'The Bee on Honey-Dew Hath Fed'

Carbohydrate Composition and Prebiotic Potential of An Australian Commercial Honey and Some New Zealand Honeydew Honeys

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Abstract

The compositions of floral and honeydew honeys differ, *inter alia,* in that honeydew honey typically has higher oligosaccharide and acid contents. Honeydew honey is made from honeydew excreted as a byproduct of plant sap digestion by aphids, including the Giant Willow Aphid (GWA), an invasive pest insect in New Zealand and other countries. GWA honeydew honey is problematic to apiarists, as it crystallizes in the comb, reducing yield and making it commercially unacceptable. This crystallization has been ascribed to high levels of the oligosaccharide melezitose.

Some oligosaccharides are prebiotic; that is, indigestible to humans but selectively fermented by beneficial colonic bacteria, conferring a health benefit. This research used NMR, HPLC and GC-MS methods for identification and quantitation of carbohydrate fractions in Beeotic®, an Australian honey marketed as prebiotic, and in some New Zealand honeydew honeys, and developed methods to extract melezitose crystals and to make palatable candy from GWA honeydew honey. The acid and enzyme lability of melezitose were also determined, as an indication of its prebiotic potential.

Analysis of Beeotic® showed it to contain 2.73 ± 0.36% oligosaccharides, 12.06 ± 0.10% disaccharides, 23.11 ± 0.59% glucose, and 49.16 ± 0.82% fructose. It was estimated that to ingest a dose capable of providing a prebiotic effect, a consumer would have to eat a minimum of 73.18 ± 9.67 g of Beeotic® daily, perhaps more if the oligosaccharides present in Beeotic® have lower prebiotic activity. This mass of honey contains 64 g digestible sugars: more than double the WHO-recommended daily maximum.

Analysis of a New Zealand beech honeydew honey showed it to contain 13.83 ± 0.01% oligosaccharides, 14.56 ± 0.10% disaccharides, 22.29 ± 0.23% glucose,

and 44.61 ± 0.29% fructose. The minimum daily dose to confer a prebiotic effect would be 14.46 ± 0.44 g of beech honeydew honey, which includes 11 g digestible sugars.

Analysis of GWA honeydew honey, in the first known carbohydrate profile of this honey, showed it to contain 37.74 ± 0.19% oligosaccharides (of which 27.4% of the honey mass was melezitose), 37.11 ± 0.27% disaccharides, 16.54 ± 0.82% glucose, and 24.68 ± 1.13% fructose. The minimum daily dose to confer a prebiotic effect would be 5.30 ± 0.03 g of GWA honeydew honey, which includes 4 g digestible sugars.

Preliminary methods were developed for making GWA honeydew honey into a palatable candy, and for extracting melezitose from GWA honeydew honey. Enzyme digestion of melezitose caused minimal hydrolysis, and acid digestion no significant hydrolysis; this indicates that melezitose may fulfil the prebiotic criterion of human indigestibility and therefore GWA honeydew honey may, pending a full prebiotic activity assay, be an appropriate prebiotic functional food. This would add value to what is currently a waste product and offset the impact of GWA honeydew on New Zealand apiarists.

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List of abbreviations

AH3	The sample of Capilano Beeotic® honey tested as representative of Beeotic® (chapter 3)
C. perfringens	*Clostridium perfringens*
cf.	(Latin: *conferatur*) compare
D_2O	Deuterated water (NMR solvent)
DP	Degree of polymerization
E. aerofaciens	*Eubacterium aerofaciens*
E. coli	*Escherichia coli*
F/G	Fructose/glucose ratio in honey
F+G	Total monosaccharides in honey
Fru*f*	Fructofuranose
GC-FID	Gas chromatography with flame ionization detection
GC-MS	Gas chromatography with mass spectrometry
Glc*p*	Glucopyranose
GWA	Giant Willow Aphid (*Tuberolachnus salignus*)
GWC	The sample of crystals isolated from a honey thought to contain Giant Willow Aphid honeydew honey, supplied by 1839 Ltd. (chapter 5)
GWH	The sample of Giant Willow Aphid honeydew honey supplied by Honey NZ Ltd. (chapter 6)
HMBC	Heteronuclear multiple bond correlation spectroscopy
HND	The sample of New Zealand beech honeydew honey supplied by Honey NZ Ltd. (chapter 4)
HPLC	High-Performance Liquid Chromatography
HSQC	Heteronuclear single quantum coherence spectroscopy
L. plantarum	*Lactobacillus plantarum*
LC-MS	Liquid chromatography with mass spectrometry
M	(SI unit abbreviation) moles per liter
mw	Molecular weight
NMR	Nuclear Magnetic Resonance spectroscopy
NOE	Nuclear Overhauser Effect (or Enhancement)
PDA	Photodiode array (HPLC detector used for UV detection)
pK_a	Negative $\log_{10}$ of the dissociation constant for a given acid
RF	Response factor (of a given sugar on GC-MS analysis)
RI	Refractive index (HPLC detection)
RIRDC	Rural Industries Research and Development Corporation
S. aureus	*Staphylococcus aureus*
T. salignus	*Tuberolachnus salignus* (Giant Willow Aphid)

TIC	Total Ion Chromatogram
TMS	Trimethylsilyl
TMSi	Trimethylsilylimidazole
WHO	World Health Organization
xol	Xylitol

1 Literature review

1.1 Preamble

Honey has been known since ancient times for its health benefits, including its antibacterial activity [2], which is due *inter alia* to certain low-to-trace concentration compounds [3-4] (its acidity, peroxide content, and osmotic potential are also antibacterial factors, but not the present focus). In recent years, compounds that make up a larger proportion of honey (oligosaccharides) have been noted to selectively encourage the growth of some desirable gut bacteria as well [5], prompting more attention to be paid to the carbohydrate analysis of honeys. Throughout this thesis, the word 'oligosaccharide' is used to refer to a saccharide composed of 3-10 sugar residues, and 'disaccharide' to refer to a saccharide of exactly two sugar residues.

1.2 Carbohydrate compositions of floral and honeydew honeys

Bees make floral honey from floral nectar, a sugar solution produced by flowering plants to encourage pollination [6], and honeydew honey from honeydew, a sugar-based solution that is excreted by insects feeding on the sugars and proteins in plant sap [7].

Insect honeydew honeys typically exhibit a higher fructose/glucose ratio (F/G) than most floral honeys: ~1.3-2.0 [8] cf. floral honey range 1.0-1.2 [8-9]. However, some floral honeys, particularly heather and thyme, have a similar F/G to some honeydew honeys [10], so F/G is not independently diagnostic. Honeydew honey generally also has lower total monosaccharide content (F+G), typically ~45%, cf. floral honey 60-80% [8-9], due to higher concentrations of disaccharides and oligosaccharides including melezitose, erlose, and maltose [11]. Floral honeys typically contain ~3-10% oligosaccharides by total mass of honey [9, 12-15], whereas

honeydew honeys may contain ~8-20% oligosaccharides by total mass of honey [7, 16-17] *.

The variety of di- and oligosaccharides in floral honey (with the occasional exception of sucrose) are formed by bee-originated α- and β-glucosidases (enzymes that hydrolyze α and β forms of glucosides, respectively), as the nectar of flowers that may be visited by bees comprises chiefly monosaccharides, with varying amounts of sucrose and trace or no other sugars. Nectar composition, concentration, and volume are influenced by a host of factors, including water availability, day or night flowering, climate, herbivory, availability of pollinators, and flower orientation; these traits are also heritable to a degree [18]. The ratio of sucrose to glucose and fructose in floral nectar, particularly, varies depending on the degree of specialization to particular pollinators [6]. Generally speaking, night-flowering and long-tubed flowers, more suited to pollination by long-tongued moths or small birds or mammals, tend to produce largely sucrose-based nectar; such flowers are not generally pollinated by bees, and their nectar is not generally an important factor in bee honey composition [19].

Insect honeydew already contains high proportions of oligosaccharides, particularly melezitose and erlose (up to 60% of total sugars or 84 g/L of honeydew) [20-21], because sucrose at concentrations of ~0.2-0.3 M in sap is converted to oligosaccharides at 0.5-1.0 M within the insect's gut to relieve feeding-induced osmotic pressure [22-23]. Some analyses consider high levels of the trisaccharide melezitose a defining characteristic of honeydew honeys [24]. The formation of melezitose is ascribed to the activity of an enzyme that has

* Honey analyses in the literature generally do not state whether the 'total honey mass' refers to the mass of solids, or the mass including water. Unless explicitly stated otherwise, it is assumed that 'total honey mass' refers to the mass including water.

been found to convert sucrose to melezitose under laboratory conditions [22]; it is established that this enzyme is actually an α-glucosidase or α-glucoside hydrolase, and is only active in synthesizing oligosaccharides in a solution of sucrose in water when the sucrose concentration is >0.2 M, a process known as reversion [23, 25]. The enzyme originates from the sap-feeding insect or aphid, not from any microsymbiont [23, 25]; microsymbionts have been observed to have an, as yet unclear, effect on the composition of the honeydew of some aphids [26].

Analysis of oligosaccharides in honey is complicated by lower sugars being further converted to oligosaccharides on ambient-temperature storage. White [14], and Kalimi *et al*. [27], ascribe this to the activity of a transglucosylase, which splits sucrose to form free monosaccharides and from them forms di- or trisaccharides, depending on what substrate sugars are available [28]. However, most hexoside hydrolases will exhibit reversion (formation of higher sugars) when the concentration of sugars is sufficiently high and the water content sufficiently low, as in honey. Insect honeydew also contains higher concentrations of invertases (β-D-fructofuranosidase) than nectar does, so as a honeydew honey 'ripens' in the comb, it undergoes a heightened oligosaccharide formation rate [29], due to the hydrolysis of sucrose and associated enzyme reversion at high sugar concentrations. When sugars are formed by acid reversion, their identities are chiefly governed by their stability in acid environments, and 1→2 and 1→3 linked α forms are favored [30]. However, in enzyme reversion reactions, such as Huber *et al.*'s study of β-galactosidase reversion, and the reversion reactions that occur in honey, the higher sugars formed, and the proportions in which they occur, are governed by the specific enzymes present [31-32].

Physically, honeydew honeys tend to be darker, less sweet, and more viscous than floral honeys [33-34]. Most trisaccharides are ~0.3-0.6 times the sweetness of

sucrose [35], and sweetness decreases with chain length [36]. The sweetness of sugars also depends on the monosaccharide units involved, the anomeric configuration, and the probability of hydrogen bonding [37].

The tendency of honey to crystallization can be partly predicted by the sugar/water ratio. A dry honey (significantly less than 15-20% water), or a honey with a high (>0.8) F/G ratio, or with a high (>1.7) glucose/water ratio, or with a high oligosaccharide concentration, is more likely to crystallize than one without these properties [8, 15, 34, 38-39]. Honeydew honeys generally contain either erlose or melezitose as the dominant oligosaccharide and as the basis upon which higher oligosaccharides are built [29]. Oligosaccharide concentration alone does not cause crystallization, as evidenced by New Zealand beech honeydew honey, which contains up to 17% oligosaccharides, the majority of which are erlose-based [40-42], yet, due to low glucose levels, crystallizes slowly or not at all [43]. However, 8-20% melezitose has been identified as causing crystallization [7, 44-45], and some aphid honeydews contain up to 40% melezitose by weight, and some honeydew honeys up to 20% by weight [7].

The synthesis in the insect's gut of melezitose and erlose from the sucrose native to tree sap is not clearly understood. Structures of melezitose and erlose are shown as Figure 1-1, where it is clear how both are derived from the sucrose present in tree sap, with an additional glucose moiety (attached to the glucose unit to form erlose and to the fructose unit to form melezitose).

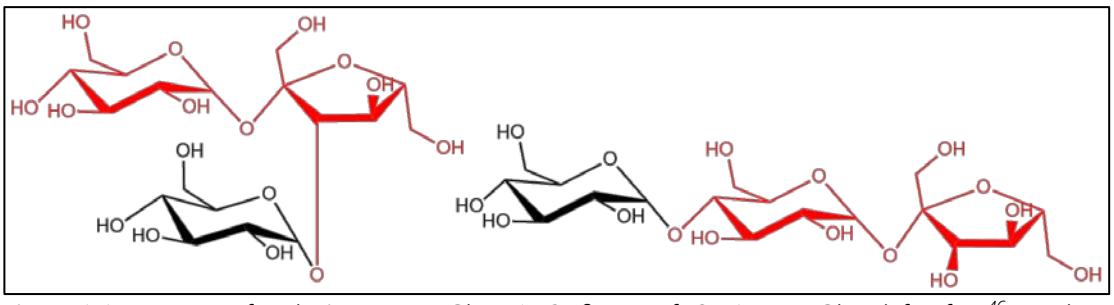

Figure 1-1: structure of melezitose, α-D-Glcp-[1→3]-β-D-Fruf-[2→1]-α-D-Glcp (left, after [46]), and structure of erlose, α-D-Glcp-[1→4]-α-D-Glcp-[1→2]-β-D-Fruf (right, after [47]). Sucrose moieties are colored red, and 'additional' glucose moieties black.

1.3 Prebiotic compounds

Probiotics are gut microflora that benefit the health of the host when they are present in high concentration. Prebiotics, generally oligosaccharides, are compounds that are selectively fermented by probiotic organisms (desirable human gut flora, including *Bifidobacteria*, *Lactobacilli,* some *Enterococci, Saccharomyces boulardii*, and *Bacillus coagulans*) [48], but not by undesirable microorganisms (including *Staphylococcus* spp. and *Escherichia coli*) [49], thus selectively encouraging growth of probiotic organisms. Foodstuffs incorporating both prebiotic compounds and probiotic organisms, are termed synbiotics [50-51], and to date most synbiotics are enriched dairy foods.

While the exact definition remains under some debate, common criteria for a compound to be considered prebiotic are that it must have a demonstrable positive effect on the health of the host organism, survive the digestion process of the host organism to reach the gut bacteria, and be selectively fermented by desirable gut flora [5, 52-54]. Studies have indicated that prebiotic compounds in foodstuffs, including some honey oligosaccharides, can contribute to increased *Bifidobacteria* and *Lactobacilli* populations, and improved blood glucose regulation [55-56] and lipid metabolism [56-57].

Oligosaccharides with demonstrated prebiotic activity include inulin, kestose, nystose, isomaltose, and raffinose [53, 58]. Fermentation of saccharides by human

gut bacteria is governed, in order of decreasing influence, by substitution of hydroxyl groups with other groups, monomeric composition, linkage, and chain length [59]. Melezitose specifically was identified by Gietl *et al*. as being metabolized significantly more slowly than other di- and oligosaccharides studied [59], making it more available toward the distal colon; this effect is ascribed to the Glc[1→3]Fru linkage, which is unusual in naturally-occurring sugars. In a 2005 study of glucooligosaccharides with 1-8 glucose units, trisaccharides were found to have a significantly higher prebiotic index than any other chain length [60]. Bifidobacteria, one of the two major probiotic genera discussed in the literature, have been reported to selectively utilize the di- and oligosaccharides sucrose, maltose, melibiose and raffinose [61]. In *in vitro* tests, some floral honeys (sourwood, alfalfa, and sage) were found to retard growth of *Clostridium perfringens* and *Eubacterium aerofaciens*, and promote growth of bifidobacteria and lactobacilli [13].

The potentially prebiotic properties of melezitose are uncertain. It has been noted to induce glycogen production in a rat liver eight hours or nine hours after feeding melezitose to a fasted rat, indicating the capability of an α-glucosidase present in rats to cleave melezitose into glucose and sucrose moieties [62]. It is utilized by certain strains of the beneficial bacterium *Lactobacillus plantarum* [63] but may not be utilized by other strains of *L. plantarum* [64], other *Lactobacilli* species [65], and may or may not be utilized by both human-derived and bovine-derived strains of various *E. coli*-inhibiting *Bifidobacteria* species [66-68]. However, melezitose does not appear to be entirely selective, as it may also be utilized by some human *Staphylococcus* and *Clostridia* species [69-72].

Lane *et al*. suggested in a 2019 paper [73] that some of the health benefits of honey oligosaccharides may be due to their property of reducing the adhesion

of *E. coli* and *S. aureus* to human epithelial cells (such as colon wall cells). However, the methodology of this study had some flaws: the honey oligosaccharides used were extracted using Sanz *et al.*'s activated-charcoal slurry method [74] based on a 1967 method [75] discussed further in section 1.5.1 of this thesis, which, as Lane *et al.* recognized, did not fully separate the oligosaccharides from the monosaccharides present in New Zealand mānuka honey. Furthermore, the Lane *et al.* study introduced the oligosaccharides and the infecting bacteria to the cells in the same solution *in vitro*, making the test a poor representation of any real-world scenario, so whether any anti-adhesive effect of oligosaccharides has implications for prebiotic activity is still unclear. This assay could be repeated using purer oligosaccharides and following Letourneau's model [76], where either the cells' binding sites are 'protected' with oligosaccharides prior to infection, or oligosaccharides are introduced very shortly after bacterial infection, to measure their effectiveness in preventing the incidence or spread of bacterial infection of cells [76-77].

Oligosaccharides can be added to foodstuffs as indigestible dietary fiber, filler, or to make a prebiotic 'functional food' [57]. Some dairy products naturally containing prebiotics, and some honeys, can qualify as functional foods without modification[50-51, 74, 78]. Appropriate dosage of prebiotics is still under discussion; however, a daily dose of up to 10 g galactooligosaccharides, 4 g fructooligosaccharides, or 8-10 g isomaltooligosaccharides, is required to produce a bifidogenic effect [57]. This is equivalent to 20-45 g of a high-oligosaccharide honeydew honey daily, using White's [17] and Bogdanov's [16] values for oligosaccharides in honeydew honey. Such a honey intake also entails consumption of 12-28 g of monosaccharides and sucrose; since government health organizations and WHO typically recommend a daily dose of no more

than 30 g free sugars for adults [79-82], this daily honey dose represents 40-93% of recommended daily free sugar intake.

1.4 Aphids, honeydew, and 'concrete honey'

The Giant Willow Aphid (*Tuberolachnus salignus*, GWA), shown in Figure 1-2, is a noted invasive pest to willows, and occasionally to some apple, pear, and black poplar species in New Zealand [83]. It has long been observed in many regions of the world where suitable host trees are present, but was first identified in New Zealand in late 2013 [83] and in northern Tasmania in early 2014 [84].

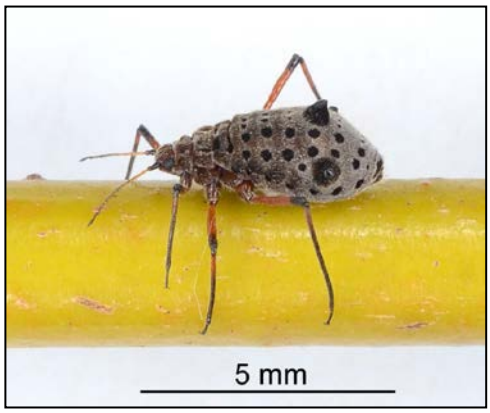

Figure 1-2: Giant Willow Aphid, T. salignus*, with scale, from reference [83].*

All species of willow studied by various researchers are affected by *T. salignus*. However, some willows can impair aphid breeding as a form of herbivory defense [85]. Climate factors may also affect the prevalence of *T. salignus* in an area, as the adult aphid typically appears in late summer and persists until late autumn [86]. Aphid infestation reduces the biomass of affected saplings, increases desiccation of new shoots, and increases overall water requirements, all of which can endanger the tree [87]. In New Zealand, willows are widely used in riparian or anti-flooding plantings and as livestock shelter trees, and to provide food for bees in early spring when usual floral sources are unavailable [44].

In feeding from the tree, *T. salignus* ingests sap containing water, sucrose, and proteins, and excretes a glucose, fructose, sucrose, and melezitose syrup [88] (honeydew) to maintain its osmotic equilibrium [44]. As discussed previously, melezitose is synthesized in the aphid's gut [22-23, 25], by enzymes native to the aphid [26, 89]. The sugar composition of aphid honeydew can alter in response to the needs of non-obligate symbionts such as ants [90], or to reduce the attractiveness of the aphid to predators such as some wasps [91-92], but in *T. salignus* and related aphids it is almost always high in melezitose [44, 83-84].

Honey made from *T. salignus* honeydew is popularly termed 'concrete honey', referring to its rapid-crystallization behavior. The high melezitose content of concrete honey reduces its saleable value by reducing the sweetness [35] and contributing to the unusual and generally-unacceptable taste produced by the salicylic and malic acids that are considered reliable chemical markers for willow aphid honeydew honey [44]. Melezitose-induced crystallization makes concrete honey unsaleable as liquid honey. Literature values for the solubility of melezitose in water vary between 50 and 781 mg/mL [93-95], so its solubility in honey is unpredictable, although it has been suggested that melezitose may begin to crystallize out of honey at a concentration of 14% or 140 mg/mL [96]. Examination of the crystal structure of melezitose monohydrate indicates that inter- and intramolecular hydrogen bonding is more extensive than that displayed in other trisaccharides studied, and the conformation of the Glc*p*[1→2]Fru*f* bond is similar to other low-solubility sugars [97]; these factors may contribute to the low solubility of melezitose in water.

Crystallization also occurs in the comb, as shown in Figure 1-3 and Figure 1-4, making concrete honey difficult or non-cost-effective to extract by usual methods, and reducing honey yield [44, 83-84, 98]. Some researchers consider high-melezitose honey a suitable overwintering food for bees, thereby reducing

expenditure on sugar syrups, but other recent research suggests that melezitose causes what some sources call 'dysentery' in bees [44, 99-101]. (As dysentery is caused by a bacterial infection, the deleterious effects of high melezitose consumption on bees is more properly ascribed to the noted laxative properties of large doses of oligosaccharides [102].)

To reduce or mitigate the effects of concrete honey on beehives, it has been suggested that beekeepers be alert for hot, dry weather in late summer through late autumn, which produces heightened honeydew production [44], and move beehives away from areas with high concentrations of willows in such weather.

Figure 1-3 (left): High-melezitose 'concrete honey' crystallized in the comb, from Swiss source [103].

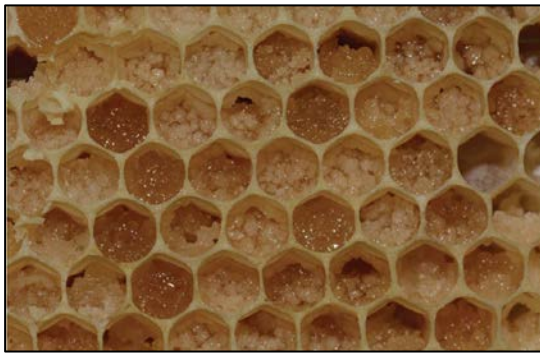

Figure 1-4 (right): High-melezitose 'concrete honey' crystallized in the comb, from NZ source [44].

1.5 Analysis of honey

1.5.1 Methods for analyzing honey oligosaccharides

Various methods have been used to separate and quantify the sugars in honey. In 1961, White *et al.* [104] used a selective adsorption method based on their 1959 method [105], using a charcoal/celite packed bench-top column eluted with aqueous ethanol to analyze Canadian clover honeys. Low *et al.* [6] in 1988 and Sporns *et al.* [106] in 1992 each analyzed a range of Canadian honeys by HPLC, both using amino-bonded stationary phases eluted with aqueous acetonitrile. Low *et al.* reported fructose and glucose as percentages of honey solids and

reported combined di- and oligosaccharides as a single figure. Sporns *et al.* reported detection of no higher sugars at all. Flores *et al.* [107] in 2014 used HPLC on a 4° ammonium functionalized ion column with an aqueous NaOH mobile phase to analyze Spanish eucalyptus honey. Makhloufi *et al.* [108], and Benaziza-Bouchema & Schweitzer [109], both in 2010, used HPLC on an ion exchange column to study Algerian eucalyptus honey.

Siddiqui and Furgala [75, 110], in 1967, analyzed Canadian clover/alfalfa honeys, by adsorbing the honey carbohydrates onto a charcoal slurry, washing off the quicker-desorbing monosaccharides until no traces of monosaccharides remained [75], fractionating, on a charcoal/celite column eluted with aqueous ethanol, the di- and oligosaccharides collected in later washings, and further fractionating the results by preparative paper chromatography. Their reported figures seem unusually low in comparison with other researchers' work, both later and earlier. Siddiqui and Furgala [75, 110] made no mention of yield calculations or tests in their method, and it seems likely that their method significantly underestimates the amount of di- and oligosaccharides present. Completely removing all traces of monosaccharides before collecting the disaccharide fraction, combined with the similar solubilities of the sugars in question, suggests that much of the disaccharide and possibly higher oligosaccharide compounds present may have been washed away before the fraction collection commenced.

In 2006, Morales *et al.* [111] revisited the charcoal slurry method of oligosaccharide extraction, varying concentrations of charcoal, ethanol, and water to determine relative recovery of oligosaccharides, and reported less than 70% recovery for any oligosaccharide in an artificial mixture. For sugars of fewer than five hexose units, recoveries as low as 5.1% were reported.

1.5.2 Chromatographic separation and quantification of sugars

High performance liquid chromatography (HPLC) can be used to separate sugars based on several attributes. An HPLC standard curve quantifies the separated sugars [112]. Trace- and semi-trace sugars may also be detected by HPLC [113].

Ion exclusion chromatography, using a stationary phase that repels the conjugate base of a dissociated acid so that low pK_a compounds elute first, is typically used to separate organic acids by their degree of dissociation in the eluent, [114]. Sugars in basic solution act as weak acids, and can be separated as acids [115]. An example is Ouchemoukh *et al*.'s separation of nine mono-, di-, and trisaccharides on a Carbopac PA1 (4 × 250 mm) anion-exchange column with pulsed amperometric detection and 0.5 mL/min gradient elution with ultrapure water/0.2 M NaOH [115]. Ouchemoukh *et al*. do not discuss the quality of the separation, but the chromatograms they provide show glucose and fructose not separated to baseline, and other sugars appearing just above baseline.

Size exclusion chromatography separates compounds by physical size, using a porous stationary phase by which smaller molecules are retained in the pores and elute later, and molecules larger than the pore size elute in the void volume [116]. Pore size is determined by the level of cross-linking in the polymer stationary phase. The Shodex KS-801 [117] is a size-exclusion/ligand-exchange column, used for sugars as a size-exclusion column, so larger molecules elute earlier. An example is Adams *et al*.'s separation of mānuka honey (sucrose, glucose, fructose, and another unidentified di- or oligosaccharide) on a Shodex KS-801 and KS802 in series, with Waters photodiode array and refractive index detection and 0.1 mL/min isocratic elution at 50 °C with HPLC grade water [118]. Unlike the higher molecular mass, less concentrated, earlier-eluting di- and

oligosaccharides, glucose and fructose are not resolved to baseline by this method.

1.5.3 Gas chromatography and mass spectrometry

Gas chromatography (GC) can also be used to separate sugars, but requires derivatization, typically per-*O*-methylation or per-*O*-trimethylsilylation, to make the sample volatile. Reducing sugars must also be reduced prior to derivatization for analysis for gas chromatography with mass spectrometry (GC-MS), to simplify the mass spectrum, which otherwise is complicated by different anomers and ring sizes arising from intramolecular rearrangements of unreduced sugars. Trimethylsilylation may be achieved using trimethylsilyl (TMS) azide [112-113], or Tri Sil HTP (a mixture of hexamethyldisilazane, trimethylchlorosilane, and pyridine) applied to reduced sugars [119-120]. An example is Chan *et al.*'s analysis of mānuka honey for flavonoids on an HP 6890 GC with a Phenomenex ZB-5 phenyl-methylsiloxane (30 m × 0.25 mm × 0.025 µm) column on a temperature gradient from 120 to 295 °C, over 27 minutes, and total ion chromatogram or selected ion monitoring modes [121]. This method did not separate low-concentration and trace compounds to baseline but was apparently adequate for quantitation. Another example is Ruiz-Matute *et al.*'s separation of trimethylsilyl derivatives of sugars in honey on an HP 6890 GC with a cross-linked methyl silicone (25 m × 0.25 mm × 0.25 µm) column on a temperature gradient from 200 to 300 °C, over 85 minutes, and Hewlett-Packard flame ionization detector or Agilent quadrupole mass analyzer detector [122]. This method resolved the major monosaccharides in honey to baseline and resolved the di- and trisaccharides present almost to baseline and without peak overlap, but was primarily focused on detection of honey adulteration, and identified only a limited number of tri- and tetrasaccharides.

Liquid chromatography with mass spectrometry (LC-MS) can be used instead of GC-MS for analysis of carbohydrates without requiring derivatization for volatility, but, as seen in Zhou *et al.*'s 2014 analysis, requires much higher carrier gas flow (9 L/min for electrospray ionization after liquid chromatography, cf. this analysis' 1.8 mL/min for gas chromatography), temperatures up to 350 °C, and potentials up to 3500 V to ensure ionization [123]. Shen & Perreault in 1998 used electrospray ionization after liquid chromatography of derivatized carbohydrates and required only 100 V ionization potential [124]; however, the need for derivatization outweighs the benefit of low-voltage ionization.

Determining the identity of a sugar from the electron impact mass spectrum of its trimethylsilyl ether is less than straightforward: large carbohydrates are too massive for a molecular ion to appear, and derivatized carbohydrates are generally too unstable to produce a distinct molecular ion [125]. Mass spectra of trimethylsilyl ethers of sugars can, however, be identified by comparison to standards.

1.5.4 Nuclear Magnetic Resonance spectroscopy sugar identification

Structural analysis of carbohydrates is complicated by the multiplicity of possible linkages: 38,016 distinct trisaccharides can potentially be made from combinations of three given hexoses [126]. Nuclear Magnetic Resonance spectroscopy (NMR) can be used to identify the stereochemistry of saccharides and determine linkages [127]. The relatively narrow spectral width in NMR, and similar chemical environments of nuclei involved, make 1D spectra hard to interpret, but 2D spectra can be used to assign non-anomeric carbons and protons [128]. HSQC and HMBC techniques are particularly useful for identifying sugar rings, as they can be more sensitive than 1D ^{13}C NMR [129]. Because oligosaccharides are important in biology, as cellular 'markers', significant attention has been paid to determining their conformation. For larger

oligosaccharides, paramagnetic lanthanide tags have been successfully used to produce a new peak for each CH group on a fully-^{13}C-labelled glucose and mannose dodecasaccharide, which were compared to analogous 2D NMR peaks of the untagged sugar to provide geometrical information about the conformation of ^{1}H and ^{13}C nuclei up to 40 Å distant from the lanthanide tag, and the conformation thus identified compared to a computer-simulated NMR spectrum [130-131]. However, such complex methods are not always necessary for the oligosaccharides considered here: identification of α and β anomers of glucose, galactose, and other sugars in which H-2 is axially disposed is possible due to the difference in ^{3}J coupling and Nuclear Overhauser Effect (NOE) enhancement of *gauche* and *anti* protons on the pyranose ring. ^{3}J coupling is greater, and NOE less, for *anti* protons, which reveals the relative orientation of OH groups and glycoside linkages [132]. Specifically, NOE enhancement of the signal of the axial C1 proton is reported to be significantly stronger for β anomers [133].

1.5.5 Bioassay for prebiotic activity

In vitro testing of compounds for prebiotic activity has been conducted with authentic samples of gut microflora in a liquid nutrient medium, and using an approximation of gut microflora (1:1 ratio of *E. aerofaciens* or *C. perfringens* with *Bifidobacteria* species), inoculated with whole honey rather than individual sugar fractions and with bacteria growth counted on agar [13, 134]. Populations of bifidobacteria (which are strict anaerobes [135]) and lactobacilli (some strains of which are oxygen-tolerant anaerobes, while others are partially aerobic [135-136]) are measured by filtered biomass before and after anaerobic incubation with samples of potential prebiotic compound [74]. Testing whether a given sugar inhibits growth of undesirable species can be accomplished by a standard well diffusion assay [137], typically with glucose as the control.

1.6 Research Aims

Detailed analysis of commercial prebiotic honeys to determine their composition for purposes of comparison, and of GWA honeydew honey for candidate prebiotic oligosaccharides, is both possible and potentially of great benefit to mitigate the effects on New Zealand apiary of the current GWA problem. This research set out to determine the carbohydrate compositions of Beeotic® (an Australian honey marketed as prebiotic), a sample of New Zealand beech honeydew honey, and a sample of GWA honeydew honey, to compare New Zealand GWA honeydew honey to a previously-quantified and better-known New Zealand honeydew honey, and to compare the potentially-prebiotic oligosaccharide content of both to a commercially-available 'prebiotic honey'. The acid and enzyme lability of the principal oligosaccharide of GWA honeydew honey were also determined, as a preliminary indication of its prebiotic potential.

2 Methods

2.1 Materials

Samples of Capilano Beeotic® honey (hereafter referred to as samples AH1-AH5) were purchased from a retail store in Australia and imported to New Zealand, arriving on February 28, 2018; they were stored in a refrigerator at 4 °C, and later in a freezer at -18 °C. Sample AH3 (manufacturer's batch number Q28.09.2018) was confirmed by HPLC to be sufficiently similar to the other samples to be considered representative of Beeotic® (as shown in Figure 3-2 and Appendix 1) and selected to use as a representative sample for sugar identification.

A sample of crystals filtered from a New Zealand honey believed by the supplier to contain Giant Willow Aphid honeydew honey (hereafter referred to as sample GWC) was provided by 1839 Honey Ltd. on May 6, 2019, and stored in a refrigerator at 4 °C.

A sample of New Zealand beech honeydew honey (hereafter referred to as sample HND) was provided by Honey New Zealand on November 28, 2018, and stored in the same refrigerator.

A sample of New Zealand Giant Willow Aphid honeydew honey (hereafter referred to as sample GWH), intact in the wax honeycomb, was scraped from frames provided by Honey New Zealand Ltd. on September 24, 2019, and stored in the same refrigerator.

Melezitose and 6-kestose standards previously isolated in this laboratory were confirmed by NMR before use (spectra shown in Appendix 2). Standards of sucrose, raffinose, and fructose, α-glucosidase from *Saccharomyces cerevisiae* (≥10 units/mg), and D_2O for NMR analysis, were obtained from Sigma-Aldrich; glucose, maltose, 250-grade apple pectin, xylitol, salicylic acid, and mannose

from BDH/AnalaR; cellobiose from Difco; and Blue Dextran from Pharmacia Fine Chemicals. An alternate sucrose standard used for GC-MS was obtained from a sealed sachet of Chelsea® White Sugar, as New Zealand food standards require anything sold as 'white sugar' to be ≥99.7% pure sucrose [138], cf. Sigma-Aldrich's ≥99.5% sucrose standard. This alternate sucrose standard was used to overcome a difficulty in completely dissolving the Sigma-Aldrich standard in *N*-trimethylsilylimidazole (TMSi); the sucrose was finely ground and held in a vacuum oven at 40 °C for two days to ensure dryness. Type 1 water for HPLC was made in-house. *N*-trimethylsilylimidazole for GC-MS derivatization was obtained from ThermoFisher Scientific. A Werther's Original™ cream candy to determine HMF concentration in commercially acceptable candy and a Vicks™ honey cough lozenge to determine the mass of a commercially acceptable para-medicinal candy were obtained from PAK'nSAVE Mill St., Hamilton, on October 27th, 2019.

2.2 Sample preparation

Dr. McLean's 'kitchen' method of separating honeycomb from GWH honey [139] was adapted to laboratory conditions: the GWH honey and wax honeycomb scraped from the frame were placed in a beaker in a heated dry bath, and stirred periodically until a thermometer submerged in the beaker read 75 °C and the honey-wax mixture became noticeably liquid. The dry bath was switched off, and the melted mixture removed to an oven (50 °C, overnight) to allow the liquid honey and solid wax to fully separate, before cooling to room temperature. Due to beaker size constraints, the honeycomb was processed in three 'batches'. The discrete top layer of solidified wax and other debris was scooped off and stored separately. The honey portion of each 'batch' was rewarmed to soften it, and the three GWH 'batches' were thoroughly mixed and

poured into jars. A portion of the GWH honeycomb was reserved, and the wax removed, outside the laboratory, so the honey could be tasted.

For quantitative analytical HPLC analysis, HND, GWH, AH3, and standards of sugars and acids were dissolved in Type 1 water (Appendix Table 2 and Appendix Table 3). For GC-MS analysis, HND, GWH, and AH3 were each dried in a vacuum oven (40 °C) and an ordinary oven (50 °C), for a total of three days, to reduce the water content. Because honey is a super-saturated sugar solution containing a not-irrelevant amount of di- and oligosaccharides, it is extremely hygroscopic and very difficult to dry, so complete drying was not attempted. Aliquots of the partially dried honey and the standards were fully dissolved in TMSi with a xylitol internal standard (Appendix Table 4) and held in a dry bath at 50 °C for one hour to derivatize before analysis. Samples were weighed out, derivatized, and tested, on the same day.

For preparative HPLC separation for sugar/acid identification, aliquots of AH3, HND, and GWH were dissolved in Type 1 water to give 1 g/mL solutions, and filtered through 0.45 µm filters before analysis.

Samples and standards for NMR were dissolved in D_2O (Appendix Table 1). The glucose standard was allowed to equilibrate in a water bath at 40 °C for three hours, and the fructose/glucose mixture standard in a water bath at 50 °C for one hour.

2.3 Giant Willow Aphid honeydew honey crystal identification

To identify whether the crystals present in sample GWC were melezitose, as Swiss, New Zealand, and German sources suggested [45, 98, 140-141], or glucose, the sample was thoroughly stirred. A spoonful of the crystals, still in the thin sticky honey matrix, was placed in a large vial and shaken with ice-cold Type 1 water, and the water drained off, leaving the crystals behind. This washing was

repeated until the water ran off clear. After a final wash with ice-cold ethanol, the crystals were vacuum-filtered, washed with more ice-cold ethanol, and dried overnight at 50 °C. Washed crystals (15.85 mg) were dissolved in D_2O (0.750 mL) and held in a waterbath (50 °C, 1 h) so that, if they were susceptible to mutarotation, the α and β forms could fully equilibrate before NMR analysis.

After the honey frames of sample GWH were scraped to remove the honeycomb and honey, fine white crystals of solid sugar crystallized out of the honey remained in the wells of the plastic frame, as shown in Figure 6-16 in section 6. The cleanest-looking of these crystals were scooped from the wells for analysis by NMR. The frame crystals were also analyzed for melting point on a Reichert Thermovar melting-point microscope.

2.4 Water content

The water contents of Beeotic® sample AH3, Giant Willow Aphid honeydew honey sample GWH, and New Zealand beech honeydew honey HND were measured using a Misco Palm Abbe digital refractometer calibrated with Type 1 water. Once the temperature of the sample had equilibrated (indicated by refractive index readings stabilizing), five stable readings were taken over five minutes, using the Honey Water % setting of the instrument.

2.5 High-Performance Liquid Chromatography (HPLC)

Some of the freeze-dried HND tetrasaccharide fraction **2a**, found by NMR to be tetrasaccharides α-D-Glc*p*(1→4)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→2)-β- D-Fru*f* and α-D-Glc*p*(1→6)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→2)-β-D-Fru*f*, was used for HPLC quantitation of the tetrasaccharide fraction in whole honey. HPLC standards, and quantitative solutions of HND, GWH, AH3, candy made from GWH (section 2.9), and a Werther's Original ™ cream candy, as detailed in Appendix Table 2, were eluted isocratically with Type 1 water at 0.8 mL/min on Shodex KS-801 and

KS-802 columns plumbed in series with a KS-G guard column, using a Waters 515 HPLC pump and 2414 RI detector, column oven temperature 80 °C, injection volume 20 µL, with data collection and oven temperature controlled using Empower 3 software. All samples of Beeotic® (including AH5 prior to its crystallization) were also analyzed on this system to confirm that AH3, which was chosen for preparative chromatography to identify sugars, was a representative sample; analytical chromatograms of Beeotic® samples AH1, AH2, AH4, and AH5 are included as Appendix 1.

Salicylic acid quantitative standards for Giant Willow Aphid honeydew honey (Appendix Table 3) were eluted on the same system, using a Waters 2996 PDA detector instead of the RI detector, with detection between 210-400 nm and 3D data collection. The quantitative honey solutions for sample GWH (Appendix Table 2) were also injected on this system to quantify salicylic acid in GWH. Analytical HPLC of sample AH3 was performed, for comparison, on a Waters Alliance e2695 HPLC system with KS-801 and KS-802 plumbed in series, a column temperature of 65 °C, flow rate 0.4 mL/min, and Waters 2414 RI detector, with injection volume 20 µL.

RI-detected chromatograms were processed using the default settings on the Empower 3 software, with the integration threshold adjusted to 10.00 area units to capture the very small early fractions. PDA-detected 3D chromatograms were sliced at 296 nm and the slice processed as for the 2D RI-detected chromatograms.

Preparative chromatography of AH3, HND, and GWH was initially eluted isocratically with Type 1 water at 1.400 mL/min on Shodex KS-2001 and KS-2002 columns plumbed in series, with the same pump and detector as used for analytical HPLC. The injection volume was 200 µL, and 12 injections were

performed. Fractions were collected in individual flasks, with a slight delay between fractions to reduce cross-contamination, and stored at -18 °C.

Fractions were reduced to ~1-2 mL on a rotary vacuum evaporator, and each fraction was re-injected for further separation. Second separation fractions were also collected, reduced in volume, and freeze-dried to remove residual water.

2.6 Nuclear Magnetic Resonance spectroscopy (NMR)

Freeze-dried AH3 preparative fractions, HND fractions **1**, **2**, and **2a**, GWH fractions **1**, **2**, **2a**, **2b**, **2c**, and **6**, the washed and filtered GWC crystals, and the GWH frame crystals, were analyzed by NMR on a Bruker Avance DRX400 FT-NMR 400 MHz spectrometer controlled with TopSpin 3.5 pl7 software. Samples were dissolved in D_2O for NMR analysis (Appendix Table 1). ^{13}C and ^{1}H spectra were acquired and compared to NMR spectra of pure standards for identification of sugars.

2.7 Gas Chromatography with Mass Spectrometry (GC-MS)

GC-MS was used for supplementary identification and quantitation of individual disaccharides and trisaccharides in AH3 (fractions **2b** and **3**), HND (fractions **2** and **3**), and GWH (fractions **2a**, **2b**, and **3**). Samples, and standards for retention time and quantitation, were prepared (Appendix Table 4); all were dissolved in *N*-trimethylsilylimidazole (TMSi) silylating agent. The whole honey samples were weighed out of the aliquots of honey that were vacuum-dried as discussed in section 2.2. Dissolved samples were placed in a dry bath to derivatize (50 °C, 1 hour) before analysis, and stored at -18 °C when not in use.

GC-MS was carried out using an Agilent 7890B GC interfaced with an Agilent 7000D triple-quadrupole mass spectrometer and PAL autosampler. The derivatized solution (1.5 µL/injection) was injected at 250 °C onto a 29.8 m × 0.25 mm × 0.25 µm Agilent J&W HP-5ms Ultra Inert column. The carrier gas was

helium at 1.8 mL/min. The oven temperature program was 55 °C (1 min) + 30 °C/min to 150 °C + 10 °C/min to 300 °C and held at 300 °C for 40 min to ensure complete elution. The instrument was controlled with Agilent MassHunter GC-MS Acquisition B.07.06 software. In data processing, chromatograms were smoothed, integrated, and the peak mass spectra extracted using the default settings and an integration peak height limit of 50,000 counts, in the Agilent MassHunter Qualitative Analysis Navigator B.08.00 software.

2.8 Calculations used in HPLC and GC-MS quantitation of sugars

2.8.1 HPLC

Standard calibration curves were constructed using analytical HPLC, with either three or four standards of different known concentrations (Appendix Table 2 and Appendix Table 3). The calibration curves were linear and constrained to pass through the point (0 mg/mL, 0 peak area), and are included as Appendix 3. The equation and coefficient of determination of the calibration curve for each compound are also included in the tables of HPLC standards in Appendix 5. The quantitation calculations for AH3 fractions **1a**, **1b**, **4**, and **5**, HND fractions **1**, **4**, and **5**, and GWH fractions **1**, **4**, and **5** (fractions consisting of a single compound) were performed according to Equation 2-1 and Equation 2-3. The calculations for AH3 fractions **2b** and **3**, HND fractions **2, 2a, 2b**, and **3**, and GWH fractions **2, 2a, 2b**, and **3** (fractions containing several compounds) were performed according to Equation 2-2 and Equation 2-3. The Excel AVERAGE function was used to calculate averages, and STDEV.S for standard deviations. The uncertainty in the concentration of each sugar or fraction in the whole honey was calculated using Excel's CONFIDENCE.T function, using *alpha* = 0.05, *standard_dev* = the standard deviation of the concentrations calculated for each of the HPLC quantitative runs, and *size* = the number of quantitative runs,

multiplied by the number of different sugar standards used in quantifying the fraction.

To calculate the concentration of a specific sugar or acid in the solution of whole honey in water, Equation 2-1 was used:

$$compound\ concentration\ in\ honey\ solution\ (mg/mL) = \frac{A}{m} \qquad \textit{Equation 2-1}$$

where *A* is the area of the fraction peak and *m* the gradient of the relevant calibration curve. To calculate the concentration of total disaccharides and total trisaccharides (fractions 3 and 2, respectively) in the solution of whole honey in water, Equation 2-2 was used:

$$compound\ concentration\ in\ honey\ solution\ (mg/mL) = \left(\frac{A}{m_{sugar_1}} + \cdots + \frac{A}{m_{sugar_n}}\right) \div n \qquad \textit{Equation 2-2}$$

where *A* is the area of the fraction peak, m_{sugarx} the gradient of the *x*th concentration curve, *n* the number of different sugar standards used, and the sugar standards are raffinose, kestose and melezitose (for estimating trisaccharides) and sucrose, maltose, and cellobiose (for estimating disaccharides).

To determine the concentration of each sugar in the whole honey, the concentration of that sugar in the known whole-honey-in-water solution used for quantitation was divided by the known concentration of the solution in mg/mL, as in Equation 2-3:

$$\begin{aligned} &compound\ concentration\ in\ honey\ (mg\ per\ mg\ honey) \\ &\quad = compound\ concentration\ in\ honey\ solution\ (mg/mL) \\ &\quad \div honey\ concentration\ in\ honey\ solution\ (mg\ honey/mL) \end{aligned} \qquad \textit{Equation 2-3}$$

where *compound concentration in honey solution* is the concentration of the sugar or acid in question in the quantitative whole-honey-in-water solution, given by Equation 2-1 or Equation 2-2; *honey concentration in honey solution* is the concentration of whole honey in the solution, and *compound concentration in honey* is the concentration of the sugar or acid in question in the whole honey.

2.8.2 GC-MS

GC-MS quantitation was applied to the disaccharide and trisaccharide fractions of AH3, HND, and GWH, to supplement and compare to the HPLC quantitation. Response factor curves, included as Appendix 4, were constructed with standards run on the same GC-MS method as was used for the samples. The compositions of the standards are given in Appendix Table 4.

