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THE UNIVERSITY OF ALBERTA

OLIGOSACCHARIDES IN HONEY; THEIR ORIGIN AND ANALYSIS

by

Nicholas Hansen Low

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Doctor of Philosophy

IN

Food Chemistry

Department of Food Science

EDMONTON, ALBERTA

Fall 1986

ISBN 0-315-32482-1

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR

Nicholas Hansen Low

TITLE OF THESIS

OLIGOSACCHARIDES IN HONEY, THEIR ORIGIN AND ANALYSIS

DEGREE FOR WHICH THESIS WAS PRESENTED Doctor of Philosophy

YEAR THIS DEGREE GRANTED Fall 1986

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **OLIGOSACCHARIDES IN HONEY, THEIR ORIGIN AND ANALYSIS** submitted by **Nicholas Hansen Low** in partial fulfilment of the requirements for the degree of **Doctor of Philosophy in Food Chemistry.**

Peter Speers
Supervisor

Date October 8, 1980

ABSTRACT

The origin of the minor carbohydrates in honey has been the subject of much speculation. It was our goal to trace the origin of these carbohydrates.

In order to determine the origin of the minor carbohydrates in honey, new methodology for the separation and analysis of honey was required. By employing high performance liquid chromatography (HPLC), capillary gas chromatography (GC) and carbon-thirteen nuclear magnetic resonance spectroscopy (^{13}C-nmr), these minor carbohydrates could be identified and quantitated.

HPLC afforded a rapid bulk separation of the minor oligosaccharide fraction (≅3%) from the major monosaccharide fraction (≅95%). This type of separation has numerous advantages over the previous laborious charcoal-celite column chromatography method. Reducing the resulting oligosaccharide fraction with sodium borohydride greatly simplified the analysis of the carbohydrates in the fraction. The use of capillary GC afforded the separation of twelve derivatized disaccharides and seven derivatized trisaccharides commonly found in honey. In addition, by comparison to standards, these carbohydrates could be quantitated. The use of ^{13}C-nmr is relatively new in the field of quantitation and identification of complex mixtures. The appearance of a "finger-print" region in the nmr, together with the use of an internal standard and a relaxing agent, allowed the disaccharides present in the

oligosaccharide fraction of honey to be identified and quantitated. A comparison of this method with the capillary GC method indicated a fairly reasonable correlation.

The separation of the enzymes commonly found in honey lead to the discovery of the enzyme β-glucosidase, which had not previously been identified in this material. The optimum values for pH and temperature, together with Km and Vmax were determined for this enzyme in honey.

Unifloral honeys were collected and analyzed for botanical origin by pollen analysis. Modifications (acetolysis and proper dilution) of the accepted pollen analysis method allowed for easier identification of the botanical origins of these honeys; five of the six honeys were determined to be unifloral.

Nectar from the floral source of each of these honeys was collected and analyzed by capillary GC. Results from the analysis demonstrated the presence of fructose, glucose and sucrose only.

Analysis of the six honeys indicated the presence of a variety of oligosaccharides. By incubating the enzymes found in honey (from the honeybee) and a typical nectar solution, the same oligosaccharide profile was apparent.

These results lead us to the belief that the minor oligosaccharides in honey originate by transglucosylation action of the enzymes in the honeybee (and in honey) with the carbohydrates present.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Dr. P. Sporns, for his helpful suggestions. In addition, I appreciated the opportunity to pursue this research project in the manner I desired.

I would also like to thank the members of my examining committee, Dr. M. Palcic, Dr. F. Wolfe, Dr. [redacted] Dr. R. Gooding and my external examiner, Dr. J. deMan, for their suggestions and constructive comments. Special thanks go to Dr. M. Palcic for her suggestions in the enzyme area.

In addition, use of the nmr spectrometer was kindly supplied by the Chemistry Department in conjunction with T. Brisbane and G. Bingham with whom many useful discussions took place.

I also thank Dr. C. Schweger and E. Simons for their help in pollen analysis and electron microscopy.

I appreciate the agencies that funded this research: Alberta Agriculture and the University of Alberta (dissertation fellowship).

Most importantly, I express my sincere thanks to my wife, Jill, for her total support during this time.

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1. INTRODUCTION TO HONEY

1.1 Historical Aspects

In the long history of humans, honey was used for thousands of years before cane or beet sugar. In early history, humans were virtually dependent on honey for sweetness. Cane sugar became a direct competitor, and largely a successor, as the trade routes between the East and Europe were established (Baxa and Bruhns, 1967).

Honey is made by bees, and the raw material for practically all the world's supply of honey is the nectar produced in the nectaries of flowers. A smaller amount arises from plants which have nectaries elsewhere (extra-floral nectaries). Nectar produced by many plants may be regarded as a bait to entice the insect to visit them, thereby collecting pollen in addition to nectar. Nectar is, however, produced by some plants (dandelion, buttercup) which require no pollinating agent. Bees are by far the most important pollinating insects, therefore the production of honey is a by-product of the survival of plant species by insect pollination.

Generally, only the social bees that establish permanent colonies store honey in sufficient quantities to be of economic importance. These social bees are termed honeybees (*Apis*), and the tropical stingless bees, meliponins. There are four species of *Apis*, of which *Apis mellifera*, commonly known as the honeybee or hive bee, is

responsible for the production of the bulk of the world's honey. The other species of *Apis* are: *Apis dorsata*, *Apis cerana* and *Apis florea*. These bees are also responsible for honey production of economic importance.

1.2 Uses of Honey

The major portion of the world production of honey (90%), is consumed as table honey, the remainder (10%) is used as an ingredient in other products.

The use of honey in baking is universal; there are specialties made with honey, some of which have ancient origins. The Egyptians fed honey cakes to sacred animals. In Rome, libum was a sacrificial honey cake. "Wafers made with honey" were familiar to the ancient Israelites; this is how they described the manna they found in the desert after their flight from Egypt (Willson and Crane, 1975).

The present industrial uses of honey in baking include breads made with honey, honey buns, fruit cake, doughnuts, cookies, etc. Graham crackers are thin biscuits made of coarse flour and honey, named after Sylvester Graham (1794-1851), an American health food promoter. Honey in bread, to the amount of .6%, in addition to enhancing the flavour and giving a deeper colour to the crust, also adds to its keeping qualities (Smith and Johnson, 1952).

Due to the relative inexpensiveness and ease of use of cane sugar, honey has an inconspicuous role in the confectionery field. However, a few products, made

exclusively with honey, such as halvah (Turkey) and pasteli (Greece), remain.

Honey has a variety of minor uses such as a sprayed-on sweetening agent for breakfast cereals, sweetener in baby foods, uses in prepared meats (hams baked in honey), sweetener in yoghurt, sprayed coating for coffee beans during roasting, and a fermented product (mead or honey wine) (Willson and Crane, 1975).

In addition to food uses, honey is also used as a sweetening agent in tobacco processing, as a component of many commercially-manufactured pharmaceutical products, and in the cosmetics industry.

In the pharmaceutical industry, honey has found a wide range of uses, from a palatable sweetening agent in general pharmaceuticals (cough mixtures, etc.), to treating burns (Osaulko, 1954; Phillips, 1933). Bulman (1955) reported honey's successful use as a surgical dressing for open wounds and septic infections. Other researchers (Albanese, 1952; Martensen-Larsen, 1954) found that the rapid utilization of fructose, and the increased rate of metabolism of alcohol in the presence of fructose, led to the use of honey for sobering drunken patients.

1.3 Honey Production

The world production of honey was estimated at 912,653 metric tonnes (Anon., 1984a). The production of honey in Canada and Alberta in 1984 was 44,135 and 12,545 metric

tonnes, respectively (Anon., 1984b). The Alberta and Canadian honey production in 1984 represented a record high.

According to government reports (Anon., 1985), total honey production in Alberta for 1985 will show a sharp decline to approximately 8,392 metric tonnes. This represents a decrease of about one-third when compared to the 1984 level, and is the second lowest crop in the last 10 years.

The main reason for this dramatic decline is the reduced yield in most of the province from unfavourable weather conditions during the honey flow. In the Peace River region, where many of Alberta's commercial honey producers are located, yield is estimated at about 55 kg (121 pounds) per colony, compared to a five-year average yield of 71.8 kg (158 pounds) per colony from 1980 to 1984.

Alberta remains the largest honey producing province in Canada, and accounts for approximately one-third of the total production in the country.

The major export markets for Canadian honey include the United States, the European Economic Community, Japan, Sweden and the Caribbean. Developments affecting the export production of honey in 1984 included the high production of honey in China (125,605 metric tonnes) and the inadequate United States production (93,000 metric tonnes). In addition, Canadian honeys must compete in both price and quality with honey from China, Argentina, Brazil and Mexico, the major honey producers and exporters (Anon., 1984a).

1.4 Honey Composition

Honey as produced by the bees has its origin in a thin, sweet liquid called nectar. By enzymatic hydrolysis of sucrose in nectar, the bee increases the attainable density of the final product, and raises the efficiency of the process in terms of caloric density. In addition, the resistance of the stored product to spoilage by micro-organisms is greatly increased due to the higher osmotic pressure attained.

Determining the composition of honey has been the subject of numerous analytical studies (White *et al.*, 1952, 1958a, 1961b, 1962; White, 1961a, 1964, 1967; White and Riethof, 1959a; White and Hoban, 1959b). The average composition of 490 samples of honey from the United States is shown in Table 1.1 (White *et al.*, 1962).

The two major carbohydrates in honey are fructose and glucose. In nearly all honey types fructose predominates (White *et al.*, 1962); these two carbohydrates together account for 85-95% of honey carbohydrates. More complex sugars (oligosaccharides) composed of two or more molecules of glucose and fructose constitute the remainder. These more complex carbohydrates, together with methods for their analysis, will be dealt with in detail in another section of this introduction.

The flavour of honey is due in part to the slight tartness or acidity. The major acid found in honey is gluconic acid. It has been shown that it is produced by the

Table 1.1 Average composition of United States honey, including ranges of values.

Component	Average	Range
Moisture	17.2	13.4-22.9
Fructose	38.2	27.2-44.3
Glucose	31.3	22.0-40.7
Sucrose	1.3	0.2-7.6
Higher Sugars	1.5	0.1-8.5
Free Acid (as gluconic)	0.43	0.13-0.92
Lactone (as gluconolactone)	0.14	0.0-0.37
Total acid (as gluconic)	0.57	0.17-1.17
Ash	0.169	0.020-1.028
Nitrogen	0.041	0.000-0.133
pH	3.91	3.42-6.10
Diastase Value	20.8	2.1-61.2

action of glucose oxidase upon glucose (Stinson *et al.*, 1960; Maeda *et al.*, 1962). In addition, the following acids have been unequivocally shown to be present in honey (Stinson *et al.*, 1960): acetic, butyric, lactic, pyro-glutamic, citric, malic and succinic. Oxalic (von Philipsborn, 1952), maleic (Goldschmidt and Burkert, 1955) and formic (Farnsteiner, 1908) acids have also been found in honey. Except for gluconic acid, the sources of the various honey acids are unknown. Many of the acids present are also present as intermediates in the Krebs cycle, and they may also be present in the nectar.

In addition to the complex oligosaccharides in honey, some of the most interesting components are enzymes. The enzymes in honey will be introduced and discussed in another section of this thesis.

Excellent reviews on all aspects of honey, including production, processing, constituents and analysis are available (Crane, 1979; Siddiqui, 1970; White, 1961a, 1962, 1978).

1.5 Sugars in Honey

The Canadian Food and Drug Act Regulations indicate that honey should be derived from the nectar of flowers and other sweet exudations of plants by the work of bees. Honey should not contain more than 20% moisture, 8% sucrose, and 0.25% ash. In addition honey should not contain less than 60% invert sugar (50:50, glucose/fructose).

All definitions of honey deal with two chemically and physically distinct commodities: nectar honey and honeydew honey. A comparison between the above shows that honeydew honey is lower in D-fructose and D-glucose and higher in pH, oligosaccharides, acidic components, ash, and nitrogen than nectar honey (White *et al.*, 1962). The trivial and systematic names for the minor sugars (disaccharides and trisaccharides), and their reduced products can be found in Tables 1.2 and 1.3, respectively.

One distinct feature of honeydew honey is the trisaccharide melezitose, which has been identified in the exudate manna of Douglas fir, European larch and scrub pine (Hudson and Sherwood, 1918, 1920).

Foraging bees carry nectar in their honey sac, mix it with enzyme-rich secretions from their hypopharyngeal glands, carry it to the hive, and pass it over to the house bees. The house bees, in turn, transmit the sweet liquid among themselves, carrying out the process of ripening by mixing with glandular secretions and by removing water (Crane, 1979). The process of ripening is continued and the raw material loses approximately 50% of its water content; further ripening occurs in the cells of the comb, in the dry air that constitutes the ventilation system of the hive. When a moisture content of approximately 20% is reached, the cells which contain honey are capped by the bees (Park, 1933; Reinhardt, 1939).

Honey, therefore, consists of a concentrated solution

Table 1.2 Trivial and systematic names of honey oligosaccharides.

Trivial Name	Systematic Name
Disaccharides	
Cellobiose	O-β-D-Glucopyranosyl-(1→4)-D-glucopyranose
Gentiobiose	O-β-D-Glucopyranosyl-(1→6)-D-glucopyranose
Isomaltose	O-α-D-Glucopyranosyl-(1→6)-D-glucopyranose
Kojibiose	O-α-D-Glucopyranosyl-(1→2)-D-glucopyranose
Laminaribiose	O-β-D-Glucopyranosyl-(1→3)-D-glucopyranose
Leucrose	O-α-D-Glucopyranosyl-(1→5)-D-fructopyranose
Maltose	O-α-D-Glucopyranosyl-(1→4)-D-glucopyranose
Maltulose	O-α-D-Glucopyranosyl-(1→4)-D-fructose
Nigerose	O-α-D-Glucopyranosyl-(1→3)-D-glucopyranose
Palatinose	O-α-D-Glucopyranosyl-(1→6)-D-fructose
Sucrose	α-D-Glucopyranosyl β-D-fructofuranoside
α,β-Trehalose (Neotrehalose)	α-D-Glucopyranosyl β-D-glucopyranoside
α,α-Trehalose	α-D-Glucopyranosyl α-D-glucopyranoside
Turanose	O-α-D-Glucopyranosyl-(1→3)-D-fructose

Table 1.2 (*cont.*)

Trivial Name	Systematic Name
Trisaccharides	
Erlose	O-α-D-Glucopyranosyl-(1→4)-α-D-glucopyranosyl β-D-fructofuranoside
Isomaltotriose	O-α-D-Glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-D-glucopyranose
Isopanose	O-α-D-Glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→6)-D-glucopyranose
1-Kestose	O-α-D-Glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2) β-D-fructofuranoside
Maltotriose	O-α-D-Glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucopyranose
Panose	O-α-D-Glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→4)-D-glucopyranose
Theanderose	O-α-D-Glucopyranosyl-(1→6)-α-D-glucopyranosyl β-D-fructofuranoside

Table 1.3 Trivial and systematic names of reduced honey oligosaccharides.

Trivial Name	Systematic Name
<u>Disaccharides</u>	
Cellobiitol	O-β-D-Glucopyranosyl-(1→4)-D-glucitol
Gentiobiitol	O-β-D-Glucopyranosyl-(1→6)-D-glucitol
Isomaltitol	O-α-D-Glucopyranosyl-(1→6)-D-glucitol
Kojibiitol	O-α-D-Glucopyranosyl-(1→3)-D-glucitol
Laminaribiitol	O-β-D-Glucopyranosyl-(1→3)-D-glucitol
Leucritol	O-α-D-Glucopyranosyl-(1→5)-D-glucitol, and O-α-D-Glucopyranosyl-(1→5)-D-mannitol
Maltitol	O-α-D-Glucopyranosyl-(1→4)-D-glucitol
Maltulitol	O-α-D-Glucopyranosyl-(1→4)-D-glucitol, and O-α-D-Glucopyranosyl-(1→4)-D-mannitol
Nigeritol	O-α-D-Glucopyranosyl-(1→2)-D-glucitol
Palatinitol	O-α-D-Glucopyranosyl-(1→6)-D-glucitol, and O-α-D-Glucopyranosyl-(1→6)-D-mannitol
Turanitol	O-α-D-Glucopyranosyl-(1→3)-D-glucitol, and O-α-D-Glucopyranosyl-(1→3)-D-mannitol

Table 1.3 (*cont.*)

Trivial Name	Systematic Name
<u>Trisaccharides</u>	
Isomaltotriitol	O-α-D-Glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-D-glucitol
Isopanitol.	O-α-D-Glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→6)-D-glucitol.
Maltotriitol	O-α-D-Glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucitol
Panitol	O-α-D-Glucopyranosyl-(1→6)-O-α-D-glucitol

of two monosaccharides, D-glucose and D-fructose, and the combination of these constitutes approximately 95% of the honey solids. Carbohydrate analysis of honey dates back to 1892, when 500 commercial samples of honey were analyzed (Wiley, 1892). The method employed was low and high temperature polarization and reducing sugar analysis. This analytical method was used, with certain modifications, by several researchers for the quantitation of levulose (fructose) and dextrose (glucose) in honey (Wiley, 1896; Browne, 1908; Lothrop and Holmes, 1931; Eckert and Allinger, 1939; Elegood and Fischer, 1940). These methods of determination were used until a critical study of sugar analysis was made by White *et al.* (1952).

Following the development of carbon-celite column chromatography (Whistler and Durso, 1950), and the application of this method to sugar analysis of honey (White *et al.*, 1954), a new procedure for the analysis of honey emerged and was called the selective-adsorption method. This new method provided a more accurate determination of glucose and fructose and revealed the presence of other honey sugars, the reducing disaccharides and higher oligosaccharides. Honey, at this time, had been considered a mixture of glucose, fructose and sucrose, and methods using polarimetry and reducing sugar analysis were unknowingly interfered with by a number of other sugars, both reducing and non-reducing.

The selective adsorption method consists of a charcoal-celite (1:1) column, upon which 0.8-1.0 g of honey is

subjected to adsorption, followed by elution under pressure with aqueous solutions of increasing ethanol content. Monosaccharides are eluted with 1% ethanol, disaccharides with 7% and higher oligosaccharides with 50% aqueous ethanol (White, 1957, 1959, 1960).

The presence of maltose in honey was suggested by Elser (1924) on the basis of its characteristic osazone formation. Liggett (1941) separated 8 honey sugars by distillation of the corresponding propionate esters and obtained a crystalline material from the disaccharide fraction which had a melting point close to that of authentic maltose octapropionate. Van Voorst (1941) fermented several honey samples with a maltase-free yeast and found that all sugars, except maltose, were removed. Hurd *et al.* (1944) indicated that maltose is a component of all honeys.

White and Hoban (1959b) used a combination of selective adsorption chromatography, preparative paper chromatography and stearic acid-treated charcoal column chromatography to confirm the presence of sucrose and maltose. This latter method had previously been used by Hoban and White (1958) to resolve dissaccharide pairs (turanose-sucrose; isomaltose-gentiobiose; maltulose-nigerose; melibiose-lactose) which could not be separated by paper chromatography.

By the use of paper chromatography, Keup (1957) demonstrated the presence of as many as 15 components in carbon-celite separated honey. Aso *et al.* (1958, 1960) detected 22 components, 15 of which were classified as

ketoses, following carbon-celite column chromatography and paper chromatography. Other investigators, including Goldschmidt and Burkert (1955), Pourtallier (1964), Flechtmann *et al.* (1963) and Cuffylo (1962), used paper chromatography to indicate honey oligosaccharides.

Hoban and White (1958) used carbon-celite chromatography to isolate 2.6 g of disaccharides from honey. This fraction was further purified using stearic acid-treated charcoal column chromatography to obtain five purified sugars. These sugars were converted to their corresponding octaacetates and the infrared spectrum of each sugar and its acetate was then compared with the spectra of known disaccharides and their acetates. Characteristic differences in the 650-1500 cm^{-1} IR absorption region (White *et al.*, 1958b) allowed the identification of isomaltose, maltulose, turanose, maltose and nigerose. The identification of these carbohydrates was confirmed by paper electrophoresis with standards (White *et al.*, 1958b).

Watanabe and Aso (1959, 1960) confirmed the presence of nigerose, maltose and isomaltose in honey, and also identified kojibiose. Using successive elutions of a charcoal-celite column with seven solvents of increasing ethanol content (2.5-30%), partial fractionation of disaccharides occurred. The first fraction was rechromatographed on a charcoal-celite column containing pH 10 borate buffer; this effected the separation of two sugars, which were acetylated. The octaacetates would not crystallize

until they were further purified by magnesol-celite column chromatography (McNeeley *et al.*, 1945). The same methodology was used to separate the octaacetates of nigerose and maltose. Melting points and specific rotations of the octaacetates were compared with literature values for authentication. The authors also indicated the presence of leucrose [O-α-D-glucopyranosyl-(1→5)-D-fructopyranose], however, the only evidence was the appearance of one spot on a paper chromatogram when mixed with an authentic sample.

Large scale fractionation of honey was accomplished by Siddiqui and Furgala (1967, 1968a,b) on a carbon-celite column. Typical reaction conditions involved the use of 2000 g of honey mixed with equal volumes of charcoal and celite (1300 mL). Water (12 L) was then added, the mixture was stirred for 2 h and allowed to settle, and the clear supernatant was decanted. Thirteen 12 liter extractions were required to remove the bulk of the monosaccharides. The charcoal residue was then washed successively with 50% aqueous ethanol (5, 5, 8, 4 and 4 liters), yielding material of 27.5, 24.0, 20.9, 6.5 g and negligible amounts, respectively (total oligosaccharide fraction 73 g). Lipid and acidic components were removed by centrifugation and extraction (water:methanol/chloroform, 500:1250/625 mL). The water-methanol phase was then stirred with Rexyn 201 (CO_3^{2-}, 500 mL) and the slurry was used in a column and eluted with water (5 L) to remove neutral sugars. The neutral sugar fraction was then placed on a carbon-celite column and

eluted using a stepwise aqueous ethanol gradient (0-30%). Further purification was accomplished using paper chromatography, electrophoresis and thin-layer chromatography. Following acetylation, the fractionation procedures resulted in the characterization of at least 24 oligosaccharides. The oligosaccharides maltose, kojibiose, isomaltose, nigerose, α,β-trehalose, gentiobiose, laminaribiose, melezitose and maltotriose were identified as crystalline β-octa- and hexdecaacetates. Sucrose, turanose, 1-kestose and panose were identified as the crystalline sugars, maltulose as the phenylosazone, and isomaltotriose as its crystalline β-hendecabenzoate. Erlose and theanderose were identified by the isolation and characterization of the disaccharides produced when hydrolyzed with an enzyme or [redacted]. Isomaltulose and 1-O-α-D-glucopyranosyl-D-fructose were tentatively identified by co-chromatography with standards. In addition, the authors also identified two new trisaccharides in honey, centose and O-β-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→4)-D-glucopyranose.

In the initial honey sample, the oligosaccharide fraction accounted for 3.65%. The approximate proportions of these sugars in the oligosaccharide fraction were: maltose (29.4%); kojibiose (8.2%); turanose (4.7%); isomaltose (4.4%); sucrose (3.9%); nigerose (1.7%); α,β-trehalose (1.1%); gentiobiose (0.4%); laminaribiose (0.09%); erlose (4.5%); theanderose (2.7%); panose (2.5%); maltotriose (1.9%); 1-kestose (0.9%); isomaltotriose (0.6%); melezitose

(0.3%); other tri-, tetra-, and pentasaccharides (0.45%).

This extensive analysis revealed features of honey composition which were at variance with other researchers. Aso *et al.* (1958, 1960) detected leucrose in their sample of honey by paper electrophoresis, however its presence was not confirmed by Siddiqui and Furgala (1967, 1968). In addition, the former workers failed to detect trehalose, whereas Siddiqui and Furgala isolated it. This work also clarified the confusion arising from the reports of raffinose in honey (Goldschmidt and Burkert, 1955; Flechtmann *et al.*, 1963; Pourtallier, 1964; Keup, 1957; Curylo, 1962) when it was shown that the trisaccharide theanderose had identical behaviour to raffinose on paper chromatography and towards ketose spray reagents.

The carbohydrates present in honey have been the subject of review by three authors (Siddiqui, 1970; White, 1975, 1978; Doner, 1977).

2. GENERAL EXPERIMENTAL

The carbohydrates used as standards were provided by Sigma Chemical Co. (melezitose, maltotriose, isomaltotriose, palatinose, isomaltose, turanose, nigerose, trehalose, gentiobiose); Koch-Light Ltd. (kojibiose); Fisher Scientific Ltd. (sucrose, maltose); Aldrich Chemical Co. (cellobiose); or were generous gifts from Dr. S. Chiba (erlose, isopanose, 1-kestose, theanderose) and Dr. J.R. Siddiqui (neotrehalose).

Honey was supplied by Dr. D. Nelson (Beaverlodge, Alberta), alfalfa, alsike, canola, red clover, sweet clover and trefoil honey; Dr. Manzour-ul-Haq (Dept. of Entomology, Faisalabad, Pakistan), *Apis cerana*, *Apis dorsata* and *Apis florea* honey.

The compounds p-nitrophenyl-α-D-glucopyranoside and p-nitrophenyl-β-D-glucopyranoside were supplied by Sigma Chemical Co.

Acetonitrile was HPLC grade (Terochem Laboratories Ltd.); reverse osmosis Milli-Q water filtered through 0.45 μm Millipore filter system was also used for HPLC analysis. All other chemicals employed were reagent grade or better.

3. DETERMINATION OF HONEY ORIGIN

Melissopalynology, the pollen analysis of honey, is the method most commonly used for determining its geographical and floral origin (Maurizio, 1975; Sawyer, 1975). Although this method can be reliably used by experts with the necessary experience and judgement, incorrect identification and categorization may lead to rejection of honey for import and/or lower prices for the product (Howells, 1969).

3.1 Determining the Geographical Origin of Honey by Amino Acid Analysis

Other methods that could be more widely used for characterizing honey have been sought. One of these methods is the amino acid content of honey, which was proposed by Tillmans and Kiesgen in 1927. Paper chromatography was initially used (Baumgarten and Möckesch, 1956) and later electrophoresis (Maslowski and Mostowska, 1963). Curti and Riganti (1966) were the first to use an automatic amino acid analyzer, utilizing ion-exchange chromatography, for the analysis of free amino acids in honey.

The most comprehensive investigation of honey amino acids was performed by Hahn (1970) and Bergner and Hahn (1972). In these experiments twenty-five honeys, fourteen honeydews, thirteen honeys from sugar feeding experiments, and three nectars were tested. The authors considered that the amino acid content of honey was quantitatively too variable to be used to characterize honey.

The sources of amino acids in honey have been investigated, and three possible origins have been recognized: nectar or honeydew, pollen, and the bees.

Pollen has been found to contain on the average 1,500 mg amino acids per 100 g, and proline is usually the major amino acid (Lotti and Anelli, 1970). Even though this concentration is ten times the amino acid concentration in honey (Koumaine, 1960), the large number of pollen grains in honey (2,000/10,000 g) represents only a very small quantity of pollen, which is equivalent to approximately 0.04 g pollen per 100 g honey, or about 0.6 mg amino acid per 100 g honey (Maurizio, 1951). Honey contains 50-300 mg amino acid per 100 g. Although these are approximate figures, it seems reasonable that pollen does not contribute significantly to the quantities of amino acids in honey.

It has also been found (Maslowski and Mostowska, 1963) that amino acids are present in honey when the bees are fed a pure sucrose solution. The amino acids could only have originated from the bees, and this was confirmed in later experiments by Hahn (1970) and Rogers and Davies (1972).

It was concluded that bees, nectar and honeydew are the major sources of amino acids in honey. The characterization of amino acid content in honey was therefore based on the assumption that the amino acid patterns of honeys are all generally similar (due to the influence of the bees), but that the variation would be due to the types of nectar or honeydew from which the honey is made.

Davies (1975) applied the method of amino acid analysis of 98 samples of honey, and 8 samples of non-honey sugar products. The procedure involved approximately 10 g of honey to which a standard was added (norleucine) and the mixture was diluted to 25 ml with sodium citrate buffer (pH 2.2). Then 1.0 ml of this solution was chromatographed on the ion-exchange analyser using a gradient of sodium citrate buffers (pH 3.25-6.50). His results indicated that honeys from different geographical regions may be noted. It was found that Australian honey has a higher proline content, Canadian honey has low aspartic acid and high lysine and histidine, and U.S. clover honey has higher aspartic and glutamic acid.

In 1978 Bosi and Battaglini used gas chromatography to determine the free and protein amino acids of 28 unifloral (by pollen analysis) honeys. The authors found that the amino acid spectra can vary greatly, according to the botanical and geographical origin (two unifloral samples from the same geographical area had a wide variety of amino acid concentrations). They concluded that, while the variability found for many amino acids, particularly free amino acids, shows that there are great differences between honeys, it also suggests that numerous factors are involved in determining each spectrum. Therefore, it is not possible to use amino acid spectra to characterize honeys.

It is also important to note that the amino acid spectrum of a honey sample may be different from that of the nectar or honeydew from which it is derived because the

amino acid may react differently with the sugars that are present in the nectar or in the honeysac of the bee (Maillard reaction). This reaction may have the effect of reducing the differences between the free amino acids of honeys from different sources.

Gilbert *et al.* (1981) used amino acid analysis to determine the geographical origin of 45 honey samples. The authors suggested that, by using a multivariate statistical analysis of their data, honey from Canada may be distinguished from Argentina and Europe. However, honey from Argentina and Europe could not be distinguished within the 95% confidence interval.

3.2 Determining the Geographical Origin of Honey by Pollen Analysis (Melissopalynology)

Although methods, such as amino acid analysis, have been sought for the characterization of honey, the method of choice is still pollen analysis. The world-wide standards for honey (pollen) analysis proposed by the Codex Alimentarius Commission (1983) were accepted by the joint FAO/WHO Food Standards Program. These regulations represent the standard methods for honey and pollen analysis world-wide. They are accepted as the standard to determine the geographical and botanical origin of honey. These internationally accepted regulations for pollen analysis are based on the microscopical analysis of honey from which the pollen has not been completely removed by filtration.

Preparation of a sample for botanical origin consists of weighing approximately 10 g honey and dissolving it in 20 mL hot water. The solution is then centrifuged at 2,500-3,000 rpm for 10 min, and the supernatant is decanted or drawn off. The sediment may then be resuspended in 10 mL distilled water and centrifuged (2,500-3,000 rpm) for 5 min. The entire sediment is placed on a microscope slide, spread over an area of 20x20 mm and allowed to dry. The sediment is then mounted with glycerine gelatine and analysed; identification is made by reference to the literature and comparative preparations.

During the removal of nectar from a flower, bees come into close contact with the anthers. Many pollen grains stick to the hairs on the bee's body during these visits to flowers, and these grains can enter directly into the unripe honey lying in open cells in the hive. Some of the ripe pollen on the anthers falls into the nectar, is sucked up with it into the bee's honey sac and also gets into the ripening honey in the cells of the comb. Just as the floral origin of honey is characterized by the pollen grains found in it, honeydew as a source of honey may be characterized by the appearance of minute green algal cells and spores from the surface flora of the plants.

Pollen grains can also get into the raw materials of honey, and therefore into honey itself in indirect ways. Pollen grains suspended in the air may fall on to the exposed surface of nectar, or on to sticky honeydew lying on

leaves, and needles of conifers. The pollen grains of wind-pollinated plants are introduced in this manner. Pollen may also enter directly into honey during its processing by the beekeeper (Maurizio, 1975).

The identification of pollen grains is based on size, shape, type and number of apertures and sculpturing. In honey the swollen condition of the grain is important, since many of the details are only visible in this state.

Pollen grains, the male reproductive cells of higher plants, as a rule appear as single cells, but they are often found in groups. On the pollen grain two perpendicular axes are present, the polar axis (P) and the equatorial axis (E) (see Figure 3.1). The contents of a pollen grain are mostly enclosed within a double wall. The wall is made up of two layers; the outer layer is called the exine and is composed of sporopollenin (Zetsche, 1932). The inner layer, or intine, is made up of cellulose and is very similar to an ordinary plant cell wall. Sporopollenin is extremely resistant to chemical attack, especially to acetolysis. It can be affected by oxidants, but it requires extremely strong oxidants and vigourous conditions (H_2SO_4/H_2O_2 or 40% chromic acid or ozone). In 1968 Brooks and Shaw suggested that sporopollenin was a complex polymer of carotenoid esters and carotenoids.

Sporopollenin is widespread in the plant kingdom, however, it is only in higher plants (angiosperms and gymnosperms) that it is built into the complex wall structures.

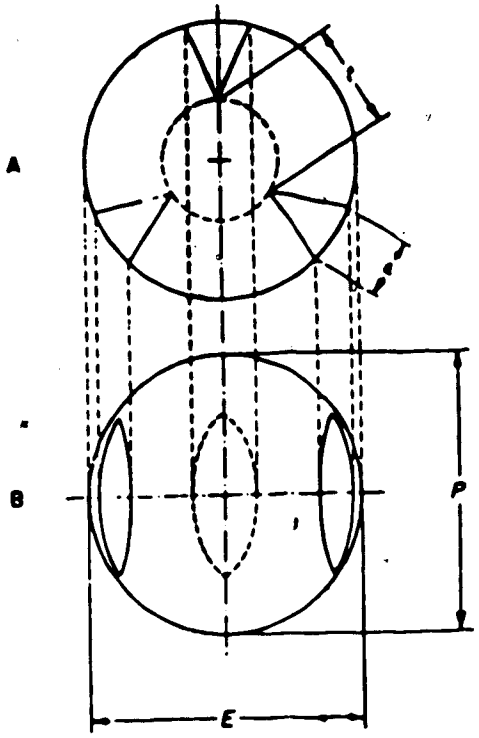


Figure 3.1 A pollen grain showing three furrows. (A) from above; (B) from the side. P = polar axis; E = equatorial axis; e = diameter of furrow; t = distance between furrows. (Crane, 1975)

The exine of pollen grains is further divided into an outer sculptured exine or ektaxine and an inner sculptured nexine or endexine (Erdtman, 1966) (see Figure 3.2). The sexine commonly takes the form of a set of radially-directed rods, supporting a roof. This roof may be complete, partially dissolved or entirely absent. According to Reitsma (1970) this roof is termed a tectum, a rod which supports a tectum or any part of it, a columella, and a rod which is not supporting anything, a baculum.

In some plant species the exine forms a completely closed layer around the contents of the pollen grain, however, most pollen grains possess openings in or thin parts of the exine, through which the pollen tubes generally emerge. These missing or thin parts of the exine are termed aperture; there are two types of aperture, named pori (pores) and colpi (furrows). Colpi are believed to be more primitive than pori and may be distinguished from the latter by being long and boat shaped with pointed ends. Pori are normally isodiametric holes; they can be slightly elongated with rounded ends. Grains with pori are labelled porate and those with colpi are colpate; with both pori and colpi combined in the same aperture, colporate.

As only one aperture is used for the pollen tube, the excess in number found on some grains suggests that they must have an additional function. Heslop-Harrison (1971) found that the thick intine region under the aperture has a store of easily leached proteins which may function in the

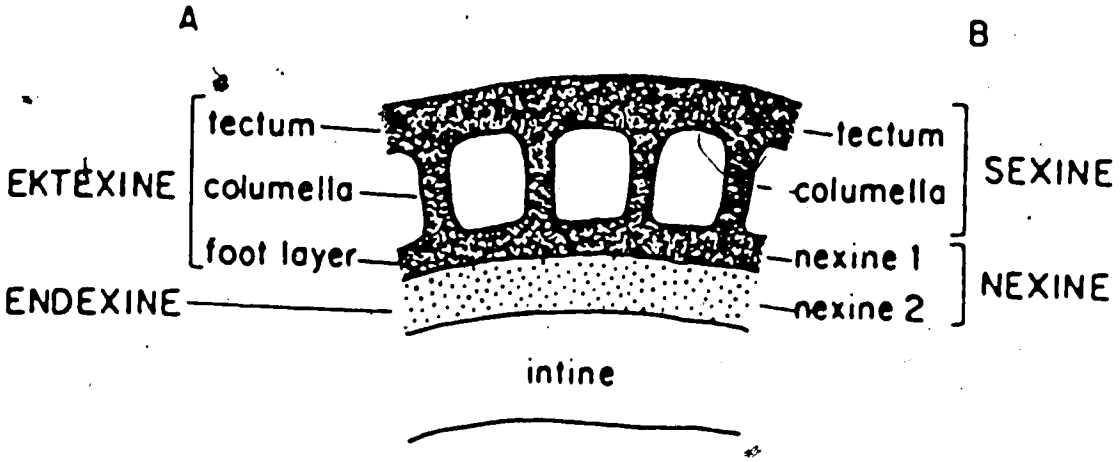


Figure 3.2 Terminology for pollen grain exine. (Moore and Webb, 1978). A. As defined by Faegri and Iversen (1974); B. As defined by Reitsma (1970).

recognition reaction between pollen grain and stigma. Wodehouse (1935) suggested that the apertures were regulatory devices, controlling the movement of water into and out of the grain. In his experiments dehydration caused an infolding of the intine at the colpus, resulting in its margins coming close together. It appears from the work of these two authors that these apertures could and probably do have more than one function.

As a result, number, position, formation, and size of the apertures constitute characteristics for determining the pollen.

Following the characterization of the pollen grains on the basis of their apertures, they can be identified conclusively by observation of the sculpturing (fine structure) of the exine. As has been previously mentioned, the sexine has been described as being composed of radially directed rods which sit on the nexine and are labelled columellae (if they support the tectum) and bacula (if they do not support anything and are cylindrical in shape). In certain cases the rods are free at their heads, but are non-cylindrical in shape. They are called gemmate (short and globular), clavate (club shaped), pilate (swollen heads) and echinate (sharply pointed); a grain with free rods is described as intectate (Figure 3.3).

In other pollen grains the sexine elements which sit on the nexine do not resemble rods. In these cases they may appear as small hemispherical warts (verrucae), tiny flakes

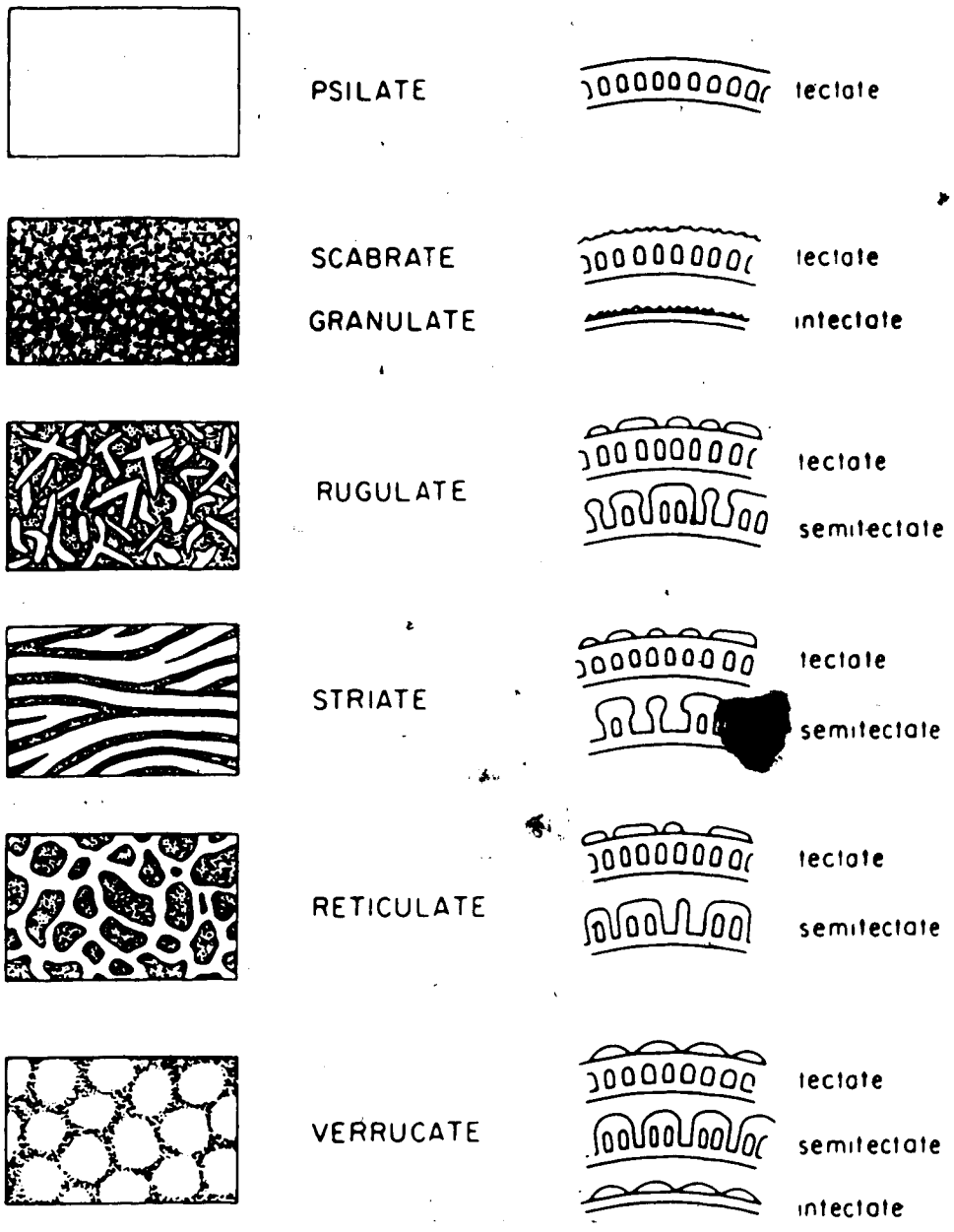


Figure 3.3 Diagrams of sculpturing types visible in a surface view of a pollen grain. In the sculpturing types shown all raised areas are light, all lower areas or holes are dark. (Moore and Webb, 1978)

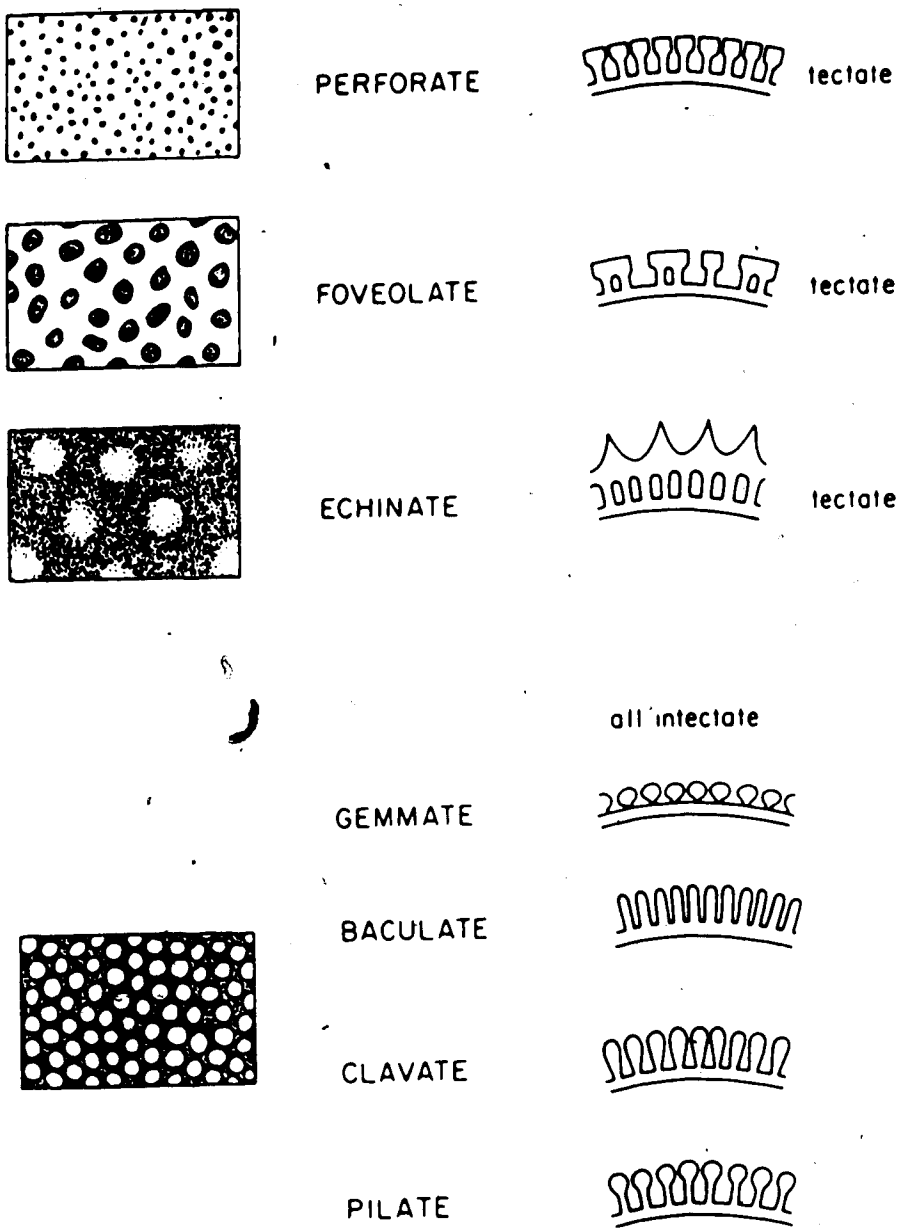


Figure 3.3 (*cont.*) Diagrams of sculpturing types visible in a surface view of a pollen grain. In the sculpturing types shown all raised areas are light, all lower areas or holes are dark. (Moore and Webb, 1978)

(scabrae), or as small grains (granules). When the heads of the columellae are joined by a complete tectum, then the grain is described as tectate. Intectate and tectate represent the two extremes and there are grains which have only a partial tectum; these grains are referred to as semitectate (Figure 3.3). (Moore and Webb, 1978). Descriptions of the various sculpturing types are given in Table 3.1.

With the information on size of the pollen grain, number of apertures and types, surface sculpturing, and finally the number of specific pollen types in the honey, both floral origin and geographical location can be identified, keeping in mind that the results must be checked against reference slides to ensure that mistakes in identification don't occur.

It should be noted that in the study of pollen grains, the complexity of their structure and patterning has necessitated a formidable terminology. In addition, structures have been assigned different names by various investigators. This can be seen even in the naming of the layers of the exine (Figure 3.2). There are in the literature at the present time two major works for pollen grain analysis (Faegri and Iversen, 1974; Erdtman, 1963) which incorporate different terminologies. Reitsma (1970) has attempted to unify these terminologies, and his incorporation of the two terminologies will be used for this thesis.

Table 3.1 Descriptions of sculpturing types (Moore and Webb, 1978).

