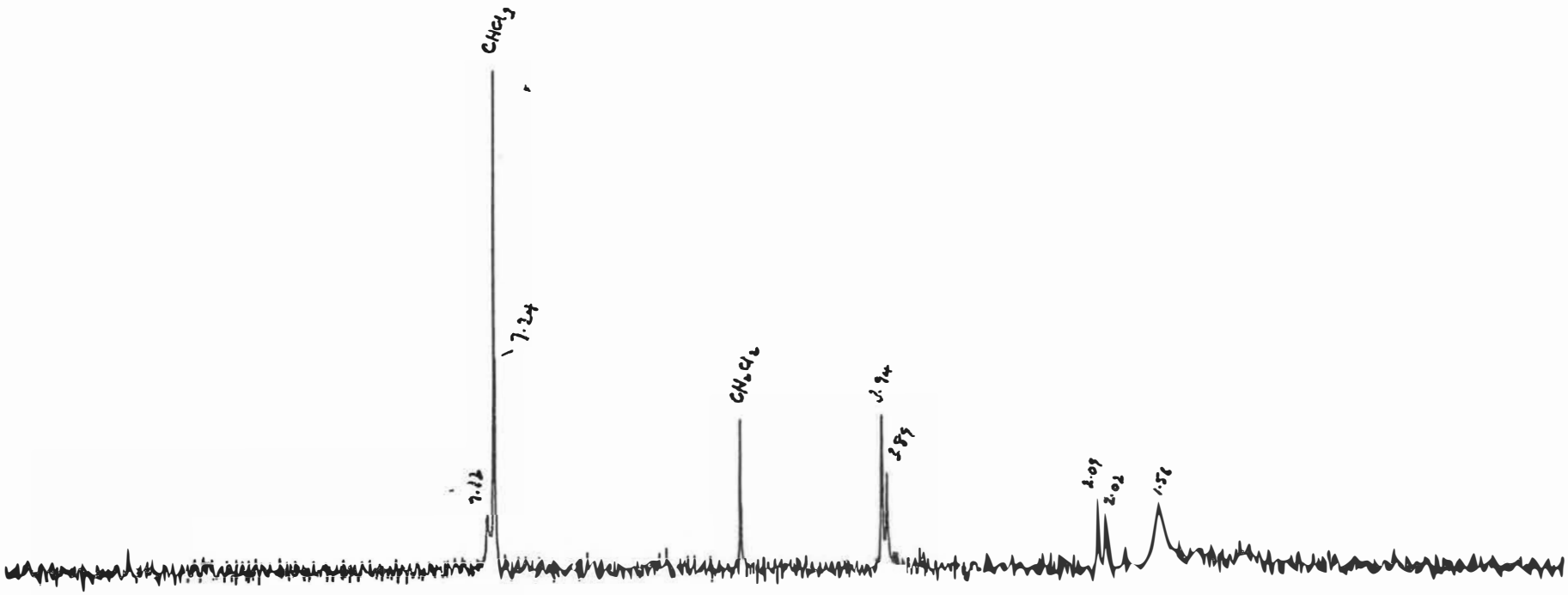


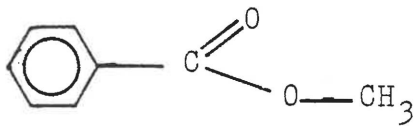
Figure 19: Proton n.m.r. spectrum of fraction 3 d



6.4 Mass Spectrometry of Fraction 3 d

Mass spectral analysis of 3 d was carried out on a Varian Mat CH 5 mass spectrometer. This revealed M^+ to have m/e (mass to charge ratio) 212 and that this peak had M + 1 and M + 2 isotope peaks corresponding to a formula of $C_{10}H_{12}O_5$. This data also showed peaks indicating loss of methyl and methoxy groups from the molecular ion.

It the compound was fully saturated a formula of $C_{10}H_{22}$ would be expected. The $C_{10}H_{12}O_5$ formulation found indicated a deficit of ten hydrogens and therefore the presence of five double bonds and/or ring(s). This is consistent with the aromatic - conjugated ester suggested by the I.R. and n.m.r analyses.



Hence the remaining substituent (x) of the aromatic compound, 3 d, was concluded to be an -OH group (Table 25). Proof of this was obtained by methylation and further mass spectrometry. Fraction 3 d was reacted with diazomethane at room temperature, in a sealed vessel (Schlenk and Gellerman, 1960, modified by P. Holland, personal communication). This gave a permethyl compound of m/e 226, indicating the replacement of a single -OH group by a $-OCH_3$ group.

$$-OH + CH_2N_2 \longrightarrow -OCH_3 + N_2$$

TABLE 25: Deduction of substituent X, of the aromatic compound 3 d, utilising mass spectral analysis.