Xylitol was chosen as an internal standard because it did not appear in the qualitative GC-MS runs of AH3, elutes sufficiently earlier than the sugars present in the sample to preclude confusion, and was available. The GC-MS response factor for each sugar standard was determined by Equation 2-4, where *peak area sugar* is the GC-MS peak area of the sugar standard, *peak area xylitol* is the peak area of the xylitol internal standard, and *mg sugar* and *mg xylitol* are the masses of those standards dissolved and derivatized in the TMSi derivatizing agent used.

$$Response\ factor = \frac{\left(\frac{peak\ area\ sugar}{peak\ area\ xylitol}\right)}{\left(\frac{mg\ sugar}{mg\ xylitol}\right)} \qquad \textit{Equation 2-4}$$

The peak area ratios and mass ratios for each sugar/xylitol standard were plotted (included as Appendix 4) and the response factor (RF) for each sugar was given by the gradient of the relevant trend line and recorded in Appendix Table 4.

The initial and final masses and water contents of the aliquots of AH3, HND, and GWH that were dried in a vacuum oven ahead of silylation for GC-MS analysis are recorded in the *Water Content* sections of the relevant chapters (3.2.1, 4.1.1, and 6.2.2, respectively). It is possible that the mass loss on drying also included some of the volatiles present in honey; however, as these are typically present only in trace amounts, the mass decrease can be treated as entirely due to water loss.

The GC-MS quantitation calculations for fractions 2 and 3 were performed according to Equation 2-5 and Equation 2-6, using the response factors of sucrose, maltose, and cellobiose as RF_1 – RF_3 for disaccharides, and those of raffinose, kestose and melezitose as RF_1 – RF_3 for trisaccharides. To estimate the quantities of disaccharides and trisaccharides present in AH3, HND, and GWH, the total peak area for each fraction was summed, and used as *A*, the area of the sample fraction peak, in Equation 2-5, where *n* is the number of different sugar standards used.

The sucrose, maltose, kestose, melezitose, raffinose, and cellobiose standard response factor curves shown in Appendix 4 were each used individually in Equation 2-5 (with *n*=1) and Equation 2-6, to determine the quantity of that sugar present in each honey.

$$sugar\ mass\ in\ dried\ honey = \left(\frac{(A)(mass\ xyl.)(RF_1)}{peak\ area\ xyl.} + \cdots + \frac{(A)(mass\ xyl.)(RF_n)}{peak\ area\ xyl.}\right) \div n$$

Equation 2-5

$$sugar\ concentration\ in\ whole\ honey\ (\%) = \frac{mass\ sugar\ in\ dried\ honey}{mass\ dried\ honey} \times \frac{mass\ dried\ honey}{mass\ whole\ honey}$$

Equation 2-6

2.9 Making candy from Giant Willow Aphid honeydew honey

Three different recipes for making candy consisting entirely of honey were identified [142-144], and the common elements extracted and combined to synthesize a generic honey-candy recipe. GWH (100 mL) was heated in a metal pan over a hot plate (250 °C) and stirred constantly. When the honey candy reached 'hard crack' stage (a drop of boiling honey placed in ice-water immediately formed a hard ball), it was taken off the heat, poured into a dish greased with rice bran oil, and allowed to cool to a point where it could safely be handled, but was still flexible. The cooled candy was taffy-pulled into a rope and cut into pieces. Some pieces were placed in a desiccator under vacuum, and others were placed in a beaker that was left open to the atmosphere but closed from dust. The condition of the pieces was checked after a week.

2.10 Separating melezitose crystals from Giant Willow Aphid honeydew honey sample GWH

Several strategies for separating the solid crystals from Giant Willow Aphid honeydew honey sample GWH were explored. These all initially exploited the property of the crystals to sink rapidly to the bottom of an aliquot when the honey was exposed to heat, and the viscosity of the liquid portion was reduced, as observed in Figure 6-5 in section 6.2.1. An aluminum tube, for good heat conductivity with minimal weight, with a narrow bore to maximize vertical separation of a small aliquot, was chosen and cut to a convenient length for laboratory use. A short length of one end of the tube had been bored out further, allowing it to accommodate a rubber stopper. A glass rod was chosen as a plunger, and the diameter of one end adjusted with Parafilm to ensure a good seal in the aluminum tube. Figure 2-1 is a schematic illustrating the operation of this apparatus.

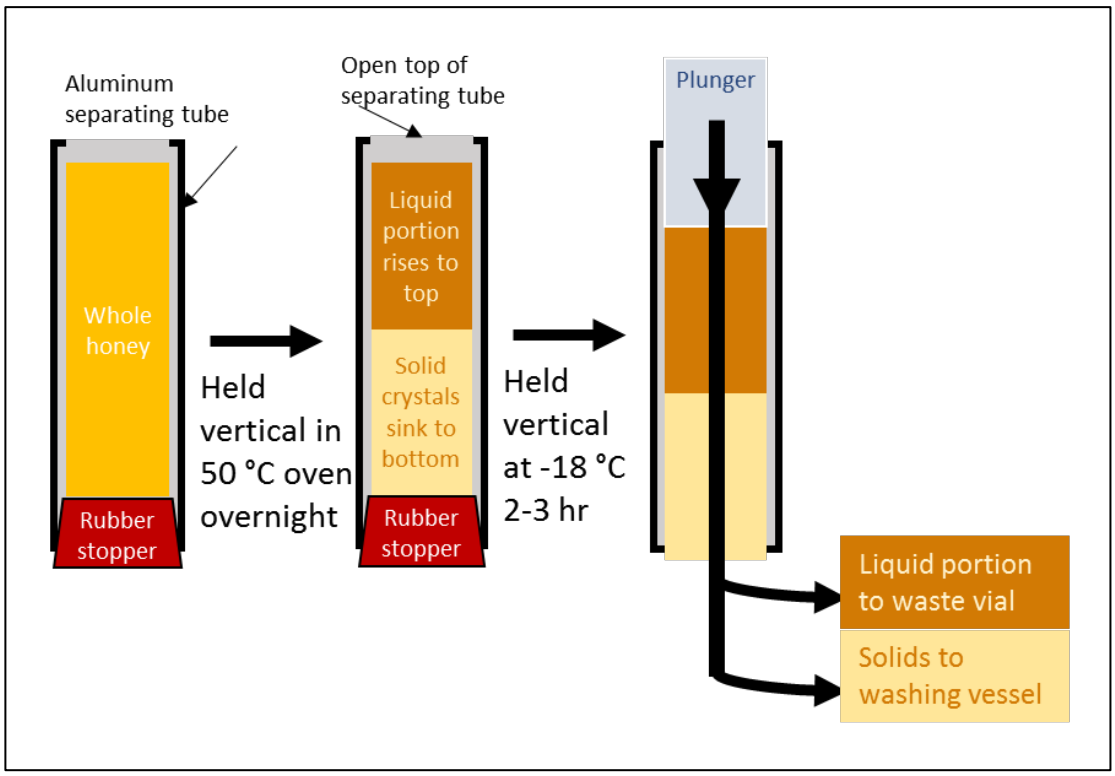

Figure 2-1: Illustration of initial separating apparatus for GWH crystals: honey is weighed into the stoppered aluminum tube, and held vertical at 50 °C overnight to allow the crystals to settle, then frozen 'solid' to reduce remixing of the settled crystal layer. The stopper is removed, and a plunger used to force the settled crystal portion out into a collecting vessel, and the liquid portion into a different vessel.

Seven individual samples of GWH were separated, the latter four in this apparatus, and are referred to as Aliquots 1-7.

2.10.1 GWH separation Aliquot 1

Honey (5.01939 g) was transferred from a thoroughly-stirred GWH jar to a vial and placed in an oven (70 °C, 21.5 h). The liquid upper portion was poured off, and the settled crystals washed with ice-cold Type 1 water and placed in the refrigerator (4 °C) to settle. The supernatant was drawn off.

2.10.2 GWH separation Aliquot 2

Honey (5.72923 g) was transferred from a thoroughly-stirred GWH jar to a vial and placed in an oven (70 °C, 21 h). The liquid upper portion was poured off, and the settled crystals shaken with ice-cold ethanol.

2.10.3 GWH separation Aliquot 3

Honey (6.05009 g) was transferred from a thoroughly-stirred GWH jar to a vial and placed in an oven (70 °C, 21 h). The liquid upper portion was drawn off and the settled crystals chilled in a freezer (-18 °C), vortexed with Type 1 water (0.500 mL) until fully suspended, and placed in a refrigerator (4 °C) to settle. The supernatant was drawn off, and the crystals suspended in ice-cold ethanol. This suspension was filtered on a Büchner funnel, and the residue placed in an oven (50 °C) to dry. The dry residue/crystals were placed in a vial and redissolved in boiling Type 1 water (12 drops), with vigorous and frequent vortexing, and holding in a waterbath at 100 °C. The vial of dissolved Aliquot 3 was heavily insulated with cotton wool, and allowed to cool slowly first at room temperature and then at -18 °C. The recrystallized Aliquot 3 was suspended in ice-cold ethanol, filtered, washed with ice-cold ethanol, and dried in an oven at 50 °C.

2.10.4 GWH separation Aliquot 4

The aluminum separation apparatus shown in Figure 2-1 was filled with thoroughly-stirred GWH and held vertical in an oven (50 °C, 15 h). The settled crystals were collected in a vial, shaken with ~⅓ their volume of ice-cold Type 1 water, and placed in a freezer (-18 °C) to settle. The liquid was poured off, and the same amount of ice-cold water added again. The vial was heated in a dry bath to 60 °C until the crystals were dissolved, and then heavily insulated and placed back in the freezer to cool.

2.10.5 GWH separation Aliquot 5

Thoroughly-stirred GWH (16.21262 g) was placed in the separating apparatus and held vertical in an oven (50 °C, 17.5 h), then removed to a freezer (-18 °C). The settled crystals were collected in a vial. Type 1 water (1.000 mL) was added, and the vial shaken until the crystals were suspended, then allowed to settle. The supernatant was drawn off, and another 1.000 mL water added. The crystals

were allowed to settle in a refrigerator (4 °C), and the supernatant poured off. The crystals were suspended in ice-cold ethanol (2.000 mL), filtered, washed with ice-cold ethanol, and dried in an oven at 50 °C.

2.10.6 GWH separation Aliquot 6

Thoroughly-stirred GWH (16.69499 g) was placed in the separating apparatus and held vertical (50 °C, overnight), then removed to a freezer (-18 °C) to chill. The settled crystals (4.64777 g) were collected in a vial and placed back in the oven (50 °C) to allow the residual liquid honey to rise, before cooling in the freezer. The contents were suspended in Type 1 water (1.000 mL), allowed to settle in the refrigerator, and the supernatant drawn off. The settled crystals were suspended in ethanol, filtered, washed with ice-cold ethanol, and dried in an oven at 50 °C. Aliquot 6 was sub-sampled (0.31220 g) at this point, and the sub-sample shaken with methanol (1.000 mL), filtered, washed with methanol, and dried in an oven at 50 °C.

2.10.7 GWH separation Aliquot 7

Thoroughly-stirred GWH (18.87795 g) was placed in the separating apparatus and held vertical (50 °C, 16 h), then removed to a freezer (-18 °C) to chill. The settled crystals (10.81383 g) were collected in a conical flask placed on a magnetic stir plate, and Type 1 water (8.0 mL) was added, with stirring, until the mixture became visibly clear and no solid crystals remained. Ethanol (103.45 mL) was slowly added at the edge of the stirring vortex until a precipitate appeared. The mixture was filtered into a clean Büchner flask, and the residue washed with ethanol (8.0 mL) and dried in an oven at 50 °C.

2.11 Acid and enzyme lability of melezitose crystals isolated from Giant Willow Aphid honeydew honey

To determine the acid lability of melezitose, and thus its likelihood to be hydrolyzed in the human stomach, a method was adapted from Skinner (2001) [145]. HCl (0.0024 M, pH 2.62), was used to approximate stomach acid [146]. GWH separation Aliquot 6 was sub-sampled for triplicate acid digestion for 18 hours, 4.5 hours, and a control sample with no acid or incubation. Masses of samples and volumes of acid and water used are detailed in Table 7-2 in section 7.3. Digestion solutions were neutralized with NaOH (0.1 M) and quantified by HPLC, using the conditions and standard calibration curves discussed in section 2.5 and Appendix 3.

To determine the enzyme lability of melezitose, and thus its likelihood to be hydrolyzed in the human small intestine, a method was adapted from Granados-Guzmán *et al*. [147] to allow an incubation time appropriate for simulation of small intestine digestion [148]. Sucrose was used as the control, to confirm that the α-glucosidase used was capable of hydrolyzing digestible sugars. For the control, α-glucosidase (55 units/mL) and sucrose (0.5 mg/mL) were dissolved in 5.000 mL of Type 1 water, and the pH adjusted to 6 (approximate average pH of the small intestine [149]) with very dilute NaOH as required. For the melezitose digestion, α-glucosidase (55 units/mL) and melezitose (0.78 mg/mL) were dissolved in 5.000 mL of Type 1 water. pH adjustment was not required. For both sucrose and melezitose, the vials containing the digestion solutions were placed in a dry bath with a water-filled dummy vial to monitor the actual temperature of the solutions, and held at 37 °C for 4.5 hours, before being heated to 90 °C and held at that temperature for 30 minutes to deactivate the enzyme. Masses of samples and enzyme and volumes of water used are detailed in Table 7-2 in section 7.3. Digestion

solutions were quantified by HPLC, using the conditions and standard calibration curves discussed in section 2.5 and Appendix 3.

3 Prebiotic honey in an Australasian context (sample AH3)

3.1 Introduction: Food standards and prebiotic honey

3.1.1 Legal requirements to claim a food has prebiotic activity

In Australia and New Zealand, the health claims ('a claim which states, suggests or implies that a food or a property of food has, or may have, a health effect' [150]) that may be made about foods are limited by Standard 1.2.7 of the Australia New Zealand Food Standards Code. Prebiotic activity is considered a general level health claim under this standard, because it is a health claim that does not 'refer to a serious disease or a biomarker of a serious disease' [150]. In order to make a general level health claim in Australia or New Zealand, a foodstuff must fulfil a 'Condition for permitted general level health claims' [151] in S4–5 of Schedule 4 of the Australia New Zealand Food Standards Code. Prebiotic claims are not included in Schedule 4. Alternatively, a foodstuff may make a general level health claim under Standard 1.2.7–18 subparagraph (3)(b), and 1.2.7–19 [152], by satisfying the systematic review requirements presented in Schedule 6. These requirements include an *in vivo* study, and, importantly, a consideration of whether the foodstuff in question, in the amount necessary to substantiate the health claim, is likely to be consumed as part of a normal diet [153].

3.1.2 Research that led to the marketing of an Australian honey as a prebiotic functional food

In September 2016 [154], Australian honey company Capilano launched their new product Beeotic®, a "clinically tested prebiotic honey" [155]. The product page on Capilano's website cited a "clinical study conducted by Conway *et al*. in 2012-13, funded by Rural Industries Research & Development Corporation" [155]. Reference to this "clinical study" was removed from Capilano's Beeotic® web page by February 7, 2018 [156]. In interviews given to Nutritional Outlook and Nutrition Insight magazines in 2016 and Bee Culture magazine in 2018, Capilano

spokespeople said Beeotic® is "guaranteed to have a minimum level of naturally occurring prebiotics" [157], "tested… to guarantee its prebiotic content" [158] and each batch is tested for "sugar profiles" [157, 159] to ensure this. The 2018 interview also stated that research leading to the production of Beeotic® began in the late 2000s, "with the first clinical study with UNSW [the University of New South Wales] in 2012" [158].

No published study matching this description has been found.

Capilano filed an entry for Beeotic® with the Australian Register of Therapeutic Goods, on August 22, 2018. This entry claims that Beeotic® is a medicine, and permits its labeling and marketing within Australia to indicate that it has therapeutic uses to "maintain/support general health and wellbeing", "decrease/reduce/relieve throat irritation", and "maintain/support wound healing" [160]. Coinciding with this filing, reference to Beeotic® as a prebiotic was removed from the Australian version of Capilano's Beeotic® web page by September 3, 2018 [161]. As of April 27, 2020, Beeotic®'s claim of prebiotic activity remained on the US version of Capilano's Beeotic® web page [162], and Beeotic® remained available for sale in America through Walmart, where it was described as "the world's first clinically tested honey with naturally occurring prebiotic oligosaccarides [sic]… that helps support a healthy microflora balance" [163-164]. As of April 29, 2020, it was also available internationally through Amazon.com, with the same description as used by Walmart [165]. All reference to Beeotic® had been removed from the Australian version of Capilano's website by March 30, 2020.)

A thesis submitted at the University of New South Wales in 2015, and supervised by Conway, was released from embargo in September 2019. This thesis stated that oligosaccharides that are present in certain Australian floral honeys, and

survive human digestion simulations, affect the bacterial composition of colonic microbiota. It also stated that an increase in some potentially beneficial gut bacteria could be attributed to the fructose content of these honeys [166]. Part of the research in this thesis was also presented at the 2013 Apimondia Congress [167]. Fructose, however, is a readily-assimilable monosaccharide that, in humans, is absorbed before reaching the large intestine, and thus would be unlikely to encounter potentially beneficial bacteria in the large intestine.

A report by Dawes *et al.* (2014) [168], published by the Rural Industries Research & Development Corporation (RIRDC), reported an *in vitro* trial of some Australian honeys to examine their prebiotic potential. The trial was conducted by Conway, who was at the time working for ProBiOz Ltd., and funded by the RIRDC. The Dawes report, which names Conway as a contributing researcher, describes a study of Australian monofloral eucalyptus honeys, and reports that oligosaccharides present in these honeys survived a digestion simulation, suggesting that honey oligosaccharides might fulfil the prebiotic criterion of human indigestibility. The report also recognized that the high sugar content of honey makes it difficult to plausibly market honey products as quasi- or para-medicinal and recommended the seeking out of an independent not-for-profit expert endorsement of Australian eucalyptus honeys as prebiotics.

The Dawes report follows on from a 2010 report by Conway, also published by the RIRDC [169]. This earlier Conway report identified honey from ironbark and stringybark eucalypts, and the non-eucalypts leatherwood and banksia, as possible prebiotic functional foods. Conway's 2010 report referenced papers by Persano Oddo (1995) [170] and Weston (1999) [42] as saying that honey has a high content of potentially prebiotic oligosaccharides. However, the Persano Oddo and Weston papers identify only honeydew honeys as containing appreciable quantities of potentially prebiotic oligosaccharides, and do not include reference

to the carbohydrate content of the Australian floral honeys considered in Conway's 2010 report.

3.1.3 Potential issues with Beeotic® as a prebiotic functional food

Conway's 2010 report [169], on which Beeotic®'s prebiotic claim is based, focused on Australian leatherbark, stringybark, and banksia honey, for which no peer-reviewed articles describing oligosaccharide composition studies are available. The floral or honeydew origin of Beeotic® is not stated on the packaging of the retail-purchased samples used for this research, or on Capilano's website or other online marketing.

As the trial methodology only determined whether the oligosaccharide fraction displays prebiotic behavior, and not whether the whole honey may be considered prebiotic, a case could be made for honeys intended for market as prebiotic functional foods to undergo quantitative analysis to determine the amount of potentially prebiotic oligosaccharides present per unit of honey.

Beeotic®'s tagline, "the honey your tummy will love" [162], is somewhat disingenuous, since the health benefits of genuine prebiotics are due to selective fermentation of oligosaccharides in the colon, and a key criterion of prebiotic foodstuffs is their indigestibility in the stomach and small intestine [5].

3.2 Results and discussion

3.2.1 Water content and fraction numbering

The water content of AH3 was 17.3%, which suggests nothing in particular about its floral or honeydew origin, as blossom honeys typically contain 15-20% water [8], with 16-18% most usual [8, 15, 171], cf. honeydew honeys, which typically contain 13-18% water [15, 171]. 1327.46 mg of whole honey was weighed out to dry for GC-MS analysis; after drying, this aliquot weighed 1156.66 mg, so the water content of the dried honey was 4.4%.

For GC-MS, all sugars used were derivatized as their unreduced per-*O*-trimethylsilyl ethers; in description of GC-MS results, the name of a sugar implies the per-*O*-trimethylsilyl ether of that sugar. For qualitative analysis of fractions **2b** and **3**, the concentrated, freeze-dried, preparative HPLC fraction was used. For quantitative analysis, whole honey dried as described in section 2.2 was used.

Figure 3-1 shows a typical preparative HPLC trace of Beeotic® sample AH3 and introduces the numbering system used to identify its fractions throughout this chapter.

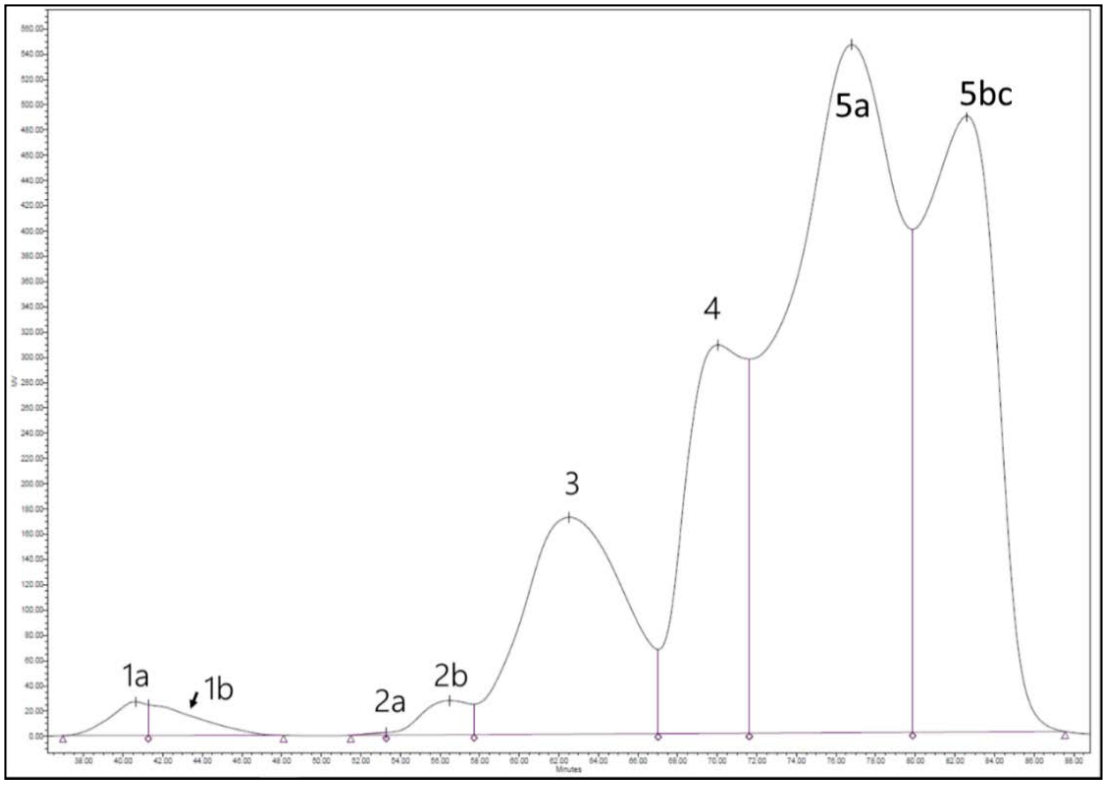

*Figure 3-1: Preparative HPLC chromatogram of Beeotic® honey AH3 (Shodex KS-G, KS-2001, and KS-2002 columns plumbed in series and eluted in Type 1 water at 1.4 mL/min at room temperature, with RI detection), showing fraction divisions with the numbering system used throughout. Fraction **5** appeared as a single peak on analytical HPLC (Figure 3-2), and as two peaks, **5a** and **5bc**, on preparative HPLC. **5bc** split into two peaks, **5b** and **5c** (not shown), when the fraction was re-injected for further separation.*

Figure 3-2 shows the analytical HPLC trace of Beeotic® honey AH3 that was used for quantitation of fractions. The other Beeotic® samples were

chromatographically identical with respect to their sugar composition, as shown by a comparison of their HPLC chromatograms (presented as Appendix 1) to that of AH3.

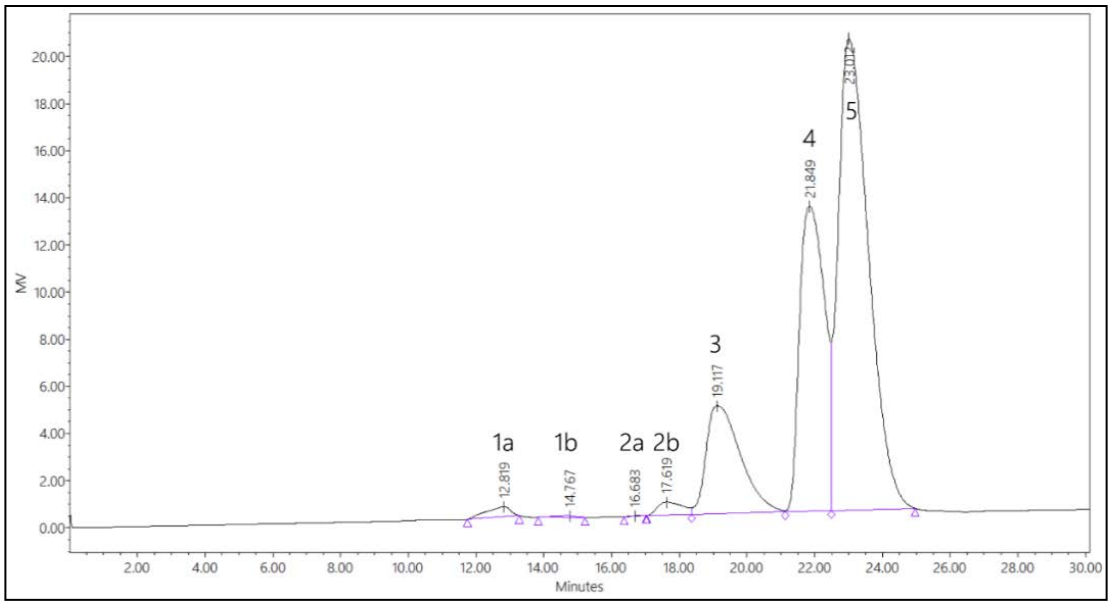

*Figure 3-2: Analytical HPLC trace of AH3 (Shodex KS-G, KS-801, and KS-802 columns plumbed in series and eluted in Type 1 water at 0.8 mL/min at 80 °C, with RI detection), identifying peaks with the fraction divisions introduced in Figure 3-1. On analytical HPLC, fraction **5** appears as a single peak, whereas on preparative HPLC it is split into **5a** and **5bc**.*

3.2.2 Fraction 1a

Figure 3-3 shows the ^{13}C NMR spectrum of **1a**. On preparative HPLC, there was some overlap with **1b**, leading to contamination of **1a** with **1b**; the **1b** signals are indicated with arrows in Figure 3-3. The pattern of the upfield signals, with a close cluster of a few intense peaks upfield representing –$\underline{C}H_2OH$ branches and a broader cluster of intense peaks slightly further down representing ring -$\underline{C}$HOH- groups (the latter expanded in the inset), and a discrete downfield group of less intense peaks representing anomeric or linking carbon atoms, resembles that of available oligosaccharide standards kestose and melezitose, but does not match either of them exactly.

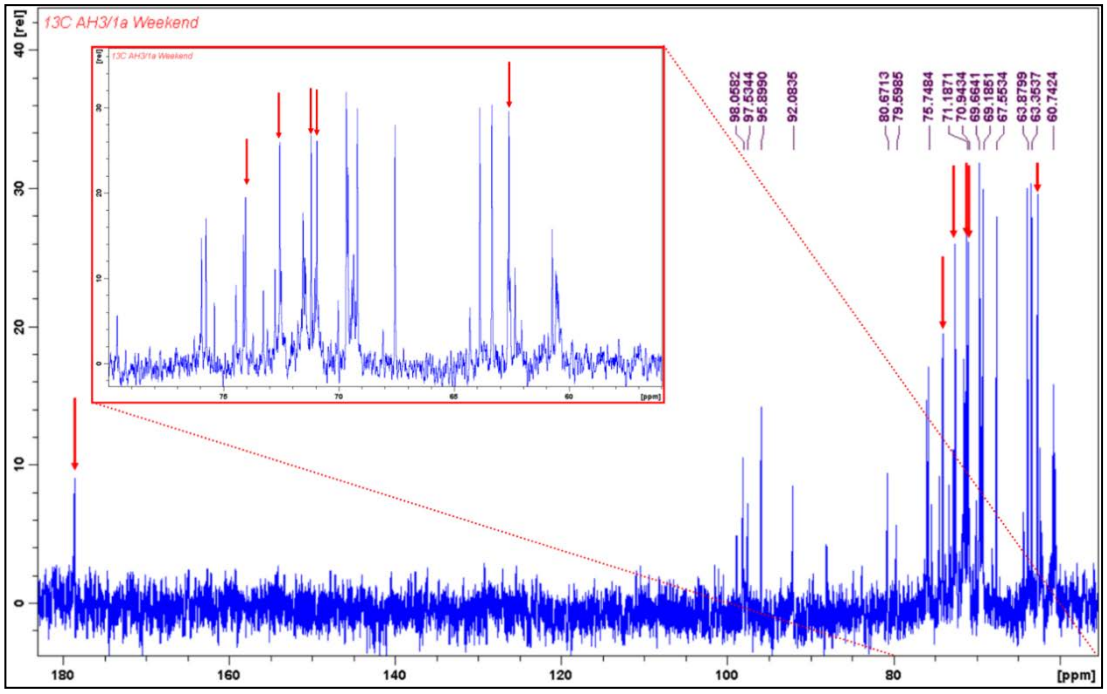

*Figure 3-3: ^{13}C NMR spectrum of AH3 fraction **1a**. D-gluconic acid signals arising from HPLC peak overlap with AH3/**1b**, identified by comparison with a standard, are marked with arrows. Inset: an expansion of the upfield portion of the spectrum, 60-80 ppm. The remaining signals show a pattern similar to large oligosaccharides, confirmed by comparison to ^{13}C spectra of melezitose and kestose (the latter shown with fraction AH3/**2b** in Figure 3-9).*

KS-series HPLC columns operate in dual size-exclusion and ligand-exchange modes, with KS-801 excluding molecules >1,000 daltons, and KS-802 excluding molecules >10,000 daltons [172-173]. As the molecular mass of a linked hexose unit is 162 daltons, the HPLC setup used for this study excludes hexasaccharides and larger sugars. The ligand-exchange aspect of these columns excludes charged species, including aqueous acids, so D-gluconic acid is excluded from the column and appears at the beginning of the chromatogram; if the column were size-exclusion only, D-gluconic acid would elute near glucose, among the monosaccharides. Compounds excluded from the column to elute at the start of the chromatogram are thus those that are larger than or equal to hexasaccharides, charged, or both. 1 mg/mL retention-time standards of Blue Dextran 2000 (mw = 2,000,000 daltons, charge-neutral glucose polymer excluded from both KS-801 and KS-802) and apple pectin (mw >10,000 daltons,

charged galacturonic acid polymer excluded from both KS-801 and KS-802) were compared to **1a** to help determine its likely properties. **1a** elutes (on average over the five Beeotic® samples tested) at 12.8 ± 0.04 minutes, Blue Dextran at 10.56 minutes, and apple pectin at 12.05 minutes. For the KS-series columns used, then, it appears that a very large molecular weight has more effect on retention time than a high charge, and the unknown fraction **1a** is likely a charged species, as it elutes closer to the charged-species retention time standard than to the large-molecule retention time standard. Pectin was used as a quantitation standard to approximate the amount of unidentified large or charged compound **1a** in sample AH3.

The literature studies found to refer charged species in honey chiefly examined amino acids, some of which retain their charge at typical honey pH ranges. These studies reported a wide range of possible amino acid concentrations: 0.0014 ± 0.0017% in floral honeys [174], 0.11 ± 0.021% in Hungarian honeydew honeys [175], 0.060 ±0.010% in floral honeys, [176]. However, identifying **1a** as consisting of amino acids does not explain why it appears to comprise >1% of the mass of AH3, an order of magnitude higher than the most liberal estimates of amino acids in honey, or why it does not appear at all in the honeydew honeys studied in chapters 4 and 6, despite honeydew honeys generally having higher amino acid contents than floral honeys [175].

Heterogeneous saccharide oligomers incorporating various proportions of galactouronic acid units are also not unknown in nature, and occur in various fruits (as pectin) and notably in alhagi honey, which is not honey, but a plant honeydew excreted by *Alhagi* species in Persia and southern Central Asia [177-178]; such polysaccharides would, however, be unusual in honey, especially at the concentration at which AH3 fraction **1a** is present.

D-gluconic acid, the major acid in honey and identified in section 3.2.1 as AH3 fraction **1b**, exists as both an open-chain acid and a lactone, in equilibrium (Figure 3-4). This equilibrium is pH-independent between pH 3 and 5 [179-180] (cf. typical honey pH range 3.2-4.5 [181]). Presence of another charged species like **1a** might affect the pH, but, given its low concentration, should not have an effect sufficient to alter the D-gluconic acid-lactone equilibrium. It appears that some property of **1a** retards elution of D-gluconic acid from the Shodex KS columns used, because in other honey samples (New Zealand beech honeydew honey HND and Giant Willow Aphid honeydew honey GWH, chapters 4 and 6 respectively), D-gluconic acid eluted at or near 12.8 minutes (cf. 14.6 ± 0.14 min in AH3), and the compound or compounds comprising AH3 fraction **1a** were not present. Ultimately, **1a** remains unidentified.

3.2.3 Fraction 1b

Figure 3-5 shows ^{13}C NMR spectra comparing **1b** to a standard of equilibrated D-gluconic acid; as the spectra are almost identical, this fraction can be confidently identified as D-gluconic acid. As additional confirmation, the chemical shifts of the peaks also correspond to literature values for D-gluconic acid [182]. D-gluconic acid, Figure 3-4, is formed from glucose by a glucose oxidase, and is a major honey acid, comprising up to 0.6% of floral honeys or 1% of honeydew honeys [10, 17, 181].

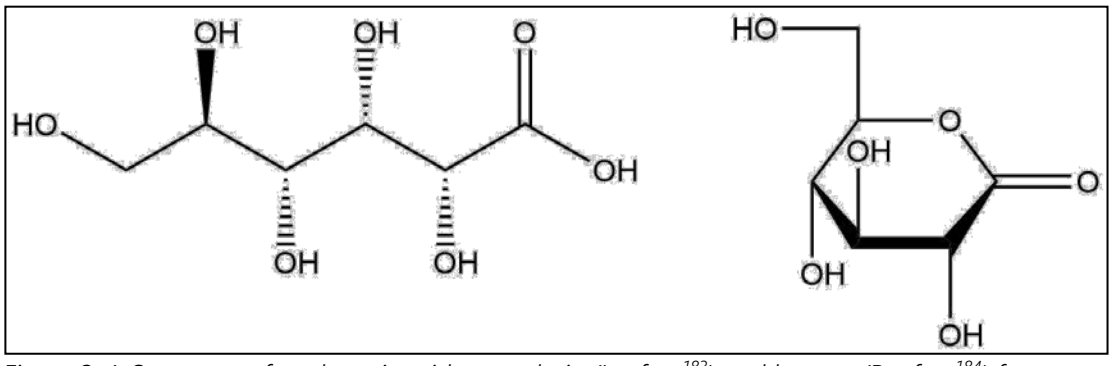

Figure 3-4: Structures of D-gluconic acid open-chain (L, after [183]) and lactone (R, after [184]) forms.

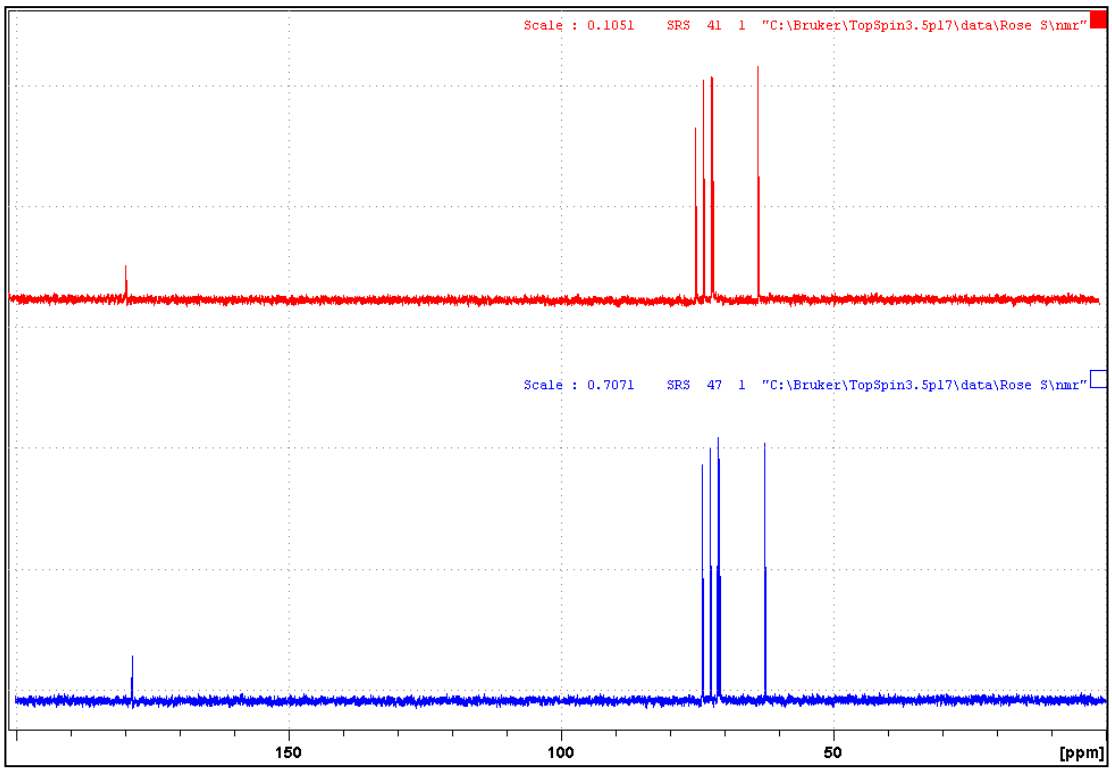

*Figure 3-5: ^{13}C NMR spectra of **1b** (top) and 21 mg/mL D-gluconic acid that was allowed to equilibrate at room temperature for three days (bottom).*

3.2.4 Fraction 2a

Figure 3-6 shows the ^{1}H NMR spectrum of **2a**; this fraction was extremely small, and not enough of it could be collected to identify by ^{13}C NMR or GC-MS. However, the overlapping doublets at ~5.4 ppm, and the small doublet at ~4.9 ppm, are reminiscent of the doublet pattern in the anomeric region of the ^{1}H spectrum of a mixture of trisaccharides (fraction **2b**, Figure 3-8), suggesting that **2a** may be one or more other large sugars present in trace amounts.

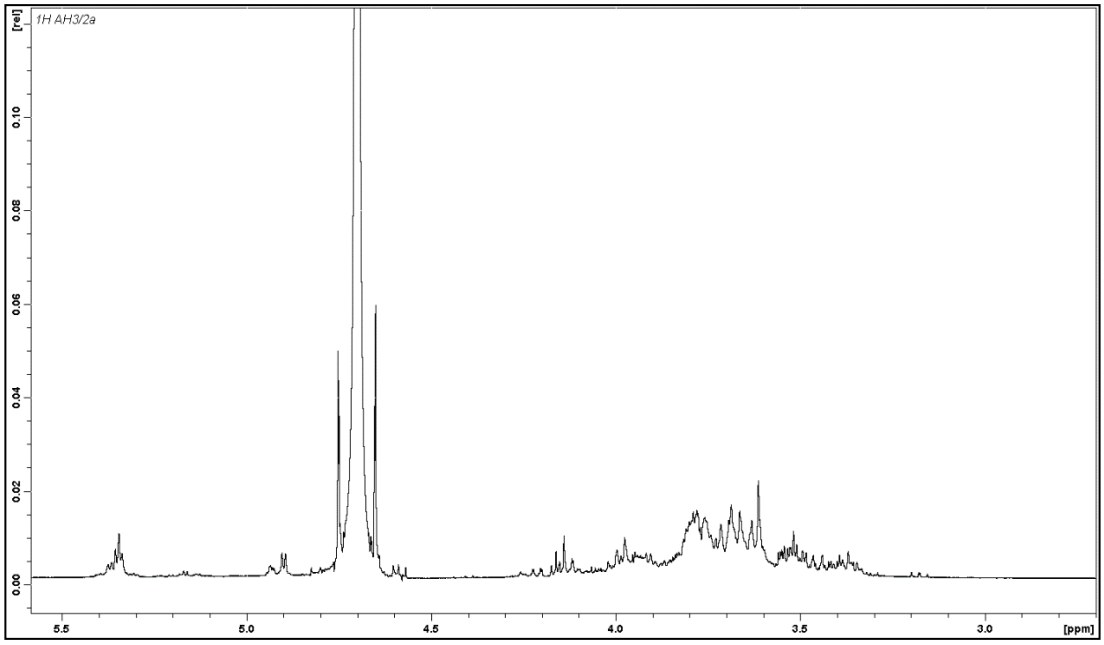

*Figure 3-6: [1]H NMR spectrum of AH3 preparative-HPLC fraction **2a**; downfield is seen a cluster of doublets reminiscent of anomeric protons of oligosaccharides. The HOD peak was not suppressed because it occurs in the anomeric region and suppression would affect these signals.*

Figure 3-7 shows [1]H NMR spectra of fraction **2a** (top; HPLC elution time 16.7 ± 1.8 minutes) compared to the synchronously-eluting fraction **2a** of HND, a sample of New Zealand beech honeydew honey (bottom: elution time 16.7 ± 0.27 minutes). As discussed at length in section 4.1.3, HND fraction **2a** is a mixture of tetrasaccharides α-D-Glc*p*(1→4)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→2)-β- D-Fru*f* and α-D-Glc*p*(1→6)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→2)-β- D-Fru*f*.

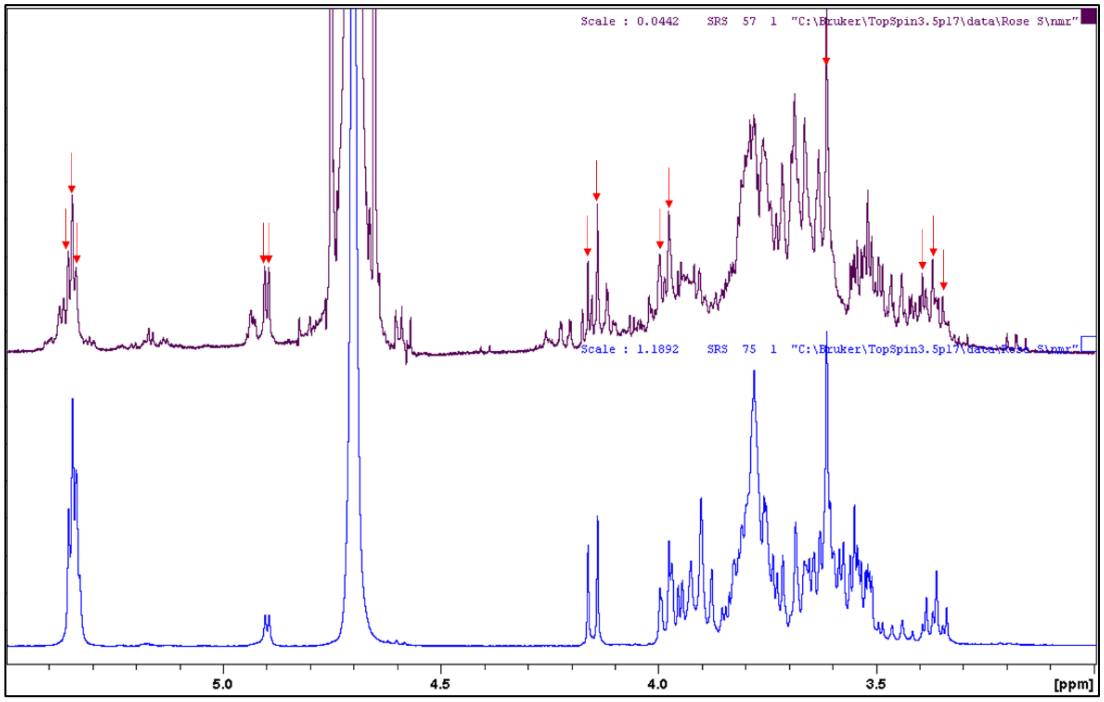

*Figure 3-7: ^{1}H NMR spectra of AH3 fraction **2a** (top) and beech honeydew honey HND fraction **2a** (bottom); HND peaks that also recognizably appear in the AH3 spectrum are marked in the AH3 spectrum with red arrows. The HOD peaks were not suppressed because they occur in the anomeric region and suppression would affect these signals.*

Because the HPLC retention times of all the compounds in AH3 fraction **2a** are similar enough for them to appear as a single peak, it can be concluded that fraction **2a** represents the tetrasaccharides present in AH3. A purified sample of HND fraction **2a** was used as a quantitative standard for AH3/**2a**.

3.2.5 Fraction 2b

Figure 3-8 and Figure 3-9 show ^{1}H and ^{13}C NMR spectra comparing **2b** to a standard of 6-kestose. These spectra show that **2b** is a mixture of several different sugars, one of which is, due to numerous coincident peaks between **2b** and the standard, deemed likely to be 6-kestose. The elution times of this mixture on HPLC and GC-MS, compared to standards, suggest that fraction **2b** contains all or most of the trisaccharides present in AH3. The mass spectra of synchronously-eluting peaks of **2b** and melezitose and 6-kestose (included in Appendix 6) confirm this identification.

