Development of a Method for the Quantitative Detection of Honey in Imported Products.

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ABSTRACT

The carbohydrate composition of Asian honeys was determined using analysis of per-*O*-trimethylsilylated sugar alditols by GC-FID. This method was established to detect the presence and quantify honey in imported products scheduled for investigation by the Ministry of Agriculture and Fisheries (MAF) Biosecurity, because the import of honey products is regulated.

The Asian honeys analysed had a carbohydrate composition within the limits set for honey by the Codex Alimentarius Commission, and had a disaccharide profile similar to honeys from elsewhere in the world.

Kojibiose, and peaks corresponding to turanose/nigerose and turanose/maltulose, which are carbohydrates not common in nature, were present in all the honey samples analysed. A reference database of the sugar content of these honeys was created; and the presence of these disaccharides together in imported products under investigation would indicate that the product contains honey.

Several samples were found to be adulterated, mostly with sucrose syrup and also with glucose syrup through improper bee-feeding.

This method is suitable for detection of the presence of honey in a product being investigated but might encounter problems when quantitation of the honey at low levels of honey addition is required, due to the poor precision of the method. This low precision resulted from the difficulty in getting a homogeneous honey sample and quantifying the small or poorly resolved peaks in the chromatograms. A report on the analysis of actual samples supplied by MAF is presented in Appendix A; quantitation of the monosaccharides, the ratio of glucose:fructose and ratio of disaccharides to monosaccharides could be used to quantitate the amount of honey present and this method is recommended for future use.

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α	lower face of a D-sugar in the Haworth convention
β	upper face of a D-sugar in the Haworth convention
CS	corn syrup
D	D series sugar
DFAs	difructose anhydrides
DP	degree of polymerisation
EGS	polyethylene glycosuccinate
ELSD	evaporative light scattering detector
F/G	fructose/glucose ratio
Fru	fructo
FTIR	fourier-transform infrared
GC	gas chromatography
GC-ArID	gas chromatography-argon ionisation detector
GC-FID	gas chromatography-flame ionisation detector
GC-MS	gas chromatography-mass spectrometry
Glc	gluco
HDMS	hexamethyldisilazane
HFCS	high fructose corn syrup
HMF	hydroxymethylfurfural
HPAE	high pressure anion exchange chromatography
HPLC	high pressure liquid chromatography
IS	invert syrup
IUPAC	International Union of Pure and Applied Chemistry
LC	liquid chromatography
MAF	Ministry of Agriculture and Forestry
MALDI-TOF	matrix-assisted laser desorption/ionisation time-of-flight
NIR	near-infrared
NMR	nuclear magnetic resonance
NOE	nuclear overhauser effects

NZ	New Zealand
p	pyranosyl
PAD	pulsed amperometric detector
PC	paper chromatography
PCA	principal component analysis
PLE	pressurised liquid extraction
RF	response factor
RI	refractive index
SCIRA	stable carbon isotopic ratio analysis
SEC	size-exclusion chromatography
SIM	selective ion monitoring
SPE	solid-phase extraction
SS	sucrose syrup
TFA	trifluoroacetic acid
TLC	thin-layer chromatography
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
UV	ultraviolet
w/w	relative percentage (weight/weight)

1 Chapter One:

INTRODUCTION

1.1 Honey

"Honey is the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature." [1]

Honey has been used by humans for thousands of years for nourishment or medicinal purposes, and it is also the only sweetening material that requires no manipulation or processing to make it ready to eat.

1.2 Honey Precursors and Production

Nectar and honeydew are the raw materials used by the honeybee for the production of honey. Both originate in the sap of vascular plants, the fluid that distributes nutrients throughout the plant. [2]

1.2.1 Nectar

The floral nectaries are supplied through the phloem (the sieve tube, cells and fibers that form the food conducting tissue of a plant) and xylem (the supporting and water-conducting tissue of vascular plants). These nectaries contain large amounts of sugar and are the bees' source of raw material from which to produce floral or nectar honey. [2]

1.2.2 Honeydew

Honeydew is a sweet liquid which comes mainly from excretions produced by hemipterous insects (plant lice such as aphids and scale insects) which feed on phloem sap and excrete the sweet liquid which is then collected by the bee. [2, 3]
Honeydew honey is variously prized relative to floral honey: in certain regions of central Europe it is more valued than floral honey but on the contrary, in North America it is considered of inferior quality. [4]

1.2.3 Honey Production

The honeybee (*Apis mellifera*) produces honey by collecting the sugar-containing raw material using its proboscis and transporting it in the honey sac. In the honey sac, the raw material is mixed with secretions from the bee, adding water and enzymes to the raw material. The foraging bee then returns to the hive and passes its load to the house bees, who undertake the process of ripening the honey. The house bee alternately expels and ingests the honey sac fluid and adds further enzyme secretions and reduces the water content of the fluid until it is ripe. Honey is ripe when the nectar sucrose has been “inverted” or transformed mostly into glucose and fructose and concentrated to about 82% solids. The bees then store the honey as a food source in the comb cells. [2]

Honey bees generally show preference for floral nectar over honeydew, but when nectar is not as easily available such as during drought or as a consequence of changed agricultural practices which have restricted bee pastures, bees gather honeydew instead. [2, 4]

1.3 Composition of Honey

The following details regarding the composition of honey are derived from *Apis mellifera* honey.

The chemical composition of honey can vary depending on the floral origin, seasonal and climatic variations, and geographical origin (see Table 1). Some of the components (carbohydrates, water, traces of organic acids, minerals, enzymes, amino acids, proteins, pigments, pollen and wax) are due to maturation of the honey, some are added by the bees and some of them are derived from the plants. [3, 5]

Table 1: Relative percentage (w/w) of glucose, fructose, oligosaccharides and water in four floral types of Canadian honeys. [3]

Honey type	% glucose	% fructose	% oligosaccharides	% water
Alfalfa	33.9 ± 0.4	37.0 ± 0.4	7.5	19.6 ± 0.6
Alsike	35.6 ± 0.4	38.5 ± 0.4	7.5	17.1 ± 0.6
Canola	40.0 ± 0.4	36.2 ± 0.4	2.0	18.9 ± 0.6
Trefoil	33.2 ± 0.4	37.1 ± 0.4	6.2	21.9 ± 0.6

1.3.1 Moisture Content

Moisture content is one of the most important characteristics of honey as it influences its keeping quality, granulation, and body. Its concentration is a function of the factors involved in ripening, including weather conditions, original moisture of nectar, its rate of secretion, and strength of the bee colony (as the bees use their wings to create a stream of dry air that constitutes the ventilation system of the hive). [3, 4]

The average moisture content of honey is 17.2%, but the range of moisture content varied from 12.2-22.9% in a survey of 490 samples of USA honey. [3]

A similar survey found the average moisture content of honeys from the Madrid province of Spain was 16.13% with a range from 13.00-18.30%. [6]

1.3.2 Carbohydrates

More than 95% of the solids of honey are carbohydrate in nature. The monosaccharides fructose and glucose are the building blocks for the more complex oligosaccharides; and represent about 85-95% of the sugar content. The remainder of the sugar content is composed of disaccharides, trisaccharides and a few higher oligosaccharides. [3, 4]

The higher sugars are formed by transglycosilation due to enzymes from the bee. Glycosyl hydrolase enzymes will, in conditions of high concentration act as transglycosidases. (Section 1.4.)

Doner (1977) [2] wrote a comprehensive review of the sugars of honey and indicated the presence of about 10-13 disaccharides and 8-9 trisaccharides but more recently, Ruiz-Matute, Brokl, Soria, Sanz & Martinez-Castro (2010) [7] claim to have characterised 25 trisaccharides (12 unequivocally) and 9 tetrasaccharides.
The names and formulæs of the oligosaccharides found in honey are given in Table 2.

Table 2: Names and formulæ of the oligosaccharides found in honey.

Trivial Name	MW	Nomenclature [8]	References
Disaccharides	342.3	$C_{12}H_{22}O_{11}$	
Cellobiose		β-D-Glc*p*-(1→4)-D-Glc*p*	*[9, 10]*
Gentiobiose		β-D-Glc*p*-(1→6)-D-Glc*p*	*[9-12]*
Inulobiose		β-D-Fru*f*-(2→1)-D-Fru*f*	*[13]*
Isomaltose		α-D-Glc*p*-(1→6)-D-Glc*p*	*[9-12, 14, 15]*
Laminaribiose		β-D-Glc*p*-(1→3)-D-Glc*p*	*[9, 11, 12]*
Leucrose		α-D-Glc*p*-(1→5)-D-Fru*p*	*[6, 15]*
Kojibiose		α-D-Glc*p*-(1→2)-D-Glc*p*	*[9, 11, 12, 15]*
Maltose		α-D-Glc*p*-(1→4)-D-Glc*p*	*[9-12, 14, 15]*
Maltulose		α-D-Glc*p*-(1→4)-D-Fru*p*(*f*)	*[11, 12, 14]*
Melibiose		α-D-Gal*p*-(1→6)-D-Glc*p*	*[10]*
Neotrehalose (α,β-Trehalose)		α-D-Glc*p*-(1↔1)-β-D-Glc*p*	*[9, 11, 12]*
Nigerose (Sakebiose)		α-D-Glc*p*-(1→3)-D-Glc*p*	*[9, 11, 12, 14, 15]*
Palatinose (Isomaltulose)		α-D-Glc*p*-(1→6)-D-Fru*f*	*[9-12]*
Sophorose		β-D-Glc*p*-(1→2)-D-Glc*p*	*[16]*
Sucrose		β-D-Fru*f*-(2↔1)-α-D-Glc*p*	*[9-12, 14]*
Trehalose (α,α-Trehalose)		α-D-Glc*p*-(1↔1)-α-D-Glc*p*	*[10]*
Turanose		α-D-Glc*p*-(1→3)-D-Fru*f*(*p*)	*[9-12, 14]*
Trehalulose		α-D-Glc*p*-(1→1)-D-Fru*f*(*p*)	*[13]*

Table 2: Names and formulæ of the oligosaccharides found in honey. (continued)

Trisaccharides	504.4	$C_{18}H_{32}O_{16}$	
Centose		α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→2)-D-Glc*p*	*[17]*
Erlose		α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	*[7, 9, 10, 12, 17]*
Isomaltotriose		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-Glc*p*	*[7, 10, 12, 17]*
Isomelezitose		α-D-Glc*p*-(1→6)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*	*[18]*
Isopanose		α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-D-Glc*p*	*[9, 12, 17]*
1-Kestose		β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*	*[7, 12, 17]*
6-Kestose		β-D-Fru*f*-(2→6)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*	*[7]*
Laminaritriose		β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-D-Glc*p*	*[12]*
Maltotriose		α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p*	*[7, 9, 10, 12, 17]*
Melezitose		α-D-Glc*p*-(1→3)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*	*[7, 10, 12, 17]*
Neokestose		β-D-Fru*f*-(2→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	*[7]*
Panose		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*	*[7, 9, 10, 12, 17]*
Planteose		α-D-Gal*p*-(1→6)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*	*[7]*
Raffinose		α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	*[7, 10]*
Theanderose		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	*[7, 9, 12, 17]*
3-α-isomaltosylglucose *		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-D-Glc*p*	*[17]*
4-α-gentiobiosylglucose (sorborose) *		β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*	*[17]*

Table 2: Names and formulæ of the oligosaccharides found in honey. (continued)

Tetrasaccharides	666.6	$C_{24}H_{42}O_{21}$	
α-4'-glucosyl-erlose		α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-*β*-D-Fru*f*	*[19]*
α-6'-glucosyl-erlose		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-*β*-D-Fru*f*	*[19]*
Fructosyl-isomelezitose *		Fru-(?→?)-α-D-Glc*p*-(1→6)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*	*[18]*
Isomaltotetraose		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-Glcp	*[17]*
Maltotetraose		α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p*	*[19]*
Nystose		β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*	*[7, 18]*
Stachyose		α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	*[18]*
Pentasaccharides	828.7	$C_{30}H_{52}O_{26}$	
Isomaltopentaose		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-Glc*p*	*[17]*
*		α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	*[19]*
*		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-*β*-D-Fru*f*	*[19]*
Hexasaccharides	990.9	$C_{36}H_{62}O_{31}$	
*		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-*β*-D-Fru*f*	*[19]*

* No trivial name

1.3.2.1Floral honey carbohydrates

In the early years of honey research, honey was believed to be a simple mixture of dextrose (glucose), levulose (fructose), and sucrose, with an undefined carbohydrate material called “honey dextrin”, believed to be analogous to starch dextrin. Over the years, improvements in analytical and separation procedures have revealed honey to be a highly complex mixture of sugars of which glucose and fructose account for 85% of the honey solids. [2, 3]

White (1992) [3] analysed 490 samples of USA honeys, the results are summarised in Table 3.

Table 3: Average composition of honey for 490 samples of USA honey. [3]

Component	Average (%)	Standard Deviation	Range (%)
Moisture	17.2	1.5	12.2 – 22.9
Fructose	38.4	1.8	30.9 – 44.3
Glucose	30.3	3.0	22.9 – 40.7
Sucrose	1.3	0.9	0.2 – 7.6
Reducing Disaccharides	7.3	2.1	2.7 – 16.0
Higher Sugars	1.4	1.1	0.1 – 3.8

Siddiqui & Furgala (1967, 1968) [11, 17] analysed the oligosaccharide content of a honey produced by bees foraging on alfalfa and red clover in Canada. The results of their analysis are shown in Table 4.

Table 4: Yields of the principal sugars in the oligosaccharide fraction (3.65%) of honey. [11, 17]

Disaccharides	%	Trisaccharides	%	Higher oligosaccharides	%
Maltose	29.4	Erlose	4.5	Isomaltotetraose	0.33
Kojibiose	8.2	Theanderose	2.7	Isomaltopentaose	0.16
Turanose	4.7	Panose	2.5		
Isomaltose	4.4	Maltotriose	1.9		
Sucrose	3.9	1-Kestose	0.9		
Maltulose and Isomaltulose (and an unidentified ketose)	3.1	Isomaltotriose	0.6		
Nigerose	1.7	Melezitose	0.3		
α,β-Trehalose	1.1	Isopanose	0.24		
Gentiobiose	0.4	Centose	0.05		
Laminaribiose	0.09	3-α-Isomaltosylglucose	trace		
Total*	56.99		13.69		0.49

*Quantitative recoveries were calculated after allowing for loss of material during separation, they are therefore approximate.

A similar analysis of the oligosaccharide content of honey was performed by Low and Sporns (1988). The oligosaccharide content (~ 3%) of an Alsike honey from Canada was analysed. [9]

More recently, Ruiz-Matute, Sanz & Martinez-Castro (2007) analysed 35 honey samples purchased in Spain but from various origins and identified a new disaccharide from honey, inulobiose, ranging in concentration from 0.93 to 6.14 mg/g. The authors stated that this disaccharide could be formed by transfructosylation. [13]

1.3.2.2 Honeydew honey carbohydrates

Honeydew passes through the digestive system of insects, and, in the process is altered and honeydew honey (also called forest honey) is subsequently produced by the bees. [4]

On average, compared to floral honey, honeydew honey is lower in glucose by 5.2%, lower in fructose by 6.4% but higher in reducing disaccharides and higher sugars. [2]

An average composition of honeydew honey was determined by White et al. (1962) and is given in Table 5. [20] It is based on 14 samples, including alfalfa, cedar, hickory, oak, and several unidentified types of honey (as cited in [3], p. 914).

Table 5: Average composition of 14 honeydew honeys. [20]

Component	Average	Standard Deviation	Range
Moisture (%)	16.3	1.74	12.2 – 18.2
Fructose (%)	31.8	4.2	23.9 – 38.1
Glucose (%)	26.0	3.0	19.2 – 31.9
Sucrose (%)	0.8	0.2	0.4 – 1.1
"Maltose" (%)	8.8	2.5	5.1 – 12.5
Melezitose[a] (%)	2.3	4.6	0.0 – 13.4
Higher sugars (%)	4.7	1.0	1.3 – 11.5

[a] Eight samples

White (1992) [3], points out that these averages compare well with those obtained for 38 Swiss honeydew honeys by Bogdanov and Baumann (1988) [21]. He also stated that there are at least two types of honeydew honeys, containing erlose or melezitose or mixtures of both, depending upon the insect(s) involved. The melezitose type can granulate rapidly (frequently in the comb itself), and the erlose type does not granulate. [4]

New Zealand honeydew honey was analysed by Astwood, Lee & Manley-Harris (1998) [19] and their results are summarised in Table 6.

Astwood *et al.* (1998) noted that, compared to White (1962)'s American honeydew honeys, the New Zealand honeydew honey showed smaller amounts of sucrose and significantly smaller amounts of maltose. Conversely, the total percentage of higher sugars (without maltose and sucrose) was greater in the New Zealand honeydew honey. Argument for the differences included a difference in the enzymatic activity of the scale insect or the bee, or seasonal or atmospheric changes.

A distinctive feature of honeydew honey when compared with floral honey is its optical rotation. Honeydew honeys are dextrorotatory, while floral honeys are invariably levorotatory. [2]

The specific rotation power depends on the amount and quality of the sugars present in honey including oligosaccharides. It was shown by Battaglini & Bossi (1972) [22] that this physical property is particularly linked to the fructose to glucose ratio and the percentage of di- and trisaccharides. Honeys containing high levels of fructose and glucose along with low di- and trisaccharide levels are levorotatory. Conversely, low fructose, glucose levels with large quantities of di- and higher saccharides (as in honeydew honey) are dextrorotatory. [2]

D-Glucose is dextrorotatory whereas D-fructose is levorotatory and therefore the specific rotation of honey depends upon their relative proportions.

Table 6: Components of the oligosaccharide fraction of a New Zealand honeydew honey. [19]

Component	Mean % of honey solids[a]	Standard Deviation	Range
Sucrose	0.55	0.12	0.40 – 0.77
Trehalose	0.044	0.0036	0.042 – 0.051
Cellobiose	0.33	0.046	0.25 – 0.37
Turanose	1.5	0.26	1.2 – 1.8
Nigerose	1.1	0.13	0.94 – 1.3
Maltose	2.2	0.20	1.9 – 2.6
Gentiobiose	0.85	0.26	0.45 – 1.3
Palatinose	1.3	0.34	0.66 – 1.8
Isomaltose	0.32	0.072	0.24 – 0.42
Erlose	1.2	0.33	0.87 – 1.8
Melezitose	0.085	0.0080	0.069 – 0.093
Maltotriose	0.54	0.19	0.32 – 0.84
Panose	0.51	0.16	0.27 – 0.73
Maltotetraose	0.51	0.23	0.18 – 0.85
α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-*β*-D-Fru*f*	3.9	1.2	1.8 – 5.5
α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-*β*-D-Fru*f*	0.46	0.13	0.21 – 0.63
α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	1.1	0.89	0.11 – 2.9
α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-*β*-D-Fru*f*	0.33	0.15	0.08 – 0.60
α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-*β*-D-Fru*f*	0.28	0.13	0.07 – 0.53
Higher sugars (except maltose and sucrose)	14.31	3.5	8.71 – 20.57

[a] Mean of duplicate (GC) or triplicate (LC) samples of six different honeydew honey samples.

1.3.3 Acids and Other Minor Components

Organic acids are present in honey, the predominant acid being gluconic acid, which is derived from glucose. Other acids identified were lactic and pyroglutamic acid. In honey, pH is not only influenced by the amount of acid present but also by the mineral content; the average pH of honey being about 3.9, ranging from 3.2 to 4.5. [3]

Honey contains minerals such as potassium, sodium, calcium, magnesium, iron, copper, manganese, chlorine, phosphorus, sulfur and silicon; and these generally average about 0.17% of its weight (ranging from 0.02 – 1.0%). In general, dark honeys are richer in minerals than light-coloured honeys. [3]

Other minor components of honey are proteins, amino acids and vitamins. [3]

1.4 Enzymes in Honey.

α-Glucosidase is the enzyme responsible for the hydrolysis of nectar sucrose to fructose and glucose. This enzyme is added to the honey by the bee and of all enzymes added by the bee, it is the most important as it converts nectar sucrose to glucose and fructose. This enzyme also has transglycosylating action and has been shown by White & Maher (1953) [23] to transfer an α-D-glucosyl unit from the sucrose molecule either to water to form free glucose, or to other sugars to form the more complex oligosaccharides present in honey such as maltulose, nigerose, maltose, kojibiose, turanose or isomaltose. Erlose for example can be conceived as resulting from the transfer of a glucose unit from sucrose to the 4-hydroxyl of glucose in an intact sucrose molecule.

β-Glucosidase has also been found in honey. [24] It is produced in the hypopharyngeal gland of the bee, is secreted into the proboscis during feeding, passed to the honey sac and from there; it is transferred to the honey. [25] This enzyme acts upon β-(1→4) bonds hydrolysing β-D-glucosides to glucose but also catalyses the

transfer of D-glucose to suitable acceptor molecules (another glucose molecule or glucose-substituted molecules).

Yeast invertase, which is present in honey through pollen, is a fructosidase enzyme, transferring D-fructofuranosyl groups to other sugars, giving D-fructose-containing oligosaccharides. Other enzymes may also be responsible for the formation of the D-fructose-containing oligosaccharides (several oligosaccharides were prepared by the action of enzymes or yeasts on solutions of glucose, sucrose or maltose). [4] However understanding of the enzymatic mechanisms in honey is incomplete.

The enzyme glucose oxidase is added by the bee to the nectar and during ripening, the enzyme oxidizes small amounts of glucose to gluconolactone, which equilibrates with gluconic acid. As each molecule of glucose is oxidized, one molecule of hydrogen peroxide is produced. The acidity thus formed contributes to the stability of the ripening nectar against fermentation and spoilage. [3]

Diastase is also added to the nectar by the bee during ripening. This enzyme destroys starch and its function (or absence of function) in honey is not known, since nectars are not known to contain starch. [3] However, measurement of the diastase activity in honey (along with hydroxymethylfurfural (HMF) content is an international parameter for the quality control of honey. Thermal treatment temperature and time must be limited when pasteurizing and stabilizing honey as temperature destroys vitamins and bionutrients, and results in an increase in HMF content and a decrease in diastase activity. [26]

Other enzymes present in honey are catalase (which destroys hydrogen peroxide) and phosphatase (which removes phosphate from organic phosphates). [3]

1.5 Review of the Methods of Analysis of Carbohydrates in Honey

1.5.1 Introduction

Carbohydrates represent the major components of honey but complex mixtures of oligosaccharides with structural similarities make complete analysis difficult. Separation, qualitative and quantitative analysis have been performed with a multitude of instruments and techniques to try and detect all types of oligosaccharides in honey.

The first methods of analysis of honey involved polarimetry and reducing sugar analyses and were established by Wiley (1892) [27] and Browne (1908) [28] (as cited in [2], p.445) but the need for an alternative method of sugar analysis was demonstrated by White et al. (1952). [29] He stated that these techniques did not necessarily lack in precision, but they did not provide any assurances of their accuracy. [30]

1.5.2 Liquid Chromatography

Liquid chromatography is a technique in which components of a mixture are separated based on differences in the rates at which they are carried through a fixed or stationary phase by a liquid mobile phase. [31]

Techniques that use liquid chromatography are Paper Chromatography (PC), Thin-Layer Chromatography (TLC), High Pressure Liquid Chromatography (HPLC) and High Pressure Anion Exchange chromatography (HPAE).

1.5.2.1 Paper and Thin-Layer Chromatography

Taufel & Reiss (1952) [32] used paper chromatography for sugar analysis and reported a total of 9 sugars, with 5 unidentified. This technique proved valuable in application to the problem of the identity of the sugars of honey and in 1954, White and Maher developed the selective adsorption method in which they first subjected the honey to carbon (or charcoal-celite) column chromatography to separate the sugars into monosaccharide, disaccharide and higher-sugar fractions prior to paper chromatography.