Formula of 3 d: $C_{10}H_{12}O_5$

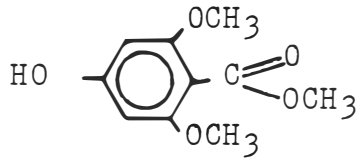
Chemical Group	Evidence	Molecular Weight
 (skeleton)	proton n.m.r signals, u.v, I.R and mass spectral data	72
$2(OCH_3)$	I.R. and proton n.m.r	62
(COO-)	I.R. and proton n.m.r	44
(CH_3)	proton n.m.r	15
		189

3 d m/e = 212

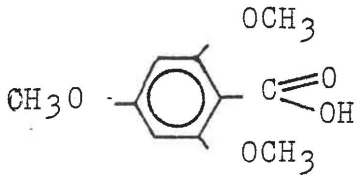
molecular weight of X is 13

and, molecular weight of -OH is 13

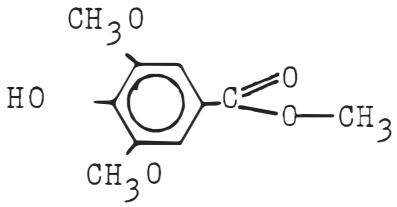
The foregoing combination of proton n.m.r and mass spectral data were consistent with four possible structural formulae. They were:



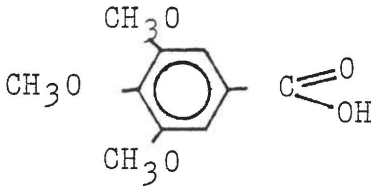
(I)



(II)



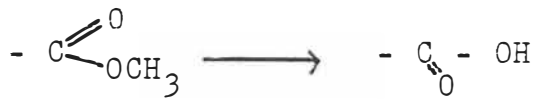
(III)



(IV)

(syringic acid methyl ester) (trimethoxy benzoic acid)

In order to reduce the number of possible formulae hydrolysis of 3 d was attempted by heating it at 130°C, overnight in methanol with 1 mol/l NaOH, in a sealed vessel. The solution was then acidified with H_2SO_4, extracted into diethyl ether and analysed. The mass spectrum indicated the formation of a hydrolysed product of m/e 198 (M^+). This was consistent with the conversion:

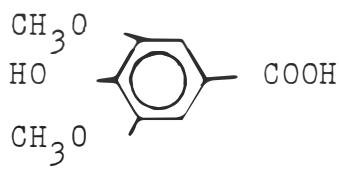


and hence structures (II) and (IV) could be eliminated.

Methyl groups in the other positions around the aromatic ring would not have been susceptible to hydrolysis owing to the effect of π-bonding orbitals around them.

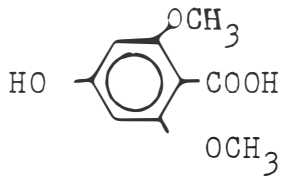
Prior to the hydrolysis at 130°C, this conversion was attempted by the same method, but at room temperature. No hydrolysis was observed. This is consistent with a conjugated "deactivated" ester, as suggested by the I.R. stretching frequency (1695 cm^{-1}), also due to the effect of π-bonding orbitals around the aromatic ring. By contrast unconjugated methyl esters typically absorb at V max 1720 cm^{-1}. (I) and (III) are conjugated "deactivated" ester structures (functional group - $COOCH_3$).

The possible products of hydrolysis were therefore:



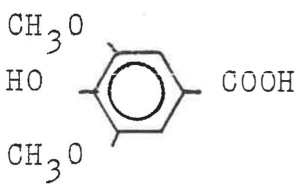
(3,5-dimethoxy-4-hydroxy benzoic acid)

or,



(2,6-dimethoxy-4-hydroxy benzoic acid)

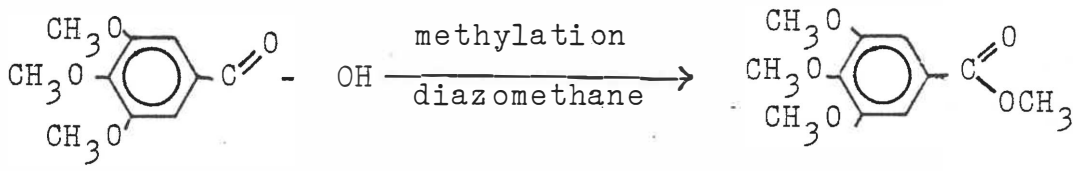
Compound (III) is a methyl ester of syringic acid, a well known plant metabolite (and constituent of lignins).



syringic acid

Therefore (III) was further investigated and subsequently established as the structure of 3 d.