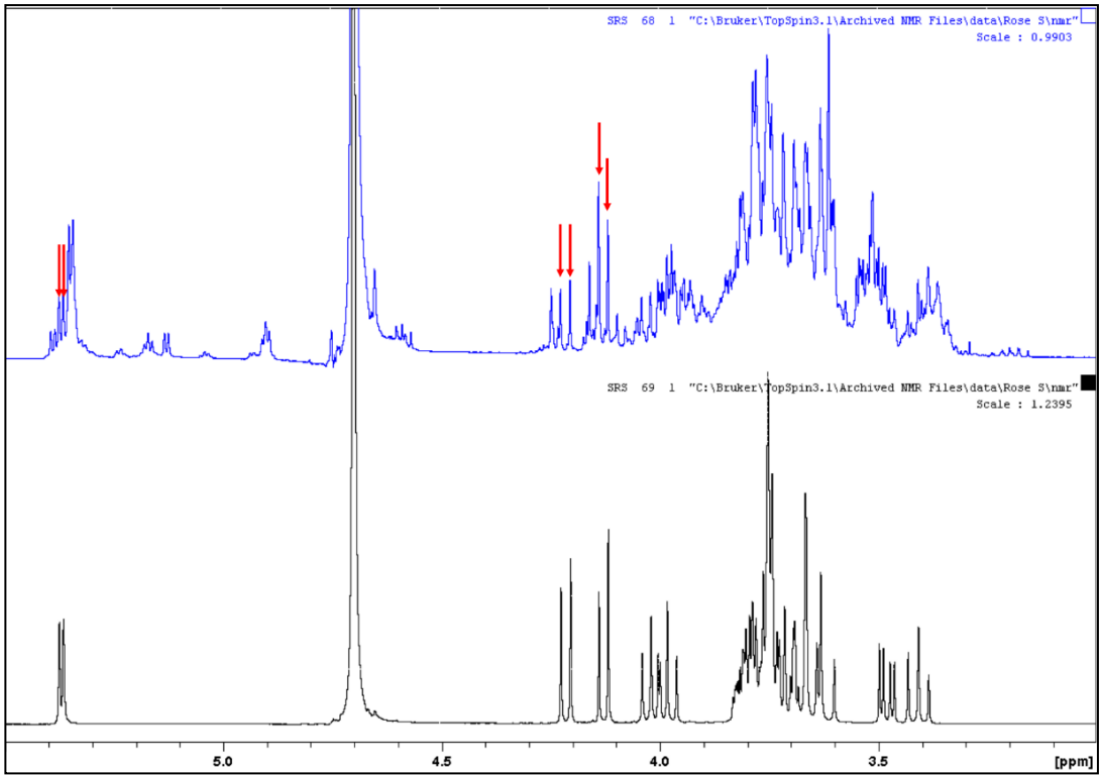

*Figure 3-8: ^{1}H NMR spectrum of AH3 fraction **2b** (top) and 13 mg/mL 6-kestose (bottom). Significant 6-kestose peaks, identified by comparison with the standard, are highlighted with red arrows in the **2b** spectrum. The HOD peaks were not suppressed because they occur in the anomeric region and suppression would affect these signals.*

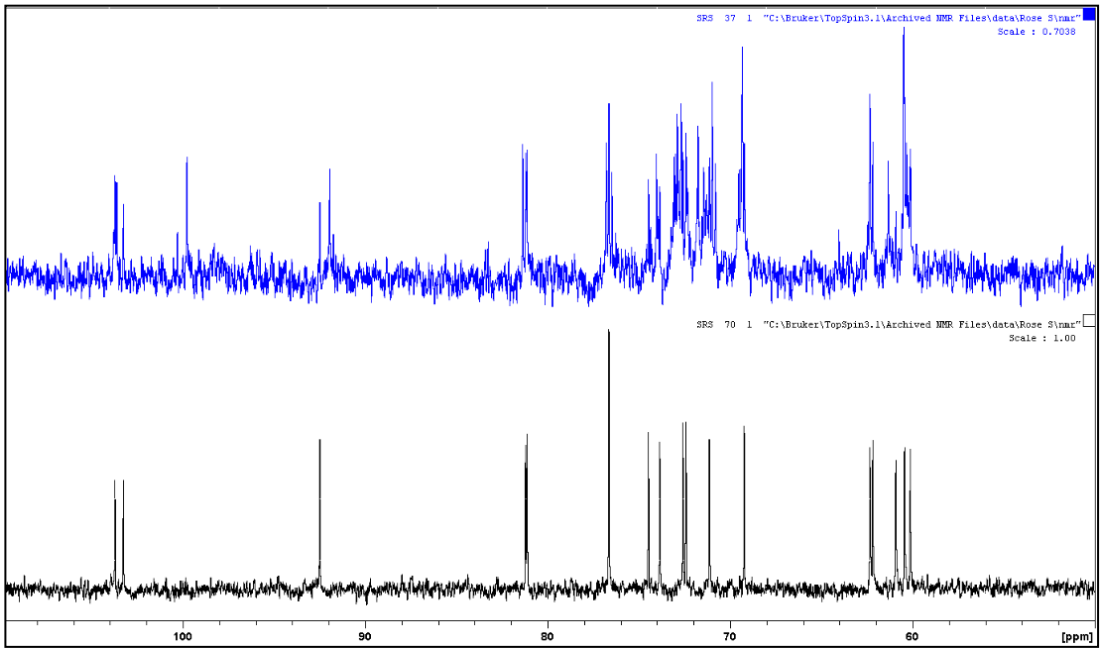

*Figure 3-9: ^{13}C NMR spectrum of AH3 fraction **2b** (top) and 13 mg/mL 6-kestose (bottom).*

Melezitose and 6-kestose standards are identified by retention time in their GC-MS total ion chromatogram, Figure 3-10, which compares total ion chromatograms of melezitose, raffinose, 6-kestose, AH3 fraction **2b**, and the fraction of some New Zealand beech honeydew honey eluting between 50 and 60 minutes on preparative HPLC. Beech honeydew honey was included because its high erlose content is well-established, so it could be safely assumed that the major trisaccharide of the honeydew honey was erlose [41], which provided a tentative erlose retention-time standard.

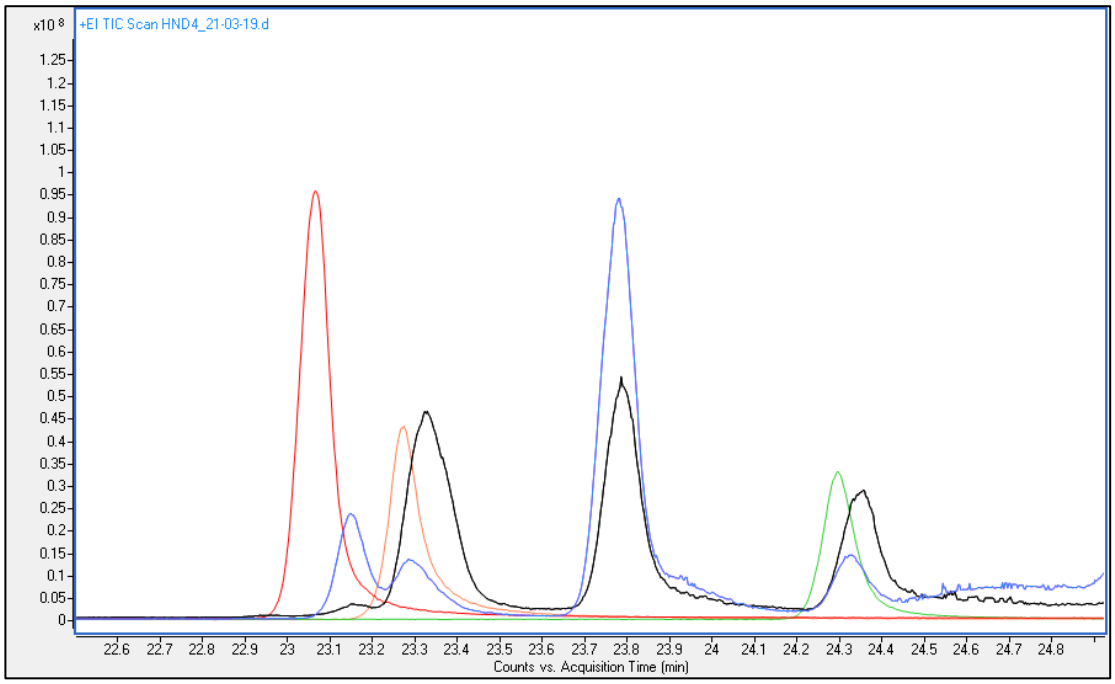

*Figure 3-10: Enlargement of major peaks in the trisaccharide region of total ion chromatograms of per-O-trimethylsilylated raffinose (red), the trisaccharide fraction of beech honeydew honey (blue), melezitose (green), kestose (orange), and AH3 fraction **2b** (black).*

Sanz *et al.* [185] and Ruiz-Matute *et al.*[186] reported a trimethylsilyl (TMS) oxime of erlose eluting between neo-kestose (the latest-eluting of the kestose group 1-kestose, 6-kestose, and neo-kestose) and melezitose. These researchers reduced their sugars with hydroxylamine hydrochloride prior to derivatization, whereas the current research derivatized the unreduced sugars, so the elution times can only be compared for the non-reducing sugars sucrose, melezitose,

erlose, raffinose and the various kestoses, upon which reduction or oximation prior to derivatization have no effect. Mateo *et al*. reported TMS ethers of unreduced kestose and melezitose eluting adjacent to each other [187], but did not test erlose. New Zealand beech honeydew honey has previously been shown to contain erlose as its main trisaccharide constituent [33, 41-42], accounting for 1.2% of the total honey mass, cf. 0.085% melezitose, ~0.5% other trisaccharides, and up to 1% of some tetrasaccharides [41]. Wu's GC-FID analysis of mānuka honey oligosaccharides [188], under similar GC conditions to those used in analysis of AH3, showed a trimethylsilylated erlose standard eluting immediately after trimethylsilylated 1-kestose and immediately before trimethylsilylated melezitose. Therefore, in the beech honeydew trisaccharide chromatogram seen in Figure 3-10, the major peak that coincides with the central trisaccharide peak of **2b** is thought to be erlose. 6-kestose and melezitose, identified by standards, also appear in the honeydew sample. (The ^{13}C NMR spectrum of the kestose standard used most closely resembles the ^{13}C NMR spectrum of 6-kestose in the literature [189], and is thus concluded to be 6-kestose.) Some researchers have noted that 1-kestose may coelute with raffinose, very shortly before 6-kestose, [185] or it may coelute with neo-kestose [186]. The small honeydew honey peak eluting just before the 6-kestose standard in Figure 3-10 eluted synchronously with a raffinose retention time standard, but the mass spectrum of this peak was considerably different to that of the raffinose standard.

The similarity of the **2b** peak elution times to known 6-kestose, raffinose, and melezitose standards, and the erlose peak, suggests that **2b** contains all or most of the trisaccharides present in AH3. 6-kestose and melezitose were positively identified by comparison of their mass spectra to the mass spectra of the synchronously-eluting **2b** peaks. Because the trisaccharides in the honey standards used were dissolved in water (in the honey) for some time prior to

drying and derivatization, any reducing sugars present would have had the opportunity to mutarotate, resulting in distinct peaks from the α and β anomers. The trisaccharide standards used were all non-reducing sugars and not susceptible to mutarotation.

Quantitative HPLC calibration curves constructed with standards of 6-kestose, raffinose, and melezitose were averaged, as discussed in section 2.8.1, to approximate the quantity of trisaccharides present in the sample, as no erlose standard was available to quantify erlose suggested by GC-MS. GC-MS quantitation results are discussed in section 3.2.9.

3.2.6 Fraction 3

Fraction **3** produced highly convoluted NMR spectra from which no useful information was gained. On HPLC, this fraction eluted between the trisaccharide fraction and glucose, and synchronously with the disaccharide standards used. It was therefore deduced to contain all or most of the disaccharides present in AH3, as confirmed by GC-MS. **2b** and **3** both appeared as single peaks in both preparative and analytical HPLC and as mixtures of sugars in NMR; these fractions were studied by GC-MS to determine their composition.

Figure 3-11 compares total ion chromatograms of sucrose, maltose, cellobiose, and **3**; the similarity of the elution times suggests that **3** contains most or all of the disaccharides in AH3. The mass spectra of synchronously-eluting peaks of **3** and sucrose, maltose, and cellobiose (included in Appendix 6) confirm this identification. Quantitative HPLC calibration curves constructed with standards of cellobiose, maltose, and sucrose were averaged, as discussed in section 2.8.1, to approximate the total quantity of disaccharides present in the sample.

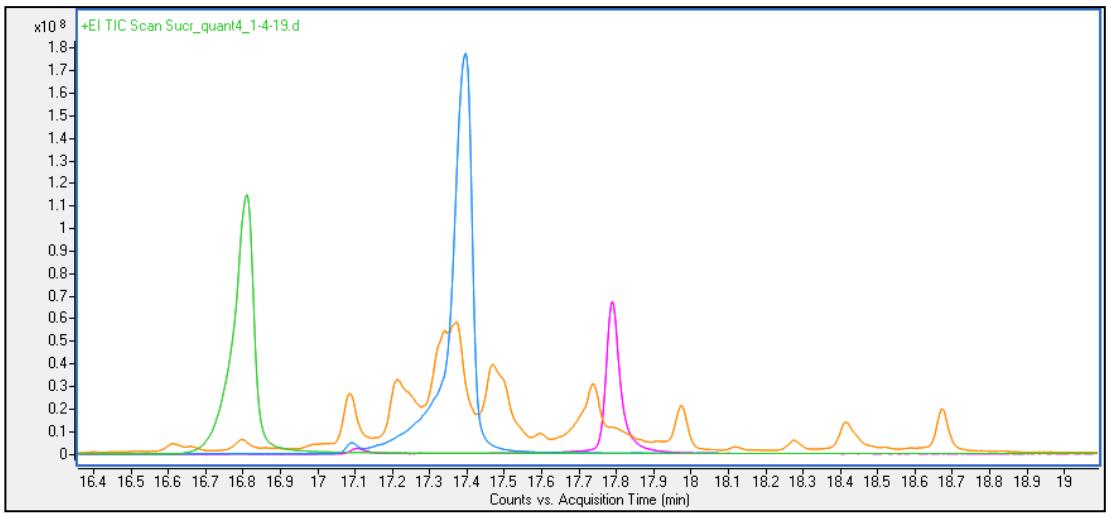

*Figure 3-11: Total ion chromatograms of freeze-dried AH3 fraction **3** (orange), and (L-R) sucrose (green), maltose (blue), and cellobiose (pink).*

The upper chromatogram in Figure 3-12 shows the disaccharide (16.5-19.0 minutes) and trisaccharide (23-25 minutes) fractions of AH3, compared to sucrose, maltose, cellobiose, kestose, and melezitose standards. The relative intensities of the fraction peaks indicate significantly more disaccharides than higher sugars: this is typical for floral honey. Figure 3-12 compares the total ion chromatogram of AH3 to that of a sample of New Zealand beech honeydew honey, with axes scaled to match. AH3 has a lower trisaccharide:disaccharide ratio than the beech honeydew honey does, and the disaccharides of AH3 are more clearly dominated by maltose, with relatively little contribution from the two late-eluting disaccharides (~18.4 and 18.7 min).

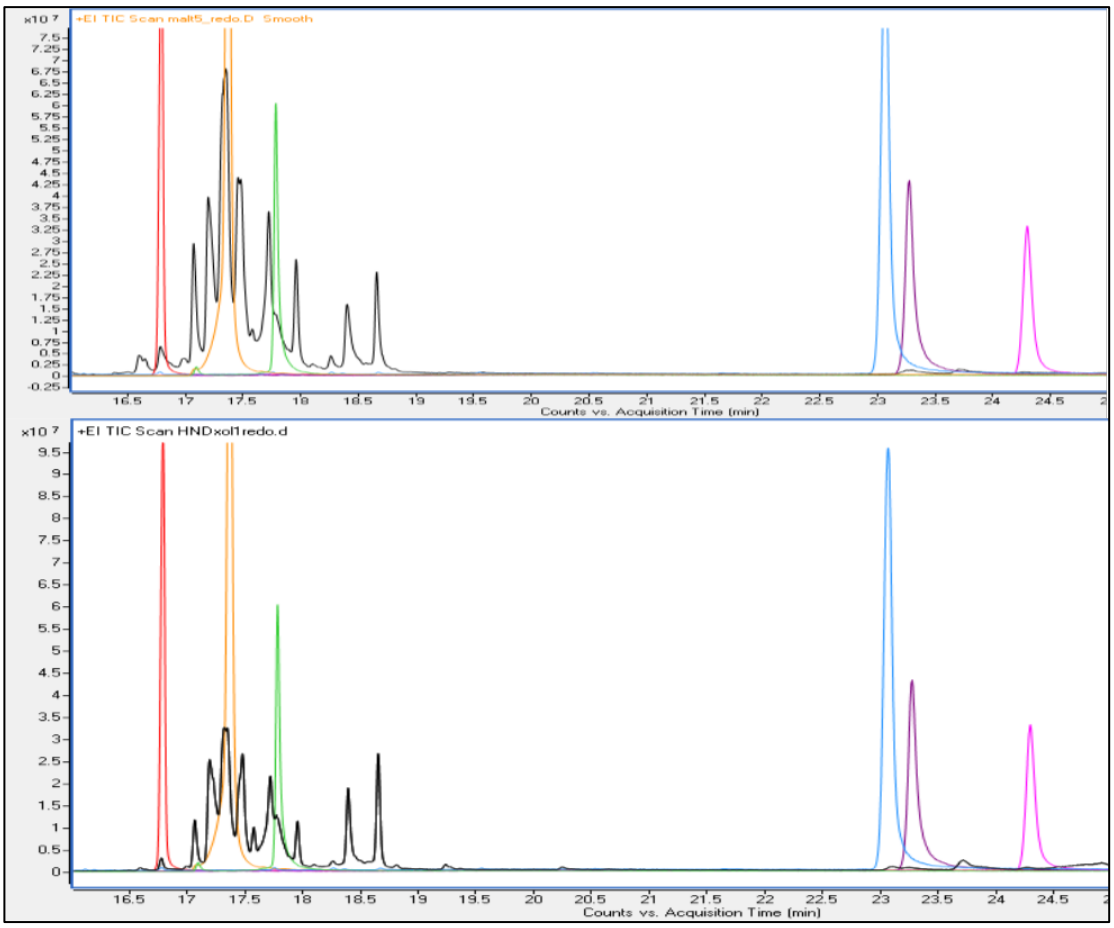

Figure 3-12: Total ion chromatograms of (top) freeze-dried AH3 (black), and (L-R) sucrose (red), maltose (orange), cellobiose (green), raffinose (blue), kestose (purple), and melezitose (pink); and (bottom) freeze-dried New Zealand beech honeydew honey sample HND (black, discussed fully in chapter 4) and the same standards. Both chromatograms are shown at the same scale.

3.2.7 Fraction 4

Figure 3-13 and Figure 3-14 show NMR spectra comparing **4** to a standard of equilibrated glucose of similar concentration; as the spectra are practically identical, this fraction can be confidently identified as glucose.

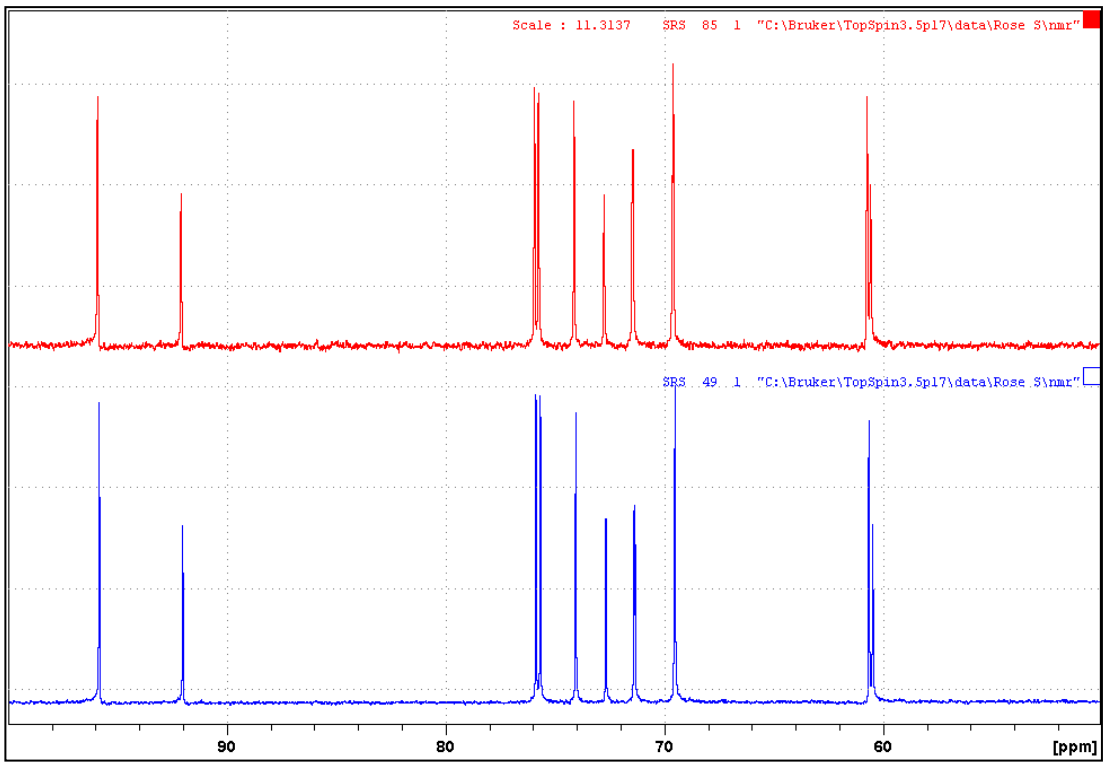

*Figure 3-13: ^{13}C NMR spectra of 20 mg/mL AH3 fraction **4** (top) and 20 mg/mL glucose that was allowed to equilibrate at 40 °C for three hours (bottom).*

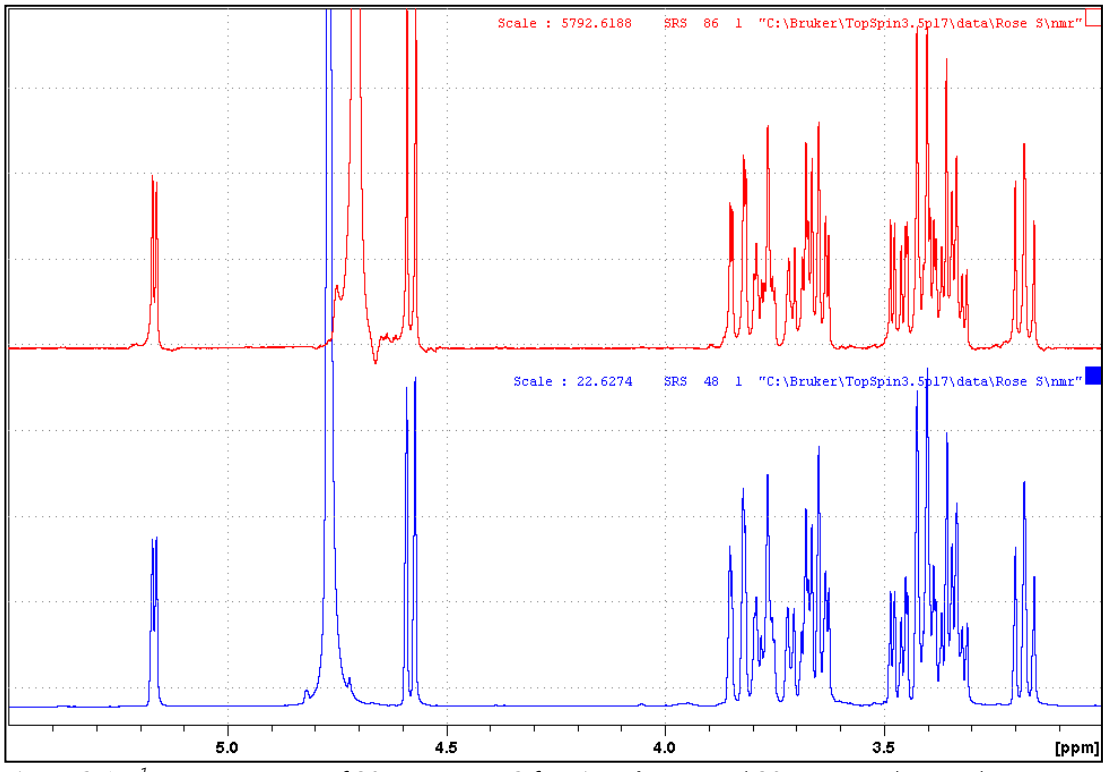

*Figure 3-14: ^{1}H NMR spectra of 20 mg/mL AH3 fraction **4** (top) and 20 mg/mL glucose that was allowed to equilibrate at 40 °C for three hours (bottom). The HOD peaks were not suppressed because they occur in the anomeric region and suppression would affect these signals.*

3.2.8 Fraction 5 (5a, 5b, 5c)

Figure 3-15 shows ^{13}C NMR spectra comparing **5a** to a standard of 2:1 fructose:glucose, with glucose peaks labeled by comparison to the standard shown in Figure 3-13. The spectra show that **5a** is a mixture of fructose and glucose. The glucose peaks in the sample are stronger relative to fructose than the glucose peaks in the standard, so the sample contains a fructose:glucose ratio rather lower than 2:1, indicating sample contamination of a fructose fraction with the earlier-eluting glucose fraction **4** discussed previously.

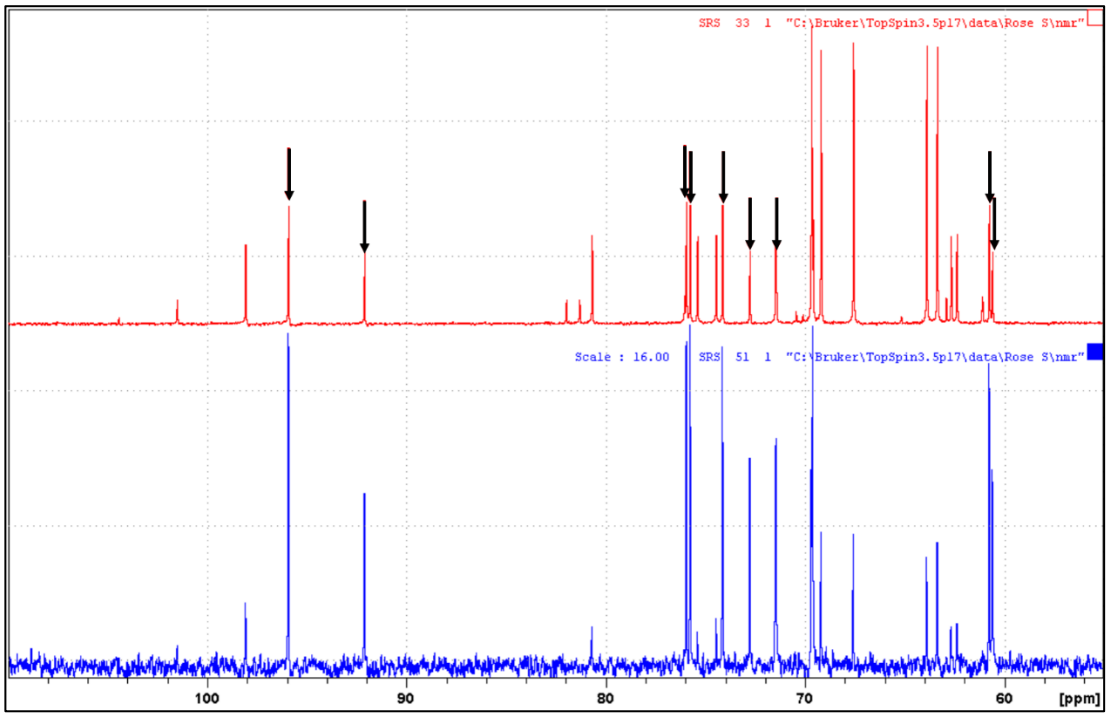

*Figure 3-15: ^{13}C NMR spectra of a mixture of 20 mg fructose and 10 mg glucose in 1 mL D_2O that was allowed to equilibrate at 50 °C for three hours (top) and 20 mg/mL AH3 fraction **5a** (bottom). Glucose peaks, identified by comparison with the glucose standard shown in Figure 3-13, are marked with black arrows in the standard.*

Fraction **5bc** as shown in Figure 3-1 proved upon second separation to contain two sub-fractions, **5b** and **5c**. Figure 3-16 shows NMR spectra comparing **5b** and **5c** to the same fructose-glucose standard used in Figure 3-15, with glucose peaks marked. The sample spectra are practically identical to each other and

coincide with the fructose peaks of the standard; therefore, fraction **5bc** is fructose.

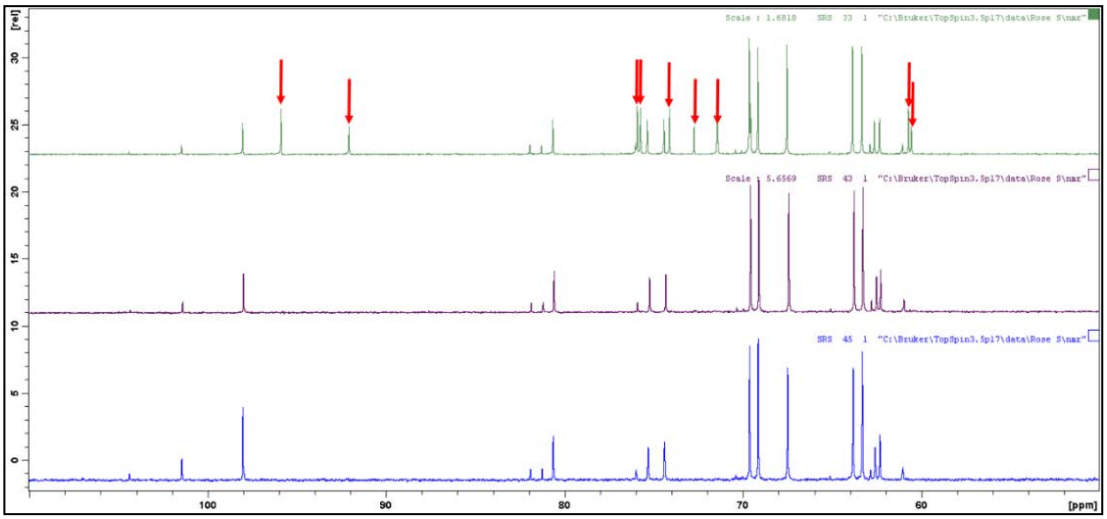

*Figure 3-16: ^{13}C NMR spectra of a mixture of 20 mg fructose and 10 mg glucose in 1 mL D_2O that was allowed to equilibrate at 50 °C for three hours (top), 16 mg/mL **5b** (middle) and 16 mg/mL **5c** (bottom). Glucose peaks, identified by comparison with the glucose standard shown in Figure 3-13, are marked with arrows in the standard spectrum.*

3.2.9 Composition of Beeotic® honey

The concentrations of each fraction in the honey, presented in Table 3-1, are not adjusted for water content, and represent the amount of the fraction present in a given unit mass of the honey as it is sampled.

*Table 3-1: Analytical HPLC and GC-MS concentrations as percent mass of whole honey, of **1a** through **5**, comprising the overall carbohydrate composition of AH3. Absolute error is reported as '95% CI'. The bottom row ('water') gives the result described in section 3.2.1. Quantities as a percentage of whole honey for **2b** and **3** are reported for both HPLC and GC-MS quantitation.*

		HPLC/GC-MS run				
Fraction	Identity	1	2	3	Average	95% CI
1a honey %	Large/charged saccharide	1.554	1.463	1.744	1.587	0.357
1b Honey %	D-gluconic acid	0.136	0.120	0.122	0.126	0.021
2a Honey %	Tetrasaccharide	0.010	0.012	0.011	0.011	0.002
2b Honey % HPLC	Trisaccharides	1.107	1.142	1.149	1.133	0.024
2b Honey % GC-MS		0.376	0.508	0.554	0.479	0.040
3 Honey % HPLC	Disaccharides	12.062	11.960	12.152	12.058	0.101
3 Honey % GC-MS		19.177	23.999	40.195	27.790	8.890
4 Honey %	Glucose	26.041	25.913	26.375	26.110	0.593
5 Honey %	Fructose	49.133	48.842	49.503	49.160	0.823
Water	Analyzed separately				17.3	-

Fraction 2a was quantified as tetrasaccharide because it eluted from the HPLC column synchronously with a peak that was observed in some New Zealand beech honeydew honey and identified by NMR as one or more tetrasaccharides (discussed fully in section 4.1.3).

The agreement between GC-MS and HPLC quantitation is poor, as shown numerically in Table 3-1, and visually in Figure 3-18. Even when derivatized samples are stored in a freezer, silylated sugars are inherently unstable. Delay between derivatization and analysis, particularly when the sample is left at room temperature, degrades the silylated sugars. Figure 3-17 shows disaccharide and trisaccharide quantities as a percentage of whole honey, calculated from the GC-MS peak areas obtained when the silylated sample was analyzed the same day, and when it was kept at -18 °C for a week after derivatization. This difference is more apparent for trisaccharides than for disaccharides, but in neither case is it statistically significant at $p \leq 0.05$.

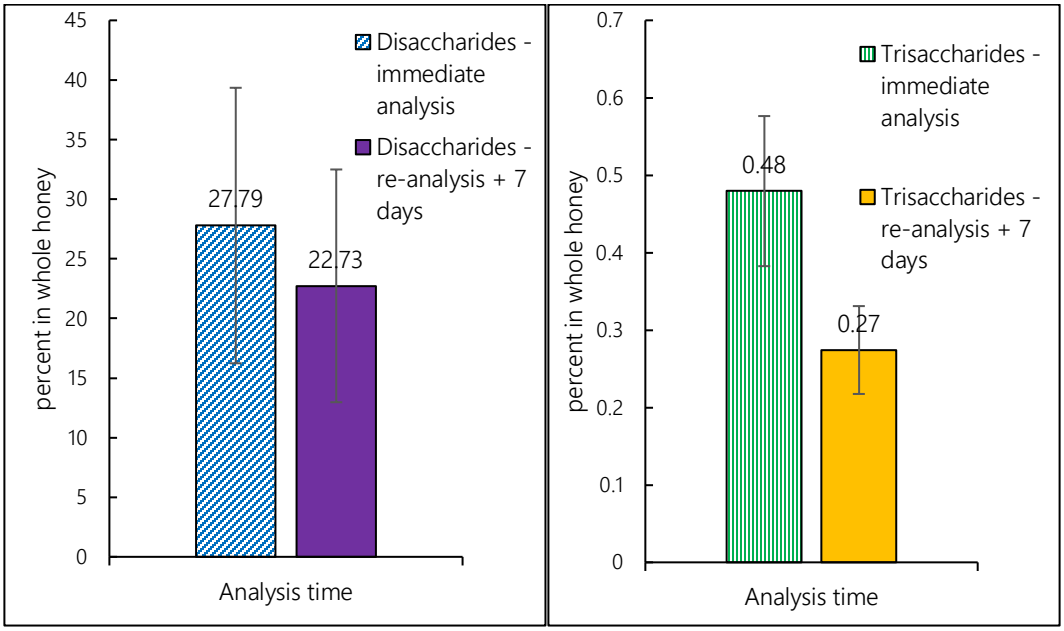

Figure 3-17: Comparison of GC-MS quantitation results for disaccharide (left) and trisaccharide (right) fractions of AH3, when the silylated sample was analyzed the same day as it was prepared (left-hand bar in each chart) and when it was re-analyzed after seven days stored at -4 °C (right-hand bar in each chart).

As shown in Figure 3-18, GC-MS quantitation appears to return a higher disaccharide percentage than HPLC; however, the uncertainty in GC quantitation of disaccharides is high, and the difference is not significant at $p \leq 0.05$. GC-MS also returns a lower trisaccharide figure than HPLC, even when it is assumed that **1a** cannot be treated as an oligosaccharide for HPLC quantitation. This difference is statistically significant. It is thought that larger sugars may be less amenable to trimethylsilylation, or less stable when derivatized, or both, than smaller sugars. Trimethylsilylated trisaccharides have masses over 1200 daltons, so their analysis by GC-MS requires high temperatures, long elution times, and mass spectral detection specifically tuned to detect large fragments; these factors also contribute to low GC-MS response from trisaccharides. A possible further factor that has not been fully explored is the possibility that ionization potentials differ between nonreducing sugars (such as those used as standards to derive the trisaccharide response factor for this GC-MS analysis) and reducing sugars (such as exist alongside nonreducing sugars in honey).

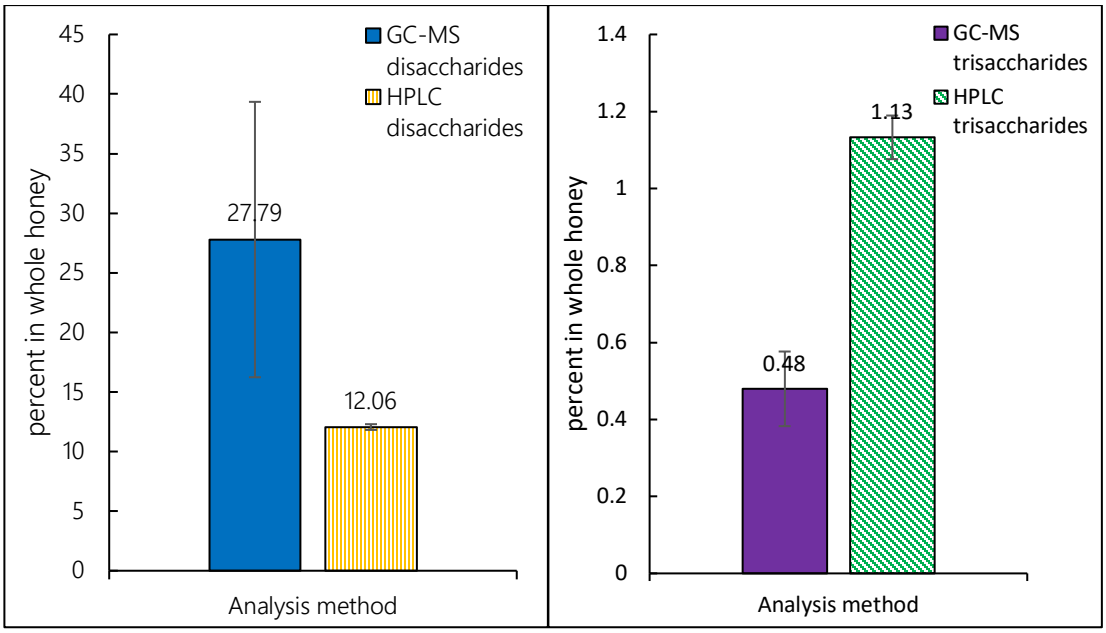

Figure 3-18: Comparison of method results, showing percent in whole honey and uncertainty (error bars) for GC-MS (leftmost bar in each chart) and HPLC (rightmost bar in each chart) quantitation of the disaccharide (left) and trisaccharide (right) fractions.

Only HPLC quantitation figures are considered in comparison with the literature for bulk fractions; individual sugars are necessarily compared using the GC-MS results, with the caveat that individual trisaccharides may be under-estimated, and individual disaccharides may be over-estimated.

3.2.10 Comparison of Beeotic® carbohydrate composition to results reported in the literature

A selection of researchers' results for di- and oligosaccharide contents of various honeys, discussed previously, are summarized in Table 3-2 for easy comparison. AH3's disaccharide content is not particularly different to Sanz's analysis of honeydew honey or floral honey [9], or Low's analysis of clover honey [6]. Conversely, literature values for disaccharides in floral honey vary widely enough that it is reasonable to observe that AH3 is not unusual.

Table 3-2: Comparison of literature values for di- and oligosaccharides in honey, determined as discussed in section 3.1.3, to current HPLC analysis of AH3. All values are given as percent mass of whole honey.

Researcher	Honey type	Disacch. (%)	Higher sugars (%)	Ref.
AH3 by HPLC	**Unknown**	**12.06 ± 0.24**	**1.14 ± 0.02**	
			~2.73 ± 0.36 if **1a** included	
White 1961	Old clover	17.25	2.06	[104]
	New clover	7.18	0.82	[105]
White 1959	Clover	8.4 ± 1.6	1.1 ± 0.4	
Siddiqui 1967-68	Clover/alfalfa	2.1	0.52	[75, 110]
Low 1988	Alfalfa	20 (combined disacch. and higher)		[6]
	Clover	12-23 (combined disacch. and higher)		
	Trefoil	10 (combined disacch. and higher)		
	Alsike	22 (combined disacch. and higher)		
Sporns 1992	Various	4.5	-	[106]
Sanz 2004	Various floral	11.1 ± 2.8	0.85 ± 0.27	[185]
	Honeydew	12.1	4.2	
Weston 1999	Heather	8 (combined disacch. and higher)		[42]
	Honeydew	17 (combined disacch. and higher)		
Pita-Calvo 2017	Honeydew			[8]
Astwood 1998	Honeydew	8.2 ± 0.6	8.9 ± 1.6	[41]
Bogdanov 2008	Honeydew	2.1	2.1	[16]
	Various floral	5.7	>4.5	

AH3's content of higher sugars, however, returns some features of interest when compared to honeys in general: depending on whether fraction **1a** is considered a large sugar of DP>6 and included, or a charged acid oligomer and excluded, AH3 may contain about the same amount of oligosaccharides as a clover honey, or an amount beginning to approach that measured by Sanz in a honeydew honey, but not close to the oligosaccharide load other researchers identified in honeydew honey.

Although the total ion chromatogram for the whole AH3 honey shows it to contain very little sucrose (0.66%), its total disaccharide content is high, similar to a clover or honeydew honey. The Conway report [169] cited by Capilano in the initial marketing for Beeotic® [155] reports some prebiotic activity in Australian leatherwood, banksia, grey and mugga ironbark, and yellow stringybark honeys. Banksia honey can contain 10.7% disaccharides [190], noticeably lower than AH3. No literature value for the total disaccharide content of the other honeys is available. Some blended Western Australian floral honeys have been found to contain 10.1-15.2% disaccharides [190]; AH3 falls within this range and is therefore not unusual for an Australian floral honey.

Incidentally, some of the honeys identified by Conway as potential prebiotic functional foods were also identified by Arcot *et al*. (2005) [191] as having a low glycemic index value, and thus being more suitable than other honeys for consumption by people with blood glucose regulation issues. Arcot's paper, citing a report by Chandler *et al*. in 1974 [192], also reported glucose, fructose, and sucrose (rather than total di- or monosaccharide) contents of a range of Australian honeys. Some African and European eucalyptus honeys, and the honeys considered by Conway, are compared to AH3 in Table 3-3. A direct comparison of oligosaccharides was not possible, as no data on oligosaccharide

or total disaccharide content of these specific Australian floral honeys was available. However, the monosaccharide, water, and sucrose content of AH3 can be compared directly to the honeys that the Conway report cited in Beeotic®'s original marketing deemed potentially prebiotic. (No data was available for yellow stringybark; red and white stringybarks, and some 'generic' eucalyptus species, are considered instead.)

Table 3-3: Comparison of glucose, fructose, and 'sucrose' content of AH3 to some honeys identified by Conway [169] as potentially prebiotic. Comparison values for specific Australian eucalyptus, leatherwood, and banksia species were obtained from Arcot et al. (2005) [191], Chandler et al. (1974) [192], and Ajlouni et al. (2010) [190]. All values are shown as percent mass of whole honey.

Honey	Glucose	Fructose	Sucrose	Water	Ref
AH3	**26.1 ± 0.59**	**49.2 ± 0.82**	**0.66 ± 0.2***	**17.3**	
Leatherwood (*Eucryphia lucida*)	30.2	43.5	2.4	15.55	191-192
Ironbark (*Eucal. sideroxylon*)	30.4	44.4	1.4	17.4	191-192
Red Stringybark (*Eucal. macrorhyncha*)	30.0	44.0	2.0	16.5	191-192
White Stringybark (*Eucal. globoidea*)	20.2	45.9	11.6	14.9	191-192
Banksia spp.	26.5	30.8	<10.7	17.8 ± 0.4	190
Algerian *Eucalyptus* spp.	29.7 ± 2.9	42.1 ± 3.3	1.1 ± 0.5	16.5 ± 1.7	108
Iberian *Eucal. globulus*	26.9 ± 1.1	39.3 ± 1.6	0.84 ± 0.3	17.8 ± 0.4	193
Algerian *Eucalyptus* spp.	31.8 ± 0.6	40.2 ± 2.3	2.8 ± 2.2	14.9 ± 0.1	109
Andalusian *Eucalyptus* spp.	27.9 ± 3.9	34.7 ± 5.0	3.4 ± 1.5	16.6 ± 0.6	193

** total disaccharides in AH3: 12.1 ± 0.24%*

The papers referenced indicated that these figures represented the glucose, fructose, and sucrose contents of the honeys. However, particularly in the case of the white stringybark (sucrose = 11.6%), it is thought that these may instead represent the concentration of total disaccharides, as sucrose is not usually a major sugar of honey, and is required by the Codex Alimentarius to comprise <5% of the total honey mass of the honeys named (except Leatherwood, which is permitted up to 10% sucrose by mass) [194].

Glucose and fructose figures for AH3 were taken from HPLC quantitation, and sucrose from GC-MS; it is possible that the sucrose content of AH3 is somewhat over-estimated, due to the HPLC/GC-MS quantitation disparity previously discussed.

AH3 appears to have higher fructose and lower glucose and sucrose than the identified monofloral honeys. Its water and sucrose contents are most like ironbark or *E. globulus* honey, but overall, it is not particularly like any of the identified honeys, suggesting it may be a different honey, possibly one of the eucalyptus honeys for which carbohydrate composition data was not found. If this is the case, the high total monosaccharide (75.3%) and very low trisaccharide (<2.7% cf. literature 8-30% oligosaccharides in honeydew honeys [16-17]) contents indicate that little if any of AH3 is honeydew honey [8-9].

The high fructose content of AH3 (49.2 ± 0.82%) is particularly notable, as fructose content of foodstuffs (including di-and oligosaccharides) has been demonstrated to be inversely proportional to its glycemic index and insulin index (that is, consuming fructose causes a lower blood glucose level and blood insulin level response than consuming other sugars) [191]. Thus, a high-fructose honey may be more suitable for consumption by people with blood glucose and blood insulin regulation issues than a lower-fructose honey. However, this is a different consideration to the concept of a high-fructose honey being prebiotic by virtue of its fructose content, as was suggested in the 2015 thesis supervised by Conway [166].

3.2.11 Suitability of Beeotic® as a prebiotic functional food

For the following calculations, in order to obtain the highest possible estimation of Beeotic® sample AH3's prebiotic oligosaccharide content, it is assumed that **1a** is an oligosaccharide. The Beeotic® prebiotic label recommends a dose of 14 g (1 metric tablespoon) of honey daily, which would supply a total oligosaccharide dose calculated by Equation 3-1 and Equation 3-2:

$$\left(0.000111 \pm 0.00002163 \ \frac{\text{g tetrasacch.}}{\text{g honey}}\right) + \left(0.01133 \ \pm \ 0.00024 \ \frac{\text{g trisacch.}}{\text{g honey}}\right) + \left(0.01587 \pm 0.00036 \ \frac{\text{g other oligosacch.}}{\text{g honey}}\right) = 0.02731 \pm 0.00357 \ \frac{\text{g oligosacch.}}{\text{g honey}}$$

Equation 3-1

$$0.02731 \pm 0.00357 \frac{\text{g oligosacch.}}{\text{g honey}} \times 14 \text{ g honey} = 0.3826 \pm 0.0505 \text{ g oligosacch.}$$ *Equation 3-2*

The Equation 3-2 value of 382.6 mg oligosaccharides per 14 g honey is fairly close to the figure of "340 mg+ per serving" quoted in Walmart's product description of Beeotic®[163-164]. The recommended dose of prebiotic oligosaccharides to produce a measurable effect is 2-10 g daily [57, 195-197]; the manufacturer-recommended serving of Beeotic® does not, therefore, deliver the recommended daily dose of prebiotic oligosaccharides. To obtain an effective prebiotic dose, a Beeotic® consumer would have to ingest a minimum honey dose calculated by Equation 3-3:

$$2 \text{ g oligosacch.} \div 0.027329 \pm 0.00361 \frac{\text{g oligosacch.}}{\text{g honey}} = 73.2346 \pm 9.6806 \text{ g honey}$$ *Equation 3-3*

Consumption of this quantity of honey would also involve ingestion of large amounts of so-called 'free sugars' (defined by the World Health Organization as "monosaccharides and disaccharides… naturally present in honey, syrups, fruit juices and fruit juice concentrates" [82]). The free sugar dose that unavoidably accompanies the quantity of honey necessary to produce a prebiotic effect using Beeotic® alone is calculated by Equation 3-4.

$$\left((0.2611 \pm 0.0059) \frac{\text{g Glc}}{\text{g honey}} + (0.4916 \pm 0.0082) \frac{\text{g Fru}}{\text{g honey}} + (0.1298 \pm 0.0026) \frac{\text{g Disacch}}{\text{g honey}} \right) \times 73.2346 \pm 9.6806 \text{ g honey}$$
$$= 63.9537 \pm 9.2171 \text{ g free sugars}$$ *Equation 3-4*

The 63.95 g of free sugars contained in the mass of Beeotic® required to produce a prebiotic effect should be considered in the light of the WHO recommended daily maximum dose of free sugars, which is 30 g [82].