Siddiqui & Furgala (1967 and 1968) [11, 17] also used the selective adsorption method and reported isolation and characterisation of 22 oligosaccharides in honey from Ottawa, Canada, by paper chromatography –electrophoresis and TLC.

Paper Chromatography and TLC have been useful in the past for the identification and quantitation of the sugars of honey. However, there are limitations, mostly due to the lack of resolution between oligosaccharides which does not permit determination of individual components.

1.5.2.2 High Pressure Liquid Chromatography (HPLC) and High Pressure Anion Exchange Chromatography (HPAE)

HPLC determination of glucose, sucrose and maltose was reported in 1977 by Thean and Funderburk and in 1979 a collaborative study recommended that the HPLC method for glucose, fructose and sucrose be adopted as an interim official first action by the Association of Official Analytical Chemists. [32, 33]

HPLC for carbohydrate analysis has become an accepted technique as relatively little sample preparation is required and the carbohydrates can be isolated after separation for further analysis. Amino-bonded silica columns which use acetonitrile/water as the mobile phase have been used for the separation of carbohydrates [12], but ion exchange resins with pure water and NaOH phases as in HPAE chromatography have proven useful for carbohydrate analysis. [34]

Swallow and Low (1990) [12] used HPLC to separate 20 structurally similar carbohydrates using anion-exchange chromatography in conjunction with a pulsed amperometric detector (PAD) in four honeys of known botanical origin. They noted that although relative oligosaccharide concentration varied from one honey to the next, the overall oligosaccharide pattern did not differ significantly and therefore these oligosaccharide patterns could be used as a "fingerprint" for honey authenticity. Difficulties in using HPLC for honey carbohydrate analysis are related to separation and detection of structurally similar compounds: the oligosaccharides present in honey are comprised mainly of disaccharides with either glucose-glucose or glucose-fructose linked units. Detection limit problems also exist due to the low concentration of the minor oligosaccharides in honey as increasing the concentration of honey not only increases the concentration of these oligosaccharides but also results in dramatic increases in glucose and fructose which swamp the active sites in the column, inhibiting oligosaccharide analysis. [12]

To solve some of the problems mentioned above, several techniques have been used. Morales, Sanz, Olano & Corzo (2006) [35] analysed three types of samples: an aqueous solution of a mixture of maltodextrin standards, an aqueous solution of honey and an aqueous solution of honey with maltopentaose and maltoheptaose added. The mono- and disaccharides were removed through adsorption onto activated charcoal which was then washed with water/ethanol solutions to desorb the mono- and disaccharides. The oligosaccharides thus adsorbed were extracted using a water/ethanol solution and subsequently analysed by HPAE-PAD and Size Exclusion Chromatography (SEC) coupled with Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight (MALDI-TOF) mass spectrometry. Oligosaccharides with a degree of polymerisation (DP) from 3 to 14 were recovered with this method but for the honey analysis, tri- and tetrasaccharides constituted most of the oligosaccharide fraction after removal of mono- and disaccharides. Noticeable variations of the oligosaccharide content were observed and the authors were able to describe the elution order of some tetra-, penta- and hexasaccharides.

1.5.3 Nuclear Magnetic Resonance Spectroscopy

Uses of Nuclear Magnetic Resonance (NMR) spectroscopy for the analysis of carbohydrates in honey are few. Some of the problems associated with the use of ^{13}C NMR for quantitation of carbohydrates include differences in carbon relaxation times and Nuclear Overhauser Effects (NOE), viscosity effects, temperature effects, solubility, digital resolution, and the low sensitivity of the ^{13}C nuclei. [36]

Low *et al*. (1988) [36] investigated the use of ^{13}C NMR spectroscopy for the qualitative and quantitative analysis of the oligosaccharides found in honey and solved some of the above mentioned problems by employing a relaxing reagent. Due to the inherent lack of sensitivity of ^{13}C nuclei to NMR detection when compared to ^{1}H nuclei (approximately 6000 times less sensitive), either concentrated carbohydrate solutions or long accumulation times were required to obtain reasonable spectra. However, the use of concentrated carbohydrate solutions may be very difficult or impossible with rare or expensive carbohydrates.

HPLC was applied to achieve concentration of the oligosaccharide fraction by the removal of the large monosaccharide fraction. The concentrated fraction was reduced with sodium borohydride and ^{13}C NMR analysis allowed for the rapid identification and quantitation of the disaccharide alditols present.
However, limitations of this methodology were realized during the examination of the ^{13}C NMR spectra of the trisaccharides. Since the positions and types of linkages of the monosaccharide units in trisaccharides were the same as in the disaccharides, many of the ^{13}C chemical shifts of the anomeric carbons were very close to the disaccharide values. Therefore, spectra containing comparable amounts of similarly linked di- and trisaccharides would present increasingly difficult problems in resolution and trisaccharides could influence the final disaccharide results. [36]

NMR was also used by Astwood, Lee & Manley-Harris (1998) [19] for characterisation of individual oligosaccharides after their isolation from Beech honeydew honey from New Zealand.

1.5.4 Gas Chromatography

Carbohydrates are not volatile and must be derivatised before Gas Chromatography (GC) analysis. In 1956, Schwarz, Baronetsky & Schoeller [37] introduced the preparation of persilylated glucose. This work was extended to the silylation of sucrose by Chang & Hass (1958) [38] and by Hedgley & Overend (1960) [39] for persilylated maltose (as cited in [9], p.558.). Successful derivatisation of the carbohydrates allowed the application of GC to the separation of carbohydrate derivatives which was first reported by McInnes, Ball, Copper & Bishop in 1958 (as cited in [40], p.35). Sweeley, Bentley, Makita & Wells (1963) [41] developed a simple and efficient method for the preparation of trimethylsilyl (TMS) ethers and since then GC has proven to be a popular and useful technique for the separation and analysis of carbohydrates.

1.5.4.1 Separation of the Mono- and Oligosaccharides as their O-Trimethylsilyl Ethers

Carbohydrates must be derivatised prior to GC analysis and were first converted into their *O*-trimethylsilyl ethers. In 1966, Brobst & Lott [40] analysed corn syrup by dissolving the syrup in pyridine and forming the *O*-trimethylsilyl ethers of the sugars with hexamethyldisilazane (HDMS) and trifluoroacetic acid (TFA); and in 1971, Haverkamp, Kamerling & Vliegenthart [42] studied a model solution of an oligosaccharide mixture, converting the disaccharides to the TMS derivatives using HDMS and trimethylchlorosilane (TMCS) in pyridine prior to GC analysis on packed columns.

However, the above methodology has proven problematic in the analysis of complex carbohydrates mixtures (like honey) due to the reducing sugars' abilities to develop tautomeric forms in solution. A non-reducing sugar exists in one tautomeric form in solution and therefore exhibits only one peak in GC. However, when dissolved in solution, reducing sugars equilibrate to up to six tautomeric forms: two pyranoses, two furanoses, an acyclic carbonyl form and its hydrate; consequently exhibiting several peaks in GC which makes peak separation difficult in a complex mixture.

To clarify identification and quantitation of a complex mixture of disaccharides, mutarotation equilibrium data for the component sugars have been used when overlapping occurred by Nikolov & Reilly (1983) [43]. They investigated the separation of seventeen TMS ether disaccharides by Gas Chromatography with Flame Ionisation Detector (GC-FID) on a fused-silica capillary column and determined equilibrium compositions of the reducing disaccharides in pyridine. All seventeen disaccharides could be identified and quantified but this method required precise control of temperature of derivatisation which occurred over 15 hours and was applied to a mixture of standards and not a natural matrix like honey.

More recently, capillary column GC analysis by Cotte *et al.* (2003) [34] using retention indexes to identify and quantify di- and trisaccharides in honey from various countries was performed. The authors reported quantitation of 10 disaccharides and 7 trisaccharides as their TMS ethers. However, overlapping of some peaks could still occur (for example, only one peak for turanose is mentioned in the disaccharide profile).

1.5.4.2 Modification of the Mono- and Oligosaccharides before Trimethylsilylation and GC Analysis.

Mono- and oligosaccharides can be modified prior to trimethylsilylation to alleviate separation problems. Oximation and reduction have been used as means to reduce the number of peaks in the gas chromatogram.

1.5.4.2.1 Oximation

The oximation reaction involves the reaction of the sugars with hydroxylamine which by producing the open chain sugar oxime suppresses the anomeric centre, and only two forms (E and Z) are obtained for every reducing sugar. Therefore for each reducing sugar, two peaks appear in the gas chromatogram. [6]
Toba & Adachi (1977) [44] investigated the separation of ten disaccharides as the sugar oxime TMS ethers by GC-FID on a packed column and TLC. They reported the unsuccessful GC analysis of the sugar oximes as they commonly gave two peaks which did not significantly improve the separation in a complex mixture.

Another variation of the oximation method was to analyse the TMS ether and TMS ether oxime derivatives in honey by parallel GC analysis from two stock solutions analysed successively on a fused-silica capillary column coated with OV-101 using temperature programming, seventeen sugars could be identified and quantitated. [43, 45]
TMS ether oximes of Hungarian honey sugars were also studied by Horváth & Molnár-Perl (1997) [10] by gas chromatography-mass spectrometry (GC-MS) using a methodology developed with model solutions. [46] The methodology involves the preparation of TMS oxime/methoxime derivatives and their separation by careful temperature programming of both the column and the injector.

1.5.4.2.2 Reduction

Monosaccharides and reducing oligosaccharides can be easily converted quantitatively into their corresponding sugar alcohols by reaction with sodium or potassium borohydride ($NaBH_4$ or KBH_4, respectively) in aqueous solution. Sugar alcohols have an open chain structure: only one peak appears in the GC for aldoses and two peaks for ketoses; as aldoses yield only one compound (the glucitol for glucose or substituted glucitol for gluco-oligosaccharides) whereas ketoses (like fructose or fructo-oligosaccharides) yield approximately equimolar parts of two C-2 epimeric sugar alcohols, glucitol and mannitol in the case of fructose (see Figure 1).

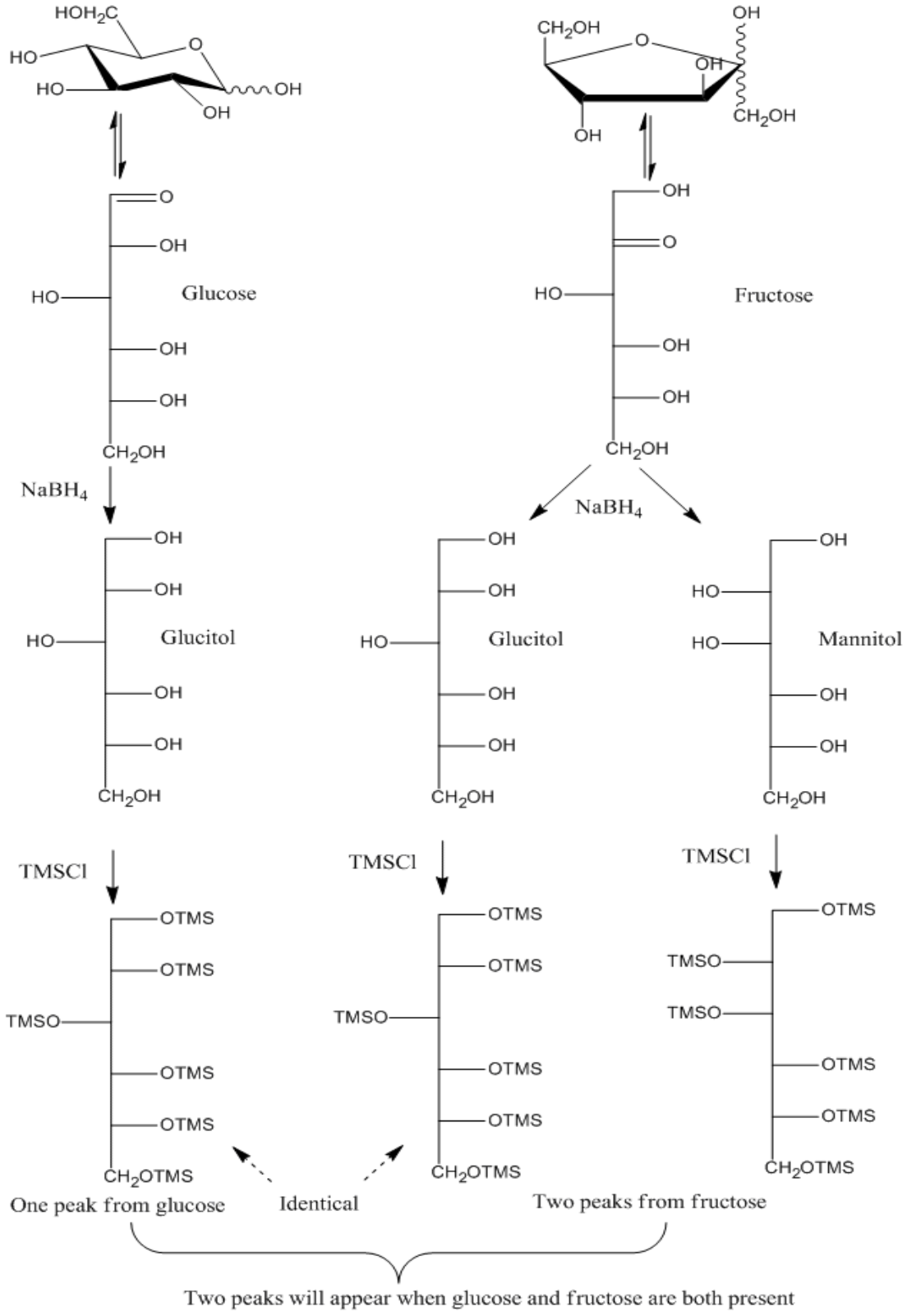


Figure 1: Reduction of Glucose and Fructose.

Sweeley *et al.* (1963) [41] developed their innovative trimethylsilylation method of sugars by reducing standard solutions of aldoses to their corresponding alcohols using potassium borohydride prior to trimethylsilylation and GC analysis. The stationary phases used were: a non-polar SE-52 column and a polar 15% polyethylene glycosuccinate (EGS) column fitted onto a GC-FID and a gas chromatograph-argon ionisation detector (GC-ArID) respectively.

Another variation of the reduction method involves carrying out the quantitation by two injections, following various elution temperatures. Nineteen sugars from a Canadian Alsike honey were identified and quantitated. [9] HPLC was used to separate the monosaccharide fraction (using an acetonitrile-water 80:20; v:v mobile phase) and then the oligosaccharide fraction (using an acetonitrile-water 50:50; v:v mobile phase). The oligosaccharide fraction was reduced using sodium borohydride and Tri-sil Z for trimethylsilylation and analysed by GC-FID on an open-tubular fused-silica capillary column coated with DB-5. Twelve disaccharides were identified and quantified by temperature programming (210°C for 12 min followed by an temperature increase of 2°C/min to 290°C), but the authors claimed that a second injection employing isothermal elution at 250°C was required to identify and quantitate the disaccharide maltulose; its glucitol peak overlapping with the identical maltose reduction peak and the mannitol peak overlapping with the glucitol peak of turanose. The seven trisaccharides were separated and quantitated using isothermal conditions of 290°C. [9]

Low & Sporns' ability to separate the maltulose peaks from the maltose and turanose peaks is doubtful due to the reduction products obtained for each of these disaccharides. Reduction of nigerose affords the D-gluctitol substituted product α-D-Glc*p*-(1→3)-D-Glucitol (see Figure 2). Maltose affords the D-glucitol substituted product α-D-Glc*p*-(1→4)-D-Glucitol (see Figure 3); reduction of turanose affords both the D-glucitol and D-mannitol substituted products α-D-Glc*p*-(1→3)-D-Glucitol (which is the same as the product of the reduction of nigerose) and α-D-Glc*p*-(1→3)-D-Mannitol (see Figure 4). Reduction of maltulose also produces two products (see Figure 5): α-D-Glc*p*-(1→4)-D-Glucitol (identical with the product from maltose) and

α-D-Glc*p*-(1→4)-D-Mannitol; but in fact this last disaccharide and α-D-Glc*p*-(1→3)-D-Mannitol (produced from turanose) are identical molecules because of symmetry; and therefore can never be separated and when these disaccharides are present together in a sample, only three peaks will appear. [47]

In parallel to this "HPLC with GC analysis", Low & Sporns (1988) [9] also reduced the honey directly without prior HPLC separation and using the same GC conditions as above, were able to eliminate the HPLC oligosaccharide purification step and analyse honey samples directly. This speeded up the analysis procedure considerably but could lead to shorter column life because of the larger amounts of material that are injected into the column. This also meant the method had higher detection limits. [9]

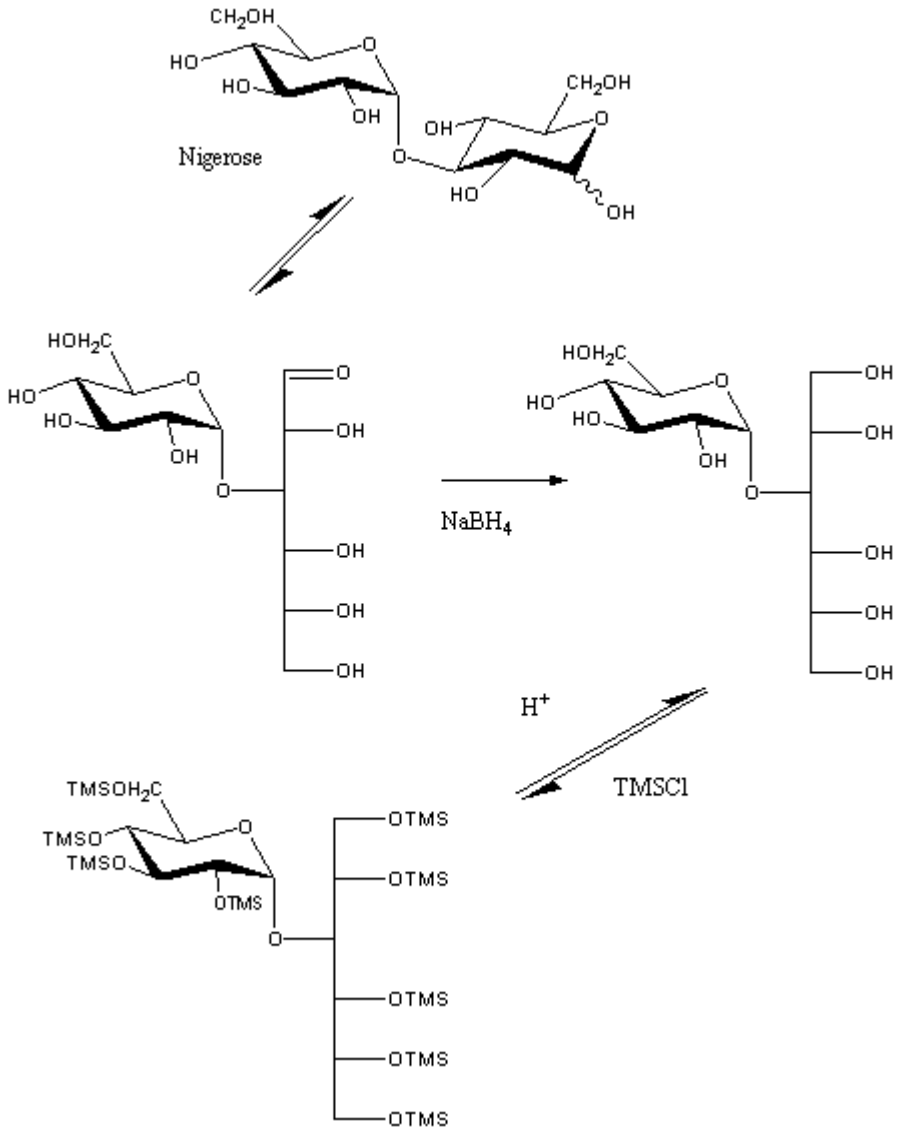


Figure 2: Reduction of Nigerose.

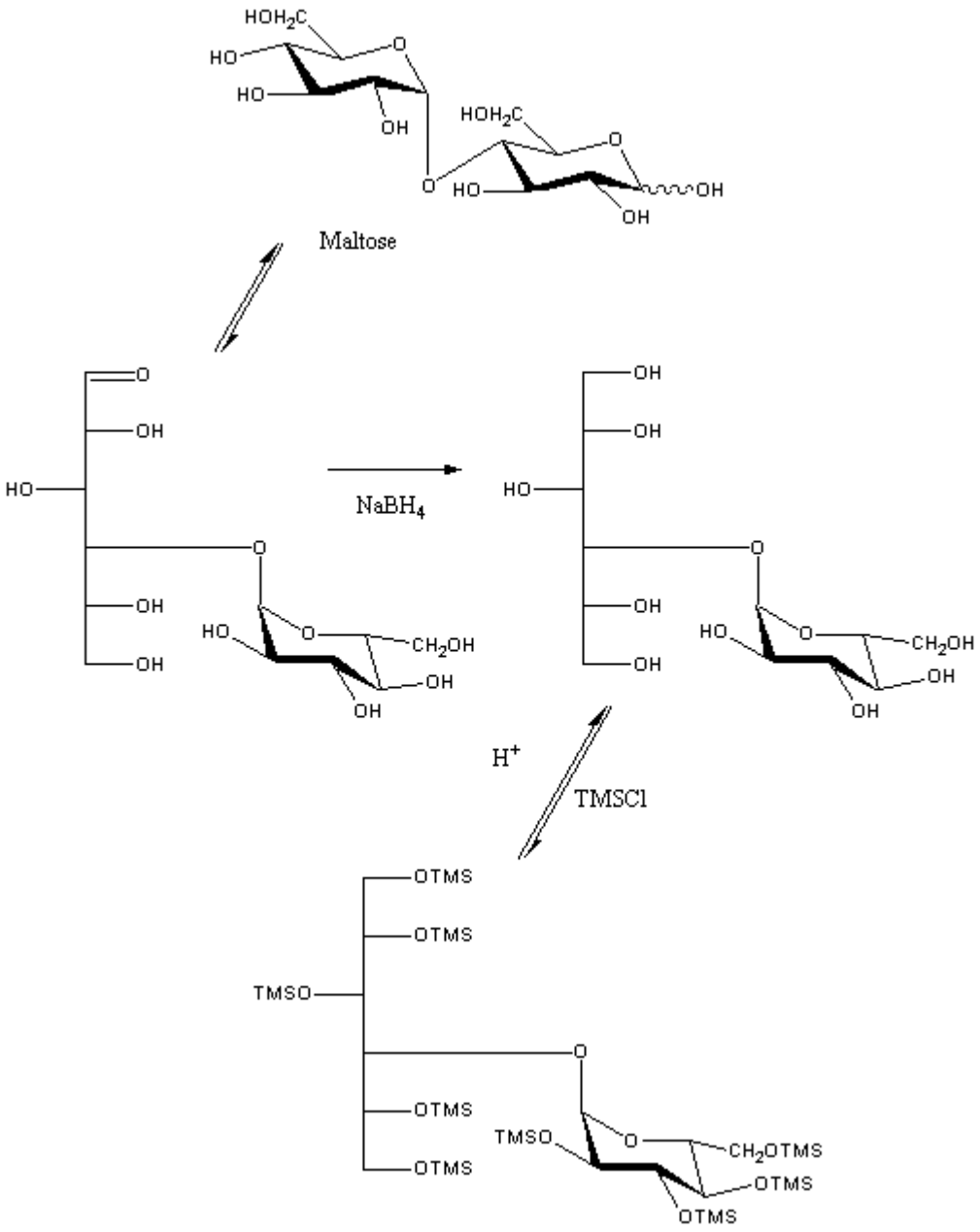


Figure 3: Reduction of Maltose.