A sample of (IV) (3,4,5-trimethoxy benzoic acid) was available in the laboratory. It was methylated with diazomethane,



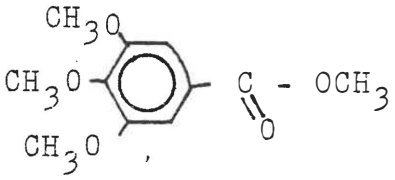
(IV) (V)

and analysed by mass spectrometry. The mass spectrum obtained was identical with that produced by the permethylation of 3 d. Since (IV) was not considered a possible structure for 3 d because of the evidence from hydrolysis and mass spectrometry and insolubility in chloroform, but produced the same methylation product, then compound (III) (methyl 4-hydroxy - 3,5-dimethoxy-benzoate or syringic acid methyl ester) was concluded to be the structure of 3 d (i.e. both (III) and (IV) had methyl groups substituted at positions 3 and 5 on the aromatic ring).

6.5 Mass Spectrometry of Other Active Fractions

Mass spectrometry was carried out on the other isolated active fractions from manuka honey in order to see if any additional evidence could be obtained on their structures.

Fraction 1 was found to have an m/e of 226 and was hence concluded to be methyl 3,4,5-trimethoxy-benzoate (o-methyl syringic acid methyl ester), which had the same structure as (III) or (IV) after methylation i.e:

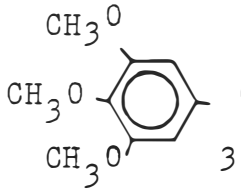


Fractions 2 and 3 a contained mostly a compound of m/e 212 which was concluded to be the same as 3 d (methyl 4-hydroxy - 3,5-dimethoxybenzoate).

Fraction 3 b contained mainly a compound of m/e 226, concluded to be methyl 3,4,5-trimethoxybenzoate.

Fractions 3 c and 3 e were also shown to contain mainly methyl 4-hydroxy - 3,5-dimethoxy benzoate.

Fractions 6, 7 and 8 (from the application line) also showed mainly m/e 212. Owing to their low R_f values they were thought to be the free benzoic acid of methyl 3,4,5-trimethoxybenzoate (i.e. 3,4,5-trimethoxybenzoic acid), rather than methyl 4-hydroxy - 3,5-dimethoxybenzoate. Elution is a displacement process and firmness of phenol binding depends on the number and position of hydroxyl groups. The possible structure of 6,7 and 8 would therefore be:



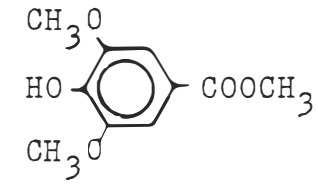
3,4,5-trimethoxybenzoic acid

Fraction 5 was very impure and gave peaks of molecular weight 212, 213, 206, 207, 181 and 182. Signals 206 and possibly 207 would have been due to the presence of fraction 4 (Figure 14) which was also analysed by mass spectrometry, although not itself antibacterial. Fraction 4 was concluded to be an involatile compound giving no obvious molecular ion and a strong peak at m/e

206. Fraction 5 was concluded to be mainly either 3,4,5-trimethoxybenzoic acid or methyl 4-hydroxy - 3,5-dimethoxybenzoate.

6.6 Conclusions

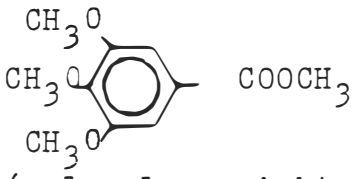
The major antibacterial components of manuka honey, separated by thin-layer chromatography, were concluded to be:



(molecular weight 212)

methyl 4-hydroxy - 3,5-dimethoxybenzoate (syringic acid methyl ester)

and,

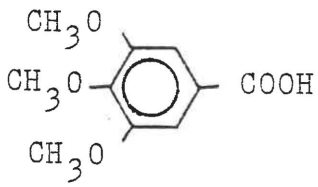


(molecular weight 226)

methyl 3,4,5-trimethoxy-benzoate (O-methyl syringic acid methyl ester)

Fractions 1 and 3 b were mainly methyl 3,4,5-trimethoxy-benzoate.

Either methyl 4-hydroxy - 3,5-dimethoxybenzoate was dissolving slowly off the application line, extending up the TLC plate to include fractions 2, 3 a, 3 d, 3 c and 3 e and the lower fractions 5, 6, 7 and 8, or it extended across the upper fractions only and the fractions with lower R_f (5, 6, 7 and 8) were 3,4,5-trimethoxybenzoic acid which would bind more firmly to the adsorbant.

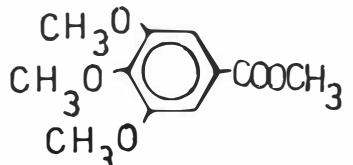
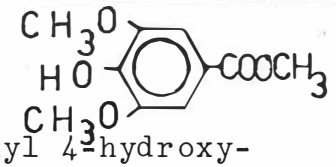
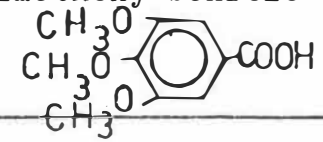


3,4,5-trimethoxybenzoic acid

The impression from the band patterns (seen under ultraviolet illumination) that there were possibly eleven different fractions may have been due to the presence of the impurities. There were actually two or three only.