The prebiotic claim was removed from Beeotic® sold in Australia in late 2018 [161], due to new labeling restrictions requiring *in vivo* trials to be completed before this claim can be made; however, as of April 30, 2020 [162], the claim of prebiotic activity remains on Beeotic® sold in the United States.

The 2010 Conway report [169], and the 2014 Dawes report [168], both of which recommend Australian eucalyptus honeys as potential prebiotic functional foods, record testing the prebiotic properties of the honeys in question by extracting the oligosaccharide fraction with an activated-charcoal adsorption-desorption method [74], and using the oligosaccharide fraction thus obtained as the sole carbohydrate source in the growth medium for an *in vitro* bioassay for prebiotic activity [169]. Omitting to test whole honeys for prebiotic activity *in vivo* overlooks the fact that when a whole honey is sold as a prebiotic functional food, the human consumer ingests the whole honey. While it is possible that only the prebiotic oligosaccharides survive digestion to reach the colon, the di- and monosaccharides present are still digested and absorbed, and the health benefits of the oligosaccharides should be weighed against the potential detriment of consuming the large quantities of 'free sugars' that accompany the relatively minute quantities of oligosaccharides in Beeotic®.

Furthermore, the carbohydrate profile of Beeotic® sample AH3, as discussed here, does not particularly resemble the carbohydrate profiles, as discussed in various literature sources, of any of the honeys (Australian eucalyptus, leatherwood, and banksia floral honeys) suggested by Conway and Dawes as potential prebiotic functional foods. The floral or honeydew origin of Beeotic® is uncertain; however, its high monosaccharide and low oligosaccharide content do not distinguish it from an ordinary floral honey.

4 New Zealand beech honeydew honey (sample HND)

4.1 Results and discussion

4.1.1 Water content and fraction numbering

The water content of the New Zealand beech honeydew honey sample HND was 15.6%. The aliquot of whole honey weighed out to dry for GC-MS analysis weighed 3330.76 mg before drying and 3158.43 mg after drying, so the water content of the dried honey was 10.3%.

Figure 4-1 shows the analytical HPLC trace of an aliquot of HND and introduces the numbering system used to identify HND fractions.

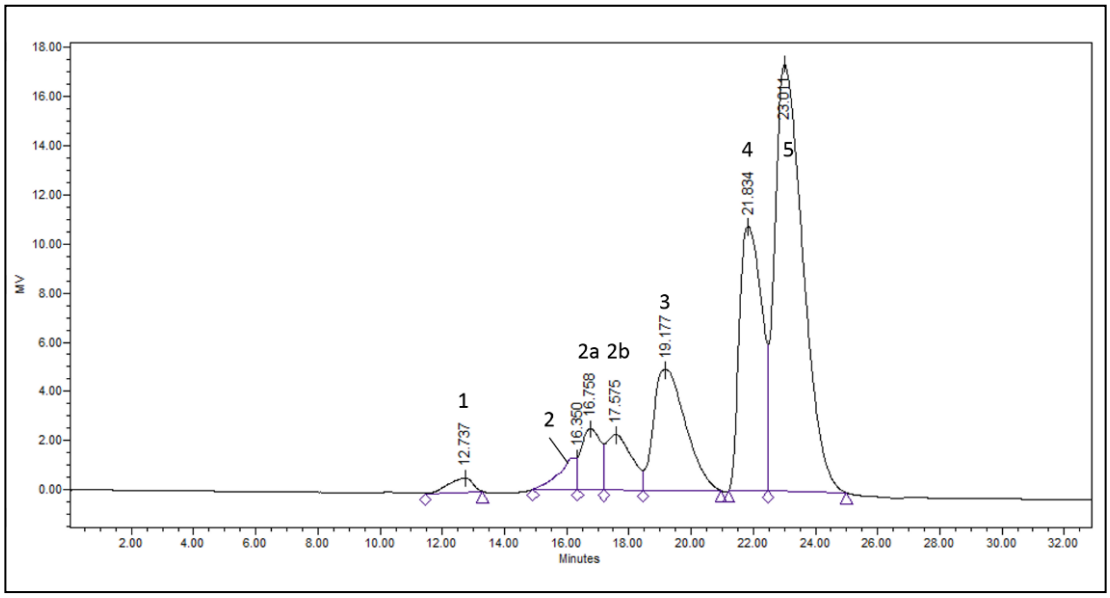

Figure 4-1: Analytical HPLC chromatogram of HND, (Shodex KS-G, KS-801, and KS-802 columns plumbed in series and eluted in Type 1 water at 0.8 mL/min at 80 °C, with RI detection) showing fraction divisions with the numbering system used throughout this chapter.

Table 4-1 lays out the retention time and identities of each HPLC peak for HND. Most fractions in the sample could be identified by their HPLC retention time. The AH3 fraction eluting at 14.7 ± 0.2 minutes and identified by NMR as D-gluconic acid (fraction AH3/**1b**, discussed in section 3.2.3) was not observed in HND. However, the first HND fraction, HND/**1**, which was identified, as discussed in section 4.1.2, as D-gluconic acid, eluted synchronously with AH3/**1a**

(12.7 ± 0.27 minutes HND/**1** cf. 12.8 ± 0.15 minutes AH3/**1a**), which was identified (section 3.2.2) as a very large or charged carbohydrate species. HND also contained an additional peak not found in AH3, fraction **2**.

Table 4-1: HND peaks and retention times (HPLC conditions: Shodex KS-G, KS-801, and KS-802 columns plumbed in series and eluted in Type 1 water at 0.8 mL/min at 80 °C, with RI detection), with peak identities. Retention times given are the mean of three determinations, and the error reported is the 95% confidence interval of these determinations.

HND peak	Peak identity	Ret. Time (min)
1	D-gluconic acid	12.7 ± 0.27
2	Tetrasaccharide/s	16.2 ± 0.25
2a	Trisaccharides	16.7 ± 0.27
2b	Trisaccharides	17.5 ± 0.28
3	Disaccharides	19.1 ± 0.27
4	Glucose	21.8 ± 0.26
5	Fructose	23.0 ± 0.27

As the large or charged saccharide discussed in section 3.2.2 did not appear in HND, the D-gluconic acid's open-chain/lactone equilibrium and retention time are not disrupted, and D-gluconic acid elutes earlier, as a charged species.

4.1.2 Fraction 1

Figure 4-2 shows ^{13}C NMR spectra of HND fraction **1** and a standard of D-gluconic acid. The ^{13}C spectra of HND fraction **1** is sufficiently similar in chemical shift and peak intensity to D-gluconic acid for positive identification. The **1** spectrum has a lower signal-to-noise ratio than the standard (largely due to the small mass of the freeze-dried fraction available) and the carbonyl signal far downfield is partially obscured in the baseline. Weak signals were observed at ~70 and ~42 ppm and may belong to a co-eluting acid, which would, however, be a very minor fraction.

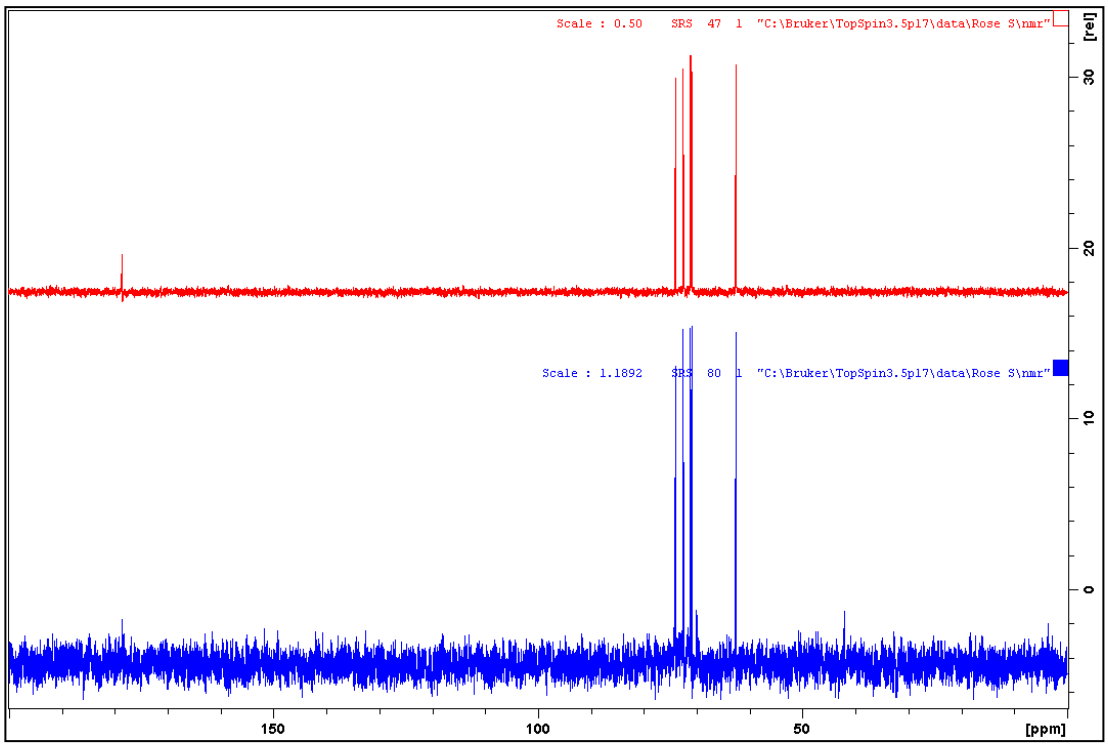

*Figure 4-2: ^{13}C NMR spectra of D-gluconic acid (top) and HND fraction **1** (bottom).*

4.1.3 Fractions 2 and 2a

Figure 4-3 compares ^{13}C NMR spectra for HND fractions **2** and **2a**; while the spectrum for the latter is noisier, the chemical shift patterns and peak intensity ratios are similar enough to identify these fractions as the same or similar compounds. The sample of New Zealand beech honeydew honey tested by Astwood *et al.* [41] contained two series of chiefly [1→4] linked tetra- and pentasaccharides. The only difference between these series was that one maintained [1→4] linkage on the terminal residue, while the other displayed [1→6] linkage. It is suggested that the tetrasaccharides of these series might elute separately (as seen in HND fractions **2** and **2a**) but produce very similar NMR spectra. The differences between the spectra shown in Figure 4-3 are not sufficiently pronounced to distinguish the tetrasaccharides either from each other or as having particular linkages on the terminal residue without recourse to detailed 2D spectroscopy; it is thought that HND fraction **2a** may be α-D-Glc*p*(1→6)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→2)-β-D-Fru*f*, as the relative intensities of

the hydroxymethyl carbons are distinctly different and closer to 1:1:3:1 than they are to the approximately 1:1:2:1 ratio observed in spectra of α-D-Glc*p*(1→4)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→2)-β-D-Fru*f* (spectrum in Figure 4-4) and HND fraction **2**; however, the spectra are not sufficiently resolved to allow any firm distinction to be drawn from the shifts of C-4 and C-6 on the glucose moiety to which the terminal glucose residue is attached.

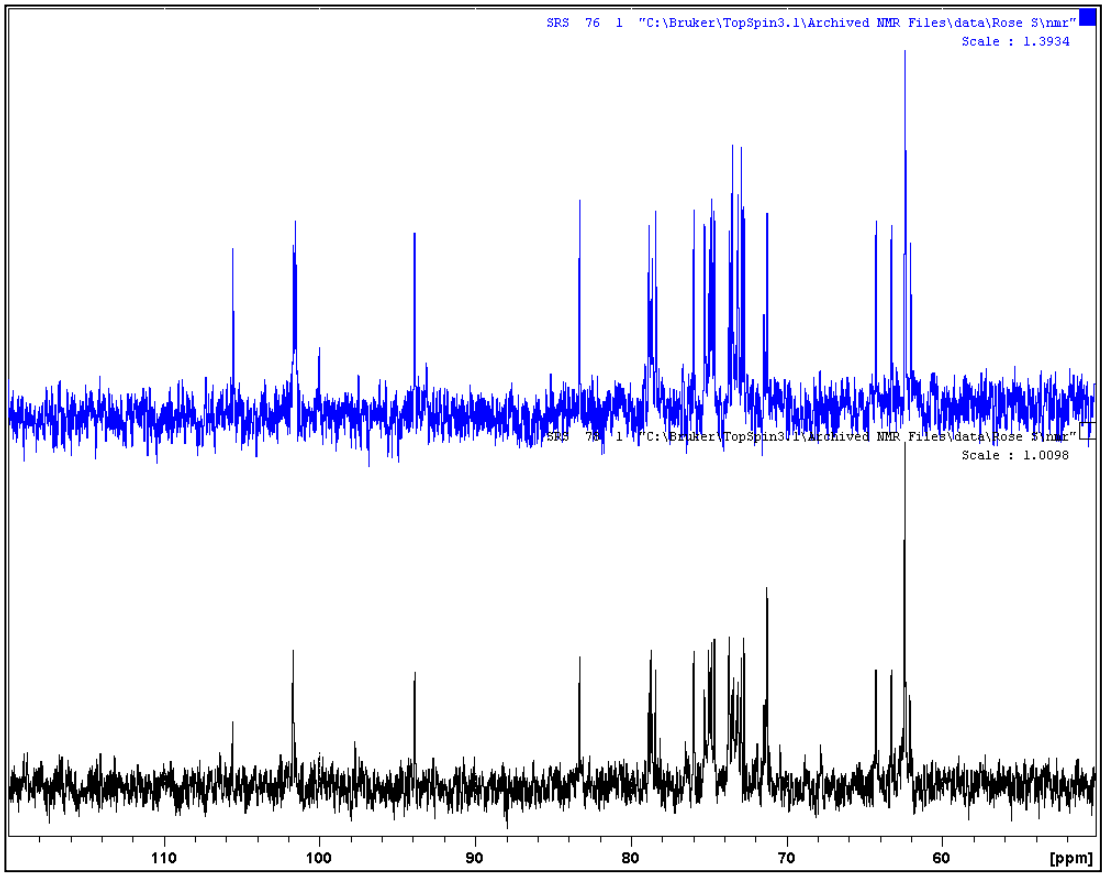

*Figure 4-3: ^{13}C NMR spectrum of HND fractions **2** (top) and **2a** (bottom).*

Figure 4-4 shows the ^{13}C spectrum of a tetrasaccharide, α-D-Glc*p*(1→4)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→2)-β-D-Fru*f*, (structure given in Figure 4-5) that was isolated from a sample of New Zealand beech honeydew honey, and is identified in a 1998 paper by Astwood *et al.* [41] as their fraction *2*.

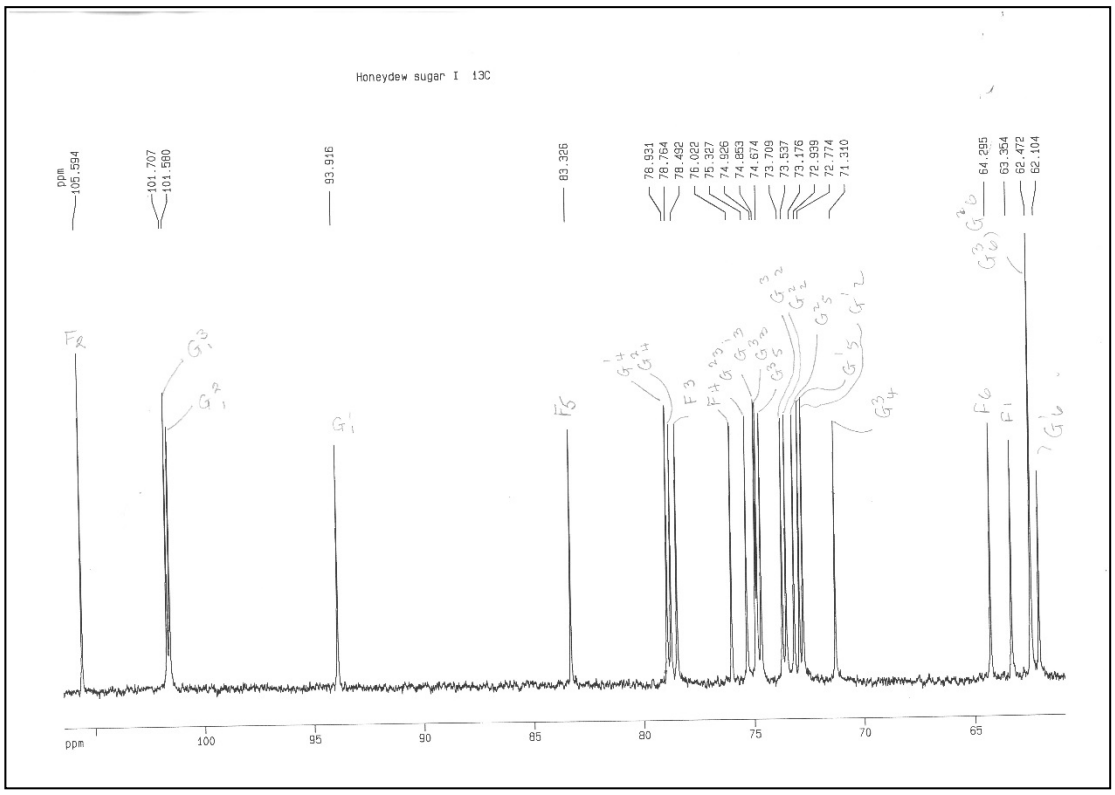

Figure 4-4: ^{13}C NMR spectrum of a tetrasaccharide identified in a sample of New Zealand honeydew honey in a 1998 paper by Astwood et al. [41].

The chemical shifts of Astwood *et al.*'s tetrasaccharide (105.594 C2 on Fru; 101.707, 101.580, 93.916 C1 on various Glc; 83.362, 78.931, 78.764, 78.492, 76.022, 75.327, 74.926, 74.853, 74.674, 73.709, 73.537, 73.176, 72.939, 72.774, 71.730 ring carbons; 64.295, 63.354, 62.472, 62.104 hydroxymethyl carbons) correspond to the chemical shifts of HND fractions **2** and **2a** (105.5462 C2 on Fru; 101.6472, 101.5311, 93.8801 C1 on various Glc; 82.2809, 78.8281, 78.6321, 78.3952, 75.9479, 75.2681, 74.9919, 74.8864, 74.7900, 74.6282, 73.6532, 73.4747, 73.1165, 72.8800, 71.2432 ring carbons; 64.2480, 63.2581, 62.3953, 62.0338 hydroxymethyl carbons), sufficiently closely to identify HND fractions **2** and **2a** as being at least closely related to Astwood *et al.*'s tetrasaccharides of structures as shown in Figure 4-5.

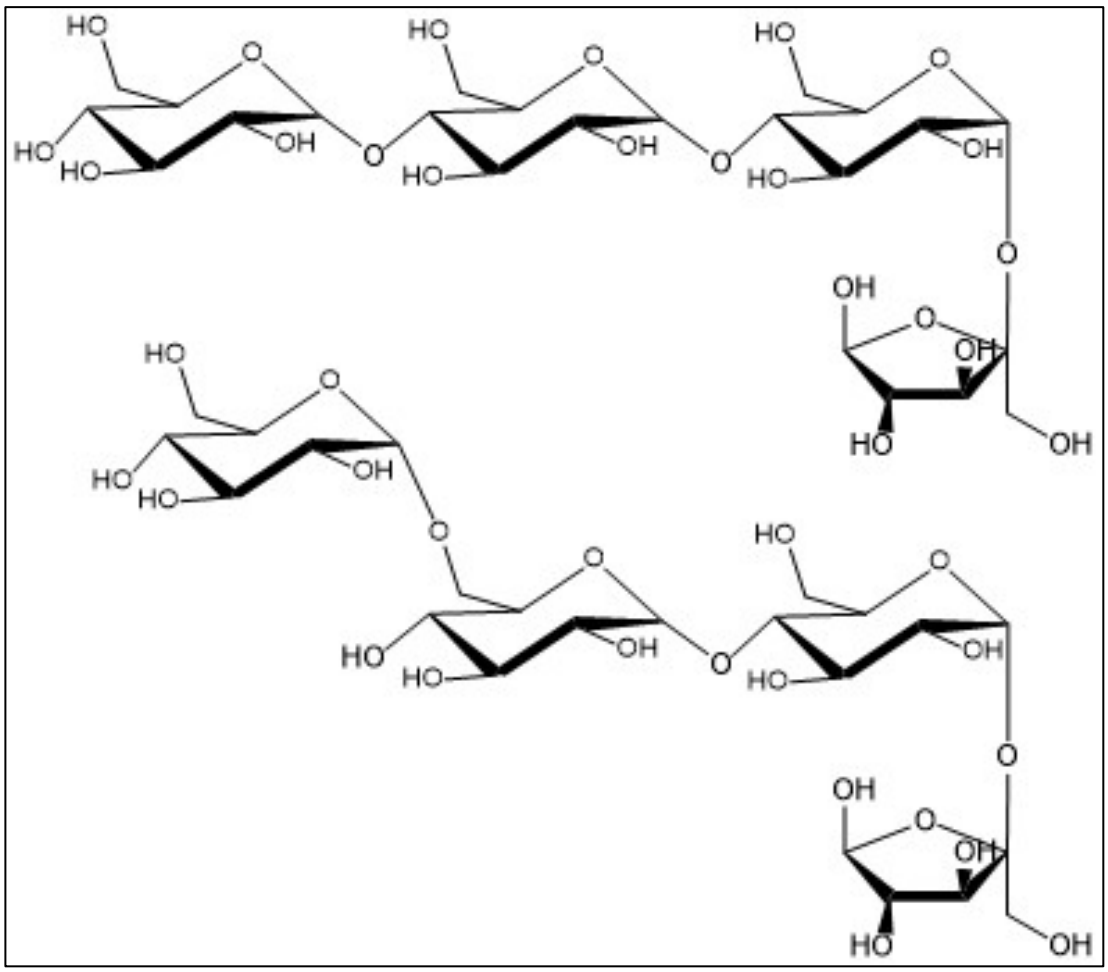

*Figure 4-5: Structures of α-D-Glcp(1→4)-α-D-Glcp(1→4)-α-D-Glcp(1→2)-β-D-Fruf (top) and α-D-Glcp(1→6)-α-D-Glcp(1→4)-α-D-Glcp(1→2)-β-D-Fruf (bottom), identified as HND fractions **2** and **2a** (not respectively).*

4.1.4 Fraction 2b

HND fraction **2b** was identified as consisting of trisaccharides by its analytical HPLC retention time as compared to raffinose, 6-kestose, and melezitose standards. The HND **2b** retention time was also compared to that of AH3 fraction **2b**, which was previously (section 3.2.5) identified as trisaccharides by NMR and GC-MS analysis of the freeze-dried preparative fraction and found to contain a mixture of trisaccharides including melezitose and 6-kestose. The electron impact mass spectra of synchronously-eluting peaks of AH3 **2b** (for comparison) and melezitose and 6-kestose (included in Appendix 7) confirm this identification. The total ion chromatograms of the derivatized fraction

compared to standards, shown in Figure 4-6 and Figure 3-10, show that HND contains comparatively little of 6-kestose or melezitose. It has larger amounts of a later-eluting sugar, and a discrete erlose peak between 6-kestose and melezitose. (Identification of this peak is discussed in section 3.2.5; no erlose standard was available). Like AH3 fraction **2b**, HND fraction **2b** resisted further HPLC separation.

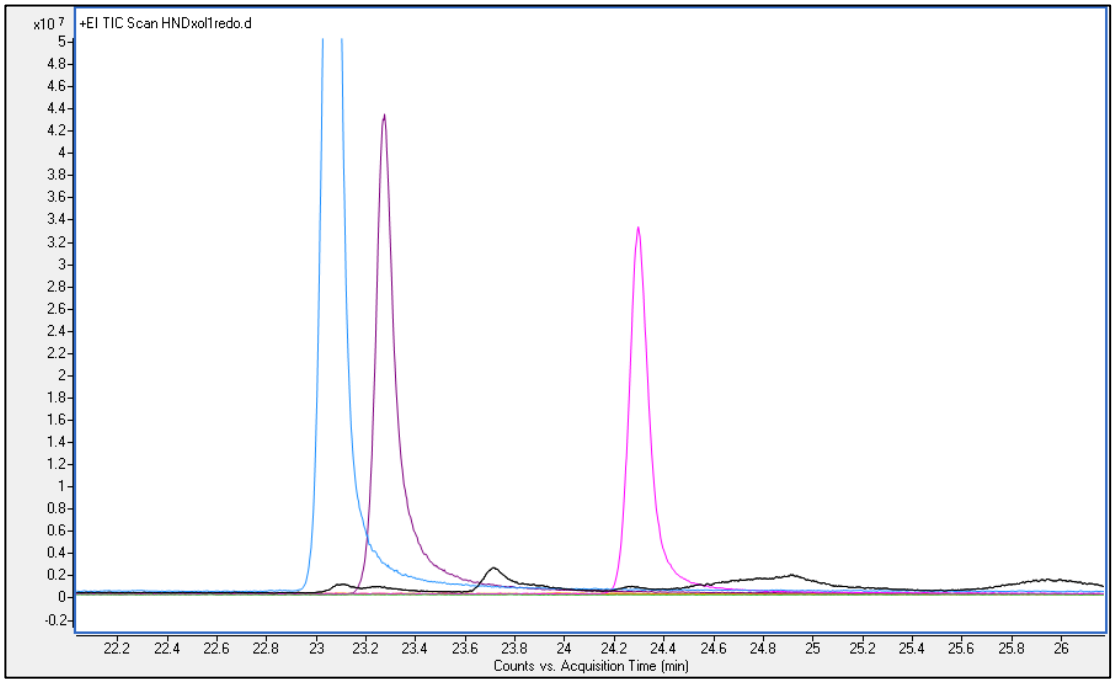

Figure 4-6: Overlaid total ion chromatograms of HND (black), raffinose (blue), 6-kestose (purple) and melezitose (pink).

4.1.5 Fraction 3

HND fraction **3** was identified mainly from its retention time (19.1 ± 0.27 minutes) in comparison to AH3 fraction **3** (19.2 ± 0.05 minutes) and disaccharide standards; therefore, this fraction comprises most or all of the disaccharides present in HND. The mass spectra of synchronously-eluting peaks of **3** and sucrose, maltose, and cellobiose (included in Appendix 7 as Appendix Figures 35-39) confirm this identification. Figure 4-7 shows the total ion chromatogram of the disaccharide portion of HND, overlaid with disaccharide standards. Figure 4-8 places this in context with the trisaccharides in HND.

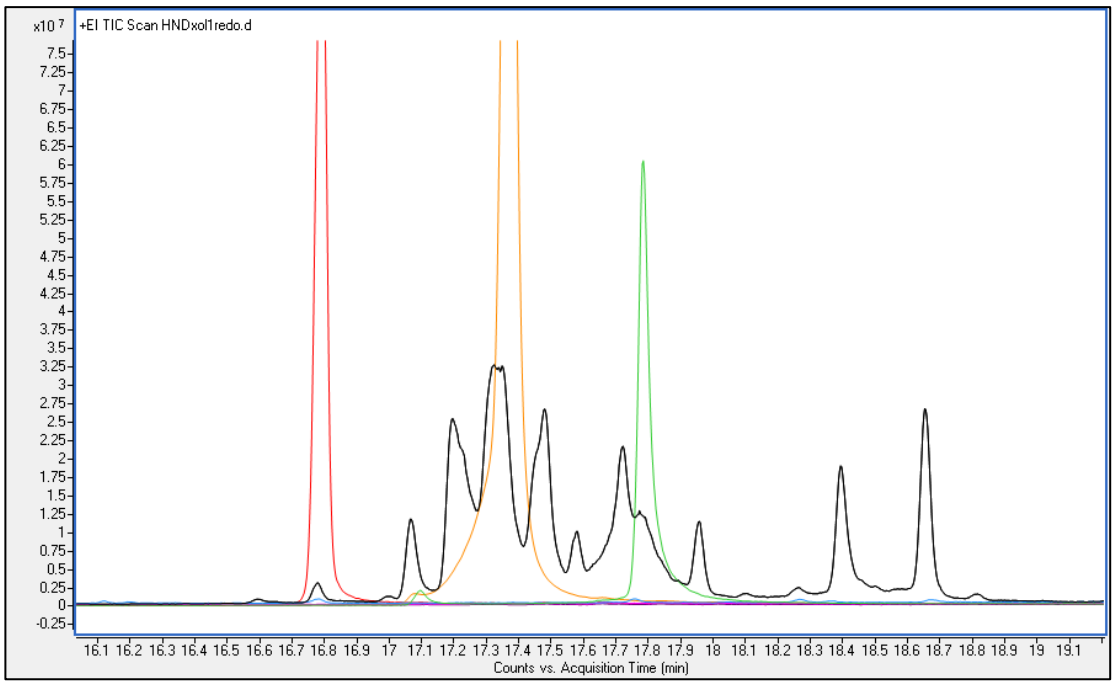

Figure 4-7: Overlaid total ion chromatograms of HND (black), sucrose (red), maltose (orange), and cellobiose (green), showing the disaccharide region of the chromatograms.

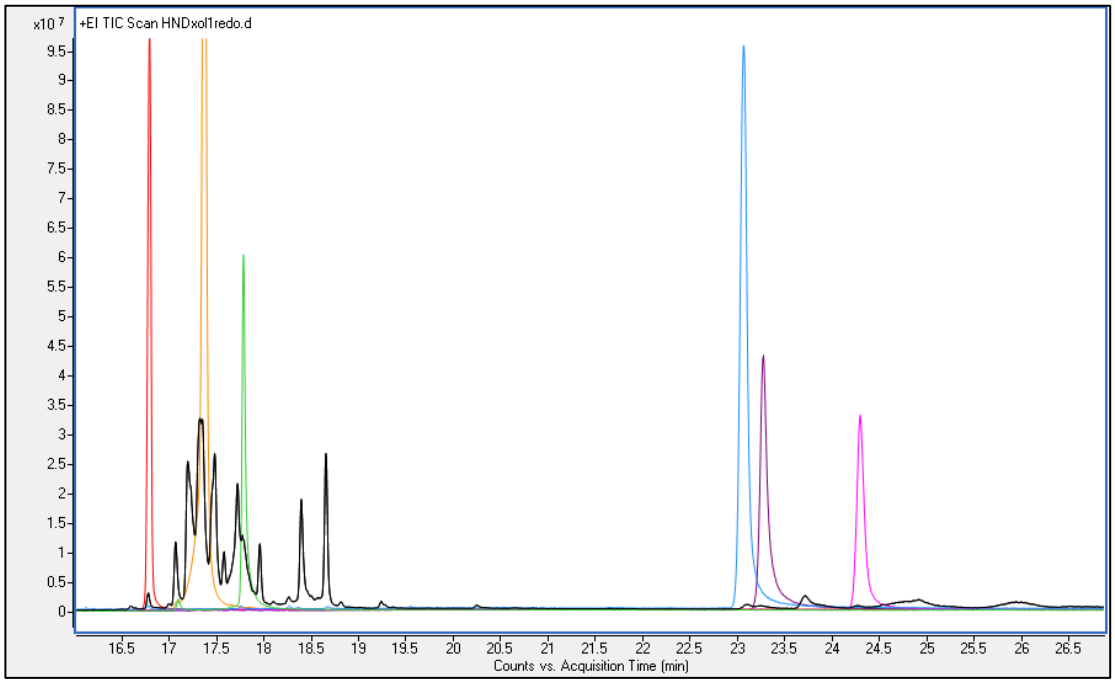

Figure 4-8: Overlaid total ion chromatograms of HND (black), sucrose (red), maltose (orange), cellobiose (green), raffinose (blue), 6-kestose (purple) and melezitose (pink) showing the disaccharide and trisaccharide regions of the chromatograms.

HND fraction 3 contained very little sucrose, and a larger amount of middle-eluting disaccharides, chiefly maltose and later-eluting disaccharides.

4.1.6 Fractions 4 and 5

These fractions were identified by their analytical HPLC average retention times compared to the retention times of AH3 fractions that were conclusively identified by NMR. HND fraction **4** eluted at 21.796 ± 0.264 minutes, cf. AH3's glucose fraction at 21.895 ± 0.215 minutes. HND fraction **5** eluted at 22.969 ± 0.265 minutes, cf. AH3's fructose fraction at 23.063 ± 0.208. It was therefore concluded that HND fraction **4** is glucose and HND fraction **5** is fructose.

4.1.7 Composition of beech honeydew honey

Figure 4-9 shows the difference between analytical results for HPLC and GC-MS methods for analysis of di-, tri-, and tetrasaccharides, with 95% confidence intervals shown as error bars.

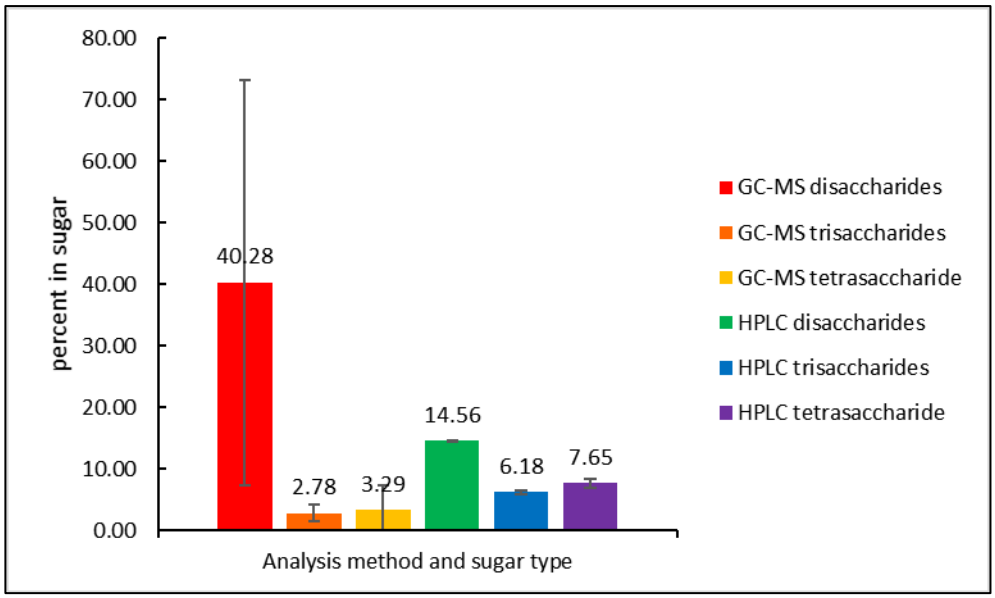

Figure 4-9: Comparison of method results, showing percent in whole honey and 95% confidence interval (error bars) for GC-MS and HPLC triplicate quantitations of the disaccharide, trisaccharide, and tetrasaccharide fractions of HND.

The figure indicates that, as for analysis of AH3 (section 3.2.9), GC-MS may overestimate disaccharides compared to HPLC. The error in GC-MS is high, and the difference in disaccharide estimates between GC-MS and HPLC is not statistically significant at $p \leq 0.05$. GC-MS analysis also underestimates

trisaccharides and tetrasaccharides (these underestimations are statistically significant at $p \leq 0.05$), which should be taken as a caveat in considering individual sugar quantitation results.

The concentrations of each fraction in the honey, presented in Table 4-2, are not adjusted for water content, and represent the amount of the fraction present in a given unit mass of the honey as it is sampled.

*Table 4-2: Analytical HPLC and GC-MS concentrations as percent mass of whole honey, of fractions **1** through **5**, comprising the overall carbohydrate composition of HND. Absolute error is reported as '95% CI'. The bottom row ('water') gives the result obtained in section 3.2.1. Quantities as a percentage of whole honey for **2b** and **3** are reported for both HPLC and GC-MS quantitation.*

		HPLC/GC-MS run				
Fraction	**Identity**	**1**	**2**	**3**	**Average**	**95% CI**
1 Honey %	Gluconic acid	2.527	2.503	2.602	2.544	0.129
2 Honey % HPLC	Tetrasaccharide	2.866	2.392	2.349	2.535	0.301
2 Honey % GC-MS		**2** and **2a** not analyzed separately on GC-MS				
2a Honey % HPLC	Tetrasaccharide	5.146	5.164	5.025	5.112	0.079
2a Honey % GC-MS		**2** and **2a** not analyzed separately on GC-MS				
2+2a Honey % HPLC	Tetrasaccharide	8.012	7.556	7.374	7.647	0.345
2+2a Honey % GC-MS		1.449	4.308	4.121	3.293	1.679
2b Honey % HPLC	Trisaccharides	5.968	6.152	6.431	6.183	0.244
2b Honey % GC-MS		1.008	2.775	4.557	2.780	1.862
3 Honey % HPLC	Disaccharides	14.500	14.464	14.701	14.555	0.098
3 Honey % GC-MS		24.971	47.706	48.171	40.283	10.194
4 Honey %	Glucose	22.327	22.187	22.360	22.291	0.229
5 Honey %	Fructose	44.527	44.570	44.743	44.613	0.285
Water					15.6	-

It should be noted that the percentages given in Table 4-2 do not add up to 100%, as each component was calculated separately and the water content was determined by a different method altogether; however, they provide a reasonable idea of the proportions of each component of the honey.

4.1.8 Comparison of beech honeydew honey carbohydrate composition to literature

Astwood *et al*. estimated the tetrasaccharide α-D-Glc*p*(1→4)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→2)-β-D-Fru*f*, which was identified in their 1998 paper as fraction *2*, and the tetrasaccharide α-D-Glc*p*(1→6)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→2)-β-D-Fru*f*, which was identified as their fraction *3*, to comprise 3.9 and 0.46% respectively, or 4.36% combined, of the total honey mass [41]. The tetrasaccharide portion of HND, fractions 2 and 2a, is estimated by the current analysis' figures to comprise 3.3% by GC-MS, which may underreport oligosaccharides, or 7.65 ± 0.35% by HPLC, of the total honey mass. The literature figure of 4.36% is not particularly unlike the figures obtained in the current analysis, and some variability is to be expected across batches of beech honeydew honey. (Astwood *et al*. also reported 0.51% maltotetraose in the tetrasaccharide portion of New Zealand beech honeydew honey; no indications of this sugar's presence were found in the current analysis of HND.)

Airborne Honey Ltd. analyses of 400 beech honeydew honey samples [43] examined only glucose, fructose, and sucrose. For glucose in New Zealand beech honeydew honey, Airborne reported 22.9 ± 0.19%, and the present analysis returns a figure of 22.3 ± 0.23%. For fructose, Airborne reported 33.8 ± 0.21%, and the present analysis 44.6 ± 0.28%. For sucrose, Airborne reported 0.67 ± 0.08%, Astwood *et al.* [41] 0.55 ± 0.12%, and this analysis, where sucrose was quantified as a single sugar only by GC-MS, 1.09 ± 1.2%.

HND, then, has a similar glucose content to, but 32% more fructose than, the beech honeydew honeys studied in the literature. Despite the relatively high oligosaccharide content of honeydew honey, this high F/G ratio may contribute to preventing its crystallization. The sucrose content result from this analysis may be higher than the literature values, but, as noted, GC-MS analysis tends to

overestimate relative to HPLC analysis, which was not able to quantify individual disaccharides, and the error is high.

No other literature sources for carbohydrate compositions of New Zealand beech honeydew honey were found, but HND's content of oligosaccharides (11.8 ± 1.0%) is comparable to literature sources for other honeydew honeys, with oligosaccharide contents between 8-30% [16-17]. Its monosaccharide content (66.9 ± 0.36%) is somewhat higher than the 45% suggested as standard for a 'generic' honeydew honey [8-9].

4.1.9 *Suitability of beech honeydew honey as a prebiotic functional food*

The recommended dose of prebiotic oligosaccharides to produce a measurable effect is 2-10 g daily [195-197]. To obtain an effective prebiotic dose using honeydew honey alone, a consumer would have to ingest a minimum honey dose calculated by Equation 4-1:

$$2\ \text{g oligosacch.} \div \left(0.0679\ \pm 0.010 \frac{\text{g tetrasacch.}}{\text{g honey}}\right) + \left(0.0618 \pm 0.0024 \frac{\text{g trisacch.}}{\text{g honey}}\right) = 14.4605\ \pm 0.4419\ \text{g honey} \qquad \textit{Equation 4-1}$$

The free sugar dose that unavoidably accompanies the quantity of honey necessary to produce a prebiotic effect using beech honeydew honey alone is calculated by Equation 4-2.

$$\left(\left(0.2229 \pm 0.00229\right)\frac{\text{g Glc}}{\text{g honey}} + \left(0.4461 \pm 0.00285\right)\frac{\text{g Fru}}{\text{g honey}} + \left(0.1456 \pm 0.0098\right)\frac{\text{g Disacch}}{\text{g honey}}\right) \times 14.4605\ \pm 0.4419\ \text{g honey} = 11.7795\ \pm 0.4147\ \text{g free sugars} \qquad \textit{Equation 4-2}$$

The WHO recommended daily maximum dose of free sugars is 30 g [82]. Beech honeydew honey, then, may be capable of independently inducing a prebiotic effect while delivering less than the WHO recommended daily free sugar dose.

5 Identification of crystals in sample GWC

A sample provided by 1839 Ltd. (henceforth referred to as GWC) consisted of crystals filtered out of honey thought by the supplier to have crystallized due to GWA honeydew honey content.

Figure 5-1 and Figure 5-2 are NMR spectra of the washed and dried crystals taken from sample GWC, compared to an equilibrated glucose standard. Minor peaks ~4 ppm in the ^{1}H spectrum, Figure 5-1, and between 70 and 80 ppm in the ^{13}C spectrum, Figure 5-2, are ascribed to contamination with another sugar, probably fructose. The crystals analyzed were not perfectly white, suggesting some of the sticky honey matrix remained. However, the major peak patterns and chemical shifts match closely enough to confidently identify the crystals present in GWC as glucose.

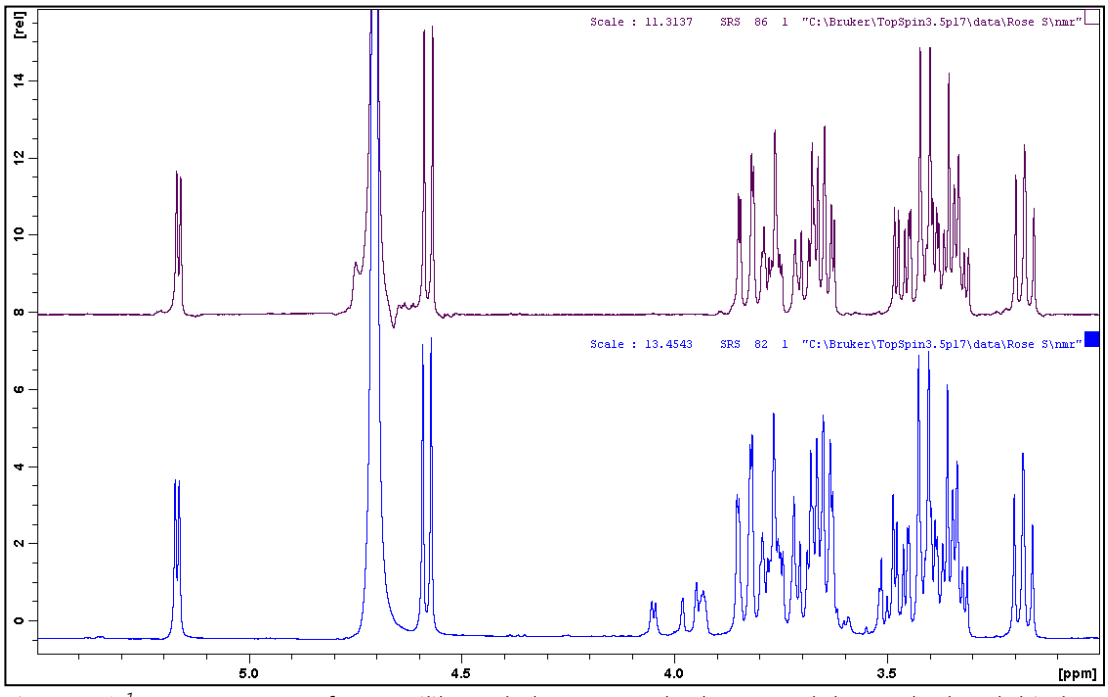

Figure 5-1: ^{1}H NMR spectra of an equilibrated glucose standard (top) and the washed and dried crystals taken from the GWC sample (bottom). The HOD peaks were not suppressed because they occur in the anomeric region and suppression would affect these signals.

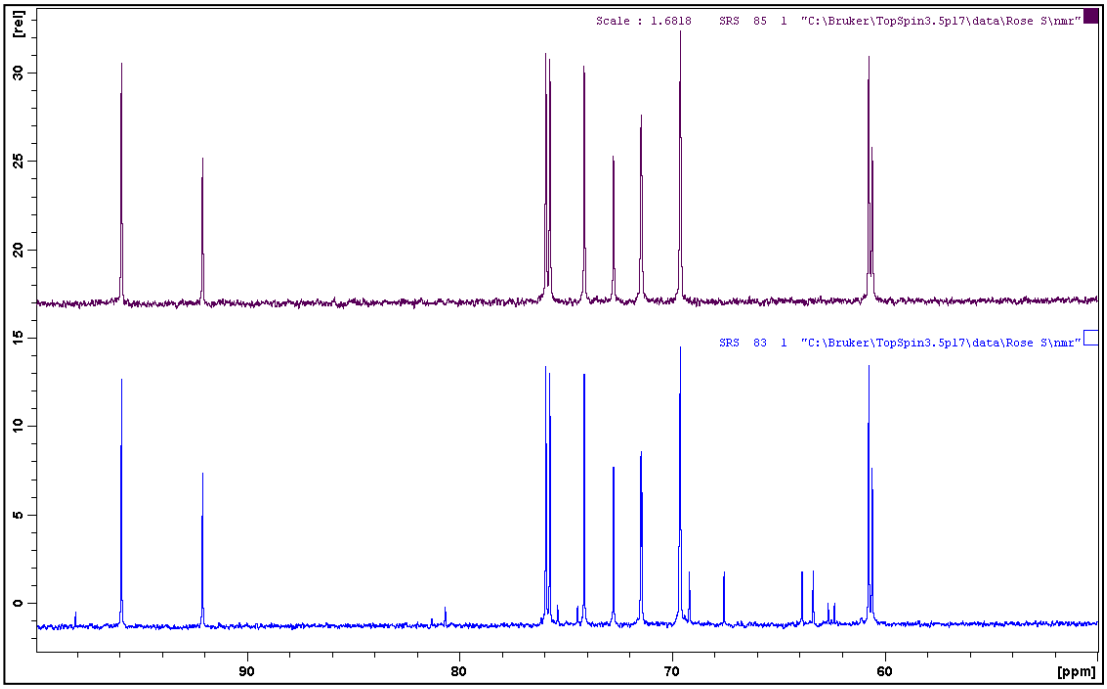

Figure 5-2: ^{13}C NMR spectra of an equilibrated glucose standard (top) and the washed and dried crystals taken from the GWC sample (bottom).