Sculpturing Type	Description
Psilate	With surface completely smooth.
Perforate	With surface pitted (holes dark at high focus). Pits < 1 μm.
Foveolate	With surface pitted (holes dark at high focus). Pits > 1 μm.
Scabrate	With projecting elements isodiametric, no dimensions greater than 1 μm (appear white at high focus).
Verrucate	With width of projecting elements as great as height (appear white at high focus).
Gemmate	With width of projecting elements same as height, but elements constricted at bases (appear white at high focus).
Clavate	With height of projecting elements greater than width, bases constricted (appear white at high focus).
Pilate	With height of projecting lements greater than width, apical parts of elements swollen (appear white at high focus).
Baculate	With height of elements greater than width, bases not constricted (appear white at high focus).
Echinate	With projecting elements pointed.

Rugulate	With projecting elements elongated sideways, length 2 x breadth and irregularly distributed (white at high focus).
Striate	With projecting elements elongated sideways and arranged ± parallel to one another.
Reticulate	With projecting elements arranged in a network pattern (net appears white at high focus).

There are three principal methods of pollen analysis used in honey control:

- Determination of the absolute pollen content (Hammer *et al.*, 1948; Maurizio, 1949; Demianowicz and Demianowicz, 1957) (see Table 3.1).
- Absolute pollen frequency. Large fluctuations occur in absolute pollen content, in addition to the percentage of certain pollen species in honey. These differences are caused by differences in the intensity of nectar coming into the colony and by secondary contamination (Demianowicz and Jablonski, 1966).
- Pollen spectrum. The pollen analysis of honey provides qualitative information about the forage plants at the collection site, and is dependent upon soil type and seasonal flowering pattern (Pritsch, 1958).

Melissopalynology has been performed by various researchers. In 1979 Feller-Demalsy and Lamontagne *et al.* analyzed 206 honey samples from the beekeeping areas of Quebec. They based their results on the absolute pollen frequency (which is the most widely used method for melisso-palynology), and found that four forms of pollen were of major importance in the honey composition. They also found geographical indicators so that the honey from Quebec could be divided into six large zones. This division was based on certain plant species being indigenous to a certain region

of the province (for example, *Lotus corniculatus* being present only in the western portion of the province). In this case not only can the floral origin of the honey be characterized but an indication of the geographical origin is given as well.

In 1981 Murrell and Szabo used the pollen frequency method to characterize pollen collected by packaged bees at Beaverlodge, Alberta. They found that, although bees visited and collected pollen from a number of sources, the major proportion of the pollen came from just a few sources. The authors used standard O.A.C. (Official Analytical Chemists) pollen traps and monitored (by weekly sampling, and cleaning of the traps) the pollen composition. They found that different pollens predominated depending on the time of year. From mid-June to August melilotus pollen was present; red clover pollen from mid-July; alsike pollen in mid-June; and rapeseed pollen was present from the second week of June to the third week in August. The relative importance of a pollen source would depend on a number of factors, including: colony preference, environmental effects on each plant species, weather effects on the flight of the foraging bees and, in the case of cultivated crops, the acreage of the crop near the apiary. Their study was carried out for two years and the authors found the same predominating pollen types for both years. However, colonies showed different preferences for numbers and types of pollen collected; for example, in 1977 one colony collected only

Liguliflorae pollen, while the other colonies were collecting six other taxa.

Lieux (1972) used melissopalynological techniques to study 54 Louisiana honeys. The purpose of this study was to determine the major plants foraged by bees, and to study honeys in regard to their geographical and botanical origin.

Other researchers have also applied microscopical analysis of the pollen grains in honey to determine its botanical origin. These include: Barth (1970a,b, 1971); Battaglini and Ricciardelli D'Albore (1971, 1972); Chaubal and Deodikar (1965); Ferrazzi (1974); Hammer (1974); Hammer *et al.* (1948); Kubisova-Kropacova and Nedbalova (1976); and Louveaux (1968). Louveaux *et al.* (1979) produced an excellent review on the use of melissopalynology for botanical identification of honey.

When characterizing a honey for its botanical origin, certain rules must be followed (Louveaux *et al.*, 1970). The analysis may be either orienting or complete. In the former, the analysis is limited to the identification of the most frequently occurring particles and to searching for significant characteristic elements in the sediment. The latter involves identification of all pollen grains and other microscopic constituents in the sediment.

Three degrees of accuracy are distinguished when counting microscopic elements in the sediment. First, estimate; this is accomplished by counting 100 pollen grains and the honeydew elements corresponding to 100 grains.

Second, determining the frequency class; this is based on counting 200-300 pollen grains and the corresponding honeydew elements. If the pollen spectrum contains only a few grains, then 200 grains are sufficient, however, 300 grains must be counted if the spectrum is rich in species. Lastly, counts expressed in percentages; presentation of frequencies as percentages is allowed only if 1,200 pollen grains have been counted.

In expressing the results of pollen analysis one must also adhere to certain guidelines. If the frequency of pollen grains is estimated, then the following terms apply: "very frequent" for pollen grains which constitute more than 45% of the total; "frequent" for grains which constitute 16-45% of the total; "rare" for grains which constitute 3-15% of the total; "sporadic" for grains which constitute less than 3%.

If the frequency classes have been determined, the following terms are used: "predominant pollen" constitutes more than 45% of the pollen grains counted; "secondary pollen" (16-45%); "important minor pollen" (3-15%); "minor pollen" (less than 3%).

In addition another important fact must be considered in the determination of the botanical origin of honey. The pollen grains of some flowers are over-represented; in those cases the percentage of pollen found in the sediment is greater than the percentage of the corresponding nectar in the honey. In other cases this trend may be reversed, they

are underrepresented.

If a honey sediment is rich in an overrepresented pollen (*Myosotis*, forget-me-not; *Castanea sativa*), then a second supplementary count is made excluding this pollen. Only honeys containing 90% of these over-represented pollens can be considered unifloral.

The following important pollens are known to be under-represented and, if the frequencies of these pollens is as high as the percentages listed in parenthesis, the honey is mainly from that source. *Lavandula spica* x *L. latifolia* (10-20%), *Salvia* (20-30%), *Robina* (30%), *Tilia* (20-30%) and *Medicago* (30%).

3.3 Pollen Analysis Experimental

Pollen slides for botanical origin determinations were prepared by modifying the method of Erdtman (1969). This approach differs substantially from the Codex Alimentarius standard method (1983). The reasoning behind the use of this method will be discussed in the results and discussion section of this chapter.

Our modification of Erdtman's (1969) method involved eight steps, omitting his first step (treatment with 10% NaOH and boiling) and replacing it with a dilution of 5 g honey in 100 mL warm (30°C) reverse osmosis Milli-Q purified water. This solution was then centrifuged for 5 min at 4,500 rpm (Method A). The resulting supernatant was decanted and re-centrifuged at 10,000 rpm to ensure that all the pollen

had sedimented. [The precipitate may then be transferred quantitatively to a 15 mL centrifuge tube and centrifuged (Method A); no further pollen was apparent.] The supernatant was decanted and the centrifugate washed (stirring thoroughly) with 10 mL acetic acid and centrifuged (Method A). The supernatant was decanted and an acetolysis reaction performed (1 mL conc. H_2SO_4/9 mL acetic anhydride). The reaction involved the addition of 10 mL of the acetolysis mixture to the centrifuge tube, stirring thoroughly, and placing the tube in a water bath (100°C) for 3-5 min. The tube was then allowed to cool (slightly) and centrifuged (Method A). The supernatant was decanted and the sediment washed with 10 mL acetic acid, stirred thoroughly, and centrifuged (Method A). Supernatant liquid was decanted and the precipitate resuspended in 10 mL distilled water to which several drops of 10% NaOH had been added (at this point a pollen stain, such as safranin-O, may be added [omitted in our work]; this stains the pollen grains pink), and centrifuged (Method A). After the supernatant had been removed, the precipitate was washed and centrifuged (Method A) with ethyl alcohol and with t-butyl alcohol. The resulting centrifugate was suspended in approximately 0.5 mL t-butyl alcohol and transferred to an Eppendorf micro-centrifuge tube containing a few drops of silicon oil. The t-butyl alcohol was allowed to evaporate (in a dust- and pollen-free environment) and a drop of this suspension was placed on a microscope slide. Following positioning of a

coverslip and sealing with paraffin wax, the pollen could then be viewed for identification.

Pollen frequency measurements were made using a Dialux laboratory microscope (Leitz Wetzlar Ltd.) at a magnification of 630x. Photomicrography of the pollen grains was accomplished using a MPS 55 Photoautomat (Wild Heerbrugg Ltd.) equipped with an MPS 51 Shutterpiece. The film used was Panatomic 32X (Kodak) for black and white, and Kodabrome 64 for colour.

Determination of the botanical origin of the honey was accomplished using the frequency method; as was presented earlier, 200-300 or more pollen grains were counted. If the results indicated that a specific pollen type constitutes more than 45% of the total, it was classed as "very frequent" and may be termed of unifloral origin (Louveaux *et al.*, 1970).

Scanning electron microscopy sample preparation:

A portion of the previously prepared pollen was further centrifuged in acetone (Method A). Following decanting of the acetone, the pollen was mixed with a few drops of acetone and transferred to folded Whatman number 1 filter paper. This filter paper was kept in a small vial or microtube with acetone to ensure that the pollen would not dry out. The pollen was then submitted to critical point drying in a Polaron Jumbo critical point dryer. SEM stubs were prepared by gluing glass coverslips to them with silver conductive paint and allowing them to dry in a dessicator

overnight.

"Pollen glue" was prepared by dissolving a small amount of "Scotch" brand tape in a small vial of chloroform (Adam and Morton, 1972). A drop of the resulting glue was placed on the previously prepared stub and the chloroform was allowed to evaporate, leaving behind a sticky residue. The pollen was attached to the stub by contacting the stub to the pollen on the filter paper containing the pollen. The pollen stub was dried overnight in a dessicator and coated with a thin layer of gold in a sputter coater for 2 min. The samples were then photographed with a Cambridge Stereoscan 250 scanning electron microscope.

3.4 Pollen Analysis Results and Discussion

As has been previously mentioned, the pollen frequency method appears to be the method of choice for determining the floral origin of honey (Townsend *et al.*, 1979; Sawyer, 1975; Louveaux *et al.*, 1970) and this was the method used in the identification of the six honeys tested.

Although great care was taken to attempt to ensure that the honey collected would be unifloral (i.e. using packaged bees, new supers for honey storage, and placing the colonies in cultivating fields of unifloral sources which were flowering), the bees still would not completely cooperate. By using the pollen frequency method, the results of the six honeys are reported in Table 3.2.

The six honeys collected and used in our experiments

Table 3.2 Honey pollen analysis.

Honey	Pollen Classification	Unifloral	Number of Pollen/ Total Pollen Counted
Alfalfa (*Medicago sativa*)	very frequent	+	225/277
Canola (*Brassica*)	very frequent	+	270/317
Red Clover (*Trifolium pratense*)	very frequent	+	202/253
Sweet Clover (*Melilotus alba*)	frequent	+	107/260
Alsike (*Trifolium hybridum*)	frequent	-	100/250
Trefoil (*Lotus corniculatus*)	very frequent	+	197/280

were subjected to pollen analysis using the determination of frequencies method (Louveaux *et al.*, 1970). As has been previously introduced, botanical origin of a honey may be specified when a predominant pollen (that is a pollen which constitutes more than 45% of the total, "very frequent") is present. For the six honeys in this study five can be considered of unifloral origin. It should be noted that sweet clover (*Medicago*) has a frequency classification of only "frequent", however, this pollen is an example of an underrepresented pollen. In this case, as the pollen is present at greater than 30%, it is considered unifloral and the botanical origin can be represented.

During preparation of pollen from honey, some major problems arose. Using the accepted procedures for pollen isolation, that is: 10 g honey in 20 mL water (<40°C) and centrifugation for 10 min and decanting, which may be followed by addition of water and recentrifugation for 5 min, very little pollen was isolated. Initially, it was surmised that the honeys collected contained minute levels of pollen, however upon centrifugation at 10,000 rpm for 20 min a significant pollen sedimentation resulted. Research conducted by Brush and Brush (1972) listed the specific gravity of fourteen pollen grains, ranging from 0.8-1.55. These authors indicated that the diameter of pollen grains (18-73 μm) and the specific gravity of the pollen grains play a significant role in their distribution in sediment layers. For our purposes, when we use a 10 g sample of

honey, which is composed of approximately 85% carbohydrates, a density gradient is introduced in the centrifuge tube. An assumption is made that of the 10 g of honey (assuming 15% water) 8.5 g is fructose and glucose (>90% of honey carbohydrates are these two monosaccharides; White *et al.*, 1962). The density of a 36% invert sugar solution is approximately 1.155 at 20°C (CRC Handbook, 1983). This would indicate that pollen grains with specific gravities lower than this value could be difficult to precipitate. To avoid this problem we diluted 5 g honey in 100 ml water; this gives a 3% solution of carbohydrates with a specific gravity of approximately 1.0. By centrifuging at 3,500-4,000 rpm for 5-10 min, a sediment was collected. Decanting the supernatant and recentrifugation at 10,000 rpm for 20 min yielded no further sedimentation of pollen.

Following sedimentation of the pollen grains, the sediment was placed on a microscope slide and allowed to dry before mounting with glycerine or silicon oil (Louveaux *et al.*, 1970). It was recommended to us (Schwager, 1985, personal communication) that acetolysis of the sediment be performed. This is the recommended procedure for all other forms of pollen analysis (Erdtman, 1963). This procedure was used by Lieux (1972) in his melissopalynological study of 54 Louisiana honeys. We found that, if acetolysis was not performed, the absolute identification of the pollen grains was difficult and erroneous identifications could occur. To illustrate this point, unacetolyzed and acetolyzed pollen

grains of *Salix*, *Trifolium pratense* and *Brassica* are shown in Figures 3.4-3.6, respectively. The pollen morphology of each of these grains and the grains of the honeys studied are shown in Table 3.3.

When acetolysis is not performed upon the pollen grains, it is quite obvious that difficulties in the identification of the pollen can occur. From the untreated pollen grains no clear representation of the apertures (furrows and/or pores) could be seen. When the acetolyzed pollen grain was viewed (all grains are at the same magnification), the apertures and sculpturing became more apparent (see Figures 3.6a and 3.6b).

Proteins and carbohydrates constitute part of the actual pollen grain. In addition these components are bound to the surface of the grain. The principle behind the viewing of a pollen grain under a microscope is the refraction of light from the surface of the grain. If proteins and carbohydrates mask the surface of the grain, no differentiation in refraction of light can occur and the surface shows no sculpturing. By submitting the grain to acetolysis, carbohydrates and proteins are removed and the sculpturing of the surface of the grain is revealed. Acetolysis also makes the grain translucent and dyes the grain for easier viewing (Erdtman, 1960; Moore and Webb, 1978).

By implementing these two modifications in the preparation of pollen for analysis, representative pollen

Table 3.3 Morphology of pollen grains.

Pollen grains of	Morphology
Trifolium pratense	tricolporate, coarsely per-reticulate heterobrochate, Amb semi-angular margo present; endopore visible, large colpus membrane with granules; costae colpi present; subproloate.
Trifolium hybridum	tricolporate; per-reticulate; coarse heterobrochate; subprolate margo; colpus constricted just at the equator.
Medicago sativa	tricolporate; subspheroidal; subprolate; colpus not split; colpus edge jagged; pore indistinct; psilate.
Lotus corniculatus	tricolporate; psilate or scabrate-varrucate; columellae are fine to invisible.
Melilotus alba	tricolporate; reticulate; grains are rectangular obtuse in an equatorial view; lumina in the center of a mesocolpium is slightly smaller in diameter than those towards the apolcolpia.
Brassica	tricolpate; reticulate; no margo present.
Salix	tricolpate; per-reticulate; thin walled when compared to *Brassica*; has a psilate margo.

-see Appendix 1 for a glossary of terms used in melissopalynology.

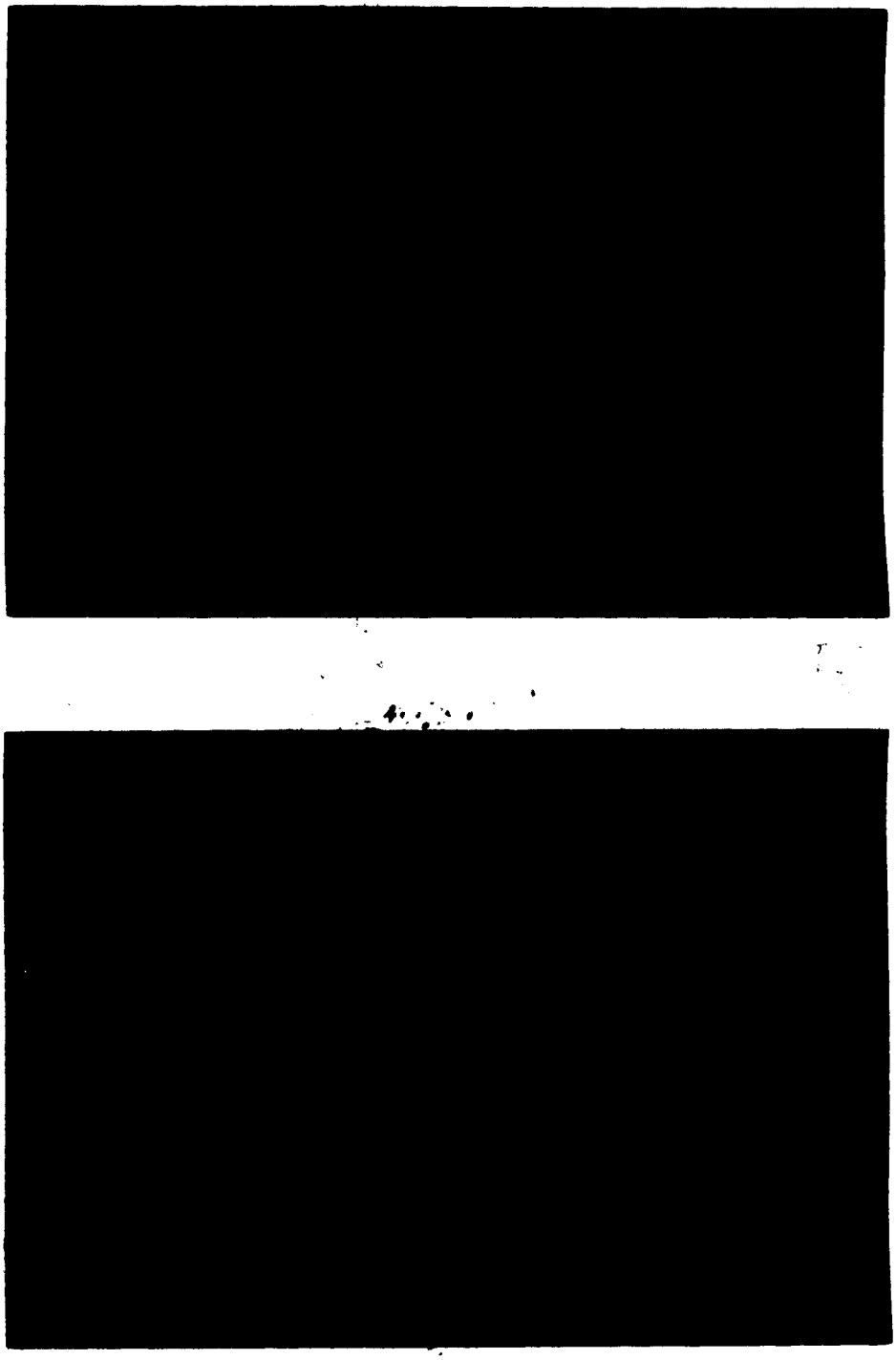


Figure 3.3 Acetylated and unacetylated pollen grains of *Salix*. a. Acetylated; b. unacetylated. (magnification 630x).

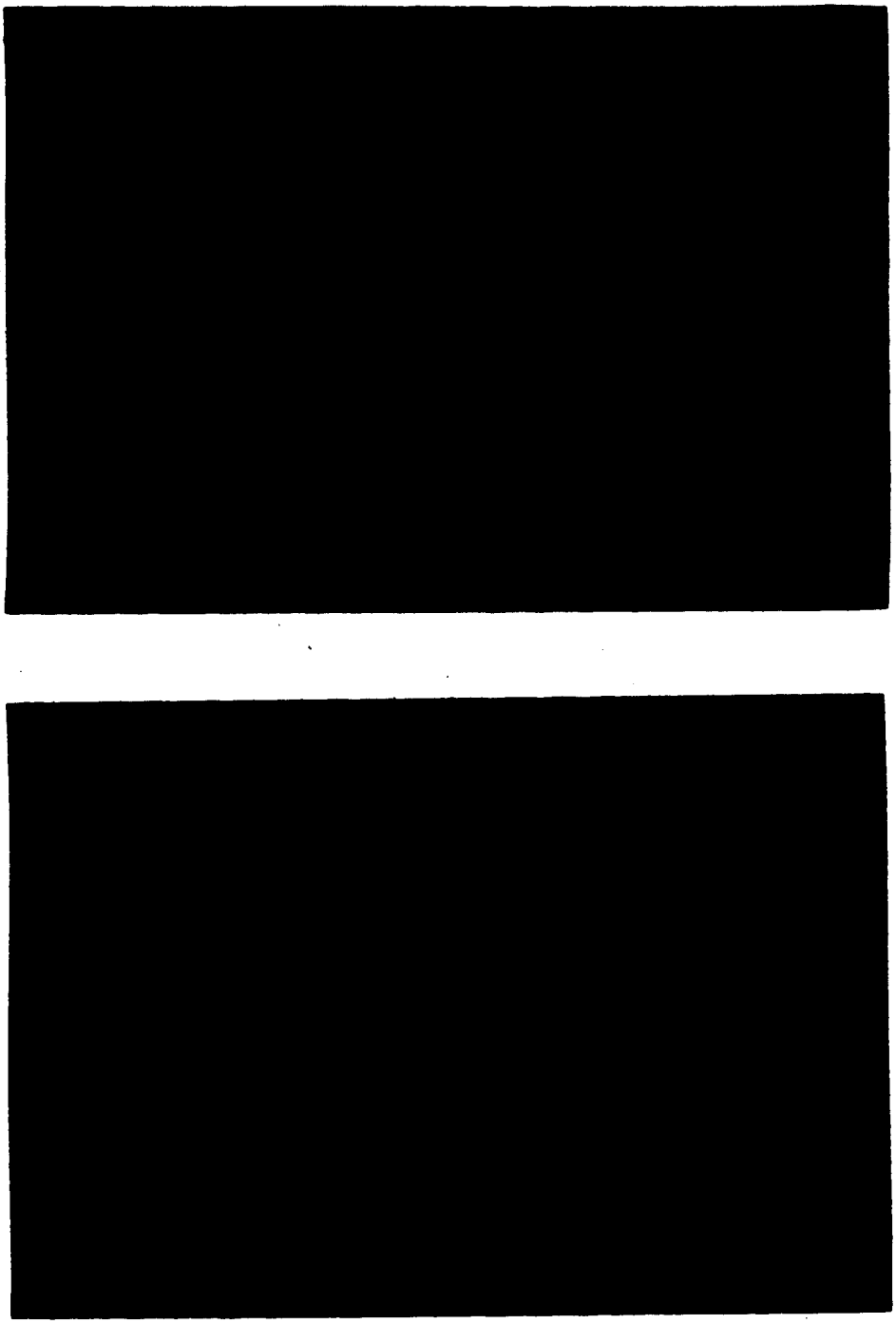


Figure 3.4 Acetylated and unacetylated pollen grains of *Trifolium pratense*. a. Acetylated; b. unacetylated. (magnification 630x).

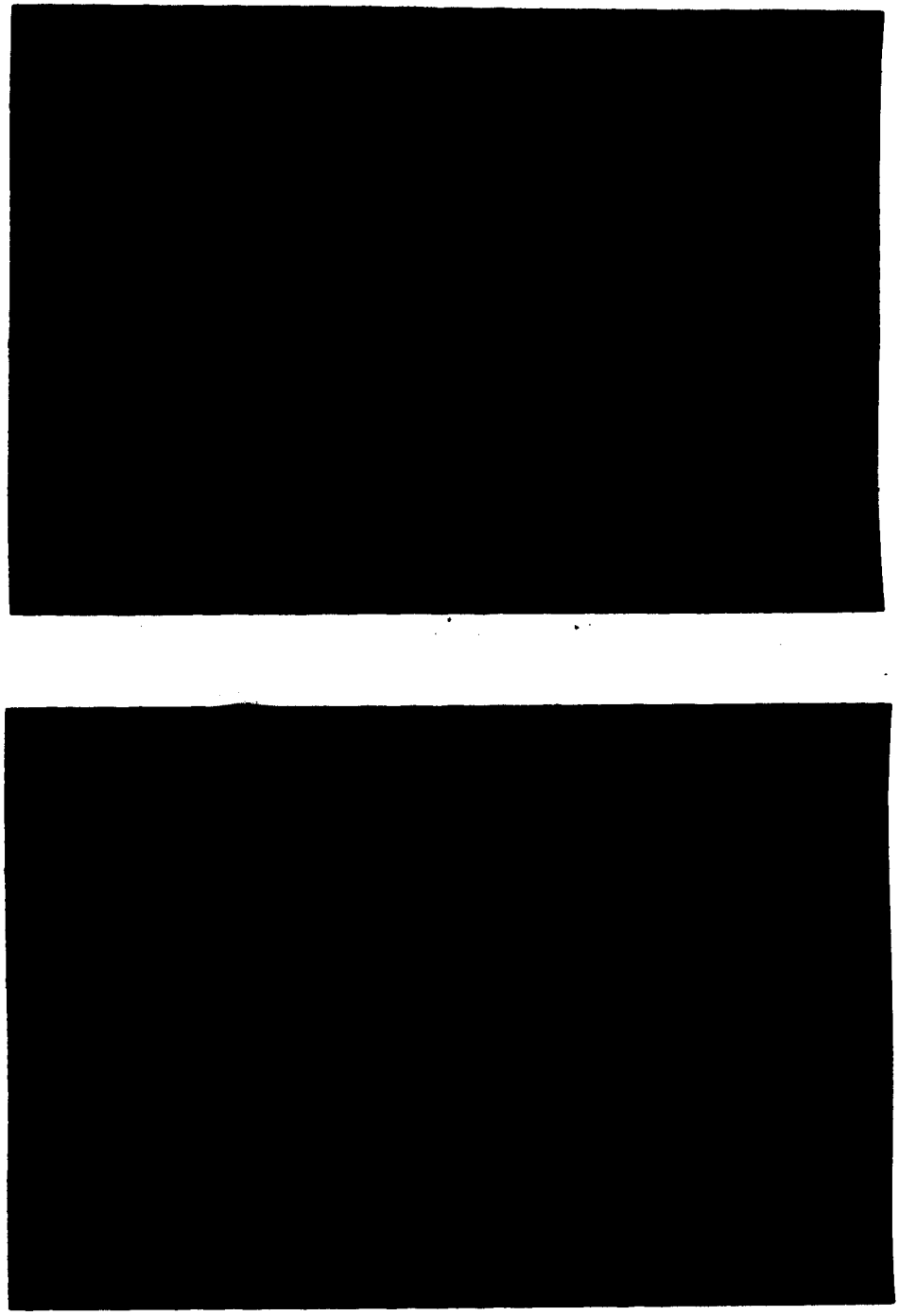


Figure 3.5 Acetylated and unacetylated pollen grains of *Brassica*. a. Acetylated; b. unacetylated. (magnification 630x).

sedimentation is assured, in addition to reduction in the number of false identifications. These factors are extremely important when exporting honeys and in the fact that clover honey is considered to be of better quality and therefore of greater value than canola honey.

Examination of pollen grains with an optical microscope can yield imperfect images due to opaqueness of the grain and its highly refractive properties. In addition, the depth of focus of an optical microscope is limited. Scanning electron microscopy (SEM) provides a considerable depth of focus and yields an extremely good representation of the surface structure of the pollen grain. Scanning electron micrographs of representative pollen grains for each of the six honeys can be seen in Figures 3.7-3.9. SEM has been applied (van Laere *et al.*, 1969) to the identification of the floral source of honey, however this methodology is expensive and time consuming.

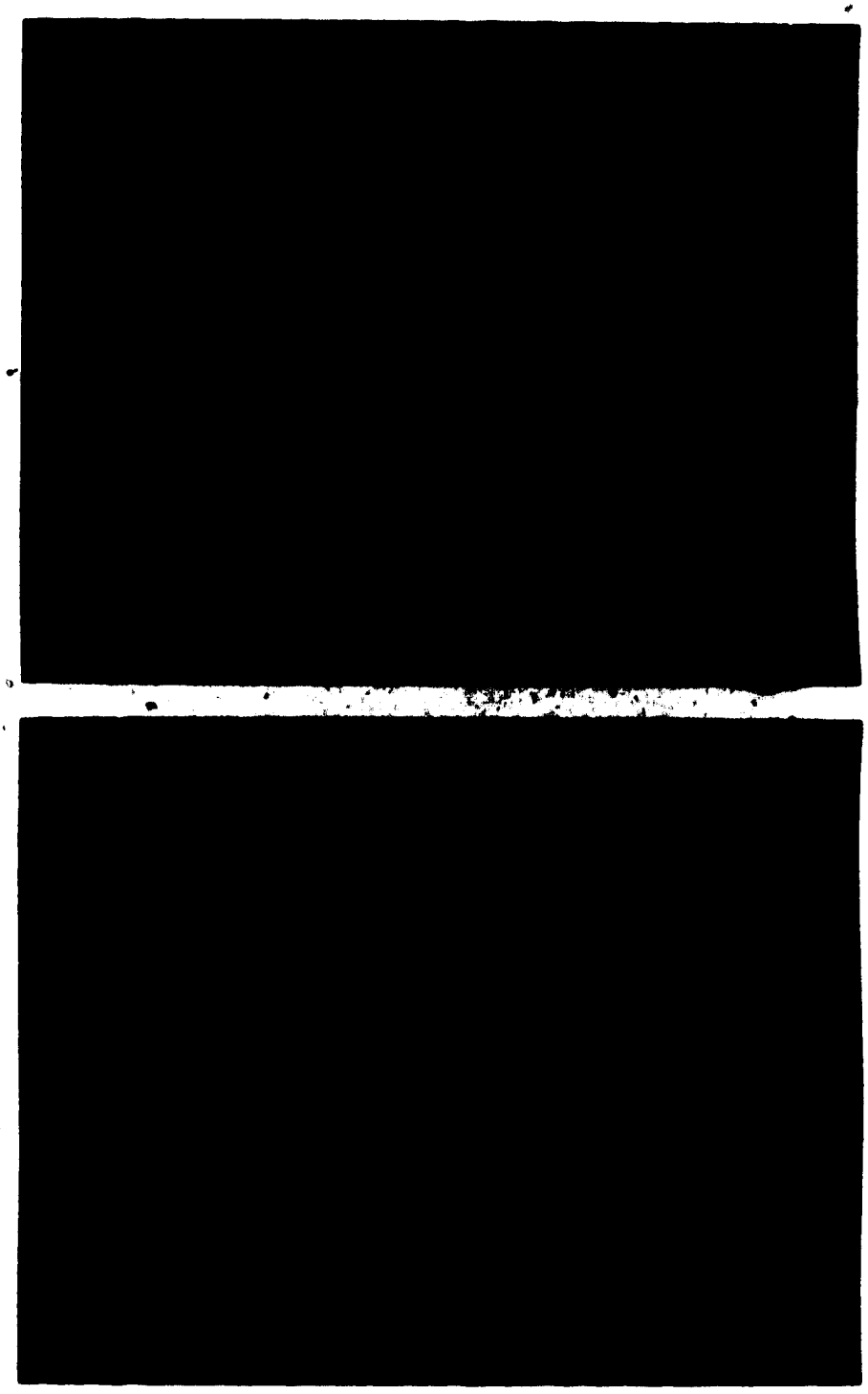


Figure 3.7 Electron micrographs of pollen grains of: a. *Lotus corniculatus*; b. *Melilotus alba*.

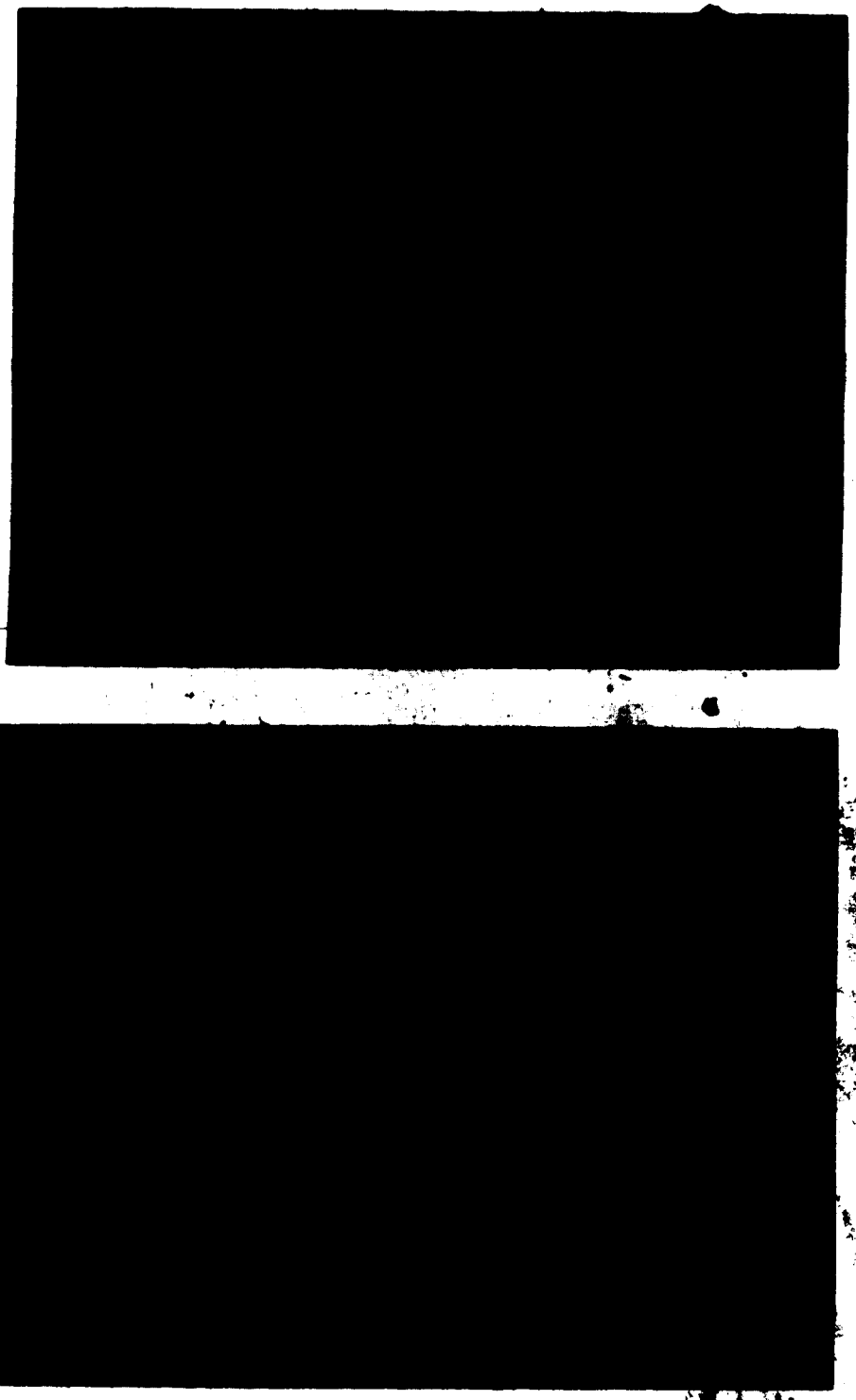


Figure 3.8 Electron micrographs of pollen grains of: a. *Trifolium pratense*; b. *Trifolium hybridum*.

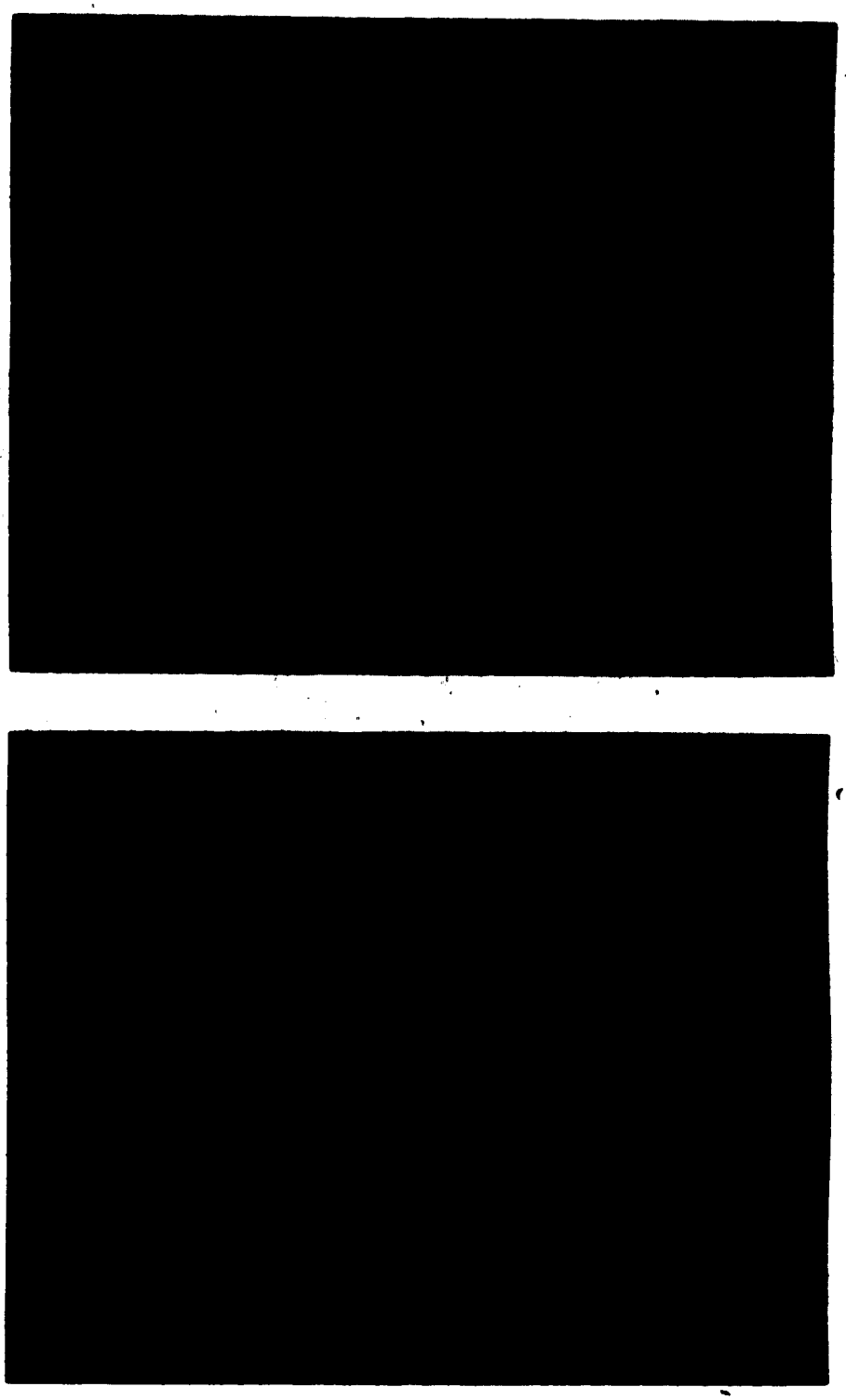


Figure 3.9 Electron micrographs of pollen grains of: a. *Brassica*; b. *Medicago sativa*.

4. NECTAR CARBOHYDRATE ANALYSIS

4.1 Introduction

The presence of sweet exudations in various flowers and their importance to bees has been known for many centuries (Aristotle, fourth century B.C.).

Initially, the liquid found in the nectary was referred to as "honey" (Müller, 1883), however, Knuth (1906) used the term "nectar" for this floral product. This term is now accepted as the correct representation for the sweet liquid produced by the nectaries of flowers.

There is considerable diversity in the location and structure of the nectary. Floral nectaries may occur on the receptacle of the flower, at the base or apex of the ovary, on the sepals, on petals, on stamens, or on combinations of the above. Extra-floral nectaries may also be present and these may be found on the leaves, stipules, stalk, and in other areas.

Caspary (1848) observed that this liquid contained in the nectary was sweet, therefore recognizing the sugar content of the nectar.

Studies on the carbohydrate composition of nectars initiated with Bonnier (1878) and were performed by many researchers (von Planta, 1886; Beutler, 1930). Wykes (1952, 1953) performed an extensive study on the composition of nectar of eighty floral species, by applying paper chromatographic techniques and comparisons to standards. Fructose,

glucose, sucrose, maltose, melibiose and raffinose were identified by this methodology. Perc[illegible] (1961), applied these same techniques to the carbohydrate identification of nectar of 900 floral species. In her work she concluded that fructose, glucose and sucrose are the main carbohydrates found in nectar. In some dicotyledons other oligosaccharides were also isolated, but only in plants with "completely broken down" nectars. She defined those nectars as those which are fructose-glucose dominant. In these nectars maltose, raffinose, melibiose and an unknown oligosaccharide were found. Studies on a variety of nectars by other researchers employing paper chromatographic techniques (Mauritzio, 1959, 1962; Bailey *et al.*, 1954; Lüttge, 1961; Zimmermann, 1953, 1954; Stockhouse, 1975; Hainsworth and Wolf, 1976) found fructose, glucose and sucrose.

Furgala *et al.* (1958) analyzed nectar from four different clovers by paper chromatography and identified fructose, glucose, sucrose and maltose.

Watt *et al.* (1974) determined the carbohydrates present in nectar by paper chromatography. The authors identified fructose, glucose, galactose, sucrose and raffinose in their nectar samples. In addition, mannose and an unknown oligosaccharide were present in a few of the nectars studied.

Jeffrey *et al.* (1969) analyzed the carbohydrates present in both the floral and extrafloral exudates of orchid species. By chromatographing on borate-impregnated silica gel G plates and by comparison with standards,

fructose, glucose, sucrose, maltose, gentiobiose, stachyose, raffinose, melibiose, lactose, melezibiose and a number of unidentified oligosaccharides were found. The authors indicated that the sensitivity of this method (1 μg) allows for the qualitative detection of all important saccharides.

Elias and Gelband (1975) applied thin-layer chromatography to the analysis of nectar from the trumpet creeper. This woody vine contains five distinct nectary systems (one floral nectar and four extrafloral) and the nectar from each was studied. The ratios of fructose, glucose and sucrose varied between the floral and extrafloral nectaries but these were the only carbohydrates isolated and identified.

Kleinschmidt *et al.* (1968) applied gas-liquid chromatography (SE-30, 5 ft x 1/8 inch) to the identification of carbohydrates in alfalfa nectar. Following collection of the nectar, the water was removed and the resulting syrup silylated (Sweeley *et al.*, 1963). This method was extremely sensitive (detecting μg quantities of carbohydrate), and α,β D-fructose, α,β D-glucose and sucrose were the only carbohydrates found. Baskin and Bliss (1969) applied gas-liquid chromatography to the analysis of orchid nectar (30 species). Following preparation of the TMS-ethers, the authors identified fructose, glucose, sucrose, maltose, cellobiose, gentiobiose, raffinose and lactose by comparison of retention times of the samples with standards.

Brewer *et al.* (1974) applied thin-layer and gas-liquid chromatography to the analysis of carbohydrates in

pistillate flowers. The authors indicated that the nectar collected was high in soluble solids (58-92%). The major carbohydrate components were identified as fructose (39%), glucose (48%) and sucrose (11%), and traces of other carbohydrates were present but unidentified.

Finch (1974) identified fructose, glucose, and sucrose as their trimethyl silyl ethers by gas-liquid chromatography of a variety of floral nectars. Two other peaks were present in a few of the nectars, but not identified.

Butler *et al.* (1972) applied gas-liquid chromatography for the identification of the TMS-carbohydrate derivatives of cotton nectar. Fructose, glucose and sucrose were the only carbohydrates present.

Van Handel *et al.* (1972) used specific quantitative assays for fructose, glucose and sucrose. The methodology involved treatment of a nectar sample with anthrone; fructose and any component containing a fructose moiety reacts with anthrone, whereas glucose and other aldehydic sugars do not (Van Handel, 1967). Sucrose can then be determined by treatment with 1 drop of 30% KOH at 100°C for 10 min, which destroys the anthrone sensitivity of all the reducing sugars, including free fructose. Sucrose remains intact under these conditions and may be quantitated by subsequent reaction with anthrone (Van Handel, 1968). Fructose concentration was then determined by the difference in optical density before and after potassium hydroxide treatment. Glucose in the nectar was determined by glucose

oxidase. From the above treatments the total carbohydrate value could be determined. When a duplicate nectar sample was treated with reducing copper reagent, the values agreed (±2%) with the sum of fructose and glucose previously determined. The authors suggested that this indicates that no other major carbohydrates are present in nectar.

Nectar analysis studies have also been carried out by Kartashova and Novidava (1964), Yakovleva (1969), Zauralov and Yakovleva (1973), Käpylä (1978) and Macior (1978). Baker and Baker (1982, 1983a,b) have reviewed the analysis of nectar carbohydrates extensively.

Baker and Baker (1979, 1982, 1983a,b) have contributed extensively to the identification of carbohydrates in nectar. These authors introduced a terminology to correlate the carbohydrate contents in nectar. By their definition a perfectly balanced nectar would contain equal quantities of fructose, glucose and sucrose, and would have a sucrose/hexose ratio of 0.5. Nectars with a sucrose/hexose ratio of more than 0.999 are labelled "sucrose dominated"; those with ratios between 0.5 and 0.999 are "sucrose rich"; nectars with ratios between 0.1 and 0.499 are "hexose rich"; and those with ratios of less than 0.1 are "hexose dominated".

Baker and Baker (1982, 1983a,b) indicated that their paper chromatographic studies support the claims that maltose, melibiose, melezitose, and raffinose may be found in nectar.

Nectar is generally derived from the phloem sap (Gunning and Steer, 1975); this viscous liquid has undergone a number of modifications while passing from the seive elements to the secretory cells. The major component of the phloem sap is sucrose, however small quantities of polysaccharides, amino acids, vitamins, lipids and inorganic ions are also present (Zeigler and Zeigler, 1962; Zimmermann and Brown, 1977; Fahn, 1979). During the process of nectar secretion, varying amounts of these compounds are removed, resulting in the less concentrated amounts found in nectar (Gunning and Steer, 1975; Simpson and Neff, 1983).

Zimmermann and Brown (1977) have shown that fructose and glucose, the two common carbohydrates found in nectar, are not found in phloem sap. The results of gas chromatographic studies (Zimmermann, 1977) of phloem have shown that sucrose accounts for >95-100% of the translocation (phloem) sugars; minor oligosaccharides such as raffinose and stachyose comprise the remainder. Baker and Baker (1983b) acknowledged that fructose and glucose in nectar may arise from sucrose hydrolysis, but also postulated that fructose and glucose are secreted from the nectary independently.