Table 26 shows the probable structural groupings and evidence for these.

TABLE 2b: Probable structural groups of antibacterial fractions from manuka honey and evidence for these groupings.

	Sample No.	U.V. absorbance spectrum	I.R. absorbance spectrum	M.S. data	Proposed structure and name
A	1	Plateau at 289 nm	High peak at 1100 cm^{-1}, relatively low peak at 1400 cm^{-1}, few peaks below 800 cm^{-1}.	Molecular weight 226, fairly pure	 methyl 3,4,5-trimethoxy-benzoate (O-methyl syringic acid methyl ester)
	3 b	Plateau at 250 nm	"Noisier" peak pattern than sample 1 - probably due to presence of some material of 212 m.wt.	Molecular weight 226, with some 212	
B	3 a 3 d 3 c 3 e	Peak or plateau within range 268-273 nm	Peaks at 1100 cm^{-1} and 1400 cm^{-1} of relatively equal height. Few peaks below 800 cm^{-1}.	Molecular weight 212, fairly pure	 methyl 4-hydroxy-3,5-dimethoxybenzoate (syringic acid methyl ester)
	2	Plateau at 256 nm	Peak at 1100 cm^{-1} high relative to peak at 1400 cm^{-1} probably due to presence of material of m.wt 226.	Molecular weight 212, with some 226	
C	6	Three plateaux	Peaks at 1100 cm^{-1} and 1400 cm^{-1} of relatively equal height.	Molecular weight 212, fairly pure	Mixture of group B and D
D	5 7 8	Three plateaux	High peak at 1100 cm^{-1}, relatively low peak at 1400 cm^{-1}. "Noisy peak pattern below 800 cm^{-1}.	Molecular weight 212, fairly pure	3,4,5-trimethoxy-benzoic acid 

CHAPTER SEVEN:

DISCUSSION

Antibacterial properties have been found in a diverse range of natural materials, many of which have been used in medicine for centuries. Synthetic substances or the purified active components of natural material are preferred because they are more concentrated and it is easier to measure an accurate dose. However, it must first be found which compound(s) in a natural antibacterial material is responsible for the antibacterial activity and find its structure and mode of action before synthetic imitations or related compounds can be manufactured. Many of the antibiotics used today are relatively short-lived due to their misuse and over-use and there has been much interest shown in recent years in returning to the use of substances derived from natural sources.

Hive products, such as propolis and honey, have been utilised by bees for 10-20 million years and are still effective for their needs. The pharmacologically active compounds present in these substances can only have altered in as much as the bee itself or plant from which the raw materials were obtained has evolved. Much conclusive research has been carried out on the active components of propolis and bee venom but there are many contradictions in the literature resulting from previous honey research. In this work an attempt was made to isolate and characterise the compounds which gave manuka honey its apparent non-peroxide antibacterial activity.

All non-peroxide antibacterial activity in manuka

honey could be extracted into absolute alcohol. A 100% v/v honey solution i.e. undiluted honey, or alcohol extract of equivalent concentration produced a zone of inhibition of 4 mm diameter in agar diffusion tests with S.aureus. In comparison though, 50% of the total activity eluted from 5 ml of undiluted honey run on a primary thin-layer chromatogram (i.e. eluent was dried, redissolved in 0.2 ml distilled water, 0.1 ml of which was assayed for activity) produced a 6 mm diameter zone of clearing. Assuming an average plate agar depth of 2 mm, the clearing given by the honey would have been produced by only 0.1 ml of undiluted honey, whereas the agar well measuring inhibition eluted from the chromatogram should have contained the equivalent of 2.5 ml of undiluted honey antibacterial activity. Hence more than half of the antibacterial activity was being lost during attempted isolation of these compounds. However the extent of bacterial inhibition is not linearly related to the amount of antibacterial activity present in agar diffusion tests and it was therefore not possible to conclude from this that 0.1 ml of undiluted honey run on a primary TLC plate and eluted would only have produced a 0.24 mm diameter zone of clearing (equivalent to 6% recovery of activity during the attempted isolation of activity). Also some activity may have been trapped in the water-insoluble compounds still present at this stage (i.e. elution of a primary TLC plate) and some may have adhered to the equipment used. Assay of the total material eluted from the rest of the silica of a primary plate developed with extract of 5 ml of honey (and excluding the active area already assayed) produced a clearing of diameter

0.7 mm. Therefore tailing of the activity over the rest of the plate could not account for the remainder of the activity loss. As only the major antibacterial components were being investigated in this thesis, this slight amount of activity which was spread over the rest of the primary TLC plate was not investigated further.