This is interesting, as GWA honeydew honey is widely understood (and has previously been shown) to crystallize due to its high melezitose content. This suggests that the GWC honey sample from which the crystals were filtered did not contain GWA honeydew honey as originally supposed. Since either, or both, of high melezitose content stemming from GWA honeydew in the honey, and a low F/G ratio, can cause a honey sample to crystallize, the crystallization behavior of a low F/G sample may be mistakenly ascribed to GWA honeydew, or vice versa, adding to the confusion in the issue facing New Zealand apiarists.

6 Giant Willow Aphid (GWA) honeydew honey (sample GWH)

6.1 Introduction: problems posed by GWA to New Zealand apiarists

As discussed in section 1.4, the honey produced from Giant Willow Aphid (*Tuberolachnus salignus*, GWA) honeydew is high in melezitose [44, 96, 100, 103, 140-141], which makes it unsuitable as winter food for bees [38, 85-87], and causes it to crystallize in the comb, making it difficult to remove [44, 83-84, 98], gritty, and less sweet than commercially-acceptable honey in New Zealand. However, it has been suggested that honeydew honey in general, and GWA honeydew honey in particular, may be preferred to floral honey in some parts of Europe [9, 96].

6.2 Results and discussion

6.2.1 Observations regarding physical properties

The honey was provided on the frame, so the level of crystallization within the comb could be assessed. Figure 6-1 shows a frame with some areas heavily crystallized and almost dry (expanded in Figure 6-2), and others more liquid (Figure 6-3).

Figure 6-1: Giant Willow Aphid honeydew honey in intact honeycomb, showing heavily crystallized areas at upper right and areas of more liquid honey cells at lower left (Author's photo).

Figure 6-2: Close-up of a heavily crystallized area of Giant Willow Aphid honeydew honey in intact honeycomb, showing fine white crystals in the comb cells (author's photo).

Figure 6-3: Close-up of a less crystallized area of Giant Willow Aphid honeydew honey in intact honeycomb, showing partially-crystallized liquid honey (author's photo).

When the honeycomb scrapings were melted down as detailed in section 2.2, the wax rose to the surface, but initially retained small cells or pockets of the dark-amber liquid honey within the wax layer. Stirring the mixture thoroughly

and holding it at 50 °C overnight overcame this problem. Figure 6-4 shows a batch of GWH immediately after removal from the dry bath, illustrating the discrete layers and approximate ratio of wax and honey. Figure 6-5 shows a different batch, after overnight settling. The paler layer of solid crystals set at the bottom of the beaker as a solid 'concrete' layer that was very difficult to stir in and settled back out within a day. However, when they had been stirred into the liquid once, subsequent settlings did not set hard and were easy to stir in.

From the sample of GWH prepared to be suitable for human consumption, it was observed that the honey was thin, 'runny', and noticeably gritty in texture, because the white crystals seen in Figure 6-5 did not dissolve, despite the high water content (discussed in section 6.2.2). The taste of the honey was fresh, refreshing, slightly woody and acidic, and not overly sweet. The high water content may have allowed some fermentation in the time Honey NZ had the frames in cold storage, and a slight alcohol scent was detected in the whole honey. However, the extent of the fermentation was below the threshold at which ethanol would appear on an HPLC chromatogram, since no such HPLC signal was observed.

Figure 6-4: GWH (separation batch 1) immediately after removing from dry bath, showing wax and debris layer above, and apparently pale opaque honey below.

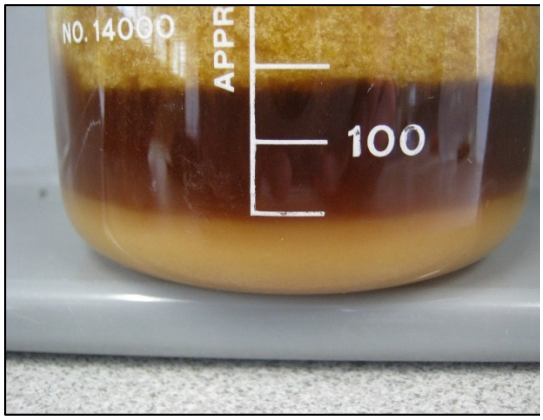

Figure 6-5: GWH (separation batch 2) after overnight settling at 50 °C, showing wax layer above, translucent dark amber liquid honey, and a layer of pale crystals settled out.

6.2.2 Water content and fraction numbering

The water content of whole, creamed GWH was 27.8%. 3706.97 mg of whole honey was weighed out to dry for GC-MS analysis; after drying, this aliquot weighed 2922.77 mg, so the water content of the dried honey was 6.6%.

Figure 6-6 shows the analytical HPLC trace of an aliquot of GWH and introduces the numbering system used to identify GWH fractions.

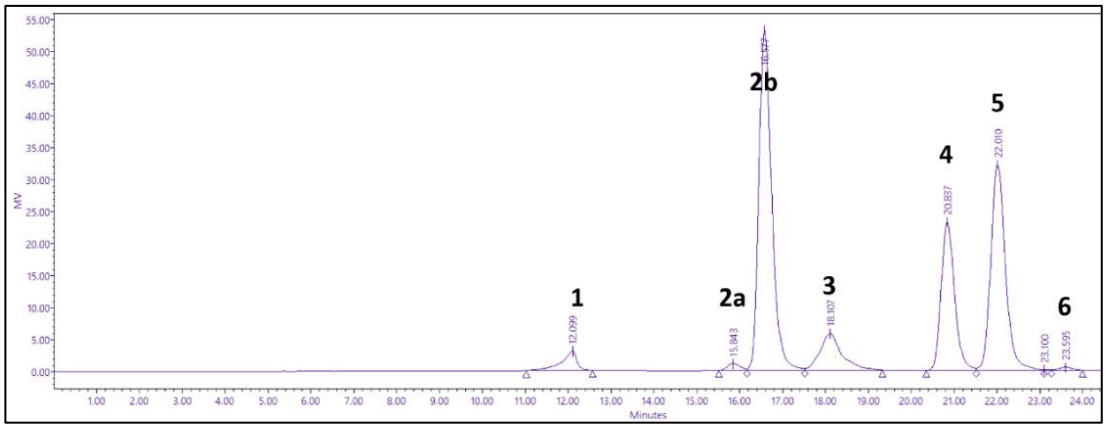

Figure 6-6: Analytical HPLC chromatogram of GWH, showing fraction divisions with the numbering used throughout this chapter.

Figure 6-7 shows the preparative HPLC trace of an aliquot of GWH, with the same numbering system as was used in Figure 6-6. As seen by comparison of Figure 6-7 to Figure 6-8, the preparative trace was not entirely consistent over multiple runs, and the peak labeled as fraction **3** varied in size over several runs. The smaller peak appearing at 70.782 minutes in Figure 6-8 was collected together with the labeled fraction **3**.

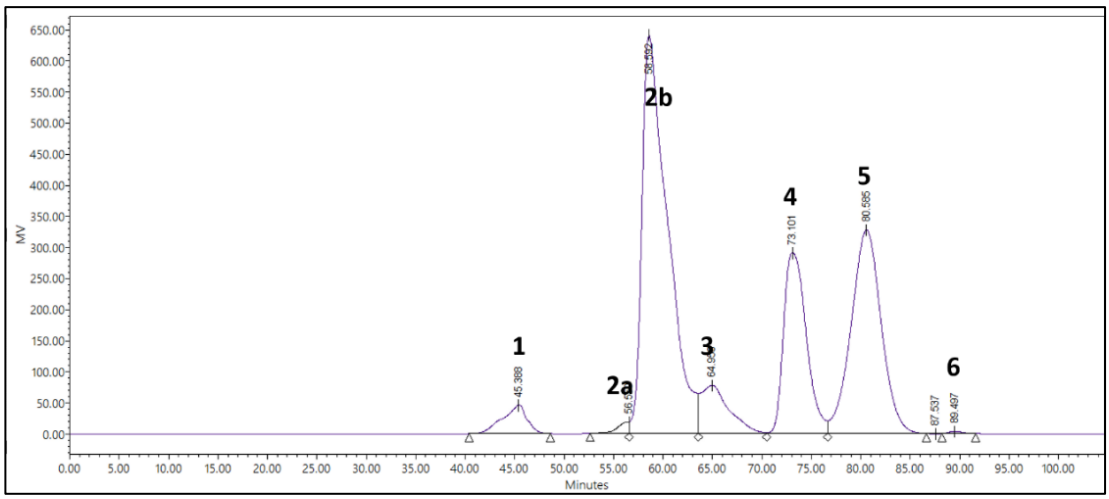

Figure 6-7: Preparative HPLC chromatogram of GWH, showing fraction divisions.

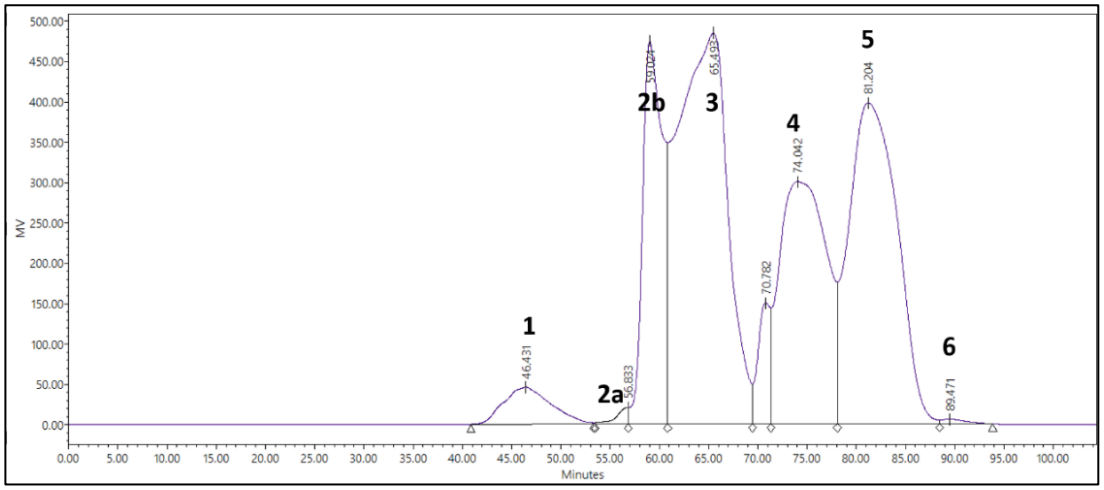

Figure 6-8: A different preparative HPLC chromatogram of GWH, showing fraction divisions.

Table 6-1 lays out the retention time of each HPLC peak for GWH. It was noted that the GWH retention times had all shifted by 0.96 ± 0.16 minute earlier than the AH3 times: this is ascribed to the effects of adhesion of 'clutter' and small non-polar chains on the column packing material. The shift in retention time indicates that the column was almost due for cleaning; however, reinjection of selected standards confirmed that fractions' retention times relative to each other were unchanged, so fractions in GWH could still be identified by their HPLC retention time and order.

The first GWH fraction, GWH/**1**, which was identified by NMR as D-gluconic acid as discussed in section 6.2.3, eluted synchronously with HND/**1**, which was also identified (section 4.1.2) as D-gluconic acid.

Table 6-1: GWH peaks and retention times (HPLC conditions: Shodex KS-G, KS-801, and KS-802 columns plumbed in series and eluted in Type 1 water at 0.8 mL/min at 80 °C, with RI detection), with peak identities. Retention times given are the mean of three determinations, and the error reported is the 95% confidence interval of these determinations.

GWH peak	Peak identity	Ret. time (min)
1	D-gluconic Acid	12.11 ± 0.03
2a	Tetrasaccharides	15.85 ± 0.02
2b	Trisaccharides	16.59 ± 0.01
3	Disaccharides	18.12 ± 0.01
4	Glucose	20.85 ± 0.02
5	Fructose	22.02 ± 0.02

6.2.3 Fraction 1

On reinjection of **1** collected by preparative HPLC, the fraction split into two peaks, which were collected separately as **1** (retention time 44.63 minutes) and **1a** (retention time 47.15 minutes). As seen in Figure 6-9, the ^{13}C NMR spectra of both **1** and **1a** match that of a D-gluconic acid standard. It is thought that preparative fractions **1** and **1a** (not resolved on analytical HPLC) may represent the acid and lactone forms of D-gluconic acid, respectively; however, these are essentially the same compound and quantified as such.

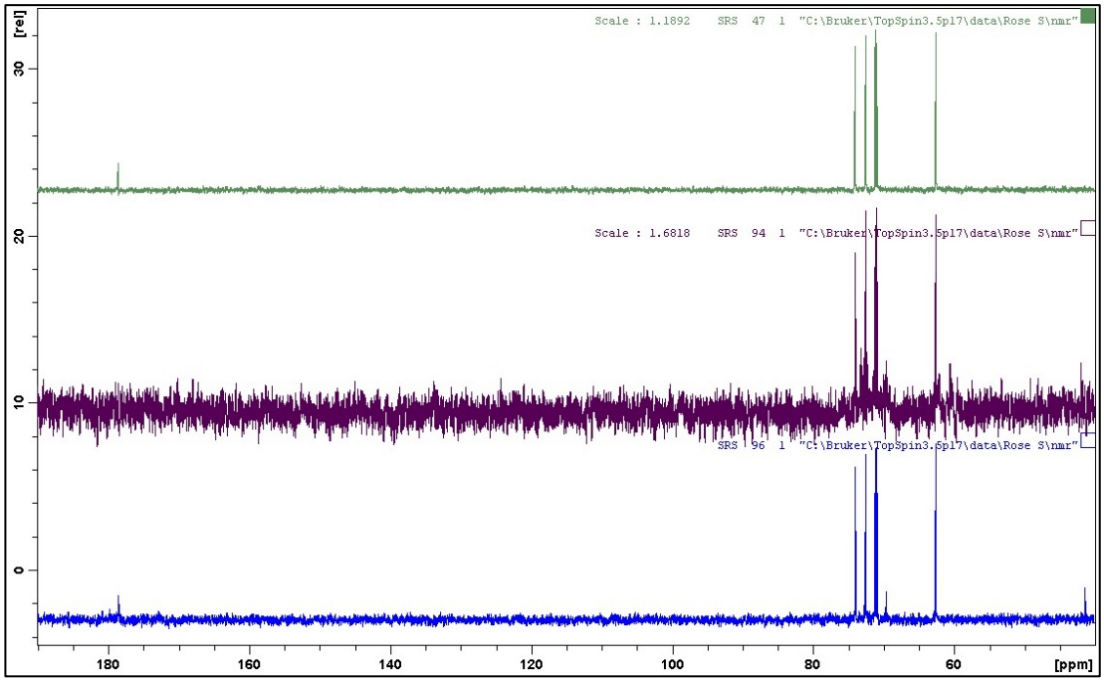

*Figure 6-9: ^{13}C NMR spectra of D-gluconic acid (top), GWH fraction **1** (middle) and GWH fraction **1a** (bottom).*

Although salicylic acid is suggested as a biomarker for GWA honeydew honey, it has previously been estimated to be present in concentrations 0.0014-0.0041% [44], and does not appear on the chromatogram using RI detection. Detection and quantitation of salicylic acid in GWH were achieved using UV-band detection on a Waters PDA detector, as the aromatic ring and conjugated carbonyl in the acid act as UV chromophores in a way sugars do not. 296 nm was selected as the wavelength at which the 3D UV chromatogram was sliced, in order to avoid the D-gluconic acid peak (12.11 ± 0.03 min, maximum peak intensity at 210 nm) and consider only the salicylic acid peak (12.29 ± 0.09 min, maximum peak intensity at 296 nm). However, the nearness of these elution times suggests that on the RI chromatograms used for quantitation of D-gluconic acid, the salicylic acid and D-gluconic acid peaks were not resolved. The concentration of salicylic acid in each of the triplicate honey solutions analyzed was subtracted from the concentration of D-gluconic acid in the same solution, to ensure no 'double-counting' occurred. As the salicylic acid

concentration was almost two orders of magnitude less than the D-gluconic acid concentration, this did not make a significant difference to the D-gluconic acid quantitation results. The salicylic acid concentration was also too low for it to appear on the NMR spectra of GWH fraction **1**.

The quantitation results (summarized in Table 6-2 on page 91) return a figure of 0.054% salicylic acid in GWH, considerably higher than the previous estimate [44]. Depending on the species of willow, the concentration of salicylic acid in the whole willow tissue ranges from 0.05-0.30% [198]. Thus, 0.054% in willow sap, and therefore in willow-sap-based honey, does not seem unreasonable, particularly since the willow sap and the GWA honeydew contain more water than the honey, and the honey-making process in the comb concentrates the salicylic acid originally present in the honeydew.

6.2.4 Fraction 2a

Not enough of GWH fraction **2a** could be collected by preparative HPLC to identify by NMR. The retention time coincided with HND fraction **2a** (16.7 ± 0.27 minutes), identified in section 4.1.3 as a mixture of tetrasaccharides including α-D-Glc*p*(1→4)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→2)-β-D-Fru*f* and α-D-Glc*p*(1→6)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→2)-β-D-Fru*f*, so GWH fraction **2a** is also identified as consisting of tetrasaccharides.

6.2.5 Fraction 2b

Fraction **2b** came off as a single discrete peak on preparative HPLC injection, and, on reinjection, could easily be separated from residual traces of fractions **2a** and **3**. Figure 6-10 and Figure 6-11 show NMR spectra of GWH fraction **2b** compared to a standard of melezitose. The chemical shifts and relative peak heights are similar enough to confidently identify fraction **2b** as consisting

entirely or mostly of melezitose. (Expanded and labeled versions of the spectra in Figure 6-11 are shown in Appendix Figure 8 and Appendix Figure 9.)

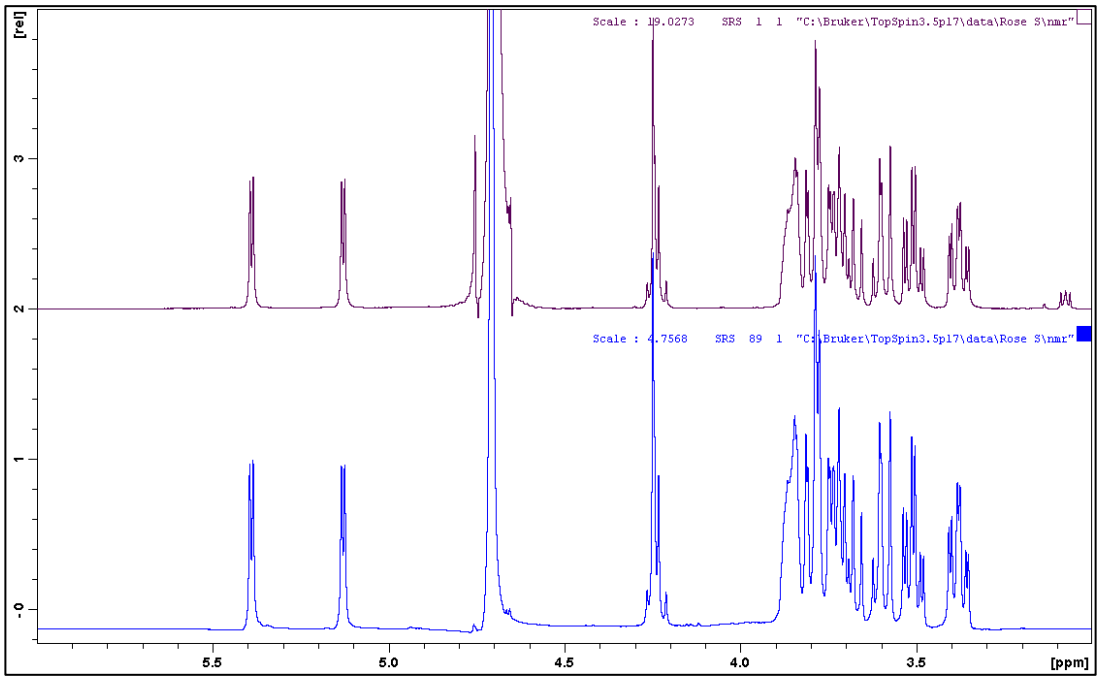

*Figure 6-10: ^{1}H NMR spectra of melezitose standard (top) and GWH fraction **2b** (bottom). The HOD peaks were not suppressed because they occur in the anomeric region and suppression would affect these signals.*

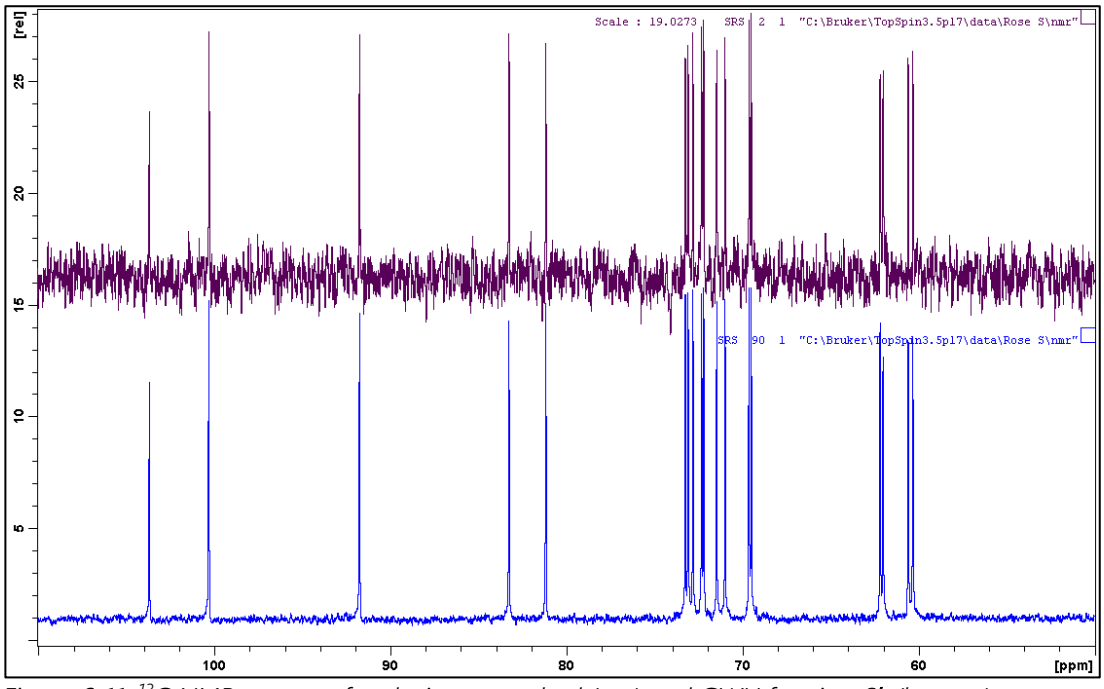

*Figure 6-11: ^{13}C NMR spectra of melezitose standard (top) and GWH fraction **2b** (bottom).*

Figure 6-12 shows the GC-MS total ion chromatogram of the disaccharide and trisaccharide regions of GWH; the tall black peak to the far right of the chromatogram, coinciding with the peak of the melezitose standard, is clearly visible, as is the very low level of other trisaccharides. Melezitose and 6-kestose were also identified by comparison of standard mass spectra to the mass spectra of synchronously-eluting GWH peaks.

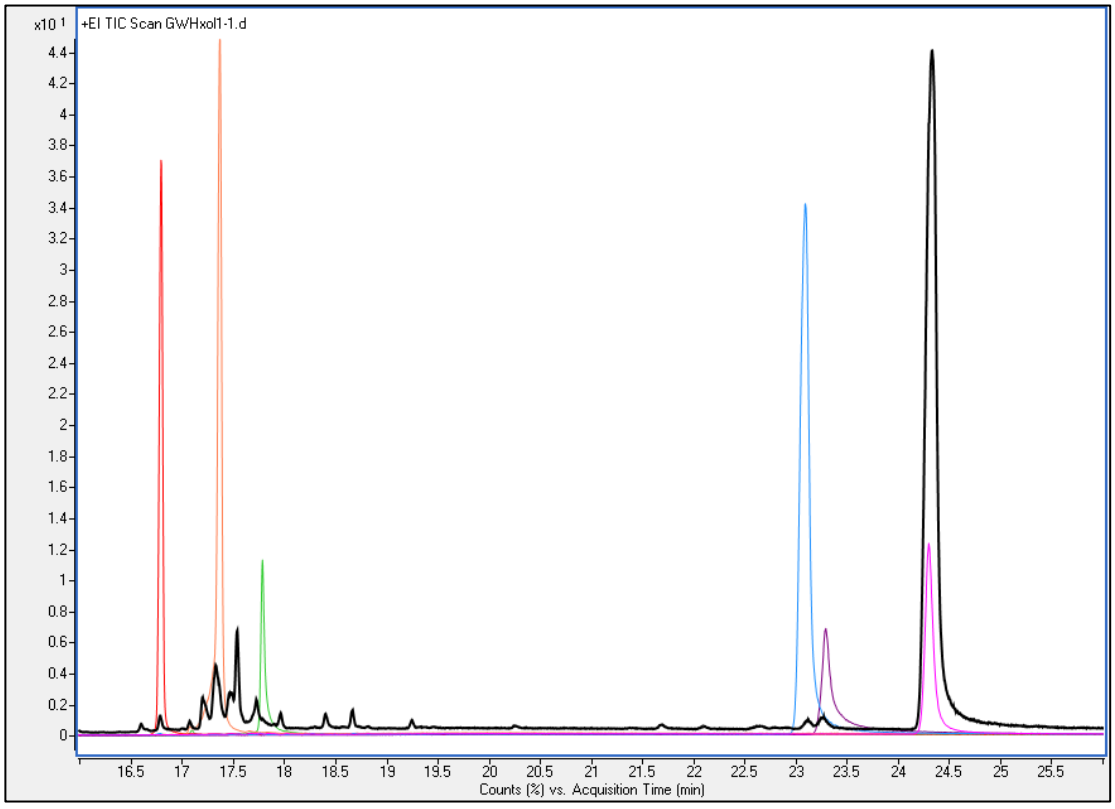

Figure 6-12: Overlaid total ion chromatograms of GWH (black), sucrose (red), maltose (orange), cellobiose (green), raffinose (blue), 6-kestose (purple) and melezitose (pink), showing the disaccharide and trisaccharide regions of the chromatograms.

6.2.6 Fraction 3

The HPLC retention time of fraction **3** compared to the disaccharide fraction of AH3, and to disaccharide standards, identifies it as containing the GWH disaccharides. On second preparative injection, the first peak to elute proved to be melezitose, indicating contamination from the much higher GWH/**2b**; the

second peak, appearing at the same retention time as the original fraction **3**, was collected as GWH/**3**. A ^{1}H NMR spectrum of **3** is shown in Figure 6-13.

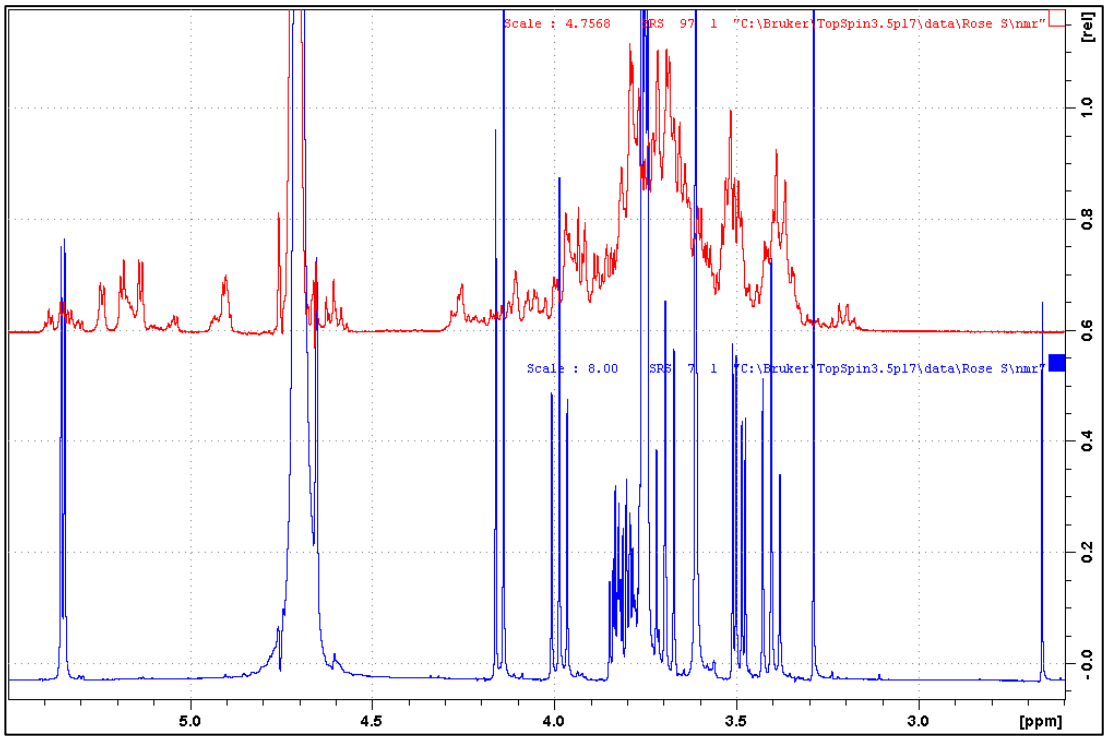

*Figure 6-13: ^{1}H NMR spectra of GWH fraction **3** (top) and sucrose (bottom, expanded to overlay the **3** spectrum). The HOD peaks were not suppressed because they occur in the anomeric region and suppression would affect these signals.*

The downfield doublet in the sucrose spectrum (representing the proton on C1 of the glucose moiety) coincides with one of the collection of doublets seen downfield in the **3** spectrum. As this doublet is so small, most of the rest of the sucrose spectrum is probably lost among the convoluted ring-proton signals. As shown by the chromatogram in Figure 6-15, sucrose is a very minor disaccharide in GWH, so the low intensity of the relevant NMR signals is unsurprising. Figure 6-14 compares ^{13}C NMR spectra of maltose, sucrose, and GWH fraction **3**.

The ^{13}C NMR spectrum of the GWH disaccharide region has major peaks that superficially resemble maltose in the three distinct, evenly-spaced peaks far downfield, and the upfield -$\underline{C}H_2$- peaks very close to each other. This is different to the three -$\underline{C}H_2$- peaks characteristic of sucrose. The GC-MS chromatogram of

the disaccharide portion of GWH (shown as Figure 6-12 on page 85) show that the major disaccharides are maltose, and a sugar eluting very shortly after it; this may be isomaltose, based on its literature retention time [188] and its structural similarities to maltose, which would produce a similar NMR spectrum.

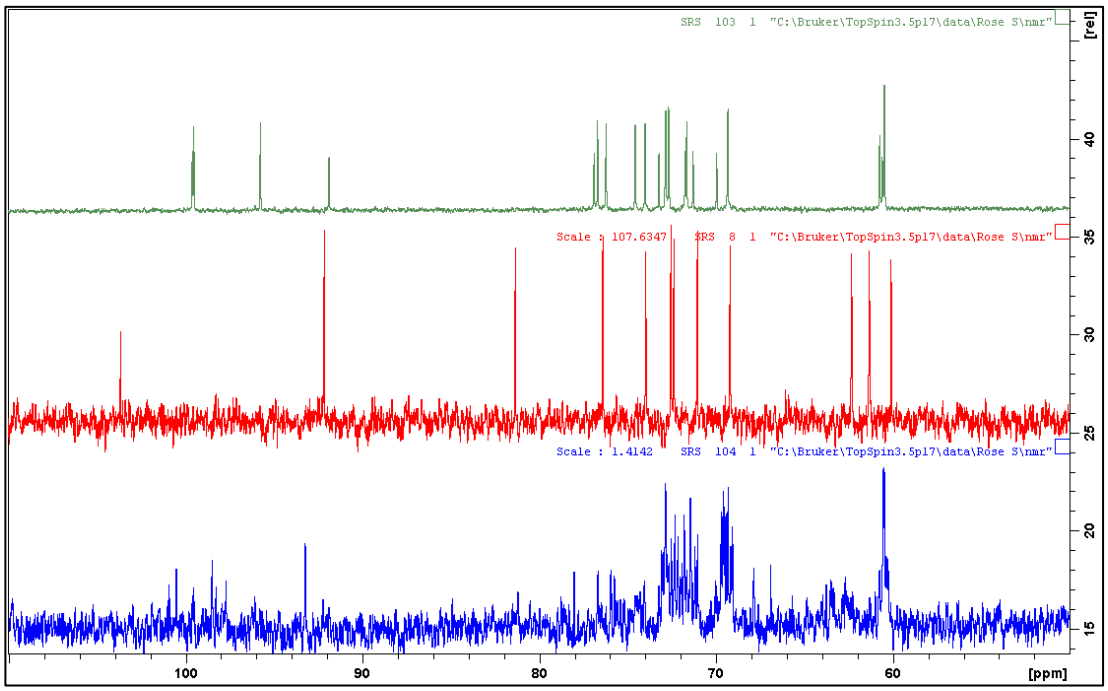

*Figure 6-14: ^{13}C NMR spectra of maltose (top), sucrose (middle), and GWH fraction **3** (bottom).*

Figure 6-15 is an enlargement of the disaccharide region of the total ion chromatogram of GWH. This honey contains very little sucrose, some maltose, and rather more of a disaccharide eluting between maltose and cellobiose (~17.55 minutes). The mass spectrum of this later-eluting disaccharide could not be conclusively identified; however, it is tentatively thought that it may be maltulose, due to peaks in the mass spectrum indicative of a glucose 1→4 linkage and a fructofuranose ring [125, 199]. Its retention time immediately after maltose under similar chromatography conditions [200] supports this identification. Alternatively, as maltose is a reducing sugar and undergoes mutarotation in solution (including in honey), this disaccharide peak and the

peak identified as maltose may simply represent the α and β anomers of maltose.

In this sense the disaccharide region of GWH is distinctly dissimilar to AH3 and HND (Figure 3-11 and Figure 4-7, respectively), both of which feature maltose at ~17.3 min predominantly, with less of the later-eluting disaccharide peak.

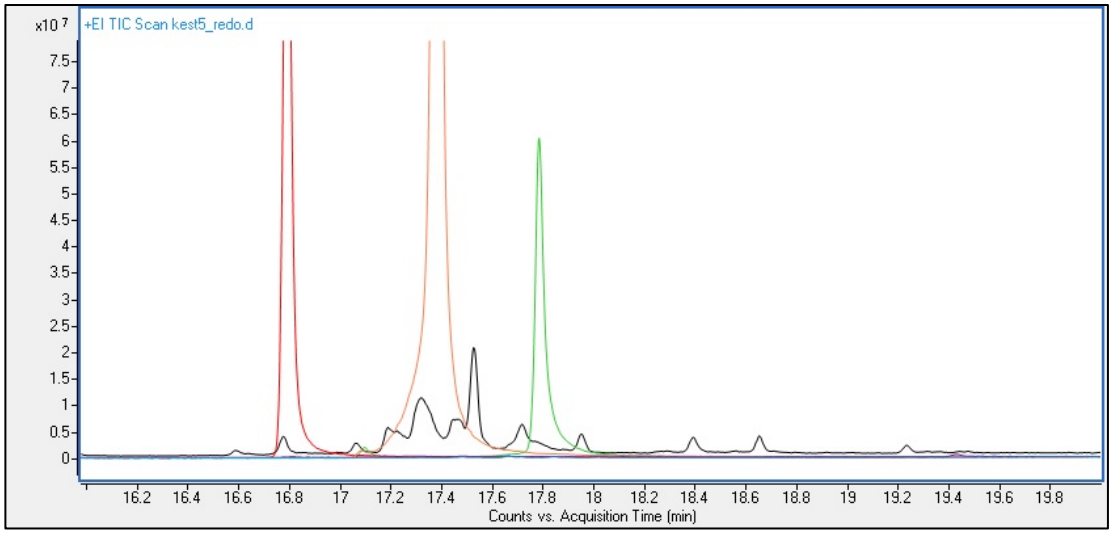

Figure 6-15: Enlargement of the disaccharide region of Figure 6-12; overlaid total ion chromatograms of GWH (black), sucrose (red), maltose (orange), and cellobiose (green).

6.2.7 Fraction 4

Fraction **4** was identified by its retention time (both absolute and relative to other identified GWH fractions) as glucose.

6.2.8 Fraction 5

Fraction **5** was identified by its retention time (both absolute and relative to other identified GWH fractions) as fructose.

The fraction identified in Figure 6-6 as **6** could not be collected in sufficient quantity for NMR identification.

6.2.9 Crystal identification

After the wax and honey of sample GWH were scraped off level with the plastic frame, fine white crystals remained in the frame wells, as shown in Figure 6-16, and were collected for analysis.

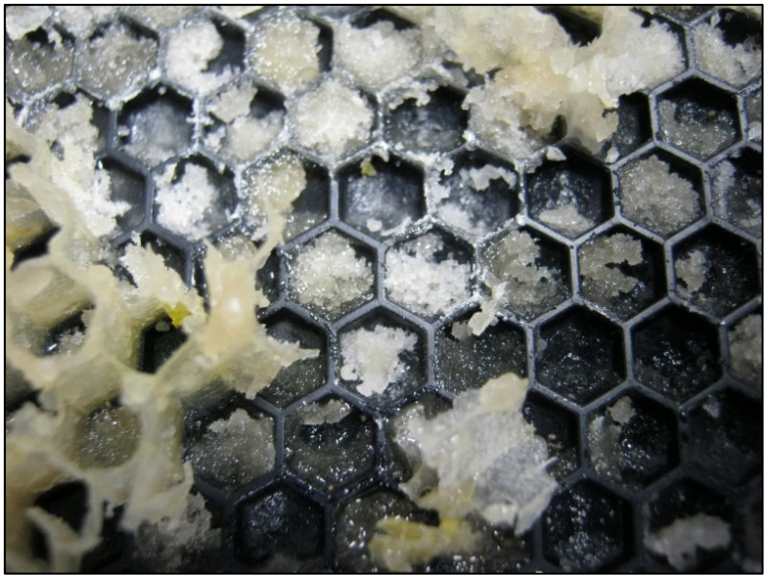

Figure 6-16: Close up of the heavily-crystallized area of GWA honeydew honey comb, after the wax was scraped off, showing white crystals collected in the frame wells (author's photo).

On a melting-point microscope, the GWH frame crystals began to show signs of softening at 140 °C, and were fully liquid at 156 °C, close to the literature value of 153 °C for the melting point of melezitose [201-202].

NMR spectra of these crystals, compared to a melezitose standard, are shown in Figure 6-17 and Figure 6-18. As with the GWC crystals, the GWH frame crystals were not entirely clean of honey, and some minor contamination peaks appeared at similar shifts to the GWC spectra (~4 ppm on ^{1}H and 70-80 ppm on ^{13}C spectra). However, there are sufficient similarities in chemical shifts and relative peak heights to identify the sugar that crystallized out of the GWH sample into the frame wells as melezitose.

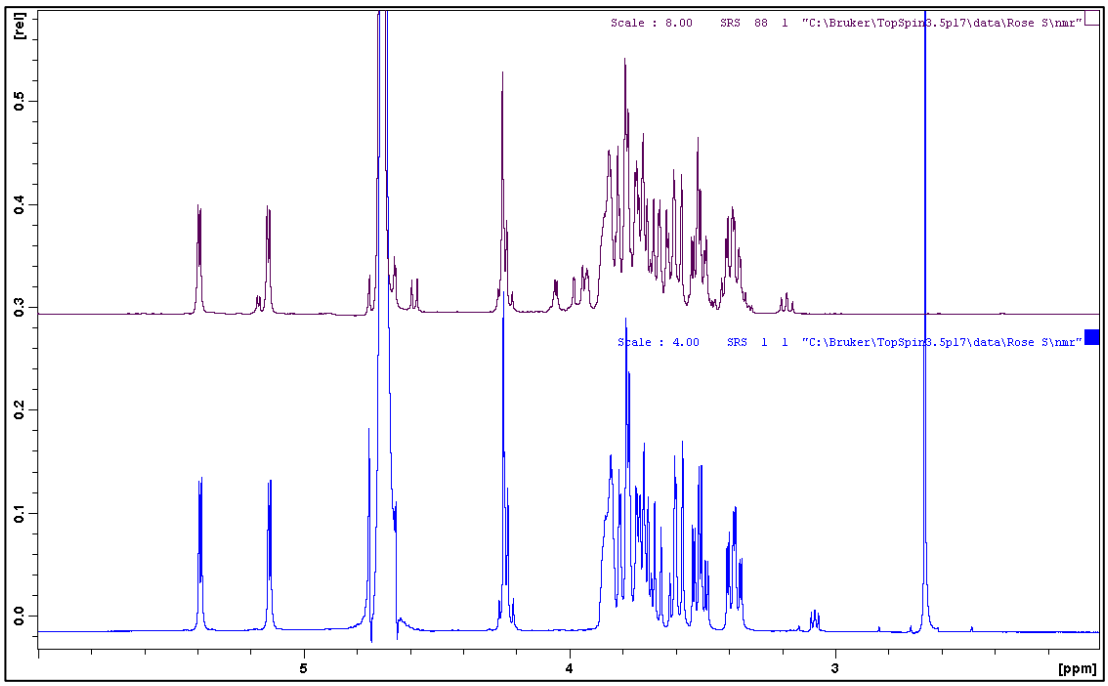

Figure 6-17: ^{1}H NMR spectra of the white crystals taken from the GWH honeycomb frame (top) and a melezitose standard (bottom). The HOD peaks were not suppressed because they occur in the anomeric region and suppression would affect these signals.

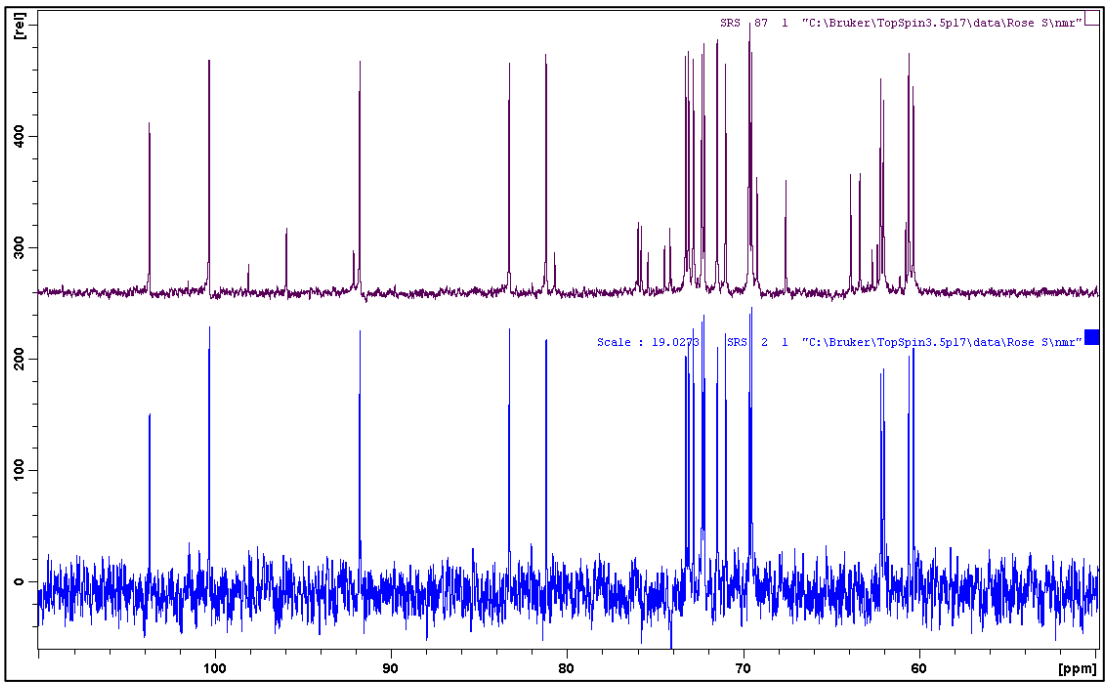

Figure 6-18: ^{13}C NMR spectra of the white crystals taken from the GWH honeycomb frame (top) and a melezitose standard (bottom).

6.2.10 Composition of Giant Willow Aphid honeydew honey

The concentrations of each fraction in the honey, presented in Table 6-2, are not adjusted for water content, and represent the amount of the fraction present in a given unit mass of the honey as it is sampled.

*Table 6-2: Analytical HPLC and GC-MS concentrations as percent mass of whole honey, of fractions **1** through **5**, comprising the overall carbohydrate composition of GWH. Absolute error is reported as '95% CI'. The bottom row ('water') gives the result obtained in section 3.2.1). Quantities as a percentage of whole honey for **2b** and **3** are reported for both HPLC and GC-MS quantitation. Salicylic acid was quantified by HPLC with UV detection using the same honey solutions used for the other HPLC quantitations.*

		HPLC/GC-MS run				
Fraction	**Identity**	**1**	**2**	**3**	**Average**	**95% CI**
1 honey %	Gluconic acid	2.496	2.393	2.444	2.445	0.128
2 honey %	Tetrasaccharides	0.728	0.731	0.748	0.735	0.011
2a honey % HPLC	Trisaccharides	36.823	37.187	36.998	37.003	0.191
2a honey % GC-MS	Trisaccharides	16.134	1.425	1.965	6.508	8.753
3 honey % HPLC	Disaccharides	36.894	36.913	37.514	37.107	0.271
3 honey % GC-MS	Disaccharides	52.823	23.024	19.836	31.894	13.986
4 honey %	Glucose	16.391	16.311	16.917	16.540	0.818
5 honey %	Fructose	24.509	24.342	25.201	24.684	1.131
Salicylic acid honey %	Salicylic acid	0.057	0.057	0.048	0.054	0.013
Water					27.8	-

Figure 6-19 shows the difference between analytical results for HPLC and GC-MS methods, with 95% confidence intervals shown as error bars.

The figure indicates that, unlike the analysis of AH3 (section 3.2.9) and HND (section 4.1.8), GC-MS underestimates both disaccharides and trisaccharides in GWH compared to HPLC. However, the error in GC-MS is higher than that in HPLC and the GC-MS analysis' underestimate of disaccharides is not statistically significant at $p \leq 0.05$. However, the difference in trisaccharide estimates between GC-MS and HPLC is statistically significant at $p \leq 0.05$, which should be taken as a caveat in considering individual sugar quantitation results.