Of the majority of the nectars studied, few contain only sucrose; in addition very few contain only glucose, and no nectars studied contain only fructose (Baker and Baker, 1983b). Nectars containing only sucrose and fructose are known in a few instances (Percival, 196[illegible]insworth and Wolf, 1976; Macior, 1978), whereas, su[illegible] glucose

nectars (no fructose) and glucose and fructose nectars (no sucrose) occur moderately frequently (Baker and Baker, 1983b).

It is generally accepted that the major carbohydrates found in nectar are fructose, glucose and sucrose. Quite often, however, other sugars have been presented in the literature as being found in nectar. These include lactose and arabinose (Gottsberger *et al.*, 1973; Watt *et al.*, 1974), mannose (Watt *et al.*, 1974; Crane, 1977), cellobiose, gentiobiose and lactose (Baskin and Bliss, 1969), stachyose and mannotriose (Jeffrey *et al.*, 1970), trehalose (Percival, 1965), maltose and melibiose (Percival, 1961; Jeffrey *et al.*, 1970), melezitose (Mauritzio, 1959, 1962; Percival, 1961), and raffinose (Baker and Baker, 1983a,b and references therein).

The presence of invertase in floral nectar has been established (Beutler, 1935; Frey-Wyssling, 1954), and in other nectar species (Matile, 1956). Zimmermann (1953, 1954) suggested the presence of transglucosidase and transfructosidase enzymes in some nectars. The presence of these enzymes in nectar could account for fructose, glucose and some of the oligosaccharides isolated from nectar.

There are a variety of factors influencing the amount and composition of nectar produced. Both internal (genetic) and external factors have been shown to affect both nectar secretion and concentration (Shuel, 1955; Percival, 1965; Corbet, 1978). External factors include sunlight, a

temperature, soil conditions, humidity, altitude and the time of day the nectar is collected (Shuel, 1955).

4.2 Nectar Carbohydrate Analysis Experimental

Nectar from the floral nectaries of some flowering cultivars (canola, trefoil, alfalfa, alsike, red clover, and sweet clover) were collected (between 10-11 a.m.) using 10 μL capillary tubes connected to a water aspirator vacuum apparatus. Approximately 30 μL of nectar could be collected from 200 flowers. The nectar was partitioned ($CHCl_3/H_2O$, 1:1, v/v) and the aqueous phase was passed through a C_{18} sep-pak (Waters). Evaporation on a Buchii rotavapour R was followed by the addition of 2 mL of reverse osmosis Milli-Q purified water (HPLC-grade water). This solution was filtered through a Swinney-25 syringe adapter using a 0.45 μm nylon-66 membrane filter (25 mm diameter, Rainin Instr. Co.). The filtered solution was analysed by high performance liquid chromatography (HPLC).

The HPLC system consisted of a Whatman Partisil PXS PAC 10/25 column (250 mm x 4.60 mm) equipped with a guard column (60 mm x 4.60 mm) packed with Whatman CO: Pell PAC media (30-38 μm). The columns were in series with a Whatman differential refractometer (Waters model R401) maintained at 25.5°C by means of a circulating water bath. The sample was introduced by means of a Rheodyne (model 7120) equipped with a 50 μL injection loop. Elution was afforded with a mobile phase consisting of acetonitrile-water-1% ammonium hydroxide

(80/20/1, v/v/v) at a flow rate of 1.0 mL/min (Beckman model 110A pump). A permanent copy of the analysis was obtained using a chart recorder (Terochem Laboratories) set at a chart speed of 20 cm/hr.

For sweet clover the flowers were so small that the nectaries could not be penetrated without a significant amount of plant material being included. To collect the nectar in this case, 300 flowers were collected and 10 mL of HPLC-grade water was added, and the mixture shaken gently for 2 min. Following centrifugation at 2000 rpm for 3 min, the supernatant was removed and passed through a C_{18} sep-pak. The remainder of the analysis was identical to that used previously.

Evaporation (Buchi Rotavapour R) to dryness of 1 mL of the HPLC nectar sample (in a 10 mL pear-shaped flask B14/20) was followed by co-evaporation with toluene. To this dried sample (approx. 0.5 mg) was added 1.5 mL of tri-sil Z (Pierce Scientific Co.; 1.5 meq/mL) through a rubber septum. The resulting solution was stirred at 60°C for 30 min, then the temperature was raised to 80°C for a further 30 min. Gas chromatographic (Varian 3700) analysis of 1.0 μL of this sample was accomplished using a DB5 capillary column (30 m x 0.25 μm; J&W Scientific Co.) with a splitter ratio of 30:1. The carrier gas was 99.999% pure helium with a flow rate of 0.27 m/sec. Elution was carried out using a temperature program of 210°C/10 min, 2 C° min/295°C. The injector port and detector (FID) temperatures were maintained at 300°C.

4.3 Nectar Carbohydrate Analysis Results and Discussion

Nectars of the six floral sources (canola, trefoil, alfalfa, alsike, sweet clover and red clover) representing the six honeys collected were subjected to carbohydrate analysis, employing both high performance liquid chromatography (HPLC) and gas chromatography (GC).

Table 4.1 shows the carbohydrates present in each of the nectars analyzed (in duplicate) and their corresponding concentrations. In addition, the nectars from various canola species were analyzed by HPLC, and the results from these analyses are shown in Table 4.2.

The data in Table 4.1 indicate that fructose is the major carbohydrate in each nectar studied, with the exception of canola. In addition, each nectar contains the carbohydrate sucrose, except for canola. No other carbohydrates were found by this HPLC methodology. It should be mentioned, however, that refractive index detection is not extremely sensitive. The results do indicate that reproducibility of results is excellent.

The nectars collected from the different canola species indicate a variance in the absolute concentration of fructose and glucose among species. However, in each case tested no sucrose was detected by this methodology. The detection limits for glucose, fructose and sucrose were approximately 28 ppm, 25 ppm and 42 ppm, respectively.

The nectars for each of the canola species were collected at various times (morning, afternoon, early and

Table 4.1 Nectar carbohydrate analysis.

Floral source	Sugars in mg/3 μL		
	Fructose	Glucose	Sucrose
Alfalfa	0.53 0.51	0.35 0.38	0.14 0.15
Alsike	0.27 0.26	0.21 0.20	0.13 0.13
Canola	0.25 0.27	0.31 0.31	- -
Red Clover	0.35 0.35	0.30 0.32	0.10 0.12
Sweet Clover	0.29 0.27	0.22 0.21	0.20 0.21
Trefoil	0.24 0.28	0.14 0.16	0.20 0.18

Table 4.2 Sugars in nectar from rapeseed cultivars.

Cultivar	Sugar in mg/3 µL	
	Fructose	Glucose
Altex	0.43	0.58
	0.39	0.48
	0.54	0.70
	1.00	1.13
Andor	0.70	0.83
	0.31	0.38
Candle	1.05	1.14
	1.11	0.87
	1.16	0.87
	1.11	0.78
Reagent	0.78	0.79
	0.58	0.73
	0.72	0.83
	1.21	1.42
Tobin	0.70	0.86
	0.76	0.87
	0.62	0.77
	0.74	0.78
Weston	0.46	0.71
	0.80	1.00
	0.97	1.22
	1.00	1.19

(variations in the results may be explained by nectar collection at various times)

late evening) and the variances in the absolute concentrations of fructose and glucose can be explained by the different collection times and by the fact that different flowers produce different levels of these carbohydrates, in addition to the other factors which influence nectar secretion. Shuel (1955), Percival (1965) and Corbet (1978) have shown that for most species optimum nectar secretion occurs between 10-11 a.m. and between 2-3 p.m. Furthermore, optimum soil temperature, humidity and amount of sunlight will result in a more concentrated nectar liquid.

The fructose/glucose ratio within each canola species is relatively consistent regardless of the time of nectar collection. Baker and Baker (1982, 1983a,b) indicated that the fructose/glucose/sucrose ratio in nectar is controlled by internal (genetic) factors rather than by external factors.

One of the main objectives of this work was to establish how and where the minor carbohydrates of honey are formed. As has been introduced, a number of researchers have indicated that nectar contains carbohydrates of higher complexity in addition to fructose, glucose and sucrose. It should also be mentioned that other researchers have found only the carbohydrates fructose, glucose and sucrose in nectar. As high performance liquid chromatography is limited both in resolution and sensitivity, an alternate method of carbohydrate analysis was used.

Anhydrous nectar from each of the floral sources was treated with tri-sil Z (Pierce Scientific Co.) to form their corresponding O-trimethylsilyl derivatives. Gas-liquid chromatography of these volatile carbohydrates indicated that only fructose, glucose, and sucrose were present in five of the six nectars analyzed. Alsike nectar had two very small peaks at 36.53 and 36.57 min, respectively. These values compare with the retention time of the disaccharide maltose. Spiking of the nectar sample with maltose indicated that the other carbohydrate present could be maltose. Detection levels for disaccharides were 10 ppb, and for trisaccharides 40 ppb. These values indicate that the main components (>99.8%) of the nectars analyzed were fructose, glucose, and sucrose. More complex carbohydrates appearing in the honeys arising from these botanical origins result from secretions or modifications by honeybees.

5. CARBOHYDRATE ANALYSIS BY HPLC

5.1 Introduction

A wide range of chromatographic techniques exist for the identification and quantitation of carbohydrates. The earlier methods of paper chromatography (PC), thin-layer chromatography (TLC) and column chromatography have for the most part been replaced by instrumental methods such as gas chromatography (GC) and high performance liquid chromatography (HPLC). These instrumental methods, in general, provide rapid analysis with greater specificity and precision. Recently, HPLC has replaced GC as the most commonly used technique for carbohydrate analysis. However, although HPLC does offer advantages over GC for carbohydrate analysis, both techniques are far from ideal. Thus, GC, using the flame ionization detector, is far more sensitive than HPLC with refractive index detection (Folkes and Taylor, 1982). However, GC analysis requires the formation of a volatile derivative of the carbohydrate, while HPLC analysis requires no such modification.

In attempts to increase the sensitivity of carbohydrate analysis by HPLC, a number of different detection systems have been employed. Conventional refractometers require column loadings of approximately 40 µg (Folkes and Taylor, 1982) for precise quantification. In most food products this is not a serious problem as carbohydrates are often present as the major components and levels of individual sugars as

low as 0.5% can be determined (Folkes and Taylor, 1982). However, if trace analysis of carbohydrates is required, refractive index detection can be a liability.

Refractive index is a bulk physical property and anything which alters this parameter results in a detector response. This may lead to problems if interfering materials are eluted at similar retention times to desired carbohydrates. In addition, the refractive index changes dramatically with temperature, pressure, composition and levels of dissolved air. In order to minimize these problems, the detector should be thermostatically controlled, solvent delivery systems pulse free and all solvents degassed before use. As each solvent system used will have a different refractive index, the use of gradient systems for elutions with this detector is severely restricted. In addition, if the temperature of the system changes, the equilibrium between solvent components absorbed onto the stationary phase and remaining in the mobile phase may be altered. Refractive index detectors based on interference effects have been marketed and have increased sensitivity, yielding good results with column loads of 1-5 µg (Berglund and Thente, 1983).

Attempts have been made to increase the sensitivity of HPLC determinations of carbohydrates by using different detectors. These include ultraviolet, mass, electrochemical, flame ionization (Wells and Lester, 1979) and polarimetric detectors. Reviews on the applicability and problems with

these detectors can be found in various articles (Macrae, 1985; Edwards and Haak, 1983).

Another method used to increase the sensitivity in HPLC for carbohydrate determination is the use of postcolumn labeling. The initial methods of postcolumn labeling of carbohydrates involved colour reactions with chromogenic reagents in strong mineral acid (Martinsson and Samuelson, 1970). Examples of these colour reactions include carbohydrate reaction with resorcinol (Smith *et al.*, 1978); orcinol (Simatupang, 1979) and anthrone (Kramer *et al.*, 1978). Other postcolumn labelling methods have been used, including oxidative methods (Larsson and Samuelson, 1976), fluorogens (Mopper *et al.*; 1980; Kato and Kinoshita, 1980), lutidine formation (Samuelson and Strömberg, 1966; Carlsson *et al.*, 1968), high efficiency electrodes [for the measurement of oxidizable compounds] (Baker *et al.*, 1983c; Watanabe and Inoue, 1983; Rocklin and Pohl, 1983), and using the reducing power of carbohydrates (Mopper and Gindler, 1973; Mopper and Degens, 1973).

In addition, other selective methods may be used (Honda, 1984). Precolumn derivatization may also be employed to increase the sensitivity in carbohydrate detection. Derivatives which fluoresce include dansylhydrazones, dansylamides and 1-(N-2-pyridylamino)deoxyalditols (Honda, 1984). Those derivatives which can be detected by absorption chromatography are presented in that section.

Recently, nuclear magnetic resonance spectroscopy has been used as an on-line detector for HPLC (Dorn, 1984). In addition, improvement in the vacuum nebulizing interface allowed direct coupling of HPLC and mass spectrometry (Alborn and Stenhagen, 1985; Liu *et al.*, 1985).

The separation of carbohydrates by HPLC has been performed using a number of different stationary phases. A successful separation of carbohydrates by HPLC was accomplished by Cerning-Bervard and Filiatre (1976) using a gel filtration column. These authors were able to separate mono-, di- and trisaccharides. For simple sugar mixtures where the molecular weight of each component differs significantly, adequate separation can be obtained using gel filtration (Sephadex and Bio-Gel) columns. Problems arise when this technique is applied to carbohydrates of similar molecular weights, and very long analysis times are required using gel fitration HPLC techniques (Macrae, 1985). This method is used to determine molecular weight profiles of polysaccharides (Macrae, 1982).

Ion-exchange resins have been applied to carbohydrate separations since the early 1970's (Hobbs and Lawrence, 1972). In some cases strong cation-exchangers are employed, often in the calcium or lithium form. In these cases the mechanism is one of partition rather than ion-exchange (Hobbs and Lawrence, 1972). Carbohydrates may also be complexed with borate ions and separated on anion-exchange columns (Floridi, 1971). Ion-exchange techniques suffer from

a number of disadvantages, including long analysis times (the anion-exchange separation of a few carbohydrate-borate complexes required as long as 20 days; Khym and Zill, 1952), acid hydrolysis of oligosaccharides (anion-exchange resins), and the need to operate columns at elevated temperatures. Recent advances in ion-exchange materials have allowed higher flow rates to be used, which results in shorter analysis times. An advantage of ion-exchange chromatography is the use of water as the mobile phase, which avoids the use of toxic solvents such as acetonitrile.

Adsorption chromatography has been applied where silica gel has been used both directly and after modification of the phase or the carbohydrate. Rocca and Rouchouse (1976) use a polar mobile phase of ethyl formate/methanol/water (6:2:1, v/v/v) to separate fructose, sorbitol, sucrose and lactose. Other researchers have employed precolumn derivatisation, such as the formation of nitrobenzoates (Nachtmann and Budna, 1977), p-nitrobenzyloximes (Lawson and Russell, 1980), and many others (Honda, 1984). This derivatisation reduces the polarity of the carbohydrates and allows for the separation with less polar mobile phases. In addition, the derivatives now absorb stongly in the ultraviolet region, thus allowing for detection at much lower levels. The more commonly encountered use of adsorption chromatography is when this technique is used in conjunction with amine modifiers [e.g. tetraethylenepentamine (TEPA)]. Aitzetmuller (1978) was the first to use this technique and it has been

used by many researchers since that publication (Aitzetmuller, 1980; Aitzetmuller *et al.*, 1979).

The most widely applied system for the HPLC analysis of carbohydrates is partition chromatography. A variety of phases have been prepared, including cyano, amino and hybrid phases of these two types (Rabel *et al.*, 1976). Polar amino or aminocyano phases are compatible with a wide range of polar solvents, however, almost all published [illegible] employ aqueous acetonitrile (Macrae, 1985). Aqueous alcohols may be used but have been shown to yield inferior resolution, presumably related to the fact that these alcohols can hydrogen-bond with the stationary phase amino groups. If the separation of monosaccharides is desired, then elution with 15-20% water/acetonitrile is recommended, whereas higher oligomers require 40-50% water/acetonitrile (Macrae, 1985). If complex mixtures of monosaccharides and higher oligomers require analysis, then gradient systems would aid in the separation, however, when using refractive index detection, this type of separation is precluded.

Although the main use of partition chromatography is based on the use of polar-bonded phases, there is an alternative method using reverse-phase chromatography. This methodology has been mainly restricted in its use to separating carbohydrates with different monosaccharide units (Palla, 1981; Macrae, 1985). This methodology has been applied to monosaccharide separations and disaccharide separations, but the resolutions are inferior to those

achieved using polar-bonded phases (Macrae, 1985). Reverse-phase columns do have an advantage in that these phases are not fouled by polar molecules.

Honda (1984) has reviewed the literature (1975-1983) for separation of simple oligosaccharides by HPLC. In many of these papers good separation was achieved for maltooligosaccharides and fructooligosaccharides. It was obvious from the literature that separation by HPLC of more complex mixtures of disaccharides, like those found in honey for example, has not been performed (Honda, 1984; Nikolov *et al.*, 1985a,b).

5.2 HPLC in Food Carbohydrate Analysis

High performance liquid chromatography has been extensively applied to the determination of carbohydrates in foods. These determinations include the qualitative and quantitative determination of monosaccharides (mainly fructose and glucose) and disaccharides (mainly sucrose) in fruits (Wade and Morris, 1982; Reyes *et al.*, 1982), cereals (Zygmunt, 1982; Iverson and Bueno, 1981), vegetables (Martin-Villa *et al.*, 1982), coffee (Trugo and Macrae, 1982), and fruit juice (Shaw and Wilson, 1983). Many other applications of HPLC analysis of carbohydrates to food are known and have been extensively reviewed (Macrae, 1982, 1985; Honda, 1984).

Carbohydrate analysis by HPLC has found many uses in food due to rapid analysis times, ease in the preparation of

samples, injection of underivatized samples in most cases, and ease of automated analysis (Honda, 1983).

The first use of HPLC amino columns for carbohydrate (mainly monosaccharide) analysis was by Linden and Lawhead (1975) and Palmer (1975). The use of these types of columns has been extended to oligosaccharides, mainly sucrose, lactose, raffinose and maltooligosaccharides (Verhaar and Kuster, 1981; Baust *et al.*, 1983; Linden and Lawhead, 1975).

Recently, these types of columns, employing stationary phases of amino-propyl, cyano, and combinations of amino and cyano have been used to attempt the separation of more complex mixtures of carbohydrates similar to those found in honey.

Ghernat *et al.* (1982) applied HPLC, using a micro-bondapak NH_2 column with acetonitrile/water as the mobile phase, to determine the major sugars contained in Algerian honey. The authors indicated the separation of fructose, glucose, sucrose and maltose was possible using their conditions. In addition, separation of lactose and raffinose was possible, but they were not found in the honeys tested.

Nikolov *et al.* (1984) attempted the separation of honey disaccharides using amine-modified silica columns (Waters micro Bondapak-NH_2, Du Pont Zorbax-NH_2). The authors indicated that six disaccharides could be detected and identified. The chromatograms shown, however, were extremely broad, with considerable overlapping of peaks and shoulders which were identified as peaks. The authors proposed that

this methodology could be used to detect adulteration of honey by the addition of high fructose corn syrup.

Moriyasu *et al.* (1984) applied HPLC to the separation of sugar anomers at low temperatures using a Nucleosil amine column. They attempted to separate a number of sugar anomers (including glucose, maltose, lactose, fucose and mannose) at temperatures ranging from 5°C to -45°C. Optimum separations occurred below -25°C using the mobile phase acetone-water-acetic acid (100:11:1, v/v/v).

Nikolov *et al.* (1985a) applied HPLC for the separation of twenty disaccharides using two commercially prepacked aminopropyl bonded silica columns (Supelcosil LC-NH_2 and Zorbax-NH_2). They found that the elution among gluco-pyranosyl-glucose disaccharides proceed in a pattern of (1→3)-linked disaccharides, then (1→4)-, a mixture of (1→2)- and (1→1)-, and finally (1→6)-linked disaccharides. In addition, these workers found that the replacement of a glucosyl residue by galactose resulted in longer retention times, while substitution by a fructose residue gave shorter retention times. From an analysis of the retention times and chromatograms presented for the standard carbohydrates, the peaks were broad and severe overlapping occurred for both columns. Even the application of different mobile phases did not greatly improve the separation. For example, cellobiose, sophorose, α,β-trehalose, lactulose, α,α-trehalose, koji-biose, lactose, β,β-trehalose and turanose were virtually unresolvable using a Supelcosil LC-NH_2 column with a mobile

phase of 75% acetonitrile/water.

Nikolov *et al.* (1985b) extended this work to the separation of thirteen trisaccharides. They found that the replacement of a glucose unit by either fructose or xylose yielded shorter retention times, while replacement by galactose gave longer retention times. Reasonable separations of (1→3)- from (1→6)-linked trisaccharides was possible using their methodology. As was the case with disaccharides, broad peaks and severe overlapping occurred. The authors indicated that gas chromatography was advisable for separation of carbohydrates of similar structure.

Cai and Zhu (1985) used a microbondapak column for the separation of glucose, galactose, sucrose, maltose and isomaltose in various food samples. Using a mobile system of acetonitrile/water, the authors were able to qualitate the presence of these carbohydrates in honey, beets, grapes and wort.

Blanken *et al.* (1985) analyzed 65 neutral oligosaccharides by HPLC, using an amine-modified silica column (Lichrosorb-NH_2). They found that chromatographic behaviour could be linked to specific structural features of the oligosaccharides. General observation indicated that the retention time increased with the number of sugar residues. The presence of N-acetylglucosamine or a fucose residue shortened the retention time and a dramatic increase in retention time was noted if the oligosaccharide contained a (1→6)-linkage. By using an elution system of acetonitrile/

water containing 15 mM potassium phosphate (pH 5.2), followed by a linear gradient of increasing buffer, maltose, isomaltose, cellobiose, gentiobiose, mellibiose and lactose could be separated.

Applications of other stationary phases for carbohydrate analysis by HPLC have been developed recently.

Porsch (1985) reacted Separon SIX silica (reverse-phase media) with hydrochloric acid and octadecyltrichlorosilane in boiling toluene with pyridine. This was followed by reaction of the product with γ-aminopropyltriethoxysilane to yield a reverse-phase with chemically-bound primary amino groups. This stationary phase was then employed for the HPLC separation of starch hydrolates using water as the mobile phase; DP (dextrose polymer) 1-9 were separated by this methodology. Reverse-phase chromatography media for the separation of oligosaccharides are known (Honda, 1984). One of the shortcomings of these types of media is the presence of α- and β-anomers in the chromatograms, which complicates the spectra (Cheetham *et al.*, 1981). By doping (with chemically-bound primary amino groups) the surface of the reverse-phase silica, the stationary phase has a sufficient catalytic effect on the rate of anomerization so that no peak splitting occurs.

Hull and Turco (1985) analyzed malto-oligosaccharides from corn syrup by HPLC using a silica gel modified with 1,4-diaminobutane (DAB). They employed a technique that had been introduced by Ackermann and Köthe (1974) for the

analysis of monosaccharides. This involved the reaction of the neutral oligosaccharide with dansyl hydrazine to yield the corresponding sugar dansyl hydrazone as a mixture of isomers. These derivatives were then separated using acetonitrile/water/DAB as the elution solvent. Dextran polymers (DP) of 2-7 could be separated.

Bonn (1985) applied a series-connected system of different ion-exchange columns for the HPLC separation of monosaccharides, oligosaccharides, sugar degradation products and alcohols. Four stationary phases were employed in this study: a Ca-loaded sulphonated polystyrene-divinylbenzene resin (I); Ca-loaded cation-exchange resin (II); H-loaded ion-exchange resin (III); and a Ag-loaded cation-exchange resin (IV). Any two of the above stationary phase columns could be coupled with a pre-column for separation purposes. The elution solvent was water and a refractive index detector was used. The sample to be separated was obtained by hydrothermal treatment of poplar wood (Bonn *et al.*, 1983). Optimal separation was afforded when stationary phases I and IV were employed. With these columns in series DP from 1-7 could be separated in addition to other components.

Koizumi *et al.* (1985) investigated the separation of (1→2)-, (1→3)-, (1→4)- and (1→6)-linked homogenous D-glucan oligosaccharides and -polysaccharides by HPLC on a 3 μm chemically-modified amine column. The saccharide samples were prepared by the partial hydrolysis or partial

acetolysis of the following: cyclosopharose; curdlan, amylose, cellulose, and luteose. Acetonitrile/water was used as the eluent and effective resolution of these saccharide samples up to a DP of 33 was afforded.

Kennedy *et al.* (1985) analysed the oligosaccharides in the extracts of soybean seed meal by employing HPLC using Waters Dextropak C_{18} reverse-phase column. The methodology involved analysis of extracts after an initial treatment of ultrafiltration with a 25,000 molecular weight cut-off filter. By employing water as the eluent, the oligosaccharides, sucrose, raffinose and stachyose could be separated.

Iwata *et al.* (1984) prepared an aqueous column of plain silica gel (Aquasil) for the separation of neutral oligosaccharides. This type of column was prepared by packing an HPLC column with Nucleosil 50-5 silica gel in a normal slurry method. Methanol was then passed through at a flow rate of 12 mL/min for 20 min, followed by chloroform-methanol-ethanol-water (62:16:16:6) at the same flow rate for 30 min. The authors indicated that these types of columns have several advantages over reverse-phase columns, including lower operating pressures, high solubility of samples, avoidance of extremely toxic elution solvents (acetonitrile), and the lower cost of columns. In their experiment, Iwata *et al.* (1984) separated sucrose, maltose and lactose standards and a series of malto-oligosaccharide standards with the mobile phase ethyl acetate-methanol-water

(12:3:1).

Cheetham and Teng (1984) attempted the separation of malto-oligosaccharides (DP 1-13) and isomalto-oligosaccharides by reverse-phase high performance liquid chromatography. They added cationic, anionic and non-ionic surfactants, tetramethyl urea and organic solvents to the mobile phase to reduce the analysis time and sharpen the peaks. The addition of the neutral, inorganic salts allowed the separation of oligosaccharides which were poorly resolved by water alone. The authors suggested that the solvophobic mechanism is in operation during these separations in addition to hydrogen bonding.

Asakawa (1985) modified a radial-pak silica gel cartridge with 0.02% 1,4-diaminobutane for the HPLC separation of malto-oligosaccharides. This methodology was applied for the analysis of enzymatic digest of amylose.

Ohtsuki *et al.* (1984) determined the reducing sugars in kiwi fruit using Dianion (CA08S) and Shodex (S-801) stationary phases. By post-column labelling with 2-cyanoacetamide, glucose, galactose and fructose were identified.

5.3 HPLC Experimental

5.3.1 Honey collection

New honey supers together with packaged bees were introduced in six flowering cultivating fields. These fields were alfalfa, alsike, canola, red clover, sweet clover and

trefoil. The honey collected by these bees was collected and frozen until analysis.

5.3.2 Analytical carbohydrate determination

Approx. 350 mg of honey was dissolved in 5 mL of reverse osmosis Milli-Q purified water (HPLC-grade water). This solution was transferred to a 10 mL volumetric flask and the volume made up with HPLC-grade water. A portion of this solution was filtered through a Swinney-25 syringe adapter using a 0.45 μm nylon-66 membrane filter (25 mm diameter). The filtered solution was subjected to analysis by HPLC employing a Whatman Partisil PXS PAC 10/25 column (250 mm x 4.60 mm) equipped with a guard column (60 mm x 4.60 mm) packed with Whatman CO: Pell PAC media (30-38 μm). The columns were in series with a Whatman differential refractometer (model R401) maintained at 25.5°C by means of a circulating water bath. The sample was introduced by means of a Rheodyne (model 7120) equipped with a 50 μL injection loop. Elution was afforded with a mobile phase consisting of acetonitrile-water-1% ammonium hydroxide (80:20:1, v/v/v) at a flow rate of 1.5 mL/min (Beckman model 110A pump). A permanent copy of the analysis was obtained using a chart recorder set at a chart speed of 20 cm/hr. Under these conditions the elution order of monosaccharides was fructose followed by glucose, with retention times of 6.6±0.1 min and 9.5±0.1 min, respectively. This methodology was used to determine the fructose/glucose ratio for trefoil, sweet

clover, red clover, alsike, alfalfa, and canola honey. Each analysis was done in triplicate and compared to standard curves.

For the disaccharide and trisaccharide standards, 1-5 mg of each standard was dissolved in 100 µL of HPLC-grade water and the analysis was carried out as previously mentioned.

5.3.3 HPLC separation of honey monosaccharides

To approx. 10 g of honey was added HPLC-grade water to a total volume of 30 mL. This solution was then passed through a Swinney-25 syringe adapter using a 0.45 µm nylon-66 membrane filer (25 mm diameter). A 6.0 mL aliquot of this solution was injected (Rheodyne model 7120; 5 mL injector loop) onto a Whatman Partisil M 20/50 preparative HPLC column (50 cm x 18 mm) equipped with a guard column (140 mm x 11 mm) packed with CO: Pell PAC media (30-38 µm). This system was in series with a Whatman differential refractometer (model R401) maintained at 25.5°C by a circulating water bath. Elution of the monosaccharide fraction was accomplished with acetonitrile-water (80:20; v:v) as the mobile phase at a flow rate of 7.0 mL/min (Beckman model 110A pump). Approx. 850 mL of this mobile phase was required. The mobile phase was then changed to acetonitrile-water (50:50; v:v) to elute the oligosaccharide fraction (450 mL of solution was collected). This procedure was repeated four times, for a total of five passes for each

honey. Evaporation of the combined oligosaccharide fractions using a Buchi rotovapour R with water aspiration yielded approx. 430 mg of a slightly coloured syrup.

5.3.4 Acetonitrile recovery

As HPLG-grade acetonitrile is quite expensive (approx. $15/L), all HPLC waste solvent used was recycled. The solvent was distilled (boiling fraction 74-76°C for azeotrope, at a pressure of 706 torr). By measurement of the refractive index (Abbe-Refractometer, Carl Zeiss, G.D.R.) of the distillate, the acetonitrile-water percentages could be calculated as follows:

Let x be the weight in g of acetonitrile.

Let y be the weight in g of water.

$$r = (1/d)(n^2-1)/(n^2+2)$$

r = specific refractivity of the compound

n = refractive index

d = density = weight/volume

r for acetonitrile = 0.2695 (at 22°C)

r for water = 0.2057 (at 22°C)

25 mL of azeotrope is weigh = wt; and d = wt/25 mL

then,

a) $x + y = wt$

b) $0.2695x + 0.2057y = wt \times r$ (of azeotrope)

and solve the simultaneous equations.

The above procedure resulted in approx. 80% acetonitrile recovery.

5.4 HPLC Results and Discussion

Monosaccharide analysis was initially performed on the six honeys to determine the fructose to glucose ratio for each honey. These values are shown in Table 5.1, using an analytical HPLC column with acetonitrile-water-1% ammonium hydroxide as the mobile phase. This HPLC method for fructose and glucose determination was not the AOAC (Official Methods of Analysis, 1984) method for honey. The accepted method involves the preparation of an adsorption column, followed by fractionation and titration of the appropriate fraction. The official method was labourious and time consuming, whereas this HPLC method involved a simple dilution followed by filtration and analysis. As shown in Table 5.1, the reproducibility of results was high (relative standard deviation after ten injections of standard fructose and glucose solutions was 1.0% and 0.5%, respectively) and the analysis time was short (less than 14 min). In addition, baseline separation of these two monosaccharides was attainable, which greatly reduced the errors involved in quantitation.

In order to qualitate and quantitate the minor sugars present in honey, methodology was required for the bulk separation of honey into monosaccharide (approx. 95% of the total soluble solids in honey) and oligosaccharide (approx. 3-10% of the total soluble solids in honey) fractions. Although a method (Siddiqui and Furgala, 1967) for this bulk separation was known, a rapid and less cumbersome method was

Table 5.1 Monosaccharides in honey, determined by HPLC.

Honey	Fructose (%)	Glucose (%)
Alsike	43.0	45.2
	42.8	45.8
	42.8	45.0
Alfalfa	44.9	38.2
	43.6	37.5
	40.1	36.3
Canola	50.0	50.0
	49.5	49.1
	49.5	49.2
Sweet Clover	44.2	44.2
	44.5	44.5
	44.0	43.8
Red Clover	40.3	35.9
	41.2	36.8
	39.8	37.0
Trefoil	48.2	41.3
	47.5	42.1
	47.5	42.1

required. Initially, an HPLC silica gel preparative column (Whatman, Partisil M20 10/50) with an amine modifier (tetraethylenepentamine, TEPA) was used. Following numerous attempts for this separation employing a variety of mobile phases and flow rates, this method was abandoned. Although for analytical columns these separations worked well, for preparatory columns the separations could not be achieved.

In 1984 Whatman introduced a preparative HPLC column (Whatman Partisil PAC Magnum 20/50) for carbohydrates. The application of this column to the separation of honey afforded an excellent separation of mono- and oligosaccharides. Typically, 10 g of honey was diluted with water to a total volume of 30 mL and then filtered. This sample was passed through the column as 5 mL aliquots and eluted with acetonitrile-water. Most of the monosaccharides were removed after 1.75 h (approximately 800 mL). The mobile phase was then changed to a higher water content and 450 mL of eluent collected. By repeating this procedure, the entire sample could be processed, yielding approximately 430 mg of essentially monosaccharide-free honey carbohydrates. Analysis of the pooled oligosaccharide fraction by HPLC indicated that approximately 7% was glucose, with no fructose present.

This procedure was applied to each of the six honeys (canola, trefoil, alfalfa, alsike, red clover, sweet clover) collected. The oligosaccharide fractions for each individual honey were combined, evaporated and dried under vacuum.

Duplicates for each of the aforementioned honeys were also prepared.

Brons and Olieman (1983) indicated that irreversible binding of carbohydrates to the stationary phase of HPLC columns occurred. To determine if this was a problem with the preparative HPLC column in our laboratory, standard solutions of glucose, sucrose and maltose were applied and eluted under our reaction conditions. Following evaporation, drying and addition of a known volume of HPLC-grade water, these standards were analysed by HPLC. Recoveries were 94.6% (glucose), 98.2% (sucrose) and 97.4% (maltose), indicating that irreversible binding to the column was negligible.

Analysis of standard oligosaccharides using HPLC (Whatman Partisil PXS 10/25 PAC) under a variety of conditions was attempted. Under optimum conditions, the values for various disaccharide standards are given in Table 5.2. The disaccharides tested (and those found in honey) had different retention times and might have been separated by HPLC. Unfortunately, peak widths at the concentrations of the minor sugars in honey were approximately half the peak heights. This resulted in severe broadening of the peaks, which in turn leads to overlapping. Although identifications of peaks was possible, quantitation was not.

Standard trisaccharides were also run under optimum HPLC conditions, and the results are shown in Table 5.3. In the case of trisaccharides, the peak width to peak height ratio was poorer, resulting in much broader peaks. When

Table 5.2 Retention times of disaccharides.

Disaccharide	mm	min
Sucrose	44.0	
Turanose	48.0	14.4
Nigerose	53.5	16.05
Maltose	55.5	16.65
Kojibiose	60.0	18.0
Trehalose	61.0	18.3
Isomaltose	70.5	21.2
Gentiobiose	75.0	22.5

Whatman Partisil PXS 10/25 PAC 250 mm x 4.60 mm; refractive index detection (16X); acetonitrile/water/1% ammonium hydroxide (80/20/1); flow rate 1.5 mL/min.

Table 5.3 Retention times of trisaccharides.

Trisaccharide	mm	min
Melezitose	91.7	27.5
Maltotriose	103.0	30.9
1-Kestose	104.3	31.3
Erlose	106.0	31.8
Isomaltotriose	112.3	33.7

Whatman Partisil PXS 10/25 PAC 250 mm x 4.60 mm; refractive index detection (8X); acetonitrile/water/1% ammonium hydroxide (80/20/1); flow rate 1.5 mL/min.

standard solutions of trisaccharides were subjected to HPLC analysis, very broad peaks occurred, and both qualitative and quantitative analyses were difficult under these conditions.

When the oligosaccharide fraction of honey was analyzed under these conditions, a broad peak was observed (similar to a UV peak), with a number of ridges which could be attributed to various di- and trisaccharides but which could not be identified without error and could not be quantitated due to broadness of the peaks.

Other attempts were made to analyze the oligosaccharide fraction of honey by HPLC. These included acetylation of the fraction, followed by HPLC analysis on both a PAC 10 column and on a reverse-phase column under a variety of conditions, but with no success. In addition, acetylation of the oligosaccharide fraction and column chromatography on a silica gel column (Merck, CC-7), elution with chloroform to chloroform/methanol, followed by HPLC analysis of each fraction was also unsuccessful. An attempt was also made to use a reverse-phase system (Whatman Partisil 10 CCS/C8) employing water and water-methanol as the eluting solvents, again with no success.

In many of these attempts to separate the numerous oligosaccharides in the sample, standards were used to determine if the methodology was applicable. Reasonable separations for standards were possible, in many cases, however, when the sample was used or when a combination of

standards was used, severe overlapping occurred.

At this stage alternative methods were investigated for the separation and quantitation of the oligosaccharides present in the monosaccharide-free fraction from the preparative HPLC separation. This separation provided ready access to a concentrated sample of the minor sugars present in honey.

6. CARBOHYDRATE ANALYSIS BY GAS CHROMATOGRAPHY

6.1 Introduction

The quantitative detection of carbohydrates is an important step in the investigation and identification of sugars. One of the oldest and most reliable methods, due to sensitivity and speed of execution, is the colorimetric method (Dutton, 1973). Analytical methods which are based on the detection of the reducing group, the characteristic property of aldoses and ketoses, have been reviewed by Binkley and Binkley (1970). These methods usually do not discriminate one sugar from another, therefore other methods have been employed for the identification and quantitation of carbohydrates.

The first report on the use of gas chromatography for carbohydrates was by McInnes *et al*. (1958). Since that time there have been many developments, including improvements in technique, equipment, detection and application. These improvements have been documented in a number of reviews on gas chromatography (Kircher, 1962; Bishop, 1962, 1964; Dutton, 1973, 1974).

Most carbohydrates are not sufficiently volatile to be used for gas chromatography. As a result, the carbohydrate is converted into a more volatile compound. Useful derivatives for this purpose include trimethylsilyl ethers, acetates, trifluoroacetates, trifluoroacetic acid anhydrides, isopropylidene acetals, methyl ethers and butane

boronates.

In 1963 Sweeley and coworkers published what is considered a classic paper on the use of trimethylsilyl derivatives of carbohydrates in gas chromatography. The discovery that trimethylsilyl derivatives are readily formed and that the resulting carbohydrate derivative is volatile revolutionized the separation and analysis of carbohydrate mixtures. Several reviews on these volatilizing agents and their uses have been published (Sweeley *et al.*, 1966; Wells *et al.*, 1964; Sweeley, 1965; Sloneker, 1968; Ettre and Zlatkis, 1967; Clamp *et al.*, 1971; Holligan, 1971; Holligan and Drew, 1971; Brobst, 1972; Dutton, 1974; Folkes, 1985).

Although Sweeley's work revolutionized the study of carbohydrates by gas chromatography, other researchers also recognized the importance of trimethylsilyl derivatives. In 1956, Schwarz *et al.* used trimethylchlorosilane/pyridine and glucose at 100°C to prepare the completely trimethylsilylated derivative. Chang and Hass (1958) extended this reaction to prepare silylated sucrose. With this method, Hedgley and Overend (1960) prepared the trimethylsilyl ethers of maltose and injected this compound onto a 180 cm x 0.8 mm glass column with a stationary phase of celite/silicon elastomer (or Apiezon M, 20-50% w/w). Using hydrogen as the carrier gas and isothermal conditions, the authors found that the separation was poor and that multiple peaks occurred.

Conversion of carbohydrates into their O-trimethylsilyl derivatives is commonly achieved by reacting hexamethyldisilazane (HMDS) [1] and chlorotrimethylsilane (TMCS) [2] in pyridine:

$$3\ ROH + \underset{[1]}{(CH_3)_3SiNHSi(CH_3)_3} + \underset{[2]}{(CH_3)_3SiCl} \rightarrow 3\ ROSi(CH_3)_3 + NH_4Cl$$

A typical procedure consists of treating 10 mg of carbohydrate in 1 mL of dry pyridine with 0.2 mL of hexamethyldisilazane and 0.1 mL of chlorotrimethylsilane (Sweeley, 1963). The reaction is normally complete at room temperature within 5 min, however other carbohydrates, such as maltose (Bhatti and Clamp, 1968) and ketoses (Semenza *et al.*, 1967), require longer reaction times and/or higher temperatures.

Trimethylsilylation is adversely affected by moisture. When dealing with carbohydrates, trace amounts of moisture are virtually always present (Bentley and Botlock, 1967). Researchers have shown (Sabbe and Cathey, 1970; Brobst and Lott, 1966; Marinelli and Whitney, 1966, 1967) that, if trimethylsilylation is catalyzed by trifluoroacetic acid in place of chlorotrimethylsilane, moderate proportions of water may be tolerated. Bentley and Botlock (1967) have indicated that if the reaction mixture is diluted with N,N-dimethylformamide then small amounts of water did not adversely affect the reaction. Willet (1967) suggested that a precolumn packed with molecular sieves be used when

analyzing aqueous samples. Alternatively, an aqueous solution may be injected onto the column, followed by a mixture of N,O-bistrimethylsilyl acetamide, trimethylsilylamine and hexamethyldisilazane (Esposito, 1968).

A powerful trimethylsilylating reagent, which is extremely useful for hydroxyl groups, is N-trimethylsilylimidazole. It is tolerant to moisture, and has been found to react completely with D-glucose in a 50% aqueous solution (Pierce, 1968), therefore it can be used with confidence when dealing with aqueous solutions or syrups which are difficult to dry completely (van Ling *et al.*, 1967; van Ling, 1969).

Other silylating reagents may be used to prepare trimethylsilyl derivatives of carbohydrates. These include N,O-bis(trimethylsilyl)trifluoroacetamide, N-trimethylsilyldiethylamine, and the mixtures N,O-bis(trimethylsilyl)-acetamide/chlorotrimethylsilane and N,O-bis(trimethylsilyl)-acetamide/N-trimethylsilylimidazole/chlorotrimethylsilane. The latter two mixtures are recommended for hindered hydroxyl groups (Pierce, 1973). Even though the above reagents may be used for preparing trimethylsilyl ethers of carbohydrates, the method of choice continues to be N-trimethylsilylimidazole (Makita and Wells, 1963; Miettinen *et al.*, 1965). Excellent reviews on methods and reagents for silylating organic compounds can be found (Pierce, 1968, 1973).

Pyridine is the solvent most commonly used for trimethylsilylation, however, for some compounds which are

sparingly soluble, N,N-dimethylformamide or dimethylsulfoxide should be used. It has been claimed by some researchers (Zinkel *et al.*, 1968; Shyluk *et al.*, 1967; Rowland and Riegelman, 1967) that pyridine may inhibit the formation of trimethylsilyl ethers, and that acetone, petroleum ether or carbon disulfide be used instead. The main problem with the use of pyridine is the "tailing" that occurs upon injection. This may be a problem when dealing with compounds of short retention times. This problem can be minimized by extracting with chloroform and washing with dilute hydrochloric acid (Partridge and Weiss, 1970), or by evaporating to dryness and diluting with cyclohexane (or some other suitable solvent) and washing with dilute hydrochloric acid (Bahl, 1970).

In most cases, complete trimethylsilylation of the carbohydrate is desired. With the O-trimethylsilyl derivatives, steric hindrance does not arise; with larger groups, such as tricyclohexylsilyl, it may be very significant (Barker *et al.*, 1963).

In their original paper, Sweeley *et al.* (1963) silylated not only monosaccharides, but di-, tri- and tetrasaccharides. This work indicated that this methodology could be used for the identification and characterization of more complex carbohydrates. As was the case with Hedgley and Overend (1960), multiple peaks, broad peaks, and severe overlapping were observed.

Davison and Young (1969) determined the common free sugars found in plants by gas chromatography. They

implimented the methodology of Sweeley *et al.* (1963) for the identification of fructose, glucose, sucrose, raffinose and stachyose. By preparing the trimethylsilyl ethers of these compounds (HMDS/TMCS/pyridine), using a glass coiled column (3 ft x 1/8 in; liquid phase of 3% SE-54 on 80-100 mesh Gas-Chrom P). Temperature programmed elution gave retention times of 5.4 min (fructose) to 23.5 min (stachyose). The peaks were very broad and no multiple peaks were apparent.

Kimura *et al.* (1969) used the silylation method of Sweeley *et al.* (1963) to prepare the trimethylsilyl derivatives of fructose, glucose, inositol, sucrose, melibiose and raffinose. Several stationary phases were used, including SE-30 (polar), OV-1 (nonpolar), OV-17 (medium polarity). The best results were obtained by using a 2 m x 3 mm stainless steel column packed with 1.5% SE-30 on Shimalite W (60-80 mesh) and a temperature program (150°C/10 min followed by 60 C°/min to 280°C). Although the authors were able to quantitate the carbohydrates present, broadness of peaks and some overlapping occurred.

Percival (1967) prepared the trimethylsilyl ethers (Sweeley *et al.*, 1963) of cellobiose, laminaribiose and other less common disaccharides. Included in this work was the reduction (using potassium borohydride) to the alditol of the corresponding disaccharide. The column used was 1.2 m x 4 mm and, although various stationary phases were tried, the best results were obtained with SE-30 and Apiezon K. No chromatograms were shown, but the retention times of the various disaccharides were given. The tabular results

indicated that there were numerous overlapping peaks.

Bhatti *et al.* (1970) prepared the trimethylsilyl ethers (Sweeley *et al.*, 1963) of biologically-important mono-, di-, tri- and tetrasaccharides. Separations were observed using a 100 cm x 0.32 cm glass column with a stationary phase of 3-3.8% SE-30 (or UC-W98) on Diatoport S and isothermal conditions. As with other reports, multiple peaks for the carbohydrates were observed, and severe overlapping of many of the di-, tri- and tetrasaccharides occurred.

Haverkamp *et al.* (1971) summarized the preparation of trimethylsilyl derivatives of twenty-three disaccharides. The stationary phases used in the separation were OV-1 (nonpolar), OV-17 (medium-polar) and OV-25 (polar). The optimal separation of disaccharide TMS ethers was obtained on 3% OV-17 as the stationary phase. Comparison of the R_s values for the disaccharides indicated that overlapping of peaks occurred. In addition, multiple peaks for the same disaccharide were observed. The authors indicated that their method could be advantageous to analyze equilibrium mixtures of anomeric forms. However, for the preparative separation of disaccharides by GC, reduction to the corresponding alditols would greatly diminish the number of components in the mixture (Haverkamp *et al.*, 1971).