It was concluded that most of the activity not accounted for was therefore being lost during application of the alcohol extract to the first plate, during chromatography or during subsequent assay. This activity may have been chemically different to the compounds characterised (Chapter 6) and possibly more volatile. Although zones of inhibition are not additive it appeared that all the activity recovered from primary chromatography was also being recovered from secondary chromatography. A proportion of the activity may therefore have been oxidised during primary chromatography, or have evaporated or been altered by drying with the hairdryer i.e. the loss did not occur during elution or assay of the material. This would have to be investigated in future work on this topic. Lavie (1963) found an antibacterial fraction from honey which was extractable into cold ether and volatile at 95°C. The temperatures reached by the hairdryer were never measured but probably would have exceeded 100°C to be able to dry the extract applied (which still contained a percentage of sugar).

A further problem was that the proportion of the activity recovered after primary chromatography was found to be sensitive to certain handling techniques. The antibacterial activity of the manuka honey extracts was lost by

oxidation when exposed to oxygen in an impure solvent mixture, probably containing iron. Species with no antibacterial activity were also produced when the compounds adsorbed to silica after chromatography were ground up in ethanol with a pestle and mortar.

Isolation of the non-peroxide antibacterial components was also difficult because originally little was known about the class of compounds being dealt with. The finding that the presence of the activity in honey depended on the plant source suggested it was not a protein. Antibacterial substances isolated from animals are usually peptides or proteins whereas plant antibacterial and antifungal substances are usually phenolic compounds, terpenes or flavones (Booker et al., 1961; Walker, 1975).

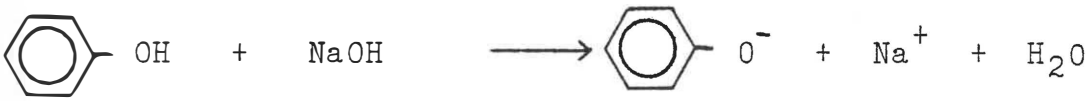
The probability that the antibacterial compounds were of non-protein nature was supported by their heat stability. Heating raw manuka honey to 95°C for up to 60 minutes did not reduce its non-peroxide antibacterial activity.

Solvent solubility and the development patterns also suggested that the compounds were not peptides. An idea of the polarity of the active substances was given by their apparent solubility in ethanol and ether mixtures after alcohol extraction of manuka honey. They were concluded to be fairly soluble because they were extractable into alcohol but not ether and remained soluble in a mixture of 20% ethanol - 80% ether. This was supported by their migration behaviour during thin-layer chromatography.

The subsequent identification by mass spectrometry of the isolated antibacterial compounds as methyl 3,4,5-trimethoxybenzoate (o-methyl syringic acid methyl ester), methyl 4-hydroxy-3,5-dimethoxybenzoate (syringic

acid methyl ester) and trimethoxy benzoic acid complied with the observations on total non-peroxide activity obtained earlier.

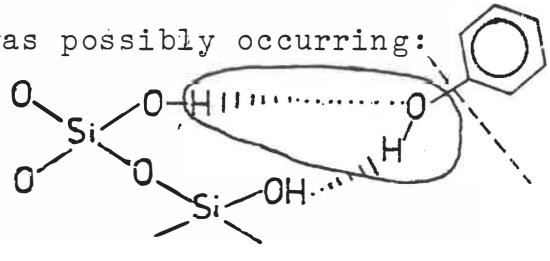
A phenol substituted with one or more hydroxy groups could explain loss of activity when alkali was added to the honey or its extract, though phenols are usually re-hydrolysable. This reaction may have been occurring:



Reacidifying should have restored the activity, though it did not appear to. Methyl 4-hydroxy-3,5-dimethoxy benzoate would have to be reacidified to approximately pH 3 to return its activity (P. Holland, personal communication). During attempted extraction with ether the solution was acidified only slightly and therefore not enough acid would have been added to rehydrolyse the molecule. Alkali could also cause loss of activity from 3,4,5-trimethoxybenzoic acid by formation of water by the hydroxyl group of the alkali with the acid group of the phenol.

It is not known if alkali would react with methyl 3,4,5-trimethoxybenzoate as this was not investigated. It is possible that this compound retained its activity but was not detected by bioassay (after addition of alkali to the extract) as it was only a minor proportion of the antibacterially active compounds present.

It is not known how loss of activity was occurring when the compounds, still bound to silica, were ground in ethanol with a pestle and mortar. This reaction, with loss of water was possibly occurring:

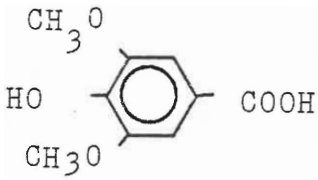


Evidence of the phenolic nature of the antibacterial compounds was also obtained when spray reagents were applied to developed thin-layer chromatograms of honey extract. The presence of phenols was shown by a stain of ferric chloride - potassium ferricyanide. A ferric chloride stain indicated that they probably did not contain two or more vicinal hydroxy groups or ortho (or peri) hydroxy-carbonyl groups.