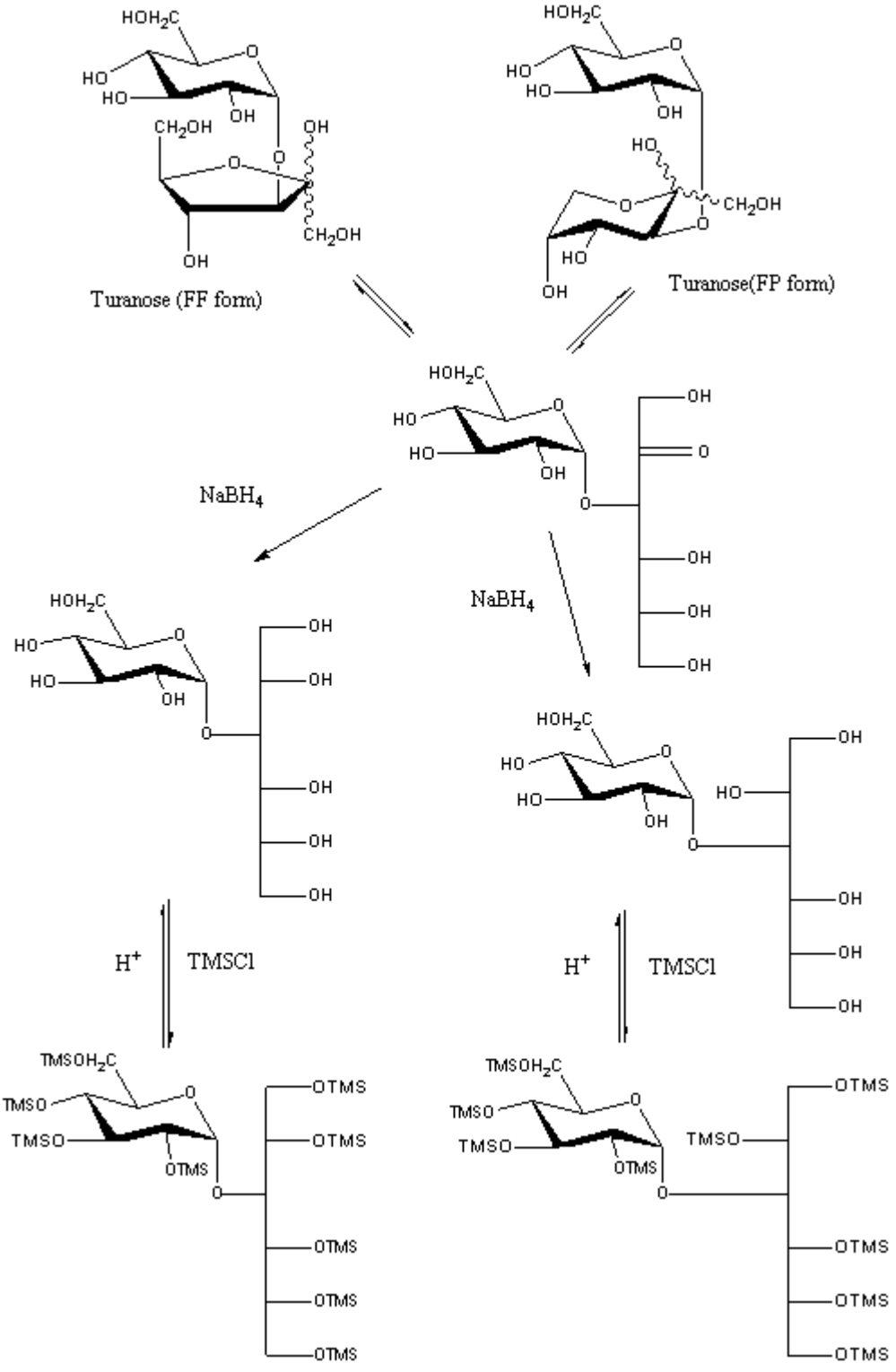


Figure 4: Reduction of Turanose.

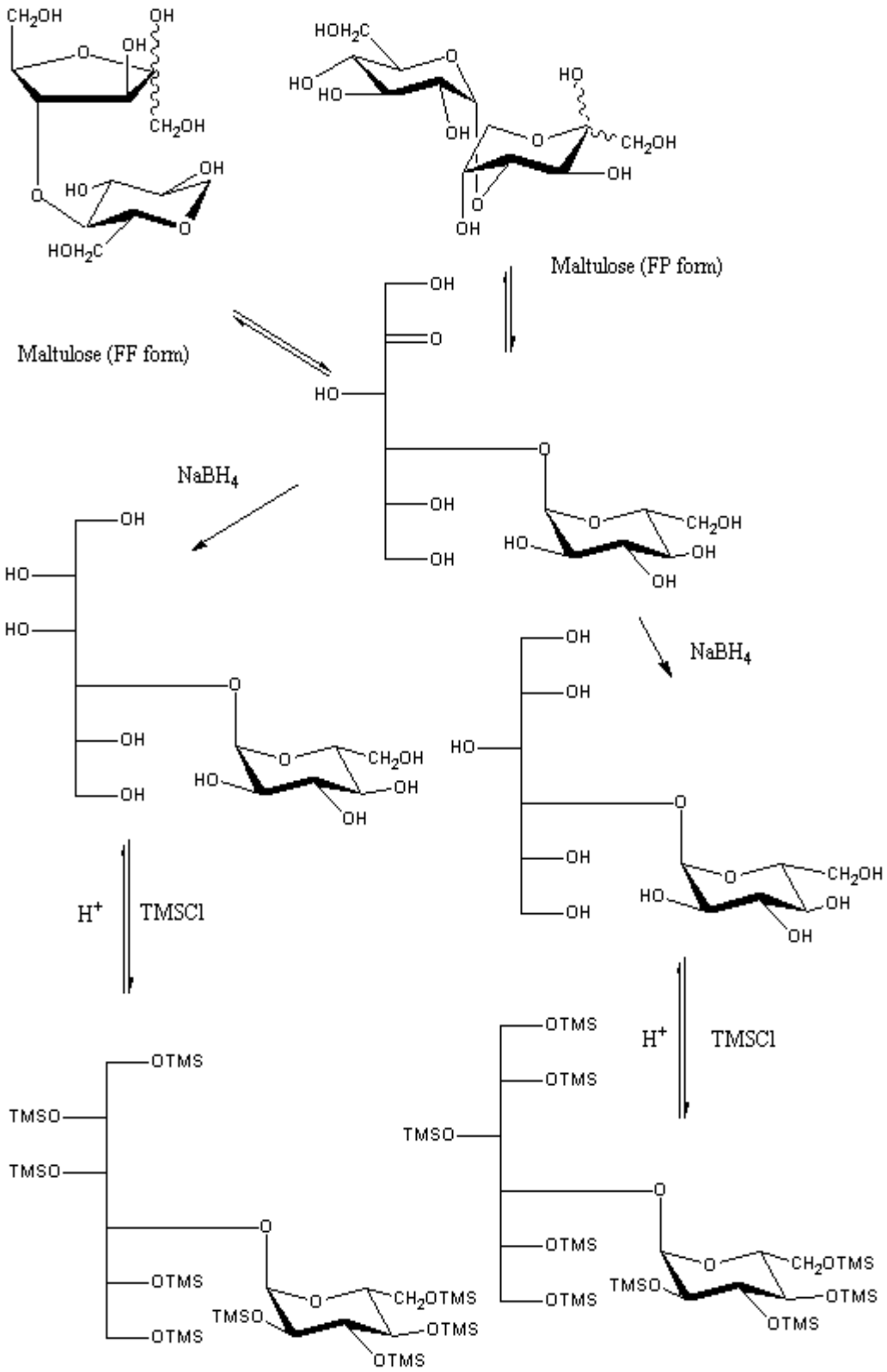


Figure 5: Reduction of Maltulose.

Reduction of honey sugars was also used in the analysis of honeydew honey in New Zealand using GC-FID (HP Ultra-2 capillary column) and GC-MS (HP-1 capillary column). The oligosaccharide profile was quantified (by LC and GC) in six samples from different sources within the South Island of New Zealand; for GC, sugars were reduced with sodium borohydride and per-*O*-trimethylsilylated. Nine disaccharides, four trisaccharides, three tetrasaccharides, two pentasaccharides and one hexasaccharide were characterised. [19]

1.5.5 Survey of the Geographical Origin of Honeys Studied and Methods of Analysis.

The oligosaccharide composition of honey has been studied in several parts of the world (see Figure 6). Geographical origin and the methods of analysis used are summarised in Table 7. This data does not intend to catalogue all the honeys ever analysed but is given to illustrate the main geographical areas in which honey has previously been analysed.

Figure 6: Geographical origin of honey oligosaccharide composition studies (grey countries are countries from which honey has been analysed).

Table 7: Geographical origin and method of analysis of honeys. Samples with oligosaccharide profiling indicated by *.

Geographical origin	Method of analysis	Reference
Algeria *	HPAE-PAD	[48]
Australia	Method not mentioned in abstract. Glucose, fructose, sucrose and maltose mentioned.	[49]
Argentina*	Solid-Phase Extraction (SPE) of monosaccharides followed by HPLC analysis of oligosaccharides with Ultraviolet (UV) detection.	[50]
Brazil *	Oligosaccharide composition analysed by HPLC-RI.	[51]

Table 7: Geographical origin and method of analysis of honeys. Samples with oligosaccharide profiling indicated by * (continued).

Brazil *	Oligosaccharide composition analysed by HPLC-RI.	[52]
Brazil *	Oligosaccharide composition analysed by HPLC-RI.	[53]
Brazil	Official methods used. Glucose, fructose and sucrose results mentioned.	[54]
Canada *	Charcoal/celite column separation followed by paper chromatography/spray reagents	[11] [17]
Canada (Alberta region)	^{13}C NMR	[36]
Canada *	HPLC for monosaccharides removal. GC-FID of reduced TMS ethers on a DB-5 column	[9]
Canada *	HPLC-PAD	[12]
France, Hungary, China, Spain, Morocco *	HPAE-PAD of monosaccharides and GC-FID of TMS ethers of di- and trisaccharides.	[34]
France, Hungary, China, Spain, Morocco, Turkey *	HPAE-PAD of monosaccharides and GC-FID of TMS ethers of di- and trisaccharides.	[55]
Greece, Italy, Spain *	TMS ether oximes by GC-FID on an SE-52 column	[56]
Hungary *	TMS ether oximes by GC-MS	[10]
Hungary and USA	TMS ethers by GC-MS on a DB-5 column Method from Molnár-Perl, Horváth & Bartha (1998)	[57]

Table 7: Geographical origin and method of analysis of honeys. Samples with oligosaccharide profiling indicated by * (continued).

India	Reducing sugar, fructose:glucose content and sucrose content according to the method of Bogdanov *et al.* (1997)	[58]
Israel	Determination of glucose, fructose and perseitol by Near-Infrared (NIR) spectroscopy.	[59]
Lithuania*	TMS ethers by GC-FID on a ZB-5 column	[60]
Lithuania *	TMS ethers by GC-FID on a ZB-5 column	[61]
Mexico	HPLC with Refractive Index (RI) detector. Results for glucose, fructose, sucrose and maltose.	[62]
Morocco *	TMS ether oximes analysed by GC-MS on a fused-silica capillary column coated with OV-1.	[63]
Morocco *	Same method as Terrab *et al.* (2001)	[64]
Nepal (Chitwan district) *	HPLC. Based on DIN 10758 at the Beekeeping Institute, Celle, Germany	[65]
Nepal	Method not mentioned in abstract. Analysis of glucose, fructose, sucrose and apparent reducing sugars mentioned.	[66]
New Zealand *	HPLC, NMR, ESMS GC-FID and GC-MS of reduced TMS ethers	[19]
New Zealand *	Monosaccharides separated and oligosaccharides analysed by HPAE-PAD	[67]
Oman	HPLC with RI detector. Glucose, fructose, sucrose and maltose results only.	[68]
Portugal *	HPLC with RI detector.	[69]

Table 7: Geographical origin and method of analysis of honeys. Samples with oligosaccharide profiling indicated by * (continued).

Romania	Sugars analysed according to the method of Bogdanov *et al.* (1997). Values for fructose, glucose, sucrose, maltose, trehalose and melezitose are reported.	[70]
Romania (Transylvania region) *	Sugar profile analysed by HPLC-RI.	[71]
Slovenia	HPAE-PAD of mono- and oligosaccharides.	[72]
Spain (Madrid region) *	TMS ether oximes by GC-FID and GC-MS on an SPB-1 column.	[73]
Spain (Madrid region) *	Adsorption onto activated charcoal and packed into a pressurized liquid extraction (PLE) cell to separate mono-, di- and oligosaccharides. TMS ether oximes used for GC-MS analysis (qualitative) and GC-FID (quantitative) analysis.	[74]
Spain (Province of Soria) *	HPLC-PAD analysis of ling, spike lavender, French lavender, thyme, forest, and multifloral honeys.	[75]
Spain (various regions) and Canary Islands *	TMS ether oximes by GC-FID and GC-MS on an SPB-1 column.	[16]
Spain (various regions) *	TMS ether oximes by GC-FID and GC-MS (2 columns used on both: Rtx-65 and SPB-1)	[6]
Spain (various regions) *	TMS ether oximes by GC-FID with OV-17 and 80-100 mesh Chromosorb W	[76]

Table 7: Geographical origin and method of analysis of honeys. Samples with oligosaccharide profiling indicated by * (continued).

Spain, Canary Islands, Italy	TMS ether oximes of monosaccharides and TMS ethers of trisaccharides (erlose and melezitose) by GC-FID and GC-MS both with an SPB-1 column.	[77]
Spain *	TMS ether oximes by GC-FID as per the method of [6]	[78]
Spain *	TMS ether oximes by GC-FID and GC-MS on SPB-1 column.	[79]
Spain and New Zealand *	TMS ether oximes by GC-FID and GC-MS on an HT-5 column. Tri- and tetrasaccharides only.	[7]
Switzerland, Germany, Italy, France and Denmark *	Method used was of the Harmonised Methods of the European Honey Commission [80]	[81]
Tenerife (The Canary Islands)	HPLC-RI based on the procedure of the European Honey Commission of [80]. Results for glucose, fructose, sucrose, maltose and erlose available.	[82]
Turkey (various regions)	HPLC-RI. Results for glucose, fructose, maltose, raffinose and saccharose available.	[83]
Unspecified (Presumably Bulgaria) *	HPLC-RI Results for glucose, fructose, turanose, maltose, sucrose, trehalose and melezitose mentioned. Isomaltose and erlose were also detected.	[84]

Table 7: Geographical origin and method of analysis of honeys. Samples with oligosaccharide profiling indicated by * (continued).

Unspecified (Presumably China)	Glucose, fructose, raffinose, stachyose mentioned. Determined by HPLC, GC, activated charcoal column chromatography, PC and mass spectrometry.	[85]
Unspecified (presumably China)	HPLC-RI. Glucose, fructose, sucrose and maltose determined.	[86]
Unspecified (presumably France)	Solid-Phase Extraction (SPE) followed by LC analysis with an evaporative light scattering detector (ELSD) and presence of polysaccharides confirmed by MALDI-TOF.	[87]
Unspecified (presumably Spain)	Activated charcoal separation of Mono- and Disaccharides. HPAE-PAD and Size-Exclusion Chromatography (SEC) of oligosaccharides followed by MALDI-TOF.	[35]
Unspecified (presumably Spain)	TMS ether oximes by GC-FID and GC-MS on both Rtx-65 and SPB-1 according to method by [6]	[13]
USA	Sugars converted into propionic esters followed by vacuum distillation of the propionates under controlled conditions.	[88]
USA	Activated charcoal column separation and determination of the fractions by various reactions	[20]

Table 7: Geographical origin and method of analysis of honeys. Samples with oligosaccharide profiling indicated by * (continued).

USA *	TMS ether oximes by GC-MS and GC-FID on and SPB-1 column.	[89]
USA (various regions), Thailand, China, Bhutan, United Kingdom, Canada, Indonesia, Japan, New Zealand, Vietnam	Fourier-Transform Infrared (FTIR) spectroscopy and HPLC-ELSD analysis of glucose, fructose, sucrose and maltose.	[90]
Venezuela *	HPLC with RI detector	[91]

1.6 Honey Adulteration

The control of honey authenticity is a major concern for consumers and authentic honey producers since adulteration can adversely affect the market.

Honey being a natural substance of relatively high commercial value and limited supply; it is more and more prone to adulteration and fraudulent practices such as selling it under a false name or origin. Adulteration of honey also involves the fraudulent modification of this natural substance by the addition of substances of lesser quality such as industrial sugar syrups.

Many adulterants have been used to adulterate honey but the most common are Corn Syrup (CS), invert syrup (IS), cane or beet sugar syrups which can be defined as Sucrose Syrup (SS) and High Fructose Corn Syrup (HFCS). The sugars are divided into two categories: the C3 type (Glucose Syrup (GS), inulin syrup and the natural sugars of honey) and C4 type (cane sugar and the sugars produced from the hydrolysis of corn starch such as HFCS). [92]

Previously, adulteration was performed by simply adding sugar and water but nowadays, specially produced syrups are manufactured to reproduce the sugar composition and ratios of natural honey. [93] This renders the detection of adulteration of honey even more difficult. Another means of honey adulteration results from improper beekeeping practices: sweeteners used to feed bees must be managed carefully so as to not adulterate the honey. [92]

1.6.1 Detection of Honey Adulteration

Several methods have been proposed to detect the adulteration of honey.

A stable carbon isotopic ratio analysis (SCIRA) can be used to detect corn or sugar cane syrup addition (C4 type syrups). The $^{13}C/^{12}C$ ratio (expressed as a δ value) of natural honey differs from the $^{13}C/^{12}C$ ratio of corn and sugar cane syrups and therefore, honey adulterated with this kind of syrup will have a different δ value. The average value for honey is -25.4% whereas for C4 syrups it is close to -10%, and this method allows the detection of 7-10% adulteration with cane sugar or corn syrup. This method has become the official control method for the detection of addition of HFCS to honeys. Deuterium NMR can also be determined to give a greater certainty in the interpretation of the $^{13}C/^{12}C$ ratio. However, SCIRA remains limited to the detection of sugar syrup from C4-plants and is not effective in detecting syrups made from C3-plants (beet, wheat, and isoglucose syrups) because the δ ^{13}C of unadulterated products will be similar to the δ ^{13}C of these types of syrups. [92, 94]

Chromatographic methods have been developed to detect honey adulteration. HPAE-PAD and GC are the most popular methods to study adulterated and non-adulterated honeys.

HFCS and IS additions in honey were detected by HPAE-PAD and GC, starting at 5% HFCS adulteration. [95, 96]

These methods have been further improved or extended to other types of sugar syrups. C3 sugar syrups, mixtures of invert sugar syrup and glucose syrup were investigated (monosaccharides analysed by HPAE-PAD and di- and trisaccharides by

GC-FID) and Principal Component Analysis (PCA) used to discriminate authentic and fraudulent samples. [34] This method was also used to assess the current state of fraud on the French market by studying three C3 sugar syrups (glucose syrup from wheat, barley or rice; inulin syrup from chicory and a medium invert sugar syrup from beet) by comparing the composition of the syrups and comparing the honey obtained by bees fed the syrups to commercial honeys. [55]

The presence of Difructose Anhydrides (DFAs) in HFCS and IS syrups was detected by GC and GC-MS. Yeast treatment was applied to remove the monosaccharide sugars and obtain an enriched fraction of DFAs. These compounds are non-fermentable pseudodisaccharides and were not present in honey samples analysed or honey samples subjected to heat treatment, and were dependent on the syrup type considered. They were identified as markers that could be used for identification of adulteration down to 5%. [97]

Another means of detecting honey adulteration is polysaccharide fingerprinting. Samples were treated with activated charcoal [98] or reversed-phase solid phase extraction [93] to remove monosaccharides and small oligosaccharides; and simultaneously concentrate traces of polysaccharides (degree of polymerisation from 3 to 17). HPAE-PAD was performed on laboratory samples doped with CS and authentic honey samples: the polysaccharides were present in the doped samples but either not detectable or present at very low concentrations in the authentic honey samples. Deliberate addition of 1% CS was readily detected. [93] HPAE-PAD was also performed on laboratory samples doped with CS and HFCS; and on authentic and commercial honeys. This method used quantitation of the malto-oligosaccharides and enabled the detection of CS adulteration down to 5% and adulterations with HFCS with different degrees of isomerisation (20 and 40%) were detected. However, adulterations with HFCS at 80% isomerisation could not be detected. [98]

1.6.2 Composition of Syrups, Effect on Honey Composition by Adulteration or Bee-Feeding.

The composition of syrups used to feed bees and their impact on honey sugar composition has been studied only recently by several research groups, but the outcomes indicate that the honeys, either adulterated or produced by bees fed with sugar syrup incorporate the sugar composition of the syrup into their composition.

1.6.2.1 Sugar Composition of Syrups

The sugar composition of several syrups used in bee-feeding; and which had been obtained from manufacturers, is presented in Table 8.

The sugar composition of syrups varies widely depending on their type and their degree of hydrolysis; they contain in variable proportion a mixture of many sugars such as glucose, fructose, disaccharides like sucrose, maltose, fructosyl-fructoses, maltotriose, dextrins, and so on. The composition can vary widely from honey (for example SS) but can also mimic honey as do some HFCS.

The sugar composition of syrups obtained from manufacturers and beekeepers can be compared and is presented in Table 9.

In terms of carbohydrate content, glucose and fructose were the main carbohydrates in HFCS and a large number of oligosaccharides are present in small quantities. Fructosyl-fructoses were the main disaccharides in HFCS, and were only observed in the HFCS samples. These disaccharides are produced during the manufacture of HFCS, from the incomplete hydrolysis of starch and are therefore characteristic of starch. Other disaccharides like sucrose, maltose and isomaltose were also present. [97]

Sucrose was the most abundant carbohydrate in SS and glucose and fructose were only present in small amounts. Disaccharides other than sucrose were not detected; and it is also noteworthy that no differences were found when syrups obtained from manufacturers or from beekeepers were compared. [89]

Table 8: Sugar Composition of Syrups.

Carbohydrates (%, unless otherwise stated)	**Erstein liquid sugar (C3 syrup) from Erstein, France.** [34]	**Erstein siroline 728:4-6. Mixture of IS and glucose syrup from Erstein, France.** [34]	**Cerestar FT 1702 (C3 syrup) from Hambourdin, France.** [34]	**CS (C4 syrup) from France Miel, France.** [93]	**Syrup A from Ickowicz S.A.** [94]	**Syrup B from Ickowicz, S.A.** [94]	**Syrup C (sucrose) from Ickowicz, S.A.** [94]
Fructose	287.9 (g/L)	108.3 (g/L)	72.8 (g/L)	10	16	15	>0
Glucose	248.2 (g/L)	159.2	269.7 (g/L)	45	20	22	>0
Sucrose	42.1	0.09	0.05	-	0	0	<100
Maltose	0.00	1.61	29.82	30	45	43	0
Maltulose	0.00	0.35	2.12	-	-	-	-
Turanose	0.18	0.37	0.00	-	-	-	-
Trehalose	0.00	0.26	0.00	-	-	-	-
Palatinose	0.06	0.12	0.07	-	-	-	-
Laminaribiose	0.00	0.25	0.17	-	-	-	-

Table 8: Sugar Composition of Syrups (continued).