Gas chromatography of disaccharides up to this time indicated that clear separation of these substances as components of a mixture would require much higher resolution than that which can be attained with normal packed columns. Larson *et al.* (1974) observed that, due to temperature

limitations and long retention times for disaccharide analysis, liquid phases of high polarity are generally unsuitable. In addition, liquid phases of lower polarity do not provide an adequate separation. Szafranek *et al.* (1974) used open tubular glass capillary columns coated with a nonpolar stationary phase to separate mixtures of monosaccharide derivatives. Adam and Jennings (1975) applied capillary chromatography (45 m x 0.25 mm; OV-101) to separate the trimethylsilyl oxime derivatives of eight disaccharides. Even though the oximes of the corresponding disaccharides were prepared, twelve peaks occurred on the spectrum, with only one overlapping peak. The reducing disaccharides gave rise to two peaks, one large and one small. the authors suggested that these probably represent the syn- and anti-forms of the oxime. This work represented a significant improvement in the separation of disaccharides and in the time necessary to analyze a sample.

Toba and Adachi (1977) investigated the separation of ten disaccharides, including two ketodisaccharides, as their sugar oxime trimethylsilyl ethers. Due to the complexity of the separation of disaccharides from each other, and as most reducing disaccharides give multiple peaks in gas chromatograms, because of the presence of tautomeric forms of reducing sugars (Sweeley *et al.*, 1963), the authors prepared the oximes of the corresponding disaccharides. They used a conventional column (2 m x 3 mm; SE-52) and found that poor resolution and multiple peaks were observed. The authors indicated that, although the analysis of disaccharides using

their oxime trimethylsilyl ethers was unsuccessful, this method could provide a convenient means to study interconversions of isomeric forms of sugar oximes.

Coduti and Bush (1977) used gas chromatography and mass spectrometry to analyze trimethylsilyl derivatives of disaccharides of glycoprotein origin. No spectrum of the separation was shown, but it appears from the tabular results that some overlapping was observed, although single peaks were observed for each derivative. Identification of the disaccharides was accomplished by their corresponding fragmentation patterns, rather than by retention times. Bush (1979) continued this work and identified the disaccharides and disaccharide methylglycosides released upon partial acid hydrolysis of glycoprotein. The carbohydrates were derivatized (Sweeley *et al.*, 1963) and were analyzed as previously mentioned.

Thompson *et al.* (1978) determined disaccharides in feces by isothermal (270°C) gas chromatography of the permethylated derivatives. They were able to separate four of the eight disaccharides attempted. Overlapping of α-lactose and β-cellobiose as well as β-lactose and β-maltose occurred. The authors found that the use of OV-17 as the stationary phase was optimal; use of SE-30 produced numerous overlapped peaks. In addition to retention times, mass spectrometry was used to aid in identification. This work was attempted so that a rapid screening method for the detection of patients suspected of having a carbohydrate intolerance secondary to intestinal disaccharide deficiency.

Bush (1979) prepared the trimethylsilyl derivatives of disaccharides from the partial acid hydrolysis and methanolysis of glycoproteins. The composition of the recovered disaccharides was determined by further hydrolysis to the trimethylsilyl monosaccharides and identification by gas chromatography-mass spectrometry (GC-MS).

Nikolov and Reilly (1983) published the relative retention times of seventeen trimethylsilyl disaccharides (cellobiose, gentiobiose, isomaltose, kojibiose, lactose, lactulose, laminaribose, maltose, maltulose, melibiose, nigerose, palatinose, sophorose, sucrose, α,α-trehalose, turanose and xylobiose) on a fused-silica capillary column (30 m x 0.26 mm) with SE-54 as the stationary phase. By conducting the separation isothermally (240°C), overlapping occurred for lactulose:β-xylobiose; sucrose:α-lactose; nigerose:turanose; α,α-trehalose:β-kojibiose; laminaribose: β-sophorose; and α-kojibiose:palatinose. The disaccharides were allowed to achieve mutarotation equilibrium by stirring in pyridine-0.2 M 2-hydroxypyridine for 15 h at 40°C. This work represented a significant breakthrough in the qualitative identification and quantitative determination of structurally similar disaccharides.

Trimethylsilylation of disaccharides (and oligosaccharides) is not the only method of derivatization for volatilization, however, it has become the method of choice. The derivatives formed are more volatile, leading to shorter retention times, and columns can be operated at lower temperatures with less baseline noise. In addition,

TMS-carbohydrate derivatives generally result in sharper peaks than corresponding methylated or acetylated carbohydrates (Laker, 1979).

Other methods have been used for the derivatization of disaccharides for gas chromatographic identification, and general reviews are available (Dutton, 1973, 1974).

6.2 Gas Chromatographic Analysis of Food Carbohydrates

A survey of the literature indicates a predominance of the use of trimethylsilyl derivatives for food carbohydrate analysis. However, both acetates and trifluoroacetates have been used. Sawardeker *et al.* (1965) reported the quantitative determination of monosaccharides as their corresponding alditol acetates. Selvendrun (1979) determined the aldoses of vegetable fibre, also as their alditol acetates. Englyst *et al.* (1982) employed the alditol acetates for the determination of sugars of nonstarch polysaccharides in foods.

Bittner *et al.* (1980) introduced an improved procedure for acetylation, using N-methylimidazole as a catalyst in place of pyridine. This resulted in shorter reaction times and lower temperatures for the reaction. Adam (1981) employed this technique to obtain the acetates of reduced monosaccharides.

Preparation of trifluoroacetates (TFA) for carbohydrates was introduced by Vilkas *et al.* (1966). Tamura and Imanari (1967) indicated that the trifluoroacetate derivatives of carbohydrates were more volatile than the

corresponding trimethylsilyl derivatives. Anderle and Kovac (1970) described a rapid preparation of TFA derivatives using trifluoroacetic anhydride with 1% pyridine as a catalyst. One of the few applications of TFA derivaties was by Luke (1971), where an improved resolution of sucrose and lactose was reported and applied to cocoa products. The TFA derivatives of carbohydrates have rarely been used for analysis due to the loss of the derivatives during chromatography (Folkes, 1985).

In 1968, Coyle *et al.* reduced the carbohydrates of corn syrup to alditols and prepared the trimethylsilyl ethers for gas chromatographic identification. Capillary column gas chromatography was employed by Demaimay and Lebouteiller (1978) and by Sennello (1971) to quantitate fructose and glucose in syrups as their corresponding trimethylsilyl oximes.

Alexander and Garbutt (1965) determined glucose in high DE (dextrose extract) syrups and sugars using trimethylsilyl derivatives. Brobst and Lott (1966) determined the carbohydrates of corn syrup and candy as their trimethylsilyl derivatives with a precision of 1.7-3.4% for glucose to maltotetrose. Beadle (1969) extended their method up to maltoheptaose for the analysis of starch hydrolysates. Trimethylsilyl glucose oligomers in low DE syrup have been analyzed by gas chromatography (Folkes and Brookes, 1984), and maltose and isomaltose were also analyzed in a similar manner. The detection of the TMS derivatives of carbohydrates in potatoes was reported by Kimura *et al.* (1969).

Davison and Young (1969) used TMS derivatives to determine glucose, fructose and sucrose in tomatoes, potatoes, apples, cabbages and carrots. The oligosaccharides of soybean meal were determined by Delente and Ladenburg (1972) as their TMS derivatives. Demaimay and Lebouteiller (1978) used both packed and capillary columns in the analysis of milk, apple juice, jam and chocolate candy for carbohydrates as their TMS oximes.

Yasui and Takenaka (1979a) determined the amount of fructose, glucose, sucrose, raffinose, and stachyose in various food products by preparing their corresponding trimethylsilyl derivatives. They carried out temperature programs on a variety of stationary phases (SE-30, OV-17, QF-1, OV-225) with capillary gas chromatography. This work was extended (Yasui *et al.*, 1979b) to chewing gum, apple juice, biscuits, and crackers. The trimethylsilyl derivatives of the oligosaccharides could be separated by their methodology and quantitation of the carbohydrates present was accomplished. Recoveries of added sucrose ranged from 95.8-100.5%.

Iverson and Bueno (1981) applied TMS carbohydrate derivatives in the analysis of a wide range of foods, including chocolate confections, ice cream, canned foods, and honey. Li and Schumann (1981) determined the TMS derivatives of sugars in breakfast cereals. Similar conditions were employed by Hoi (1983) for cane sugar products, Daniels *et al.* (1982) for chewing gum and sorbitol mint, and Li *et al.* (1983) for yogurt carbohydrates. Further

examples of the use of gas chromatography for the identification of carbohydrate derivatives (mainly TMS) can be found in the reviews of Fölkes (1985), Dickes and Nicholas (1976) and Birch (1973).

The use of gas chromatography for the analysis of carbohydrates in honey has been studied by very few researchers.

Pourtallier (1968) and Yoshiro *et al.* (1969) used gas chromatography to determine fructose and glucose in honey. Battaglini and Bosi (1972) were able to identify fructose, glucose and sucrose in honey using a 1.80 m x 3 mm glass column (OV-17) with temperature programming. In addition, two other broad peaks were apparent, but not identified. Sarra and Durio (1974) also identified fructose, glucose and sucrose in honey samples by gas chromatography. Glass capillary gas chromatography coupled with mass spectrometry has been used to detect honey sugars. Vervack *et al.* (1978) applied capillary GC to the analysis of a variety of foods, including honey. Glucose, sucrose and maltose were identified.

6.3 Gas Chromatography Experimental

To 5 mL of reverse osmosis Milli-Q purified water (HPLC-grade H_2O) was added 1 mg of oligosaccharide (2.9×10^{-6} moles [disaccharides]; 1.98×10^{-6} moles [trisaccharides]). To this solution was added an excess (50-200 meq) of sodium borohydride. The reaction mixture was stirred at room temperature for 1 h, then cooled in an ice-water bath and

neutralized by dropwise addition of 10% glacial acetic acid. The reaction mixture was then passed through a Dowex 50 WX 8 ion exchange resin (80 mm x 8 mm) and eluted well with HPLC-grade H_2O (this column could be re-used for four more samples). The eluent was then evaporated *in vacuo* (Buchi rotavapour R) and co-evaporated with methanol (5 x 5 mL). The reaction flask containing the colourless syrup was fitted with a rubber septum, 1 mL of tri-sil Z (Pierce Scientific Co.), (1.5 meq/mL) was added and the resulting slightly yellow solution was heated at 60°C for 30 min. The temperature was then raised to 80°C and the reaction mixture stirred for 30 min.

Gas capillary chromatography was carried out on a Varian (model 3700) gas chromatograph with a DB5 capillary column (30 m x 0.25 μm; J and W Scientific Co.). Typically, 1.0 μL of sample was injected with a splitter rate of 30:1. The carrier gas was 99.999% pure helium with a flow rate of 0.27 m/sec. For disaccharides, a temperature program of 210°C/10 min, followed by an increase of 2 C°/min to 290°C was used. Trisaccharides were eluted using isothermal conditions (295°C). The injector port and detector temperatures were maintained at 300°C.

Each of the following reducing oligosaccharides was derivatized and analyzed in the preceding manner: maltose, maltulose, nigerose, palatinose, isomaltose, turanose, cellobiose, gentiobiose, kojibiose, laminaribiose, isopanose, panose, maltotriose, and isomaltotriose. In addition, the following non-reducing oligosaccharides were

silylated and analyzed as previously mentioned: sucrose, trehalose, neotrehalose, 1-kestose, erlose, and theanderose.

The monosaccharide-free portions of the nine honeys mentioned in the HPLC section were also subjected to reduction and silylation. Typically, 20 mg of monosaccharide-free honey (1.11×10^{-4} moles, based on a molecular weight of 180 g/mole) was dissolved in 50 mL of HPLC-grade H_2O. The remainder of the conditions for reduction and silylation were the same as those previously used for the standards, except for employing a 100 mm x 10 mm column of Dowex 50 WX 8, co-evaporation with 10 x 10 mL of methanol, and 18 mL of tri-sil Z was used for silylation.

6.3.1 Synthesis of panose (Wolfrom and Koijumi, 1967).

To 5 g (0.0139 moles) of maltose monohydride (previously dried over P_2O_5 under reduced pressure for 24 hr at 100°C) in a 250 mL round bottom flask (B24/40) was added dry pyridine (30 mL). This solution was distilled at atmospheric pressure (with the addition of more dry pyridine) until a steady boiling point of 112-114°C was attained for the distillate. To this solution was added 4 g (0.0143 moles) of triphenylmethyl chloride (trityl chloride), and the reaction flask was stoppered, stirred and heated at 40°C for 64 h. A further 15 mL of dry pyridine was added and the flask was cooled to 0°C, then 22 mL (0.233 moles) of acetic anhydride was added and the reaction vessel was stoppered and stirred at room temperature for 72 h. The resulting solution was poured into a 1 L beaker containing

300 mL of ice and water; this emulsion was stirred vigourously for 18 h. The precipitate was collected by vacuum filtration and dried over P_2O_5 under reduced pressure. This solid was placed on a silica gel column (Merck CC-7, 2.5 x 50 cm) and the desired product was eluted (following the first 350 mL) using benzene/ethyl acetate (3:2, v/v) as the eluent. The solvent was removed *in vacuo* and the resulting solid was recrystallized from chloroform (m.p. 161-163°C; literature m.p. 164-164.5°C [Wolfrom and Koijumi, 1967]). The resulting white crystalline solid (1.3 g, 11%) [O-(2,3,4-tri-O-acetyl-6-O-trityl-α-D-Glucosyl)-(1→4)-tetra-O-acetyl-β-D-glucopyranose (compound II, Figure 6.4)] was dried under reduced pressure over P_2O_5 at 80°C for 48 h.

Silver perchlorate (0.72 g; 0.0035 moles) was dissolved in 12 mL of absolute nitromethane and 0.7 g (0.00125 moles) of anhydrous calcium sulfate was added to the solution. The solution was allowed to stand at room temperature for 15 min, and then the previously dried compound II (1.3 g; 0.0015 moles) was added. This yellow clear solution was then cooled to 0°C and 0.67 g (0.00179moles) of 3,4,6-tri-O-acetyl-2-O-nitro-β-D-glucopyranosyl chloride (III, Figure 6.4) was added and the mixture vigourously stirred. The reaction mixture was allowed to warm to room temperature, the colour of the solution was orange; at this point silver chloride and trityl perchlorate precipitate. The solution was allowed to stir at room temperature for 30 min, then was filtered. The nitromethane solution was diluted with

chloroform and dried over sodium bisulfite and, following filtration, was evaporated under reduced pressure. The resulting syrup was dissolved in 98% ethanol and hydrogenated for 3 h at 35 psi, employing a catalyst of 10% palladium on charcoal (35 mg). Filtration and concentration gave a syrup, which was deacylated with 0.05 M sodium methoxide in methanol for 18 h at 4°C. The resulting solution was placed on a celite-charcoal (1:1, w/w) column (2.5 x 40 cm) and eluted using water raising the ethanol content to 10% when the desired component was eluted. Concentration of the desired fraction *in vacuo* gave a slightly yellow syrup. This syrup was recrystallized using ethanol/ether diffusion, yielding an off-white powder (m.p. 219-220°C dec.; lit. m.p. 221°C [Wolfrom and Koijumi, 1967]). Elemental analysis calculated for $C_{18}H_{32}O_{16}$: C, 42.86; H, 6.39. Found: C, 42.41; H, 6.56.

6.3.2 Synthesis of maltulose (Hicks *et al.*, 1983)

In a 100 mL beaker were added 1.0 g (2.78×10^{-3} moles) of maltose and 85 mL of HPLC-grade H_2O. To this solution was added 0.18 g (2.91×10^{-3} moles) of boric acid. This solution was then titrated with triethylamine to a pH of 11.0. The resulting solution was transferred to a 100 mL volumetric flask and made up to volume with HPLC-grade H_2O. This mixture was transferred to a 250 mL round bottom flask fitted with a rubber septum and the flask was immersed in a 70°C water bath and heated for 3 h. The solution was cooled and chromatographed on a 80 cm x 2.5 cm column of 50 mL

IR-120 (H^+), 45 mL XAD-4, and 35 mL of A-561 resins. The effluent was collected, evaporated *in vacuo*, and re-evaporated with 3 x 30 mL of absolute methanol. The residue (1.8 g) was dissolved in water (10 mL) and placed in a water bath at 45°C. Acetone (62 mL) was slowly added to the water/carbohydrate mixture, and the entire solution was kept at 45°C for 1 h. The solution was allowed to cool to room temperature; crystallization occurred over a period of five days. The off-white powder was re-crystallized from water-acetone. The white crystals (56 mg) were isolated by filtration and dried over P_2O_5 under vacuum for 48 h (m.p. 109-110°C; literature m.p. 116-119°C [Hicks *et al.*, 1983]). Elemental analysis calculated for $C_{12}H_{22}O_{11} \cdot {}^1/_2\ H_2O$: C, 41.03; H, 6.48. Found: C, 41.14; H, 6.41.

6.4 Gas Chromatography Results and Discussion

Reducing sugars differ from most other organic compounds in one characteristic property. When a pure reducing sugar is dissolved in a solvent, the solution obtained can contain up to six compounds, whereas most organic compounds contain only one. The six compounds in solution include the two pyranoses, two furanoses, and the acyclic carbonyl form and its hydrate (Angyal, 1984).

The carbohydrates found in honey are of glucose and fructose origin, that is they are composed of multiple glucose units or glucose-fructose units (Siddiqui and Furgala, 1970).

Glucose can exist in four tautomeric forms (Figure 6.1) at 25°C in H_2O: β-furanoside (0.14%), acyclic aldehyde (0.0026%), β-pyranoside (62%) and α-pyranoside (38%) (Angyal, 1984). Fructose at 25°C in H_2O can also exist in four tautomeric forms (Figure 6.2): α-pyranoside (trace), β-pyranoside (75%), α-furanoside (4%), and β-furanoside (21%) (Angyal, 1976). From this data it becomes obvious that a sensitive analytical method, such as capillary gas chromatography, could yield multiple peaks for the same compound. When dealing with complex carbohydrate mixtures like those found in honey, the presence of multiple peaks represents a significant problem. In order to relieve this problem, the oligosaccharide fraction obtained by HPLC separation was reduced with sodium borohydride. This resulted in greatly reducing the number of tautomers representing each carbohydrate. There are at least twelve disaccharides in honey (Siddiqui, 1970), only two of which are non-reducing sugars. Assuming three detectable peaks for each of the ten remaining disaccharides yields a minimum of thirty-two peaks possible in a sensitive analytical system. This complexity could obviously lead to severe difficulties in qualitative and quantitative analysis. Reducing the carbohydrates alleviates most of these difficulties, however, reduction of a fructose moiety yields two products, glucitol and mannitol. Therefore, if a mixture of glucose and fructose was reduced with sodium borohydride, glucose would be converted to glucitol, fructose would be converted to an equimolar mixture of glucitol and mannitol. Quantitation of

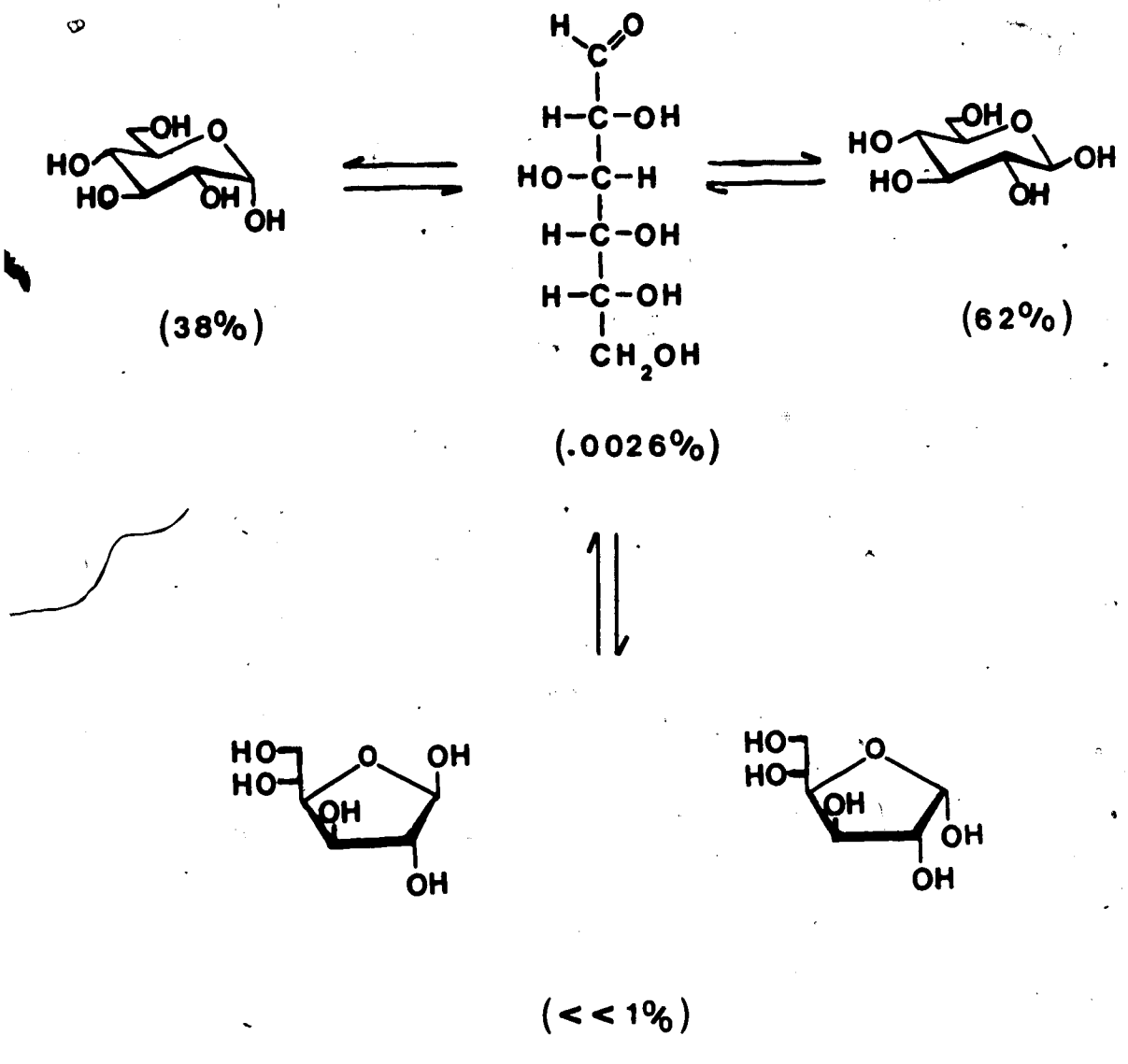


Figure 6.1 Tautomers of D-glucose in solution.

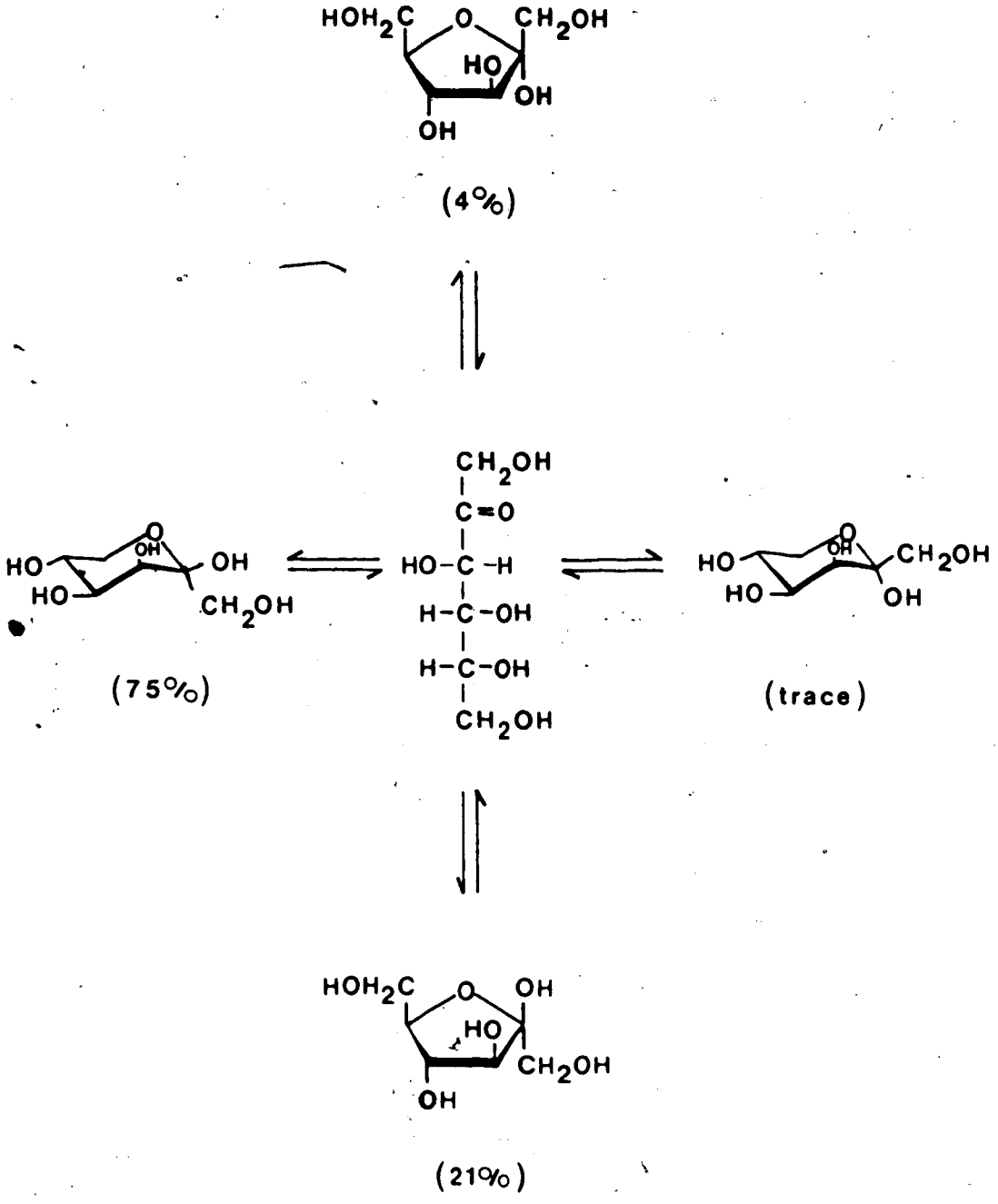


Figure 6.2 Tautomers of D-fructose in solution.

this reduction would yield a higher than expected glucitol peak and a lower than expected mannitol peak. In the honey system, this problem arises. For example, the fructose-containing disaccharides present, maltulose, palatinose and turanose, when reduced, would not only yield two peaks (which could cause peak overlapping problems) but would interfere with quantitation of maltose, isomaltose and nigerose, respectively.

An initial attempt was made to separate reduced and silylated standard carbohydrates using capillary gas chromatography employing an intermediate polarity stationary phase 30 m column (DB 1701; J and W Scientific Co.). Preparation of the standards involved reduction with 5 mg of sodium borohydride of 1 mg of each of the following carbohydrates: maltose, maltulose, turanose, nigerose, palatinose, kojibiose, laminaribiose, isomaltose, gentiobiose, cellobiose, maltotriose, isomaltotriose, panose, and isopanose. The reaction mixture was quenched and the various impurities present removed. The resulting reduced compounds were silylated using tri-sil Z (Pierce Scientific Co.) and the standards were then subjected to capillary gas chromatographic analysis.

Although separation of the disaccharide and trisaccharide series was possible using the intermediate polarity stationary phase, only limited resolution of the carbohydrates within each series was possible. Employing a variety of isothermal and temperature programming conditions did little to improve the separation of the standards.

Separation of the standard reduced and silylated carbohydrates was then attempted on a non-polar stationary phase. Employing a temperature program consisting of 210°C/12 min and 2 C°/min to 290°C gave complete separation of the ten reduced and silylated disaccharide standards. Operation of this column at isothermal temperatures of 295°C gave complete separation of the four reduced and silylated trisaccharide standards.

Preparation of a standard oligosaccharide for GC analysis is shown in Figure 6.3, using the disaccharide maltose as an example.

To ensure that this methodology could be used to quantitate as well as qualitate the carbohydrates present in honey, standard curves for sucrose, trehalose and reduced maltose were prepared, yielding linear relationships for the concentrations tested. In addition, a standard disaccharide mixture (consisting of the reduced moieties of cellobiose, laminaribiose, nigerose, turanose, maltulose, maltose, kojibiose, gentiobiose, palatinose, isomaltose, in addition to the non-reduced sucrose, trehalose and neotrehalose) was prepared at three different concentrations and results yielded linear relationships for the concentrations tested.

A standard trisaccharide mixture (consisting of the reduced moieties of isomaltotriose, maltotriose, panose, isopanose, in addition to the non-reduced erlose, melezitose and 1-kestose) was prepared at three different concentrations and was also found to be linear in the ranges studied.

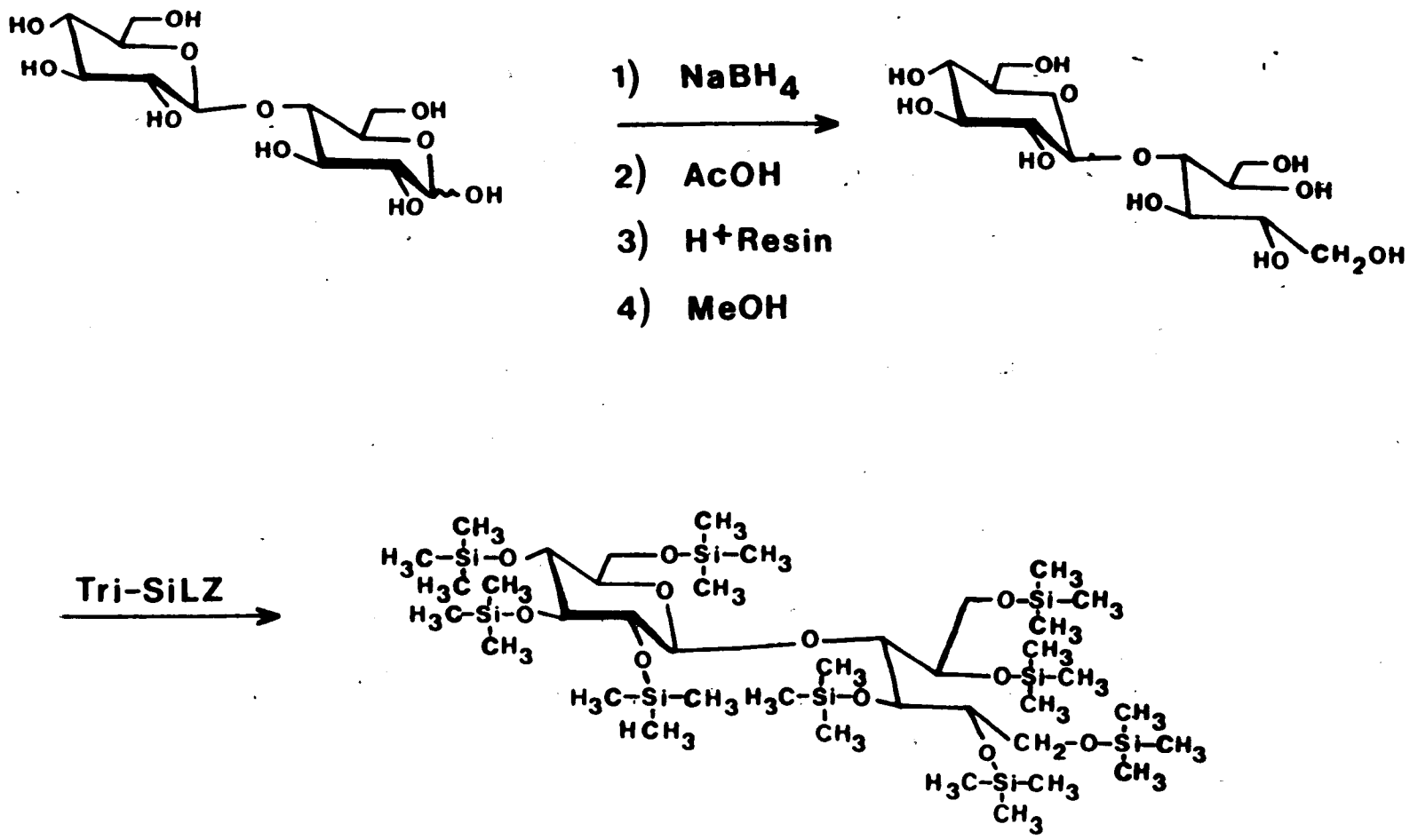


Figure 6.3 Preparation of maltose for GC analysis.

Typical retention times for these reduced (where applicable) and silylated disaccharides are shown in Table 6.1. The reduced (where applicable) and silylated trisaccharide retention times are shown in Table 6.2.

A vast majority of the oligosaccharides present in honey are not commercially available, therefore many of the standards used for GC analysis were obtained gratis from various researchers. A few of the oligosaccharides analyzed were prepared in our laboratory. Maltulose was prepared according to the methodology of Hicks *et al.* (1983) by treating maltose with boric acid and triethylamine or sodium hydroxide. The trisaccharide panose was prepared employing the methodology of Wolfrom and Koizumi (1967) (Figure 6.4).

As was mentioned previously, and as can be seen from Table 6.1, reduction of the three fructose-containing reducing disaccharides results in two peaks. It is obvious therefore that upon quantitation these three disaccharides, in addition to the three with which they coincide, must be accounted for. Maltulose, palatinose and turanose were each reduced at three different concentrations and analyzed by capillary gas chromatography. The ratios of the areas of the two compounds derived from each of these reductions indicated that for maltulose the ratio of O-α-D-glucopyranosyl-(1→4)-D-glucitol/O-α-D-glucopyranosyl-(1→4)-D-mannitol was approximately 1:1. For palatinose, O-α-D-glucopyranosyl-(1→6)-glucitol/O-α-D-glucopyranosyl-(1→6)-mannitol was approximately 1:1. For turanose, O-α-D-glucopyranosyl-(1→2)-glucitol/O-α-D-glucopyranosyl-(1→2)-

Table 6.1 GC retention times for reduced (where applicable) O-TMS disaccharides.

Disaccharide	Retention Time (min)
Sucrose	34.55
Trehalose	37.61
Neotrehalose	39.51
Cellobiose	40.03
Laminaribiose	40.56
Nigerose	41.37
Maltulose	41.85 42.23
Turanose	41.37 41.85
Maltose	42.23
Kojibiose	42.70
Gentiobiose	43.73
Palatinose	44.63 45.12
Isomaltose	45.12

(temp. program 210°C/12 min; 2 C°/min to 290°C)

Table 6.2 GC retention times for reduced (where applicable) O-TMS trisaccharides.

Trisaccharide	Retention Time (min)
1-Kestose	20.12
Isopanose	20.52
Erlose	21.89
Melezitose	23.60
Maltotriose	33.56
Panose	41.20
Isomaltotriose	44.59

(isothermal conditions, 295°C)

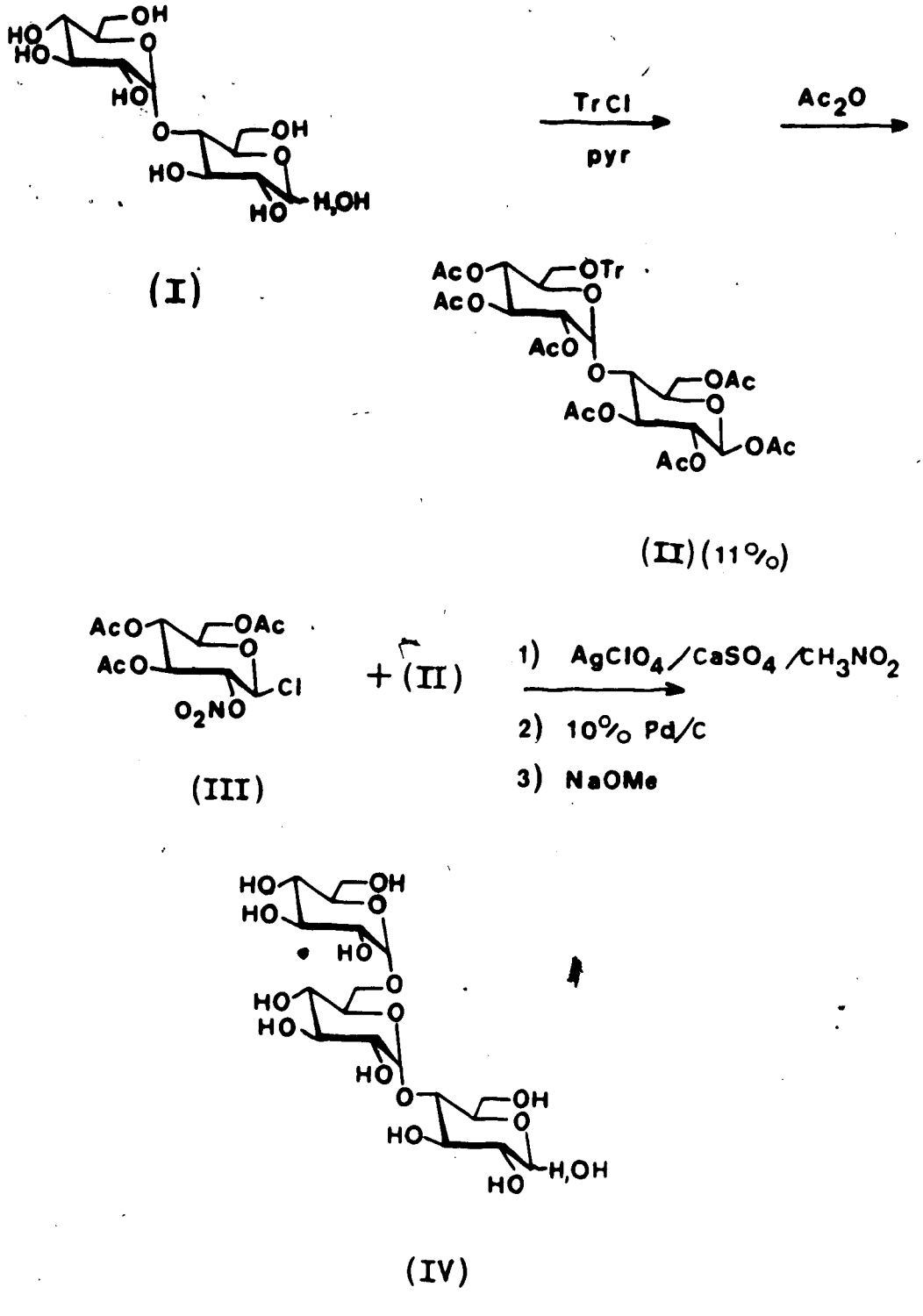


Figure 6.4 Chemical synthesis of panose and an isomeric trisaccharide (Wolfrom and Koizumi, 1967).

mannitol was approximately 1:2. Knowing this information, maltulose, maltose, nigerose, turanose, palatinose and isomaltose can be quantitated successfully employing these experimental conditions. Another problem arises however, as the two peaks for maltulose overlap with maltose (as expected) and with one of the turanose peaks. Attempts were made to vary the programming conditions to alleviate this problem, but these were unsuccessful. Siddiqui (1970) showed that the amount of maltulose present in honey (0.004%) is extremely low and therefore in our calculations it was assumed to be zero. By using both the temperature program and the isothermal conditions of capillary gas chromatography, the disaccharides and trisaccharides in honey can be both identified and quantitated.

Monosaccharide-free fractions from nine different honeys were subjected to reduction, silylation and capillary GC analysis. Figures 6.5-6.13 contain the data obtained from these analyses. In each case the ratios of the identified oligosaccharides are given with respect to maltose. The reason for this approach was that in most cases maltose represented the major oligosaccharide present (Siddiqui, 1970). In actual fact the reduced (where applicable) oligosaccharides were analyzed; their corresponding non-reduced trivial name was used in the bar graph.

The nine honeys analyzed represented the collection of four different species of honeybees. These included six honeys from *Apis mellifera* (alfalfa, alsike, canola, sweet clover, red clover, trefoil) and one honey each from *Apis*

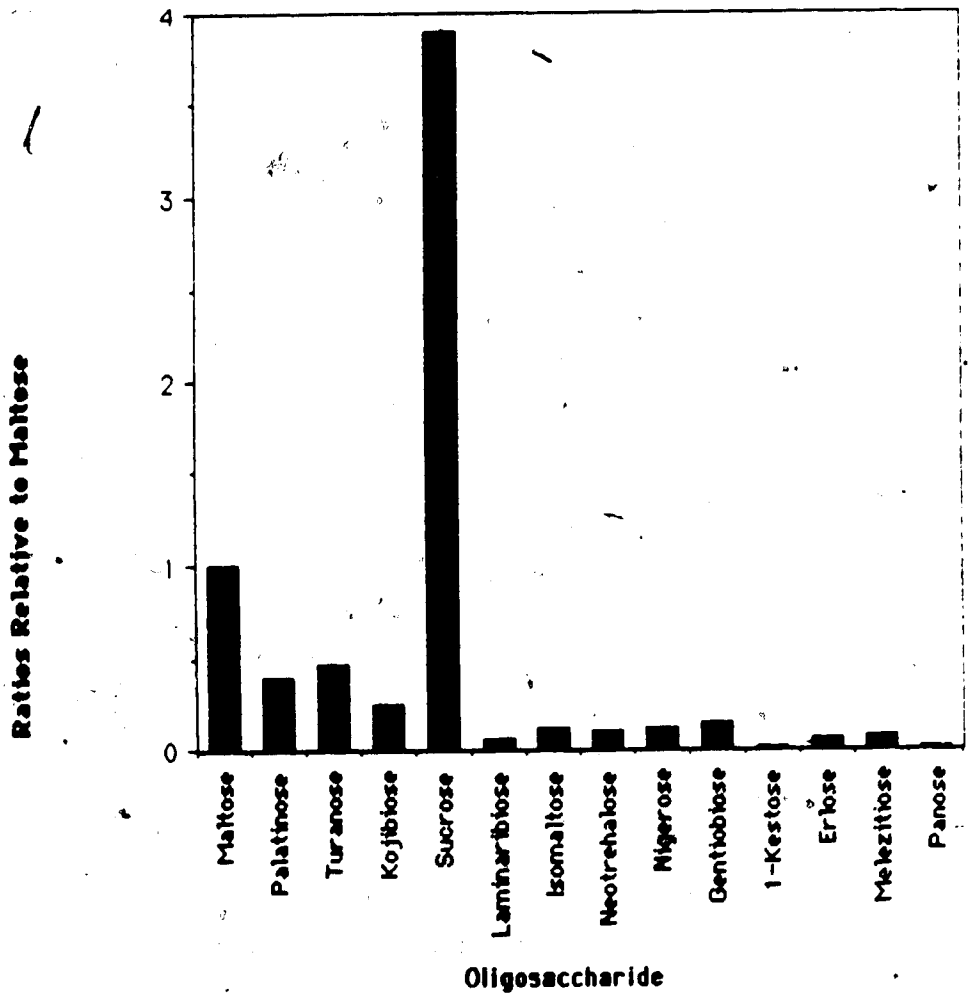


Figure 6.5 Oligosaccharides identified in alfalfa honey.

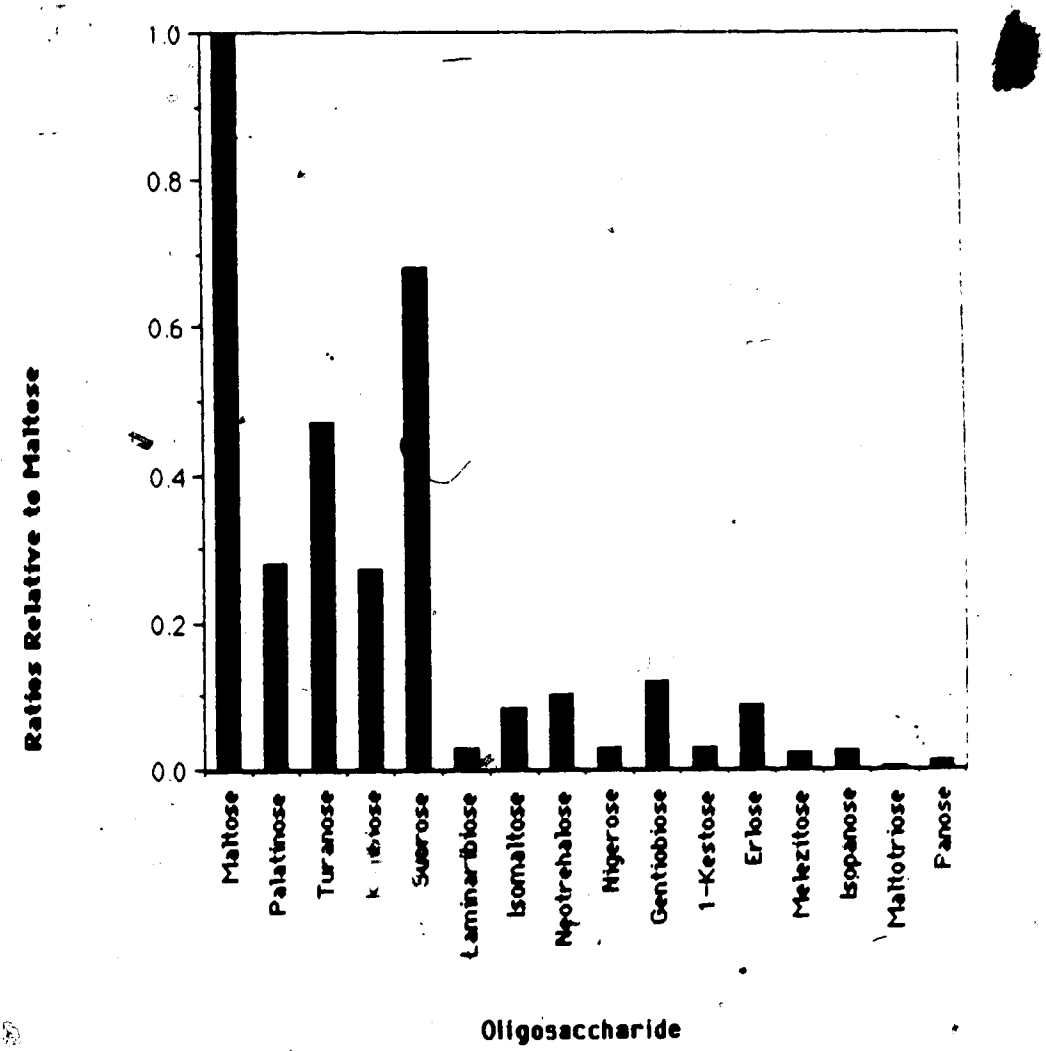


Figure 6.6 Oligosaccharides identified in alsike honey.