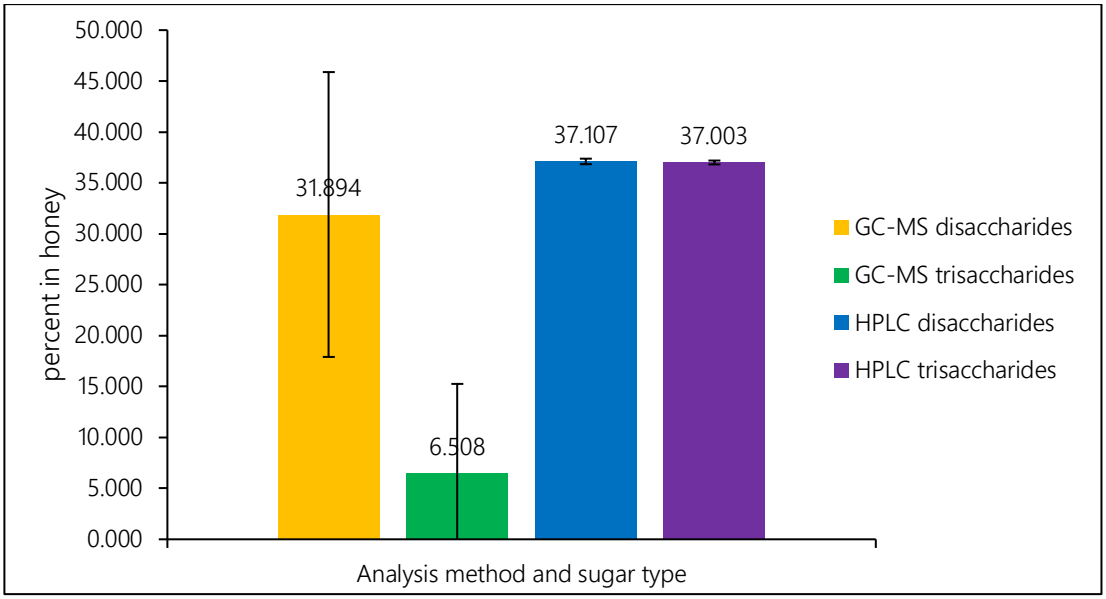

Figure 6-19: Comparison of method results, showing percent in whole honey and 95% confidence interval (error bars) for GC-MS and HPLC triplicate quantitations of the disaccharide and trisaccharide fractions of GWH.

6.2.11 Comparison of GWA honeydew honey composition to literature

No existing reliable literature for the full carbohydrate composition of GWA honeydew honey was found.

The salicylic acid content was higher than has been previously determined.

Airborne Honey Ltd. determined that honeys with a F/G ratio <1.25 will crystallize and honeys with F/G >1.64 will generally not; GWH had F/G = 1.5, so its monosaccharide composition may not be solely responsible for its crystallization behavior.

As noted by various Swiss apiarists, and in section 6.2.1, honey has been observed to crystallize when the melezitose content is ≥14% [98, 103, 140]; obviously the 37% trisaccharides in GWH, of which the vast majority is melezitose, is over this limit and makes crystallization practically certain. The monosaccharide content of GWH (41.224 ± 1.395%) is slightly lower than the expected monosaccharide content of a generic honeydew honey [8-9], and the oligosaccharide content (37.800 ± 0.192%) is well over the expected

oligosaccharide content of a generic honeydew honey [16-17], making GWH an atypical honeydew honey.

6.2.12 Suitability of GWA honeydew honey as a prebiotic functional food

The recommended dose of prebiotic oligosaccharides to produce a measurable effect is 2-10 g daily [195-197]. To obtain an effective prebiotic dose using GWA honeydew honey alone, a consumer would have to ingest a minimum honey dose calculated by Equation 6-1:

$$2\ \text{g oligosacch.} \div \left(0.0075\ \pm 0.0001 \frac{\text{g tetrasacch.}}{\text{g honey}}\right) + \left(0.3700 \pm 0.0019 \frac{\text{g trisacch.}}{\text{g honey}}\right) = 5.2997 \pm 0.0268\ \text{g honey} \quad \textit{Equation 6-1}$$

The free sugar dose accompanying the honey necessary to produce a prebiotic effect using GWA honeydew honey alone is calculated by Equation 6-2.

$$\left((0.1654 \pm 0.0082)\frac{\text{g Glc}}{\text{g honey}} + (0.2468 \pm 0.01131)\frac{\text{g Fru}}{\text{g honey}} + (0.3711 \pm 0.0027)\frac{\text{g Disacch}}{\text{g honey}}\right) \times 5.2997 \pm 0.0268\ \text{g honey} = 4.1513\ \pm 0.09634\ \text{g free sugars} \quad \textit{Equation 6-2}$$

The WHO recommended daily maximum dose of free sugars is 30 g [82]. GWA honeydew honey, then, may be capable of independently inducing a prebiotic effect while delivering less than the WHO recommended daily free sugar dose.

7 GWA honeydew honey as a prebiotic functional food product

Although, as discussed in section 6.2.12, GWA honeydew honey may be capable of delivering an effective prebiotic dose of oligosaccharides without exceeding the recommended free sugar intake, it is a thin, 'gritty' honey with a slightly acidic taste, and is unlikely to be commercially acceptable in its raw form. Two approaches were investigated in a preliminary study of the feasibility of GWH as a marketable prebiotic functional food. A method of making the honey into candy, and a method of separating out the melezitose crystals in a form that would be acceptable as a food supplement, were developed.

7.1 Making GWA honeydew honey into high-oligosaccharide toffee

Some of the sample GWH collected was made into candy, as detailed in section 2.9. Soft crack stage was reached at 138 °C, and hard crack at 142 °C. A very slight scorching smell was also noticed at this temperature. The liquid candy poured into a dish to cool is shown in Figure 7-1, to illustrate that the color is not noticeably different to the color of the liquid portion of the raw honey (middle layer in Figure 6-5) and that the melezitose crystals have all dissolved. The candy behaved in all respects like a toffee made of ordinary table sugar, but displayed a stronger tendency to stick to glassware, and was somewhat less troublesome to 'pull', than toffee made from table sugar. After 7 days, the pulled, cut-up candy exposed to atmosphere was sticky on the surface but still solid, and the candy stored under vacuum in a desiccator to mimic the airtight conditions of individually-sealed commercially-available candy, shown in Figure 7-2, remained hard and not sticky.

Figure 7-1: Toffee made from GWH, poured into a glass dish to cool. Lines on background paper are 7 mm apart, for scale.

Figure 7-2: Toffee made from GWH, after taffy-pulling and cutting into pieces, shown in the vacuum desiccator in which it was stored, with scale bars to show the size of the pieces.

Although it was expected that some water was lost in the candy-making process, so that more of both oligosaccharides and free sugars would be present per unit mass of toffee than of whole GWH honey, HPLC analysis of a crushed toffee revealed that the overall carbohydrate composition of the honey did not change. As seen in Table 7-1, there is a discernible increase per unit mass of sample of all the sugars considered, due to water loss; this increase is statistically significant at $p \leq 0.05$. However, the ratio of oligosaccharides to free sugars is unchanged between honey and toffee. The percentages provided in the table do not add up to 100: this is because each sugar was quantified individually and any possible water remaining in the toffee could not be measured. However, they provide a reasonable idea of the proportions of each component in the honey.

Table 7-1: Analytical HPLC peak areas, and concentrations as percent mass of whole honey, of trisaccharides, disaccharides, glucose and fructose, comprising the overall carbohydrate composition of toffee made from GWH. Absolute error is reported as '95% CI'. The second row of each section of the table provides a comparison between the result for whole honey (detailed in Table 6-2 in section 6.2.10) and the result for toffee.

		HPLC run				
Fraction	Identity	1	2	3	Average	95% CI
2a Toffee %	Trisaccharides/s	44.542	45.292	44.884	44.906	0.394
cf. Honey %	Trisaccharides/s	36.823	37.187	36.998	37.003	0.191
3 Toffee %	Disaccharides/s	44.768	51.249	51.373	49.130	3.965
cf. Honey %	Disaccharides/s	36.894	36.913	37.514	37.107	0.370
4 Toffee %	Glucose	19.765	17.734	18.066	18.521	2.707
cf. Honey %	Glucose	16.391	16.311	16.917	16.540	0.818
5 Toffee %	Fructose	28.438	27.741	27.680	27.953	1.045
cf. Honey %	Fructose	24.509	24.342	25.201	24.684	1.131

One toffee as shown in Figure 7-2 weighs ~5 g. This mass is considered reasonable in comparison to a commercially available Vicks™ honey and menthol cough lozenge at ~4 g; the lozenge was selected to provide an example of acceptable size for commercially available para-medicinal candy products. A 5-g GWH toffee delivers a total oligosaccharide dose calculated by Equation 7-1:

$$0.4491 \pm 0.00394 \frac{\text{g oligosacch.}}{\text{g toffee}} \times 5 \text{ g toffee} = 2.2453 \pm 0.0197 \text{ g oligosacch.} \quad \textit{Equation 7-1}$$

The required dose of prebiotic oligosaccharides to produce a measurable effect is 2-10 g daily [195-197]. To obtain an effective prebiotic dose, a consumer would have to ingest a minimum toffee dose calculated by Equation 7-2:

$$2 \text{ g oligosacch.} \div 0.4491 \pm 0.00394 \frac{\text{g oligosacch.}}{\text{g toffee}} = 4.4534 \pm 0.0175 \text{ g toffee} \quad \textit{Equation 7-2}$$

The free sugar dose that unavoidably accompanies the quantity of toffee necessary to produce a prebiotic effect using toffee alone is calculated by Equation 7-3.

$$\left((0.1852 \pm 0.02707)\frac{\text{g Glc}}{\text{g toffee}} + (0.2795 \pm 0.01045)\frac{\text{g Fru}}{\text{g toffee}} + (0.4913 \pm 0.0365)\frac{\text{g Disacch}}{\text{g toffeee}}\right) \times 4.4538 \pm 0.0175\ \text{g toffee} = 4.2580 \pm 0.0342\ \text{g free sugars} \quad \textit{Equation 7-3}$$

It is clear, then, that if melezitose proves to be prebiotic in *in vitro* and *in vivo* trials, the minimum effective prebiotic dose may be obtained from GWH toffee alone, while remaining well within the recommended free sugar intake [82] and the Food Standards requirement alluded to in section 3.1.1 [151] (that if a health claim is made for a food, an amount of that food necessary to substantiate that claim must be likely to be consumed in a normal diet).

7.2 Extracting solid melezitose from GWA honeydew honey

The aliquots of GWH and the aluminum separating apparatus discussed here were introduced in section 2.10 (*Separating melezitose crystals from Giant Willow Aphid honeydew honey sample GWH*).

7.2.1 GWH separation Aliquot 1

After washing with ice water, the crystals dissolved upon returning to room temperature, and did not recrystallize on chilling to 4 °C. This aliquot developed mold before further recrystallization attempts could be made, and was discarded.

7.2.2 GWH separation Aliquot 2

Immediately on shaking with ice-cold ethanol, the settled crystals in the sticky matrix formed a single hard ball that resisted separation by warming.

7.2.3 GWH separation Aliquot 3

The initial separation and washing of the crystals yielded 0.61360 g (10.14% of initial honey mass) of very fine solid material that formed friable clumps. The color of the material could be approximated as Pantone 2001C or Hex color #F9F0AE. Dissolution of this initial solid was difficult and required extended stirring in a water bath at 100 °C. The solution resisted recrystallization initially but crystallized on protracted freezing (-18 °C, two days). The recrystallized, filtered solids had the same texture as before, but the color had lightened to approximately Hex color #FCFCE2, as shown in the vial on the right in Figure 7-4. The HPLC chromatogram of the recrystallized sample dissolved in Type 1 water is shown below as Figure 7-3; it can be seen from this that the crystals are pure or almost pure melezitose.

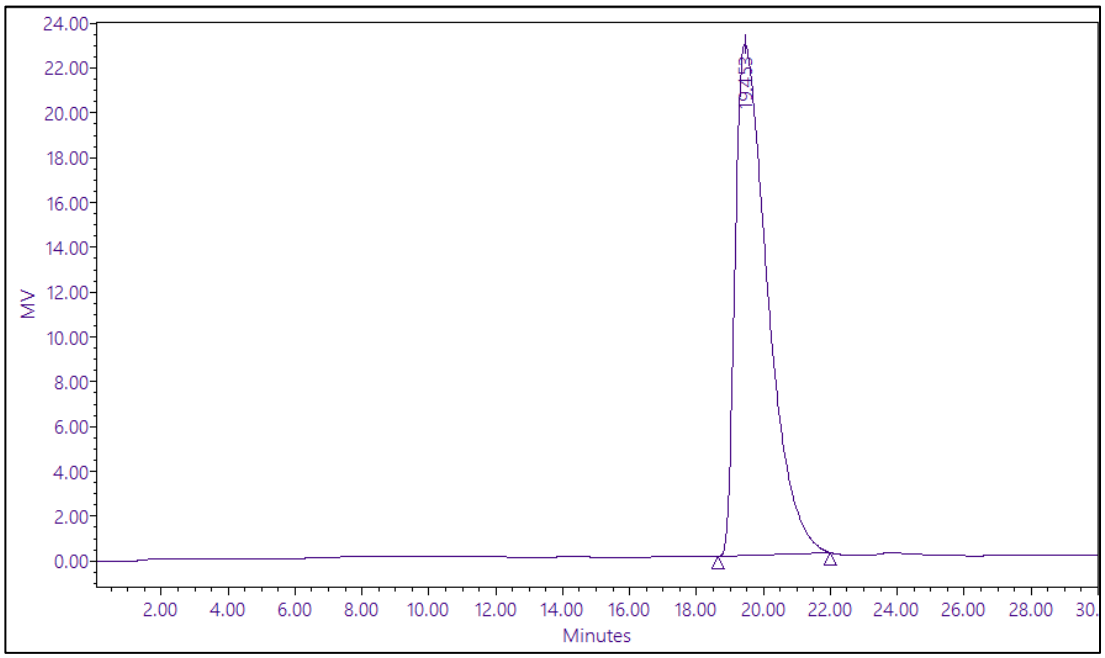

Figure 7-3: HPLC chromatogram of the recrystallized GWH Aliquot 3, showing a single peak at the retention time for melezitose.

Figure 7-4: Vials containing filtered and dried GWH crystal separation aliquots, L-R: Aliquot 7, Aliquot 6, Aliquot 6 sub-sample washed with methanol, and Aliquot 3 after recrystallization.

7.2.4 GWH separation Aliquot 4

This aliquot was dissolved for recrystallization before Aliquot 3, and enough water was added to allow the crystals to fully dissolve at 60 °C without stirring; the solution would not recrystallize even when seeded with crystals from the recrystallized Aliquot 3, so clearly the amount of water used was too much.

7.2.5 GWH separation Aliquot 5

The initial separation of the crystals yielded 0.77143 g (4.75% of starting honey mass) of very fine solid material that formed friable clumps. This aliquot was also dissolved for recrystallization before Aliquot 3, and while it dissolved with difficulty at 100 °C with stirring, the 20 drops added was also too much water, as this solution also resisted recrystallization.

7.2.6 GWH separation Aliquot 6

The initial separation of the crystals yielded 1.15431 g (6.91% of starting honey mass) of very fine solid material that formed friable clumps. The color of the material could be approximated as Pantone 2001C or Hex color #F9F0AE, as seen in the second-to-left vial in Figure 7-4. The sub-sample that was washed with methanol and re-filtered was a similar color and texture to the recrystallized Aliquot 3, and weighed 0.20796 g (66.6% of starting sub-sample

mass) after filtering, as shown in the second-to-right vial in Figure 7-4. As seen in the HPLC chromatogram in Figure 7-5, and later in Table 7-2 (section 7.3), the solids obtained by washing GWH crystals once with water and once with methanol are over 94% melezitose, with <3% each residual glucose and fructose.

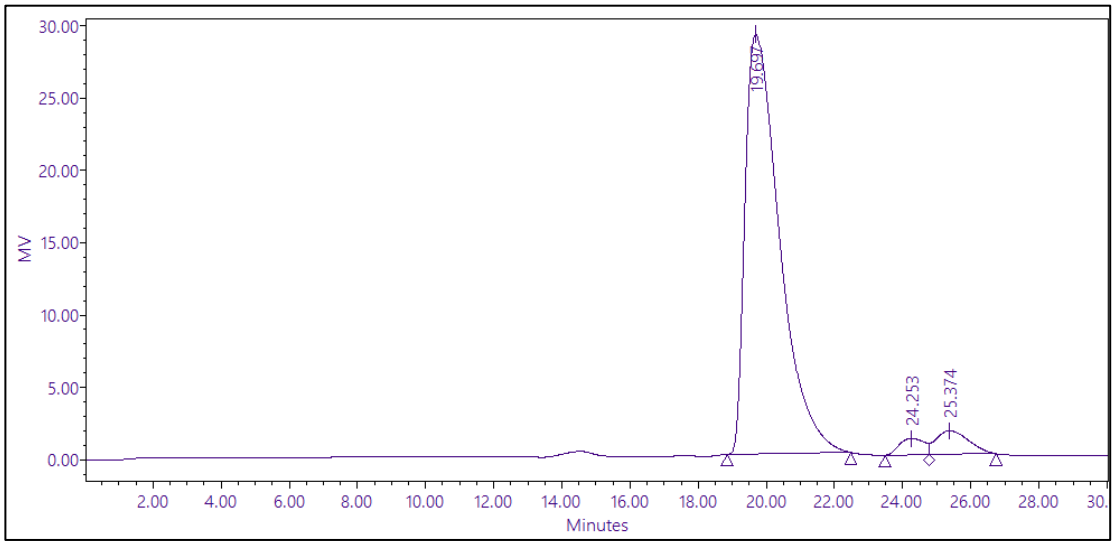

Figure 7-5: HPLC chromatogram of GWH separation Aliquot 6, showing the melezitose peak (19.697 min).

7.2.7 GWH separation aliquot 7

The settled crystals from the GWH dissolved readily in 8 mL of water with stirring and began to noticeably precipitate after 87.35 mL ethanol had been added. The remaining 16 mL ethanol was added after precipitation was noted, to increase the precipitate's displacement from the solution and maximize the yield. The precipitate appeared pale lemon yellow in solution, but when filtered, was dark brown and very sticky. After oven-drying and scraping off the filter paper, the precipitate was approximately Hex color #E6C8A0, as seen in the leftmost vial in Figure 7-4.

7.3 Acid lability of melezitose crystals isolated from Giant Willow Aphid honeydew honey

Table 7-2 shows the concentrations of melezitose, glucose, and fructose in solutions of GWH separation Aliquot 6 after digestion in HCl at pH 2.62 and 37 °C, for 18 hours, 4.5 hours, and no delay. These conditions were chosen to mimic human stomach acid pH [203] and temperature and determine whether melezitose is likely to survive this stage of digestion. Because human stomach residence time of indigestible solids averages 4.8 h if taken with food, or 1.2 h if taken fasting [204], the 4.5-hour digestion results are more appropriate as a measure of the acid lability of melezitose in the human stomach.

Table 7-2: Concentrations as percent mass of whole honey of acid digestion products after 18 hours', 4.5 hours', and no digestion of GWH separation Aliquot 6. Absolute error is reported as '95% CI'.

Treatment	Vial number			Average	95% CI
	1	2	3		
18-hour digestion					
solids in 2.000 mL HCl (mg)	28.20	29.76	29.02	28.993	1.938
Melezitose (as % of solids)	92.633	92.873	92.224	92.577	0.815
Glucose (as % of solids)	3.702	3.582	3.908	3.731	0.410
Fructose (as % of solids)	3.665	3.545	3.868	3.693	0.405
4.5-hour digestion					
solids in 2.000 mL HCl (mg)	31.43	30.67	27.52	29.873	5.150
Melezitose (as % of solids)	93.441	94.144	91.513	93.033	3.384
Glucose (as % of solids)	3.296	2.943	4.265	3.501	1.701
Fructose (as % of solids)	3.263	2.913	4.222	3.466	1.683
No digestion					
solids in 0.400 mL HCl (mg)	6.12	6.22	5.88	6.073	0.434
Melezitose (as % of solids)	94.966	92.816	94.860	94.214	3.010
Glucose (as % of solids)	2.530	3.610	2.583	2.908	1.513
Fructose (as % of solids)	2.504	3.574	2.557	2.878	1.497

After 4.5 hours digestion, melezitose was apparently depleted by 1.2%, and did not show further depletion on further digestion. The hydrolysis of melezitose, producing more glucose and fructose, did not cause changes in the concentrations of these sugars in solution that were statistically significant at $p \leq 0.05$. Figure 7-6 and Figure 7-7 can be compared to Figure 7-5 (HPLC chromatograms of, respectively, solutions of GWH crystal separation Aliquot 6

after 4.5 hours' digestion, after 18 hours' digestion, and dissolved in plain Type 1 water) to show the slightness of the effect of digestion.

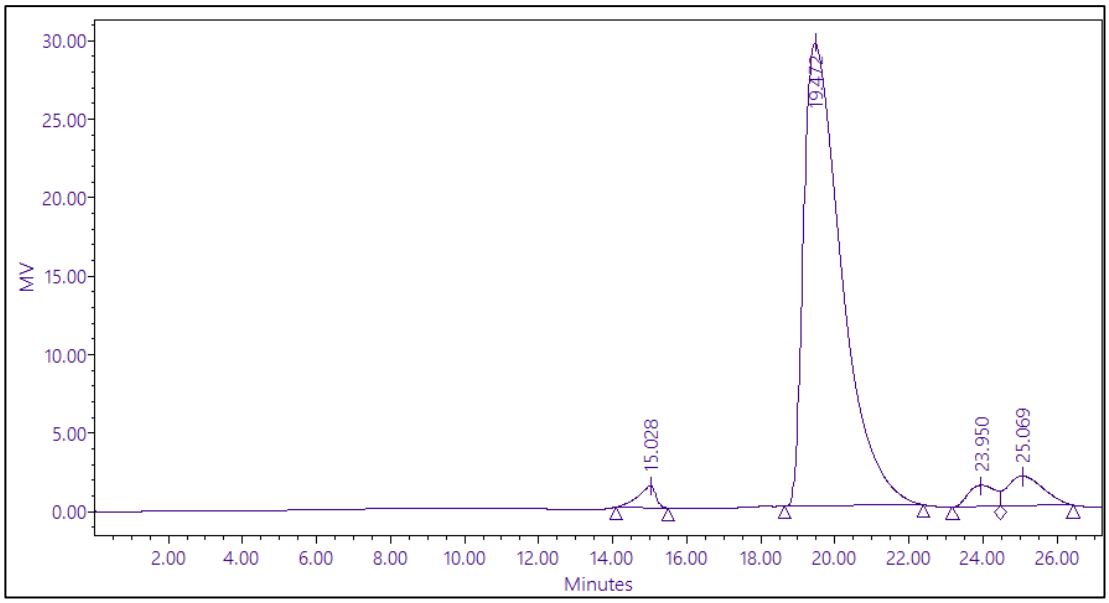

Figure 7-6: HPLC chromatogram of neutralized acid-hydrolysis solution of melezitose after 4.5 hours digestion at 37 °C; peaks L-R are salts from acid neutralization, melezitose, glucose, and fructose.

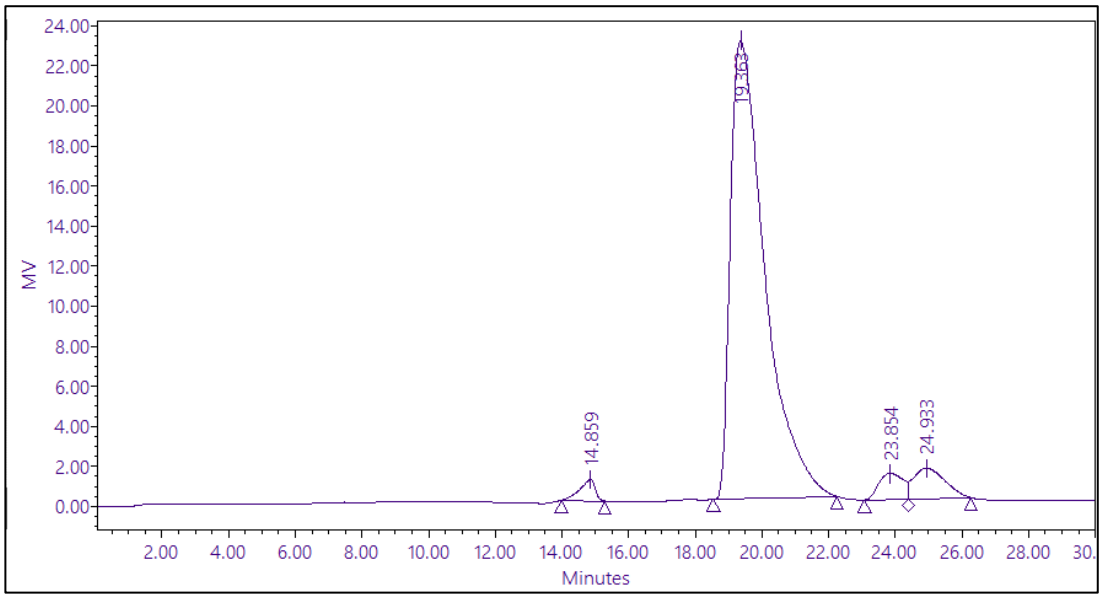

Figure 7-7: HPLC chromatogram of neutralized acid-hydrolysis solution of melezitose after 18 hours digestion at 37 °C; peaks L-R are salts from acid neutralization, melezitose, glucose, and fructose.

7.4 Enzyme lability of melezitose

Table 7-3 shows the concentrations of sucrose, melezitose, and α-glucosidase used in triplicate digestions, and the concentrations of sucrose, melezitose, and monosaccharides remaining in the solutions after digestion. The pH, temperature, and incubation times were chosen to mimic the human small intestine [148-149] and determine whether melezitose is likely to survive this stage of digestion. Sucrose was used as a control to confirm that the α-glucosidase used was in fact capable of hydrolyzing digestible sugars.

Table 7-3: Initial concentrations of sucrose (mg/mL), melezitose (mg/mL), and α-glucosidase (enzyme units per milliliter, U/mL), and final concentrations of sucrose, melezitose, and monosaccharides (mg/mL and mol%), in enzyme digestion solutions. Absolute error is reported as '95% CI'. α-glucosidase concentration is expressed as minimum U/mL because the Sigma-Aldrich-provided label description of the enzyme specified ≥10 U/mg product.

	Vial number				
Sugar	1	2	3	Average	95% CI
Sucrose					
Minimum α-glucosidase (U/mL)	0.62	0.62	0.54	0.59	0.11
Initial sucrose (mg/mL)	0.52	0.49	0.68	0.56	0.25
Final sucrose (mg/mL)	0.27	0.31	0.31	0.30	0.06
Final sucrose (as mol% of sugars)	28.91	34.77	23.78	29.15	13.66
Final monosaccharides (mg/mL)	0.35	0.31	0.52	0.39	0.27
Final monosaccharides (as mol% of sugars)	71.09	65.23	76.22	70.85	13.66
Melezitose					
Minimum α-glucosidase (U/mL)	0.54	0.52	0.60	0.55	0.10
Initial melezitose (mg/mL)	0.16	0.17	0.39	0.24	0.33
Final melezitose (mg/mL)	0.14	0.18	0.37	0.23	0.31
Final melezitose (as mol% of sugars)	88.30	89.37	94.01	90.56	7.55
Final monosaccharides (mg/mL)	0.02	0.02	0.01	0.02	0.01
Final monosaccharides (as mol% of sugars)	11.70	5.80	5.99	7.83	8.33

4.5 hours was chosen as the incubation time for the enzyme digestions, as it is the approximate mean residence time of a labeled dose in the human small intestine; this residence time is not significantly affected by whether the dose is taken fasting or with food [148]. (In simulating α-glucosidase digestion, only the small intestine residence time is considered, because this enzyme is not involved in carbohydrate hydrolysis before this point in the digestive tract.) The pH of the

α-glucosidase digestion solutions was set to 6, as this is the approximate mean pH of the small intestine [149].

After 4.5 hours digestion, melezitose was apparently depleted by 4.71%. The hydrolysis of melezitose did not cause changes in the melezitose concentration in solution that were statistically significant at $p \leq 0.05$. Under the same digestion conditions, sucrose was depleted by 47.3%, a statistically significant difference. It can thus be concluded that melezitose is not significantly hydrolyzed by α-glucosidase under conditions similar to human digestion.

7.5 Conclusion

It is possible to make a palatable high-oligosaccharide toffee from Giant Willow Aphid honeydew honey that may provide the minimum dose required to induce a prebiotic effect while delivering an amount of free sugars considerably below the recommended intake. It is also possible to extract melezitose crystals from GWA honeydew honey directly with >94% purity (with glucose and fructose impurities) by washing alone, and pure or almost pure melezitose by recrystallization, without use of solvents that would be inappropriate for use in a food product. It may be possible to market 'washed' melezitose as an oligosaccharide supplement without the recrystallization step, as the glucose and fructose contribute negligibly to the consumer's free sugar intake but may be enough to give the supplement a slightly sweet taste. Melezitose displays statistically insignificant depletion on digestion in a crude human-stomach and human-small-intestine model, and so may fulfil the prebiotic criterion of indigestibility by humans.

8 Comparison of honey compositions

Figure 8-1 is a graphical comparison of the sugar compositions of the honeys analyzed in this study. Chart data for Figure 8-1 was taken from the HPLC quantitation results presented in Table 3-1, Table 4-2, and Table 6-2, respectively, and normalized so the honey composition for each totaled 100%.

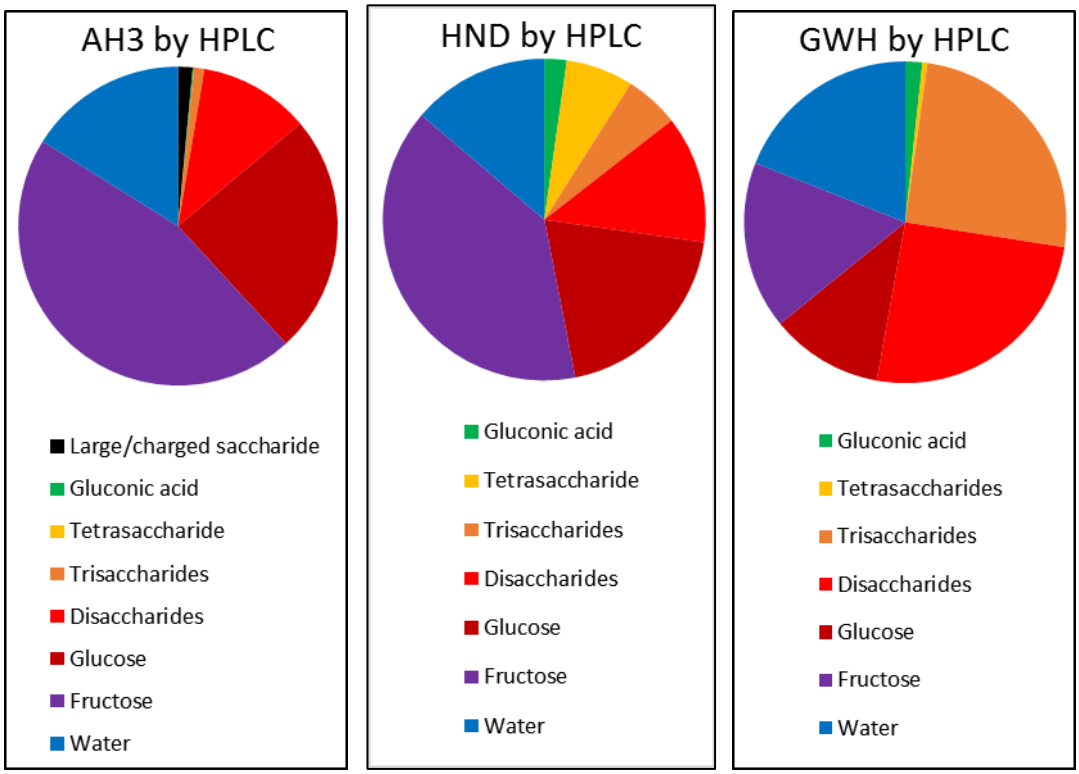

Figure 8-1: Pie charts showing relative amounts of various carbohydrates and water in (L-R) Beeotic® honey sample AH3, New Zealand beech honeydew honey sample HND, and Giant Willow Aphid honeydew honey sample GWH.

The water contents of the honeys are all similar, as are the D-gluconic acid contents, with HND slightly higher in acid as a proportion of the total honey mass, and GWH slightly wetter as a proportion of the total honey mass (both statistically significant at $p \leq 0.05$), than the other two honeys considered.

AH3 is representative of Beeotic® honey, which is commercially marketed as prebiotic. As seen in Figure 8-1, AH3 is primarily fructose, glucose, and water, with a small quantity of disaccharides and almost no higher sugars. HND has a

similar F/G ratio to AH3 (2.00 cf. AH3's 1.88), but lower total monosaccharides and disaccharides; this decrease is offset by significantly higher trisaccharides and tetrasaccharides. GWH's F/G ratio is much lower than the other honeys (1.5), which, as discussed, could be contributing to the fast-crystallizing behavior of GWH; however, this is not definite as the ratio is not within the range where crystallization is sure to occur, and low F/G ratio generally causes glucose to precipitate, as opposed to the melezitose precipitation seen in GWH.

The minimum honey doses required to induce a prebiotic effect were calculated in sections 3.2.11 (Beeotic® AH3), 4.1.9 (beech honeydew honey HND), and 6.2.12 (GWA honeydew honey GWH), using an oligosaccharide dose size of 2 g. While 1 g of prebiotic oligosaccharides is the minimum effective dose for fructooligosaccharides [197], this figure was not used in calculations because melezitose and the tetrasaccharides common to all three honeys are not fructooligosaccharides. Furthermore, the minimum effective daily dose for xylooligosaccharides is 2 g, for lactulose 3 g [197], for isomaltooligosaccharides 8-10 g [195], and other researchers' results suggest the minimum daily dose for fructooligosaccharides may be as high as 4-8 g [196].

Table 8-1 lays out the amounts of honey required to deliver the low (xylooligosaccharide) and high (isomaltooligosaccharide) literature estimates of the minimum required oligosaccharide dose to induce a prebiotic effect using honey alone, and the amounts of free sugars consumed along with these doses. As with Table 3-1, Table 4-2, and Table 6-2, it should be noted that the sugar masses do not add up to the total mass of honey, as each component was calculated separately; however, they provide a reasonable idea of the proportions of each component present. In order to give the most favorable possible interpretation of AH3's performance as a potential prebiotic functional

food, it is assumed that AH3/1a (identified as a large or charged saccharide) can be included in the estimation of potentially prebiotic oligosaccharides in AH3.

Table 8-1: High and low estimates of the mass of honey required to obtain a prebiotic functional dose, and the free sugars consumed along with this mass of honey.

Honey	Minimum dose	Honey required	Free Sugars
AH3	High (10 g)	366.17 ± 48.40	319.77 ± 46.08
	Low (2 g)	73.23 ± 9.68	63.95 ± 9.22
HND	High (10 g)	72.30 ± 2.21	58.89 ± 2.07
	Low (2 g)	14.46 ± 0.44	11.77 ± 0.41
GWH	High (10 g)	26.50 ± 0.13	20.76 ± 0.48
	Low (2 g)	5.30 ± 0.03	4.15 ± 0.10

Table 8-1 shows that AH3, the Australian honey Beeotic®, marketed as containing "prebiotic oligosaccharides" [162], can deliver the minimum known effective dose of oligosaccharides to induce a prebiotic effect, but taking this dose includes ingestion of 63.9 g of free sugars (more than twice the recommended daily intake [82]). If the oligosaccharides (including tetrasaccharides) present in AH3 require a higher dose to induce a prebiotic effect, perhaps up to the 10 g required for isomaltooligosaccharides, over 300 g of free sugars could be delivered.

New Zealand beech honeydew honey can deliver 2 g of potentially prebiotic oligosaccharides without exceeding the recommended daily intake of free sugars. However, if higher oligosaccharide doses are required (which may be the case, as there is evidence for oligosaccharides of a lower degree of polymerization having greater prebiotic activity than larger oligosaccharides [59]), inducing a prebiotic effect using beech honeydew honey alone may involve delivery of over 60 g of free sugars.

Giant Willow Aphid honeydew honey may be able to deliver even the higher oligosaccharide dose required while delivering an amount of free sugars below the recommended daily intake.

9 Conclusions, recommendations, and future work

The key differences between floral and honeydew honeys are their acid and oligosaccharide contents and fructose/glucose ratios (where honeydew honey is higher); and their monosaccharide contents (where floral honey is higher). Although the high levels of oligosaccharides in honeydew honey contribute to its lower monetary value relative to floral honey, certain oligosaccharides are prebiotic and confer certain health benefits that may add value to the honey. (A prebiotic compound is one that is not digestible to humans, but is selectively utilized by beneficial colonic bacteria, imparting a health benefit to the consumer.)

Honey made from honeydew produced by the Giant Willow Aphid (GWA) crystallizes in the comb, representing a major loss to apiarists, and has previously been estimated to contain over 14% melezitose.

This research set out to quantify the carbohydrate compositions of an Australian honey (Capilano Beeotic®) marketed as a prebiotic functional food; of a New Zealand beech honeydew honey; and of a sample of GWA honeydew honey. Further, it also sought to develop methods for adding value to GWA honeydew honey, and to determine whether melezitose, the principal oligosaccharide of GWA honeydew honey, is susceptible to acid or enzyme hydrolysis, and thus to human digestion.

Capilano's Beeotic® product, an Australian honey marketed as 'clinically tested prebiotic honey' [155] was introduced in Australia and the US in September 2016. The studies on which this claim was based examined mostly eucalyptus floral honeys. The research reported here showed that Beeotic® contains a maximum of 2.8% oligosaccharides, or 383 mg oligosaccharides per 14-g manufacturer-recommended daily dose. This concentration of oligosaccharides is not

distinguishable from the concentration usually seen in ordinary floral honey such as is sold without any special health claims or associated special price premium. The minimum recommended dose of oligosaccharides as determined by studies not associated with Beeotic® is 2 g daily. To deliver this dose of oligosaccharides, 73.23 g of Beeotic® would be required; this mass of honey would also deliver 63.95 g of 'free' or digestible sugars (cf. WHO recommendation of no more than 30 g free sugars daily). At the time of writing, Beeotic® is not advertised on the Australian version of Capilano's website but remains available and advertised as prebiotic on the US version of the website, at Walmart, and internationally through Amazon.com.

On analysis, a sample of New Zealand beech honeydew honey was found to contain considerably higher concentrations of oligosaccharides, particularly tetrasaccharides, than Beeotic®. The experimentally determined carbohydrate composition values are similar to those obtained by Astwood *et al*. in their 1998 study of New Zealand beech honeydew honey.

No full carbohydrate analysis of Giant Willow Aphid honeydew honey prior to this research was found in the literature; only estimates of its melezitose and salicylic acid content (>14%, and 0.0014-0.0041%, respectively) had been reported. Analysis of a sample of GWA honeydew honey indicated that the melezitose and salicylic acid contents were 27.4% and 0.52%, respectively, and that the crystals precipitated from the honey are melezitose. The overall oligosaccharide and monosaccharide contents (very high and quite low, respectively) of GWA honeydew honey are exaggeratedly typical of a honeydew honey.

Also for this research, prototype methods were developed for converting GWA honeydew honey into a high-melezitose candy without affecting the ratio of

oligosaccharides to free sugars, and for extracting clean crystals of melezitose from GWA honeydew honey. Melezitose has previously been shown to be fermented slowly by fecal bacteria, which would increase its availability in the distal colon. This research has shown that melezitose is also not significantly hydrolyzed in crude human stomach and small intestine simulations, suggesting it meets the prebiotic criterion of human indigestibility and, even if no other health benefits can be proven, it could still qualify as soluble dietary fiber and reduce bowel transit times.

While few recommendations for concrete action can be made from a limited study, the results presented here make a case for a survey of other honeys, including honeydew honeys and, specifically, of more examples of Giant Willow Aphid honeydew honey, for oligosaccharide content and potential prebiotic activity.

The underlying idea that high-oligosaccharide honey has value as a prebiotic functional food may have merit. However, this depends upon the results of *in vitro* assays of various common honey oligosaccharides for prebiotic activity. It is concluded that a wider range of honeys than were considered by the studies informing Capilano's production of Beeotic® (honeys of 19 different origins, all floral, including *Eucalyptus*, *Banksia*, *Brassica*, and *Eucryphia* species) should be examined. Honeydew honeys in particular should be considered, due to their generally high oligosaccharide content.

Future work could include carbohydrate analysis of various honeys; *in vivo* and *in vitro* assays of various honeys and common honey oligosaccharides for prebiotic activity; and further investigation of Giant Willow Aphid honeydew honey to transform a troublesome waste substance into a commercially viable product. Should melezitose prove to meet the prebiotic criteria of being

selectively fermented by desirable gut organisms and providing a measurable health benefit, GWA honeydew honey could also be marketed as a true prebiotic functional food.

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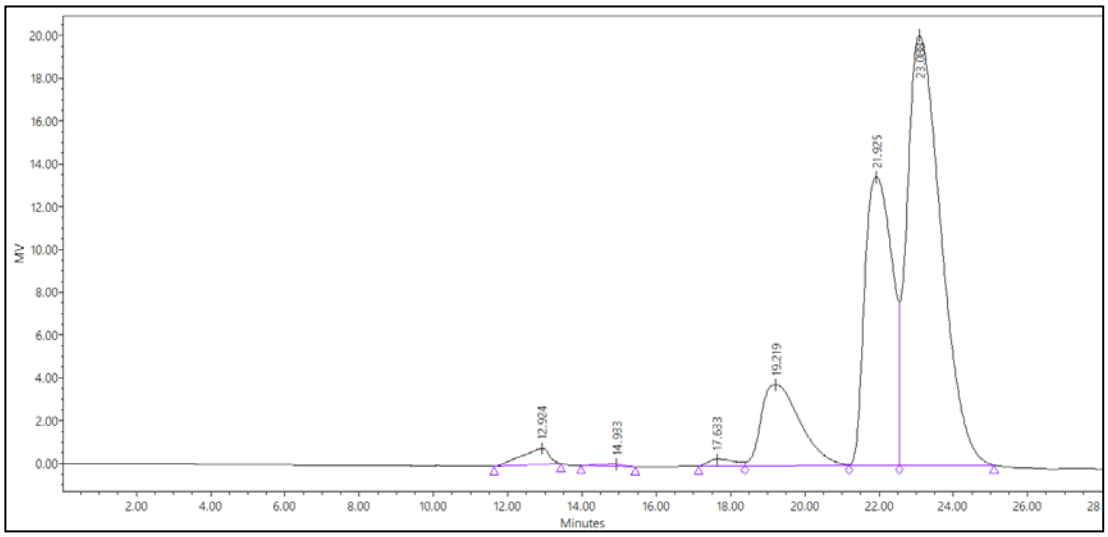
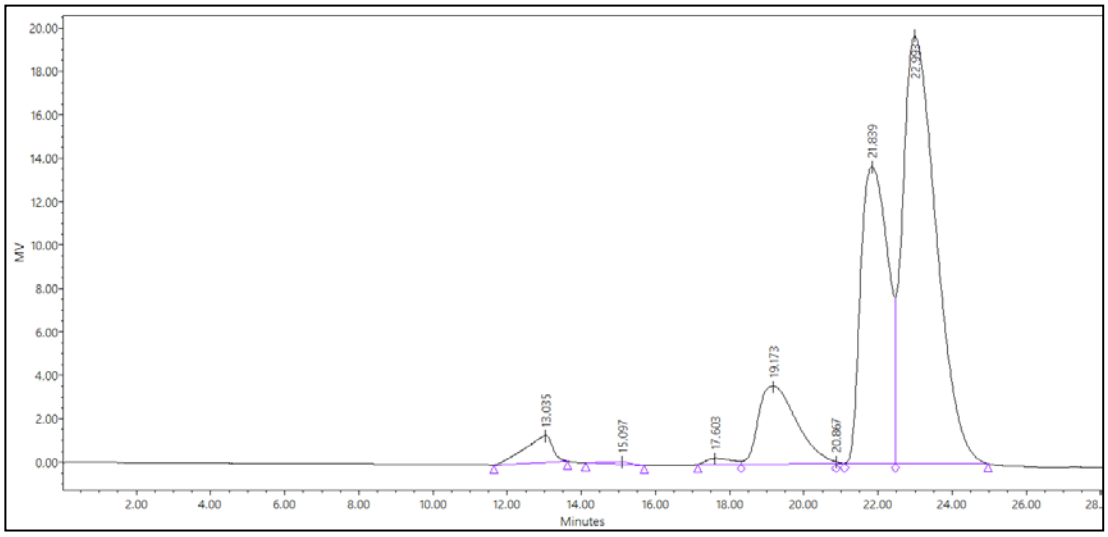
Appendices

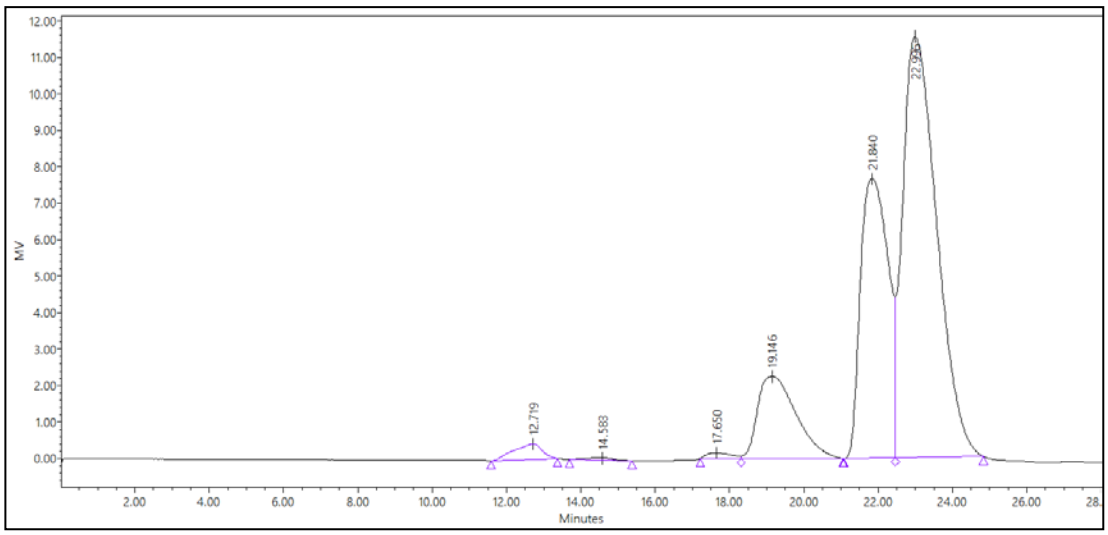
Appendix 1: HPLC chromatograms of other Beeotic® samples



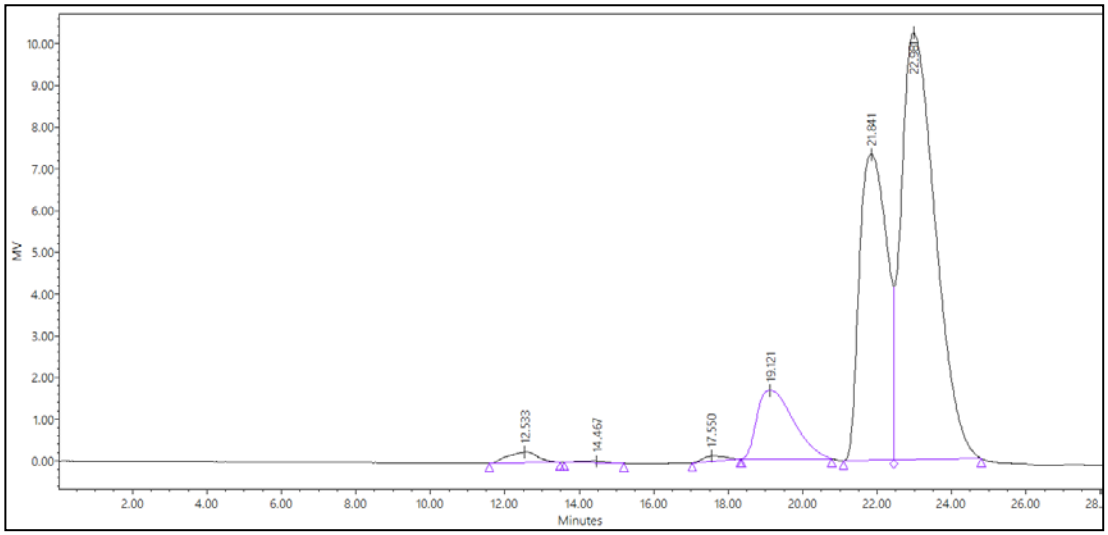
Appendix Figure 1: Analytical HPLC chromatogram of Beeotic® AH1.