Melibiose	0.00	0.02	0.00	-	-	-	-
Isomaltose	0.00	0.09	1.6	-	-	-	-
Gentiobiose	0.00	0.08	0.09	-	-	-	-
Raffinose	0.09	0.00	1.47	-	-	-	-
Neo-kestose	0.04	0.00	0.73	-	-	-	-
1-kestose	0.03	0.00	0.00	-	-	-	-
Erlose	0.00	0.05	0.04	-	-	-	-
Melezitose	0.00	0.04	0.22	-	-	-	-
Maltotriose	0.00	1.20	6.52	13	0	0	0
Panose	0.35	0.00	0.90	-	-	-	-
Oligosaccharides	-	-	-	2*	17	20	0

(-): not determined

* Higher oligosaccharides

Table 9: Carbohydrate content of HFCS and SS obtained from manufacturers and beekeepers. [97]

Carbohydrates (%)	HFCS-75* (B) (n = 2)	HFCS-55* (M) (n = 4)	HFCS-55* (B) (n = 5)	HFCS-42* (M) (n = 3)	HFCS-42* (B) (n = 1)	HFCS + SS (B) (n = 4)	SS (B) (n = 3)
Fructose	54.32	41.39	40.57	30.48	31.56	16.59	1.27
Glucose	23.44	34.69	33.18	40.22	34.22	21.26	2.51
Fructosyl-fructoses	2.98	1.53	1.73	0.95	1.35	0.84	0.0
Sucrose	0.19	0.08	0.04	0.04	0.02	35.67	72.52
Maltose + unknown disaccharide	0.8	0.91	0.92	0.90	1.15	0.37	0.0
Isomaltose	0.5	0.62	0.61	0.62	0.70	0.19	0.0
Unknown disaccharide	1.02	0.65	0.57	0.79	0.70	0.21	0.0

* = degree of isomerisation
n = number of samples
M = obtained from manufacturer
B = obtained from beekeepers

1.6.2.2 Effect on Sugar Composition of Adding Sugar Syrups to Authentic Honeys.

When additions of 10, 20 and 40% glucose-fructose-maltose syrup (see Table 8 for precise composition of the syrup) were made to honey, the sugar composition of the honey was modified: the maltose content increased and conversely a dramatic relative decrease in all other sugars was observed; leading to an impoverishment of the honey

composition. Similarly with a SS, the sucrose content dramatically increased but the relative concentration of all other sugars diminished. [94]

An artisanal honey (its composition analysed) was adulterated with CS, 20HFCS, 40HFCS and 80HFCS at levels of 5, 10 and 20% to determine if it was possible to detect the addition of these syrups. High molecular weight oligosaccharides of DP from 3 to 16 were detected. Changes in the composition from the original honey composition were observed, even at the 5% level: an increase in oligosaccharides of DP 2-6 was detected and malto-oligosaccharides of DP 7–16 could be observed. Adulteration with 20HFCS could also be detected by an increase in malto-oligosaccharides from DP 3-15. However, adulteration with 40HFCS only showed slight variations and no changes were detected for adulterations with 80HFCS. This decrease in malto-oligosaccharides of high molecular weight with increasing isomerisation degree of the syrups parallels the amounts of oligosaccharides present in the syrups. [98] The same conclusions were drawn with honey samples adulterated with CS at 1%. Adulteration at 0.1% was more difficult to detect due to the natural variability from one sample to another but such adulteration levels would be of no substantial financial benefit. [93]

From these studies it seems therefore that the compositional features of the syrup is transferred to honey when it is simply added and that it creates a relative impoverishment of the sugar composition of the honey, especially when syrups with a single main component are added.

1.6.2.3 Effect of Bee-Feeding on the Honey Produced

Bee-feeding can modify the sugar composition of the produced honey if it is done improperly.

Bee-feeding is usually practiced in three situations:

- To stimulate the queen’s egg-laying performance and maintain a high population of bees in hives to encourage a more effective and longer honey production,

- To support the bee colonies through the winter period which is a low nectar availability period, mostly due to meteorological conditions,
- To treat bee diseases by application of veterinary prescriptions via the use of sugar syrup.

Consequently, beekeepers may have to use bee-feeding several times during the year. [94]

Glucose-fructose-maltose syrups and SS commonly fed to bees by beekeepers were used in a bee-feeding experiment on a small four hive apiary. [94] The initial state of the apiary was evaluated by analysing a sample of honey from each hive collected before the first feeding. Three of the hives were fed with three different sugar syrups, the fourth was not fed any syrup and was used as control. The bee-feeding was carried out as follows:

- Increasing amount of syrup supplied each week (from 1 L/hive to 5 L/hive at the last feed), at a rate of two feedings per week,
- The bee-feedings occurred at 3-day intervals to allow sufficient time for the bees to assimilate the syrups.

HPAE-PAD was used to analyse the samples. The peak areas associated with maltose and sucrose in the control hive were relatively constant from the beginning to the end of the experiment. However, honey produced from hives fed with glucose-fructose-maltose syrup showed a decrease in the peak area associated with sucrose but the peak area associated with maltose increased regularly while the other sugars in the honey decreased. Towards the beginning of the experiment, the bees are able to assimilate the amount of syrup supplied; but later on in the experiment the amount of maltose starts to increase, paralleled by a decrease in the amount of sucrose produced. This is due to the enzymatic hydrolysis of the sugars by the bees: sucrose is usually converted to glucose and fructose but when the amount of syrup supplied reaches a saturation level for the bees; their enzymatic activity also reaches its maximum and the bees start to store the glucose (produced from hydrolysis of sucrose) in a more

elaborate form as maltose and erlose (and a dramatic decrease in sucrose is observed). Consequently, maltose comes mainly from the syrup itself but is also produced by the enzymatic hydrolysis of the bees when they are fed glucose-fructose-maltose or sucrose syrup intensively for a long period of time (the amount of maltose had increased by a factor of 10 after 10 days in this experiment). Intensive and extended bee-feeding can therefore have the same effect as adulteration by addition of syrup to honey: an accumulation of syrup products is observed along with a dilution effect which diminishes the minor sugars of honey and the other chemical compounds (proteins, amino acids and organic acids). [94]

In another feeding experiment, bees were fed glucose syrup from wheat (S1), inulin syrup from chicory (S2) and medium invert sugar syrup from beet (S3). [55]
In this study, syrups S1 and S2 were both said to be starch hydrolysates. This statement is incorrect for S2 since inulin is a polysaccharide ranging from DP 70 to 1000 consisting of a range of polymers of β-(2→1) linked D-fructofuranose, ending with a glucopyranose [99] as shown in Figure 7 and does not come from the hydrolysis of starch.

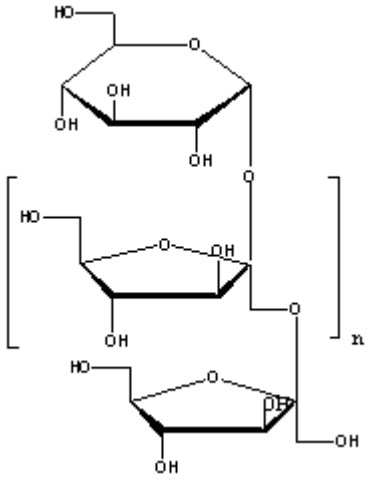


Figure 7: Structure of inulin.

Furthermore, the authors state that syrups S1 (glucose syrup) and S2 (inulin syrup) are characterised by high concentrations of maltose and maltotriose (approximately 48% maltose and 17% maltotriose in S1; 31% maltose and 4% maltotriose in S2).

Again this statement does not apply to inulin syrup since hydrolysis of this syrup would yield fructo-oligosaccharides and not gluco-oligosaccharides such as maltose and maltotriose.
It can be speculated that the authors incorrectly described glucose syrup as an inulin syrup. This is further confirmed by the apparent similarity of the results for syrups S1 and S2.

Analysis of honey produced by bees feeding on glucose syrup showed an increase in monosaccharides. This increase was mainly due to the enzymatic activity of the bees on maltose and maltotriose; transforming these di- and trisaccharide into glucose mainly (glucose formation being the major process) but also fructose. On the other hand, syrup S3 (a sucrose syrup with moderate invert sugar content) was composed of mainly sucrose (approximately 34%) and also glucose and fructose. Its hydrolysis by the bee enzymes yields equimolar amounts of glucose and fructose and consequently, honey produced by bees feeding on this syrup showed an equivalent increase in the two monosaccharides. Therefore for the syrups studied, the same conclusion can be drawn: a dilution effect of the oligosaccharide fraction is observed by an increase in the monosaccharide content. [55]

The most recent bee-feeding study involved several types of HFCS, SS and a mixture of HFCS and SS. [89] Bees were fed in an enclosed flight arena (10 nucleus colonies; each 5 frames with about 10,000 workers) and in an apiary (five nucleus colonies). The bees were fed by a regular supply of syrup solutions all at 67% solids w/v and a protein supplement. Honey samples obtained from bees naturally foraging were used for comparative purposes.
For honey produced in the flight arena, SS honeys were characterised by a large increase in the amount of sucrose present (from 5.0 mg/g of honey in the control hive to 131.7 mg/g); however adulteration with SS can only be indicated if the amount of sucrose exceeds the legal limit since some honey types can naturally have high amounts of sucrose. The use of HFCS in the flight arena resulted in honeys with an increase in the amount of sucrose from the control sample (from 5.0 mg/g of honey to 10.9 mg/g), the amount of monosaccharides did not vary much but the main

difference was the presence of DFAs (none in the control honey to 0.4-0.8 mg/g) and also carbohydrates with two fructose units which are originally present in HFCS syrups only. The enzymes secreted by the bees, again, play a role in the final composition of the honey. Honey invertase is an α-glucosidase enzyme (it hydrolyses sucrose to glucose and fructose but also transfers the glucosyl moiety of sucrose to other acceptor carbohydrates hence giving rise to the oligosaccharides of honey). Fructosidase activity in honey is small and originates from yeasts and pollen. However, the caged bees produced honey without pollen and consequently the hydrolysis of fructosyl-fructoses from the syrup is not possible and could explain the carbohydrate profile of the honey obtained. In comparison, bees fed HFCS in the apiary only showed a lower value of sucrose compared to the flight arena bees. These results may indicate that the fructosidase activity of bees alone is not sufficient to remove the fructosyl-fructoses from HFCS fed to the bees. The authors concluded that the carbohydrate profile of honeys produced by bees fed HFCS was notably different from those fed SS and from free foraging bees; and that honey from bees fed HFCS could be easily identified through the detection of the fructosyl-fructose disaccharides; but this is true only if no pollen is present. They observed a dilution effect of the glucosyl-glucoses and glucosyl-fructoses naturally present in honey.

1.7 Origins of the Current Project

1.7.1 MAF Biosecurity Requirements with Regards to Honey.

Our research group was approached by the Ministry of Agriculture and Forestry (MAF) Biosecurity division for the development of a method to measure the amount of honey in imported products.

The import of honey products into New Zealand (NZ) is regulated under the Biosecurity Act 1993, Animal Products Act 1999 and Food Act 1981 to prevent the introduction of pests and diseases not present in NZ.

In 2004, MAF conducted an analysis of the biosecurity risk posed by the importation of the following honey bee products: honey, propolis, pollen, royal jelly, beeswax and bee venom. The report considered a number of risk organisms such as viruses, bacteria (example: European Foulbrood), fungi, arthropod parasites (example: small hive beetle, *Varroa destructor*), protozoa and other honey bee races (example: africanised bees or other non *A. mellifera* honey bees); and gave recommendations of sanitary measures necessary in the treatment of each honey bee product with regard to particular organisms. [100]

An Import Health Standard (IHS) for processed bee products was issued pursuant to Section 22 of the Biosecurity Act 1993. This IHS specifies the requirements to be met for the effective management of risks associated with the importation of the specified processed bee products.
The following products require a permit to import [101] :

- processed composite foods/food ingredients containing more than 2% honey, pollen or royal jelly that have not been baked or fried
- confectionery containing more than 2% honey, pollen or royal jelly that is not boiled
- refined propolis products that have not been packaged in consumer-ready packages for direct retail sale
- dietary supplements containing more than 2% honey, pollen or royal jelly that have not been encapsulated and packaged in consumer-ready packages for direct retail sale
- medical preparations containing more than 2% honey, pollen or royal jelly
- samples of honey and other processed bee products for evaluation and subsequent destruction.

Hence, a method of analysis of honey in imported products is needed to monitor the import of bee products.

1.7.2 Legal Requirements for the Development of the Method of Analysis.

The method of analysis developed for this project needs to be a robust method, able to stand in court in NZ; as the results of an analysis of a product containing honey above the legal limit could be needed as evidence in prosecution cases, and therefore must meet the requirements needed to be admissible in court.
The admissibility of evidence in court is determined following the provisions of the Evidence Act 2006; the purpose of this act being to help secure the just determination of proceedings.

The admissibility rules for expert opinion and expert evidence are described under Part 2, Section 25 of the Evidence Act 2006:
"Part 2, Section 25: Admissibility of expert opinion evidence
(1) An opinion by an expert that is part of expert evidence offered in a proceeding is admissible if the fact-finder is likely to obtain substantial help from the opinion in understanding other evidence in the proceeding or in ascertaining any fact that is of consequence to the determination of the proceeding.
(2) An opinion by an expert is not inadmissible simply because it is about:

(a) an ultimate issue to be determined in a proceeding; or

(b) a matter of common knowledge.

(3) If an opinion by an expert is based on a fact that is outside the general body of knowledge that makes up the expertise of the expert, the opinion may be relied on by the fact-finder only if that fact is or will be proved or judicially noticed in the proceeding." [102]

With the description of an expert being defined in Section 4:
"Section 4: Interpretation
Expert means a person who has specialised knowledge or skill based on training, study, or experience.

Expert evidence means the evidence of an expert based on the specialised knowledge or skill of that expert and includes evidence given in the form of an opinion". [102]

Commonly speaking, the admissibility of expert evidence is governed by three principles:

- Common knowledge/ordinary experience

Expert evidence is rendered admissible if the subject matter of the evidence requires special study or knowledge likely to be outside the wisdom of the fact-finder.

- Ultimate issue

Expert evidence is admissible if the role of the expert is to assist the fact-finder in deciding the issue at hand.

- Novel scientific evidence

The traditional test for the admissibility of novel scientific evidence was the "general acceptance" or "recognized branch of science" rules; but newly ascertained or applied scientific theories have posed a problem for courts because in some cases, science is not a closed system and the available evidence may not rule out either of two competing but well-reasoned scientific propositions.
More recently, the admissibility of novel scientific evidence is determined by considering: falsifiability, potential error rate, existence of standards controlling the operation(s), peer review and publication and general acceptance.

1.7.3 Previous Work Leading up to this Project.

A method was developed by Tanner-Dempsey (2008) [103] at the University of Waikato to identify and quantify the presence of honey within food products using the analysis of disaccharide content. In this method, four different New Zealand honeys were used to spike (1% honey) three matrices (fruit drinks which were high in natural sugar content and contained no honey). Spiked and non-spiked samples were

trimethylsilylated and compared and analysed using GC-MS with Selective Ion Monitoring (SIM) of molecular ions *m/z* 451, *m/z* 361 and *m/z* 204 (fragments which are common to trimethylsilylated sugars). Three peaks were identified as being present in all spiked chromatograms and consistent with honey chromatograms; and these peaks were not seen in the non-spiked samples. The peaks were identified (by retention times) as kojibiose (α or β), turanose and kojibiose (α or β) and most importantly are not disaccharides used in sweeteners found in food products other than honey.

The method was also tested on five samples provided by MAF (four known to contain honey and one which the manufacturers claimed contained no honey) and showed that the three peaks were present in the samples known to contain honey and were not seen in the sample which contained no honey (supposedly).

The report discussed above was reviewed [104], and the reviewer found that the "analytical method looks very promising from the perspective of monitoring potential imports into NZ." ([104], pp.2)

However, three issues were noted:

- <u>For the quantification of honey levels in a product:</u>

  The method had not been extensively validated and more analytical method development was needed to confirm that small oligosaccharides (especially kojibiose as the method had selected) can be reliably quantified. Also the level of the small oligosaccharides must be correlated with the levels in the honey in order to calculate the amount of honey present. The reviewer stated that "This would most easily be achieved if a conservative conversion factor based upon the assay of a range of typical honeys could be experimentally determined[…]with the proviso that the levels of such small oligosaccharides, especially kojibiose, do not vary widely." ([104], pp.2)

  The reviewer also stated that while the disclosed method documented the assessment of several honeys, they are from NZ, and that data on the levels of the small oligosaccharides in honeys from other countries is needed.

- False positives

  An assessment of the occurrence of the small oligosaccharides from other sources than honey needs to be undertaken to ascertain that it is uniquely found in honey as this would reduce the risk of a false positive from another ingredient present in the product in question.

- False negatives

  The presence of the small oligosaccharides chosen to detect honey must be confirmed in all honeys so as to reduce the risk of false negatives. Published data on the presence of the small oligosaccharides in Chinese-sourced honeys is needed as the bees in China could be different to those in North America or Europe. "Probably a representative set of Chinese honeys would need to be tested, if it is expected that imports from this area would be significant." ([104], pp.3)

1.8 Aims of the Present Research

The present research has several aims:

1) Develop a robust method for the quantitative detection of honey in imported products by quantifying (using GC) disaccharide(s) or trisaccharide(s) identified as marker sugars for the presence of honey
2) Carry out a literature search to demonstrate that the "marker sugars" are not common in food products
3) Create a database of the di- and trisaccharides in Asian honeys.

Aim 1:

From Table 7, it is obvious that most of the honeys analysed with identification and quantification of the oligosaccharide profile were done so by GC. Out of all the GC (FID or MS) analyses, the derivatisation technique prevalently used was oximation of the sugars prior to trimethylsilylation. The second most-used technique was the direct

trimethylsilylation of the sugars (analysis of TMS ethers) and only a couple of research groups used reduction prior to trimethylsilylation.
Oximation allows only two forms (E and Z) to be obtained for every reducing sugar and therefore for each reducing aldose or ketose sugar, two peaks appear in the gas chromatogram. This is a significant improvement in terms of the number of peaks observed in the chromatograms compared to TMS ethers (up to six peaks for a reducing sugar), but peaks overlapping in a complex mixture like honey still make separation difficult. It seems therefore more sensible to use reduction prior to trimethylsilylation, as with this technique, the sugars are converted to their alcohol form and only one peak appears in the GC for aldoses and two peaks for ketoses.

The various methods used previously have identified and quantified the sugars of honey but these methods have not been applied in the reverse situation, where one would want to determine the amount of honey present in products containing a proportion of honey.
Our aim is to develop a robust method using GC and reduction of the sugars prior to trimethylsilylation; with which a specific disaccharide(s) or trisaccharide(s) is identified as a marker sugar and routinely quantified. The marker sugar(s) can then be rationalised against an average amount of this marker sugar in a typical honey and therefore used to determine the actual amount of honey present in the product.

Aim 2:

To select a disaccharide(s) or trisaccharide(s) as a "marker sugar", it must not be present in common foods, as this sugar could be added from a different source than honey in the product analysed and therefore contribute as a false-positive during quantitation of honey in the product.
A literature search of the occurrence of the "marker sugar" selected in common foods must therefore be undertaken to prove it is uncommon in foods other than honey, or ideally only present in honey.

Aim 3:

As can be seen from the data presented in Table 7 and Figure 6, most of the honeys studied with the most detail and occurrences were from Europe or North America. Data available for most other countries is either scarce, has an incomplete analysis of the sugar composition or the country of origin of individual honey results is not specified.

The Food and Agriculture Organisation (FAO) of the United Nations lists the top 3 honey producing countries as being China, Argentina and Turkey, with China producing 357,220 tonnes of natural honey in 2007. [105]

The main producers, exporters and importers of natural honey in 2007 are summarised in Table 10.

Table 10: Producers, exporters and importers of natural honey in 2007. [105]

Honey main producers	Quantity (tonnes)	Unit value (US$/tonne)
China	357,000	-
Argentina	81,000	-
Turkey	73,935	-
Ukraine	67,700	-
Honey main exporters		
Argentina	79,861	1,680
China	65,288	1,464
Mexico	30,912	1,826
Hungary	23,872	2,717
Honey main importers		
USA	105,438	1,544
Germany	94,077	2,036
Japan	37,887	1,776
United Kingdom	30,109	2,812

- = not specified.

As can be seen from Table 10, China and Argentina were the main producers and exporters of natural honey in 2007. However data available on the characteristics of Chinese honey (and Asian honey in general) is limited, the data specific to the Asian honeys is not mentioned separately to other honey analysed or available data is limited to publications in Chinese.

A complete analysis of the sugars of Asian honeys is essential to determine an average Asian honey sugar composition and distinguish it (or not) from its counterparts. Studies suggest that the composition of honey is affected by factors such as floral origin, seasonal and climatic variations, nectar availability, geographical origin and bee species.

Analysis of samples from different species of honey bees suggest that significant differences can be observed in the moisture content, electrical conductivity, invertase, proline content; and also in the sugar composition.

There are 11 species of honeybees (see Table 11) and their nesting behaviour determines whether or not the bees will tolerate being kept inside a man-made hive.

Table 11: Honeybee species and type of nest. [106]

Honeybee species with nest consisting of multiple combs	**Honeybee species with nest consisting of single combs**
Apis cerana	*Apis andreniformis*
A. koschevnikovi	*A. binghami*
A. mellifera	*A. breviligula*
A. nigrocincta	*A. dorsata*
A. nuluensis	*A. florea*
	A. laboriosa

Apis mellifera (also called the European bee or the hive bee) is the most common bee. It is indigenous to Africa, Europe and the Middle East and has been introduced to the Americas, Australasia and much of the rest of the world. This bee is regarded as being of medium size and other species are judged as large or small relative to it. [106]

Most of the available literature on the sugar composition of honey refers to honey produced by *A. mellifera* (but this is not always stated).

Apis cerana (also called the Asian hive bee) is indigenous to Asia; between Afghanistan and Japan, and occurs from Russia and China in the north, to southern Indonesia. This bee builds a nest similar in style to *A. mellifera*, and builds its nest within a cavity. *Apis cerana* occurs (as does *A. mellifera*) over a wide geographical area and it varies in size: tropical races are smaller and with smaller colonies.

A. koschevnikovi (also known as the red bee), *A. nigrocincta* and *A. nuluensis* have been identified in Sabah, Malaysia in Northern Borneo and in Sulawesi in Indonesia, respectively.

Apis andreniformis and *A. florea* are very small in size. These bees build a single-comb nest which is small in size and usually suspended from a branch or low down in bushes or from a rock surface for *A. florea*. "*Apis andreniformis* has been identified in South East Asia, Borneo, the Philippines and the southern Chinese peninsula, while *A. florea* is indigenous to Oman, spreading southeast through Asia as far as some of the islands of Indonesia and the Philippines. In 1985, it was identified in Sudan and lately reported in Iraq." ([106], pp. 7)

A. dorsata (or rock bee) are large bees whose nest is single-combed suspended from a branch, cliff or building. It is found only as far as Afghanistan, to Bali in the South and is limited to the Himalayas in the North.

A. binghami and *A. breviligula* are present in Indonesia and the Phillipines, respectively.

A. laboriosa is the largest of the honeybees and its colonies are often found in clusters of sometimes up to 100 combs suspended from a cliff face very near to one another. This honeybee is found in the Himalayas (Nepal, Bhutan and China) at higher altitudes than *A. dorsata*.

The distribution of the honeybees geographically is varied as shown in Table 12.

Table 12: Geographical distribution of honeybee species. [106]

Geographical area	Indigenous honeybee species	Introduced honeybee species
Africa	*A. mellifera*	*A. florea* (Sudan)
Asia *	*A. andreniformis* *A. binghami* *A. breviligula* *Apis cerana* *A. dorsata* *A. florea* *A. laboriosa* *A. koschevnikovi* *A. nigrocincta* *A. nuluensis*	*A. mellifera*
Australasia and the Pacific	No indigenous honeybees	*A. mellifera*
Europe	*A. mellifera*	
Middle-East	*A. mellifera* *A. florea*	
The American continent	No indigenous honeybees	*A. mellifera*

* Not all of the species are present in every country.