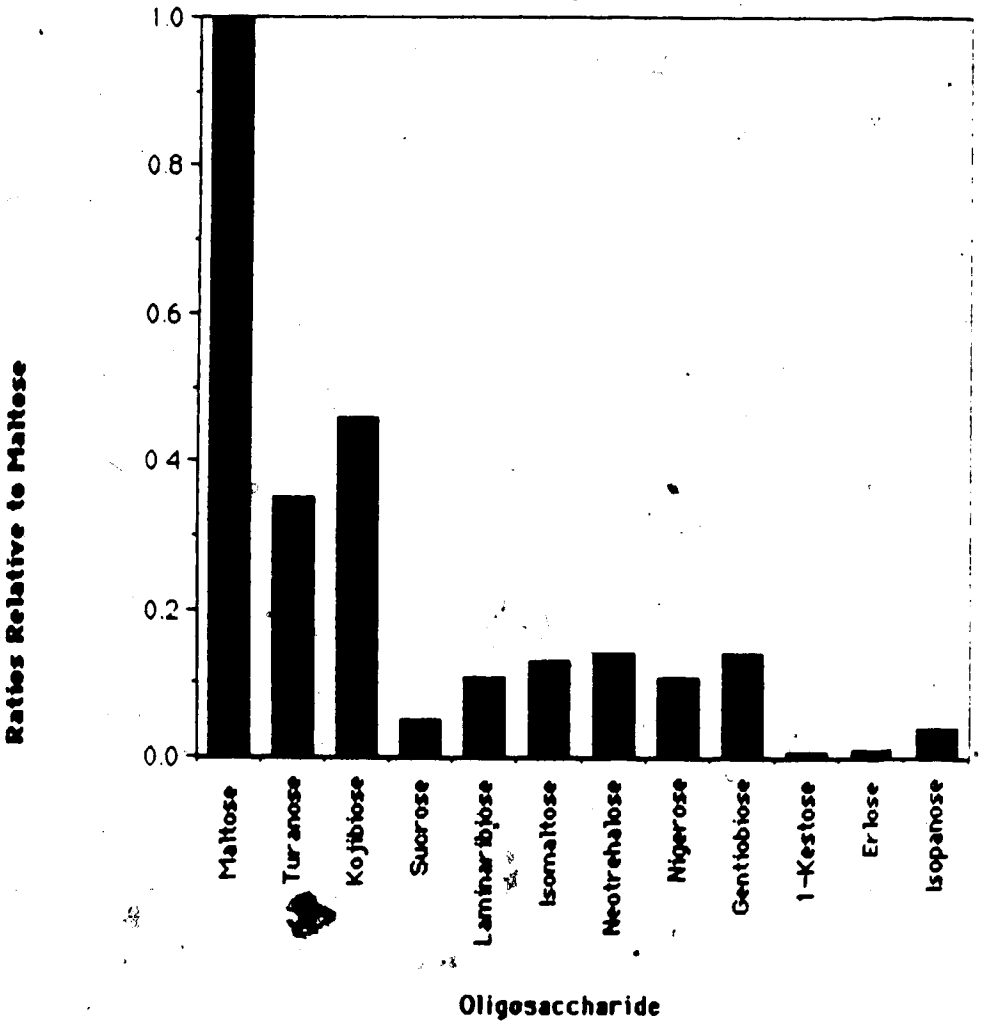


Figure 6.7 Oligosaccharides identified in canola honey.

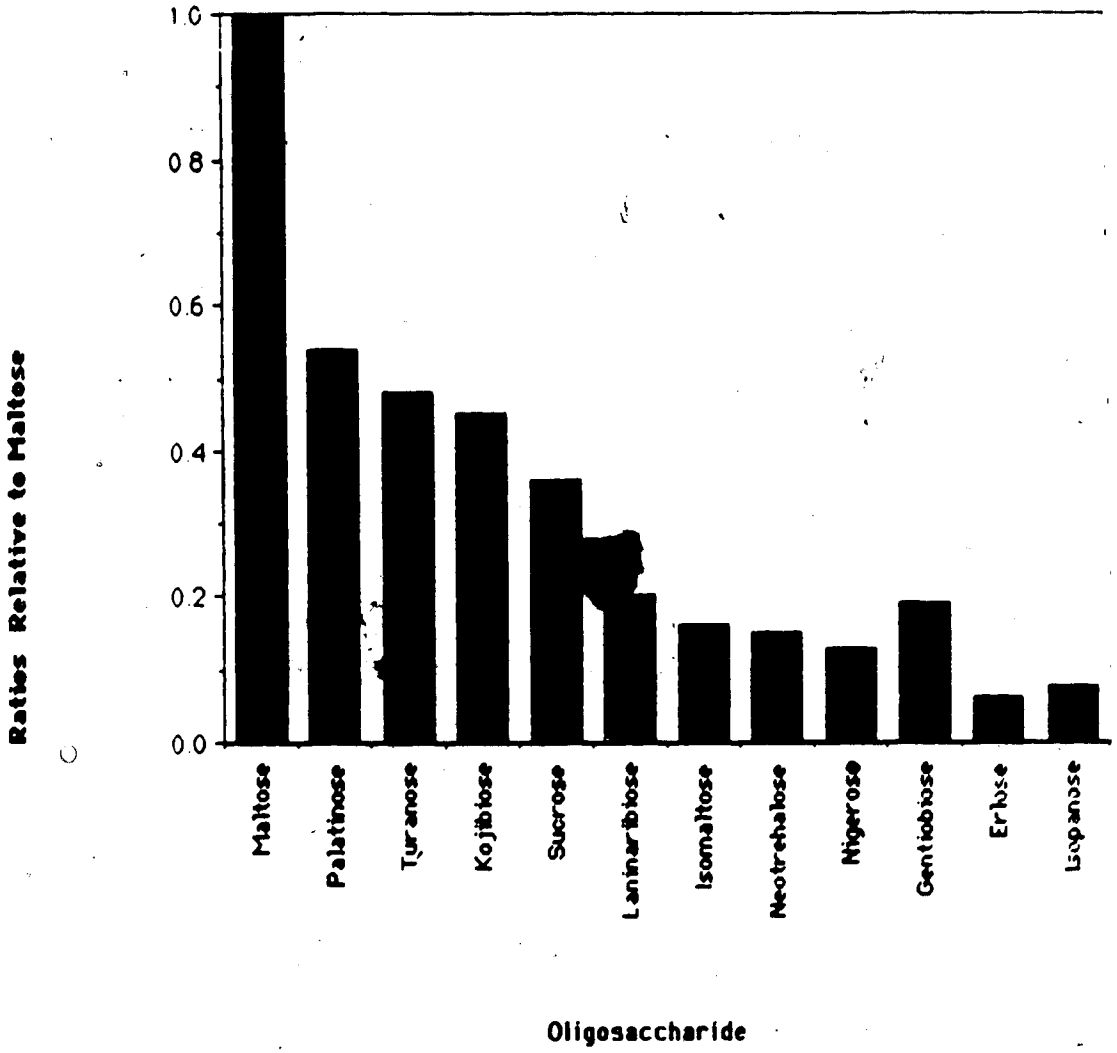


Figure 6.8 Oligosaccharides identified in red clover honey.

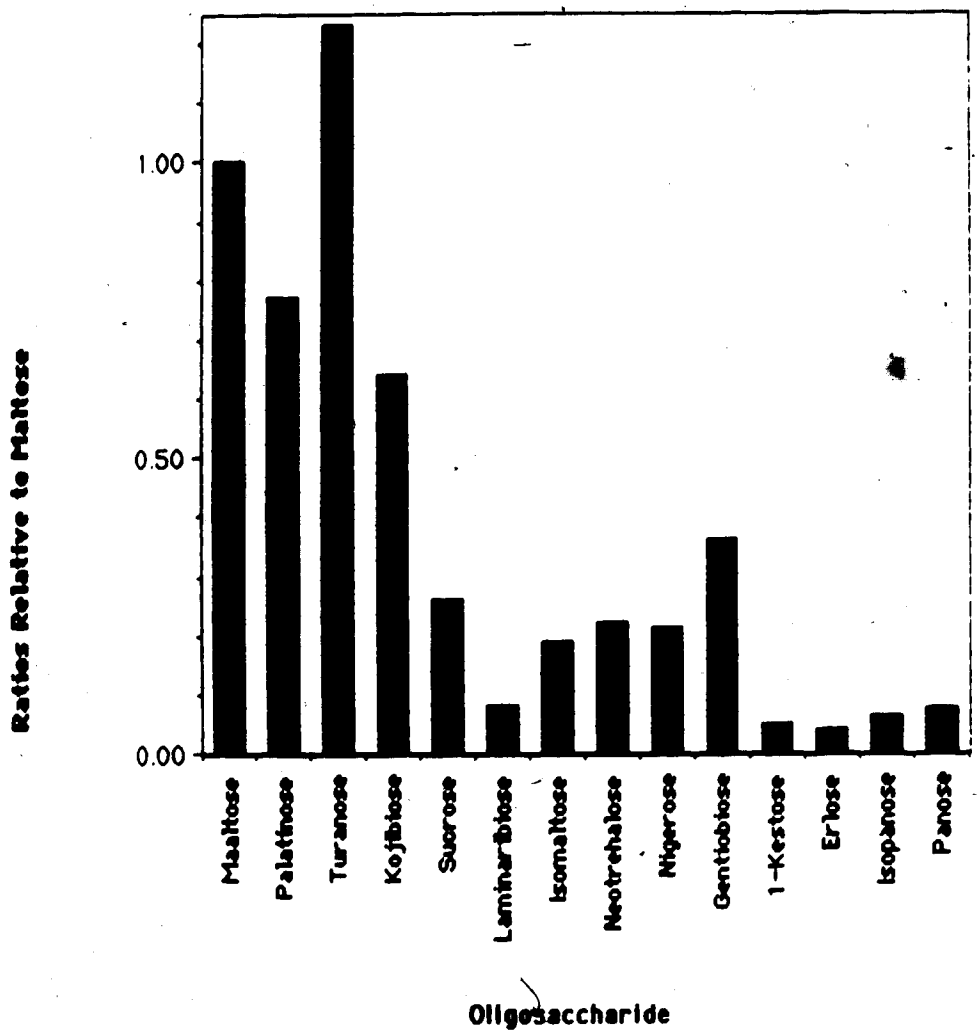


Figure 6.9 Oligosaccharides identified in sweet clover honey.

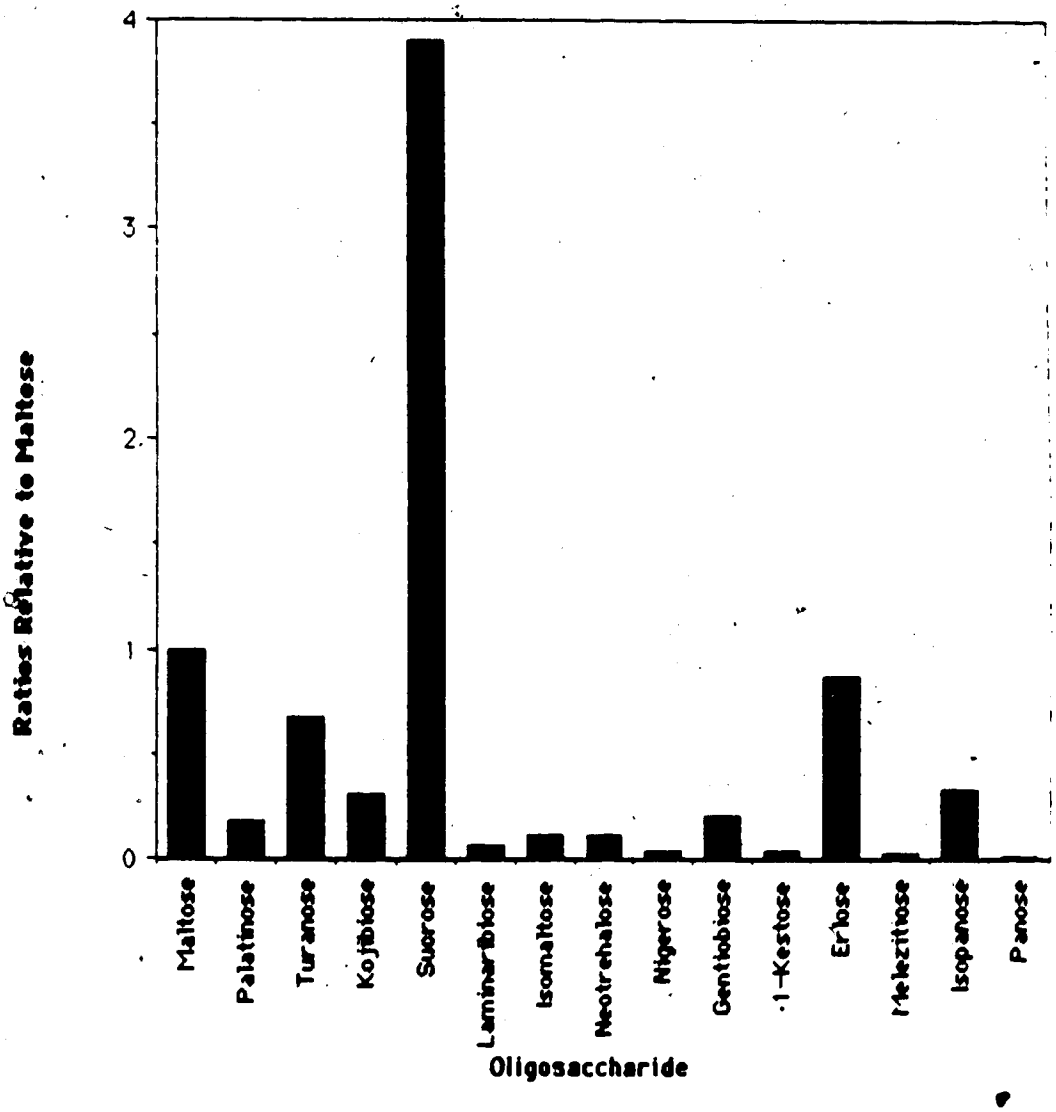


Figure 6.10 Oligosaccharides identified in trefoil honey.

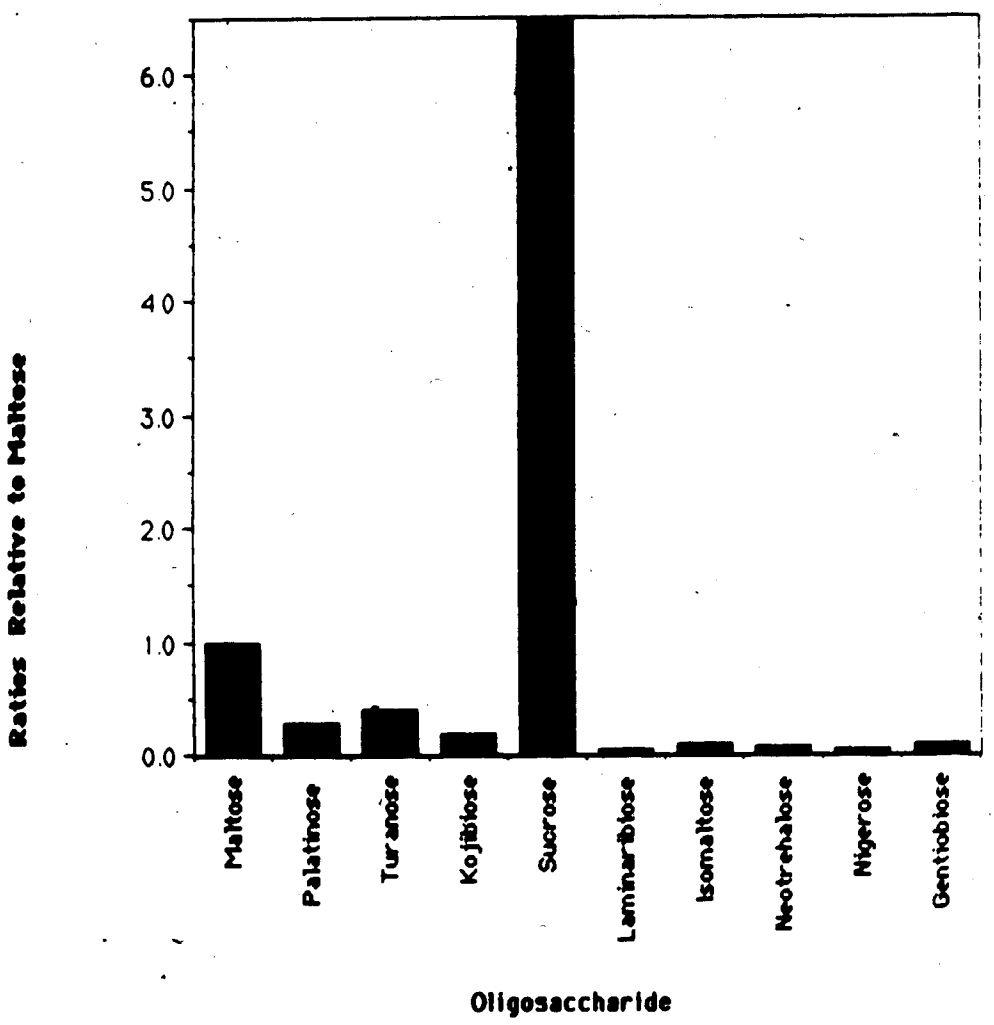


Figure 6.11 Oligosaccharides identified in *Apis cerana* honey.

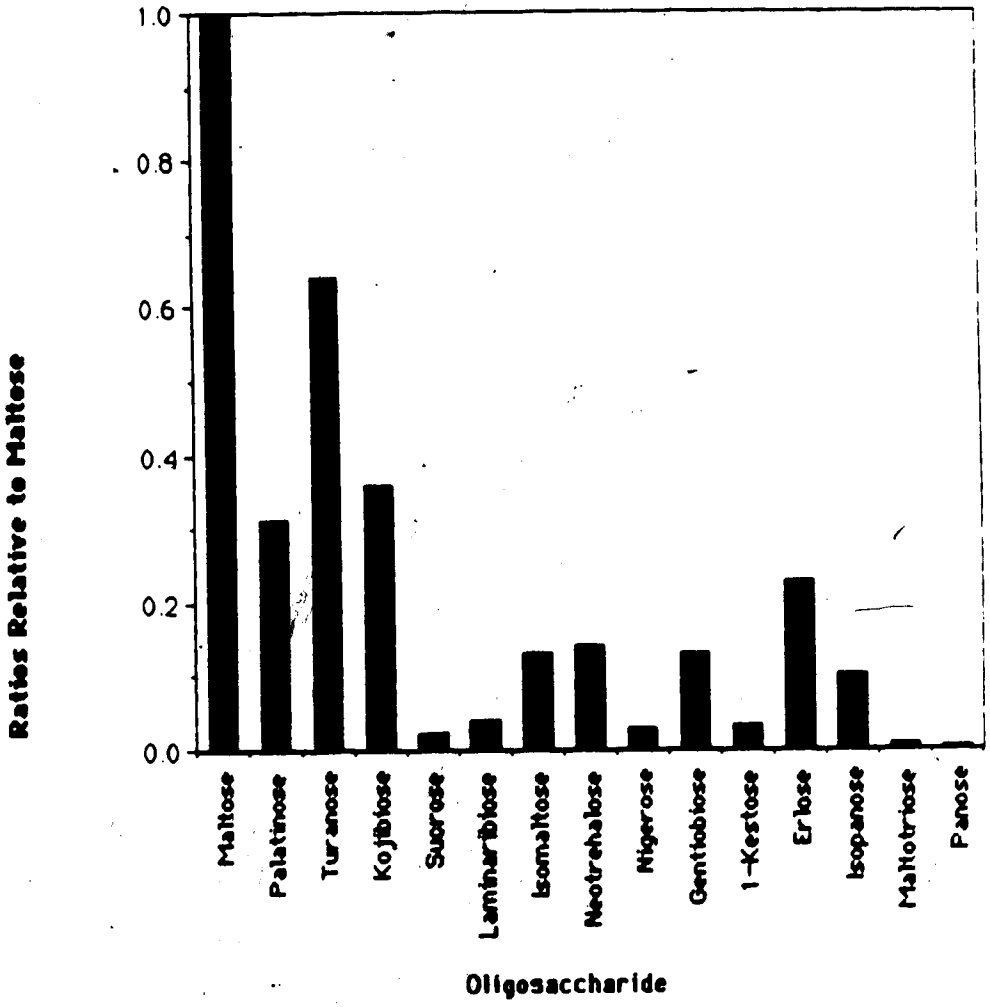


Figure 6.12 Oligosaccharides identified in *Apis dorsata* honey.

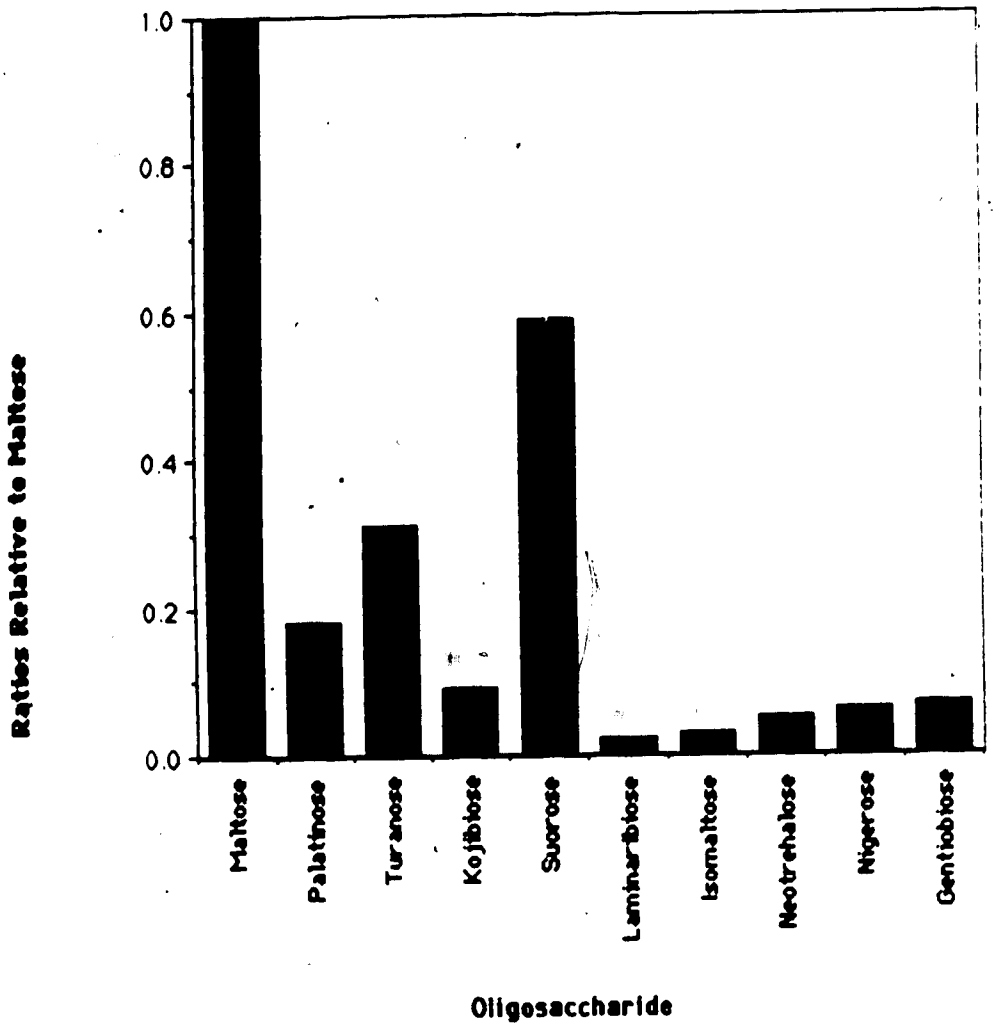


Figure 6.13 Oligosaccharides identified in *Apis florea* honey.

dorsata, *Apis cerana*, and *Apis florea*.

The major oligosaccharide found in six of the nine honeys analyzed was maltose. In two other honeys (alfalfa and *Apis cerana*), sucrose was the major oligosaccharide, whereas in sweet clover honey turanose was the major oligosaccharide. Comparison of our results with those of Siddiqui (1970) indicates a good correlation with the types of oligosaccharides present, however the percentages of oligosaccharides present in the honeys we analyzed differ from his values. The variations between our results and those of Siddiqui (1970) and the variations within the honeys that we analyzed can be explained by the enzyme concentration and activity within each honey, among other factors. In each of the nine honeys tested we also identified the oligosaccharide palatinose. Palatinose was not found by Siddiqui (1970), however it is present in fairly high concentration in each of the nine honeys we tested. In addition, some minor peaks which occurred at identical retention times in each of the honeys analyzed were not identified as no standard with an identical retention time under these experimental conditions was found. One of these minor peaks could be the oligosaccharide leucrose which Siddiqui (1970) found in honey.

Identification of the oligosaccharides present was based on comparison of the retention times of standards with those of the honeys analyzed. Spiking of the honey sample with each of the standards was also used for identification. A typical GC spectrum of the reduced (where applicable)

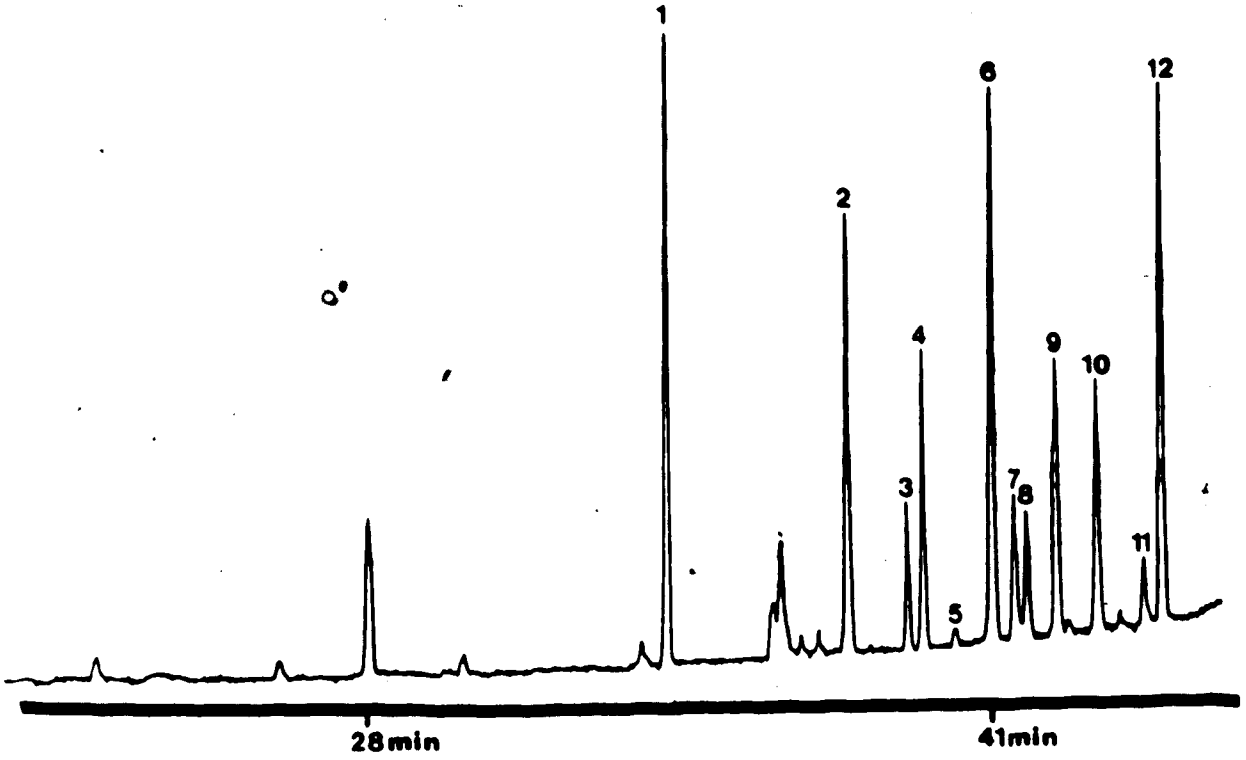
disaccharides found in honey is shown in Figure 6.14.

Typically, 20 mg of monosaccharide-free honey (1.11×10^{-4} moles; based on a molecular weight of 180 g/mole) was used for GC analysis. Assuming 6 hydroxyl groups per molecule or 6 equivalents, then 20 mg of honey requires 6.67×10^{-4} equivalents of silylating reagent. In a typical reaction, 18 mL of tri-sil Z was used, or 0.027 moles, therefore a 40-fold excess of silylating reagent was added to ensure complete silylation.

To ensure that the minor peaks present in each of the honey samples were not due to partially-silylated oligosaccharides, more silylating reagent was added and the solution re-heated. Upon analysis, the same minor peaks were present. This indicates that these peaks are not a result of partial silylation, but are due to actual compounds present in the sample.

During the experimental work-up of the reduction we were also aware that losses may occur when the oligosaccharide fraction was desalted on Dowex ion exchange resin. Recovery studies employing sucrose, maltose, turanose, and palatinose indicated that no irreversible binding was occurring as the recoveries were all >98%. As fructose-containing oligosaccharides are also acid labile (Angyal, 1984), sucrose was subjected to the reduction conditions and work-up; analysis by HPLC indicated that no hydrolysis of sucrose occurred.

From this information we feel that we have an excellent method for the identification and quantitation of the minor



1- sucrose
2- trehalose
3- neotrehalose
4- cellobiose
5- laminaribiose
6- turanose/nigerose
7- maltulose/turanose
8- maltose/maltulose
9- kojibiose
10- gentiobiose
11- palatinose
12- isomaltose/palatinose

Figure 6.14 Typical GC spectrum of the reduced (where applicable) disaccharides found in honey.

carbohydrates present in honey.

7. CARBOHYDRATE ANALYSIS BY ^{13}C NUCLEAR MAGNETIC RESONANCE

7.1 Introduction

The basic principles of nuclear magnetic resonance (nmr) result from the fact that all nuclei have a charge. In some nuclei this charge "spins" on the nuclear axis, generating a magnetic dipole along the axis. The angular momentum of this spinning charge is described by the spin number (I). Spin numbers can have the values 0, $^1/_2$, 1, etc. (I=0 denotes no spin). The intrinsic magnitude of this generated magnetic dipole is expressed in terms of a nuclear magnetic moment (Rathbone, 1985).

Each proton and neutron has its own spin; the spin number (I) is a resultant of all these spins. If the sum of the protons and the neutrons is even, then the resultant spin number is zero or an integer. If the sum is odd, then the resultant spin number is a half-integral. If the numbers of protons and neutrons are both even, then the spin number is zero (therefore no spin) (Silverstein *et al.*, 1974).

Nuclei with a spin number of one-half (i.e. ^1H and ^{13}C) have a uniform spherical charge distribution. This allows these nuclei to be easily measured by nmr. Nuclei with a spin number of one or greater have a nonspherical charge distribution which results in an electrical quadrapole moment, affecting relaxation time and coupling to neighbouring nuclei.

The spin number (I) determines the number of orientations a nucleus may assume in an external uniform

magnetic field. These orientations are expressed by 2I+1, therefore, if I=$^1/_2$, the nucleus has two possible orientations, either aligned with or against the applied magnetic field. These two orientations describe different energy states for the nucleus. As the nucleus itself is spinning, the resulting motion is actually a rotation (precession) about the imposed magnetic field. In a given applied magnetic field different nuclei precess with different frequencies. In addition, different nuclei will precess at different frequencies in a different field. With the proper frequency of electromagnetic radiation (from a radio-frequency unit), the energy may be absorbed by the spinning precessing nucleus, exciting it to the next highest energy level. Measurement of this change in energy state for each nucleus in the sample provides the basic information for nuclear magnetic resonance spectroscopy.

The exact frequency at which a nucleus will resonate is determined by the effective magnetic field at that nucleus. This magnetic field is the result of many factors. The displacement of this frequency from the hypothetical value is referred to as the screening constant. The shielding effects of the surrounding electron clouds in addition to the electronegativity of a nearby atom generally have the greatest influence on this displacement. In nuclei having postulated p or d orbitals (C, N, P), distortions from ideal geometry cause large shifts.

The above is a rather simplistic approach to nuclear magnetic resonance spectroscopy. General aspects of high-

resolution nmr have been investigated by many researchers, such as Silverstein *et al.* (1974), Harris (1983), and Fukushima and Roeder (1981).

The nmr spectrum consists of a plot of intensity of absorption versus the frequency of radiation. Basically, there are two methods to obtain an nmr spectrum: the oldest, the continuous-wave (CW) frequency sweep, and the field sweep method. In the frequency sweep method the magnetic field is kept constant while the nuclei in the sample are excited into resonance by application of radio frequency. In the field sweep method, the radio frequency is held constant while the magnetic field is varied to excite the nuclei into resonance. This methodology is useful when dealing with concentrated samples (0.1 to 0.6 M). Generally it is only applicable to ^1H as other nuclei have lower relative sensitivities, although this problem may be alleviated by repeated scanning. The other major problem with this technique is that the normal scanning time in CW-nmr is at least 250 sec, therefore the acquisition times for spectra are long (Rathbone, 1985; Silverstein, 1974; Abraham and Loftus, 1981).

The most widely used nmr methodology is Fourier-transform (FT) nmr spectroscopy, which has significantly shortened the time required to perform an nmr experiment. This technique involves the simultaneous excitation of all the nuclei in the sample by the application of a short (10-60 μsec), powerful (several kW) pulse of radio frequency power. Following application of the pulse, the spectrometer

receiver is switched on (1-10 sec) while the re-emission of absorbed energy is recorded as a free-induction decay (FID) signal. The FID signal consists of a detector response versus time. This signal is converted by Fourier transformation into a plot of signal intensity versus frequency. A spectrum obtained in this manner is virtually identical to the CW-hmr method except that the acquisition time is much faster (Hall, 1980; Rathbone, 1985).

Measurable parameters of an nmr spectrum include the following (Hall, 1980):

(1) Chemical shift - the position of the center of the resonance signal with reference to a selected, internal or external standard. Chemical shifts are normally present in dimensionless units (ppm).

(2) Integral - the relative area of each separate resonance. These areas are proportional to the relative number of nuclei resonating at each particular frequency (problems arise when dealing with different T_1 values).

(3) Spin-lattice relaxation times (T_1 values) - time constants for the transfer of absorbed energy from the resonating nuclei to the lattice (surrounding environment).

(4) Spin-spin relaxation times (T_2 values) - time constants for loss of phase coherence of the resonating nuclei through direct spin-spin interaction between different nuclei without energy transfer to the lattice. Spin-spin relaxation determines signal linewidth.

The first nmr observations of ^{13}C nuclei were by Lauterbur (1957) and Holm (1957). Early experiments were hindered by poor resolution and the requirements that the sample to be analyzed be highly soluble and of low molecular weight.

Following the breakthrough of wide-band proton decoupling and various instrumental and technical developments, ^{13}C-nmr spectroscopy was advanced to the status of a practical analytical research tool (Levy and Nelson, 1972).

7.1.1 ^{13}C-nmr and carbohydrates

Carbon-thirteen nmr spectroscopy has been applied to the structural determination of a number of monosaccharides and their derivatives (Coxon, 1980). This technique has also been applied to oligosaccharides (Dorman and Roberts, 1971; Allerhand *et al.*, 1971; Allerhand and Doddrell, 1971) and polysaccharides (Dorman and Roberts, 1971).

Dorman and Roberts (1971) used ^{13}C for the structural analysis of a number of common disaccharides (1-2 M aqueous solutions) by the continuous-wave technique. The ^{13}C chemical shifts of these compounds were referenced to carbon disulfide. Using these conditions, the authors found that interpretation of the spectra of the equilibrated anomers was complicated by peak overlaps. As an example, α,β-cellobiose displayed only 12 resonances, out of a possible 24. From this work, a number of structural assignments were made that have since been reassigned by

more definitive methods (Coxon, 1980).

With the introduction of the pulse-Fourier transform technique, ^{13}C spectra of single anomers or partially equilibrated anomers of carbohydrates could be obtained in a short time. This technique (FT-nmr) was used in the study of a number of disaccharides (Voelter *et al.*, 1971; Voelter *et al.*, 1973; Usui *et al.*, 1973; Jennings and Smith, 1973; Colson *et al.*, 1974). In addition to the determination of anomeric ratios, this technology was applied to the positions of the linkages of di- and trisaccharides (Coxon, 1980).

Nuclear magnetic resonance spectroscopy has been used extensively as a method for the structural determination of carbohydrate molecules. It allows the elucidation of both the static and dynamic structures of carbohydrates. A number of reviews have been written on the study of carbohydrates in solution and in the solid state (Rathbone, 1985; Vliegenthart *et al.*, 1983; Bock and Pedersen, 1983; Bock and Thøgersen, 1982; Gorin, 1981; Hall, 1980; Barker and Walker, 1980; Jennings and Smith, 1980; Coxon, 1980; Smith and Saito, 1980; Perlin, 1976; Durette and Horton, 1971).

Applications of ^{1}H and ^{13}C nmr for analysis of carbohydrates in foods are few. However, nmr has been used by various researchers in the food industry, and an excellent review has been published by Weisser (1975).

Kainosho (1976) used high resolution ^{13}C-nmr spectroscopy to detect sucrose in an intact seed of *Aucuba japonica* (aoki).

Mathur-De Vré (1979) applied ^{1}H- and ^{13}C-nmr spectroscopy to study polymer-solvent interactions. The ^{13}C linewidth measurements, in addition to the bound-water relaxation times, reveal important information on the flexibility of polymer chains. This methodology has been applied to the rheological properties of gels (Child and Pryce, 1972) and wheat starch pastes (Callaghan *et al.*, 1983). Structural studies employing the ^{1}H nmr relaxation times for starch-water systems have also been investigated (Labuzá and Busk, 1979; Lechert *et al.*, 1980; Nakazawa *et al.*, 1980; Schwier and Lechert, 1982). Water binding capacities of a number of food products have been reported (Hennig, 1977; Hennig and Lechert, 1977) using wide-line ^{1}H- and ^{2}H-nmr spectroscopy. Application of this methodology has been used to determine the moisture content of a variety of foodstuffs (Brosio and Di Nola, 1982). Doornbose *et al.* (1981), Altena *et al.* (1981) and Röper *et al.* (1983) applied ^{1}H and ^{13}C nmr to the identification of Amadori- and Heyns-rearrangement products formed during the processing of foods. These products arise from the reactions between amino acids (or proteins) and carbohydrates and are related to flavour and colour formation.

Introduction of the minispec pc 10/20 (Bruker) (and the newer models, the minispec 100/120), which is an automated nmr analyser for analytical laboratory use, has introduced nmr analysis into all types of laboratories.

This automated nmr spectrometer has been used to determine the total fat content of chocolate, the fat

content of dried sausage samples, cheese, margarine, butter and cocoa products. It may also be used to determine the water content of various foods and foodstuffs, including the moisture in oil seeds, glucose, tobacco, and sugar beets. The oil content of oilseeds may also be determined. As nmr is a non-destructive technique and an automated analyzer can provide consistent accurate results and rapid analysis times (minutes or seconds), this type of equipment is gaining in popularity (Bruker minispec application notes).

There are a number of reasons why ^{1}H- and ^{13}C-nmr are not used more extensively for carbohydrate analysis in foods. Even though the areas under the peaks in an nmr spectrum are proportional to the number of protons or carbons (under appropriate conditions) present in the sample (Shaw, 1976), accuracies of approximately 5% are possible. When this technique is compared to other methods (gas-liquid chromatography, high performance liquid chromatography, etc.) of higher sensitivity and lower cost, nmr spectroscopy would be used only when its unique selectivity could be applied. The sensitivities of ^{1}H- and ^{13}C-nmr spectroscopy are also significant. The most abundant isotope of carbon, atomic weight 12, has no nuclear spin and therefore is not observable by nmr. The natural abundance of ^{13}C is only 1.1%, compared to 100% for ^{1}H. The magnetogyric ratio of ^{13}C nuclei is approximately 1/4 that of ^{1}H. The sensitivity of a nucleus in an nmr experiment is proportional to the cubed magnetogyric ratio, therefore ^{1}H nuclei will give rise to a signal 64 times larger than ^{13}C nuclei. The result of both

the lower magnetogyric ratio and 1.1% natural abundance of ^{13}C produces a reduction in the sensitivity of approximately 6000 relative to a ^{1}H-nmr experiment (Levy and Nelson, 1972).

It has been recognized that ^{13}C-nmr spectroscopy could be used for the quantitative analysis of carbohydrates (Coxon, 1980; Rathbone, 1985). Problems associated with the use of ^{13}C-nmr are well documented and include relaxation times (T_1 and T_2), the nuclear Overhauser effect, viscosity effects, temperature effects, solubility, digital resolution, and the sensitivity of the ^{13}C nuclei. Wehrli and Wirthlin (1976) indicated that these problems are less severe in the ^{13}C-nmr quantitation of carbohydrates than for other organic molecules, however measurements of ^{13}C-nmr signal integrations are prone to error (Wehrli and Wirthlin, 1976). Levy and Nelson (1972) concluded that no direct correlation exists between the integrated peak areas and the number of carbon nuclei. They indicated that this is due to long spin relaxation times and variable nuclear Overhauser effects (nOe).

Coxon (1980) calculated the T_1 values for a number of the carbon nuclei in carbohydrates and found that T_1 in most instances is less than 1 sec. In order for the carbon nuclei to relax so that another pulse may be applied, it is necessary to wait a period of at least $5T_1$ (or 5-6 sec). This makes the acquisition time necessary to obtain a ^{13}C spectrum lengthy and expensive. In addition, the nOe for the carbon nuclei vary and these factors introduce errors into

the quantitation measurements (Berry *et al.*, 1977; Czarniecki and Thornton, 1977).

Bock and Pedersen (1983) found that, if ^{13}C-nmr samples were measured under suitable conditions and if the peak areas of similar carbon atoms (carrying the same number of protons) were compared, the accuracy of quantitative methods was increased.

Rathbone (1985) indicated that, in order to obtain reliable ^{13}C-nmr signal integration, it was essential to achieve good signal/noise ratio. This may be accomplished by using concentrated solutions or by multiplying the FID signal by a sensitivity-enhancing factor. In addition, the author found that a sufficiently high resolution (≥5 points per signal) was necessary to define the lines in a spectrum.

Cerbulis *et al.* (1978) noted that quantitative ^{13}C-nmr requires comparisons of resonances having different nOe's. In an attempt to prove the structure of β-lactosylurea, the gated-decoupling technique was employed. The technique requires the turning off of the frequency during long periods (≅30 sec, or 5-7 times T_1) between data acquisitions in order to remove the nOe effect.

Carbon-thirteen nmr spectroscopy also has a number of advantages over ^{1}H-nmr and other carbohydrate analysis methods. The range of chemical shifts for ^{13}C nuclei is approximately 250 ppm, compared to only 10 ppm for ^{1}H. The natural abundance of ^{13}C is only 1.1%, therefore coupling between two ^{13}C nuclei is not usually a problem. In ^{1}H nmr, ^{1}H-^{1}H coupling occurs (both short and long range coupling)

in addition to ^{1}H-^{13}C coupling which results in splitting of the nmr signals and complexes the spectrum. The preparation of a sample for ^{13}C-nmr is non-destructive and requires no derivatization. However, the most important aspect of ^{13}C-nmr spectroscopy of carbohydrates is the "fingerprint" region for the anomeric carbons (≈90-110 ppm). In this region oligosaccharides which differ only in the position of attachment ($\alpha 1 \rightarrow 1$, $\alpha 1 \rightarrow 2$, etc.) may be unequivocally identified (Coxon, 1980). In addition, disaccharides which are attached to the same position but differ anomerically ($\alpha 1 \rightarrow 1$, $\beta 1 \rightarrow 1$) can also be identified.

Blunt and Munro (1976) applied ^{13}C-nmr spectroscopy for the qualitative and quantitative determination of carbohydrates extracted from various tissues of *Pinus radiata*. They used a relaxation delay of >4T, to determine fructose, glucose and sucrose levels in these tissues, with standard deviations of 3-8%. The authors compared the values obtained by nmr to those obtained by GC and found good correlation.

Recently, Tamate and Bradbury (1985) applied ^{13}C-nmr spectroscopy for the analysis of carbohydrates in tropical root crops. They quantitated the amounts of fructose, glucose, sucrose, maltose and raffinose by measurement of the ratios of peak heights with those of an internal standard. The authors used a relaxation delay time of 2 sec and analyzed their results by comparison to standard curves. Comparison of the results obtained by nmr to those obtained by HPLC showed deviations of 3-30%.

7.2 Nuclear Magnetic Resonance Experimental

Standards (3-17 mg) were reduced with sodium borohydride (50 eq) in water (5 mL) at room temperature for 2 h. The solution was cooled in an ice water bath and neutralized by dropwise addition of glacial acetic acid. This solution was desalted by passing through a column (80 mm x 8 mm) of Dowex 50 WX 8 ion exchange resin. The column was further washed with two bed volumes of HPLC-grade H_2O. The combined washings were evaporated *in vacuo* and co-evaporated (4 x 5 mL) with methanol. The colourless oil was dissolved in 500 μL of deuterated dimethyl sulfoxide (DMSO-d6) and transferred quantitatively to a 5 mm thin-walled nmr tube. To this solution was added 11.33 mg of methyl β-D-ribofuranoside (internal standard), and 2 mg of chromium acetylacetonate (relaxing agent). Carbon-thirteen nmr analysis was carried out on a Bruker WH 400 nmr spectrometer with a superconducting magnet. Accumulation times varied from 20 min to 6 h. Standard operating conditions were: SF (spectrometer frequency) = 100.5743849; SY (synthesizer frequency) = 74.8900000; O1 (carrier frequency) = 5600.00; SW (sweep width) = 29411.765; FW (filter width) = 29000; AQ (acquisition time) = 0.5571; H_z/pt = 1.795; Pw (pulse angle) = 4.0; RD (relaxation delay) = 0; O2 (decoupling frequency) = 8300; temperature = 297°K; GS (gain set) = 116. These standard operating conditions were used for all of the compounds analyzed.

Honey (≅300 mg) was also reduced with sodium borohydride (50 eq) and the work-up was the same as that

used for the standards. The resulting colourless sample was taken up in 500 μL of warmed (<40°C) DMSO-d6 and transferred to a 5 mm thin-walled nmr tube, 3 mg of chromium acetylacetonate was added, and the nmr spectrum was run for approximately 10 h (60,000 scans), under the standard conditions listed above.

7.2.1 Preparation of O-methyl-β-D-ribofuranoside

To 2 g (0.0133 moles) of D-ribose in 75 mL of reagent grade methanol was added 1.0 mL of distilled acetyl chloride (0.0141 moles). The resulting slightly yellow solution was stirred at room temperature for 4 h and was monitored by thin-layer chromatography (Merck, Kieselgel 60 F254 plates; SSE as the solvent [upper phase of EtOAc:n-PrOH:H_2O, 4:1:2]) to ensure the greatest yield of the desired faster moving product. The reaction was quenched by adding 5 mL of Dowex 1X2 (OH^-) ion exchange resin and stirred until neutral by pH paper. The resin was filtered and the solution removed *in vacuo* (Buchi rotavapour R). The resulting yellowish oil was dissolved in the minimum amount of water and placed on a Dowex 1X2 (OH^-) ion exchange resin column (2 x 20 cm) and the minor products were removed by elution with water. The major product, O-methyl-β-D-ribofuranoside, was eluted with 3% MeOH/H_2O. Evaporation of the solvent followed by crystallization from EtOH/H_2O, yielded 1.57 g (72%) of white needle-like crystals, m.p. 77-79°C (literature 76-78°C). Elemental analysis calculated for $C_6H_{12}O_5$: C, 43.90; H, 7.37. Found: C, 43.65; H, 7.42.