Phenols tail badly during thin-layer chromatography (A. Wilkins, personal communication) and this is also consistent with the migration behaviour of the antibacterial compounds during isolation.

Confirmation of the identification of the antibacterial activity as the compounds mentioned above is still required however. This should be obtained by testing these compounds (authentic standards) for antibacterial activity by the assay method used throughout this work. It is possible that the compounds isolated and identified by mass spectrometry were present with another substance, or several others, which were not detected because they were in too small a quantity or because they were non-volatile, but which were responsible for the antibacterial activity.

Another aspect of this study to be investigated further is the true origin and synthesis of the compounds which were isolated and characterised. A possible precursor of them is syringic acid which occurs in vast amounts in lignin - in combined forms and may be liberated by alkaline hydrolysis.



Booker, Combie and Cooper (1981), however, did not find these compounds or syringic acid in manuka. They isolated from the bark of manuka the diketone, leptospermone; triterpene acids; ursolic acid acetate; mannitol; the dicoumarin, ellagic acid and its o-methyl ethers; and various terpenes. They purified and characterised the compounds which were present in a reasonable quantity in the bark and it is possible that the compounds found in this study may have been present only in the nectar of the manuka plant, or they occurred only in minute quantities which somehow became concentrated in honey. It is also possible that the compounds may exist only in the pollen (and leach out into the honey). It would be of interest to analyse the composition of manuka nectar and pollen. The compounds do not appear to have been isolated from other native plants either. The pollen, nectar and sap of these could also be investigated for antibacterial compounds. Similar structures might be expected if the origin of the compounds in this study is syringic acid or lignin.

The apparent nectar origin of the non-peroxide antibacterial factors in manuka honey implies that the antibacterial properties of a honey will depend on its floral source. This could account for some of the discrepancies in the findings of previous research concerning honey. Classification of honey floral type in New Zealand is an approximate procedure only and may be carried out through taste, aroma, pollen analysis, or a combination of these. The honey is labelled according to the apparent major nectar source. For export purposes, however, the law requires at least 51% of a specific floral type to be

present to be labelled as that floral origin. These laws vary in other countries, and the antibacterial activity will depend on its total floral composition and not just its major floral source. This was clearly demonstrated when a honey labelled "various" was assayed for activity (Chapter 3). This honey contained non-peroxide antibacterial activity although its major source (43%) was white clover according to a pollen analysis and it would have been marketed in New Zealand as clover honey. However other "clover" honeys assayed for activity gave negative results. A honey containing only a small percentage of an antibacterially active floral source may still show strong activity depending on the content and activity of the compound(s) characteristic to that nectar source.

The discrepancies between various studies on the antibacterial properties of honey can also be attributed to some extent to the assay method used. James et al. (1972) investigated the antibacterial properties of Jamaican honey using streak plates (test cultures streaked on agar plates containing 2, 10 or 20% honey dilutions), ditch plates (test organism streaked on agar plates containing ditches of 10% honey in agar), sensitivity disc method (2 or 10% honey-saturated filter discs applied to agar plates previously flooded with test culture and dried), and an agar well method (the same procedure as used in this study, with 5, 10 and 20% dilutions of honey). The disc method and well method proved to be very insensitive. None of the honey samples produced any inhibition in the disc method or the well method. However it was noted that the higher concentrations of honey often caused a mucoid

appearance of the growth immediately around the well. Inhibitory action was best seen when honey was incorporated in agar plates. (The method of Dold and Witzenhousen, 1955). Therefore the method used in the present study was relatively insensitive compared to that used in some previous research.

Much of the past research into the antibacterial properties of honey has focused on inhibine (hydrogen peroxide) and has not quantitatively taken into account the presence of other antibacterial compounds. The inhibine number of a particular honey was measured using a solution of whole raw honey, not an extract. Therefore total inhibine number was measured which would have included inhibition due to other factors present as well as H_2O_2. For instance White and Subers (1963) measured inhibine by the procedure of Dold andWitzenhousen and measured H_2O_2 by a colourimetric method using o-dianisidine. They formed a table relating inhibine number to peroxide accumulation from these results but of the 45 samples tested more than one quarter of them (14) gave anomalous results. Seven produced more peroxide than accounted for by their inhibine number and seven produced less. Their use of o-dianisidine to measure H_2O_2 may also have produced overestimations of H_2O_2 (Chapter 3) and therefore the presence of other anti-bacterial factors would have been "masked".

In 1964 White and Subers measured the effect of heat on honey inhibine and found that on heating at 70°C for ten minutes nineteen samples lost over 80% of their activity against S.aureus, five lost 60-80%, two lost 40-60% and two were more heat resistant. During the present study it was

found that heating an alcohol extract of manuka honey at 95°C for up to 1 hour did not reduce its antibacterial activity at all. This assay measured non-peroxide activity only whereas that of White and Subers would have measured all antibacterial activity present. Adcock (1962) found that heating a 50% honey solution at 90°C for 15 minutes completely destroyed its peroxide value. Only one experiment was carried out however and the inhibine value (i.e. total antibacterial effect) was not measured, and the peroxide system may have been unstable at even lower temperatures. Hence the antibacterial activity after heating, as measured by White and Subers, may have been due entirely to non-peroxide factors.