The difference in the sugar composition of honeys from different honeybee species has been scarcely studied in Asian honeys.

Physico-chemical parameters for Philippine honeys were determined. Samples of *A. mellifera*, *A. dorsata* and *A. cerana* and commercial honeys were analysed for moisture content, electrical conductivity, ash content, pH, free acidity, lactone, total acidity, glucose, fructose and sucrose content, HMF and invertase activity. [107] The results for sucrose and the fructose to glucose ratio showed variations (see Table 13).

Table 13: Water and sugar content of *A. mellifera, A. dorsata, A. cerana* and commercial honeys from the Philippines. [107]

Parameter	Water (%)	Glucose (%)	Fructose (%)	Sucrose (%)	F/G[a]
Apis mellifera (n=27)	19.5 ± 1.6	28.7 ± 4.1	34.4 ± 3.7	1.97 ± 2.81	1.21 ± 0.13
Apis dorsata (n=5)	23.1 ± 2.3	30.5 ± 2.7	31.4 ± 5.3	3.59 ± 3.90	1.02 ± 0.10
Apis cerana (n=9)	22.0 ± 3.7	27.2 ± 3.1	26.9 ± 3.9	9.51 ± 4.12	0.99 ± 0.09
Commercial honeys (n=14)	20.0 ± 3.2	30.8 ± 7.2	27.1 ± 6.6	7.27 ± 7.16	0.88 ± 0.12
Total samples (n=55)	20.3 ± 2.7	29.3 ± 5.0	31.1 ± 5.8	4.62 ± 5.39	1.07 ± 0.19

[a] Fructose/Glucose ratio
n = number of samples

The results showed that the average water content is high in bees domestic to the Philippines (*A. cerana and A. dorsata*) compared to *A. mellifera*. The authors gave several reasons for the high water content: the method of extraction, lack of equipment to dry the honey, the behaviour of the bees (the native bees tend to migrate often and therefore do not dry their honey as much) and the warm humid climate which makes it hard to dry the honey.

The fructose and glucose content of local bee honey was not that different from other honeys; the exception being the sucrose content: *A. cerana* and the commercial honeys having higher sucrose content.

The carbohydrate composition of honey from different honeybee species was studied in more details in honeys from Nepal using HPLC. 28 honey samples from *A. dorsata*, 26 from *A. cerana* and 27 from *A. mellifera* were collected from colonies on the same day and from the same floristic region of Chitwan district, central Nepal. [65] The authors performed significance tests using the Bonferroni-Holm method.

An average carbohydrate composition of Nepalese honeys from central Nepal is depicted in Table 14.

Table 14: Average carbohydrate composition of honeys from central Nepal. [65]

Sugars (g/100g honey)	Mean	Standard deviation (%)
Fructose	34.36	0.67
Glucose	22.0	0.48
Sucrose	5.55	0.17
Turanose	2.25	0.15
Maltose	2.18	0.14
Trehalose	2.11	0.08
Isomaltose	1.94	0.08
Melibiose	2.01	0.13
Erlose	2.18	0.09
Melezitose	2.20	0.08
Raffinose	2.21	0.09
Panose	2.94	0.23
Maltotetraose	3.24	0.21
Oligosaccharide L_1[a]	-	-
Oligosaccharide L_2[b]	-	-

[a] Uncharacterised oligosaccharide peak that was not quantified.
[b] Honeydew-specific sugar that was not quantified.

Comparison between the three bee species (see Table 15) showed that "there were no significant differences between the honey types in pH, glucose oxidase, and the amount of glucose. However, the amount of fructose was significantly higher in *A. dorsata* and *A. cerana* than in *A. mellifera* honeys. Similarly, the amount of oligosaccharide L_2 was significantly higher and sucrose was significantly lower in *A. dorsata* honeys than in *A. cerana* and *A. mellifera* honeys." ([65], pp. 367)

The amount of sucrose was found to be higher in *A. cerana* and the commercial honeys by Laude *et al.* (1991) [107]; which contradicts the results of Joshi *et al.* (2000) [65] who found sucrose to be higher in *A. mellifera* honeys, followed by *A.*

cerana and lastly, *A. dorsata*. However, the amount of oligosaccharide L_2 (which is a honeydew-specific oligosaccharide) was significantly higher in *A. dorsata* honeys than in *A. cerana* and *A. mellifera* honeys. [65] Astwood *et al.* (1998) [19] noted that, compared to White (1962)'s American honeydew honeys [20], the New Zealand honeydew honey showed smaller amounts of sucrose; and even though these results were from *A. mellifera* bees, the sucrose content seems to be lower in honeydew honeys compared to floral honeys. The sucrose content of the *A. dorsata* bees reported by Joshi *et al.* (2000) [65] could therefore be influenced by their honeydew characteristics.

Table 15: Carbohydrate compositions of honeys from Nepal by honeybee species. [65]

Parameters	***A. dorsata*** **(mean ± standard deviation)**	***A. cerana*** **(mean ± standard deviation)**	***A. mellifera*** **(mean ± standard deviation)**
Moisture (%)	21.51 ± 2.38	20.12 ± 2.66	17.14 ± 2.56
Fructose	48.01 ± 2.35	48.25 ± 1.62	45.93 ± 1.8
Glucose	42.23 ± 4.94	44.02 ± 4.54	41.95 ± 2.53
Sucrose	0.33 ± 0.29	1.39 ± 1.71	1.96 ± 1.93
Turanose	1.42 ± 0.49	0.97 ± 0.7	1.66 ± 0.5
Maltose	2.22 ± 0.73	2.09 ± 0.86	3.26 ± 0.61
Oligosaccharide L_2	2.16 ± 3.29	0.45 ± 0.89	0.31 ± 0.75
Others[a]	3.63	2.83	4.93
F/G ratio	1.15 ± 0.13	1.11 ± 0.13	1.1 ± 0.05

[a] Others = sum of kojibiose, trehalose, isomaltose, melibiose, erlose, melezitose, maltotriose, raffinose and maltotetraose.

As in the Philippine honeys, differences in moisture content were also observed. The authors found the moisture content to be significantly higher in *A. dorsata* than in *A. cerana* and *A. mellifera* honeys.

2 Chapter 2:

MATERIALS AND METHODS

2.1 Materials

2.1.1 General reagents

IRC-50 resin standard grade was used to neutralize excess $NaBH_4$ after reduction of the samples and was generously donated by I. Suckling and also purchased from BDH Chemicals Ltd.

$NaBH_4$ used to reduce the carbohydrates in the samples was obtained from Alfa Aesar - A Johnson Matthey Company.

Tri Sil HTP reagent was obtained from Thermo Fisher Scientific Inc. and used to sylilate the samples prior to GC analysis.

Solvents used in the course of this research were methanol, pyridine and water. Methanol was of HPLC grade and supplied by either Scharlau or Ajax Finechem Pty Ltd. (as was available). Pyridine (99+%, A.C.S. reagent) was purchased from Sigma-Aldrich Co. and dried over molecular sieve. Water was either distilled or deionised and obtained from a Crystal Pure Ultra Pure Water System.

Glacial acetic acid (analytical reagent) was used to remove leftover borate after reduction of the samples and was purchased from Ajax Finechem Pty Ltd.

2.1.2 Mono-, Di- and Oligosaccharide Standards.

Xylitol and kojibiose were obtained from Sigma-Aldrich Co.; fructose, sucrose, turanose were purchased from Aldrich Chemical Co, Inc; maltose, nigerose, trehalose, palatinose, melibiose, gentiobiose, isomaltose, melezitose, raffinose,

maltotriose, panose, isomaltotriose were from Sigma Chemical Company; glucose was purchased from BDH AnalaR; cellobiose was purchased from BDH Biochemical; maltulose was from CMS Chemicals Ltd. Kestose was isolated from oligofructose kindly supplied by Salkat New Zealand, using in-house HPLC.

2.1.3 Honey samples

Honey samples were supplied by MAF Biosecurity, the division of MAF responsible for the New Zealand biosecurity system.

The honey samples provided were intercepted at the New Zealand border by MAF Biosecurity personnel. The country of origin and names of the samples were recorded as cited on the labels of the containers of honey or as cited by MAF.

A summary of the samples used in this study is presented in Table 16, with their countries of origin, in order of decreasing number of samples.

Not all of the samples were used for carbohydrate analysis; either due to them being outside the normal moisture content range of honey or from difficulties in sample preparation.

Table 16: Honey samples' country of origin and name.

Country of origin	Name on the label	Reference number
China (n = 15)		
	Bass honey	109
	Beijing TRT	76, 77, 107
	China	80
	Chincell Tang honey	64
	Feng Chao Su	84, 98, 99
	Honey ex China	115
	Xutaishan	19
	No name "S"	20, 21
	Propolis honey yaoumei	110
	Watson's honey sugar polish	78
India (n = 14)		
	Bailley drinking water bottle	79
	Dabur honey	48, 49
	Dabur honey (guaranteed pure)	70, 104
	Dabur honey "for CSD only"	71, 95
	Dabur-Madhu	69
	Honey Agmark-Bombay	106
	Madhusagar (MMS) Pure apiari honey	75
	Miss Bee	119
	Mixed fruits with honey	97
	Nature Pure 100% honey	81
	Pure honey, Gita Bhawan Ayurved Sansthan	13

Table 16: Honey samples' country of origin and name (continued).

Vietnam (n = 5)		
	Honey from Pax hive	118
	Honey from Vietnam	117
	Mat Ong Longan honey	116
	Mâtong Nguyên Chât	111
	Mât Ong natural pure honey	89
Thailand (n = 4)		
	H & B Health & Beauty	22
	No name -10-1-068361-1-000	23
	Yellow honey tablet	72, 102
Japan (n = 3)		
	100% honey	26
	Japanese honey	93
	Pure honey	82
Malaysia (n = 3)		
	Eva Madu honey	120
	Longan honey	83, 90
Russia (n = 3)		
	Honey from Russia	86
	MED + honeycomb	91
	Unlabelled	92
"India-Malaysia-Thailand?" (n = 2)		
	Fogel's honey	87
	M-150	88
Indonesia (n = 2)		
	Tamboka segel Ituh	25
	Tolak ansin	24

Table 16: Honey samples' country of origin and name (continued).

Sri Lanka (n = 2)		
	Bees honey	74
	Sri Lanka	108
Philippines (n = 1)		
	Pure honey	47
South Korea (n = 2)		
	Jirimountain plantation pure honey	68
	Pure honey	113

2.2 General methods

Whatman 43 or Whatman 41 (7.0 cm) ashless paper was used to filter the standards and samples.

Volumes of liquid that could not fit into a sample vial were reduced using an Eyela rotary evaporator equipped with an Eyela Water Bath SB-650 and a Büchi Vac®V-500 vacuum pump. Once liquid had been reduced enough to fit into a sample vial, further evaporation was achieved using a Pierce ReactiTherm heating module set at 40°C and fitted with a Pierce Reacti-Vap Evaporating Unit blowing a stream of dry nitrogen.

Complete removal of water from the standards and samples prior to sylilation was achieved using a Lab Line Instruments, Inc. vacuum oven. The temperature in the vacuum was controlled using a Lab Line Instruments, Inc. thermometer and the vacuum created using a Büchi V-700 vacuum pump.

2.2.1 Glassware Cleaning Protocol

Glassware used in the analysis of carbohydrates (and especially for trace analysis) must be acid cleaned to remove organic residues that might be present on the glassware. All glassware was soaked overnight in a concentrated nitric acid bath, subsequently rinsed with tap water and distilled water at least three times, respectively and then oven-dried.

In a concentrated nitric acid solution, sugars are oxidized to aldaric acids and further oxidation breaks down the carbon frame of the sugars into small fragments, hence removing the carbohydrates.

2.2.2 Determination of Moisture Content

Moisture content of the samples was determined using a Misco Palm Abbe PA203 Digital refractometer. Measurements for each sample were taken every 10 seconds until three consecutive stable values were obtained.

Samples unable to be determined by this instrument were weighed into glass vials that had previously been accurately weighed, freeze-dried using a Labconco Bulk Tray Drier until complete dryness, and the vials re-weighed to determine moisture content.

Equation 1 was used to calculate the moisture content in the samples that could not be determined using the digital refractometer.

Equation 1: Calculation of % moisture in freeze-dried samples.

$$\%moisture = \frac{Wt(s) - \left(Wt(f) - Wt(v)\right)}{Wt(s)} \times 100$$

Where:

%moisture is the moisture content (in percent) of the sample

Wt(s) is the weight of the sample in mg

Wt(f) is the weight of the vial and sample after freeze-drying (mg)

Wt(v) is the weight of the empty vial prior to freeze-drying (mg)

The moisture content was expressed as an average of three values which had a standard deviation less than 5%.

2.3 Analysis and Quantitation of Oligosaccharides by GC-FID

After being provided by MAF, the honey samples were stored at 4°C until needed for analysis.

Prior to measurement of moisture content and preparation of the samples, the honey jars were allowed to warm to room temperature overnight, then warmed to 40°C for 24 hours and stirred to remove crystallisation. Samples which were heavily crystallised were warmed at 40°C for up to 48 hours and stirred several times.

An acidic methanol solution was prepared by making up a 0.5% acetic acid in methanol solution.

Three xylitol internal standard solutions were prepared by weighing xylitol (100 mg) into volumetric flasks (100 mL). The flasks were made up to the mark with deionised water to yield solutions of concentration 1.00 mg/mL. Since each sample was analysed in triplicate, a separate xylitol standard solution was used for each replicate.

2.3.1 Reduction and Sylilation of Standards

A set of standards was made up containing known amounts of sugar standards and a xylitol internal standard. These standards were made up as explained below, then analysed by GC-FID and Response Factor (RF) was calculated and then used to quantify the oligosaccharides in honey.

Solutions of standards were made up by weighing in volumetric flasks.

<u>For reducing sugar standards:</u>

$NaBH_4$ (5 mg per mg of standard) was weighed into a glass vial (7 mL) and required amounts of sugar standard was then pipetted into the glass vial. Deionised water (1 mL) was added. The vial was heated (50°C) for 4 hours, then cooled and freshly washed IRC-50 resin added to the vial to remove excess $NaBH_4$ until no gas evolved. The standards were filtered, evaporated under reduced pressure until nearly all the solvent had evaporated. The remaining liquid was transferred into a glass vial (7 mL), blown dry under a stream of dry nitrogen and maintained at 40°C and co-evaporated six times with 2 mL acidic methanol to remove leftover borate. Xylitol internal standard (100μL) was added and blown dry under a stream of dry nitrogen at 40°C until no liquid was visible then dried overnight in a vacuum oven at 40°C.

Trimethylsilylation was achieved by adding dry pyridine (900 μL) and sonicating (5 min). Tri Sil HTP (100 μL) was added and the vials heated (10 min, 75°C). The vials were left to cool and subsequently centrifuged (3 min, 3000 rpm).

Supernatant (0.5 mL) was transferred to a clean GC vial, and either analysed by GC-FID as is; or diluted appropriately using dry pyridine (1 mL) and subsequently analysed by GC-FID.

<u>For non-reducing sugar standards such as sucrose, trehalose and raffinose:</u>

Xylitol internal standard (100 μL) and sugar standard were added to a GC vial. The solution was maintained at 40°C and blown dry under nitrogen gas.

Trimethylsilylation was achieved by adding dry pyridine (500 μL) and sonicating (5 min). Then Tri Sil HTP (500 μL) was added and the vials heated (10 min, 75°C). The vials were left to cool, centrifuged (3 min, 3000rpm) and subsequently analysed by GC-FID.

2.3.2 Reduction and Silylation of Samples

Honey (approximately 15 mg) and $NaBH_4$ (60-70 mg) were weighed into a glass vial (7 mL) and deionised water (1 mL) was added. The vial was heated (50°C) for 4 hours, then cooled and freshly washed IRC-50 resin added to the vial to remove excess $NaBH_4$ until no gas evolved. The samples were filtered, evaporated under reduced pressure until nearly all the solvent had evaporated. The remaining liquid was transferred into a glass vial (7 mL), blown dry at 40°C under a stream of dry nitrogen and co-evaporated six times with 2 mL acidic methanol to remove leftover borate. Xylitol internal standard (100 μL) was added and blown to near dryness. The vials were then dried overnight in a vacuum oven at 40°C.

Trimethylsilylation was achieved by adding Tri Sil HTP (1.5 mL), sonicating for 10 mins and heating (10 min, 75°C). The vials were left to cool and subsequently centrifuged (3 min, 3000 rpm).

Supernatant (0.5 mL) was transferred to a clean GC vial and subsequently analysed by GC-FID.

2.3.3 GC-FID Parameters.

Standards and samples were analysed on a gas chromatograph (Model 6890N Series, Agilent Technologies) equipped with an autosampler (Model G2614A Series Autosampler, Agilent Technologies) and injector unit (Model 7683 Series Injector, Agilent Technologies), and operated by an HP GC ChemStation software.

Analyses were carried out with an on-column injector and using a 30m×0.32mm×0.25μm Zebron ZB-5 capillary column (phase: 5%-phenyl-95%-

dimethylpolysiloxane) and FID detection. Carrier gas was hydrogen at 2.6 mL/min. Two microliter samples were injected into the column, with the injector temperature tracking the oven temperature. Detector temperature was maintained at 325°C.
The oven temperature program used is shown in Table 17.

Table 17: Oven temperature program.

°C/min	Temperature (°C)	Hold time (min)
	150	5
3	300	
1	325	10

2.3.4 Data analysis

Chromatographic data files were evaluated and peaks integrated using the ChemStation software.

2.3.5 Quantitation of Oligosaccharides in the Honey Samples

GC-FID peak areas were integrated and used to calculate the oligosaccharide concentrations in the honey samples. Peak areas depend on the quantity of compound eluting out of the column and its relative response in the detector. Hence it is necessary to determine response factors for each oligosaccharide in order to quantitate it.

Response factors were determined by analysing each available standard in triplicate with varying amounts of compound and a consistent amount of internal standard.
Equation 2 was used to calculate the response factor.

The weight of each available oligosaccharide in the samples was determined by solving Equation 2 for Wt(o) as shown in Equation 3.

The percentage of each oligosaccharide in the sample was calculated using Equation 4.

Equation 2: Response Factor calculation.

$$RF(o) = \frac{\frac{A(o)}{A(Xyl)}}{\frac{Wt(o)}{Wt(Xyl)}}$$

Where:

RF(o) is the response factor for the oligosaccharide,

A(o) is the integrated peak area of the oligosaccharide,

A(Xyl) is the integrated peak area of the internal standard xylitol,

Wt(o) is the weight of the oligosaccharide (mg),

Wt(Xyl) is the weight of the internal standard xylitol (mg)

Equation 3: Calculation of the weight of the oligosaccharide in the honey samples.

$$Wt(o) = \left[\frac{\frac{A(o)}{A(Xyl)}}{RF(o)} \right] \times Wt(Xyl)$$

Where:

A(o) is the integrated peak area of the oligosaccharide,

A(Xyl) is the integrated peak area of the internal standard xylitol,

RF(o) is the response factor for the oligosaccharide,

Wt(o) is the weight of the oligosaccharide (mg),

Wt(Xyl) is the weight of the internal standard xylitol (mg)

Equation 4: Calculation of the percentage of each oligosaccharide in the honey samples.

$$\%(o) = \frac{Wt(o)}{Wt(s)} \times 100$$

Where:

%(o) is the percentage of the oligosaccharide in the honey sample,

Wt(o) is the weight of the oligosaccharide (mg),

Wt(s) is the weight of the honey sample (mg).

2.3.6 Contamination and Degradation of the GC-FID Column and Detector.

After a succession of runs, the sensitivity of the detector would decrease; signaled by a noisier baseline. This problem was solved by doing solvent runs, baking the column at 325°C for up to 90 minutes while running the detector at 375°C and thoroughly cleaning the detector parts.

Also the septum in the injection system needed to be replaced frequently to avoid leak problems.

Over time, loss of resolution was observed through shifting of retention times. The RTs of both the internal standard and trimethylsilylated carbohydrates shifted slowly earlier, making peak assignment in some samples difficult when comparing with standards that were analysed a long time apart.

3 Chapter 3:

RESULTS AND DISCUSSION

3.1 Aim 1: Method Development

3.1.1 Precision

The precision of an analytical method is the degree of agreement between individual results obtained when the method is applied to multiple sampling of a homogenous sample.

The sample preparation method was assessed by preparing and analysing a honey sample (sample ID: 109), ten times and determining the mean and % Relative Standard Deviation (%RSD) as shown in Equation 5, the values for sample 109 are shown in Table 18.

The precision of the instrument was also investigated by preparing a standard of known concentration and analysing it ten times using the GC-FID method.

The mean and %RSD (Equation 5) were determined and shown in Table 18 and Table 19.

Equation 5: Calculation of % RSD.

$$\%RSD = \frac{StdDev \times 100}{mean}$$

Table 18: Evaluation of the precision of the sample preparation by replicate analysis of sample 109.

Sugar	109#1	109#2	109#3	109#4	109#5	109#6	109#10	109#13	109#15	Mean	StdDev	%RSD
	% w/w											
Monosaccharides	76.81*	69.95	70.34	54.85	57.20	65.43	66.47	67.39	62.42	**64.26**	**5.69**	**8.86**
Sucrose	0.02	0.02	0.02	0.03	0.06*	0.02	0.02	0.03	0.03	**0.02**	**0.01**	**20.72**
Trehalose	0.05	0.05	0.05	0.17	0.20	0.05	0.03	0.13	0.13*	**0.09**	**0.07**	**72.13**
Cellobiose	0.50	0.45	0.48	0.44	0.46	0.42	0.41	0.45	0.43	**0.45**	**0.03**	**6.73**
Laminaribiose	0.37	0.30	0.29	0.30	0.45	0.21	0.30	0.30	0.45	**0.33**	**0.08**	**23.90**
Nigerose +Turanose1	1.65	1.21	1.49	1.81	1.63	1.39	1.23	1.92	1.99	**1.59**	**0.29**	**17.96**
Turanose2+Maltulose1	0.93	0.72	0.83	1.03	0.98	0.79	0.75	1.11	1.33	**0.94**	**0.20**	**20.95**
Maltulose2+Maltose	1.86	1.52	1.65	1.76	1.43	1.70	1.67	1.81	1.93	**1.70**	**0.16**	**9.31**
Kojibiose	0.52	0.52	0.52	0.48	0.35	0.50	0.50	0.42	0.45	**0.47**	**0.06**	**11.77**
Melibiose	0.67	0.69	0.69	0.41	0.34	0.51	0.51	0.44	0.49	**0.53**	**0.13**	**24.66**
Gentiobiose	0.29	0.32	0.32	0.21	0.18	0.28	0.28	0.21	0.21	**0.26**	**0.05**	**21.45**
Palatinose	1.07	0.78	1.04	0.62	0.59	0.83	0.79	0.74	0.74	**0.80**	**0.16**	**20.52**
Isomaltose	0.23	0.58	0.21	0.29	0.24	0.52	0.63	0.37	0.29	**0.37**	**0.16**	**43.34**
Raffinose	0.01	0.01	0.01	0.00	0.01	ND	0.00	0.01	0.01	**0.01**	**0.00**	**44.91**
Kestose	0.06	0.03	0.05	0.04	0.05	0.03	0.04	0.06	0.06	**0.05**	**0.01**	**22.23**
Erlose	0.03	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.02	**0.02**	**0.00**	**24.18**
Melezitose	0.02	0.02	0.01	0.02	0.03	0.01	0.02	0.02	0.02	**0.02**	**0.01**	**35.08**
Maltotriose	0.10	0.07	0.07	0.06	0.07	0.08	0.07	0.07	0.09	**0.08**	**0.01**	**18.08**
Panose	0.17	0.14	0.17	0.09	0.09	0.14	0.15	0.19	0.14	**0.14**	**0.03**	**24.27**
Isomaltotriose	0.03	0.02	0.03	ND	ND	0.02	0.01	0.02	0.02	**0.02**	**0.01**	**30.92**

* outlier which was not included in the calculations
ND = not detected

Table 19: Evaluation of the precision of the GC-FID by replicate analysis of a sucrose standard.