7.3 Nuclear Magnetic Resonance Results and Discussion

Sucrose and trehalose were used as standards in the initial investigation for the use of ^{13}C-nmr spectroscopy for quantitative analysis. Relaxation delay times of 6 sec were used and concentrations ranging from 3-50 mg of each compound were subjected to analysis. The solvent initially used was D_2O; as it has no carbons, the computer must be programmed to set a reference standard. Using these techniques, errors in the standard curves of 10-15% were quite common.

Investigation of the literature on ^{13}C-nmr suggested the use of paramagnetic relaxing agents (Lamar, 1971; Wehrli and Wirthlin, 1976; Williams and Fleming, 1980). Chromium acetylacetonate was used in our experiments. The Cr^{+3} ion in this compound has unpaired electrons which cause the ^{13}C nuclei to relax efficiently (Williams and Fleming, 1980). This addition results in a number of effects: the detrimental loss of the nOe will result in no enhancement of the ^{13}C nuclei signal (maximum enhancement of 2.98), and the relaxing agent reduces the spin-spin relaxation time (T_2). Line widths are proportional to the inverse of T_2, therefore, if T_2 is short, then the line widths broaden. The beneficial effects also involve the loss of the nOe; this will allow the quantitation of the ^{13}C nuclei in the spectrum. As has previously been mentioned, the maximum nOe is 2.98, however different carbon nuclei experience different nOe's, therefore, if the nOe's vary, then accurate quantitation is difficult to impossible. The addition of the

relaxation agent causes spin-lattice relaxation (T_1) to be more efficient, and it shortens the spin-lattice relaxation time, resulting in shorter T_1's and therefore enables pulsing more frequently. If no relaxing agent were used, it would be necessary to wait 5-6 T_1's (or to employ the gating technique for quantitation) or approximately 30 sec between pulses. Each pulse requires 0.65 sec, therefore it is possible to acquire >40x the number of scans in an experiment with a relaxing agent. As Coxon (1980) indicated, an experiment with a paramagnetic relaxing agent requires 9x as many scans as one without to obtain the same signal-to-noise ratio (2.98^2). Though this enhancement is lost by the addition of the relaxing agent, the signal-to-noise can be made up by the increased number of scans possible (due to T_1 being shorter). Employing this methodology allows the quantitative measurements of ^{13}C nuclei in the sample. The problem with line-width broadening can be minimized if the concentration of relaxing agent is controlled (i.e. not an excess).

Complete ^{13}C-nmr data on glucobioses and glucotrioses as well as fructose-containing di- and trisaccharides are known (Bock and Pedersen, 1983; Usui *et al.*, 1973). While reviewing this data, overlaps for the α and β anomers of various disaccharides were observed. To alleviate this ambiguity in the identification and quantitation of the various carbohydrates in honey, the samples to be tested were reduced with sodium borohydride. For example, if maltose was analyzed by ^{13}C-nmr, three peaks would be

present in the 90-110 ppm region [C1'(100.8), C1α(93.1), C1β(97.1)], whereas reduced maltose has only one peak in this region [C1'(100.8)].

An internal standard was also used in our measurements. The compound methyl β-D-ribofuranoside was used since, among other things, the anomeric carbon is structurally similar to the anomeric carbons of interest in honey (that is, a carbon covalently bonded to two electronegative oxygen atoms and one hydrogen atom). In addition the anomeric carbon of this compound can be used for an internal reference. Finally, the ratio of the anomeric carbon area of the reference compound with those of the standards can be used for quantitative analysis.

Known concentrations of trehalose, sucrose, and their mixtures were analyzed by ^{13}C-nmr (400 MHz) employing chromium acetylacetonate in deuterated dimethyl sulfoxide (DMSO-d6). Analyzing the area of the anomeric carbon (for sucrose the C1' carbon was used) versus concentration gave a linear relationship for the concentrations studied (5-45 mg; Figures 7.1 and 7.2). In addition, the linear relationship was still apparent when these two standards were mixed. Four different concentrations of maltose were reduced by sodium borohydride and analyzed by ^{13}C nmr; again the area of the anomeric carbon (C1') versus concentration was also linear for the concentrations studied (4-50 mg).

Analysis of five identical samples of reduced cellobiose under the same conditions produced a standard deviation of 0.44%. Reduced maltose was also analyzed at

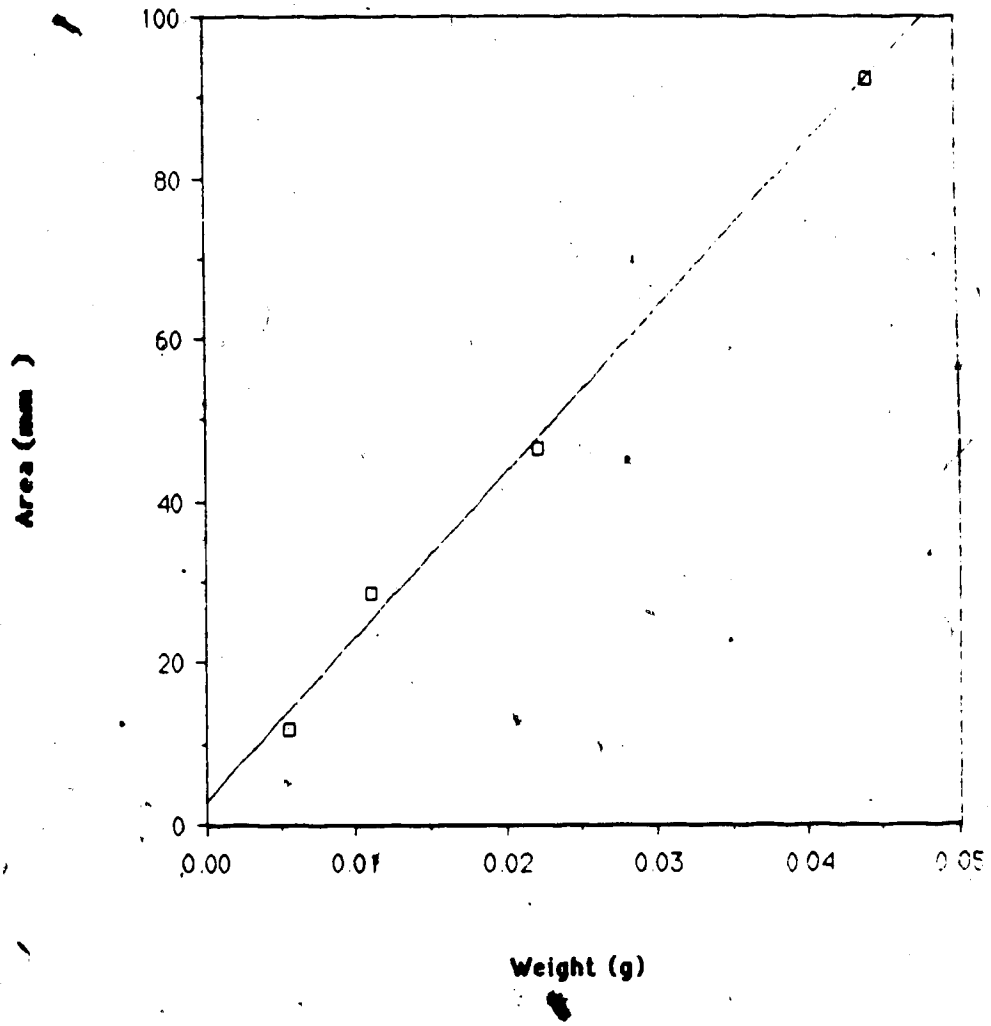


Figure 7.1 ^{13}C-nmr area of anomeric carbon versus concentration for trehalose.

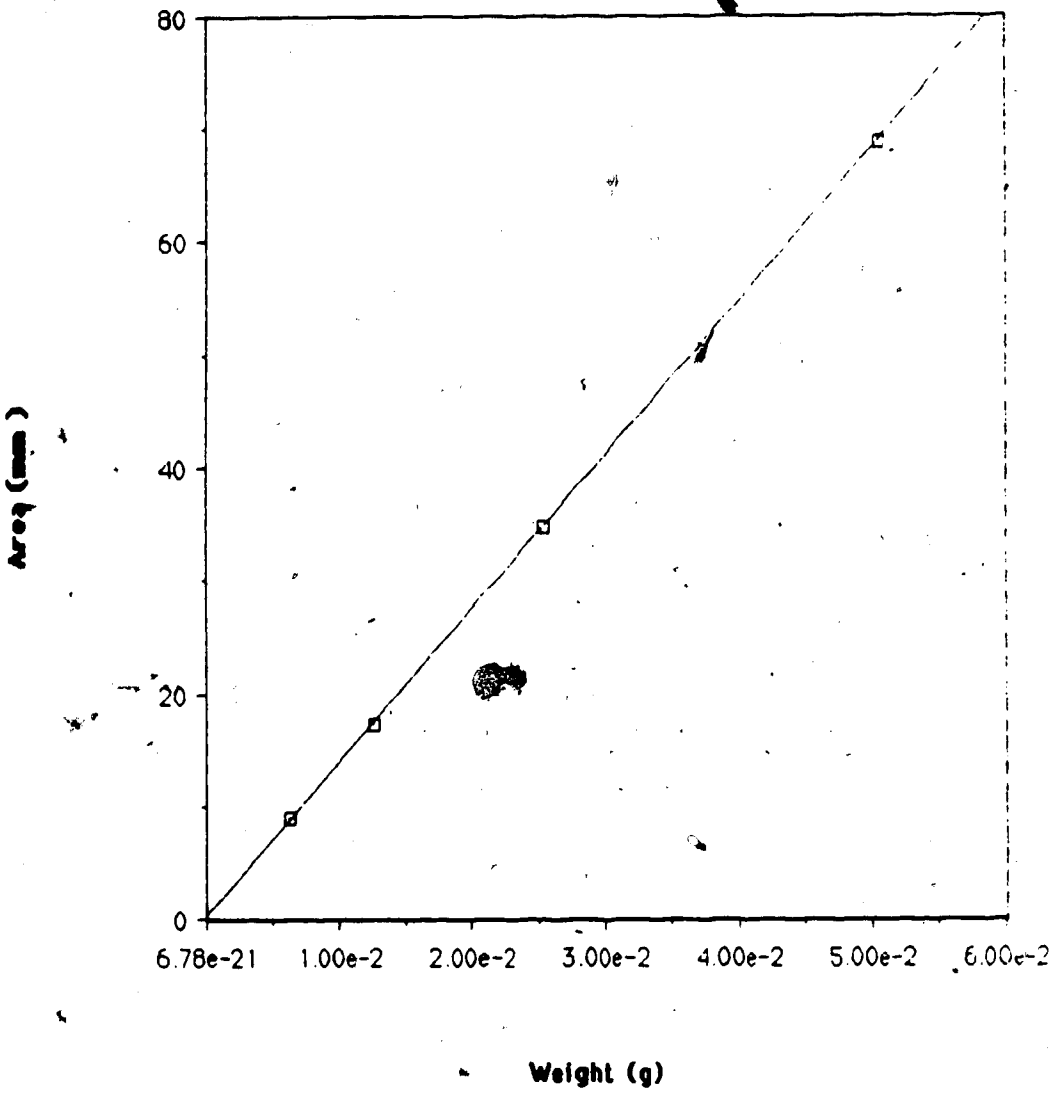


Figure 7.2 ^{13}C-nmr area of anomeric carbon versus concentration for sucrose.

various acquisition times (from 500 to 60,000 scans); the result of this experiment is shown in Figure 7.3. As can be seen, a linear relationship is apparent. The results of these experiments lead to the conclusion that ^{13}C-nmr analysis employing a relaxing agent and using DMSO-d6 would allow for reproducible quantitative determination of oligosaccharides.

Thirteen reduced (where applicable) disaccharides of known concentration, with the addition of a known concentration of standard, were analyzed by ^{13}C-nmr on a Bruker 400 MHz nmr spectrometer. Table 7.1 contains the chemical shift(s) of the anomeric carbon(s) for each disaccharide. As was the case in the GC analysis of fructose-containing reducing disaccharides, two peaks were observed by ^{13}C-nmr for these compounds. However, unlike capillary gas chromatography, each of the glucitol and mannitol peaks can be identified and quantitated as no overlapping occurs. The standard deviation associated with each of the chemical shifts is ±0.02 ppm. A peak can be identified if a separation of 0.08 ppm exists, however, accurate quantitation requires a separation of 0.5 ppm. For each of the reduced standards, a separation of at least 0.1 ppm exists, except for cellobiose and gentiobiose, whose anomeric carbons have chemical shifts of 103.59 and 103.61 ppm, respectively. This does not pose a problem for their detection in honey since cellobiose was not found by capillary gas chromatography. A comparison of ratios of the internal standard to the anomeric carbon(s) for each

Table 7.1 Assignments of the ^{13}C resonance of the anomeric carbon(s) of disaccharides (reduced where applicable) found in honey.

Disaccharide	Chemical Shift (ppm)[1]
Cellobiose	103.59
Gentiobiose	103.61
Isomaltose	98.62
Kojibiose	99.20
Laminaribiose	104.30
Maltose	100.77
Maltulose	100.79 99.56
Neotrehalose	103.56 100.68
Nigerose	99.59
Palatinose	98.70 98.61
Sucrose	103.87 91.64
Trehalose	92.93
Turanose	100.32 99.57

[1] standard deviation for all measurements was ±0.02

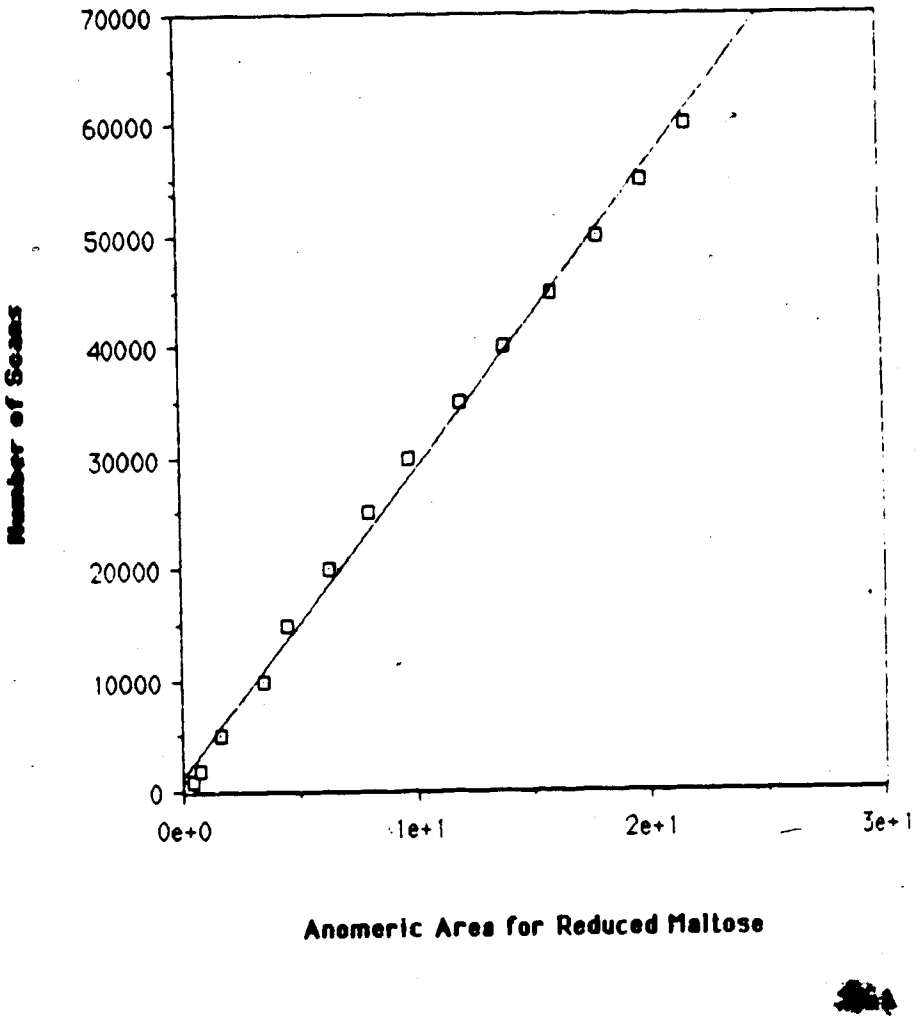


Figure 7.3 ^{13}C-nmr analysis of reduced maltose at various acquisition times.

standard will allow the quantitation of each of these disaccharides present in honey. When maltulose, palatinose, and turanose were reduced and analyzed by ^{13}C-nmr, the ratios of the glucitol/mannitol peaks were approximately 1:1, 1:1 and 1:2, respectively. These results agree with those obtained when these reduced carbohydrates were analyzed by capillary gas chromatography.

Seven reduced (where applicable) trisaccharides of known concentration (together with known concentrations of internal standard and chromium acetylacetonate) were analyzed by ^{13}C-nmr (400 MHz). These results are shown in Table 7.2.

Limitations of this methodology are realized when comparing reduced maltotriose and reduced maltose (these limitations also apply when dealing with non-reduced maltotriose and maltose) as the positions of linkage of the monosaccharide units are all $\alpha 1 \rightarrow 4$, therefore no clear distinction can be made with regards to the concentration of each carbohydrate. From the gas-liquid chromatographic information of the carbohydrates present in the honey samples analyzed, >99% of the total carbohydrates in the separated fraction are disaccharides, hence the contribution of trisaccharides and oligosaccharides can be assumed to be negligible. In addition, due to the increased molecular weight of trisaccharides (and larger oligosaccharides) as well as the way these carbohydrates rotate, a [illegible]-tration of these compounds is required in order to obtain a ^{13}C-nmr spectrum.

Table 7.2 Assignments of the ^{13}C resonance of the anomeric carbon(s) of trisaccharides (reduced where applicable) found in honey.

Trisaccharide	Chemical Shift (ppm)[1]
Erlose	103.91 100.75 91.65
Isomaltotriose	98.50 98.55
Isopanose	98.60 98.55
1-Kestose	103.85 91.67
Maltotriose	100.69 100.67
Melezitose	103.93 100.43 91.63
Panose	98.55 98.60

[1] standard deviation for all measurements was ±0.02

This procedure was applied to the high performance liquid chromatographically separated samples of alfalfa and sweet clover honey. Following drying (vacuum pump), these samples (≅300 mg) were reduced (sodium borohydride) and analyzed by ^{13}C-nmr. A typical spectrum is shown in Figure 7.4. The analyzed data, together with that obtained by gas-liquid chromatography, are shown in Tables 7.3 and 7.4. The major disaccharides found by GC were also identified by ^{13}C-nmr. In alfalfa honey the ratios determined by each method agree quite closely, except for that obtained for kojibiose, where twice the concentration was found by ^{13}C-nmr when compared to that determined by GC. In addition, the concentration of sucrose differed by ≅20%. For sweet clover honey, the values for turanose (≅30%) and gentiobiose (≅50%) did not agree with the GC data. Repetition of the analysis by ^{13}C-nmr did not improve these results. No apparent reasons for the anomalies in the results for these two honeys are known. The data, aside from these values, are in quite close agreement.

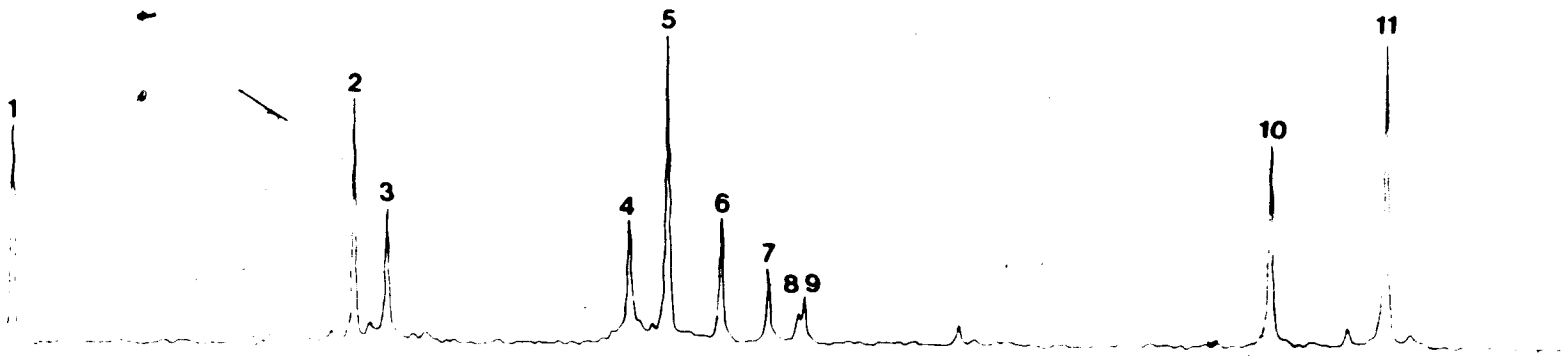
The ^{13}C-nmr method as applied to the identification of minor honey carbohydrates (disaccharides) has advantages over other techniques in that the method is relatively rapid, non-destructive, and allows for the complete identification and quantitation of the disaccharides present. Honey represents an extremely complicated case for ^{13}C-nmr analysis in the number of oligosaccharides present and their virtually identical structures. It is our opinion that in other food products where the number and complexity of

Table 7.3 Comparison of disaccharide ratios in alfalfa honey, determined by ^{13}C-nmr with comparison to gas chromatographic determination.

Disaccharide	^{13}C-nmr	Gas Chromatography
Maltose	1.0	1.0
Palatinose	.36	.39
Turanose	.41	.46
Kojibiose	.58	.25
Sucrose	3.2	3.9
Neotrehalose	.11	.10
Nigerose	.10	.12
Gentiobiose	.13	.15
Isomaltose	.096	.11

Table 7.4 Comparison of disaccharide ratios in sweet clover honey determined by ^{13}C-nmr with comparison to gas chromatographic determination.

Disaccharide	^{13}C-nmr	Gas Chromatography
Maltose	1.0	1.0
Palatinose	.68	.77
Turanose	.96	1.23
Kojibiose	.58	.64
Sucrose	.29	.26
Neotrehalose	.19	.22
Nigerose	.19	.21
Gentiobiose	.19	.36
Isomaltose	.16	.19



1- standard
2- sucrose
3- gentiobiose
4- maltose
5- turanose
6- turanose/nigerose
7- kojibiose
8- palatinose
9- isomaltose
10- trehalose
11- sucrose

Figure 7.4 Typical ^{13}C-nmr spectrum of standard oligosaccharides.

carbohydrates present are fewer and simpler, respectively, that this type of methodology could be very useful.

8. THE ENZYMES IN HONEY

8.1 Introduction

The theory that enzymes were present in honey was tested early in the twentieth century. Marpmann (1903) reported that alcoholic fermenting and proteolytic enzymes were present in honey. Gothe (1914) noted that honey did not contain lactase, lipase and proteases. His work indicated that the initial enzyme tests performed on honey were subject to error as the reagents may have reacted with the sugars and acids present in honey.

Initially, the interest in the enzymes in honey centered around the need to distinguish between natural and artificial honeys (Crane, 1977). The enzyme system diastase (α- and β-amylase) is recommended by the Codex Alimentarius Committee (1983) as a standard for honey quality. Other researchers (Duisberg and Hadorn, 1966; Hadorn and Zurcher, 1966; Sigenthaler, 1977) recommend using α-glucosidase activity as a measure of honey quality.

The major enzymes present in honey include both α- and β-amylase (diastase), α-glucosidase I and II (invertase, sucrase or saccharase), and glucose oxidase. Minor enzyme activities present include catalase, phosphorylase, and phosphatase.

Lampitt *et al.* (1930) studied the effects of temperature and pH on diastase activity in honey. They found that the pH and temperature optima for α-amylase were 5.0-5.3 and 22-30°C and for β-amylase, 5.3 and 45-50°C. Many

authors have studied the effects of both temperature and pH on diastatic activity in honey (Crane, 1979 and references therein). Schepart and Subers (1966) attempted the isolation of amylases from honey and although they were able to reach a 200-fold purification of α-amylase, attempts to further purify the enzyme were unsuccessful due to its instability.

It is not readily apparent why diastase activity should be found in honey as starch is not the primary carbohydrate source for this insect. Gothe (1914) postulated that the enzyme originated from the bees but also indicated that pollen could be the source of this enzyme activity. Phillips (1927) found that bees could not utilize raw or cooked starch or dextrins. Lotmar (1935) fed bees solutions of 8% starch dextrin/8% sucrose, 5% starch and 8% sucrose. She found that bees survived longer on the starch/dextrin solution, however, appreciable differences in the death rates did not occur for any solution. Vansell and Freeborn (1929) proposed that pollen was the source of diastase in honey. Their work was based on the relationship between pollen content and diastase activity. In a number of California honeys studied they found that both diastase activity and absolute pollen counts were low. Lothrop and Paine (1931) studied a number of honeys from different floral sources (therefore different pollen) and found a wide variation in diastase activity. They indicated that these variations support Vansell and Freeborn's hypothesis. Bartels and Fauth (1933) attempted to correlate absolute pollen content with diastase activity and found that pollen

content (regardless of floral origin) was independent of diastase activity. These authors suggested that high temperatures and low humidity resulted in low diastase activities in the honeys tested by Vansell and Freeborn.

Fiehe (1932) postulated that nectar was the major source of diastase. Weishaar (1933) ran diastase activity experiments on nectar, pollen, and bees and found that 1.5-2.5% and 0.25-0.75% of the activity resulted from the nectar and pollen, respectively, and that the remainder was due to the bees. Gorbach (1942) found that the pH optima for plant diastase and bee diastase were slightly different and that the pH optimum in honey resembled that of the bees. It is widely accepted (Rinaudo *et al.*, 1973; Crane, 1979; White, 1978) that the diastase in honey originates with the bee.

The presence of glucose oxidase in honey was first noted by Cocker (1951), based on the fact that, following neutralization, the honey became acidic after storage. White *et al.* (1958) attributed this pH increase to the hydrolysis of lactones present in honey. Further work by White *et al.* (1962, 1963) conclusively proved the presence of glucose oxidase in honey and its ability to produce gluconolactone (then gluconic acid) and hydrogen peroxide. The presence of hydrogen peroxide aided in the explanation of the antibacterial effect (inhibine) of honey. Gluconic acid is the organic acid produced by this enzyme (Stinson *et al.*, 1960) and is responsible for a portion of the taste of honey (tartness), in addition to honey's stability towards

microorganisms (Crane, 1979). Schepartz and Subers (1964) have shown that the source of this enzyme is also the bee.

8.2 Invertase in Honey.

The enzyme responsible for the major chemical changes which occur when the bee converts nectar into honey is invertase. Invertase (sucrase, saccharase, β-fructosidase, α-glucosidase) is an enzyme which is found in a great variety of organisms. The invertase in one organism may be quite different from that found in another. The hydrolysis of sucrose to fructose and glucose is the major reaction performed by this enzyme, however, the enzyme may be an α-D-glucopyranoside glucohydrolase (EC 3.2.1.20), or a β-D-fructofuranoside fructohydrolase (EC 3.2.1.26). The invertase present in honey bees is an α-D-glucopyranoside glucohydrolase (White and Maher, 1953), commonly referred to as α-glucosidase.

Nelson and Cohn (1924) were the first to work on the invertase enzyme from honey. They used alcohol to precipitate the enzyme from honey. Following dialysis, the honey invertase was adsorbed on alumina, eluted, and dialyzed. The authors compared the relative reaction rates between honey and yeast invertase. They found that honey invertase showed a different reaction rate towards sucrose, than did yeast invertase. Nelson and Sottery (1924) indicated that honey invertase was activated by D-glucose, while yeast invertase was not. In addition, they found that honey invertase would not hydrolyse raffinose, whereas yeast

invertase would. Papadakis (1929) studied the effects of honey invertase on the hydrolysis of sucrose in the presence of a variety of other carbohydrates. The author found that sucrose hydrolysis was not activated by any of the carbohydrates studied. Other researchers (Ammon, 1949; Gorbach, 1942) also studied the effects of pH and other carbohydrates on honey invertase.

8.3 Isolation and Properties of Honey Invertase

White and Maher (1953a) confirmed that the invertase enzyme in honey is a glucoinvertase (this was reported first by Gorbach, 1942) by chromatographic studies. They based this conclusion on the inactivity of the honey enzyme towards raffinose and its moderate activity towards melezitose, whereas strong activity towards maltose and sucrose was observed. The authors also found that the enzyme was inhibited by glucose and that a glucose unit was transferred to sucrose, yielding a trisaccharide (erlose). In addition, the hydrolysis of maltose by honey invertase yielded not only glucose but a series of glucooligosaccharides.

The first successful separation of the invertase enzymes in honey was performed by White and Kushnir (1967). They used dialysis, ion exchange and gel filtration chromatography in addition to starch-gel electrophoresis to purify the enzymes. The α-glucosidase present showed a single band with an approximate molecular weight of 51,000 daltons.

Once the α-glucosidase from honey was separated and purified, further work on the enzyme's physical and chemical characteristics could be performed. Huber (1975) and Huber and Mathison (1975) isolated α-glucosidase from the head, thorax and abdomen of adult honeybees (the species was not specified). The α-glucosidase was isolated using fractionation with ammonium sulfate, followed by dialysis. Hydroxylapatite, ion exchange and gel filtration chromatography were also employed. The final step involved passing the enzyme solution over a column of bound p-aminophenyl-β-D-glucopyranoside. Following these separations, the authors found that the abdomen of the bee contained the major portion of the α-glucosidase isolated; only minor amounts were found in the head and thorax regions. The molecular weight of the α-glucosidase isolated was 93,000 daltons by SDS gel electrophoresis. The Km's for sucrose and for p-nitrophenyl-α-D-glucopyranoside hydrolysis were 4 mM and 0.5 mM, respectively.

Huber (1975) noted that a significant portion of the α-glucosidase (almost 50% in the abdomen) was soluble even at 80% saturated ammonium sulfate. Gontarski (1957) had suggested that two types of sucrase were present in honeybees. This observation was based on studies which indicated that sucrase in honey and the pharyngeal glands differed from that of the intestines. Riedel and Simpson (1961) have shown that the mouth parts and heads of drones and queens do not contain sucrase, whereas the worker bee heads do. Queens and drones do not produce honey and

therefore the enzyme necessary for this production could be absent. However, all bees require a digestive or metabolic sucrase in order to utilize sucrose as an energy source.

Takenaka and Echigo (1976, 1978) isolated α-glucosidase from honey by ammonium sulfate precipitation (60% solution) and dialysis, followed by DEAE-[illegible] and Sephadex G-100 (X2) chromatography. They found that the α-glucosidase in honey was fractionated into two components, α-glucosidase I and II. The molecular weights of these two components were 73,000 and [illegible] daltons, respectively. The pH and temperature optima for these two enzymes were found to be 6.0 and 40°C for each. The Km's (sucrose) for α-glucosidase I and II were found to be 4.4 mM and 4.6 mM, respectively. These enzymes were also shown to be glycoproteins and contained 15-20% carbohydrate.

Takewaki *et al.* (1980) purified α-glucosidase I and II from honeybees. They homogenated 500 g of frozen honeybees using the head/thorax region and the abdomen region separately. Ammonium sulfate precipitation (95% saturation) and dialysis, CM-cellulose, and Sephadex G-100 chromatography were used to purify α-glucosidase I. DEAE-cellulose, CM-cellulose and Sephadex G-100 chromatography were used to separate α-glucosidase II. The locations of the α-glucosidases in the honeybee were determined; the head/thorax contained 22% α-glucosidase I and 46% α-glucosidase II, while the abdomen contained 78% α-glucosidase I and 54% α-glucosidase II. The molecular weights of α-glucosidase I and II as determined by SDS gel

electrophoresis were 98,000 and 76,000 daltons, respectively. The pH optimum (sucrose) of both α-glucosidase I and II was 5.0, but α-glucosidase I was stable over a wide pH range, while α-glucosidase II was sensitive to pH.

The origin of saccharase in honey was confirmed by Rinaudo *et al.* (1973). The possible sources of honey saccharase were studied; these included nectar, pollen and the honeybee. The authors found that honey and bee gland saccharase activities were optimal at a pH of 5.9 and temperature of 40°C, whereas pollen and nectar saccharases had respective pH optima of 4.4 and 4.7, and temperature optima of 55°C and 45°C. They also found that honey α-glucosidase is competitively inhibited by fructose, while pollen and nectar saccharases are unaffected. In addition, treatment of saccharases from honey and honeybees with a series of amino acid modifiers indicated that both enzymes behaved similarly. The authors concluded that honey saccharase is of insect origin and that it is transmitted to honey by the bee.

8.4 Transglucosylation Action of Honey Invertase

As it name implies, α-D-glucopyranoside glucohydrolase (α-glucosidase) is a hydrolysis enzyme. It can also function in the transfer of a glucose unit to another carbohydrate moiety, thus acting as a glucotransferase. This type of reaction is referred to as transglucosylation. Under normal conditions (water not the limiting reagent), α-glucosidase transfers the α-D-glucose unit from hydrolysis to water,

yielding D-glucose. However, if water is limiting, then this α-glucose unit can be transferred to any acceptor molecule in the solution, such as fructose, glucose, sucrose or any oligosaccharide.

White and Maher (1953a) were the first to observe this transglucosylation reaction by honey α-glucosidase. In their study a honey enzyme concentrate (prepared by precipitation with ethanol followed by dialysis) and sucrose were incubated at 25°C in acetate buffer of pH 5.7 for 2 h. Following chromatography on charcoal-celite into a mono-, di- and trisaccharide fraction, the di- and trisaccharide fractions were subjected to paper chromatographic separation. The authors used co-chromatography with standards to identify two carbohydrates in these fractions. Maltose and erlose were the carbohydrates identified in this manner, which conclusively proved that a D-glucose unit was transferred to sucrose (to form erlose) and glucose (to form maltose). The only other explanation for these results is the presence of the carbohydrate erlose in honey (which may still be present following enzyme precipitation of honey by ethanol, and dialysis) and the presence of fructosidase in honey, which will yield maltose from hydrolysis of erlose. The fact that raffinose was not hydrolyzed by the honey enzyme concentrate and the fact that care was take to remove all the carbohydrates upon dialysis would rule out these possibilities.

Nelson and Cohn (1924) also observed the transglucosylation properties of honey invertase, although they failed

to recognize this activity. When studying the rate of sucrose inversion by honey invertase, they reported that after an initial increase in the reaction rate to a maximum of 10-15% inversion, the velocity gradually fell during the remainder of the reaction. This was later explained to be due to the formation of the trisaccharide erlose in the amount of approximately 11% of the total sugars (White and Maher, 1953b). Erlose has a specific rotation nearly double that of sucrose, therefore the presence of erlose would lead to erroneously high values for sucrose concentration and lead to errors in the velocity calculations for sucrose inversion by honey invertase.

White and Maher (1953b) extended their work in the transglucosylation activity of honey invertase by incubating sucrose and a honey enzyme concentrate under the same conditions as their previous paper (White and Maher, 1953a). Following isolation by column and paper chromatography, a yield of 11% (based on the original weight of sucrose) of the trisaccharide erlose was obtained.

Huber and Mathison (1975) isolated α-glucosidase from the abdomens of honeybees and found that it had transglucosylation activity. They used a highly purified α-glucosidase with sucrose (0.2 M) and incubated (temperature, pH and time were not reported). Separation of the products by thin-layer chromatography indicated a band corresponding to trisaccharides. Isolation and characterization of these products was not performed, however, the authors hypothesize that the product arises from the transfer of α-D-glucose to sucrose.

Takenaka and Echigo (1978) used 100 mL of their purified honey enzyme solution with sucrose (50 g) in an acetate buffer (pH 6.0), and incubated at 30°C. Paper chromatography of the reaction solution showed six bands, three of which had mobilities similar to that of tri- and tetrasaccharides. The trisaccharide was shown to be erlose, and one of the tetrasaccharides was tentatively identified as O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside. The third band was not identified as only a minute amount was isolated. The major products isolated were fructose, glucose and sucrose, however, the other oligosaccharides either originated by chemical reactions or by transglucosylation. This work represented an extension of their earlier research (Takenaka and Echigo, 1976) on the transglucosylation activity of honey α-glucosidase.

Ono *et al.* (1981) incubated honey α-glucosidase (0.5 IU) and sucrose (2.4 g) at 30°C for 60 h. The sucrose solution was then fractionated by paper chromatography, and the trisaccharide erlose was isolated from the paper zones. The authors indicated that the major sugars present in the reaction mixture were fructose, glucose and sucrose (≈99%) and that approximately 1% of the total carbohydrate in the enzyme-sucrose reaction was the trisaccharide erlose.

Recently, Deifel *et al.* (1985) found that honeybees fed with sucrose produced honey containing maltose, erlose, maltotriose and isomaltosyl-sucrose. They also incubated honey saccharase with sucrose and found the above-mentioned

oligosaccharides along with glucose and fructose. The authors indicated that these carbohydrates are formed by the transglucosylation action of honeybee saccharase.

The transglucosylation properties of α-glucosidase are not restricted only to honeybee α-glucosidase. A few representative examples of the general activity of this enzyme will be presented.

Chiba *et al.* (1985) incubated α-glucosidase from buckwheat and sucrose at pH 5.0 and 30°C. The oligosaccharides maltose, kojibiose, nigerose and isomaltose were detected by a combination of [redacted] and gas chromatography. In addition, Chiba *et al.* (1984) isolated three trisaccharides from the incubation of buckwheat α-glucosidase and maltose/sucrose. These trisaccharides were identified as maltotriose, erlose and theanderose.

8.5 Honey Enzymes Experimental

The honey sample (1500 g) was diluted with 1 L of purified Millipore RO (reversed osmosis) water and dialyzed (Spectropor membrane tubing, 73 mm) overnight (10 h) at 4°C against cold, running tap water. To ensure that all the carbohydrates had been removed, the enzyme soltuion was tested with Benedict's solution (an orange precipitate indicates the presence of reducing carbohydrates). The dialyzed enzyme solution (2.5 L) was then treated slowly with enzyme-grade ammonium sulfate to 75% saturation, and the solution was allowed to stand at 4°C for 10 h. The resulting precipitate was collected by centrifugation

(18,890 g for 25 min at 4°C). The precipitate was dissolved in a minimum quantity of HPLC-grade water and dialyzed (Spectropor membrane tubing, 23 mm) for 8 h, changing the purified water every 30 min. The dialyzed enzyme solution was applied to a DE-52 (Whatman) ion exchange column (2.5 x 30 cm) equilibrated with 0.01 M phosphate buffer (pH 7.5). Elution was carried out with the same buffer by a linear gradient of NaCl (0-0.4 M), the flow rate was 27 mL/h, and detection was with a UV monitor at 280 nm. The active fractions, as determined by reaction with p-nitrophenyl-α-D-glucopyranose [α-PNPG] (Sigenthaler, 1977; Takewakai *et al.*, 1980), were pooled and concentrated to 3 mL with an Amicon filtering system (molecular weight cutoff at 10,000 daltons). This enzyme solution was then applied to a Sephadex G-100 column (2.5 x 80 cm) which had been equilibrated with 0.01 M phosphate buffer (pH 6.8). The protein was eluted with the same buffer and showed two major fractions. The two fractions (α-glucosidase I and II) were separately applied to another Sephadex G-100 column (2.5 x 80 cm) equilibrated with 0.01 M phosphate buffer (pH 6.8), and eluted with the same buffer. The purification procedures are summarized in Table 8.1.

8.5.1 α-Glucosidase or β-glucosidase analysis

A honey sample (2 g) was dissolved in approximately 10 mL of 0.1 M phosphate buffer of the appropriate pH (6.0 for α-glucosidase; 4.3 for β-glucosidase), transferred to 25 mL volumetric flask and made up to volume with more of the

Table 8.1 Purification of honey transglucosylatic[illegible]es.

Step	Volume (mL)	Total Units	Total Protein (mg)	Specific Activity (unit/mg)	Purification
Crude	50	8320	800	10.4	1
DE-52	100	4010	223	18.0	1.7
Sephadex G-100	50	2987	60	49.8	4.8
Sephadex G-100					
α-glucosidase I	30	120	2	60	5.8
α-glucosidase II and β-glucosidase	30	1100 600	15	73 40	6.9

1 unit will convert 1 μmole of α-PNPG (or β-PNPG) to p-nitrophenol.

buffer.

To two 25 mL, glass screw-top vials was added 5 mL of 0.002 M substrate (α-PNPG or β-PNPG) in the same phosphate buffer as the honey. The vials were allowed to equilibrate in a water bath of the desired temperature (40°C for α-glucosidase; 60°C for β-glucosidase) for 5 min before 0.5 mL of the honey solution was added to one vial (sample, and 0.5 mL of 3.0 M, pH 10.5 Tris buffer and 0.5 mL of the honey solution were added to the second vial (blank). These mixtures were rapidly shaken and incubated for 20 min in the water bath before 0.5 mL of 3.0 M, pH 10.5 Tris buffer was added to the sample vial. (When the 0.1 M phosphate buffer was used for the dilution of honey and β-PNPG was lower than pH 3.0, it was necessary to add 1.0 mL of the Tris buffer to the blank and sample vials to achieve a basic enough pH for proper colour development. In order to compare results, this extra dilution was taken into account before the results were reported.)

The absorbances of the blank and sample vials were recorded at 400 nm using a Bausch and Lomb Spectronic 21 spectrophotometer. Absorbances were reported as the difference between sample and blank readings.

Both α-glucosidase and β-glucosidase analyses were carried out in triplicate.

8.5.2 Dialysis tubing preparation

Dialysis tubing was washed in water, 0.01 M ethylenediaminetetraacetic acid, disodium salt (EDTA), and in water

again before use.

8.5.3 Protein determination

Protein concentration was determined by the method of Bradford (1976), using Bio-Rad Protein Assay, employing Bovine Serum Albumin as the protein standard.

8.5.4 Transglucosylation reactions

The crude enzyme extract following the DE-52 treatment was used for the transglucosylation reaction.

To fructose (2 g), glucose (2 g) and sucrose (1 g) was added 17 mL of purified Millipore reverse osmosis purified water (≅30% carbohydrate solution). To this solution was added 1 mL (≅40 units of α-glucosidase) of the crude enzyme mixture. The resulting solution was filtered using a sterilization filter unit (Millipore; 0.20 μm) and placed on a shaker for 48 h at 21°C. The solution was then heated at 80°C for 10 min to destroy all enzyme activity and was then subjected to HPLC separation to remove the monosaccharides present. The resulting oligosaccharide fraction (≅3 mg) was evaporated and treated with tri-sil Z (2 mL) and analyzed by gas-liquid chromatography.

Similar reactions were performed using only sucrose (2 g) in 6 mL, glucose (2 g) in 6 mL, or fructose (2 g) in 6 mL, each with 1 mL of the crude enzyme solution. Following HPLC separation of the oligosaccharides, the fractions were silylated (tri-sil Z) and analyzed by gas chromatography.

8.6 Honey Enzymes Results and Discussion

The minor carbohydrates that are present in honey have been isolated and characterized (Siddiqui, 1970). Glucose-glucose disaccharides are found in honey with $\alpha 1 \rightarrow 2$, $\alpha 1 \rightarrow 3$, $\alpha 1 \rightarrow 4$, $\alpha 1 \rightarrow 6$, $\alpha 1 \rightarrow \beta 1$, $\beta 1 \rightarrow 3$, and $\beta 1 \rightarrow 6$ linkages. In addition, glucose-fructose disaccharides and a variety of trisaccharides and oligosaccharides may be explained by the transglucosylation activity of the enzymes present in honeybees and which are transferred to honey.

As has been previously mentioned and shown, the honeys tested contained a variety of disaccharides and trisaccharides (see the gas chromatography and nuclear magnetic resonance sections of this thesis). Also, the nectars responsible for these honeys were found to be free from carbohydrates other than fructose, glucose and sucrose (see the nectar section of this thesis). It was our hypothesis that these complex carbohydrates must have been formed from the interaction of the enzymes located in the honey sac of the bee (and later in honey) and the carbohydrates found in nectar. In order to establish the veracity of those assumptions, the enzymes responsible for these transformations were isolated from honey using the techniques of Takenaka and Echigo (1978). In our isolation we used a honey which was very enzyme-active (not a unifloral honey, but mainly of clover origin as indicated by pollen analysis). Purification of the honey enzymes of interest (α-glucosidase I, II) was approximately 6-fold for α-glucosidase I and 7-fold for α-glucosidase II/β-glucosidase fractions.

Incubation of a typical nectar solution (Baker and Baker, 1983) consisting of fructose, glucose and sucrose with a crude mixture of the enzymes for 48 h at room temperature yielded both di- and trisaccharides. Following removal of the monosaccharides by HPLC, the oligosaccharides were reduced, silylated and analyzed by GC. From the chromatogram, the disaccharides sucrose, neotrehalose, turanose, maltose, kojibiose, gentiobiose and isomaltose were identified, and the trisaccharide erlose was found.

Glucose was incubated (48 h, room temp.) with a crude mixture of the enzymes, followed by the same work-up procedure and GC analysis. Maltose, gentiobiose, nigerose, isomaltose and kojibiose were the disaccharides identified; no trisaccharides were apparent.

Fructose was also incubated (48 h, room temp.) with the crude enzyme solution. Following work-up, sample preparation, and GC analysis, no carbohydrate products were found.

The results of these experiments demonstrate the ability of the enzymes present in honey to form more complex carbohydrates from the carbohydrates normally found in nectar. That these enzymes are transglucosylation enzymes is readily apparent by the lack of any oligosaccharides present in the fructose/enzyme experiment.

Following the gas chromatographic analysis of the nine honeys, it was noted that at least three beta-linked disaccharides were present in each honey tested. The existence of β-glucosidase activity in honey was discovered

by substituting β-PNPG for α-PNPG in the testing of honey for α-glucosidase activity (Sigenthaler, 1977).

The fact that an enzyme was causing the hydrolysis of β-PNPG was confirmed by heating the honey solution in boiling water for five minutes to destroy the enzyme activity, then finding that hydrolysis of β-PNPG did not occur with the boiled honey.

To determine some of the enzyme characteristics, the factors of pH and temperature for Sigenthaler's (1977) twenty minute test were optimized using a simplex optimization technique (Shavers *et al.*, 1979). The results are shown in Table 8.2. Using this procedure, the pH and temperature optima for β-glucosidase in honey were established between 4.0 and 4.3, and 58 and 60°C, respectively, for our experimental conditions. Holding the temperature at 60°C, β-glucosidase activity varied as shown in Figure 8.4.