Appendix Figure 2: Analytical HPLC chromatogram of Beeotic® AH2.

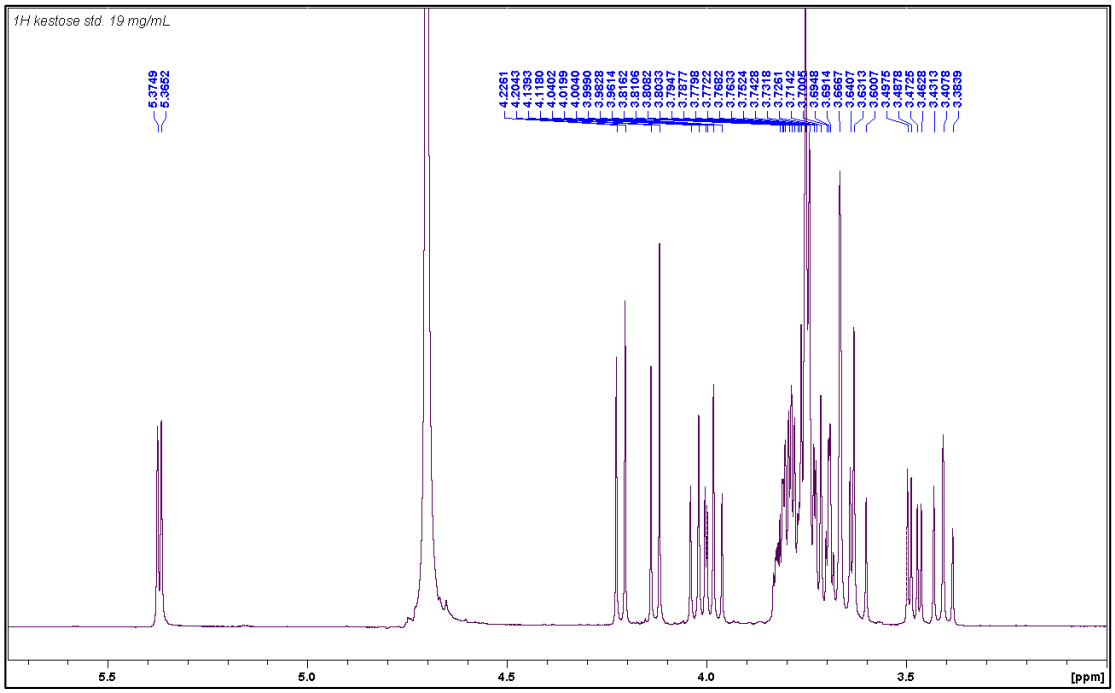


Appendix Figure 3: Analytical HPLC chromatogram of Beeotic® AH4.

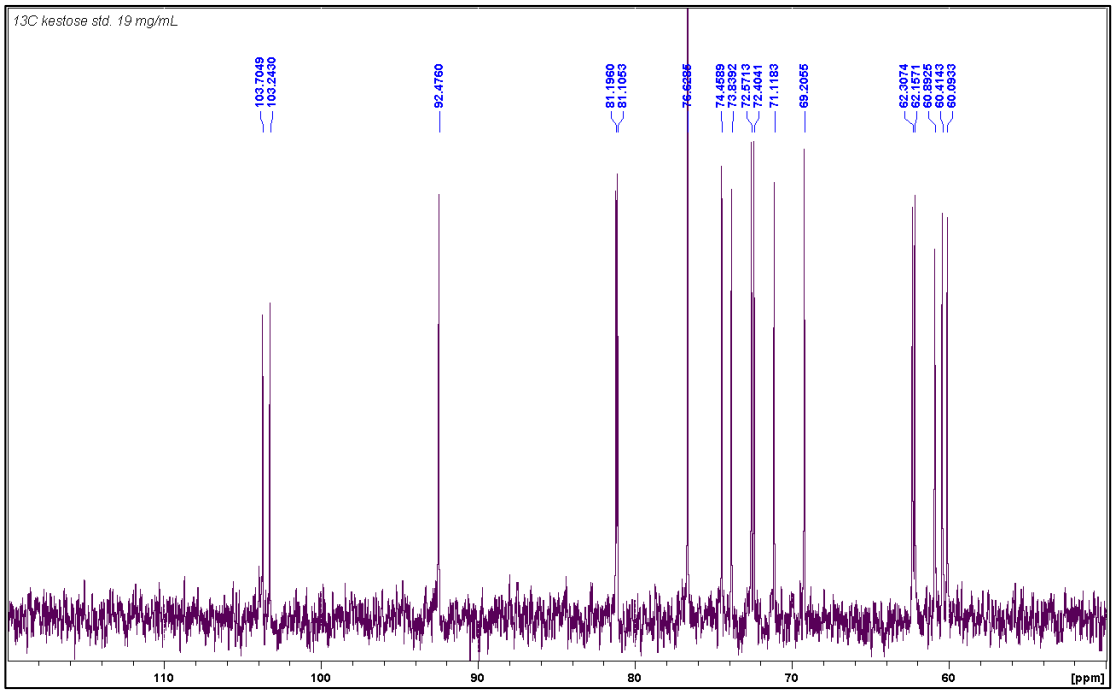


Appendix Figure 4: Analytical HPLC chromatogram of Beeotic® AH5.

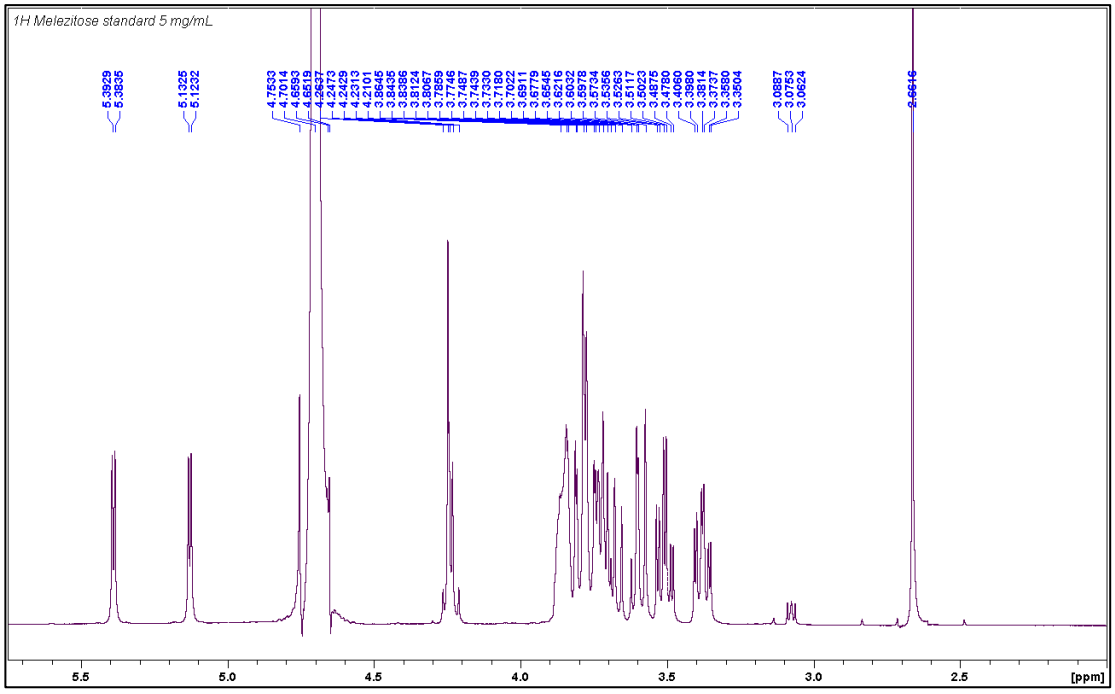
Appendix 2: ^{1}H and ^{13}C NMR spectra of 6-kestose and melezitose standards to confirm identity, and ^{13}C NMR spectrum of GWH/2b



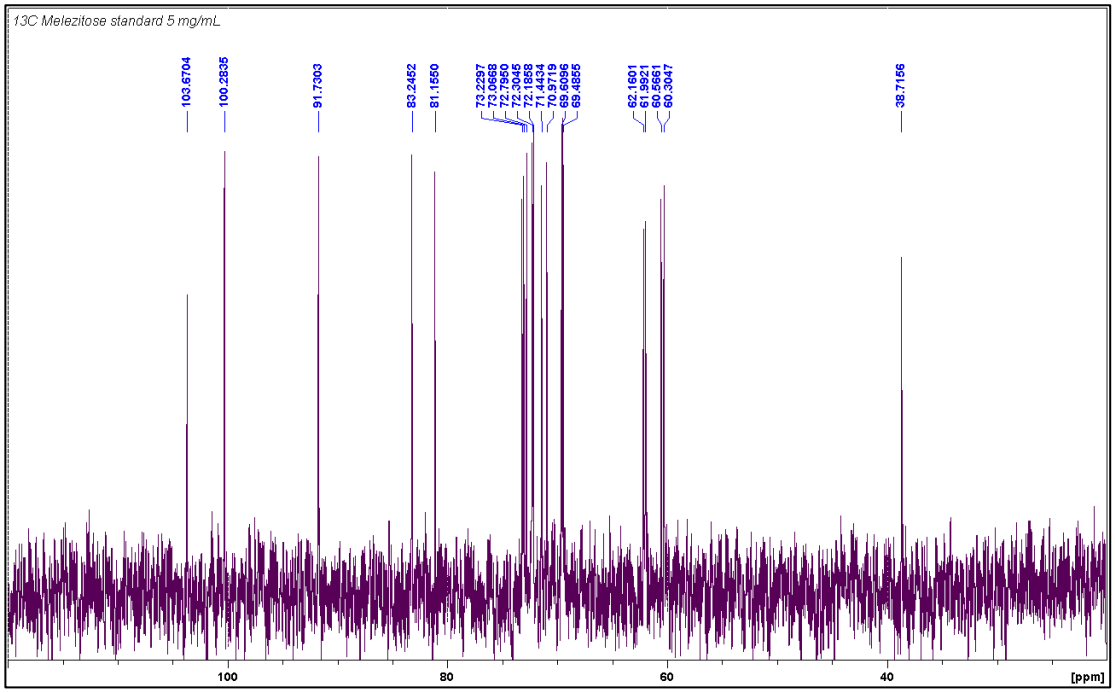
Appendix Figure 5: ^{1}H NMR spectrum of 6-kestose



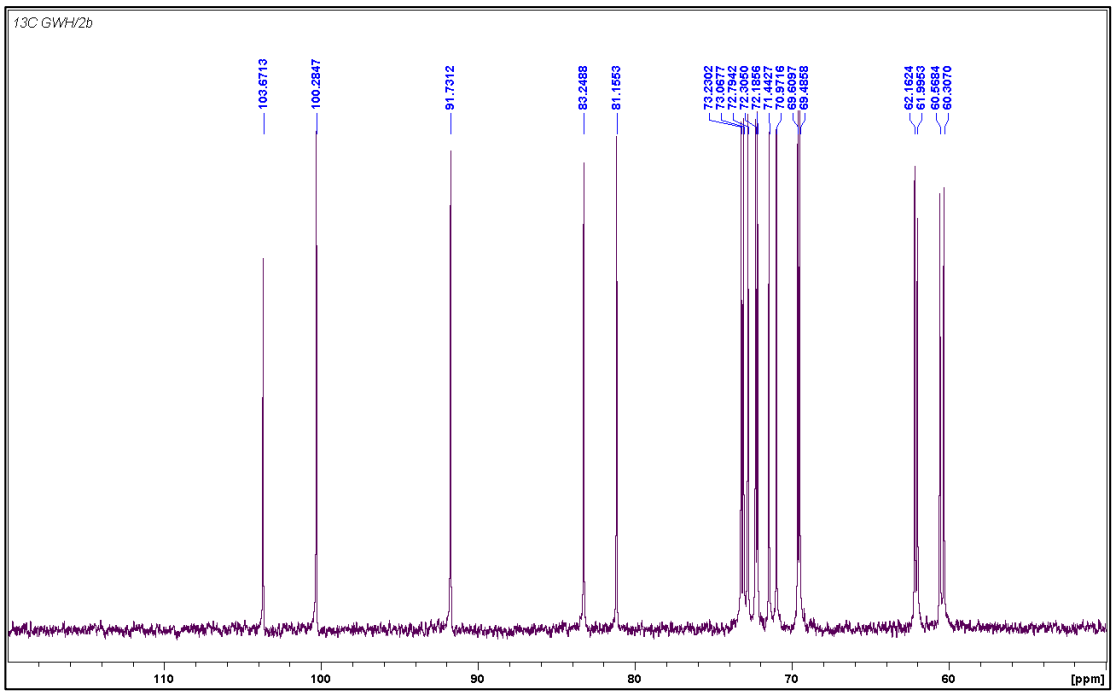
Appendix Figure 6: ^{13}C NMR spectrum of 6-kestose



Appendix Figure 7: ^{1}H NMR spectrum of melezitose

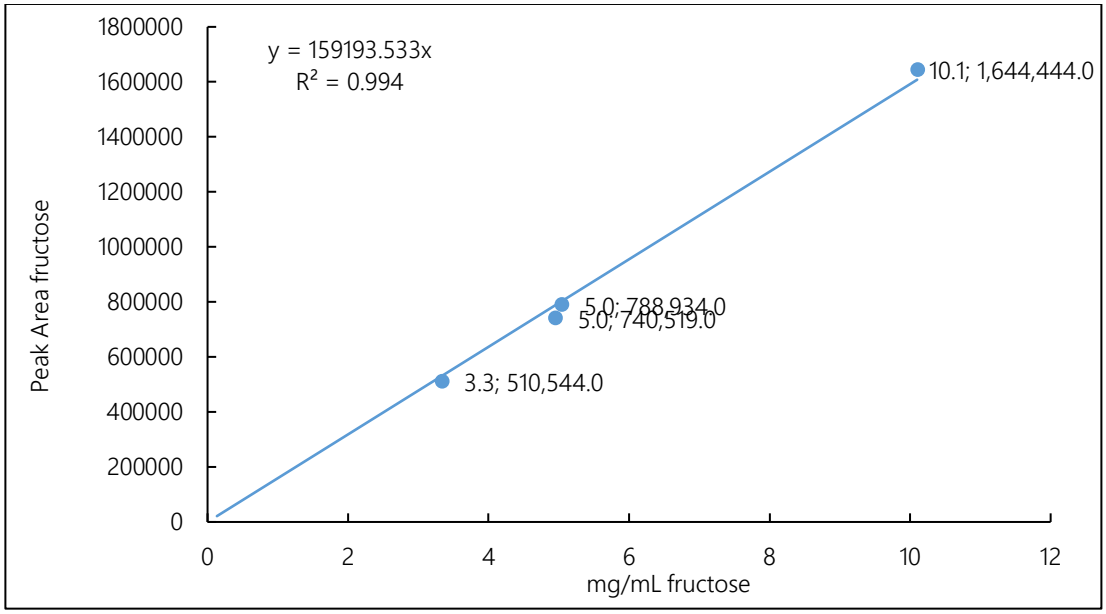


Appendix Figure 8: ^{13}C NMR spectrum of melezitose

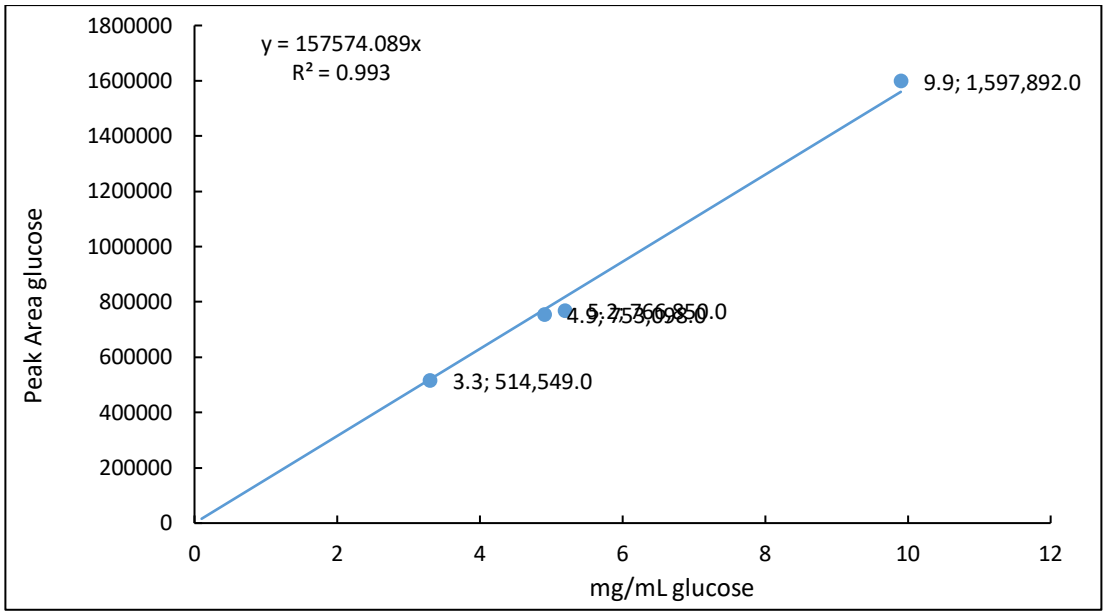


*Appendix Figure 9: ^{13}C NMR spectrum of GWH fraction **2b***

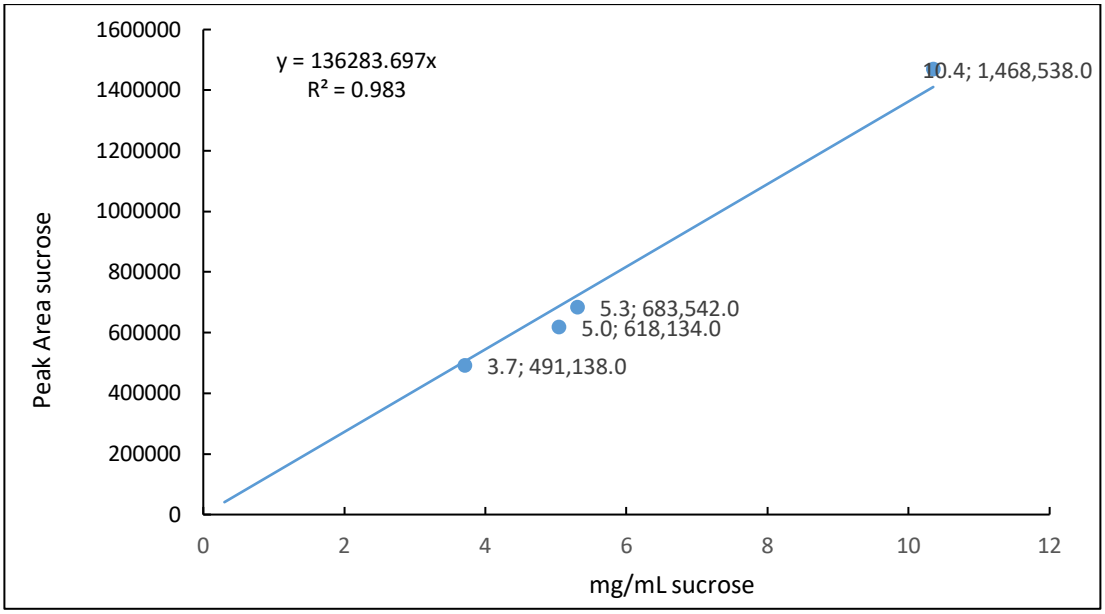
Appendix 3: Quantitation calibration curves for HPLC



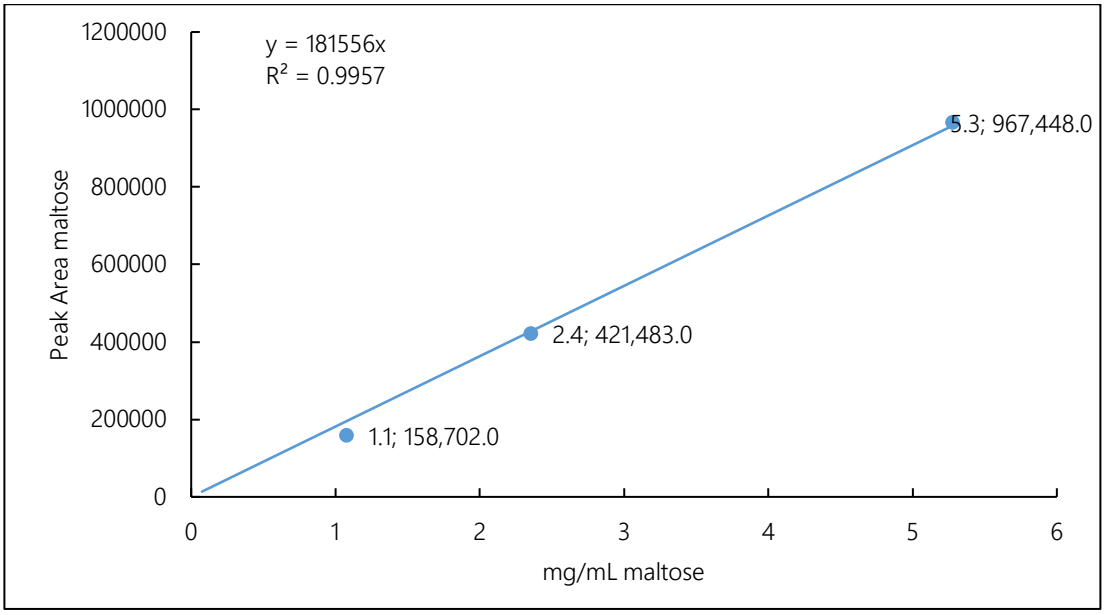
Appendix Figure 10: Fructose HPLC quantitative calibration curve.



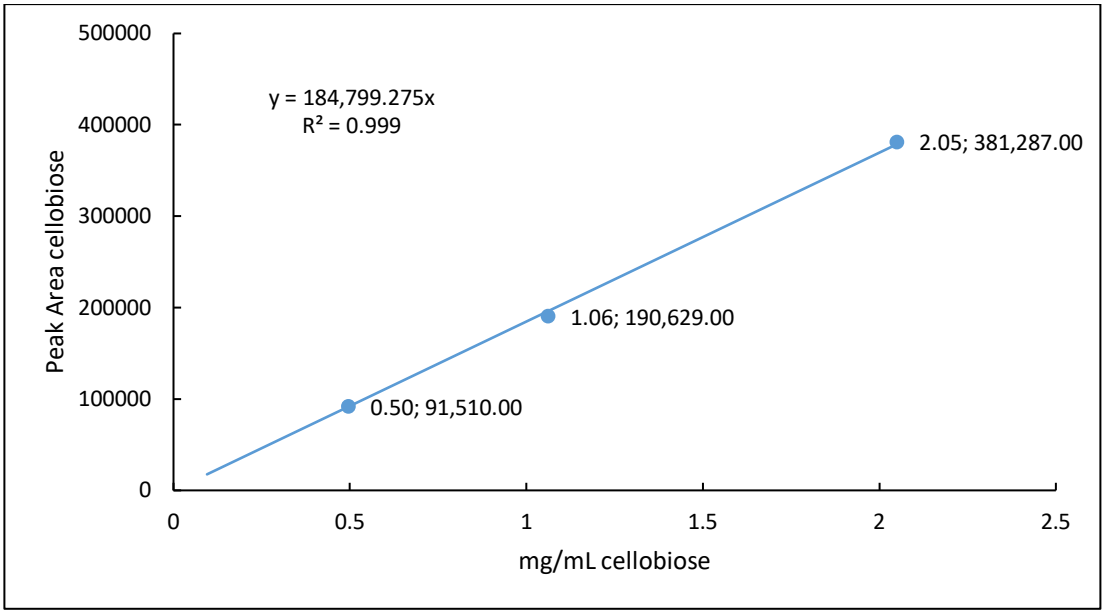
Appendix Figure 11: Glucose HPLC quantitative calibration curve.



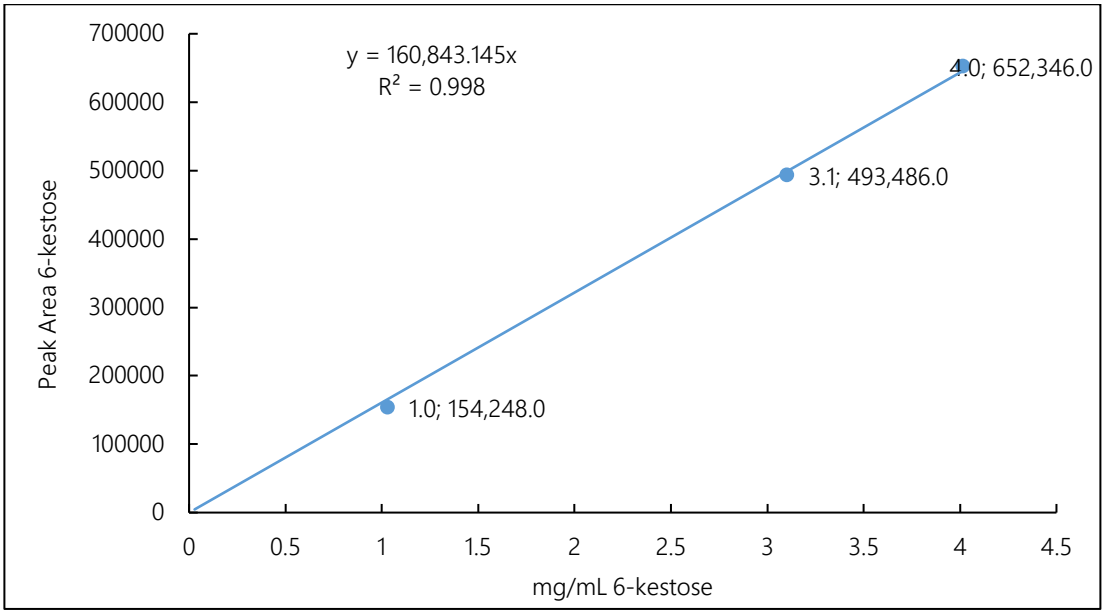
Appendix Figure 12: Sucrose HPLC quantitative calibration curve.



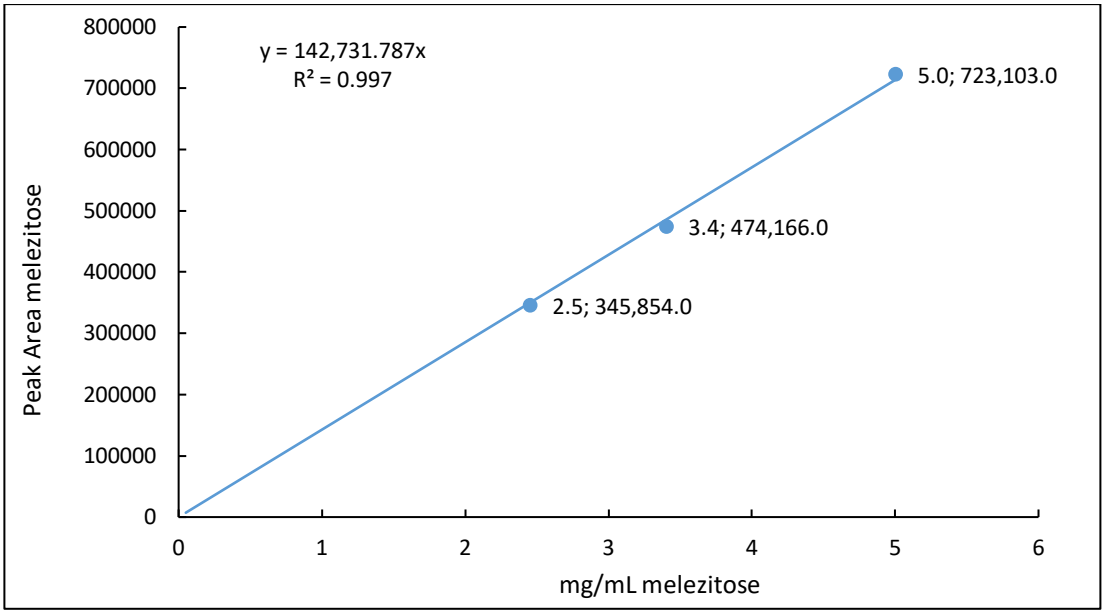
Appendix Figure 13: Maltose HPLC quantitative calibration curve.



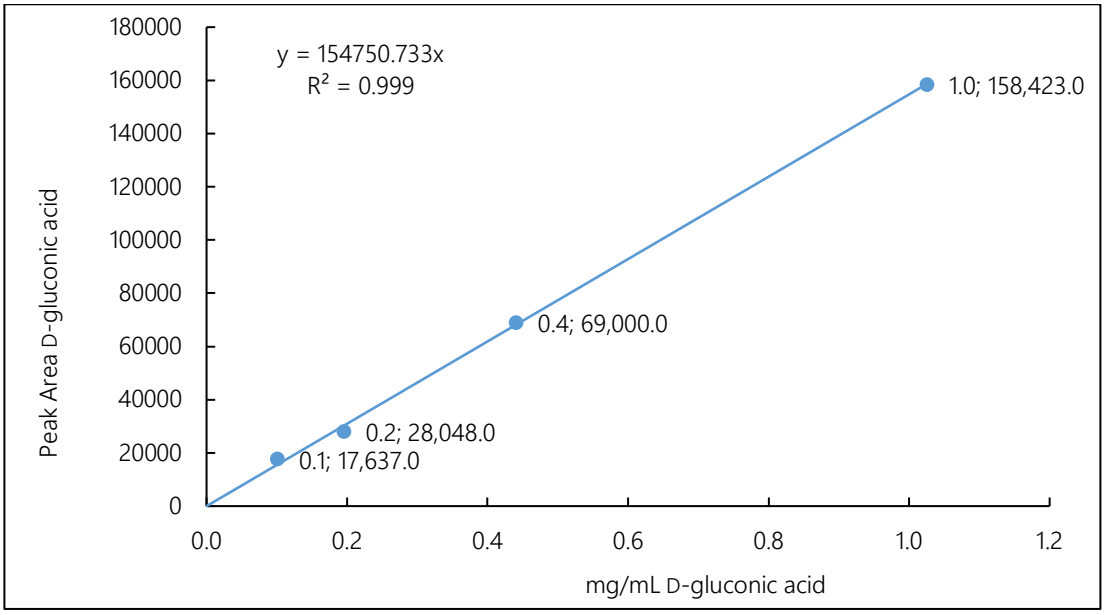
Appendix Figure 14: Cellobiose HPLC quantitative calibration curve.



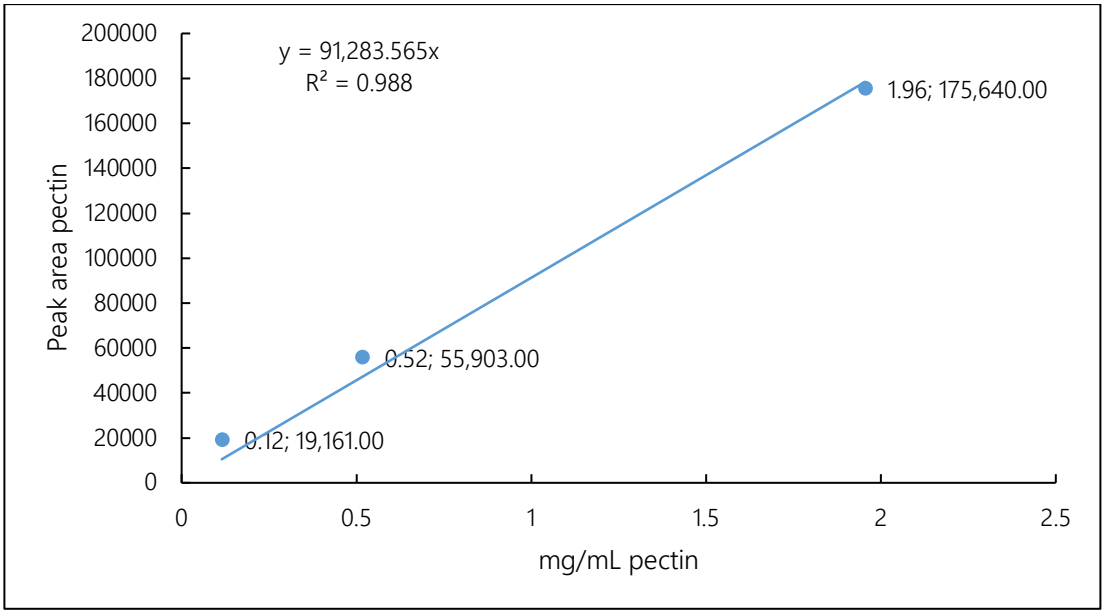
Appendix Figure 15: 6-kestose HPLC quantitative calibration curve.



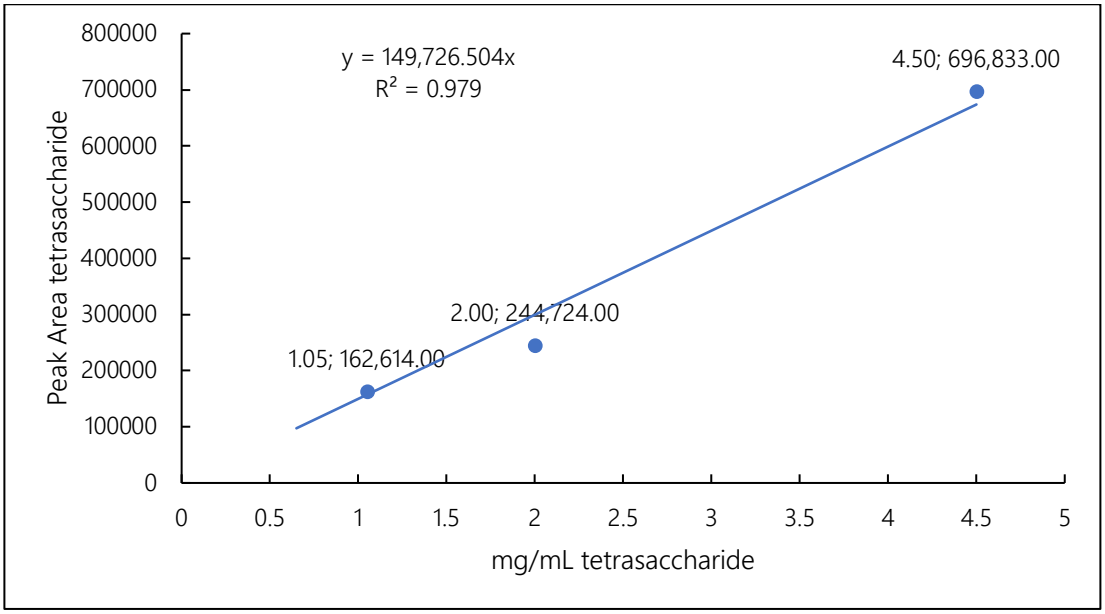
Appendix Figure 16: Melezitose HPLC quantitative calibration curve.



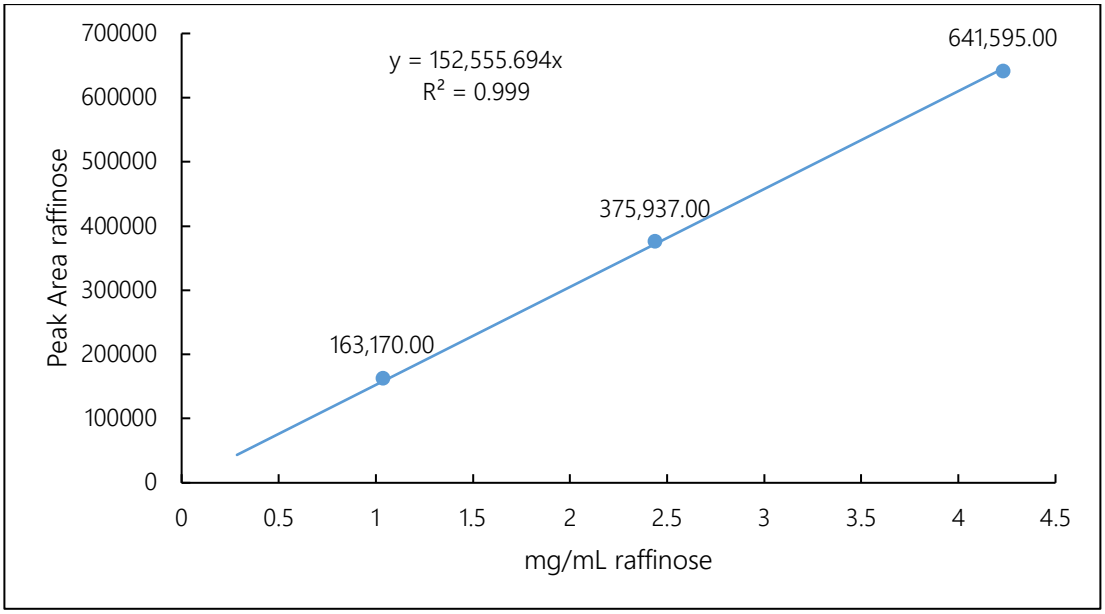
Appendix Figure 17: D-gluconic acid HPLC quantitative calibration curve.



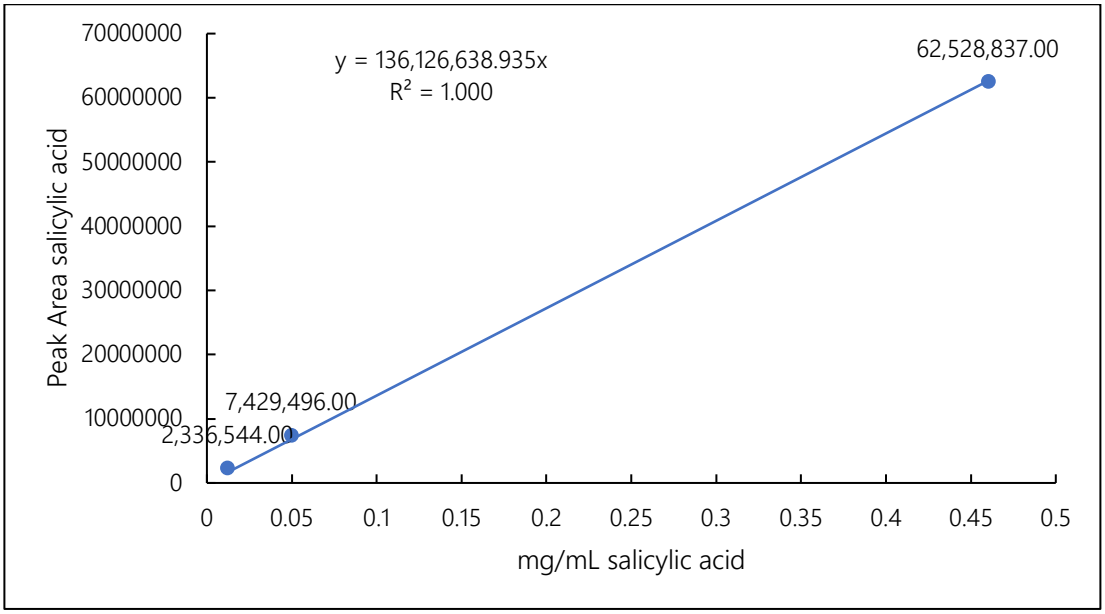
Appendix Figure 18: Pectin HPLC quantitative calibration curve.



Appendix Figure 19: Tetrasaccharide α-D-Glcp(1→4)-α-D-Glcp(1→4)-α-D-Glcp(1→2)-β-D-Fruf HPLC quantitative calibration curve

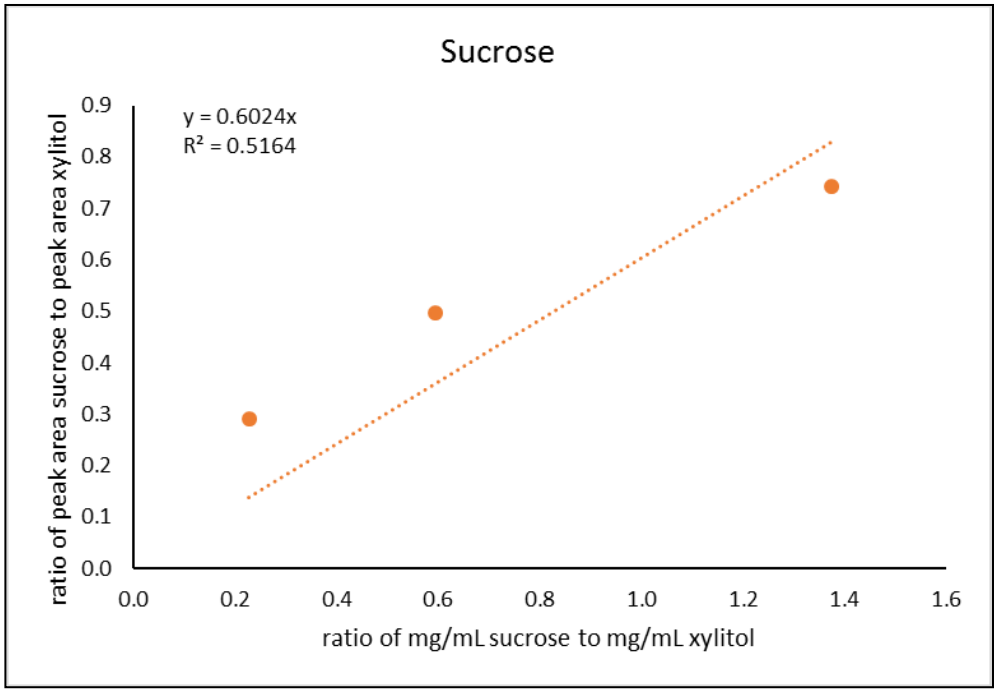


Appendix Figure 20: Raffinose HPLC quantitative calibration curve.

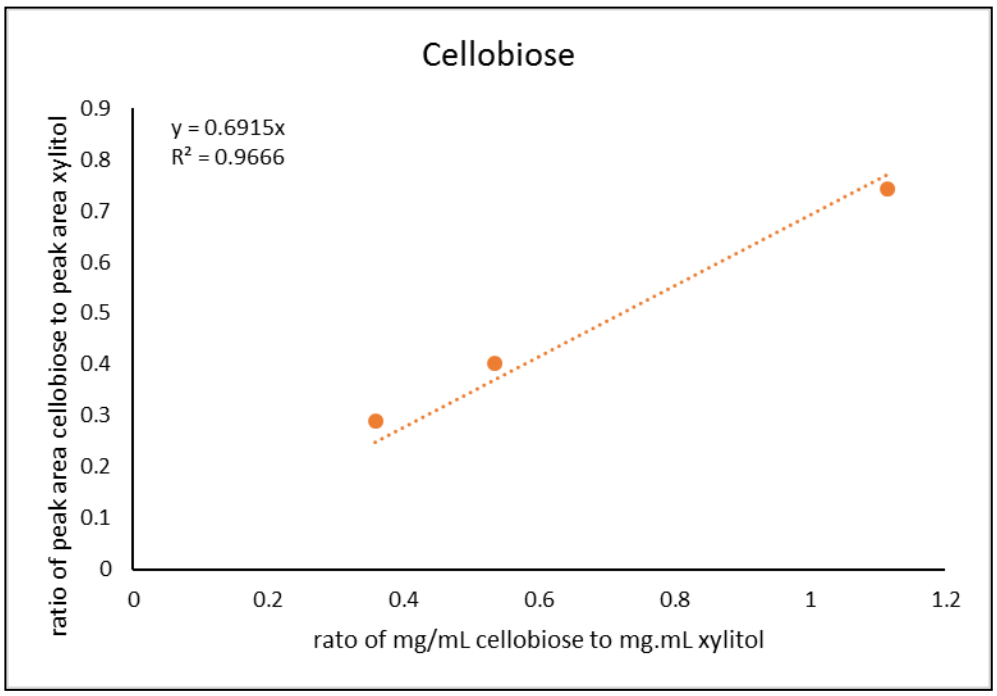


Appendix Figure 21: HPLC quantitative calibration curve for salicylic acid. Quantitation for this compound was performed with UV detection instead of RI detection.