Repeat #	Area Xylitol	Area Sucrose	Ratio (suc/xylitol)	Weight sucrose (mg)
Rept1	3194.2	109.2	0.0342	0.0045
Rept2	3420.2	118.3	0.0346	0.0046
Rept3	3378.3	117.5	0.0348	0.0046
Rept4	3126.1	107.6	0.0344	0.0046
Rept5	3106.6	107.4	0.0346	0.0046
Rept6	3214.5	105.8	0.0329	0.0044
Rept7	3292.8	113.9	0.0346	0.0046
Rept8	2595.7	82.4	0.0317	0.0042
Rept9	2910.2	101.1	0.0347	0.0046
Rept10	3889.7	136.9	0.0352	0.0047
Rept11	2893.2	93.6	0.0324	0.0043
			Mean:	0.0045
			Std Deviation	0.0002
			RSD (%)	3.3271

The %RSD for the sample preparation is quite large and varies widely between the oligosaccharides. This could be attributed in part to the difficulty in getting a homogeneous sample from honey.

The RSD for the instrumental method was about 3.3%. From the analysis of these ten replicate of sucrose, the limit of quantitation can be determined. The limit of quantitation represents the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The limit of detection is the lowest concentration in a sample that can be detected; and it is obvious that the limit of detection is constrained by the method rather than the instrument.

A generic method quantitation limit for disaccharides was calculated by analysis of a serial dilution of sucrose until the last standard with acceptable precision and accuracy was analysed. This showed that the limit of quantitation by this method was 0.33 %w/w. It is immediately obvious that many of the disaccharides are below this limit and this could also account for the large %RSD in this and subsequent reported measurements.

Many authors who detail the di- and trisaccharides of honey do not include an error estimate, for example [6, 9, 34]; possibly for the same reason. The results indicate that although di- and trisaccharides can be used to demonstrate the presence of honey, quantitation would be best achieved by looking at the monosaccharides of honey or by concentrating the oligosaccharide fraction.

3.2 Aim 2: Literature Search.

A literature search for the occurrence of several di- and trisaccharides that could be used as marker sugars was undertaken.

3.2.1 Occurrence of Kojibiose

Review of the available literature containing the word kojibiose afforded numerous articles relating to the use of kojibiose in metabolic studies of enzymes. In all these cases, kojibiose was purchased or enzymatically produced in the laboratory; and since kojibiose is prohibitively expensive to purchase, it is unlikely to be used for adulteration.

The occurrence of kojibiose in food has been mentioned only on a few occasions.

The foods in which kojibiose was detected are summarised in Table 20 and originate mostly from Japan. It was detected in the products of the fermentation of rice in several articles dating from 1954 to 1977 and detected by either paper chromatography and/or carbon column chromatography. These techniques can be described as primitive compare to modern identification techniques but the fact that several independent researchers found kojibiose in the fermentation products of rice suggest that this fact is trustworthy.

Table 20: Occurrence of kojibiose in foods.

Food type	Details	Reference
Mirin (sweet sake)	Carbon column chromatography separation followed by paper chromatography and ionophoresis. Glucose, trehalose, kojibiose, nigerose, maltose, isomaltose, maltotriose, panose, isomaltotriose, and four higher oligosaccharides were detected. A separative detection of the sugars showed glucose to be 77-91% of the total.	[108, 109]
Amasake (sweet sake)	Amasake contained (detected by paper chromatography): glucose (74-75% of sugar content), kojibiose, nigerose, maltose, isomaltose, panose, isomaltotriose, and 2 higher oligosaccharides. Sum of kojibiose, nigerose and maltose is 8-9% of sugar content.	[110]
Amazake	Detection by paper chromatography: of the total sugars 70-80% was glucose, 3.3-9.1% isomaltose + maltotriose, and 5.5-8.9% nigerose + maltose + kojibiose.	[111]
Sake	Glucose removed. Identification (using physical and chemical properties) of panose, 4-**α**-isomaltotriosyl-D-glucose, sakebiose and kojibiose.	[112]
Koji (steamed rice)	The sugars identified were: xylose, arabinose, glucose, galactose, isomaltose, kojibiose, maltose, dextrantriose, and panose.	[113]

Table 20: Occurrence of kojibiose in foods (continued).

Soybean roots	Sucrose, kojibiose, cello- and laminari-oligosaccharides were found in cultured soybean root exudates. Methylation analysis and HPLC-PAD used.	[114]
White soy sauce	Xylose, arabinose, fructose, glucose, galactose, kojibiose, nigerose, maltose, isomaltose, maltotriose, panose, isomaltotriose, and 2 unknown sugars and 2 higher oligosaccharides were detected by paper and charcoal column chromatography and paper electrophoresis.	[115]

Kojibiose has been detected in soybean roots [114], this article is more recent and uses a more modern technique of analysis which is HPLC-PAD and the authors claim that the marker of kojibiose used is based on standard retention times; however the source of the standards claimed to be used in that research is not mentioned and no measured amount of kojibiose is given.

Kojibiose was also detected by using paper chromatography/column chromatography and paper electrophoresis in white soy sauce. [115] Soy sauce is a traditional condiment in East and Southeast Asian countries. It is produced from the fermentation of soybeans with molds.

It can be concluded that unless the food material or product states specifically that it contains sake or a fermented rice product, and possibly soy sauce; it can be assumed that the kojibiose present in the food material being analysed comes only from honey present in the product.

3.2.2 Occurrence of Turanose

From research undertaken of the available literature, turanose is commonly used as a sucrose analogue in metabolic studies of enzymes. In all of these cases, turanose was purchased or manufactured in laboratory.

The food types in which turanose has been identified are given in Table 21.

Table 21: Occurence of turanose in foods.

Food type	Details	Reference
Apricot (Hungary)	Turanose detected in apricot (at less than 0.05% of dry matter) as TMS-oxime ether by GC-MS by quantitation of selective fragment ions.	[116]
Honeydew	Sugar content of 3 honeydews was investigated. Honeydew from 2 species of the whitefly genus Trialeurodes contained more than 20% turanose.	[117]
Korean Dendropanax seed	Turanose detected in small amount in the seed of the Korean Dendropanax plant (*Dendropanax morbifera Lev.*).	[118]
Rice roots	Turanose detected in cultured rice root exudates. GC-MS used.	[119]

Honeydew, Korean Dendropanax seeds and rice roots have not been found to be common foods. Honeydew is produced by aphids feeding on the sap of plants on which they deposit the viscous substance; this is harvested by bees to make honeydew honey. However harvesting of honeydew by hand for direct consumption by humans is unlikely.

Korean Dendropanax is an evergreen broadleaf tree that grows in the southern part of Korea and is mostly known for its sap being used as a lacquer [120] and therefore, it is not apparently consumed as food.

Turanose was found to be present in apricots at less than 0.05% of the dry matter. It was detected by GC-MS.

It can be concluded that unless the food material or product states specifically that it contains apricot, it can be ascertained that the turanose present in the food material being analysed only comes from honey present in the product.

3.2.3 Occurrence of Nigerose

Research of the available literature afforded only a few articles relating to the presence of nigerose in food. Most of the articles mentioning nigerose related to its manufacture or use in biochemical studies of enzymes from which it can be produced. The food types in which nigerose was found are summarised in Table 22.

Table 22: Occurrence of nigerose in foods.

Food type	Details	Reference
Mirin (sweet sake)	Carbon column chromatography separation followed by paper chromatography and ionophoresis. Glucose, trehalose, kojibiose, nigerose, maltose, isomaltose, maltotriose, panose, isomaltotriose, and four higher oligosaccharides were detected. A separative detection of the sugars showed glucose to be 77-91% of the total.	[108, 109]
Amasake (sweet sake)	Amasake contained (detected by paper chromatography): glucose (74-75% of sugar content), kojibiose, nigerose, maltose, isomaltose, panose, isomaltotriose, and 2 higher oligosaccharides. Sum of kojibiose, nigerose and maltose is 8-9% of sugar content.	[110]
Amazake	Detection by paper chromatography: of the total sugars 70-80% was glucose, 3.3-9.1% isomaltose + maltotriose, and 5.5-8.9% nigerose + maltose + kojibiose.	[111]
Beer	Maltulose was detected by paper chromatography and isolated by chromatographic analysis.	[121]
Beer	Xylose, arabinose, glucose, nigerose, maltose, isomaltose, maltotriose, maltotetraose, ribose, galactose, and fructose were detected by paper chromatography.	[122]
Rice-Koji Miso	Glucose. isomaltose, nigerose and Isomaltotriose were detected. Method not mentioned in article.	[123]

Table 22: Occurrence of nigerose in foods (continued).

White soy sauce	Xylose, arabinose, fructose, glucose, galactose, kojibiose, nigerose, maltose, isomaltose, maltotriose, panose, isomaltotriose, and 2 unknown sugars and 2 higher oligosaccharides were detected by paper and charcoal column chromatography and paper electrophoresis.	[115]

The foods in which nigerose was found are closely related to foods also containing kojibiose. Nigerose was found in products made from the fermentation of rice such as sake or mirin, and possibly rice-koji miso. It is also mentioned in an article dating from 1961 [122] which relates the identification of several sugars in beer; and in a 1960 [115] paper about the sugar compositions of white soy sauce. All these findings originate from the same authors or research groups in Japan.

It can be concluded that unless the food material or product states specifically that it contains sake or a fermented rice product, and possibly soy sauce and beer; it can be ascertained that the nigerose present in the food material being analysed only comes from honey present in the product.

3.2.4 Occurrence of Maltulose.

Because the reduction of turanose gives peaks corresponding to signals from nigerose and maltulose, the combined turanose/nigerose and turanose/maltulose peaks must be considered in the carbohydrate profile and consequently, the occurrence of maltulose in foods must be considered.

From research undertaken of the available literature, maltulose is present in sweetening syrups like the ones used for honey adulteration and bee-feeding as it is formed during the isomerisation of maltose; but also in sweetening agents used in food.

Table 23: Occurence of maltulose in foods.

Food type	Details	Reference
Beer	Maltulose was detected by paper chromatography and isolated by chromatographic analysis.	[121]
Dry pasta	Maltulose was detected in dry pasta. The method was not stated. However it was not detected in fresh pasta.	[124]
Infant formula	Maltulose was detected in infant formula. The method of analysis was not stated.	[125]

The foods (other than sweetening agents) in which maltulose was found are infant formula, dry pasta and beer as shown in Table 23.

It can be concluded that unless the food material or product states specifically that it has been artificially sweetened or contains infant formula, dry pasta, and possibly beer, it can be ascertained that the maltulose present in the food material being analysed only comes from honey present in the product.

From this literature search, the detection of kojibiose, turanose or nigerose together in a sample being analysed can be attributed to the presence of honey in the sample, unless any of the foods mentioned in Table 20, Table 21, Table 22 and Table 23 are present together.

The presence of turanose/maltulose plus kojibiose or nigerose/turanose and kojibiose could be taken as convincing proof of the presence of honey.

3.3 Aim 3: Study of the Asian Honeys.

3.3.1 Moisture Content

The moisture content of the honey samples was determined by refractometry. Samples which were unable to be determined using the digital refractometer were not included in the calculations of the average honey composition. The results are summarised in Table 24.

The Codex Alimentarius Commission standard for honey [126] states that Heather honey (*Calluna*) must not contain more than 23% moisture and all other honeys not more than 20% moisture.

Table 24: Average moisture and solids content of honeys per country.

Country of origin of honeys	Average moisture content (%)	Average solids (%)
China (n = 13)	18.6 (17.2 – 22.9)	81.4 (77.1 – 82.8)
India (n = 14)	18.8 (17.8 – 21.2)	81.2 (78.8 – 82.2)
Indonesia (n = 1)	18.4	81.6
Japan (n = 2)	17.4 (16.5 – 83.5)	82.7 (81.8 – 83.5)
Malaysia (n = 3)	16.5 (15.9 – 16.8)	83.5 (83.2 – 84.1)
Philippines (n = 1)	21.7	78.3
Russia (n = 2)	17.4 (16.7 – 18.1)	82.6 (81.9 – 83.3)
South Korea (n = 1)	18.4	81.6
Sri Lanka (n = 2)	20.75 (20.5 – 21.0)	79.25 (79.0 – 79.5)
Thailand (n = 2)	17.4 (17.1 – 17.6)	82.7 (82.4 – 82.9)
Vietnam (n = 3)	20.7 (19.3 – 22.4)	79.3 (77.6 – 80.7)
"India-Malaysia-Thailand?"(n = 2)	19.3 (15.4 – 23.2)	80.8 (76.9 – 84.6)

n = number of samples

From the results given in Table 24, average Asian honey moisture content can be calculated. The average moisture content of honeys from Asia in this study was 18.8% (81.2% solids) and ranged from 15.9 to 22.9 %.

Compared with the average moisture content of USA floral honeys of 17.2% [3], see Table 3; or the average moisture content of honeys from the Madrid province of Spain of 16.13%; the average Asian honey moisture content of 18.8% is slightly higher and the range of values wider. The limit of 20% moisture of the Codex Alimentarius Commission for honey (except Heather honey *(Calluna);* not more than 23%) was exceeded by a total of nine samples: one Chinese, two Indian, one South

Korean, one Filipino, one Sri Lankan, two Vietnamese and one of the "India-Malaysia-Thailand?" honey samples.

The moisture content of samples that were unable to be determined using the digital refractometer were measured using the freeze-drying method. The results for these samples are summarised in Table 25.

Table 25: Moisture content of non-honey samples.

Sample ID and description	Country of origin of samples	Average moisture content (%), range and standard deviation
24: colourless liquid smelling like menthol	Indonesia	75.0 (71.5 – 81.7) 4.7
26: honey coloured throat lozenge	Japan	65.8 (62.3 – 72.3) 4.6
78: crystallized honey paste (supposedly a bodywash)	China	45.6 (42.1 – 49.2) 3.5
91: honeycomb	Russia	43.7 (43.2 – 44.2) 0.5
72 and 102: honey coloured fibrous hard balls	Thailand but maybe Vietnam	8.2 (8.1 - 8.4) 0.1
110: Propolis	China	52.4 (48.2 – 56.7) 4.2
111: honey coloured liquid	Vietnam	32.0 (30.4 – 34.1) 1.6
117: honey coloured liquid	Vietnam	47.2 (44.6 – 50.5) 2.5

These samples may contain some honey but their moisture content is outside a normal honey moisture content range and therefore they will not be included in calculations of the average Asian honey composition.

3.3.2 Carbohydrate profile

The carbohydrate profile of triplicate samples of the Asian honeys was studied by GC-FID. Typical chromatograms of each sample as well as peak areas and calculated results for each replicate are presented in Appendix B on the supplementary data disc. A typical chromatogram of the disaccharide fraction wih peaks assigned is presented in Figure 8, and a chromatogram of the trisaccharide fraction in Figure 9.

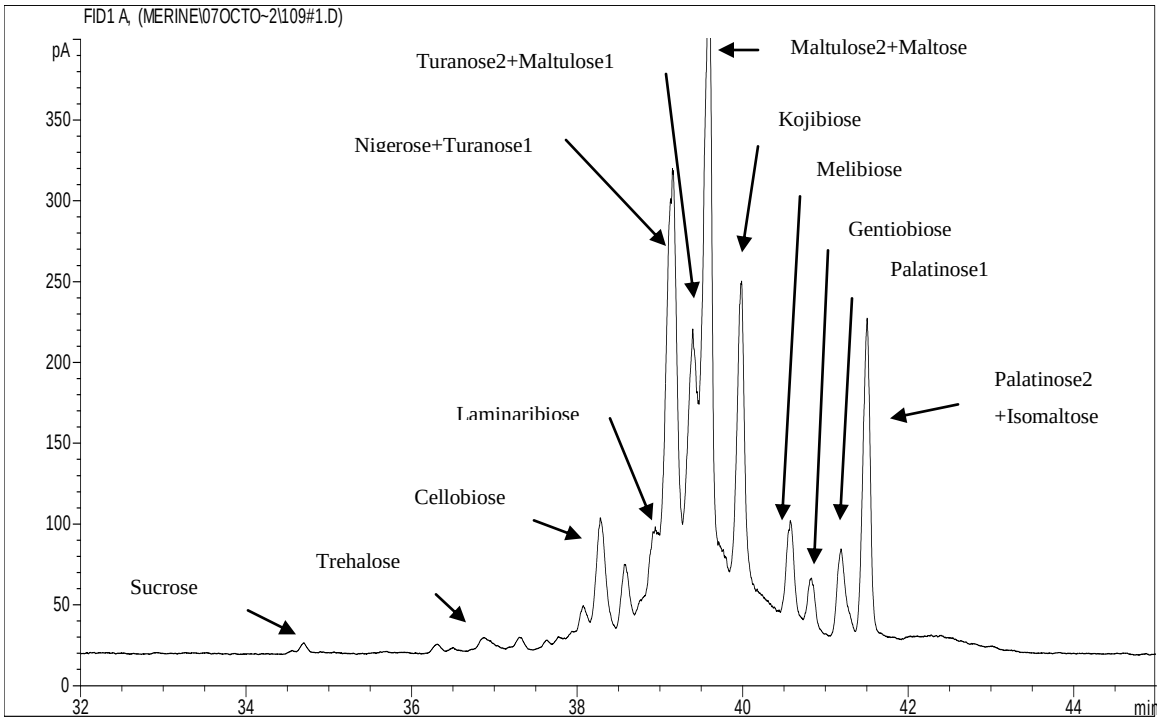


Figure 8: Typical chromatogram of the disaccharide fraction.

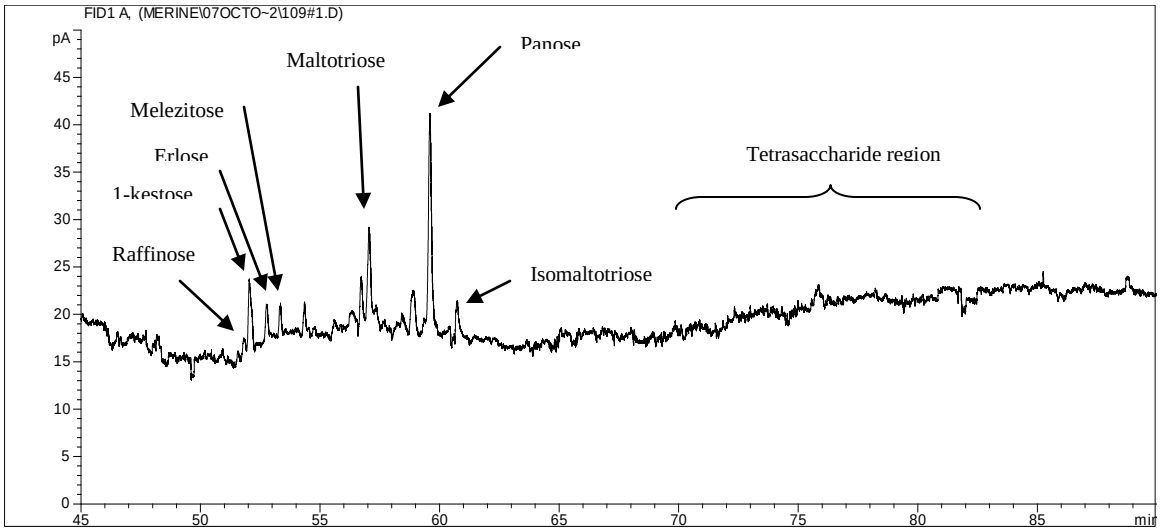


Figure 9: Typical chromatogram of the trisaccharide fraction.

A number of peaks could be seen in the disaccharide fraction of all samples that could not be identified from lack of standard.

3.3.2.1 Standard Curves and Response Factors (RF)

The carbohydrates of honey were quantified by comparison with standard curves (see Figure 10, Figure 11 and Figure 12) of standard sugars.

Equation 2, Equation 3 and Equation 4 (Section 2.3.5) show the calculations involved in the quantitation of the mono- and oligosaccharides. The area ratio of standard to internal standard was plotted against the weight ratio of standard to internal standard, and three values of each standard were fitted by a linear trendline which passed through zero. The slope of the fitted linear trendline being the RF is then used to calculate the amount of oligosaccharide in the samples. Raw data for standard sugar analyses are presented in Appendix B on the supplementary data disc.

When impurities were detected in the standard, the weight of the standard sugar was corrected by calculating the area ratio of the standard and the impurities as shown in Equation 6.

Equation 6: Calculation of purity of a standard sugar.

$$P = \frac{A(std)}{A(t)}$$

Where:

P is the level of purity of the standard sugar

A(s) is the area of the main peak(s) of the standard sugar

A(t) is the total peaks area excluding the peak area of xylitol.

The weight of the standard sugar was corrected by the purity of the standard to yield the net weight of standard sugar as shown in Equation 7.

Equation 7: Calculation of the net weight of standard sugar.

$$W(c) = P \times W(w)$$

Where:

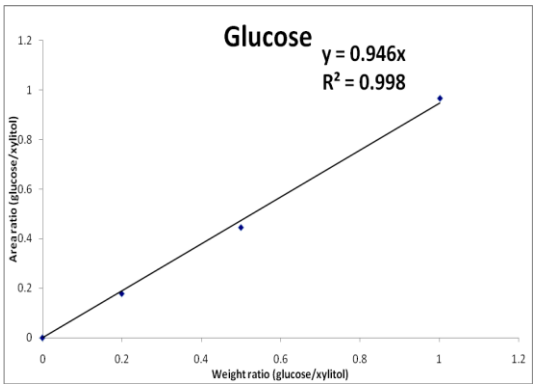
W(c) is the corrected weight of the standard (mg)

P is the level of purity of the standard sugar

W(w) is the weight of standard sugar from the balance weight (mg)

All calculated RF values are summarised in Table 26.

Since reduction of glucose and fructose gives undistinguishable products (see Section 1.5.4.2.2), monosaccharides were quantified together in the honey samples, and their average RF of 0.97 was used.



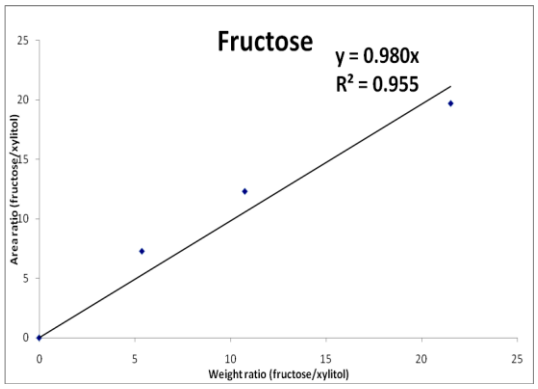
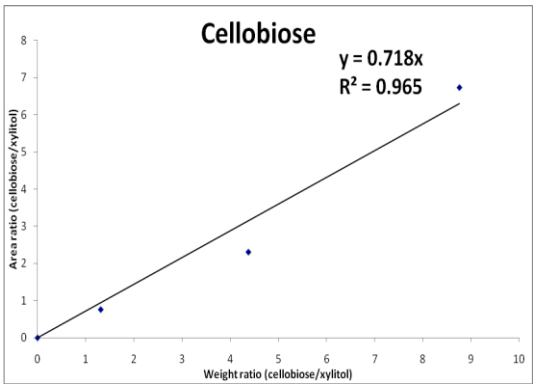


Figure 10: Standard Curves and RF of monosaccharides.