Using the optimum conditions of pH 4.3 and temperature 60°C, the six honey samples previously analyzed were shown to have β-glucosidase activities varying from 0.073 to 0.211 (Table 8.3) absorbance units at 400 nm using our testing procedure. In addition, honey samples from three other honeybee species were tested for β-glucosidase activity and these results are also shown in Table 8.3. All nine honey samples tested had β-glucosidase activity. This implies that this enzyme is added by the bee or may be present in the nectar from which the honey is derived. If this enzyme is added by the bee, its purpose is unknown since the nectar

Table 8.2 β-Glucosidase activity of a honey sample following Simplex Optimization procedure and varying temperature and pH.

pH[1]	Temperature (°C)	Absorbance (at 400 nm)
6.0	40	0.043±0.001[2]
7.5	60	0.025±0.014
4.3	60	0.177±0.025
2.8	40	0.069±0.002
4.8	45	0.093±0.005
6.3	65	0.065±0.004
5.4	59	0.115±0.006
5.0	74	0.035±0.005
4.8	52	0.136±0.002
3.7	53	0.141±0.002
3.1	60	0.035±0.017
4.4	54	0.131±0.003
5.0	61	0.159±0.009
4.9	67	0.137±0.003
4.6	58	0.150±0.005
4.6	63	0.131±0.014
4.0	58	0.174±0.003
4.0	63	0.147±0.010

[1] Values of pH and temperature are given in the order that the Simplex Optimization determined.

[2] Standard deviation.

Table 8.3 β-Glucosidase activities of nine honeys.

Honey	Absorbance (at 400nm)
Alfalfa	0.183±0.010[1]
Alsike	0.154±0.006
Canola	0.140±0.003
Red Clover	0.206±0.005
Sweet Clover	0.210±0.002
Trefoil	0.211±0.005
Apis cerana	0.110±0.006
Apis dorsata	0.073±0.003
Apis florea	0.171±0.008

[1] Standard deviation.

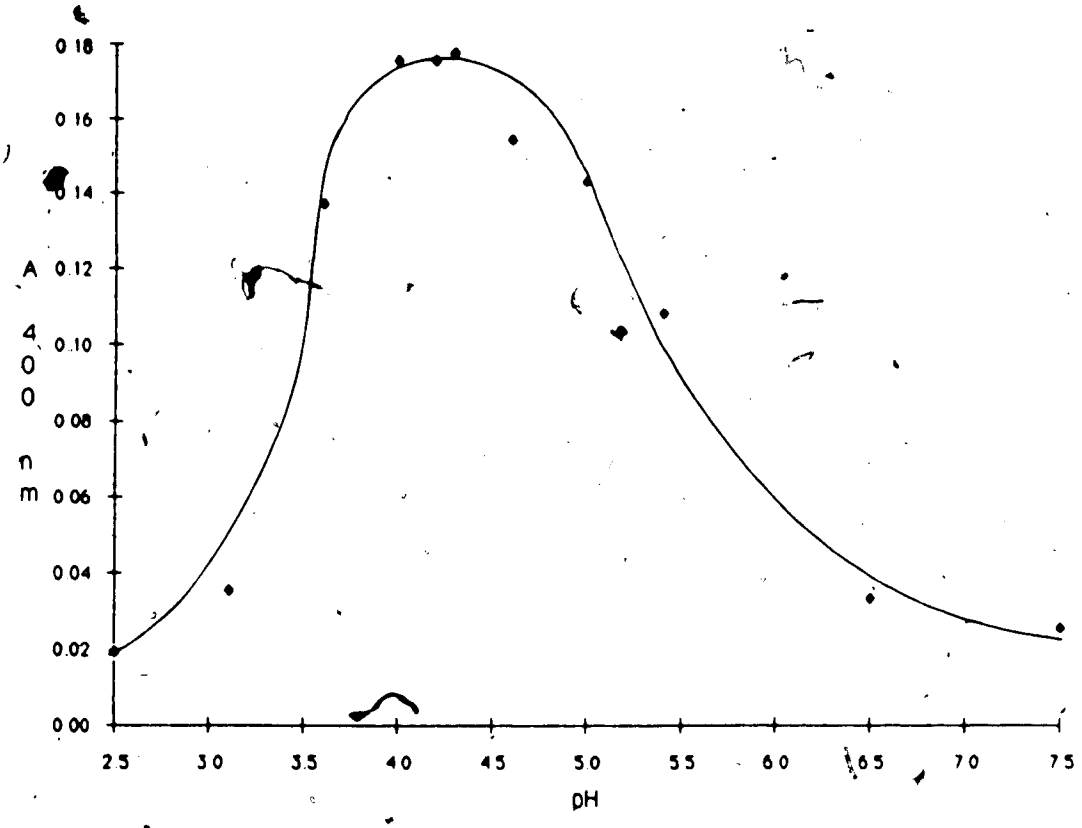


Figure 8.1 pH dependence of β-glucosidase at 60°C.

sources contain almost exclusively the monosaccharides fructose and glucose, in addition to the disaccharide sucrose (see Chapter 4 of this thesis), which contains an α-linked glucose. The presence of β-glucosidase in honey could explain the presence of the three beta-linked carbohydrates identified in our honeys: neotrehalose, laminaribiose and gentiobiose.

Although it is difficult to compare the activities of different enzymes analyzed in different manners, it appears that there is considerable β-glucosidase activity in honey compared to the closely related α-glucosidase activity measured in a similar analysis. The absorbances at 400 nm for α-PNPG and β-PNPG for sweet clover honey were 0.256 and 0.210, respectively.

To determine the possibility that other sugars may be interfering with the determination of β-glucosidase activity, a honey sample was dialyzed and the β-glucosidase analysis performed before and after dialysis. The results were an absorbance of 0.140±0.020 for the dialyzed sample and 0.140±0.008 for the undialyzed sample, indicating, as Sigenthaler (1977) had shown for α-glucosidase, that proper dilution of the honey sample removes interfering effects of the sugars present.

In addition to determining the pH and temperature optima for β-glucosidase, Km and Vmax were also determined. Employing nine different concentrations (0.0001 M - 0.04 M) of β-PNPG at the pH and temperature optima, the values for Km and Vmax were experimentally determined (Wilkinson, 1961)

to be 12.9±2.8 mM and 0.524±0.046 mM, respectively.

During the separation and purification of honey α-glucosidase I and II, it was found that honey α-glucosidase II and β-glucosidase were co-chromatographed under our separation conditions. Various attempts were made to isolate honey β-glucosidase; these included selective precipitation with ammonium sulfate, ion exchange chromatography (DE-52 and hydroxylapatite), gel filtration chromatography (Sephadex G-100 and G-200), and chromatofocusing. The isolation of β-glucosidase was also attempted by preparing an affinity column, which enabled β-glucosidase to be removed from α-glucosidase II, however, β-glucosidase could not be eluted from the affinity column.

9. CONCLUSIONS

In this work an attempt has been made for an in depth approach to determine the origin of oligosaccharides in honey.

Unifloral honeys were produced by using packaged bees and placing the colonies in cultivated fields. Identifying the botanical origin of honey by the traditionally-accepted method of pollen analysis exposed a few serious flaws in this methodology. Proper dilution of the honey and the use of the acetolysis procedure facilitated identification of the pollen.

Representatives of the floral varieties from which the honey originated were collected. Following nectar extraction, derivatization (silylation) of the carbohydrates present in the nectar converted them to more volatile compounds which were analyzed by capillary gas chromatography. The results showed that, for the six floral sources analyzed, the nectars were virtually free of all oligosaccharides except sucrose.

Methods were required for the identification and quantification of the oligosaccharides in honey. The use of high performance liquid chromatography for the fractionation of honey yielded an oligosaccharide fraction that was practically free of monosaccharides. Reduction of this fraction with sodium borohydride and derivatization with tri-sil Z prepared the oligosaccharides for capillary gas chromatography. The development of this relatively rapid methodology resulted in the identification and quantitation

of all but one of the oligosaccharides previously found (Siddiqui, 1970) in honey using more laborious methods.

In addition to capillary gas chromatographic analysis of the monosaccharide-free and reduced fraction, carbon-thirteen nuclear magnetic resonance was performed. The use of this technique on the reduced oligosaccharides in conjunction with the addition of a relaxing agent resulted in identification of the oligosaccharides in honey and the ability to quantitate these carbohydrates. A comparison of the two methods provided information on the usefulness of ^{13}C-nmr analysis for identification and quantitation in the extremely complex problem of the analysis of honey oligosaccharides.

Investigations into the enzymes in honey have shown the presence of β-glucosidase, which was not seen by other researchers. The presence of this enzyme in honey may explain the beta-linked oligosaccharides identified in this work and by others (Siddiqui and Furgala, 1970).

Incubation of a typical nectar secretion with partially purified honey enzymes produced a number of oligosaccharides which were also identified in honey.

From the above data then, it seems that the presence of minor oligosaccharides in honey is due to transglucosylation activity of the enzymes added by the bee.

10. FUTURE RESEARCH IN HONEY

It would be interesting and very useful to compare the AOAC official method for honey carbohydrate analysis (carbon-celite chromatography) with our high performance liquid chromatographic method. The HPLC method is very rapid and precise and would likely correlate well with the accepted method and could (even should?) replace it.

Incubation of a typical nectar solution with individually purified α-glucosidase I and II would be another project that could yield some interesting results. It would be of interest to see if the oligosaccharides produced by each of these enzymes were the same. In conjunction with this, incubation at various times, pH's, temperatures, and using a variety of solvents to yield specific products (oligosaccharides) could be economically important (i.e. could replace difficult chemical synthesis).

Finally, completing the isolation of β-glucosidase would be an interesting project.

11. REFERENCES

Abraham, R.J. and Loftus, P. 1981. Proton and Carbon-13 N.M.R. Spectroscopy an Integrated Approach. London: Heyden.

Adam, S. and Jennings, W.G. 1975. Gas chromatographic separation of silylated derivatives of disaccharide mixtures on open tubular glass capillary columns. J. Chromatogr. 115: 219-221.

Adam, S. 1981. Separation of mono- and disaccharide derivatives by high-resolution gas chromatography. Z. Lebensm.-Unters. Forsch. 173: 109-112

Aitzetmuller, K. 1978. Sugar analysis by high-performance liquid chromatography using silica columns. J. Chromatogr. 156: 354-358.

Aitzetmuller, K., Boehrs, M. and Arzberger, E. 1979. Separation of higher sugars using HPLC amine modifier I. J. High Res. Chromatogr. Comm. 2: 589-590.

Aitzetmuller, K. 1980. Anwendungen eines HPLC amine modifiers zur Zuckeranalyse in der Lebensmittelchemie. Chromatographia 13: 432-436.

Albanese, A.A. 1952. Utilization and protein-sparing action of fructose in man. Metabolism 1: 20-25.

Alexander, R.J. and Garbutt, J.T. 1965. Use of sorbitol as internal standard in determination of D-glucose by gas liquid chromatography. Anal. Chem. 37: 303-305.

Alborn, H. and Stenhagen, G. 1985. Direct coupling of packed fused-silica liquid chromatographic columns to a magnetic sector mass spectrometer and application to

polar thermolabile compounds. J. Chromatogr. 323: 47-66.

Allerhand, A. and Doddrell, D. 1971. Strategies in the application of partially relaxed fourier transform nuclear magnetic resonance spectroscopy in assignments of carbon-13 resonances of complex molecules. J. Am. Chem. Soc. 93: 2777-2779.

Allerhand, A., Doddrell, D. and Komoroski, [redacted] 1971. Natural abundance carbon-13 partially relaxed fourier transform nuclear magnetic resonance spectra of complex molecules. J. Chem. Phys. 55: 189-198.

Altena, J.H., Van den Ouweland, G.A.M., Teunis, C.J. and Tjan, S.B. 1981. Analysis of the 220-mHz, NMR spectra of some products of the Amadori and Heyns rearrangements. Carb. Res. 92: 37-49.

Ammon, R. 1949. Der Ursprung der Diastase des Bienenhonigs. Biochem. Z. 319: 295-299.

Anderle, D. and Kovac, P. 1970. Gas chromatography of partially methylated alditols as trifluroacetyl derivatives I. Separation of mono-O-methyl-per-O -TFA-D-glucitols. J. Chromatogr. 49: 419-424.

Angyl, S.J. 1976. Conformational analysis in carbohydrate chemistry III. The ^{13}C NMR spectra of hexuloses. Aust. J. Chem. 29: 1249-1265.

Angyl, S.J. 1984. The composition of reducing sugars in solution. Adv. Carbohydr. Chem. Biochem. 42: 15-68.

Anonymous. 1976. Composition determination of mixtures with glass capillary GC-MS. JEOL News 13A: 16-19.

Anonymous. 1984a. FAO Production Yearbook. 38: 250.

Anonymous. 1984b. Canada Yearbook. Ottawa: Minister of Supply and Services Canada.

Anonymous. 1985. Market Commentary, Agriculture Canada. Ottawa: Minister of Supply and Services Canada.

Aristotle. Natural History. Oxford translation (1910). Oxford: Oxford University Press. (quoted in Lorch, 1978).

Asakawa, M. 1985. Determination of malto-oligosaccharides by high-performance liquid chromatography using a silica column. Kaseigaku Zasshi. 36: 293-298.

Aso, K., Watanabe, T. and Yamao, K. 1958. Honey I. The sugar composition of honey. Hakko Kogaku Zasshi 36: 39-44.

Aso, K., Watanabe, T. and Yamao, K. 1960. Honey I. The sugar composition of honey. Tohoku. Agr. Res. 11: 101-108.

Bahl, O.P. 1970. Glycosidases of *Aspergillus niger*. J. Biol. Chem. 245: 299-304.

Bailey, M.E., Fieger, E.A. and Oertel, E. 1954. Paper chromatographic analyses of some southern nectars. Glean. Bee Cult. 82: 401-403, 472-474.

Baker, H.G. and Baker, I. 1979. Sugar ratios in nectars. Phytochem. Bull. 12: 43-45.

Baker, H.G. and Baker, I. 1982. Chemical constituents of nectar in relation to pollination mechanisms and phylogeny. In "Biochemical Aspects of Evolutionary Biology." p. 131-171. Chicago: University of Chicago

Press.

Baker, H.G. and Baker, I. 1983a. Floral nectar sugar constituents in relation to pollinator type. In "Handbook of Experimental Pollination Biology." p. 117-141. New York: Van Nostrand Reinhold.

Baker, H.G. and Baker, I. 1983b. A brief historical review of the chemistry of foral nectar. In "The Biology of Nectaries." p. 126-152. New York: Columbia University Press.

Baker, J.K., Kapeghian, J. and Verlangieri, A. 1983. Determination of ascorbic acid and dehydroascorbic acid in blood plasma samples. J. Liq. Chromatogr. 6: 1319-1332.

Barker, R. and Walker, T.E. 1980. C-13 NMR-spectroscopy of istopically enriched carbohydrates. Methods Carbohdr. Chem. 8: 151-165.

Barker, S.A., Brimacombe, J.S., Harnden, M.R. and Jarvis, J.A. 1963. Sucrose derivatives. Part II. Some silyl and cyanoethyl ethers and a hepta-acetal. J. Chem. Soc.: 3403-3406.

Bartels, W. and Fauth, A. 1933. Beobachtungen bei der Untersuchung californischer Honige. Z. Unters Lebensmittel 66: 396-407.

Barth, O.M. 1970a. Microscopical analysis of honey samples. An. Acad. Brasil. Cienc. 42: 351-366.

Barth, O.M. 1970b. Pollen spectrum of some honey samples of the state Rio de Janeiro. Rev. Brasil. Biol. 30: 575-582.

Barth, O.M. 1971. Pollen spectrum of some honey samples of the states Bahia and Ceara. Rev. Brasil. Biol. 31: 431-434.

Baskin, S.I. and Bliss, C.A. 1969. Sugar occurring in extrafloral exudates of the Orchidaceae. Phytochemistry 8: 1139-1145.

Battaglini, M. and Ricciardelli D'Albore, G. 1971. Pollen spectrum of some Sicilian honeys. Ann. Facolta Agraria Univ. Studi Perugia 26: 277-303.

Battaglini, M. and Ricciardelli D'Albore, G. 1972. Preliminary studies on Italian unifloral honeys. Ann. Facolta Agraria Univ. Studi Perugia 27: 219-224.

Battaglini, M. and Bosi, G. 1973. Chemical-physical characterization of monofloral honeys on the basis of the sugar spectrum and specific optical rotation. Sci. Technol. Alimenti 3: 217-221.

Baumgarten, F. and Mockesch, I. 1956. Uber die papier-chromatographische Auffindung frier Aminosauren in Bienenhonig. Z. Bienenforsch. 3: 181-184.

Baust, J.G., Lee, R.E. Jr., Hendrix, D.L., Friday, D. and James, H. 1983. Comparative separations of low-molecular weight carbohydrates and polyols by high-performance liquid chromatography: radially compressed amine modified silica versus ion exchange. J. Chromatogr. 261: 65-75.

Baxa, J. and Bruhns, G. 1967. Zucker im Leben der Volker. Eine Kultur- und Wirschaftsgeschichte (Berlin).

Beadle, J.B. 1969. Gas chromatographic determination of starch hydrolyzate saccharide distribution through maltoheptaose. J. Agric. Food Chem. 17: 904-906.

Bentley, R. and Botlock, N. 1967. A gas chromatographic method for analysis of anomeric carbohydrates and for determination of mutarotation coefficients. Anal. Biochem. 20: 312-320.

Bergner, K.G. and Hahn, H. 1972. Zum Vorkommen und zur Herkunft der freien Aminosauren in Honig. Apidologie. 3: 5-34.

Berry, J.M., Hall, L.D. and Wong, K.F. 1977. Concerning tumbling motion of disaccharides in aqueous solution. Carbohydr. Res 56: C16-C20.

Beutler, R. 1930. Biologisch-chemische Untersuchungen an Nektar von Immenblumen. Zeit. Vergleich. Physiol. 12: 72-176.

Beutler, R. 1935. Nectar. Bee World 24: 106-116, 128-136, 156-162.

Bhatti, T. and Clamp, J.R. 1968. Identification and estimation of monosaccharides and disaccharides in urine by gas-liquid chromatography. Clin. Chim. Acta. 22: 563-567.

Bhatti, T., Chambers, R.E. and Clamp, J.R. 1970. The gas chromatographic properties of biologically important N-acetylglucosamine derivatives, monosaccharides, disaccharides, trisaccharides, tetrasaccharides and pentasaccharides. Biochim. Biophys. Acta. 222: 339-347.

Biino, L. 1970. Ricera alcuni aminoacidi in due varieta di miele. Riv. Ital. Essenze Profumi 53: 80-84.

Binkley, W.W. and Binkley, R.W. 1970. Photolysis of d-galactose oxime - a photochemical Wohl degradation. Tetrahedron Lett. 39: 3439-3442.

Birch, G.G. 1973. Gas-liquid chromatography of food carbohydrates with special reference to trimethylsilyl derivatives: a review. J. Food Technol. 8: 229-246.

Bishop, C.T. 1962. Separation of carbohydrate derivatives by gas liquid partition chromatography. Methods Biochem. Anal. 10: 1-42.

Bishop, C.T. 1964. Gas-liquid chromatography of carbohydrate derivatives. Advan. Carbohydr. Chem. 19: 95-147.

Bittner, A.S., Harris, L.E. and Campbell, W.F. 1980. Rapid N-methylimidazole-catalyzed acetylation of plant cell wall sugars. J. Agric. Food Chem. 28: 1242-1245.

Blanken, W.M., Bergh, M.L.E., Koppen, P.L. and Van den Eijnden, D.H. 1985. High-pressure liquid chromatography of neutral oligosaccharides: effects of structural parameters. Anal. Biochem. 145: 322-330.

Blunt, J.W. and Munro, H.G. 1976. An automated procedure for qualitative and quantitative analysis of mixtures by means of carbon magnetic resonance spectroscopy: applications to carbohydrate analysis. Aust. J. Chem. 29: 975-986.

Bock, K. and Pedersen, C. 1983. Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides. Adv. Carbohydr. Chem. Biochem. 41: 27-66.

Bock, K. and Thorgersen, H. 1982. Ann. Rep. NMR Spec. 13:1.

Bonn, G., Concin, R. and Bibleter, O. 1983. Hydrothermolysisa new process for the utilization of biomass. Wood Sci. Technol. 17: 195-202.

Bonn, G. 1985. High-performance liquid chromatographic elution behavior of oligosaccharides, monosaccharides and sugar degradation products on columns using water as the mobile phase. J. Chromatogr. 322: 411-424.

Bonnier, G. 1878. Les nectaires. Ann. Sci. Nat. Serie 6, Bot. 8: 5-212.

Bosi, G. and Battaglini, M. 1978. Gas chromatographic analysis of free and protein amino acids in some unifloral honeys. J. Apic. Res. 17: 152-166.

Bradford, M. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Brewer, J.W., Colyard, K.J. and Lott, C.E., Jr. 1974. Analysis of sugars in dwarf mistletoe nectar. Can J. Bot. 52: 2533-2538.

Brobst, K.M. and Lott, C.E. 1966. Determination of some components in corn syrup by gas-liquid chromatography of the trimethylsilyl derivatives. Cereal Chem. 43: 35-43.

Brobst, K.M. 1972. Gas-liquid chromatography of trimethylsilyl derivatives. Analysis of corn syrup. Methods Carbohydr. Chem. 6: 3-8.

Brons, C. and Olieman, C. 1983. Study of the high-performance liquid chromatographic separation of reducing sugars, applied to the determination of lactose in milk. J. Chromatogr. 259: 79-86.

Brooks, J. and Shaw, G. 1968. Identity of sporopollenin with older kerogen and new evidence for the possible biological source of chemicals in sedimentary rocks. Nature 220: 678-679.

Brosio, E. and Di Nola, A. 1982. Trends Anal. Chem. 1: 284.

Browne, C.A. 1908. Chemical analysis and composition of American honeys. U.S. Bur. Chem. Bull. 110: 1-93.

Brush, G.S. and Brush, L.M.,Jr. 1972. Transport of pollen in a sediment-laden channel: a laboratory study. Am. J. Sci. 272: 359-381.

Bulman, M.W. 1955. Honey as a surgical dressing. Middx. Hosp. J. 55: 188-189.

Bush, C.A. 1978. Excitation coupling in circular-dichroism spectrum of glycopeptide linkage compound, beta-aspartyl glucosylamine. Biophys. J. 21: A161.

Bush, C.A. 1979. Gas chromatography and mass spectrometry of disaccharides from glycoproteins. Biochem. Biophys. Methods 1: 227-235.

Butler, G.D., Loper, G.M., McGregor, S.E., Webster, J.L. and Margolis, H. 1972. Amounts and kinds of sugars in the nectars of cotton (*Gossypium* spp.) and the time of their secretion. Agron. J. 64: 364-368.

Cai, X. and Zhu, Y. 1985. Determination of carbohydrates by high performance liquid chromatography. Shipin Yu Fajiao Gongye 5: 9-17.

Callaghan, P.T., Jolley, K.W., Lelievre, J. and Wong, R.B.K. 1983. Nuclear magnetic resonance studies of wheat-starch pastes. Colloid. Interface. Sci. 92: 332-337.

Carlsson, B., Isaksson, T. and Samuelson, O. 1968. Automatic chromatography of hydroxy acids on anion-exchange resins. Anal. Chim. Acta 43: 47-52

Caspary, J.X.R. 1848. Dissertatio inauguralis de nectariis. Bohn. (quoted in Lorch, 1978).

Gayle, T., Viebrock, F. and Schiaffino, J. 1968. Gas chromatography of carbohydrates. Cereal Chem. 45: 154-161.

Cerbulis, J., Pfeffer, P.E. and Farrell, H.M. Jr. 1978. Reaction of lactose with urea. Carbohydr. Res. 65: 311-313.

Cerning-Bervard, J. and Filiatre, A. 1976. A comparison of the carbohydrate composition of legume seeds: horsebeans, peas, and lupines. Cereal Chem. 53: 968-978.

Chang, C.D. and Hass, H.B. 1958. Synthesis of a silicon derivative of sucrose. J. Org. Chem. 23: 773.

Chaubal, P.D. and Deodikar, G.B. 1965. Morphological characterization of pollen grains of some major honey yielding plants of the Western Ghats (India). Indian Bee J. 27: 1-28.

Cheetham, N.W.H., Sirimanne, P. and Day, W.R. 1981. High-performance liquid chromatographic separation of carbohydrate oligomers. J. Chromatogr. 207: 439-444,

Cheetham, N.W.H. and Teng, G. 1984. Some applications of reverse-phase high-performance liquid chromatography to oligosaccharide separations. J. Chromatogr. 336: 161-172.

Chiba, S., Asada-Komatsu, Y., Kimura, A. and [illegible]shima, K. 1984. A new trisaccharide, synthesized by the transglucosidase action of immobilized buckwheat α-glucosidase. Agric. Biol. Chem. 48: 1173-1178.

Chiba, S., Kimura, A., Kobori, T. and Saitoh, K. 1985. Quantitative determination of disaccharides produced from soluble starch through transglucosylation by buckwheat α-glucosidase. Depun Kagaku 32: 213-216.

Child, T.F. and Pryce, N.G. 1972. Steady-state and pulsed NMR studies of gelatin in aqueous agarose. Biopolymers 11: 409.

Clamp, J.R., Bhatti, T. and Chambers, R.E. 1971. Determination of carbohydrate in biological materials by gas-liquid chromatography. Mthods Biochem. Anal. 19: 229-334.

Cocker, L. 1951. The enzymic production of acid in honey. J. Sci. Food Agric. 2: 411-414.

Codex Alimentarius Commission. 1983. Proposed draft Codex Standard for honey (world-wide standard). Rome: Joint FAO/WHO Food Standards Programme (CX/PFV 84/13).

Coduti, P.L. and Bush, C.A. 1977. Structure determination of N-acetyl amino sugar derivatives and disaccharides by gas chromatography and mass spectroscopyy. Anal. Biochem. 78: 21-38.

Colson, P., Jennings, H.J. and Smith, I.C.P. 1974. Composition, sequence, and confirmation of polymers and oligomers of glucose as revealed by carbon-13 nuclear magnetic resonance. J. Am. Chem. Soc. 96: 8081-8087.

Corbet, S.A. 1978. Bees and the nectar of *Echium vulgare*. In "The Pollination of Flowers by Insects." p. 21-30. London: Academic Press.

Coxon, B. 1980. Carbon-13 nuclear magnetic resonance spectroscopy of food-related disaccharides and trisaccharides. Dev. Food Carbohydr. 2: 351-390.

Crane, E. 1977. Dead bees under lime trees. Bee World 58: 129-130.

Crane, E. 1979. Honey a Comprehensive Survey. London: William Heinmann Ltd.

Curti, R. and Riganti, V. 1966. Ricerche sugli aminoacidi del miele. Rass. Chim. 18: 278-282.

Curylo, J. 1962. Chromatographic separation of carbohydrates from nectar and leaf honey. Pszczel. Zeszyty Nauk 6: 1-14.

Czarniecki, M.F. and Thornton, E.R. 1977. C-13 nuclear magnetic resonance spin-lattice relaxation in N-acylneuraminic acids: probes for internal dynamics and conformational analysis. J. Am. Chem. Soc. 99: 8273-8279.

Daniels, D.H., Warner, C.R. and Fazio, T. 1982. Gas chromatographic determination of sorbitol, mannitol, and xylitol in chewing gum and sorbitol in mints. J. Assoc. Off. Anal. Chem. 65: 588-591.

Davies, A.M.C. 1975. Amino acid analysis of honeys from eleven countries. J. Apic. Res. 14: 29-39.

Davison, P.K. and Young, R. 1969. Gas chromatography of carbohydrates: the quantitative determination of the free sugars of plants as their trimethylsilyl ethers. J. Chromatogr. 41: 12-21.

Delente, J. and Ladenburg, K. 1972. Quantitative determination of the oligosaccharides in defatted soybean meal by gas-liquid chromatography. J. Food Sci. 37: 372-374.

Demaimay, M. and Lebouteiller, M.J. 1978. Method simplifee d'analyse des derives silyles d'oximes de sucre par chromatographie en phase gazeuse application aux denrees alimentaires. Ann. Technol. Agric. 27: 455-467.

Demianowicz, Z. and Demianowicz, A. 1957. Nowe podstawy analizy pylkowej miodow. Pszczel Zesz. Nauk. 1: 69-78.

Demianowicz, Z. and Jablonski, B. 1966. Nectar secretion and honey yield of four species of plants with small flowers. Pszczel Zesz. Nauk. 10: 87-94.

Dickes, G.J. and Nicholas, P.V. 1976. Gas Chromatography in Food Analysis. London: Butterworths.

Diefel, A., Gierschner, K. and Vorwohl, G. 1985. Saccharose im honig. Deutsche Lebensmittel-Rundschau 81: 280-285.

Doddrell, D. and Allerhand, A. 1971. Study of anomeric equilibria of ketoses in water by natural-abundance carbon-13 Fourier transform nuclear magnetic resonance. D-fructose and D-turanose. J. Am. Chem. Soc. 93: 2779-2781.

Doner, L.W. 1977. The sugars of honey - a review. J. Sci. Food Agric. 28: 443-456.

Doornbos, T., Van den Ouweland, G.A.M. and Tjan, S.B. 1981. Amadori compounds, derived from 6-deoxy sugars, as flavour precursors. Prog. Food Nutr. Sci. 5: 57-63.

Dormán, D.E. and Roberts, J.D. 1971. Nuclear magnetic resonance spectroscopy. Carbon-13 spectra of some common oligosaccharides. J. Am. Chem. Soc. 93: 4463-4472.

Dorn, H.C. 1984. ^{1}H-NMR: a new detector for liquid chromatography. Anal. Chem. 56: 747A-758A.

Duisberg, H. and Hadorn, H. 1966. Welche anforderungen sind an handelshonige zu stellen? Mitt. Geb. Lebensmittel-unters Hyg. 57: 386.

Durette, P.L. and Horton, D. 1971. Conformational analysis of sugars and their derivatives. Adv. Carbohydr. Chem. Biochem. 26: 49-125.

Dutton, G.G.S. 1973. Applications of gas-liquid chromatography to carbohydrates: Part I. Advan. Carbohyd. Chem. Biochem. 28: 11-160.

Dutton, G.G.S. 1974. Applications of gas-liquid chromatography to carbohydrates: Part II. Advan. Carbohyd. Chem. Biochem. 30: 9-110.

Eckert, J.B. and Allinger, H.W. 1939. Physical and chemical properties of California honeys. Cal. Agr. Sta. Bull. 631: 3-27.

Edwards, P. and Haak, K. 1983. A pulsed amperometric detector for ion chromatography. Am. Laborator. 15: 78.

Elegood, J.A. and Fischer, L. 1940. Composition of fireweed honey. Food Res. 5: 559-561.

Elias, T.S. and Gelband, H. 1975. Nectar: its production and functions in trumpet creeper. Science 189: 289-291.

Elser, E. 1924. Maltose in bee-honey. Mitt. Geb. Lebensmittelunters Hyg. 25: 92-96.

Englyst, H., Wiggins, H.S. and Cummings, J.H. 1982. Determination of non-starch polysaccharides in plant foods by gas-liquid chromatography of constituent sugars as alditol acetates. Analyst 107: 307-318.

Erdtman, G. 1960. The acetolysis method. Svensk. bot. Tidgkr. 54: 561-564.

Erdtman, G. 1963. Palynology. Advances in Botanical Research 1: 149-208.

Erdtman, G. 1966. Sporoderm morphology and morphogenesis. A collocation of data and suppositions. Arana Palynol. 6: 318-322.

Erdtman, G. 1969. Handbook of Palynology. Morphology. Taxonomy. Ecology. Copenhagen: Munksgaard.

Esposito, G.G. 1968. On column formation and analysis of trimethylsilyl derivatives. Anal. Chem. 40: 1902-1904.

Ettre, L.S. and Zlatkis, Z. 1967. The Practice of Gas Chromatography. New York: Wiley.

Faegri, K. and Iversen, J. 1974. Textbook of Pollen Analysis. Oxford: Blackwell.

Fahn, A. 1979. Ultrastructure of nectaries in relation to secretion. Am. J. Bot. 66: 977-985.

Farnsteiner, K. 1908. Der Ameisensauregehalt des Honigs. Z. Unters. Nahr.-U. Genussmittel. 15: 598-604.

Feller-Demalsy, M., Lamontagne, Y, Marquis, D. and Parent, J. 1979. Apidologie 10: 313-340.

Ferrazzi, P. 1974. Dandelion honeys from Piedmont and Lombardy, 1. Mellisopalynological analysis. Apicolt. mod. 65: 21-26.

Fiehe, J. 1932. Uber die Herkunft der Honigdiastase. Z. Unters. Lebensmittel 63: 329-331.

Finch, S. 1974. Sugars available from flowers visited by the adult cabbage root fly, *Erioschia brassicae*. Bull. Ent. Res. 64: 257-263.

Flechtmann, C.H.W., Felho, C.F. Caldas, Amaral, E. and Arzolla, J.D.P. 1963. Analysis of honey from the state of Sao Paulo. Bol. Ind. Animal 21: 65-73.

Floridi, A. 1971. An improved method for the automated analysis of sugars by ion-exchange chromatography. J. Chromatogr. 59: 61-70.

Folkes, D.J. and Taylor, P.W. 1982. "HPLC in Food Analysis." London: Academic Press.

Folkes, D.J. and Brookes, A. 1984. Glucose Syrups: Science and Technology. London: Elsevier Applied Science.

Folkes, D.J. 1985. Gas-liquid chromatography. In "Analysis of Food Carbohydrates." p. 91-123. London: Elsevier Applied Science.

Food and Agriculture Organization of the United Nations. 1984. Production of Honey. 38: 250.

Frei, R.W. and Lawrence, J.F. 1973. Fluorigenic labelling in high-speed liquid c omatography. J. Chromatogr. 83: 321-330.

Frey-Wyssling, A., Zimmermann, M. and Mauriz 1954. Ube den enzymatischen Zuckerumbau in Nektarien. Experimentia. 10: 490-492.

Fukushima, E. and Roeder, S.B.W. 1981. Experimental Pulse N.M.R. - A Nuts and Bolts Approach. Reading: Addison-Wesley.

Furgala, B., Gochnauer, T.A. and Holdaway, F.G. 1958. Constituent sugars of some northern legume nectars. Bee World 39: 203-205.

Ghernati, H.M., Abdeddaim, K. and Guermouche, M.H. 1982. Rapid high-performance liquid chromatography methods for the separation and quantification of a mono-, di-, and trisaccharide mixture and applications. J. Liq. Chromatogr. 5: 1725-1748.

Gilbert, J., Shepherd, M.J., Wallwork, M.A. and Harris, R.G. 1981. Determination of the geographical origin of honeys by multivariate analysis of gas chromatographic data on their free amino acid content. J. Apic. Res.

20: 125-135.

Goldschmidt, S. and Burkert, H. 1955. Die Hydrolyse des cholinergischen Honigwirkstoffes und anderer Cholinester mittels Cholinesterasen und deren Hemmung im Honig. Hoppe-Seyler's Z. physiol. Chem. 301: 78-89.

Gontarski, H. 1957. Eine Halbmikromethode zur quantitativen Bestimmung der Invertase in Bienenhonig. Z. Bienenforsch. 4: 41-45.

Gorbach, G. 1942. Zur Kenntnis der Starkeverdauung durch die Biene. 2. Mitteilung in der Reihe: Ernahrungsphysiologische Studien an der Biene. Forschungsdienst 13: 67-78.

Gorin, P.A. 1981. Carbon-13 nuclear magnetic resonance spectroscopy of polysaccharides. Adv. Carbohydr. Chem. Biochem. 38: 13-104.

Gothe, F. 1914. Die Fermente des Honigs. Z. Unters. Nahr.-U. Genussmittel 28: 273-286.

Gottsberger, G., Schrauwen, J. and Linskens, H.F. 1973. Die Zucker-bestandteil des Nektars einiger tropischer Bluten. Portug. Acta. Biol. Ser. A 13: 1-8.

Gunning, B.E.S. and Steer, M.W. 1975. Ultrastructure and the Biology of Plant Cells. London: Arnold.

Hadorn, H. and Zurcher, K. 1966. Eine verbasserte polarimetrische methode zur saccharasenzal-bestimmung in honig. Deut. Lebensmittel Rundschau 62: 195-198.

Hahn, H. 1970. Zum Gehalt und zur Herkunft der freien Aminosauren in Honig. Universitat Stuttgart: Dissertation.

Hainsworth, F.R. and Wolf, L.L. 1976. Nectar characteristics and food selection by hummingbirds. Oecologia 25: 101-103.

Ha L.D. 1980. The Carbohydrates. Volume 1B. p. 1299-1306. New York: Academic Press.

Hammer, B. 1974. The pollen content of honey samples from the Botanical Garden in Copenhagen. p. 122-127. In "Report of the Activity of the Botanical Garden in the Years 1972-1974."

Hammer, O., Jorgensen, E.G. and Mikkelsen, V.M. 1948. Studier over danske honnigprovers indhold af blomsterstov. Tidsskr. PlAvl. 52: 293-352.

Handbook of Chemistry and ysics. 1978. 59th edition. Florida: CRC Press.

Harris, R.K. 1983. Nuclear Magnetic Resonance Spectroscopy. London: Pitman.

Haverkamp, J., Kamerling, J.P. and Vliegenthart, J.F.G. 1971. Gas-liquid chromatography of trimethylsilyl dissacharides. J. Chromatogr. 59: 281-287.

Hedgley, E.J. and Overend, W.G. 1960. Trimethylsilyl derivatives of carbohyd es. Chem. Ind. 378-380.

Hennig, H.-J. 1977. NMR-investigations of the role of water for structure of native starch granules. Starke 29: 1-4.

Hennig, H.-J. and Lechert, H. 1977. NMR study of D_2O in native starches of different origins and amylose of type-B. J. Colloid. Interface Sci. 62: 199-204.

Heslop-Harrison, J. 1971. The pollen wall: structure and development. In "Development and Physiology." p. 75-98. London: Butterworths.

Hicks, K.B., Symanski, E.V. and Pfeffer, P.E. 1983. Synthesis and high-performance liquid chromatography of maltulose and cellobulose. Carb. Res. 112: 37-50.

Hoban, N. and White, J.W. Jr. 1958. Gradient elution of disaccharides in a stearic acid-treated charcole column. Anal. Chem. 30: 1294-1296.

Hobbs, J.S. and Lawrence, J.G. 1972. Determination of carbohydrates by liquid chromatography: lactose in milk. J. Sci. Food Agr. 23: 45-51.

Holligan, P.M. 1971. Routine analysis by gas-liquid chromatography of soluble carbohydrates in extracts of plant tissues. New Phytol. 70: 239-269.

Holligan, P.M. and Drew, E.A. 1971. Routine analysis by gas-liquid chromatography of soluble carbohydrates in extracts of plant tissues. New Phytol. 70: 271-297.

Holm, C.H. 1957. Observation of chemical shielding and spin coupling of carbon-13 nuclei in various chemical compounds by nuclear magnetic resonance. J. Chem. Phys. 26: 707-708.

Honda, S., Suzuki, S., Takahashi, M., Kakehi, K. and Ganno, S. 1983. Automated-analysis of hexosamines by liquid-chromatography with photometric and fluorimetric postcolumn labeling using 2-cyanoacetamide. Analyt. Biochem. 134: 483-488.

Honda, S. 1984. Review. High-performance liquid chromatography of mono- and oligosaccharides. Anal. Biochem. 140: 1-47.

Howells, V.W. 1969. Some reflections on the pollen analysis of honey. J. Assoc. Publ. Analysts 7: 88-93.

Huber, R.E. 1975. The purification and study of a honey bee abdominal sucrose exhibiting unusual solubility and kinetic properties. Arch. Biochem. Biophys. 168: 198-209.

Huber, R.E. and Mathison, R.D. 1975. Physical, chemical, and enzymatic studies on the major sucrose of honey bees (*Apis mellifera*). Can. J. Biochem. 54: 153-164.

Hudson, C.S. and Sherwood, S.F. 1918. The occurrence of melezitose in a manna from the Douglas Fir. J. Am. Chem. Soc. 40: 1456-1460.

Hudson, C.S. and Sherwood, S.F. 1920. The occurrence of melezitose in honey. J. Am. Chem. Soc. 42: 116-125.

Hull, S.R. and Turco, S.J. 1985. Separation of dansyl hydrazine-derivatized oligosaccharides by liquid chromatography. Anal. Biochem. 146: 143-149.

Hurd, C.D., Englis, D.T., Bonner, W.A. and Rogers, M.A. 1944. Carbohydrate analysis as applied to honey. J. Am. Chem. Soc. 66: 2015-2017.

International Commission for Bee Botany of I.U.B.S. 1970. Methods of melissopalynology. Bee World 51: 125-138.

Iverson, J.L. and Bueno, M.P. 1981. Evaluation of high pressure liquid chromatography and gas-liquid chromatography for quantitative determination of sugars

in foods. J. Assoc. Off. Anal. Chem. 64: 139-143.

Iwata, S., Narui, T., Takahashi, K. and Shibata, S. 1984. Separation of carbohydrates by liquid chromatography in an aqueous, silica gel column. Carbohydr. Res. 133: 157-162.

Jeffrey, D.C., Arditti, J. and Ernest, R. 1969. Determination of di-, tri-, and tetrasaccharides in mixtures with their component moieties by thin layer chromatography. J. Chromat. 41: 475.

Jeffery, D.C., Arditti, J. and Koopowitz, N. 1970. Sugar content in floral and extrafloral exudates of orchids: pollination, myrmecology and chemotaxonomy implications. New Phytol. 69: 187-195.

Jennings, H.J. and Smith, I.C.P. 1973. Determination of composition and sequence of a glucan containing mixed linkages by C-13 nuclear magnetic resonance. J. Am. Chem. Soc. 95: 606-608.

Jennings, H.J. and Smith, I.C.P. 1980. Determination of polysaccharide structures with C-13 NMR. Methods in Carbohydr. Chem, 8: 97-105.

Kainosho, M. 1976. C-13 NMR studies of intact plant-tissues cytoplasmic aucubin and sucrose in a single seed of *Aucuba Japonica*. Tetrahedron Lett. 4279-4282.

Kapyla, M. 1978. Amount and type of nectar sugar in some wildflowers of Finland. Ann. Bot. Fennici 15: 85-88.

Kartashova, N.N. and Novidava, T.N. 1964. A chromatographic study of the chemical composition of nectar. Isv. Tomskogo Otd. Vses. Botan. Obschestva 5: 111-119.

Kato, T. and Kinoshita, T. 1980. Fluorometric detection and determination of carbohydrates by high-performance liquid chromatography using ethanolamine. Anal. Biochem. 106: 238-243.

Kennedy, I.R., Mwandemele, O.D. and McWhirter, K.S. 1985. Estimation of sucrose, raffinose and stachyose in soybean seeds. Food Chem. 17: 85-93.

Keup, N. 1957. Study of the glucides of honey by paper chromatography. Inst. Grand Duccal Luxemb. Sect. Sci. Nat. Phys. Math. Arch. 24: 91-97.

Khym, J.X. and Zill, L.P. 1952. The separation of sugars by ion exchange. J. Am. Chem. Soc. 74: 2090-2094.

Kimura, M., Tohma, M., Okazawa, Y. and Muria, N. 1969. Quantitative determination of mono- and oligosaccharides in potato tubers by gas-liquid chromatography. J. Chromatogr. 41: 110-112.

Kircher, H.W. 1962. Gas-liquid partition chromatography of sugar derivatives. Methods Carbohydr. Chem. 1: 13-20.

Kleinschmidt, M.G., Dobrenz, A.K. and McMahon, F.G. 1968. Gas chromatography of carbohydrates in alfalfa nectar. Plant Physiol. 43: 665-667.

Knuth, P. Handbook of Flower Pollination. 1906-1909. Oxford University Press.

Koizumi, K., Utamura, T. and Okada, Y. 1985. Analysis of homogeneous D-gluco-oligosaccharides and -polysaccharides (degree of polymerization up to about 35) by high-performance liquid chromatography and thin-layer chromatography. J. Chromatogr. 321: 145-157.

Koumaine, A. 1960. Amino acids in honey. Suom. Kemistil. B.33: 185-187.

Kramer, K.J., Spiers, R.D. and Childs, C.N. 1978. A method for separation of tetralose from insect hemolymph. Anal. Biochem. 86: 692-696.

Kubisova-Kropacova, S. and Nedbalova, V. 1976. Pollen analysis of honey from Northeast Moravia. Polnohospodarstvo 22: 943-959.

Labuza, T.P. and Busk, G.C. 1979. Analysis of the water binding in gels. J. Food Sci. 44: 1379-1385.

Laere, O. van., Lagasse, A. and Mets, M. de. 1969. Use of the scanning electron microscope for investigating pollen grains isolated from honey samples. J. Apic. Res. 8: 139-145.

Laker, M.F. 1979. Estimation of disaccharides in plasma and urine by gas-liquid chromatography. J. Chromatogr. 163: 9-18.

Lamar, G.N. 1971. Problem with C-13 intensities in proton-decoupled nuclear magnetic resonance spectra-undermining Overhauser effect with free radicals. J. Am. Chem. Soc. 93: 1040.

Lampitt, L.H., Hughes, E.B. and Rooke, H.S. 1930. The diastatic activity of honey. Analyst, Lond. 55: 666-672.

Larson, P.A., Honold, G.R. and Hobbs, W.E. 1974. Gas chromatographic separation of α-lactose and sucrose as the trimethysilyl derivatives. J. Chromatogr. 90: 345-349.

Larsson, K. and Samuelson, O. 1976. Anion-exchange chromatography of alditols in borate medium. Carbohdr. Res. 50: 1-8.

Lauterbur, P.C. 1957. Carbon-13 nuclear magnetic resonance spectra. J. Chem. Phys. 26: 217-218.

Lawson, M.A. and Russell, G.F. 1980. Trace level analysis of reducing sugars by high-performance liquid chromatography. J. Food Sci. 45: 1256-1258.

Lechert, H., Maiwald, W., Kothe, R. and Basler, W.-D. 1980. NMR-study of water in some starches and vegetables. J. Food Proc. Preserv. 3: 275-299.

Leung, H.K. and Steinberg, M.P. 1979. Water binding of food constituents as determined by NMR, freezing, sorption and dehydration. J. Food Sci. 44: 1212-1216.

Levy, G.C. and Nelson, G.L. 1972. Carbon-13 Nuclear Magnetic Resonance for Chemists. New York: Wiley-Interscience.

Li, B.W. and Schuhmann, P.J. 1981. Gas chromatographic analysis of sugars in granola cereals. J. Food Sci. 46: 425-427.

Li, B.W., Schumann, P.J. and Holden, J.M. 1983. Determination of sugars in yogurt by gas-liquid chromatography. J. Agric. Food Chem. 31: 985-989.

Lieux, M.H. 1972, A melissøpalynogical study of 54 Louisiana honeys. Rev. Palaeobot. Palynol. 13: 94-124.

Liggett, R.W. 1941. Ph. D. thesis, Northwestern University.

Linden, J.C. and Lawhead, C.L. 1975. Liquid chromatography of saccharides. J. Chromatogr. 105: 125-133.

Ling, G. van, Ruijterman, C. and Vlugter, J.C. 1967. Catalytic hydrogenolysis of saccharides. Carbohydr. Res. 4: 380-386.

Ling, G. van. 1969. Silylation and gas-liquid chromatographic analysis of an aqueous polyhydric alcohol mixture. J. Chromatogr. 44: 175-177.