Gonnet and Lavie (1960), also using alcohol extracts of various honeys (and therefore assaying non-peroxide antibacterial factors), found that heat only slightly decreased activity, depending on the type of honey. All retained some activity after autoclaving at 120°C for 15 minutes. They also found that heating (80°C, 30 minutes) and neutralising honey caused a complete loss of activity but re-acidfying restored it. The present study with manuka honey gave similar results. It is to be expected that the non-peroxide antibacterial factors of other honeys will have different properties depending on their origin.

The composition of a honey and its pollen content will vary according to the flowers in the vicinity of the hive. The composition of bee venom, royal jelly and propolis is less variable however and it is therefore unlikely that the antibacterial compounds present in manuka and certain other honeys will have come from any of these. Bee venom

has only slight antibacterial activity (Lavie, 1960), although melittin (the largest single component, by weight) has a stronger effect and penicillin-resistant S.aureus strain 80 is sensitive to it, (Fennell et al, 1968). It also contains phospholipase A (Orlov, 1979) which can degrade cell membranes. Both these compounds are proteins and not related to the structures isolated in this study and it would be unlikely for any substance in bee venom to enter honey. The acid fraction of royal jelly is weakly antibacterial, is soluble in water, alcohol and ether, but is very unstable (Lavie, 1960). It is also unlikely that royal jelly would come into contact with the honey in the hive. Propolis is strongly antibacterial and is soluble in water and alcohol (Lavie, 1960). During its collection some of the constituents may come into contact with nectar or pollen, also being collected. Propolis is mainly collected from the boughs, leaves and buds of poplar, birch, elm, alder, beech and horse chestnut trees (Ghisalberti, 1979). Therefore, if this was the origin of honey's antibacterial compounds it would be expected that most honey samples assayed, irrespective of floral origin, would possess some antibacterial activity. Also the compounds which have been shown to contribute toward propolis' antibacterial action are galangin, pinocembrin (both flavones), caffeic acid and ferulic acid (both cinnamic acid derivatives) (Ghisalberti, 1979); which were not detected in this study.

Antibacterial activity, soluble in water and alcohol, has also been found in some types of pollen, increasing with time and temperature during storage (Lavie, 1960). Hence the possibility of the antibacterial compounds in

manuka honey coming from its pollen content requires further investigation. The bees wax in the hive also contains thermostable, acetone- and water-soluble antibacterial activity (Lavie, 1960) and is in constant contact with the honey. Compounds may therefore leach from the wax into the honey and be present after extraction. However Lavie (1960) reports that it has no activity against Salmonella (manuka honey does) and its composition is unlikely to vary greatly, irrespective of honey type (Harman, 1983).

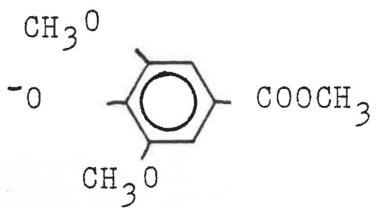
It is not yet known if the isolated compounds are synthesised in the manuka plant and are present in nectar (or pollen) in the same form as they are in honey, or whether precursors of them exist in nectar (or pollen) which are converted to these antibacterial compounds by reaction in the bees saliva (i.e. converted to more or less complex compounds during the ripening of nectar into honey). The acidic environment of the honey (also effected by bee salivary enzymes) may also be involved in the conversion. This is thought to occur with some of the compounds present in propolis (Cannon et al. 1973, cited by Ghisalberti, 1979). A sample of Western Australian propolis was found to contain pterostilbene which had not been reported in Eucalyptus. It was thought possible that bees transform naturally-occurring stilbenes by methylation or demethylation to pterostilbene. Benzyl alcohol was also in the propolis and may be a degradation product of the pterostilbene.

Several glands are connected to the oral cavity in the head and thorax of the worker honeybee. The pharyngeal (which contain antibiotic activity of their own; Lavie, 1960) or hypopharyngeal glands, the post cerebral, thoracic and labial glands (salivary glands) and possibly also the

mandibular glands are the important ones for the processing and ripening of honey. The hypopharyngeal glands secrete bee milk (part of the food for brood rearing), and also produce a secretion rich in diastase, invertase and glucose oxidase, used in elaborating honey. The salivary glands are believed by many to provide the liquid needed to dissolve solid food but produce no enzymes to break down carbohydrate. One person however (Inglesent, 1940; cited by Crane, 1975) has claimed that they produce diastase and invertase. The mandibular glands mostly contribute to the "brood-food" but their secretions have been discovered in the honey sac contents so it is possible that they may contribute toward the ripening of honey. The composition of these gland secretions could be more closely investigated and the individual components, particularly the enzymes, could be tested for their possible effect on the antibacterial compounds of this study and their potential precursors.