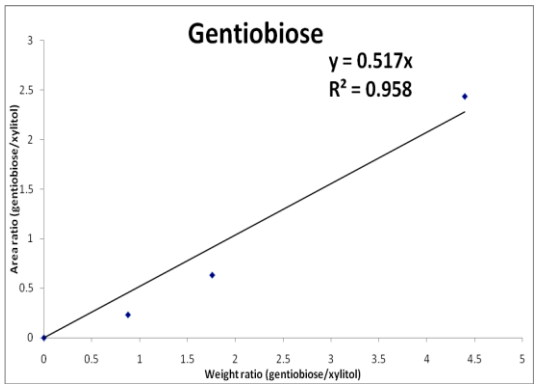


Figure 11: Standard Curves and RF of disaccharides.

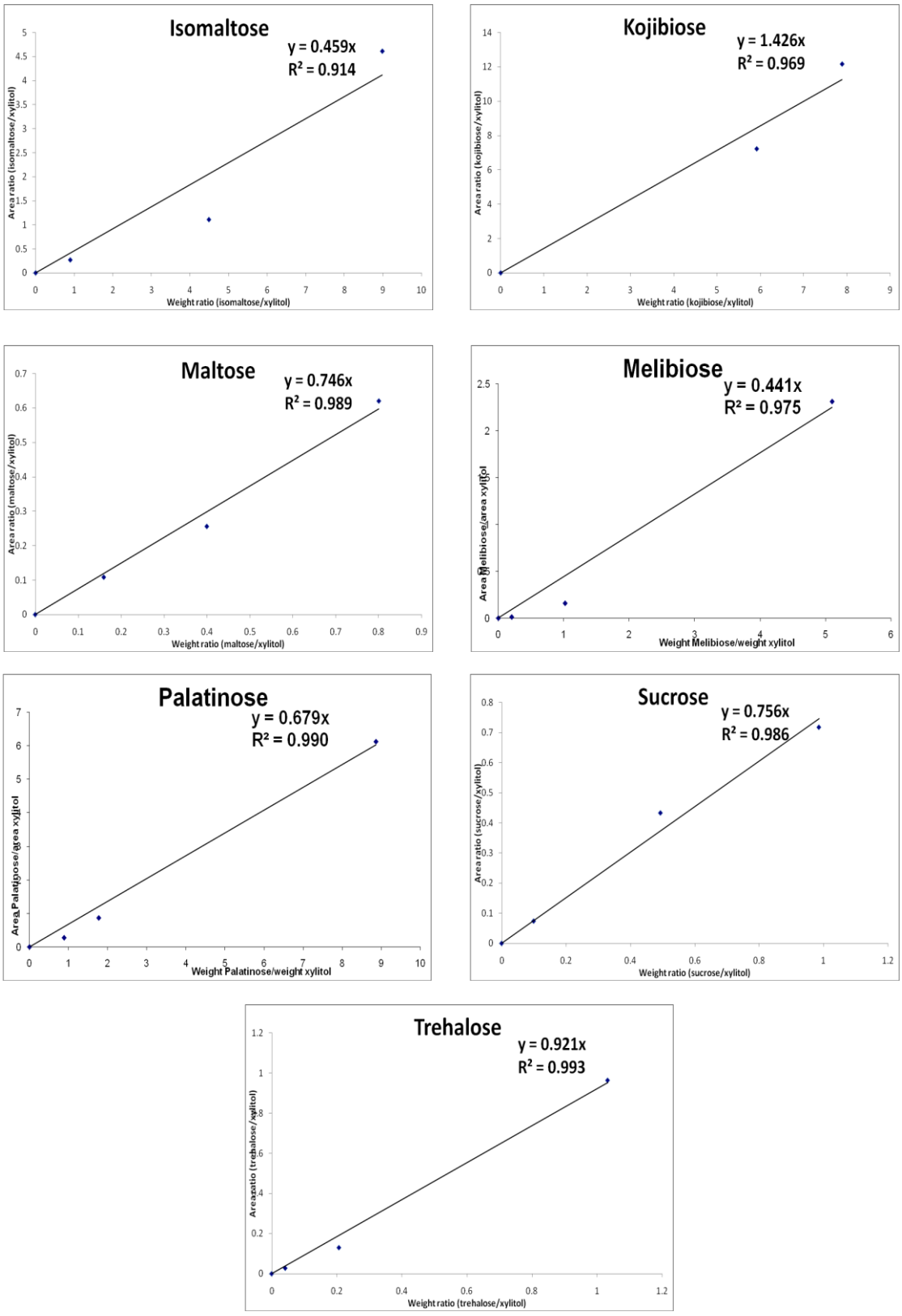


Figure 11: Standard Curves and RF of disaccharides. (continued)

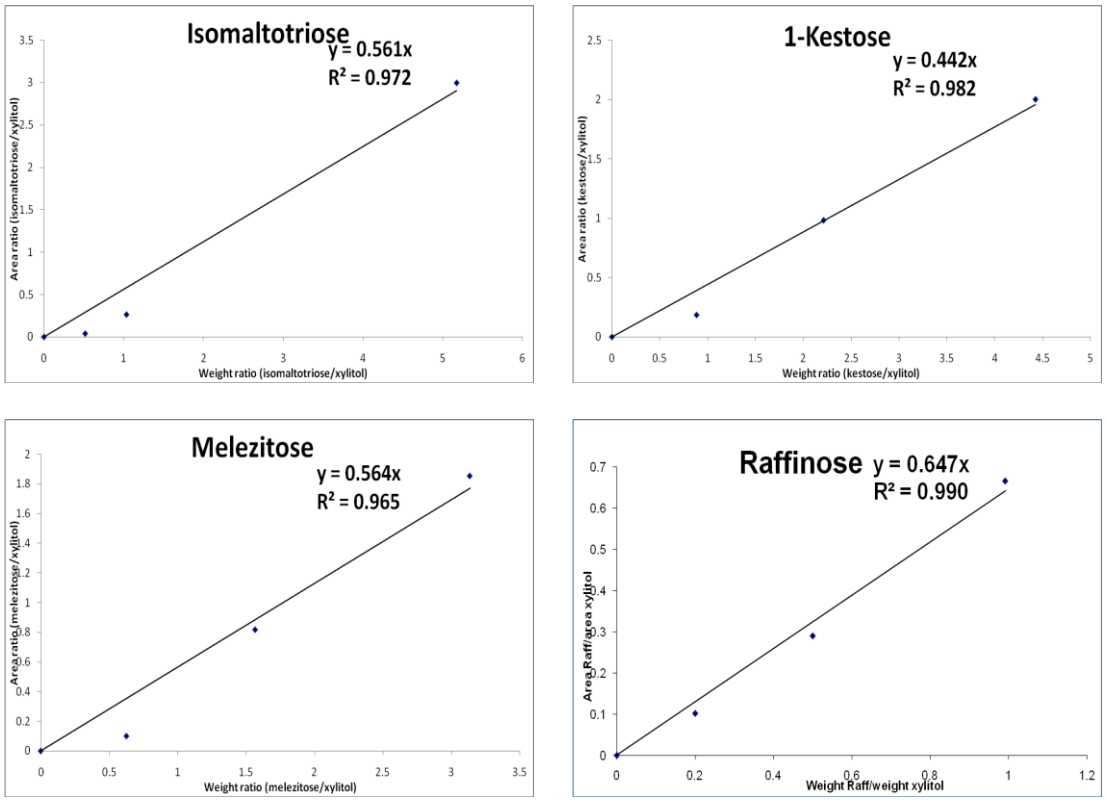


Figure 12: Standard Curves and RF of trisaccharides.

Table 26: RF of standard sugars.

Disaccharides	RF	Trisaccharides	RF
Sucrose	0.76	Raffinose	0.65
α,α-Trehalose	0.92	1-kestose	0.44
Cellobiose	0.72	Melezitose	0.56
Kojibiose	1.43	Isomaltotriose	0.56
Melibiose	0.44		
Gentiobiose	0.52		
Palatinose	0.68		
Isomaltose	0.46		
Mean	0.80	Mean	0.55
Std. Dev.	0.32	Std. Dev.	0.09
Range	0.44 - 1.43	Range	0.44 - 0.65

The peaks for nigerose, turanose, maltulose and maltose coincided in the chromatograms (Section 1.5.4.2.2), the mean RF for disaccharides of 0.80 was used to quantitate these peaks.
The peaks for isomaltose and the glucitol-substituted reduction product of palatinose coincided in the chromatograms. To distinguish whether the peak only accounted for the presence of palatinose or isomaltose/palatinose, the ratio of the mannitol-substituted palatinose reduction peak area and the glucitol-substituted palatinose reduction peak area was used. This ratio was found to be 0.505 and therefore the contribution of the glucitol reduction product of palatinose in the peak was substracted. If the peak area became less than zero, it was concluded that the peak only arose from palatinose and no isomaltose was present.

A standard for laminaribiose was not available and therefore the mean RF for disaccharides was used to quantitate this sugar in the chromatograms. Identification of the peak belonging to this sugar was achieved by comparison of the elution order in the work carried out by Jundong Wu (2000) who also used GC-FID. [47]
Similarly, standards for panose, maltotriose and erlose were not available and therefore the mean RF for trisaccharides was used to quantitate these sugars, along with identification of the peaks by comparison with elution orders from Jundong Wu (2000). [47]

3.3.2.2 Honeys from China

Several samples from China were found to be adulterated and were not included in the calculations of the average Asian honey composition.
Figure 13 shows the chromatogram of a typical Chinese honey compared to a Chinese honey adulterated with sucrose in Figure 14.

The average Chinese honey composition based on samples 19, 64, 76, 77, 107, 109 and 115 is presented in Table 27.

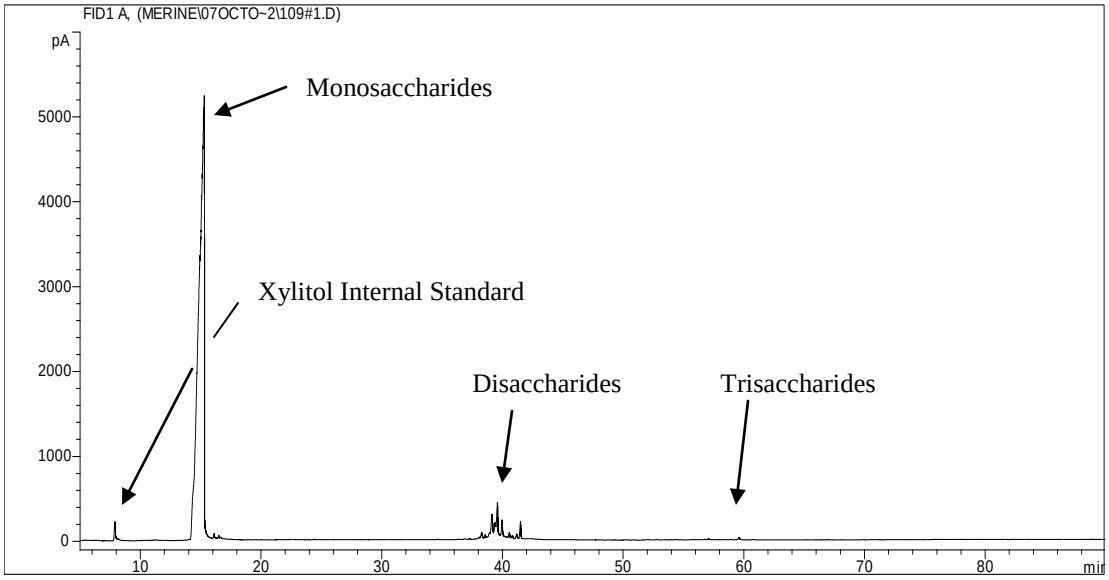


Figure 13: GC chromatogram of a typical Chinese honey.

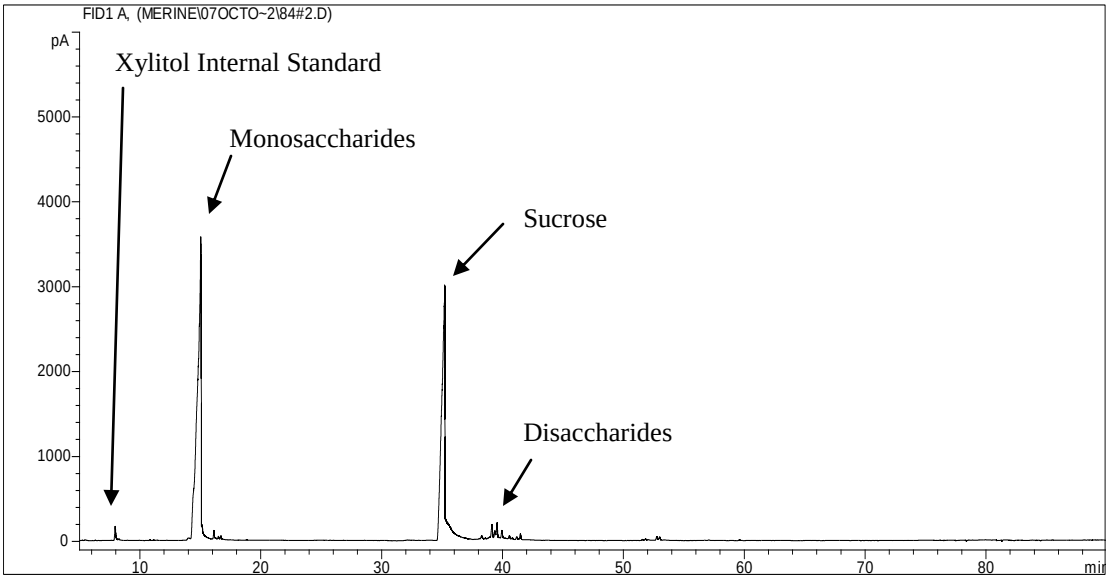


Figure 14: GC chromatogram of an adulterated Chinese honey.

The results shown in Table 27 show that the honeys analysed are in accordance (within experimental uncertainty) with the Codex Alimentarius Standard for Honey (2001) [126] which states that the fructose and glucose content must not be less that 60g/100g and the sucrose content no more than 5g/100g.

The results also demonstrate that some di- and trisaccharides were not detected but the marker sugars are present and that the di- and trisaccharide composition is similar to that of honeys reported in the literature, [3, 9, 34, 78].

Table 27: Average sugar composition of Chinese honeys.

Sample ID:	107	109[a]	64	76**	77	115	80	19*	Mean	Std. Dev.	Range
	% w/w (mean of three determinations except *,**)										
Monosaccharides	75.28**	64.45	75.37	68.74	69.13**	54.06	57.43	60.06	**65.56**	**7.96**	**54.06 - 81.33**
Sucrose	0.21	0.02	0.22	0.32	0.46	0.30	0.96	3.13	**0.70**	**1.02**	**0.02 - 0.96**
Trehalose	0.05	0.09	0.10	0.09	0.10	0.16	0.03	0.14	**0.10**	**0.04**	**0.05 - 0.16**
Cellobiose	0.42	0.45	0.26	0.42	0.38	0.19	0.15	0.35	**0.33**	**0.11**	**0.19 - 0.45**
Laminaribiose	0.41	0.33	0.47	0.33	0.43	0.23	0.21	0.12	**0.32**	**0.12**	**0.23 - 0.47**
Nigerose +Turanose1	1.18	1.59	0.90	1.19	1.43	0.76	0.50	0.70	**1.03**	**0.38**	**0.90 - 1.59**
Turanose2+Maltulose1	0.70	0.94	0.56	0.62	0.80	0.64	0.22	0.33	**0.60**	**0.23**	**0.56 - 0.94**
Maltulose2+Maltose	1.54	1.70	0.80	1.51	1.93	0.88	1.20	0.87	**1.31**	**0.43**	**0.80 - 1.93**
Kojibiose	0.36	0.47	0.24	0.41	0.36	0.14	0.14	0.08	**0.28**	**0.14**	**0.14 - 0.47**
Melibiose	0.34	0.53	0.35	0.50	0.51	0.20	0.18	0.15	**0.35**	**0.16**	**0.20 - 0.53**
Gentiobiose	0.15	0.26	0.27	0.24	0.26	0.03**	0.07	0.10	**0.17**	**0.10**	**0.03 - 0.27**
Palatinose	0.45	0.80	0.52	0.73	0.68	0.23	0.28	0.15	**0.48**	**0.24**	**0.23 - 0.80**
Isomaltose	ND	0.37	ND	ND	ND	ND	ND	ND	**0.37**	-	-
Raffinose	ND	0.01	0.03	0.00	0.01	0.01	0.01	0.10	**0.03**	**0.03**	**0.00 - 0.05**
1-Kestose	0.05	0.05	0.34	0.11	0.15	0.08	0.14	0.03	**0.12**	**0.10**	**0.05 - 0.34**
Erlose	0.31	0.02	0.08	0.57	0.65	0.94	0.49	ND	**0.44**	**0.33**	**0.02 - 0.94**
Melezitose	0.03	0.02	0.01	0.07	0.05	0.09	0.03	0.01	**0.04**	**0.03**	**0.01 - 0.09**
Maltotriose	0.09	0.08	0.04	0.12	0.14	0.10	0.12	ND	**0.10**	**0.03**	**0.04 - 0.14**
Panose	0.07	0.14	0.04	0.10	0.08	0.02	0.04	ND	**0.07**	**0.04**	**0.02 - 0.14**
Isomaltotriose	ND	0.02	0.01*	0.01	0.01*	0.00*	0.00	ND	**0.01**	**0.01**	**0.00 - 0.02**

[a] 109 reproducibility 9 replicates
ND = not detected
* One replicate
** Two replicates

3.3.2.2.1 Adulterated Chinese Honey Samples.

Eight out of the 14 Chinese honey samples were found to be adulterated, mostly by SS and also glucose syrup.

Samples adulterated with SS showed a large sucrose peak in the GC chromatograms relative to the monosaccharides. The quantitation of the disaccharides in these samples was sometimes rendered difficult due to the presence of the large sucrose peak which made the baseline rise significantly, sometimes obstructing some of the smaller disaccharide peaks as can be seen in Figure 15. Because of this, not all of the disaccharides in these samples were quantified. The results are summarised in Table 28.

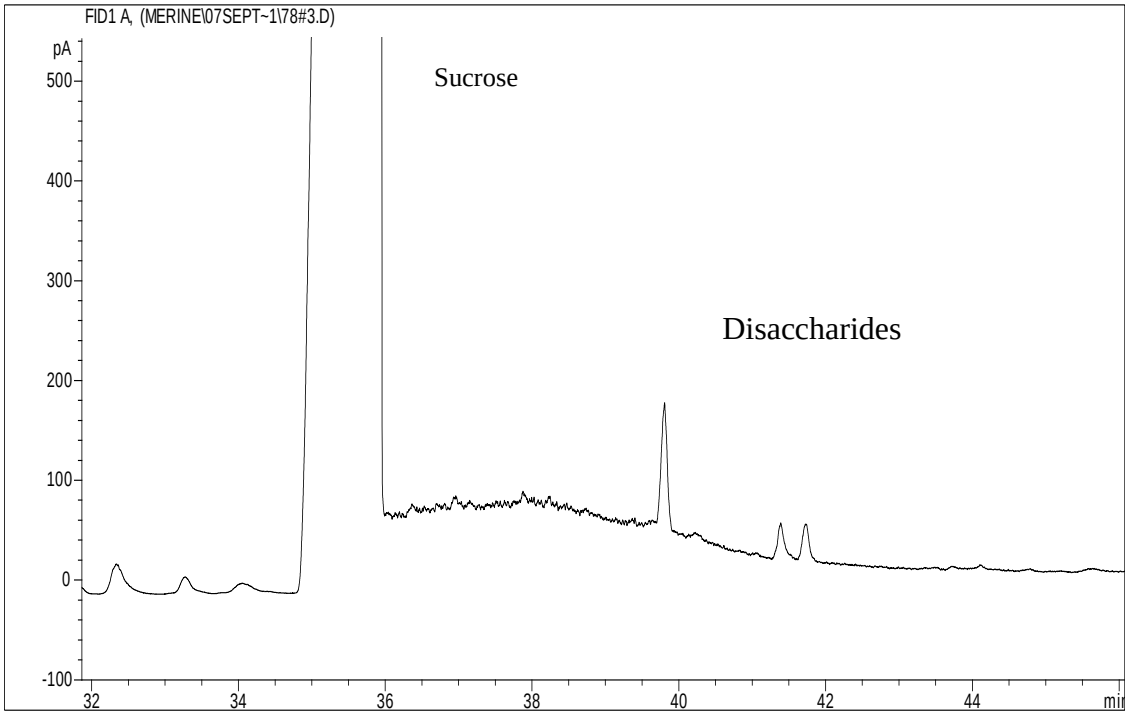


Figure 15: Small disaccharide peaks obscured by sucrose in a honey adulterated with SS.

Table 28: Composition of the adulterated Chinese honeys.

Sample:	84**	98	99*	78**	20	21
	% w/w (mean of 3 determinations, except *,**)					
Monosaccharides	36.82	26.40	34.41	1.07	50.02	41.46
Sucrose	33.14	26.58	41.91	30.78	22.98	30.06
Trehalose	ND	0.17	ND	ND	0.19	0.24
Cellobiose	0.30	0.41	0.64	ND	0.24	0.27
Laminaribiose	0.16	0.18	0.28	ND	0.09	0.26
Nigerose +Turanose1	0.76	0.73	1.11	ND	0.21	0.31
Turanose2+Maltulose1	0.44	0.42	0.54	ND	0.18	0.20
Maltulose2+Maltose	0.77	0.68	0.95	0.21	0.36	0.41
Kojibiose	0.31	0.26	0.33	ND	0.09	0.08
Melibiose	0.50	0.51	0.70	ND	0.18	0.17
Gentiobiose	0.25	0.29	0.38	ND	0.15	0.16
Palatinose	0.62	0.85	1.02	0.15	0.15	0.19
Isomaltose	ND	ND	ND	ND	ND	ND
Raffinose	0.10	0.06	0.10	0.00	0.10	0.13
1-Kestose	0.08	0.05	0.10	0.01	0.08	0.11
Erlose	0.26	0.27	0.44	0.02	0.15	0.79
Melezitose	0.04	0.06	0.18	0.00	0.02	0.03
Maltotriose	0.07	0.06	0.06	0.01	0.01	0.01
Panose	0.09	0.09	0.11	0.02	0.01	0.00
Isomaltotriose	ND	0.01	0.02	0.00	ND	ND

ND = not detected
* One replicate
** Two replicates

Most of these adulterated honey samples have been adulterated with SS and since the amount of sucrose present in the honey (approximately 30% in all the adulterated honeys) is similar to the amount of monosaccharides present, this suggests these honeys were not adulterated by addition of the syrup but by feeding the bees with large amounts of SS. The bees transformed some of the sucrose to glucose and fructose by enzymatic hydrolysis but sucrose also accumulated in the honey. If the honey had been adulterated by addition of the syrup, a relative reduction of all the peaks across the carbohydrate profile including monosaccharides would have occurred. (Section 1.6.2.2)

3.3.2.3 Honeys from India.

Seven honey samples from India were analysed. Their average sugar composition is summarised in Table 29.
None of the honey samples from India were found to be adulterated.

The results shown in Table 29 show that the honeys analysed are in accordance (within experimental uncertainty) with the Codex Alimentarius Standard for Honey (2001). [126]
The results show that some di- and trisaccharides were not detected but the marker sugars are present and that the di- and trisaccharide composition is similar to that of honeys reported in the literature, [3, 9, 34, 78].

Table 29: Average carbohydrate composition of honeys from India.