Liu, H., Matsumoto, K. and Tsumie, S. 1985. Improvement of vacuum nebulizing interface for direct coupling of high-performance liquid chromatography with a mass spectrometer and some applications. Zhejiang Gongzueyuan Xuebao 26: 1-11.

Lorch, J. 1978. The discovery of nectar and nectaries and its relation to views on flowers and insects. Isis 69: 514-533.

Lothrop, R.E. and Holmes, R.L. 1931. Determination of dextrose and levulose in honey by use of iodine-oxidation method. Ind. Eng. Chem. Anal. Ed. 3: 334-339.

Lothrop, R.E. and Paine, H.S. 1931. Diastatic activity of some American honeys. Ind. Eng. Chem. Anal. Ed. 23: 71-74.

Lotmar, R. 1935. Abbau und Verwertung von Starke und Dextrin durch die Honigbiene. Arch. Bienenk. 16: 195-204.

Lotti, G. and Anelli, G. 1970. Gli aminoacidi liberi des pollini. Ind. agrari. 8: 239-245.

Louveaux, J. 1968. L'analyse pollinique des miels. In "Traite de biologie de l'abeille." p. 325-362. Paris: R. Chauvin.

Louveaux, J., Maurizio, A. and Vorwohl, G. 1970. Methods of mellisopalynology. Bee World 51: 125-131.

Louveaux, J., Maurizo, A. and Vorwohl, G. 1979. Methods of mellisopalynology. Bee World 59: 139-157.

Luke, M.A. 1971. Gas and thin layer chromatographic determination of sugars in cocoa products. J. Assoc. Off. Anal. Chem. 54: 937-939.

Luttge, U. 1961. Uber die Zusammensetzung des Nektars und der Mechanismus Seiner Sekretion. I. Planta 56: 189-212.

Macior, L.W. 1978. Pollination interactions in sympatric *Dicentra* species. Am. J. Bot. 65: 57-62.

Macrae, R. and Dick, J. 1981. Analysis of carbohydrates using the mass detector. J. Chromatogr. 210: 138-145.

Macrae, R. 1982. HPLC in Food Analysis. p. 322. London: Academic Press.

Macrae, R. 1985. High performance liquid chromatography. In "Analysis of Food Carbohydrates." p. 61-89. London: Elsevier Applied Science.

Maeda, S., Mukai, A., Kosugi, N. and Okada, Y. 1962. The flavour components of honey. J. Food Sci. Tech. 9: 270-274.

Makita, M. and Wells, W.W. 1963. Quantitative analysis of fecal bile acids by gas-liquid chromatography. Anal. Biochem. 5: 523-530.

Marinelli, L. and Whiteny, D.J. 1966. Gas-liquid chromatography of wort and beer carbohydrates I. The characterization of wort, beer, and brewing syrup

carbohydrates. J. Inst. Brewing 72: 252-256.

Marinelli, L. and Whitney, D.J. 1967. Gas liquid chromatography of wort and beer carbohydrates II. Quantitative determination of wort, beer, and brewing syrup carbohydrates. J. Inst. Brewing 73: 35-39.

Marpmann, G. 1903. Beitrage zur Prufung und Beurteilung des Bienenhonigs. Pharm. Ztg. Berl. 48: 1010.

Martensen-Larsen, O. 1954. Detoxification of drunkenness. Brit. Med. J.: 464.

Martin-Villa, C., Videl-Valverde, C. and Rojas-Hidalgo, E. 1982. High-performance liquid chromatographic determination of carbohydrates in raw and cooked vegetables. J. Food Sci. 47: 2086-2088.

Martinsson, E. and Samuelson, O. 1970. Partition chromatography of sugars on ion-exchange resins. J. Chromatogr. 50: 429-435.

Maslowski, P. and Mostowska, I. 1963. Elecktrochromatograficzne badania skladu wolnych aminoklwasow w niektorych odmianach miodow. Pszczel. Zesz. Nauk 7: 1-6.

Mathur-De Vre, R. 1979. NMR-studies of water in biological systems. Prog. Biophys. Mol. Biol. 35: 103-134.

Matile, P. 1956. Uber den Stoffwechsel und die Auxinabhangigkeit der Nektosekretion. Ber Schweiz. Botan. Ges. 66: 237-296.

Maurizio, A. 1949. Beitrage zur quantitativen Pollenanalyse des Honigs. Beih. Schweig. Bienenztg 2. 18: 320-421.

Maurizio, A. 1951. Pollen analysis of honey. Bee World 32: 1-5.

Maurizio, A. 1959. Papierchromatographische untersuchungen an Blutenhonigen und Nektar. Ann. de l'Abeille 2: 291-341.

Maurizio, A. 1962. From the raw material to the finished product: honey. Bee World 43: 66-81.

Maurizio, A. 1975. Microscopy of honey. In "Honey: a Comprehensive Survey." p. 240-252. London: Heinemann.

McInnes, A.G., Ball, D.H., Cooper, F.P. and Bishop, C.T. 1958. Separation of carbohydrate derivatives by gas-liquid partition chromatography. J. Chromatogr. 1: 556-557.

McNeeley, W.H., Brinkley, W.W. and Wolfram, M.L. 1945. Separation of sugar acetates by chromatography. J. Am. Chem. Soc. 67: 527-529.

Miettinen, T.A., Ahrens, E.H. and Grundy, S.M. 1965. Quantitative isolation and gas-liquid chromatographic analysis of total dietary and fecal neutral steroids. J. Lipid Res. 4: 411.

Moore, P.D. and Webb, J.A. 1978. An Illustrated Guide to Pollen Analysis. London: Hodder and Stoughton Ltd.

Mopper, K. and Degens, E.T. 1973. A new chromatographic sugar autoanalyzer with a sensitivity of 10^{-11} moles. Anal. Biochem. 45: 147-153.

Mopper, K. and Gindler, E.M. 1973. A new noncorrosive dye reagent for automatic sugar chromatography. Anal. Biochem. 56: 440-442.

Mopper, K., Dawson, R., Liebezeit, G. and Hansen, H.-P. 1980. Borate complex ion exchange chromatography with fluorimetric detection for determination of saccharides. Anal. Chem. 52: 2018-2022.

Moriyasu, M., Kato, A., Okada, M. and Hashimoto, Y. 1984. Kinetic studies of fast equilibrium by means of HPLC separation of sugar anomes in a very low temperature region. Anal. Lett, 17: 689-699.

Muller, H. 1883. The Fertilisation of Flowers. London: Macmillian.

Murrell, D.C. and Szabo, T.I. 1981. Pollen collection by honey bees at Beaverlodge, Alberta. Am. Bee J. 121: 885-888.

Nachtmann, F. and Budna, K.W. 1977. Sensitive determination of derivatized carbohydrates by high-performance liquid chromatography. J. Chromatogr. 136: 279-287.

Nakazawa, F., Noguchi, S., Takahashi, J. and Kato, M. 1980. Kaseigaku Zasshi 31: 541.

Nelson, J.M. and Cohn, D.J. 1924. Invertase in honey. J. Biol. Chem. 61: 193-224.

Nelson, J.M. and Sottery, C.T. 1924. Influence of glucose and fructose on the rate of hydrolysis of sucrose by invertase from honey. J. Biol. Chem. 62: 139-147.

Nikolov, Z.L. and Reilly, P.J. 1983. Isothermal capillary column gas chromatography of trimethylsilyl disaccharides. J. Chromatogr. 254: 157-162.

Nikolov, Z.L., Meagher, M.M. and Reilly, P.J. 1985a. High-performance liquid chromatography of disaccharides on amine-bonded silica columns. J. Chromatogr. 319: 51-57.

Nikolov, Z.L., Meagher, M.M. and Reilly, P.J. 1985b. High-performance liquid chromatography of trisaccharides on amine-bonded silica columns. J. Chromatogr. 321: 393-399.

Ohtsuki, K., Kawabata, M. and Taguchi, K. 1984. Analysis of sugars in kiwifruit. Kyoto-furitsu Daigaku Gakiyutsu Hokoku Rigaku Seikatsu Kagaku: 21-25.

Ono, K., Nuessle, D.W. and Smith E.E. 1981. Isolation of oligosaccharides formed from sucrose by α-D-glucosyl-transferases. Carb. Res. 90: 158-164.

Osaulko, G.K. 1954. Uses of honey in ophthalmology. Glean. Bee Cult. 82: 739.

Palla, G. 1981. C_{18} reversed-phase chromatographic determination of invert sugar, sucrose, and raffinose. Anal. Chem. 53: 1966-1967.

Palmer, J.K. 1975. A versatile system for sugar analysis via liquid chromatograph. Anal. Lett. 8: 215-224.

Papadakis, P.E. 1929. Further findings on invertase from honey. J. Biol. Chem. 83: 561-568.

Park, O.W. 1933. Studies on the rate at which honeybees ripen honey. J. Econ. Entomol. 26: 188-193.

Partridge, R.D. and Weiss, A.H. 1970. Extraction procedure for TMS carbohydrate ethers. J. Chromatogr. Sci. 8: 553.

Percival, M.S. 1961. Types of nectar in angiosperms. New Phytol. 60: 235-281.

Percival, M.S. 1965. Floral Biology. p. 85-87. Oxford: Pergamon Press.

Percival, E. 1967. Gas chromatography of the trimethysilyl ethers of some less-common disaccharides. Carbohydr. Res. 4: 441-443.

Perlin, A.S. 1976. MTP International Review of Science, Organic Chemistry Series 2. Volume 7. p. 1-34. London: Butterworths.

Philipsborn, H. von. 1952. Uber Calciumoxalat in Pflanzenzellen. Protoplasma 41: 415-424.

Phillips, C.E. 1933. Honey for burns. Glean. Bee Cult. 61: 284.

Phillips, E.F. 1927. The utilization of carbohydrates by honeybees. J. Agric. Res. 35: 385-428.

Pierce, A.E. 1968. Silylation of Organic Compounds. Illinois: Pierce Chemical Company.

Pierce, A.E. 1973. Gas-phase analysis of nucleic acid components as their trimethylsilyl derivatives. In "Synthetic Procedures in Nucleic Acid Chemistry." Volume 2, p. 125-14[illegible] New York: Wiley-Interscience.

Planta, A. von. 1886. Uber die Zusammensetzung einiger Nektar-Arten. Hoppe-Seyler's Z. physiol. Chem. 10: 227-247.

Porsch, B. 1985. High-performance liquid chromatography of oligosaccharides in water on a reverse phase doped with primary amino groups. J. Chromatogr. 320: 408-413.

Pourtallier, J. 1964. Sugars in honey analysis. Bull. Apic. Doc. Sci. Tech. Inf. 7: 197-202.

Pourtallier, J. 1968. Use of gas chromatography for determining sugars in honey. A. Bienenforsch. 9: 217-221.

Pritsch, G. 1958. Untersuchungen uber die Bienenweideverhaltnisse verschiedener Standorte auf leichteren Boden unter besonderer Berucksichtigung der Untersuchungen mittels Honig- Pollenanalysen. Arch. Geflugelz. Kleintierk. 7(3/4): 184-247, 7(5/6): 282-310.

Rabel, F.M., Caputo, A.G. and Butts, E.T. 1976. Separation of carbohydrates on a new polar bonded phase material. J. Chromatogr. 126: 731-740

Rathbone, E.B. 1985. Nuclear magnetic resonance spectroscopy in the structural analysis of food-related carbohydrates. In "Analysis of Food Carbohydrates." p. 149-223. London: Elsevier Applied Science.

Recondo, E.P. and Rinderknecht, H. 1959. Eiene neue, einfache syntheses des 1-O-Acetyl-2,3,5-Tri-O-benzoyl-β-D-Ribofuranosides. Helv. Chim. Acta 42: 1171-1173.

Reinhardt, J.F. 1939. Ventilating the bee colony to facilitate the honey ripening process. J. Econ. Entomol. 32: 654-660.

Reitsma, T. 1970. Suggestions towards unification of descriptive terminology of angiosperm pollen grains. Rev. Paleobot. Palynol. 10: 39-60.

Reyes, F.G.R., Wrolstad, R.E. and Cornwell, C.J. 1982. Comparison of enzymic, gas-liquid chromatographic, and high performance liquid chromatographic methods for determining sugars and organic acids in strawberries at three stages of maturity. J. Assoc. Off. Anal. Chem. 65: 126-131.

Riedel, I.B. and Simpson, J. 1961. Absence of salivary invertase in queen and drone honeybees. Experientia 17:365.

Rinaudo, M.T., Ponzetto,C., Vidano, C. and Marletto, F. 1973. The origin of honey saccharose. Comp. Biochem. Physiol. 46B: 245-251.

Rocca, J.L. and Rouchouse, A. 1976. Separation de sucres sur microparticules de silica par chromatographie en phase liquid a haute performance. J. Chromatogr. 117: 216-221.

Rocklin, R.D. and Pohl, C.A. 1983. Determination of carbohydrates by anion exchange chromatography with pulsed amperometric detection. J. Liq. Chromatogr. 6: 1577-1590.

Rogers, P.E.W. and Davies, A.M.C. 1972. Unpublished results.

Roper, M., Roper, S., Heyns, K. and Meyer, B. 1983. NMR spectroscopy of N-(1-deoxy-D-Fructos-1-yl)-L-amino acids ("Fructose-amino acids"). Carb. Res. 116: 183-195.

Rowland, M. and Riegelman, S. 1967. Use of carbon disulfide as a solvent for the silylation of submicrogram amounts of carboxylic acids. Anal. Biochem. 20: 463-465.

Sabbe, W.E. and Cathey, G.W. 1970. Adaptation of gas-liquid chromatography to analysis of certain sugars of cotton leaves. Agron. J. 62: 36-39.

Samuelson, O. and Stromberg, H. 1966. Separation of alditols and aldoses by partition chromatography on ion-exchange resins. Carbohydr. Res. 3: 89-96.

Sarra, C. and Durio, P. 1974. Bromatological evaluation of honey. Investigations on the carbohydrate content by gas chromatography. Atti Soc. Ital. Sci. Vet. 28: 679-682.

Sawardeker, J.S., Sloneker, J.H. and Jeanes, A. 1965. Quantitative determination of monosaccharides as their alditol acetates by gas-liquid chromatography. Anal. Chem. 37: 1602-1604.

Sawyer, R.W. 1975. Melissopalynology in the determination of the geographical and floral origin of honey. J. Assoc. Publ. Analysts. 13: 64-71.

Schepartz, A.I. and Subers, M.H. 1964. The glucose oxidase of honey. I. Purification and some general properties of the enzyme. Biochem. Biophys. Acta 85: 228-237.

Schepartz, A.I. and Subers, M.H. 1966. Observations on honey diastase. J. Apic. Res. 5: 45-58.

Schwarz, R., Baronetsky, E. and Schoeller, K. 1956. Zum Silicoseproblem. Nueu Verbindungen von Kieseksaure-derivaten mit Monosacchariden. Agnew. Chem. 68: 335.

Schwier, I. and Lechert, H. 1982. X-ray and nuclear magnetic resonance investigations on some structural problems of starch. Starke 34: 11-16.

Selvendran, R.R., March, J.F. and Ring, S.G. 1979. Determination of aldoses and uronic acid content of vegetable fiber. Anal. Biochem. 96: 282-292.

Semenza, G., Curtis, H.-C., Kolinska, J. and Muller, M. 1967. Studies on intestinal sucrose and intestinal sugar transport VI. Liberation of α-glucose by sucrose and isomaltose from the glycone moiety of the substrates. Biochim. Biophys. Acta 146: 196-204.

Sennello, L.T. 1971. Gas chromatographic determination of fructose and glucose in syrups. J. Chromatogr. 56: 121-125.

Shavers, C.L., Parsons, M.L. and Deming, S.W. 1979. Simplex optimization of chemical systems. J. Chem. Ed. 56: 307-309.

Shaw, D. 1976. Fourier Transform N.M.R. Spectroscopy. Amsterdam: Elsevier Applied Science.

Shaw, P.E. and Wilson, C.W. 1983. Separation of fructose, glucose and sucrose in fruit by high performance liquid chromatography using UV detection at 190nm. J. Sci. Food Agric. 34: 109-112.

Shuel, R.W. 1955. Nectar secretion in relation to nitrogen supply, nutritional status, and growth of the plant. Can. J. Agric. Sci. 35: 124-138.

Shyluk, J.P., Youngs, C.G. and Gamborg, O.L. 1967. Gas chromatography of the trimethylsilyl derivatives of shikimic acid and biochemically related compounds. J. Chromatogr. 26: 268-269.

Siddiqui, I.R. and Furgala, B. 1967. Isolation and characterization of oligosaccharides from honey. Part I. Disaccharides. J. Apic. Res. 6: 139-145.

Siddiqui, I.R. and Furgala, B. 1968a. Isolation and characterization of oligosaccharides from honey. Part II. Trisaccharides. J. Apic. Res. 7: 51-59.

Siddiqui, I.R. and Furgala, B. 1968b. Centrose: A branched trisaccharide containing α-D-(1→2) and α-D-(1→4) glycoside bonds. Carbohydr. Res. 6: 250-252.

Siddiqui, I.R. 1970. The sugars of honey. Adv. Carbohydr. Chem. Biochem. 25: 285-309.

Siegenthaler, V. 1977 Eine einfache und rasche methode zur bestimmung der _-glucosidase (saccharase) in honey. Mitt. Geb. Lebensmittelunters Hyg. 68: 251-258.

Silverstein, R.M., Bassler, G.C. and Morrill, T.C. 1974. Magnetic resonance spectrometry. In "Spectrometric Identification of Organic Compounds." p. 159-196. Toronto: John Wiley and Sons.

Simatupang, M.H. 1979. Hochdruckflussigkeitschromatographische Trennugen von Kohlenhydraten mit einem colorimetrischen Nachweisverfahren. J. Chromatogr. 180: 177-183.

Simpson, B.B. and Neff, J.L. 1983. Evolution and diversity of floral rewards. In "Handbook of Experimental Pollination Biology." p. 142-159. New York: Van Nostrand Reinhold. p. 159-196. Toronto: John Wiley and Sons.

Sloneker, J.H. 1968. "Biomedical Applications of Gas Chromatography." Volume 2. New York: Plenum Press.

Smith, D.F., Zopf, D.A. and Ginsburg, V. 1978. Fractionation of silyl oligosaccharides of human milk by ion-exchange chromatography. Anal. Biochem. 85: 602-608.

Smith, I.C.P. and Saito, H. 1980. I.U.P.A.C. Proceedings Int. Congress Pure Applied Chem. 27: 213.

Smith, L.B. and Johnson, J.A. 1952. The use of honey in bread products. Bakers Digest 25: 103-106.

Stinson, E.E., Subers, M.H., Petty, J. and White, J.W. Jr. 1960. The composition of honey. V. Separation and identification of the organic acids. Arch. Biochem. 89: 6-12.

Stockhouse, R.E. 1975. Nectra composition of hawk moth-visited species of Onagraceae. Gr. Basin Natur. 35: 273-274.

Sweeley, C.C., Bentley, R., Makita, M. and Wells, W.W. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Am. Chem. Soc. 85: 2497-2507.

Sweeley, C.C. 1965. Analysis of carbohydrates by gas chromatography. Bull. Soc. Chim. Biol. 47: 1477-1494.

Sweeley, C.C., Wells, W.W. and Bentley, R. 1966. Gas chromatography of carbohydrates. Methods Enzymol. 8: 95-108.

Szafranek, J., Pfaffenberger, C.D. and Horning, E.C. 1974. Separation of aldonic, deoxyaldonic, hexuronic and hexaric lactones and acids using thermostable open

tubular glass capillary columns. J. Chromatogr. 88: 149-156.

Takenaka, T. and Echigo, T. 1976. The properties and trans-glucosidation action of honey α-glucosidase. Tamagawa Daikaku Nogakubu Kenkyu Hokoku 16: 1-9.

Takenaka, T. and Echigo, T. 1978. Properties and trans-glucosidation action of α-glucosidase of honey. Part 2. Tamagawa Univ. Fac. Agric. Bull. 18: 22-31.

Takewaki, S., Chiba, S., Kimura, A., Hirokazu, M. and Kioke, Y. 1980. Purification and properties of α-glucosidases of the honey bee *Apis mellifera* L. Agric. Biol. Chem. 44: 731-740.

Tamate, J. and Bradbury, J.H. 1985. Determination of sugars in tropical root crops using ^{13}C N.M.R. spectroscopy: comparison with the h.p.l.c. method. J. Sci Food Agric. 36: 1291-1302.

Tamura, Z. and Imanari, T. 1967. Nanogram order analysis of sugars by gas chromatography. Chem. Pharm. Bull Tokyo 15: 246.

Thente, K. 1983. HPLC analysis of component sugars in fiber polysaccharides from cereals. Dev. Food Sci. 5A: 477-482, 34-41.

Tillmans, J. and Kiesgen, J. 1927. Die Formoltitration als mittel zur Unterscheidung von Kunstlichen und naturlichen Lebensmittelen. Z Unters. Lebensmittel 53: 131-137.

Thompson, R.M., Coffin, R.D. and Glick, M.R. 1978. Determination of disaccharides in feces by permethylation and gas chromatography: rapid screening for carbohydrate intolerance in children. Clin. Chim. Acta. 84: 185-197.

Toba, T. and Adachi, S. 1977. Gas-liquid chromatography of trimethyl silylated disaccharide oximes. J. Chromatogr. 135: 411-417. Agric. Biol. Chem. 44: 731-734.

Townsend, G.F., Smith, M.V. and Adams, R.J. 1979. Identification of honey sources by pollen analysis of nectar from the hive. J. Apic. Res. 18: 292-297.

Trugo, L.C. and Macrae, R. 1982. The determination of carbohydrates in coffee products using high-performance liquid-chromatography. In "Proceedings of the Tenth International Scientific Colloquium on Coffee." Salvador, Brazil. p. 187-192. Paris: Association Scientifique Internationale Cafe.

Usui, T., Yamaoka, N., Matsuda, K., Tuzimura, K., Sugiyama, H. and Seto, S. 1973. C-13 nuclear magnetic-resonance spectra of glucobioses, glucotrioses and glucans. J. Chem. Soc. Trans. Perkins I 20: 2425-2432.

Van Handel, E. 1967. Determination of fructose and fructose-yielding carbohydrates with cold anthrone. Anal. Biochem. 22: 280-283.

Van Handel, E. 1968. Direct microdetermination of sucrose. Anal. Biochem. 22: 280-283.

Van Handel, E., Haeger, J.S. and Hansen, C.W. 1972. The sugars of some Florida nectars. Am. J. Bot. 59: 1030-1032.

Vansell, G.H. and Freeborn, S.B. 1929. Preliminary report on the investigations of the source of diastase in honey. J. Econ. Ent. 22: 922-926.

Verhaar, L.A.Th. and Kuster, B.F.M. 1981. Liquid chromatography of sugars on silica-based stationary phases. J. Chromatogr. 220: 313-328.

Vervack, W., Foulon, M., Vanbelle, M. and Descrumps, S. 1978. Determination of mono-, di-, and trisaccharides in some foods using gas chromatography. Rev. Ferment. Ind. Aliment. 33: 48-58.

Vilkas, M., Hiu-I-Jan, Boussac, M. and Bonnard, M. 1966. Chromatographie en phase vapeur de sucres a l'etat de trifluoracetates. Tetrahedron Lett. 14: 1441-1446.

Voelter, W., Breitmaier, E. and Jung, G. 1971. Fourier transform C-13-NMR-Spectroscopy. 10. Pulse Fourier-transform C-13 NMR spectroscopy of mutarotating sugars. Agnew. Chem. Int. Ed. 10: 935.

Voelter, W., Bilik, V. and Breitmaier, E. 1973. Configuration and mutarotation influences on C-13 NMR-spectra of some disaccharides. Coll. Czech. Chem. Comm. 38: 2054-2071.

Voorst, F.T. Van. 1941. Biochemical sugar determination. XII. Honey. Chem. Weekblad 38: 538-542.

Wade, N.L. and Morris, S.C. 1982. Rapid determination of sugars in canteloupe melon juice by high-performance liquid chromatography. J. Chromatogr. 240: 257-261.

Watanabe, T. and Aso, K. 1959. Isolation of kojibiose from honey. Nature 183: 1740.

Watanabe, T. and Aso, K. 1960. Isolation of kojibiose, nigerose, maltose and isomaltose from honey. Tohoku J. Agr. Res. 11: 109-115.

Watanabe, N. and Inoue, M. 1983. Amperometric detection of reducing carbohydrates in liquid chromatography. Anal. Chem. 55: 1016-1019.

Watt, W.B., Hoch, P.C. and Mills, S.G. 1974. Nectar resource used by *Colias* butterflies. Oecologia 14: 353-374.

Wehrli, F.W. and Wirthlin, T. 1976. Interpretation of Carbon-13 N.M.R. Spectra. London: Heyden.

Weishaar, H. 1933. Untersuchungen uber Bestimmung, Mindestwert, und Herkunft der Honigdiastase. Z. Unters. Lebensmittel 65: 369-399.

Weisser, H. 1975. NMR spectroscopy in the food industry. Bruker minispec application note 7.

Wells, W.W., Sweeley, C.C. and Bentley, R. 1964. Biomedical Applications of Gas Chromatography. Volume 1. New York: Plenum Press.

Whistler, R.L. and Durso, D.F. 1950. Chromatographic separation of sugars on charcoal. J. Am. Chem. Soc. 72: 677-679.

White, J.W., Jr., Ricciuti, C., and Maher, J. 1952. Determination of dextrose and levulose in honey. Comparison of methods. J. Assoc. Off. Agric. Chem. 35: 859-872.

White, J.W., Jr. and Maher, J. 1953a. Transglucosidation by honey invertase. Arch. Biochem. 42: 360-367.

White, J.W., Jr. and Maher, J. 1953b. α-maltosyl-β-D-fructofuranoside, a trisaccharide enzymatically synthesized from sucrose. J. Am. Chem. Soc. 75: 1259-1260.

White, J.W., Jr. and Maher, J. 1954. Selective adsorption method for determination of the sugars of honey. J. Assoc. Off. Agr. Chem. 37: 466-478.

White, J.W., Jr., Petty, J. and Hager, R.B. 1958a. The composition of honey. II. Lactone content. J. Assoc. Off. Agric. Chem. 41: 194-197.

White, J.W., Jr., Eddy, C.R., Petty, J. and Hoban, N. 1958b. Infrared identification of disaccharides. Anal. Chem. 30: 506-510.

White, J.W., Jr. and Riethof, M.L. 1959a. The composition of honey. III. Detection of acetylandromedol in toxic honeys. Arch. Biochem. 79: 165-167.

White, J.W., Jr. and Hoban, N. 1959b. The composition of honey. IV. Identification of the disaccharides. Archs. Biochem. Biophys. 80: 386-392.

White, J.W., Jr. 1961a. A survey of American honeys. Glean. Bee Cult. 89: 230-233.

White, J.W., Jr., Riethof, M.L. and Kushnir, I. 1961b. The composition of honey. VI. The effect of storage on carbohydrates, acidity, and diastase content. J. Food Sci. 26: 63-71.

White, J.W., Jr. and Subers, M.H. 1963. Studies on honey inhibine 2. A chemical assay. J. Apic. Res. 2: 93-100.

White, J.W., Jr., Reithof, M.L., Subers, M.H. and Kushnir, I. 1962. Composition of American honeys. Tech. Bull. U.S. Dept. Agric., No. 1261.

White, J.W., Jr. 1964. Diastase in honey: the Schade method. J. Assoc. Off. Anal. Chem. 47: 486-488.

White, J.W., Jr. 1967. Measuring honey quality - a rational approach. Am. Bee J. 107: 374-375.

White, J.W., Jr. and Kushnir, I. 1967. The enzymes in honey: examination by ion-exchange chromatography, gel filtration, and starch-gel electrophoresis. J. Apic. Res. 6: 69-89.

White, J.W., Jr. 1975. Composition of honey. In "Honey: a comprehensive survey." p. 157-206. London: Heinemann.

White, J.W., Jr. 1978. Honey. Adv. Food Res. 24: 287-375.

Wiley, H.W. 1892. Sugar analysis of North American honeys. U.S. Bur. Chem. Bull. 13: 744-768.

Wiley, H.W. 1896. On the estimation of levulose in honeys and other substances. J. Am. Chem. Soc. 18: 81-9.

Wilkinson, G.N. 1961. Statistical estimations in enzyme kinetics. Biochem. J. 80: 324-332.

Willet, J.E. 1967. The analysis of aqueous samples by gas chromatography. Chem. Ind.:1701-1702.

Williams, D.H. and Fleming, I. 1980. Nuclear magnetic resonance spectra. In "Spectroscopic Methods in Organic Chemistry." p. 74-152. London: McGraw-Hill.

Willson, R.B. and Crane, E. 1975. Uses and products of honey. In "Honey: a Comprehensive Survey." London: Heinemann

Wodehouse, P.P. 1935. Pollen Grains. New York: McGraw-Hill.

Wolfram, M.L. and Koijumi, K. 1967. A chemical synthesis of panose and an isomeric trisaccharide. J. Org. Chem. 32: 656-660.

Wykes, G.R. 1952. An investigation of the sugars present in the nectar of flowers of various species. New Phytol. 51: 210-215.

Wykes, G.R. 1953. The sugar content of nectars. Biochem. J. 53: 294-296.

Yakovleva, L.P. 1969. The qualitative sugar composition of the nectar of some honey plants. Apic. Abstr. 22: 91.

Yasui, T. and Takenaka, R. 1979a. Quantitative determination of saccharides in foods by gas chromatography. Part I. Separation of mono- and disaccharides as their trimethylsilyl derivatives. Shokuhin Sogo Kenkyusho Kenkyu Hokokou 34: 105-109.

Yasui, T., Nikkuni, I. and Hase, S. 1979b. Quantitative determination of saccharides in foods by gas chromatography. Part II. Determination of mono- and disaccharides in jams, Yokan, chewing gums, biscuits,

crackers and apple juices. Shokuhin Sogo Kenkyusho Kenkyu Hokokou 34; 110-115.

Yoshiro, H., Keiji, I. and Takehisa, S.T. 1969. Analysis of fructose and glucose in honey by gas chromatography. Yakugaku Zasshi 89: 734-736.

Zauralov, O.A. and Yakovleva, L.P. 1973. Sugar composition of the nectars of some nectariferous plants. Rast. Resur. 9: 444-451.

Zetsche, F. 1932. Kork und cuticularsubstanzen. In "Handbuch der Pflanzen-analyse." Berlin: Springer-Verlag.

Ziegler, H. and Ziegler, I. 1962. Die wasserloslichen Vitamine in den Siebrohrensaften einiger Baume. Flora, Jena. 152: 257-278.

Zimmermann, M. 1953. Papierchromatographische Untersuchungen uber die pflanzliche Zuckersekretion. Ber. Schweig. bot. Ges. 63: 402-409.

Zimmermann, M. 1954. Uber die Sekretion saccharosespaltender Transglukosidasen in pflanzlichen Nektar. Experientia 10: 145-149.

Zimmermann, M.H. and Brown, C.L. 1977. Trees: Structure and Function. New York: Springer-Verlag.

Zinkel, D.F., Lathrop, M.B. and Zank, L.C. 1968. Preparation and gas chromatography of the trimethylsilyl derivatives of resin acids and the corresponding acids. J. Gas Chromatogr. 6: 158-160.

Zygmunt, L.C. 1982. High pressure liquid chromatographic determination of mono- and disaccharides in presweetened cereals: a collaborative study. J. Assoc.

Off. Anal. Chem. 65: 256-264.

12. APPENDIX 1 -- GLOSSARY OF MELISSOPALYNOLOGY

Amb: the outline of a grain when the polar axis is directed towards the observer.

Colpus (pl. colpi): a furrow or elongated aperture which serves as an exit for the pollen tube, the ratio of length to breadth of which is >2.

Columellae: small, rod-like elements, radially directed and forming the inner layer of the sexine. They are attached at their bases to the nexine and at their heads to the tectum (when present).

Costa colpi (pl. costae colpi): a thickening of the endexine surrounding the colpus or furrow.

Endopore: the inner pore formed by separation of the ektexine and endexine in the area of the aperture.

Heterobrochate: a reticulate sculpturing type in which the brochi are uniform in size.

Lumen (pl. lumina): a gap or space between the walls of a reticulate, striate or rugulate sculpture.

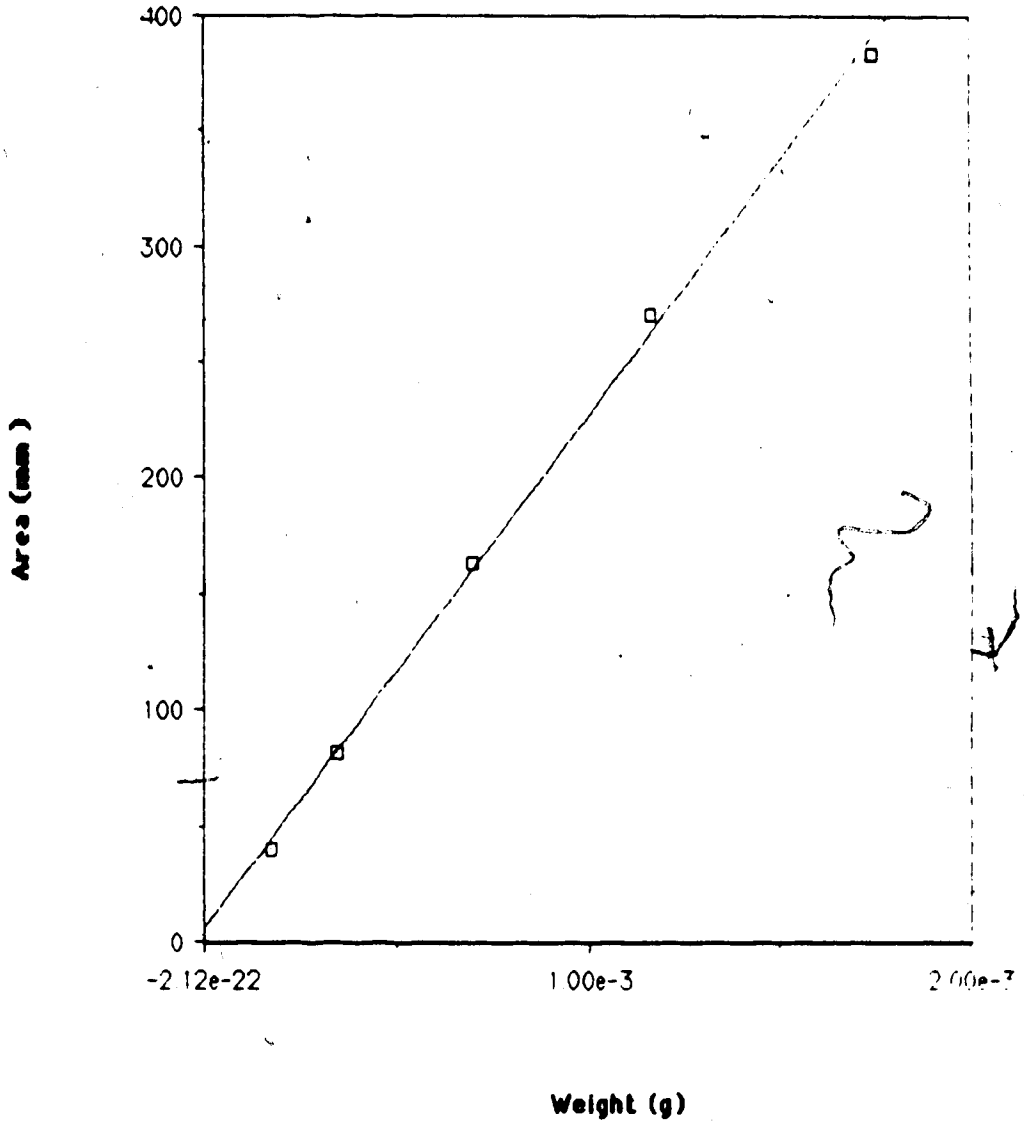
Margo: the area surrounding the colpus, the sculpturing of which is different from the rest of the grain, where the ektexine is thicker or thinner than in the rest of the grain.

Mesocolpium: area between the poles and the colpi.

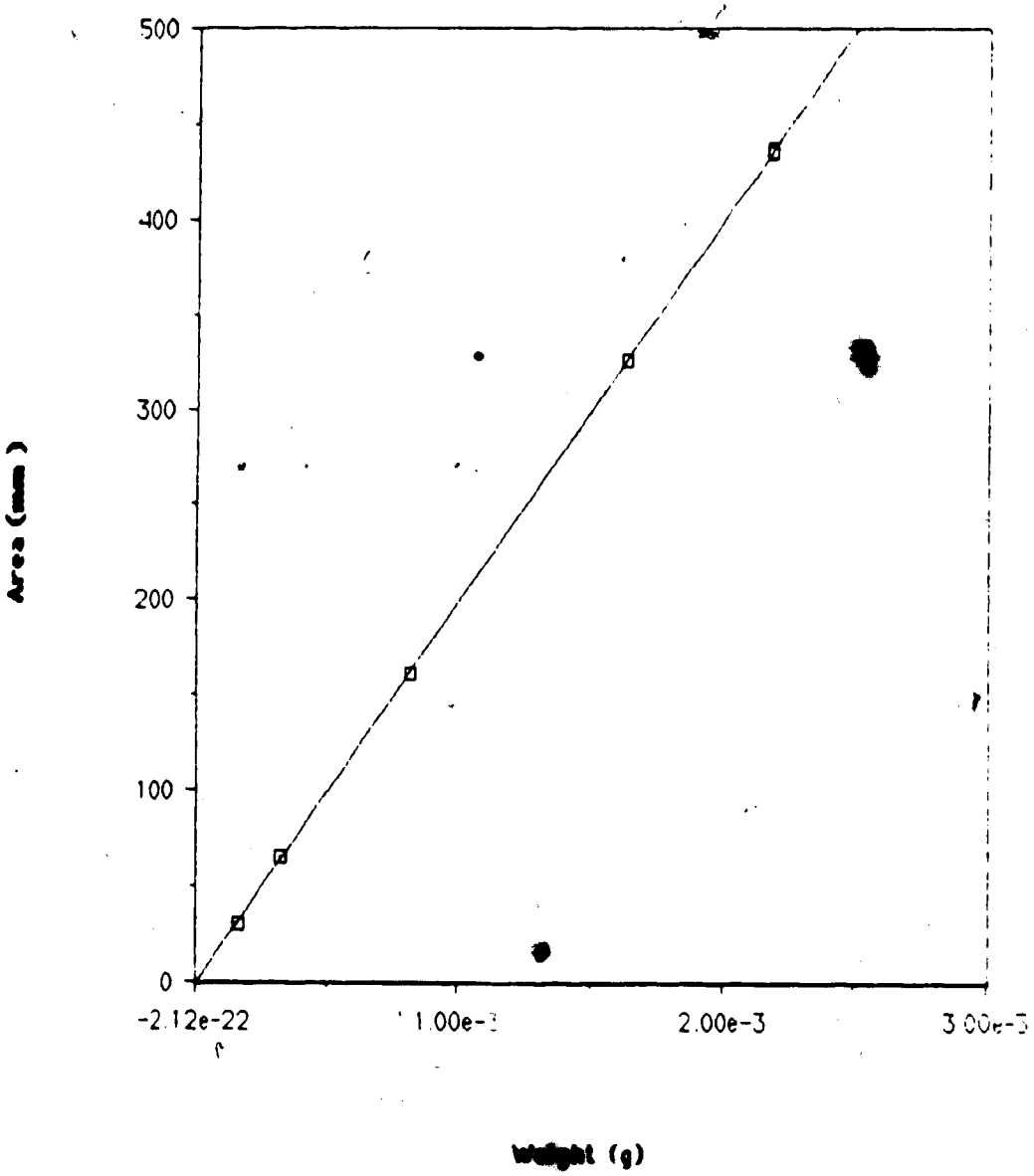
Porus (pl. pori): a circular or slightly elliptic aperture with a length/breadth ratio smaller than 2.

Tricolporate: with three colpi, each with a pore.

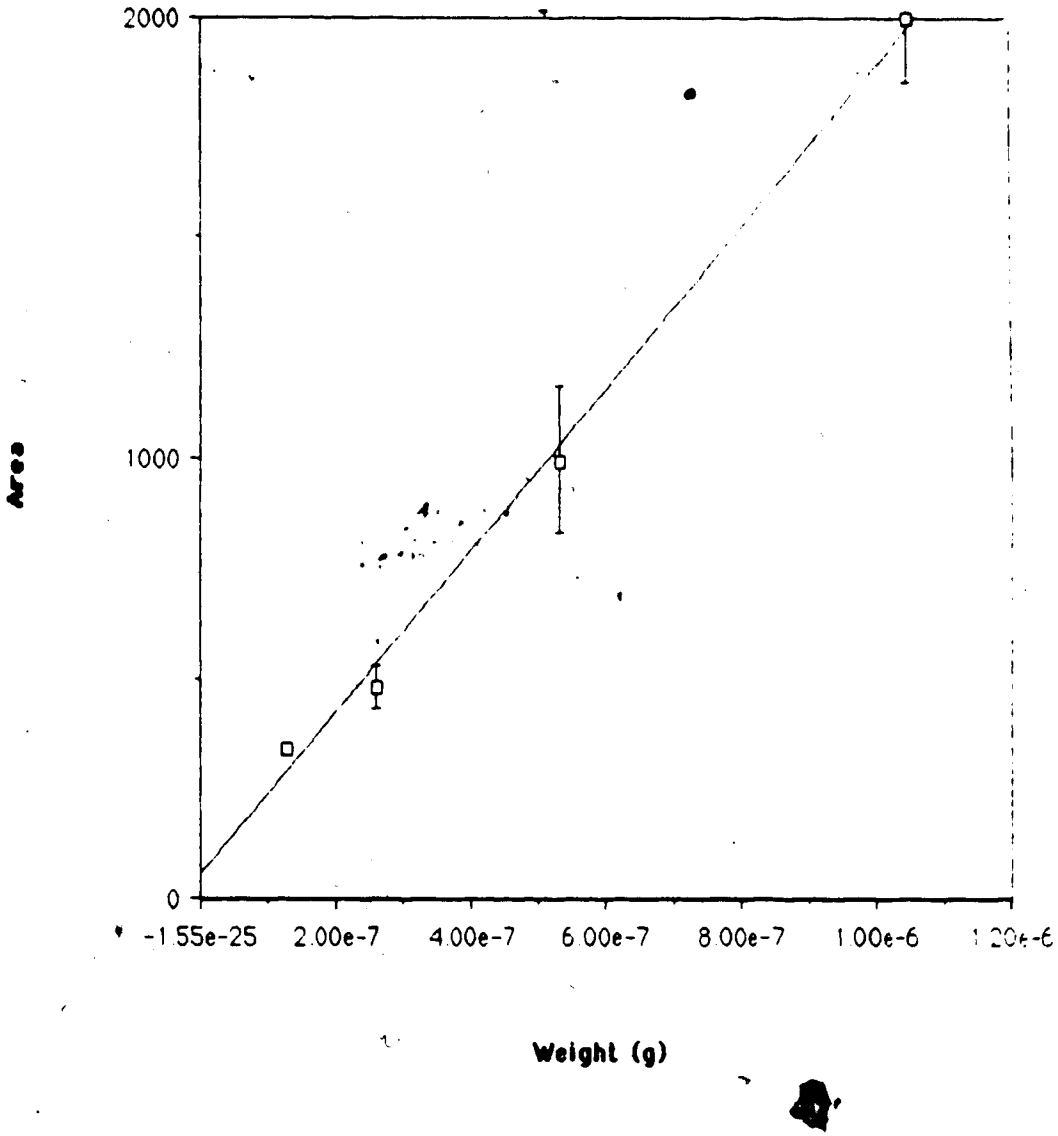
13. APPENDIX 2 -- HPLC STANDARD CURVE FOR FRUCTOSE



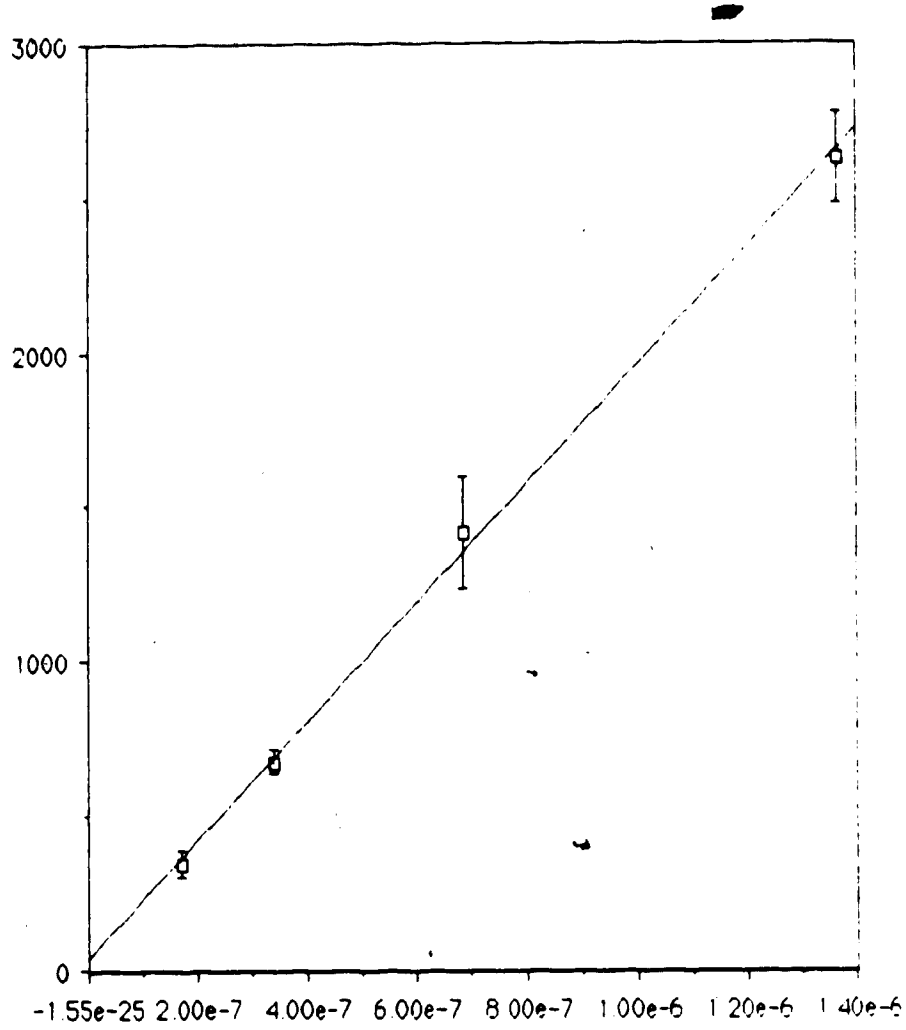
14. APPENDIX 3 -- HPLC STANDARD CURVE FOR GLUCOSE



15. APPENDIX 4 -- GC STANDARD CURVE FOR TREHALOSE



16. APPENDIX 5 -- GC STANDARD CURVE FOR SUCROSE



17. APPENDIX 6 -- GC STANDARD CURVE FOR REDUCED MALTOSE