Inactive precursors of manuka compounds may enter unripened honey as more simple unsubstituted molecules (such as syringic acid which may exist free in the plant as a lignin precursor) or as more complex molecules which become degraded as the result of enzyme action (e.g. enzymes produced by white rot fungi growing on sawdust will produce various compounds by oxidation and demethoxylation, including syringic acid and syringaldehyde from lignin (Walker, 1975; Schubert, edited by Miller, 1973)). The decreasing pH of ripening honey may be an important influence. For instance, it seems likely that the content of syringic acid methyl ester (methyl 4-hydroxy - 3,5-dimethoxybenzoate will increase as the manuka honey becomes more acid and therefore the antibacterial potential of

manuka honey should increase. (Activity is restored to this compound after alkali-inactivation by decreasing the pH to approximately pH 3). It is possible therefore that this compound will not exist at all in unripened honey. O-methyl syringic acid methyl ester (methyl 3,4,5-trimethoxy benzoate) may be produced in localised areas of unripened honey where methylation is possible by substitution of the syringic acid methyl ester molecule after alkaline hydrolysis. This compound may originally exist in this form as phloem sap is usually slightly alkaline:



Discovery of the origin and synthesis of these antibacterial compounds would be aided by complete analysis of manuka plant nectar, contents of the bees honey sac and unripened honey samples.

The mode of action of the isolated compounds from manuka honey should also be investigated. Little work has been done on the actual mode of action of antibacterial factors in honey against bacteria. Mohrig and Messner (1968) found honey had a bacteriolytic component which acted on most gram positive organisms and had a generally bacteriostatic effect. They claimed that all the characteristic features of inhibines in honey described in the literature could be explained by the lysozyme. James et al., (1972), found the samples of honey they tested had a bacteriostatic effect only. It caused increased pleomorphism and variability in gram reaction and indistinct cell margins in both gram negative and gram positive organisms.

The mode of action of the non-peroxide antibacterial factors in this study may be related to their phenolic nature. Phenolic hydroxyl groups permit stable cross-links with proteins and this may be the way in which syringic acid methyl ester and possibly 3,4,5-trimethoxybenzoic acid inhibit S.aureus. They could bind to the cell membrane proteins, inhibiting their biochemical functions, such as ATP production and potassium uptake; or simply cause disruption and therefore leakage by binding to anionic sites on the cell wall, as do cationic detergents. They may also be transported across the cell membrane owing to their small size, and interrupt cellular functions.

It will be necessary to investigate whether the anti-bacterial compounds are bacteriostatic or bactericidal. This can be carried out by growing cultures of S.aureus in broth tubes containing different concentrations of honey or an isolated factor. After an incubation period loopfuls of the test organisms could be spread on nutrient agar plates or transferred to fresh nutrient broth to check for viability. Lower concentrations may be bacteriostatic and higher concentrations may be bactericidal.

Further work will also be required to then find the mode of action of the compounds. Information on this could be obtained by turbidity tests to measure the release of cell constituents (indicating disruption of the cell membrane) or by electron microscopy. Disruption of the cell membrane could also be investigated by measuring release of radioactively-labelled cell components. Radio-active labelling of the antibacterial substances could be used to find if they bind to or enter the bacterial cell.

Specific assays can be used to determine which biochemical functions of the cell are inhibited by the antibacterial factors.

The isolated antibacterial compounds should be re-tested against a range of bacteria in different concentrations. Their mode of action against each, and whether they are bacteriostatic or bactericidal also requires investigation.

The mode of action of the compounds and their spectrum of action will indicate if they may have further use as antibiotics. For instance if they have a wide spectrum of action, especially against such species as penicillin-resistant S.aureus strain 80, and their mode of action is such that they will not have adverse effects on the eucaryotic cell (as phenols usually do) then they may have potential.

Other compounds separated from manuka honey and not thought to be antibacterial could be investigated for inhibitory activity when in higher concentrations.

Further work could also be done on investigating other compounds from manuka honey which may be antibacterially active. The active compound(s) apparently volatile at the temperatures reached by the hairdryer used in this study have yet to be isolated - and may constitute the major portion of the non-peroxide manuka activity. Other active compounds may be present too which were not detected by mass spectroscopy because they were involatile or were present in minute quantities (though very active) and therefore may have only appeared as contiminants in the samples. Seasonal variations in the manuka honey composition could also be

investigated and the method of high pressure liquid chromatography (HPLC) may be more useful for this.

Another interesting area of research which could be investigated is the isolation of antibacterial compounds from other plant nectar sources, or the honey, if this is where the compounds are in active or more concentrated form. The honey or nectar of medicinal herbs may prove a particularly interesting and useful area of research.

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