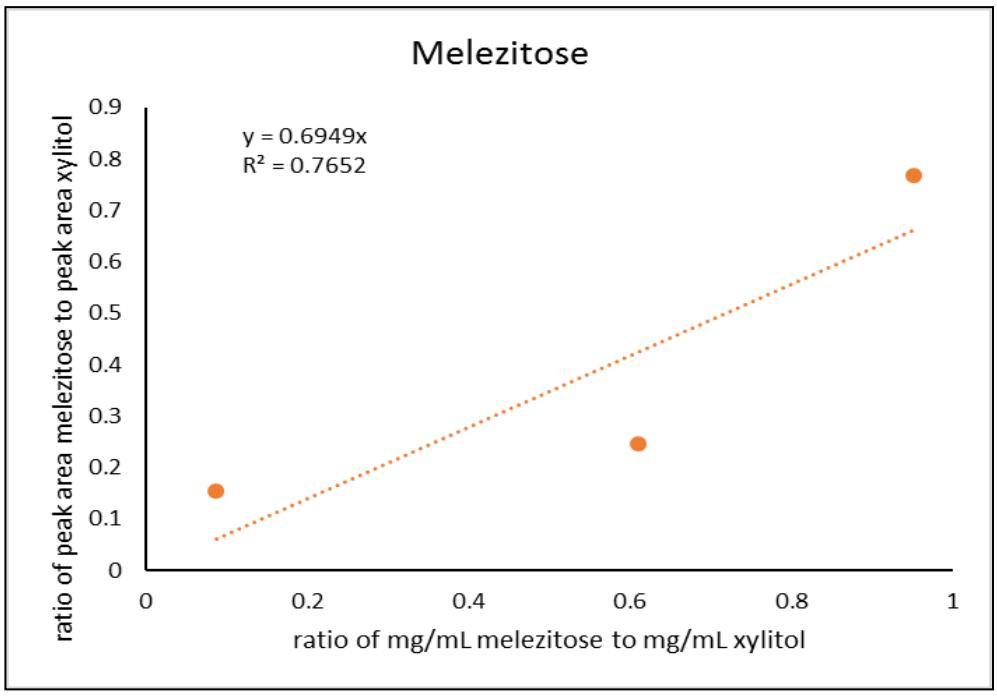
Appendix 4: Quantitation calibration curves for GC-MS



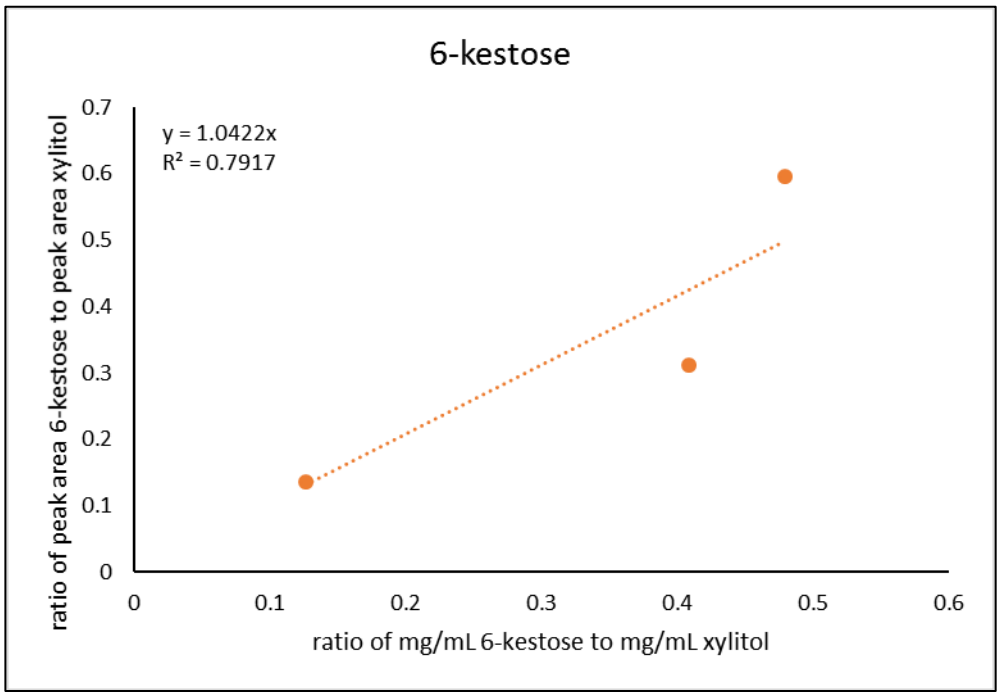
Appendix Figure 22: Sucrose (white sugar) GC-MS quantitative calibration curve; the response factor is the gradient m given in the trendline equation y=mx.



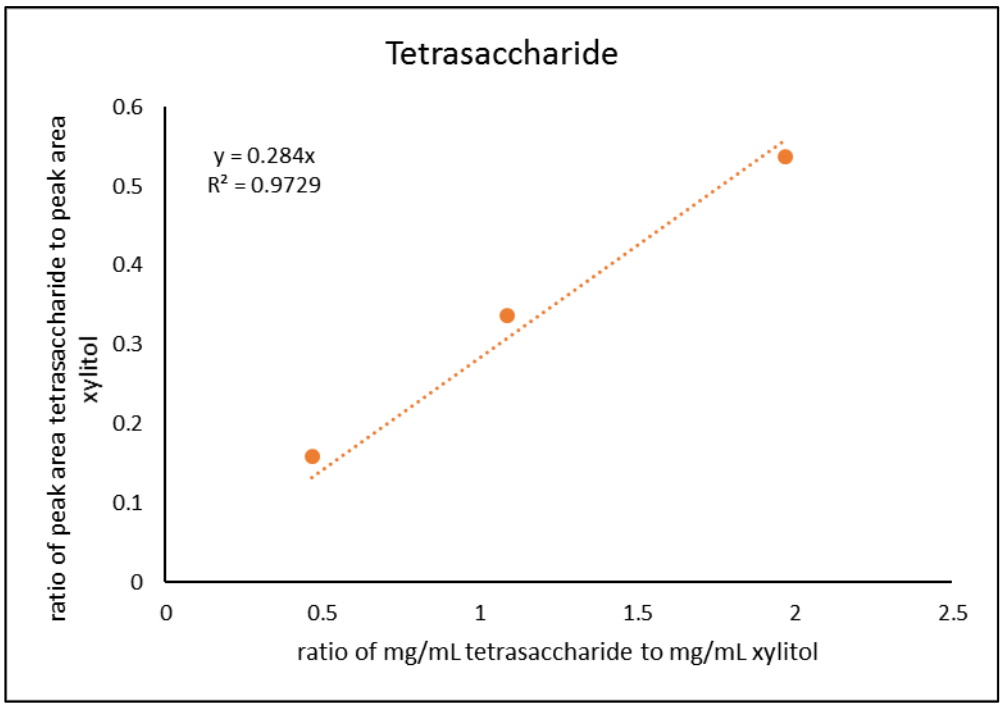
Appendix Figure 23: Cellobiose GC-MS quantitative calibration curve; the response factor is the gradient m given in the trendline equation y=mx.



Appendix Figure 24: Melezitose GC-MS quantitative calibration curve; the response factor is the gradient m given in the trendline y=mx.



Appendix Figure 25: 6-kestose GC-MS quantitative calibration curve; the response factor is the gradient m given in the trendline equation y=mx.



Appendix Figure 26: Tetrasaccharide α-D-Glcp(1→4)-α-D-Glcp(1→4)-α-D-Glcp(1→2)-β-D-Fruf GC-MS quantitative calibration curve; the response factor is the gradient m given in the trendline equation y=mx.

Appendix 5: Details of NMR, HPLC, and GC-MS qualitative and quantitative samples and standards

Appendix Table 1: Details of standards and freeze-dried preparative HPLC fractions of honey samples AH3, HND, and GWH, analyzed by NMR for identification of sugars.

Sample	Mass (mg)	D_2O (mL)
AH3		
1a	Unknown (washed sample vial with D2O) *	0.75
1b	Unknown (washed sample vial with D2O) *	0.75
2a	Unknown (washed sample vial with D2O) *	0.75
2b	Unknown (washed sample vial with D2O) *	0.75
3	13.4	0.75
4	22	1
5a	13.4	0.75
5b	12.2	0.75
HND		
1	3.99	0.75
2	Unknown (washed sample vial with D2O) *	0.75
2a	5.21	0.75
2b	5.48	0.75
GWH		
Frame crystals	14.58	1
1	3.03	0.75
1a	7.13	0.75
2b	14.95	0.75
3	14.82	0.75
GWC		
Crystals	15.85	0.75
Standards		
Glucose	9.94	1.00
Fructose	20.61	1.00
Sucrose	33.97	3.00
Melezitose	9.18	2.00
6-kestose	9.70	0.75
Glucuronic acid	16.00	0.75
D-gluconic acid	20.70	1.00

** These fractions did not leave enough dried residue to accurately weigh and dissolve; the freeze-dried vial was washed with D_2O to dissolve the sample, and the whole transferred to a clean NMR tube for analysis.*

Appendix Table 2: Details of HPLC quantitative standards and samples by RI detection, showing the standard concentrations used and the mathematical equation and coefficient of determination (R^2) of the calibration curve linear equation, where y is the peak area and x the concentration.

Sample or standard	honey/sugar (mg)	water (mL)	Concentration (mg/mL)	ret. time	peak area	calibration gradient	R^2
AH3 aliquot 1	72.17	5.000	14.43				
AH3 aliquot 2	80.55	5.000	16.11				
AH3 aliquot 3	77.01	5.000	15.4				
HND aliquot 1	80.25	5.000	16.05				
HND aliquot 2	75.63	5.000	15.13				
HND aliquot 3	91.81	6.000	15.30				
GWH aliquot 1	118.97	6.000	19.83				
GWH aliquot 2	98.17	5.000	19.63				
GWH aliquot 3	115.28	6.000	19.21				
GWH candy 1	46.32	3.000	15.44				
GWH candy 2	43.36	3.000	14.45				
GWH candy 3	48.92	3.000	16.30				
Werther candy 1	44.45	3.000	14.82				
Werther candy 2	45.76	3.000	15.25				
Werther candy 3	44.00	3.000	14.67				
Melezitose 1	4.9	2.000	2.45	16.715	345854	y = 142,731.787x	0.997
Melezitose 3	5.1	1.500	3.40	16.731	474166		
Melezitose 2	5.0	1.000	5.00	16.736	723103		
Sucrose 1	10.1	2.004	5.04	18.131	618134	y = 136283.697x	0.983
Sucrose 2	20.7	2.000	10.35	18.168	1468538		
Sucrose 3	11.1	3.000	3.70	18.135	491138		
Sucrose 4	10.6	2.000	5.30	18.133	683542		
Glucose 1	10.4	2.004	5.19	20.979	766850	y = 157574.089x	0.993
Glucose 2	19.8	2.000	9.90	21.024	1597892		
Glucose 3	9.9	3.000	3.30	20.993	514549		
Glucose 4	9.8	2.000	4.90	20.99	753098		
Fructose 1	10.1	2.004	5.04	22.156	788934	y = 159193.533x	0.994
Fructose 2	20.2	2.000	10.10	22.173	1644444		
Fructose 3	10.0	3.000	3.33	22.142	510544		
Fructose 4	9.9	2.000	4.95	22.143	740519		
D-gluconic acid 1	4.1	4.000	1.03	8.724	158423	y = 154750.733x	0.999
D-gluconic acid 2	4.4	10.000	0.44	8.569	69000		
D-gluconic acid 3	3.9	20.000	0.20	8.033	28048		
D-gluconic acid 4	5.0	50.000	0.10	8.4	17637		
Maltose 1	4.3	4.000	1.08	19.272	158702	y = 181,556.437x	0.996
Maltose 2	9.4	4.000	2.35	19.281	421483		
Maltose 3	21.1	4.000	5.28	19.227	967448		
6-kestose 1	3.6	3.500	1.03	17.51	154248	y = 160,843.145x	0.998
6-kestose 2	6.2	2.000	3.10	17.527	493486		
6-kestose 3	12.0	3.000	4.01	17.016	652346		
Blue Dextran	2.36	2.000	1.18	10.56		(retention time standard)	

Appendix Table 2 continued

Sample or standard	honey/sugar (mg)	water (mL)	Concentration (mg/mL)	ret. time	peak area	calibration gradient	R^2
Ethanol	0.40	4.000	0.10	28.629		(retention time standard)	
Pectin 1	0.2	2.000	0.12	11.797	19161	y = 91,283.565x	0.988
Pectin 2	1.6	3.000	0.52	12.386	55903		
Pectin 3	3.9	2.000	1.96	12.075	116151		
Cellobiose 1	3.2	3.000	1.06	18.89	190629	y = 84,799.275x	0.999
Cellobiose 2	7.2	3.500	2.05	18.883	381287		
Cellobiose 3	2.0	4.000	0.50	18.882	91510		
Raffinose 1	8.46	2.000	4.23	16.851	641595	y = 152,555.694x	0.999
Raffinose 2	7.31	3.000	2.44	16.85	375937		
Raffinose 3	2.07	2.000	1.035	16.836	163170		
HND/2a 1*	0.21	0.200	1.05	15.585	162614	y = 149,726.504x	0.979
HND/2a 2*	0.40	0.200	0.20	15.595	244724		
HND/2a 3*	0.90	0.200	0.45	15.5815	696833		

The freeze-dried HPLC preparative fraction HND/2a was found by NMR to be a clean sample of the tetrasaccharide/s comprising HND fractions **2a and **2b**, and was used as a GC-MS quantitative standard for the tetrasaccharide portion of HND.*

Appendix Table 3: Details of HPLC salicylic acid quantitative standards by UV detection, showing the standard concentrations used and the mathematical equation and coefficient of determination (R^2) of the calibration curve linear equation, where y is the peak area and x the concentration.

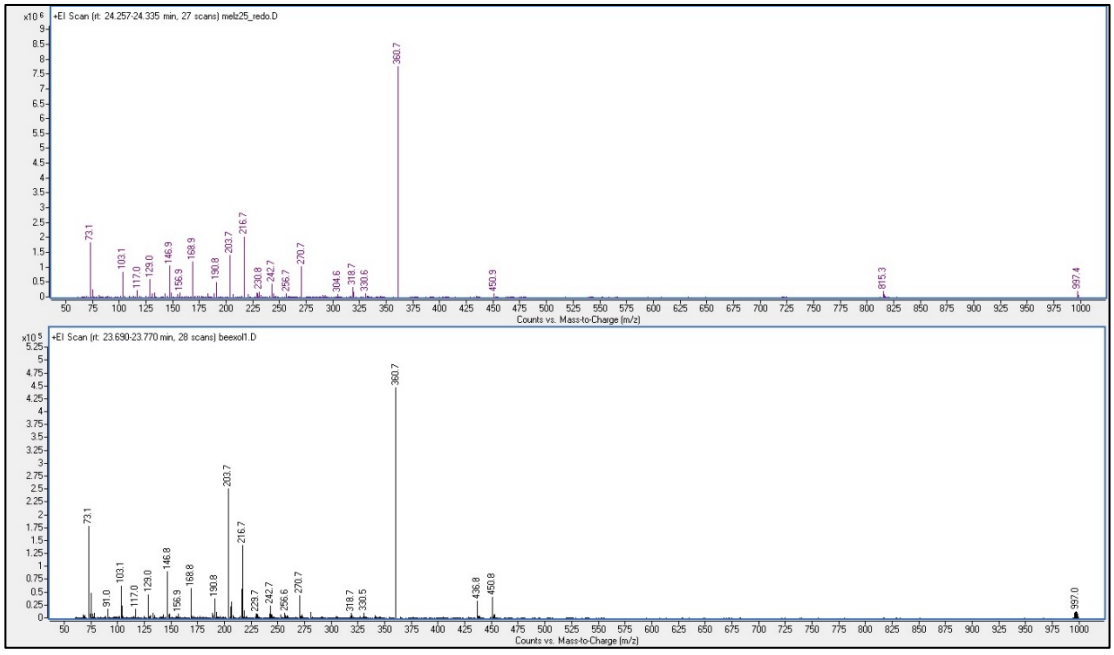
	standard (mg)	water (mL)	Concentration (mg/mL)	ret. time	peak area	calibration gradient	R^2
Salicylic acid 1	0.96	4.000	0.5888	11.776	12254531	y = 394,102,870.952x	0.981
Salicylic acid 2	3.98	4.000	0.6335	12.67	35553792		
Salicylic acid 3	1.38	3.000	0.46	11.461	179366287		

Appendix Table 4: Details of GC-MS quantitative standards, where all samples were made up in 1.0 mL TMSi, and each standard was tested at three concentrations, each with a different ratio of standard to internal standard, to produce the response factor and concentration calibration curves shown in Appendix 4. 'Sucrose' is the Chelsea White Sugar standard mentioned in section 2.1. RF is calculated by Equation 2-4, and R^2 is the fit of the trendline of which RF is the slope.

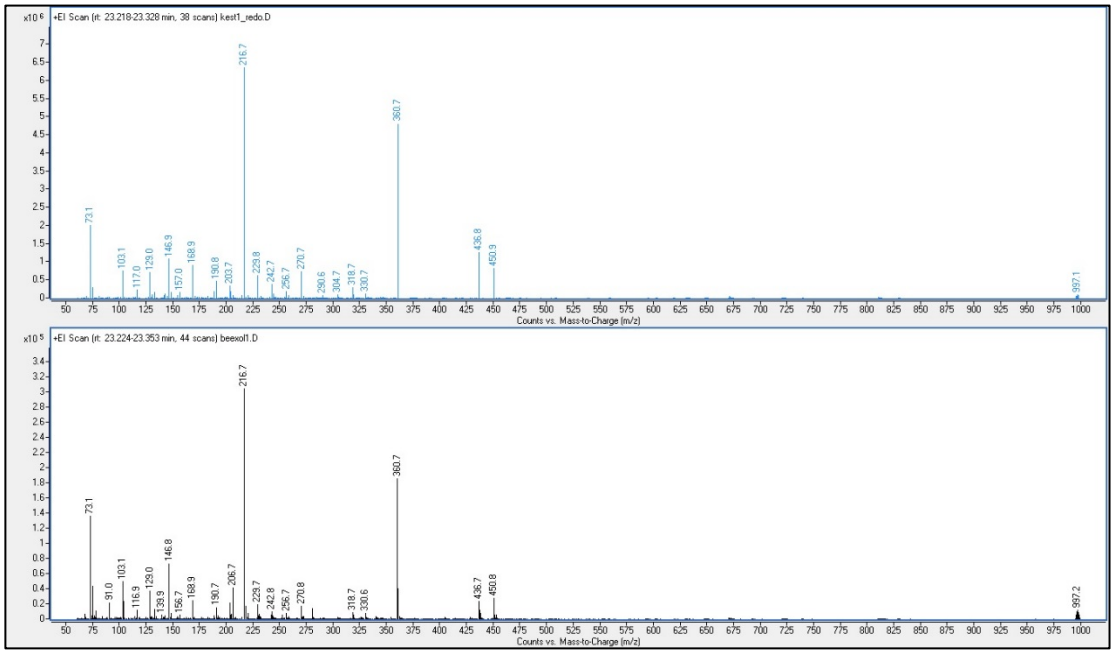
Sample	mg	mg xyl.	peak sugar	peak xyl.	RF	R^2
AH3.1	7.48	0.53				
AH3.2	8.57	1.02				
AH3.3	3.28	1.94				
GWA.1	4.86	0.52				
GWA.2	5.31	0.92				
GWA.3	5.23	2.12				
HND.1	5.77	0.37				
HND.2	4.57	1.6				
HND.3	6.2	3.01				
Melezitose 1	0.12	1.38	198,512,210.69	1,296,713,575.87	0.6949	0.7652
Melezitose 2	0.47	0.77	247,668,849.83	1,006,284,122.75		
Melezitose 3	1.39	1.46	909,575,273.40	1,185,380,748.34		
6-kestose 1	0.15	1.19	160,915,753.36	1,192,420,644.75	1.0422	0.7917
6-kestose 2	0.38	0.93	267,269,267.63	858,919,449.62		
6-kestose 3	0.91	1.9	764,005,745.80	1,281,762,814.80		
Sucrose 1	0.21	0.92	240,263,182.39	826,139,304.00	0.6024	0.5164
Sucrose 2	0.56	0.94	373,924,028.93	754,050,206.53		
Sucrose 3	1.14	0.83	539,841,015.56	727,006,432.37		
Maltose 1	0.22	0.95	304,959,718.75	704,664,042.63	1.1005	0.7900
Maltose 2	0.52	1.19	478,475,801.93	789,115,226.27		
Maltose 3	1.17	1.06	859,004,404.01	761,700,060.21		
Cellobiose 1	0.29	0.81	218,888,638.36	754,679,172.94	0.6915	0.9666
Cellobiose 2	0.48	0.9	291,201,955.97	725,674,420.73		
Cellobiose 3	0.97	0.87	648,511,579.25	874,165,438.89		
Raffinose 1	0.23	0.13	927446762.8	723941921.1	0.7294	0.983
Raffinose 2	0.17	0.42	539554844.1	1676732135		
Raffinose 3	0.1	1.92	531443161.8	3615722724		
HND/2a 1*	1.06	2.27	241175373.2	1513500307	0.284	0.9729
HND/2a 2*	1.27	1.17	389192719.6	1158497088		
HND/2a 3*	1.99	1.01	578830461	1075949982		

The freeze-dried HPLC preparative fraction HND/2a was found by NMR to be a clean sample of the single tetrasaccharide comprising HND fractions **2a and **2b**, and was used as a GC-MS quantitative standard for the tetrasaccharide portion of HND*

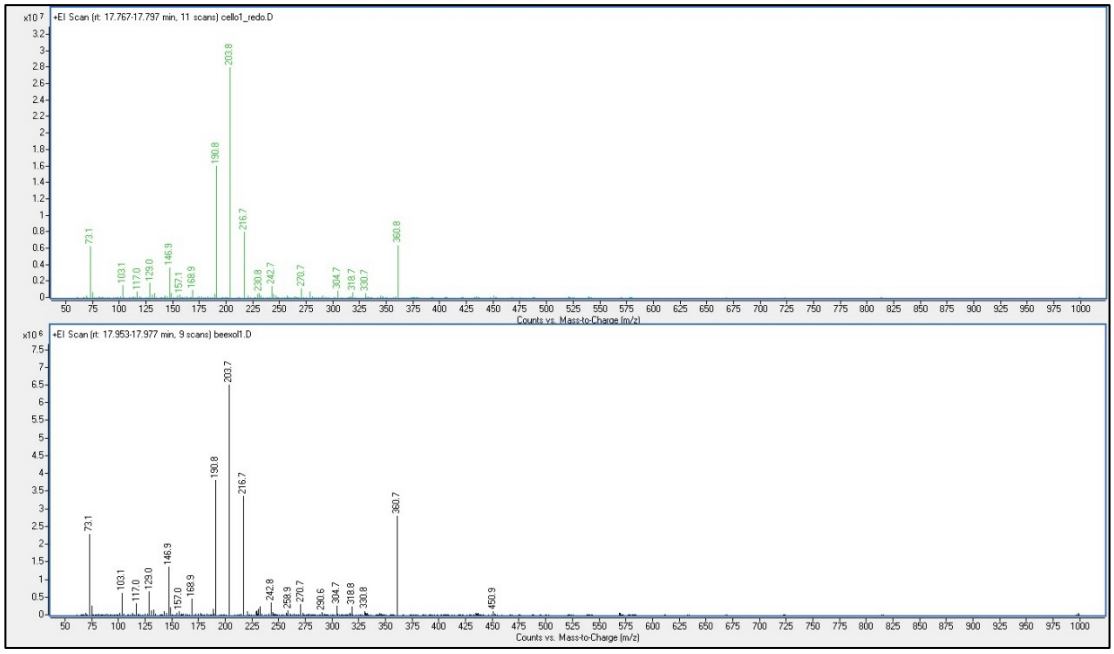
Appendix 6: AH3 mass spectra for further peak ID by GC-MS



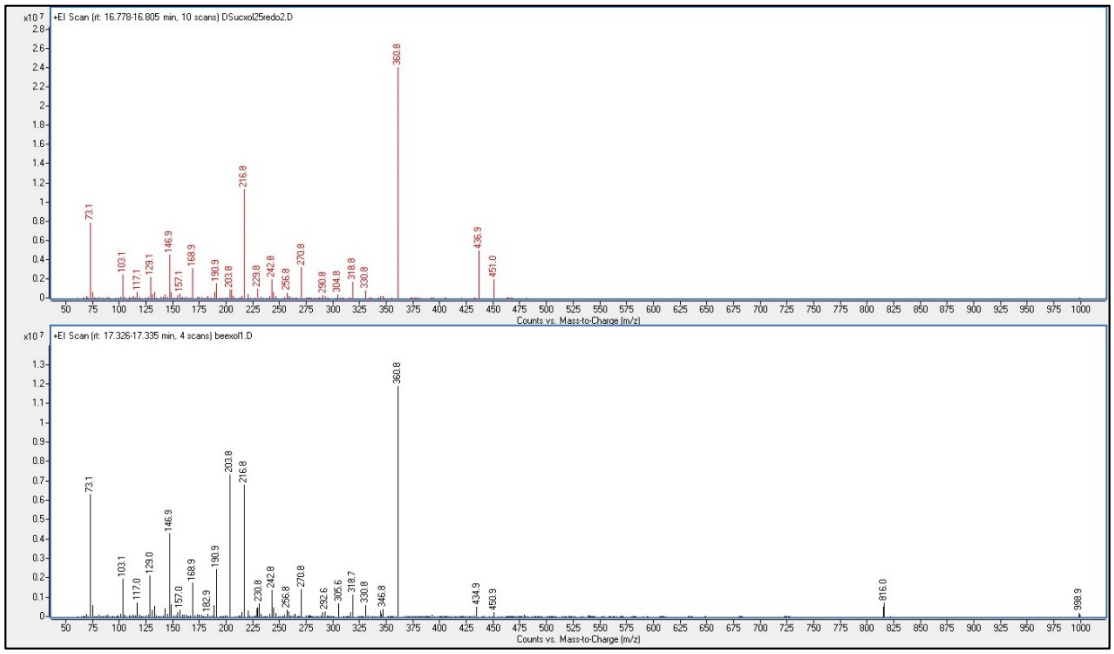
Appendix Figure 27: Mass spectra of melezitose peak 24.257-24.335 min (top) and AH3 peak 23.690-23.770 min (bottom).



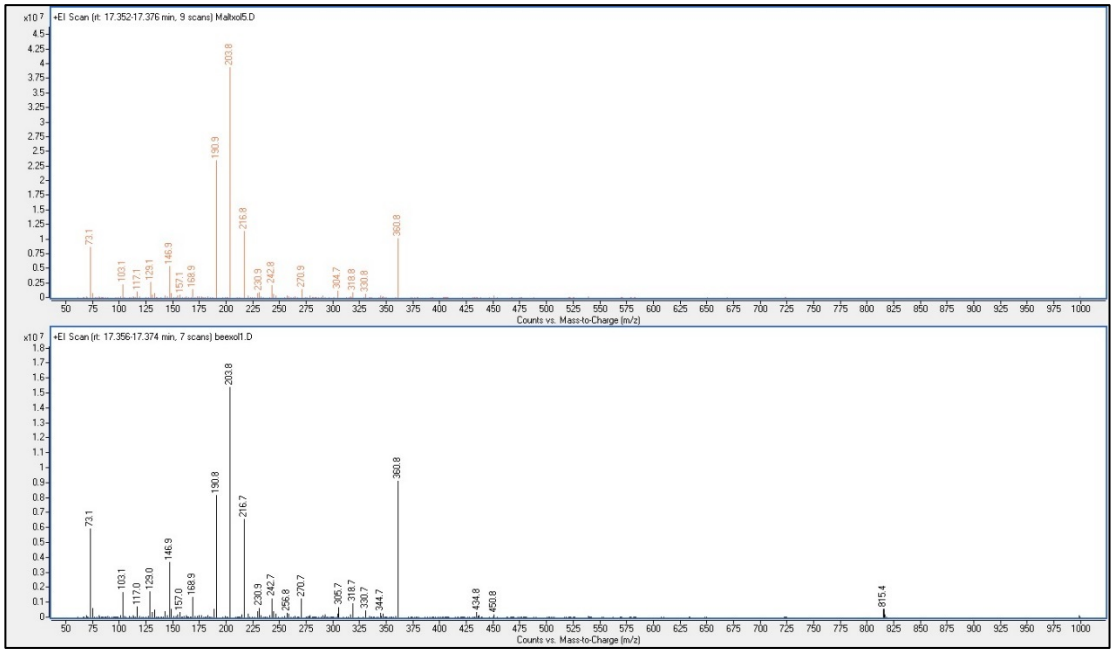
Appendix Figure 28: Mass spectra of 6-kestose peak 23.218-23.328 min (top) and AH3 peak 23.224-23.353 min (bottom).



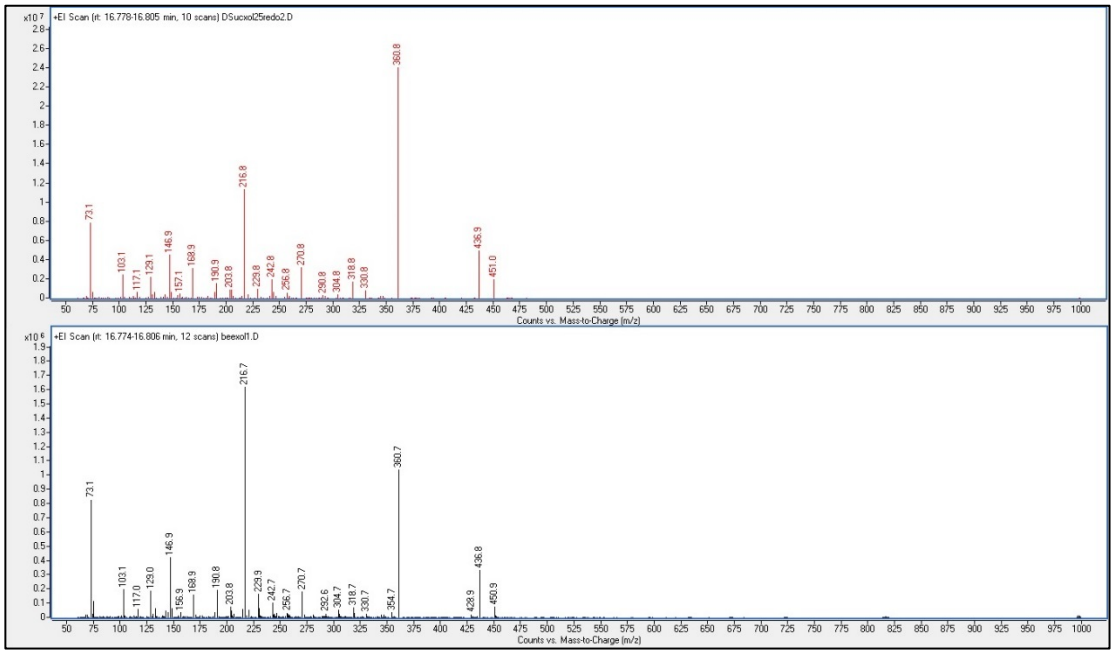
Appendix Figure 29: Mass spectra of cellobiose peak 17.767-17.797 min (top) and AH3 peak 17.953-17.977 min (bottom).



Appendix Figure 30: Mass spectra of sucrose peak 16.778-16.805 min (top) and AH3 peak 17.326-17.335 min (bottom).

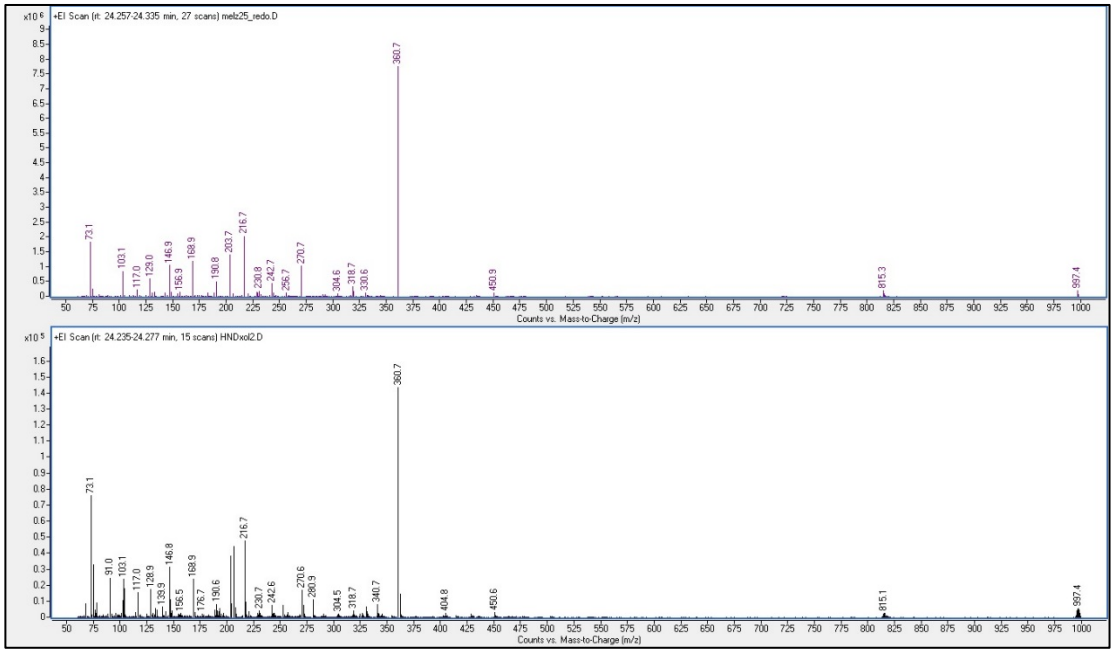


Appendix Figure 31: Mass spectra of maltose peak 17.352-17.376 min (top) and AH3 peak 17.356-17.374 min (bottom).

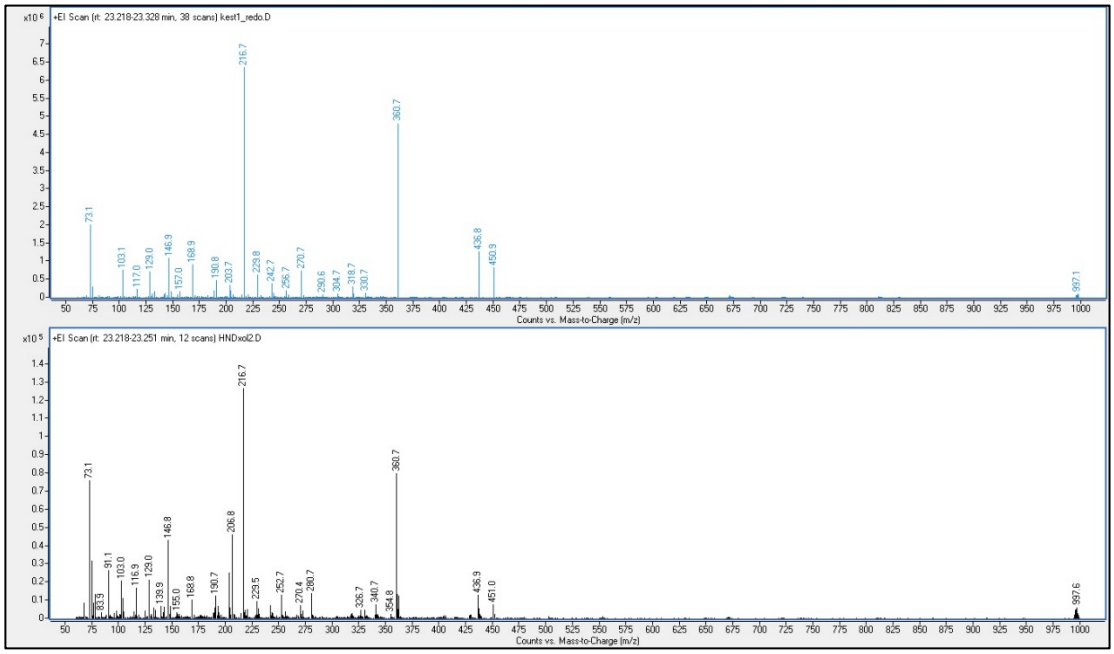


Appendix Figure 32: Mass spectra of sucrose peak 16.778-16.805 min (top) and AH3 peak 16.774-16.806 min (bottom).

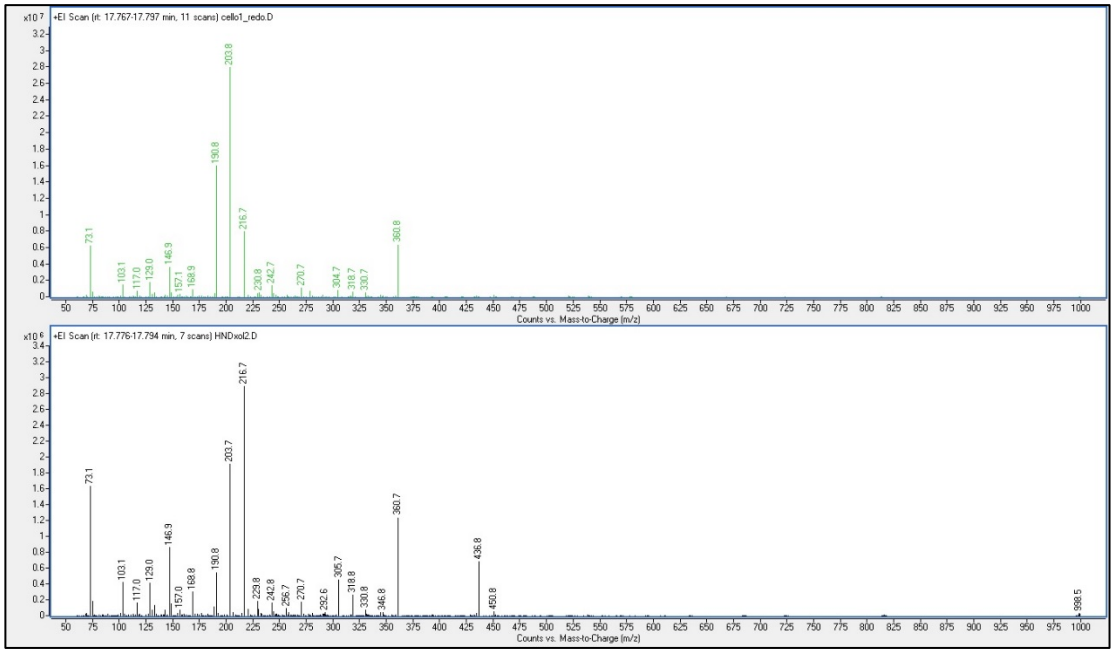
Appendix 7: HND mass spectra for further peak ID by GC-MS



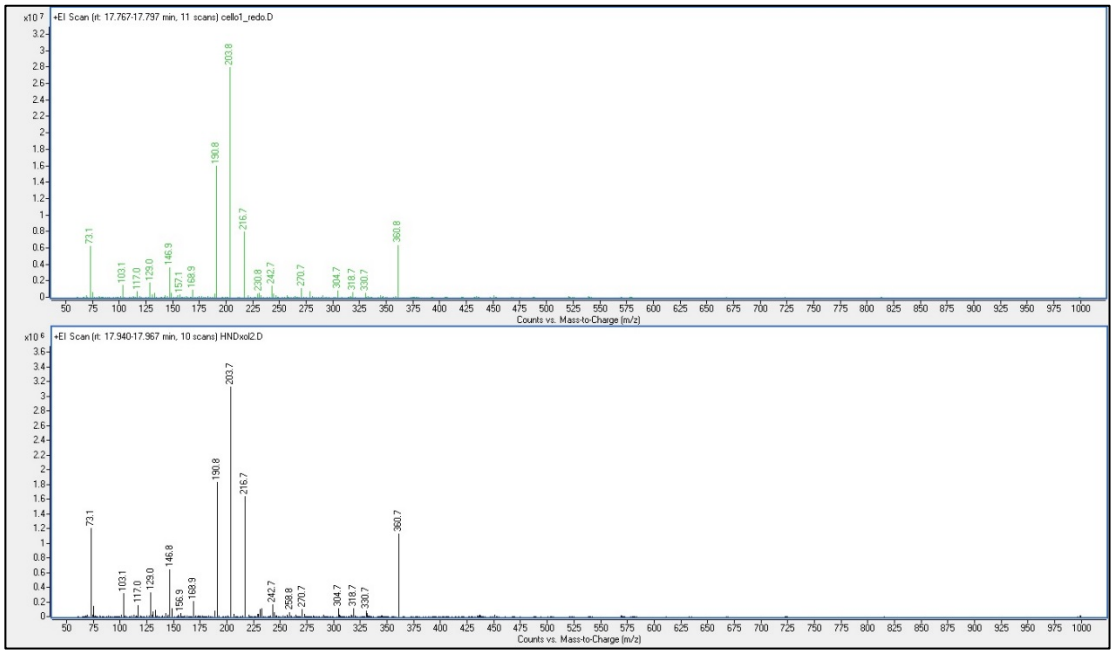
Appendix Figure 33: Mass spectra of melezitose peak 24.257-24.335 min (top) and HND peak 24.235-24.277 min (bottom).



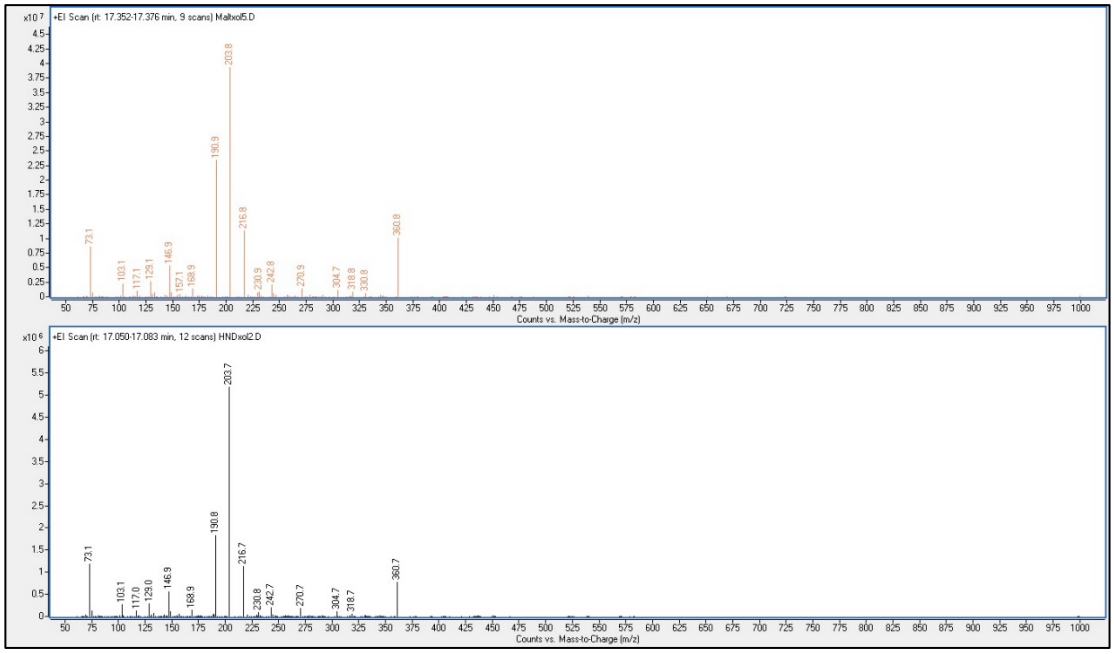
Appendix Figure 34: Mass spectra of 6-kestose peak 23.218-23.238 min (top) and HND peak 23.218-23.251 min (bottom).



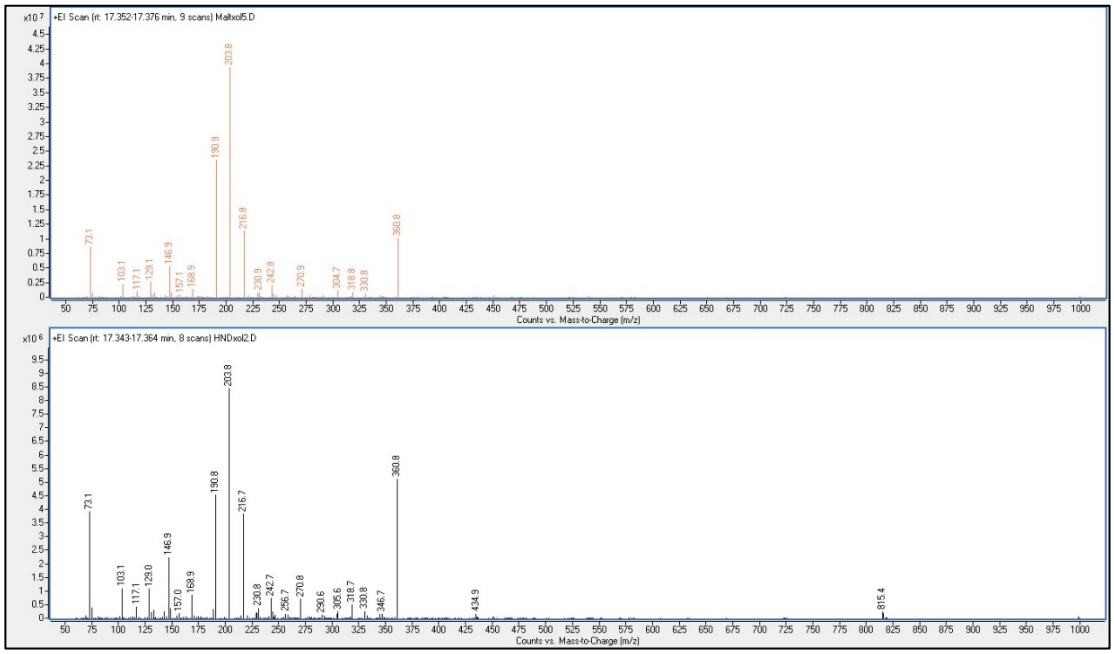
Appendix Figure 35: Mass spectra of cellobiose peak 17.767-17.797 min (top) and HND peak 17.776-17.794 min (bottom).



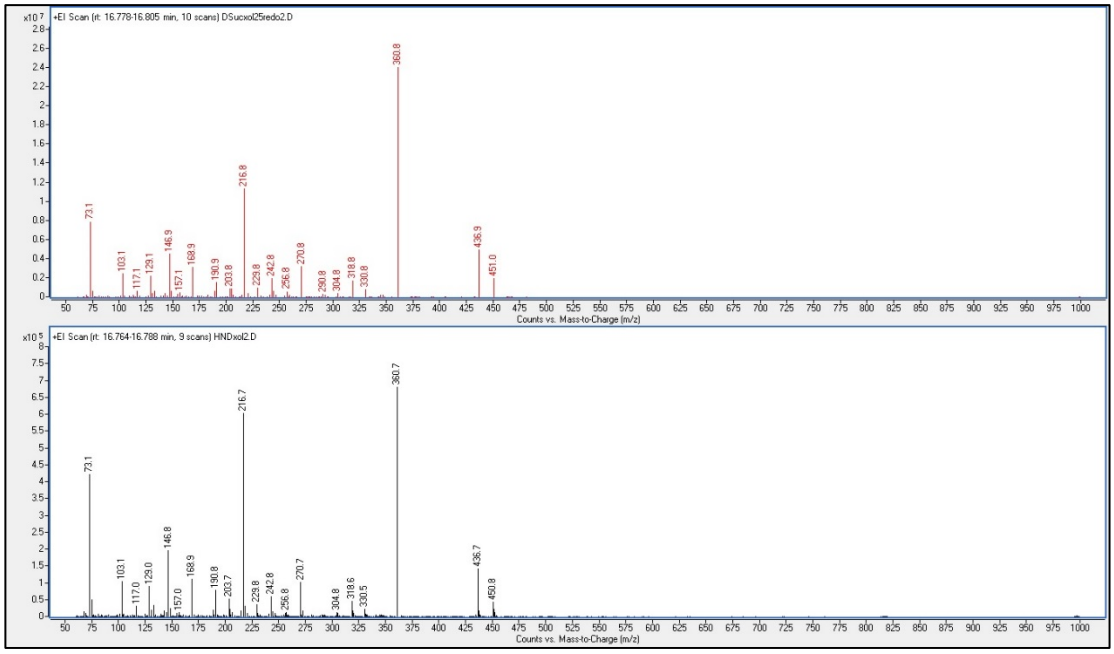
Appendix Figure 36: Mass spectra of cellobiose peak 17.767-17.797 min (top) and HND peak 17.940-17.967 min (bottom).



Appendix Figure 37: Mass spectra of maltose peak 17.352-17.376 min (top) and HND peak 17.050-17.083 min (bottom).



Appendix Figure 38: mass spectra of maltose peak 17.352-17.376 min (top) and HND peak 17.343-17.364 min (bottom).



Appendix Figure 39: Mass spectra of sucrose peak 16.778-16.805 min (top) and HND peak 16.764-16.788 min (bottom).