Sample ID	13	48**	49	75	79**	81**	119	Mean	Std Dev	Range
	% w/w (mean of 3 determinations, except *,**)									
Monosaccharides	61.80	61.23	62.98	74.78	74.52	73.41	76.73	**69.35**	**6.96**	**61.23 - 76.73**
Sucrose	0.06	0.02	0.11	0.20	0.17	0.02	0.02	**0.09**	**0.07**	**0.02 - 0.20**
Trehalose	0.22	0.07	0.05	0.15	0.19	0.09	0.03	**0.12**	**0.07**	**0.03 - 0.22**
Cellobiose	0.56	0.51	0.35	0.57	0.46	0.48	0.43	**0.48**	**0.08**	**0.35 - 0.57**
Laminaribiose	0.31	0.20	0.26	0.39	0.17	0.34	0.23	**0.27**	**0.08**	**0.17 - 0.39**
Nigerose +Turanose1	1.49	1.86	1.09	1.39	0.90	1.79	1.35	**1.41**	**0.35**	**1.09 - 1.86**
Turanose2+Maltulose1	0.72	0.98	0.54	0.86	0.54	1.04	0.75	**0.77**	**0.20**	**0.54 - 1.04**
Maltulose2+Maltose	1.56	1.99	1.21	1.42	1.15	1.70	1.79	**1.55**	**0.31**	**1.15 - 1.99**
Kojibiose	0.61	0.71	0.42	0.45	0.08	0.56	0.52	**0.48**	**0.20**	**0.08 - 0.71**
Melibiose	0.93	1.00	0.59	0.51	0.17	0.84	0.87	**0.70**	**0.29**	**0.17 - 1.00**
Gentiobiose	0.46	0.29	0.20	0.27	0.12	0.26	0.33	**0.27**	**0.10**	**0.12 - 0.46**
Palatinose	1.53	1.83	1.02	0.63	0.12	1.28	1.42	**1.12**	**0.58**	**0.63 - 1.63**
Isomaltose	ND	ND	ND	ND	ND	ND	ND	**-**	**-**	**-**
Raffinose	ND	0.01	0.02	ND	ND	ND	ND	**0.02**	**0.00**	**0.01 - 0.02**
1-Kestose	0.02	0.10	0.09	0.04*	ND	0.26	0.07	**0.10**	**0.08**	**0.04 - 0.26**
Erlose	0.02	0.07	0.26	0.21	ND	0.10	0.08	**0.12**	**0.09**	**0.02 - 0.26**
Melezitose	0.02	0.04	0.03	0.03**	ND	0.02*	0.02	**0.03**	**0.01**	**0.02 - 0.04**
Maltotriose	0.13	0.18	0.17	0.10**	ND	0.08	0.12	**0.13**	**0.04**	**0.08 - 0.18**
Panose	0.20	0.30	0.14	0.10**	ND	0.12	0.13	**0.17**	**0.07**	**0.12 - 0.30**
Isomaltotriose	0.03	0.05	0.03	ND	ND	0.02*	0.02*	**0.03**	**0.01**	**0.02 - 0.05**

ND = not detected
* One replicate
** Two replicates

3.3.2.4 Honeys from Vietnam.

Five honey samples from Vietnam were analysed using GC. However, two of these samples had moisture contents outside a normal range for honey and therefore were not included in any calculations.

The results are summarised in Table 30.

Table 30: Average carbohydrate composition of honeys from Vietnam.

Sugar	89	116	118*	Mean	Std Dev	Range
	% w/w (mean of 3 determinations, except *,**)					
Monosaccharides	61.21	60.51	64.62	**62.11**	**2.20**	**60.51 - 64.62**
Sucrose	0.02	0.01	0.28	**0.10**	**0.15**	**0.01 - 0.28**
Trehalose	0.06	0.06	0.07	**0.06**	**0.00**	**0.06 - 0.07**
Cellobiose	0.46	0.51	0.35	**0.44**	**0.08**	**0.35 - 0.51**
Laminaribiose	0.19	0.23	0.13	**0.19**	**0.05**	**0.13 - 0.23**
Nigerose +Turanose1	1.34	1.71	0.69	**1.24**	**0.51**	**0.69 - 1.71**
Turanose2+Maltulose1	0.73	0.78	0.06	**0.52**	**0.40**	**0.06 - 0.78**
Maltulose2+Maltose	1.49	1.50	0.05	**1.01**	**0.83**	**0.05 - 1.50**
Kojibiose	0.54	0.60	0.05	**0.40**	**0.30**	**0.05 - 0.60**
Melibiose	0.51	0.74	0.10	**0.45**	**0.32**	**0.10 - 0.74**
Gentiobiose	0.28	0.33	0.05	**0.22**	**0.15**	**0.05 -0.33**
Palatinose	0.75	1.21	0.09	**0.68**	**0.56**	**0.09 - 1.21**
Isomaltose	0.13**	0.17	ND	**0.15**	**0.03**	**0.13 - 0.17**
Raffinose	0.02**	ND	ND	**0.02**	-	-
1-Kestose	0.04	0.07	0.01	**0.04**	**0.03**	**0.01 - 0.07**
Erlose	0.01**	0.03	0.01	**0.02**	**0.01**	**0.01 - 0.03**
Melezitose	0.01**	ND	ND	**0.01**	-	-
Maltotriose	0.11	0.10	0.02	**0.08**	**0.05**	**0.02 - 0.11**
Panose	0.10	0.14	ND	**0.12**	**0.03**	**0.10 - 0.14**
Isomaltotriose	0.01*	0.01*	ND	**0.01**	**0.00**	**0.01 - 0.01**

ND = not detected
* One replicate
** Two replicates

The results presented in Table 30 show that the honeys analysed are in accordance (within experimental uncertainty) with the Codex Alimentarius Standard for Honey (2001). [126]

Some di- and trisaccharides were not detected in Vietnamese honeys but the marker sugars are present and the di- and trisaccharide composition is similar to that of honeys reported in the literature, [3, 9, 34, 78].

3.3.2.5 Honeys from Japan.

Three samples of honey from Japan were analysed in the course of this research. None of these samples were adulterated, the results are summarised in Table 31.

Table 31: Carbohydrate composition of Japanese honeys.

Sugar	26*	82	93	Mean	Std. dev.	Range
	% w/w (mean of 3 determinations, except *,**)					
Monosaccharides	75.62	72.48	71.79	**73.30**	**2.041646**	**71.79 - 75.62**
Sucrose	0.22	0.17	0.14	**0.18**	**0.040102**	**0.14 - 0.22**
Trehalose	0.12	0.06	0.11	**0.09**	**0.031516**	**0.06 - 0.12**
Cellobiose	0.55	0.21	0.39	**0.38**	**0.169117**	**0.21 - 0.55**
Laminaribiose	0.50	0.38	0.13	**0.34**	**0.190269**	**0.13 - 0.50**
Nigerose +Turanose1	1.57	0.98	0.62	**1.05**	**0.479258**	**0.62 - 1.57**
Turanose2+Maltulose1	0.85	0.59	0.29	**0.58**	**0.279664**	**0.29 - 0.85**
Maltulose2+Maltose	1.82	1.53	1.36	**1.57**	**0.233713**	**1.36 - 1.82**
Kojibiose	0.30	0.22	0.07	**0.20**	**0.117591**	**0.07 - 0.30**
Melibiose	0.27	0.21	0.05	**0.18**	**0.118067**	**0.05 - 0.27**
Gentiobiose	0.13	0.06	0.03	**0.07**	**0.0508**	**0.03 - 0.13**
Palatinose	0.38	0.32	0.16	**0.29**	**0.1136**	**0.16 - 0.38**
Isomaltose	ND	ND	0.64	**0.64**	-	-
Raffinose	ND	ND	ND	-	-	-
1-Kestose	0.06	0.12	0.03	**0.07**	**0.041266**	**0.03 - 0.12**
Erlose	0.17	0.17	ND	**0.17**	**0.002438**	**0.17 - 0.17**
Melezitose	0.01	0.02**	0.01*	**0.01**	**0.003272**	**0.01 - 0.02**
Maltotriose	0.09	0.06	0.05	**0.07**	**0.023449**	**0.05 - 0.09**
Panose	0.06	0.04**	0.22	**0.11**	**0.096896**	**0.04 - 0.06**
Isomaltotriose	ND	ND	0.01	**0.01**	-	-

ND = not detected
* One replicate
** Two replicates

The results shown in Table 31 show that the honeys analysed are in accordance (within experimental uncertainty) with the Codex Alimentarius Standard for Honey (2001). [126]

The results also show that some di- and trisaccharides were not detected in Japanese honeys but the marker sugars are present and the di- and trisaccharide composition is similar to that of honeys reported in the literature, [3, 9, 34, 78].

3.3.2.6 Honeys from Malaysia.

Three samples of honey from Malaysia were studied. One of the samples showed signs of adulteration and was not included in the calculations.
The results for the non-adulterated samples of honey are presented in Table 32.

Table 32: Carbohydrate composition of honeys from Malaysia.

Sugar	83	90	Mean	Std. Dev.	Range
	% w/w (mean of 3 determinations, except *,**)				
Monosaccharides	65.88	67.96	**66.92**	**1.47**	**65.88 - 67.92**
Sucrose	0.07	0.05	**0.06**	**0.01**	**0.05 - 0.07**
Trehalose	0.13	0.07	**0.10**	**0.04**	**0.07 - 0.13**
Cellobiose	0.53	0.42	**0.47**	**0.08**	**0.42 - 0.53**
Laminaribiose	0.47	0.73	**0.60**	**0.19**	**0.47 - 0.73**
Nigerose +Turanose1	0.48	0.56	**0.52**	**0.06**	**0.48 - 0.56**
Turanose2+Maltulose1	0.29	0.27	**0.28**	**0.02**	**0.27 - 0.29**
Maltulose2+Maltose	1.02	1.60	**1.31**	**0.41**	**1.02 - 1.60**
Kojibiose	0.22	0.20	**0.21**	**0.02**	**0.20 - 0.22**
Melibiose	0.36	0.30	**0.33**	**0.04**	**0.30 -0.36**
Gentiobiose	0.27	0.28	**0.28**	**0.01**	**0.27 - 0.28**
Palatinose	0.54	0.48	**0.51**	**0.04**	**0.48 - 0.54**
Isomaltose	0.77	0.95	**0.86**	**0.13**	**0.77 - 0.95**
Raffinose	ND	ND	-	-	-
1-Kestose	0.02**	0.02*	**0.02**	**0.00**	**0.02**
Erlose	0.02	0.02	**0.02**	**0.00**	**0.02**
Melezitose	0.01**	0.02*	**0.02**	**0.00**	**0.01 - 0.02**
Maltotriose	0.11	0.13	**0.12**	**0.01**	**0.11 - 0.13**
Panose	0.15	0.18	**0.16**	**0.03**	**0.15 - 0.18**
Isomaltotriose	0.03	0.03	**0.03**	**0.00**	**0.03**

ND = not detected
* One replicate
** Two replicates

The results shown in Table 32 show that the honeys analysed are in accordance (within experimental uncertainty) with the Codex Alimentarius Standard for Honey (2001). [126] The results also show that some di- and trisaccharides were not detected in honeys from Malaysia but the marker sugars are present and that the di- and trisaccharide composition is similar to that of honeys reported in the literature, [3, 9, 34, 78].

The adulterated sample exhibited slightly high monosaccharide content (approximately 70%w/w), a large maltose peak (approximately 8%w/w of the sample), a large maltotriose peak (approximately 7%w/w) and the possible presence of a maltotetraose peak eluting at 83 minutes (see Figure 16).

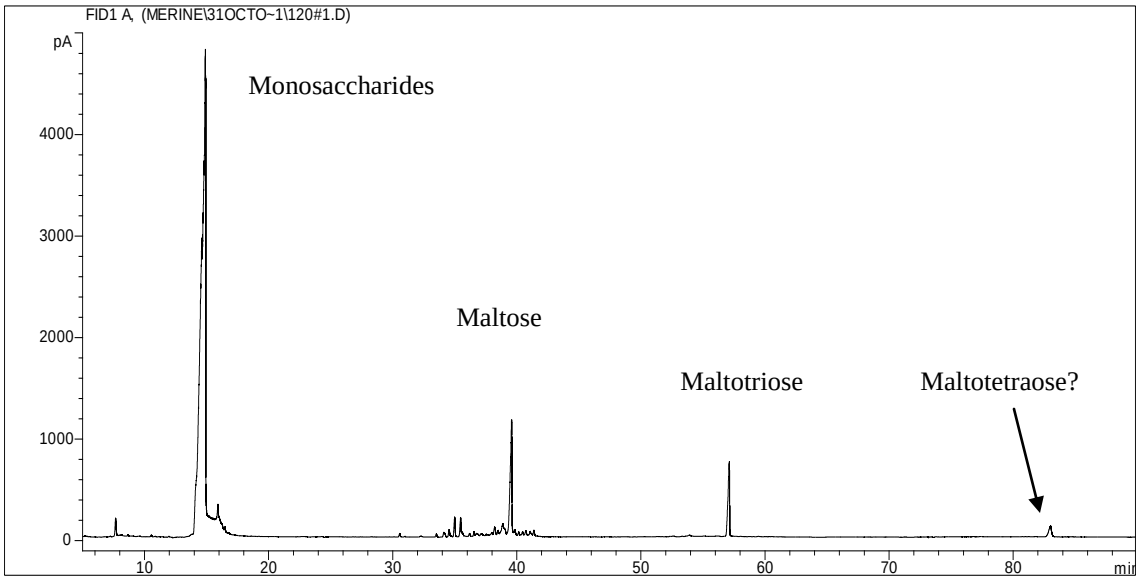


Figure 16: Adulterated honey from Malaysia.

These observations are similar to those made by Cotte et *al*. (2004) for adulteration with glucose syrup through improper bee-feeding. Sucrose fed to the bees is hydrolysed to fructose and glucose but after prolonged and intensive feeding, the bees start to store the glucose as the higher oligosaccharides maltose and maltotriose. (Section 1.6.2.3.)

3.3.2.7 Honeys from Indonesia.

Two honey samples from Indonesia were analysed using GC. Sample 24 was an adulterated sample, see Figure 17.

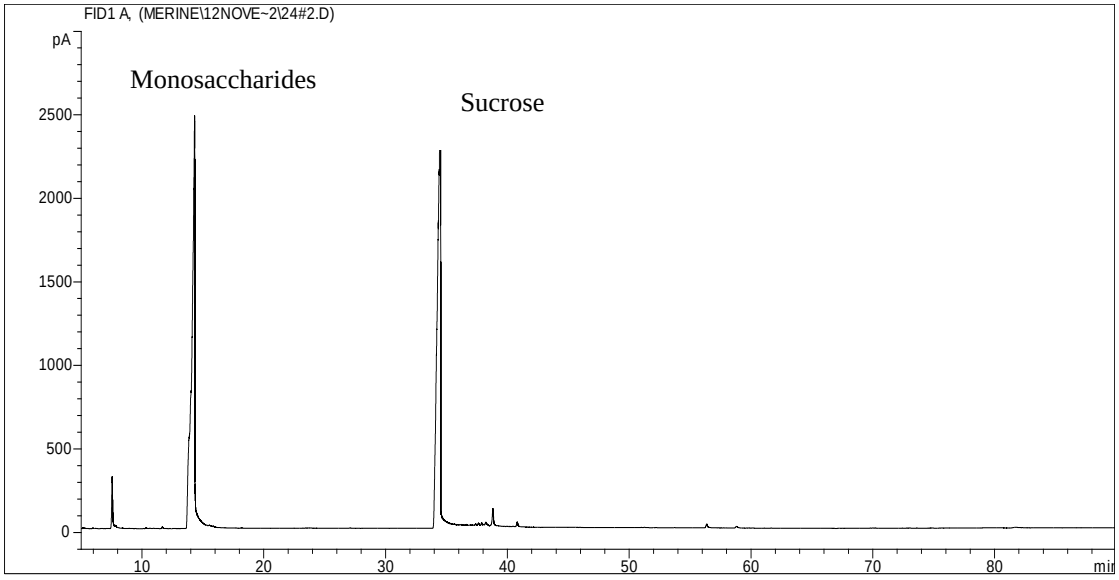


Figure 17: Adulterated honey from Indonesia.

The adulterated sample was unable to be fully quantified but its main components were monosaccharides (approximately 17%) and sucrose (approximately 27%). This suggests the adulteration might have occurred through feeding of the bees with SS.

The other sample (number 25) was quantified and the results are shown in Table 33.

Table 33: Carbohydrate composition of sample 25 from Indonesia.

Sugar	25
	% w/w (one replicate)
Monosaccharides	68.30
Sucrose	0.27
Trehalose	ND
Cellobiose	0.49
Laminaribiose	0.17
Nigerose +Turanose1	0.83
Turanose2+Maltulose1	0.14
Maltulose2+Maltose	1.04
Kojibiose	0.09
Melibiose	0.20
Gentiobiose	0.13
Palatinose	0.25
Isomaltose	ND
Raffinose	ND
1-Kestose	0.01
Erlose	0.01
Melezitose	ND
Maltotriose	0.04
Panose	0.01
Isomaltotriose	ND

ND = not detected

The results shown in Table 33 show that the honey analysed is in accordance (within experimental uncertainty) with the Codex Alimentarius Standard for Honey (2001). [126] The results also shows that some di- and trisaccharides were not detected in this honey from Indonesia but the marker sugars are present and that the di- and trisaccharide composition is similar to that of honeys reported in the literature, [3, 9, 34, 78].

3.3.2.8 Honey from the Philippines.

One sample of honey from the Philippines was analysed. At first this sample looked to be adulterated since its sucrose content was found to be slightly above the sucrose content allowed in the Codex Alimentarius Commission Standard for honey. However, only one replicate of this sample was analysed, due to sample preparation problems. Consequently, as a precaution, this sample was not used in the calculation of the average Asian honey composition.

3.3.2.9 Honeys from South Korea.

Two samples of honey from South Korea were analysed using GC (see results in Table 34), one was found to be adulterated (sample 68) and was not included here.

Table 34: Carbohydrate composition of honey from South Korea.

Sugar	113
	% w/w (mean of 3 replicates)
Monosaccharides	55.80
Sucrose	0.03
Trehalose	0.04
Cellobiose	0.49
Laminaribiose	0.11
Nigerose +Turanose1	1.76
Turanose2+Maltulose1	1.11
Maltulose2+Maltose	1.94
Kojibiose	0.66
Melibiose	1.16
Gentiobiose	0.46
Palatinose	1.47
Isomaltose	ND
Raffinose	ND
1-Kestose	0.19
Erlose	0.20
Melezitose	0.07
Maltotriose	0.16
Panose	0.29
Isomaltotriose	0.07

ND = not detected

The results shown in Table 34 show that the honey analysed is in accordance (within experimental uncertainty) with the Codex Alimentarius Standard for Honey (2001). [126] Some di- and trisaccharides were not detected in this honey from South Korea but the marker sugars are present. The di- and trisaccharide composition is similar to that of honeys reported in the literature, [3, 9, 34, 78].

3.3.2.10 Carbohydrate profile of Asian honeys.

From all the results presented above, an average carbohydrate composition for honey originating from Asia can be calculated, and is presented in Table 35.

This average composition agrees with the limits set for honey by the Codex Alimentarius Commission Standard for honey (2001). [126]
The Codex Alimentarius Commission Standard (2001) for honey states that honey must not contain less than 60g//100g fructose and glucose (honeydew honey or blends with nectar honey must not be less than 45g/100g).
The sucrose content must not be more than 5g/100g of honey; for Alfalfa (*Medicago sativa*), Citrus spp., False Acacia (*Robinia pseudoacacia*), French Honeysuckle (*Hedysarum*), Menzies Banksia (*Banksia menziesii*),Red Gum (*Eucalyptus camaldulensis*), Leatherwood (*Eucryphia lucida*), Eucryphia milligani; the sucrose content must not be more than 10g/100g of honey and for Lavender (*Lavandula spp*) and Borage (*Borago officinalis*) honeys it must not be more than 15g/100g.

Table 35: Average Asian honey carbohydrate composition.

Sugar	China (n = 8)	India (n = 7)	Vietnam (n = 3)	Japan (n = 3)	Malaysia (n = 2)	South Korea (n = 1)	Indonesia (n = 1)	Mean	Std Dev	Range
	% w/w									
Monosaccharides	65.56	69.35	62.11	73.30	66.92	54.91	68.30	**65.78**	**5.90**	**54.91 - 73.30**
Sucrose	0.70	0.09	0.10	0.18	0.06	0.03	0.27	**0.20**	**0.23**	**0.03 - 0.27**
Trehalose	0.10	0.12	0.06	0.09	0.10	0.04	ND	**0.08**	**0.03**	**0.04 - 0.12**
Cellobiose	0.33	0.48	0.44	0.38	0.47	0.47	0.49	**0.44**	**0.06**	**0.33 - 0.49**
Laminaribiose	0.32	0.27	0.19	0.34	0.60	0.11	0.17	**0.29**	**0.16**	**0.11 - 0.60**
Nigerose +Turanose1	1.03	1.41	1.24	1.05	0.52	1.69	0.83	**1.11**	**0.38**	**0.52 - 1.41**
Turanose2+Maltulose1	0.60	0.77	0.52	0.58	0.28	1.07	0.14	**0.57**	**0.31**	**0.14 - 1.07**
Maltulose2+Maltose	1.31	1.55	1.01	1.57	1.31	1.83	1.04	**1.37**	**0.30**	**1.01 - 1.83**
Kojibiose	0.28	0.48	0.40	0.20	0.21	0.63	0.09	**0.33**	**0.19**	**0.09 - 0.63**
Melibiose	0.35	0.70	0.45	0.18	0.33	1.12	0.20	**0.47**	**0.33**	**0.18 - 1.12**
Gentiobiose	0.17	0.27	0.22	0.07	0.28	0.44	0.13	**0.23**	**0.12**	**0.07 - 0.44**
Palatinose	0.48	1.12	0.68	0.29	0.51	1.06	0.25	**0.63**	**0.35**	**0.25 - 1.06**
Isomaltose	0.37	ND	0.15	0.56	0.77	ND	ND	**0.47**	**0.27**	**0.15 - 0.77**
Raffinose	0.03	0.02	0.02	ND	ND	ND	ND	**0.02**	**0.01**	**0.02 - 0.03**
1-Kestose	0.12	0.10	0.04	0.07	0.02	0.18	0.01	**0.08**	**0.06**	**0.01 - 0.18**
Erlose	0.44	0.12	0.02	0.17	0.02	0.20	0.01	**0.14**	**0.15**	**0.01 - 0.44**
Melezitose	0.04	0.03	0.01	0.01	0.02	0.06	ND	**0.03**	**0.02**	**0.01 - 0.06**
Maltotriose	0.10	0.13	0.08	0.07	0.12	0.15	0.04	**0.10**	**0.04**	**0.04 - 0.15**
Panose	0.07	0.17	0.12	0.11	0.16	0.28	0.01	**0.13**	**0.09**	**0.01 - 0.28**
Isomaltotriose	0.01	0.03	0.01	0.01	0.03	0.07	ND	**0.03**	**0.02**	**0.01 - 0.07**

ND = not detected

4 CONCLUSIONS

The Asian honeys analysed had a carbohydrate composition within the limits set for honey by the Codex Alimentarius Commission, and had a disaccharide profile similar to honeys from elsewhere in the world.

Kojibiose, turanose/nigerose and turanose/maltulose were present in all the Asian honeys analysed and are suitable markers for the presence of Asian honey in products because these disaccharides are not common in foods and therefore their presence indicates that the product contains honey.

The presence of adulterated honeys in the foodstuff or other sweeteners could cause problems with quantitation, especially for adulterations or sweeteners with SS since the small disaccharide peaks can be obstructed by the large sucrose peak.

Precision of the method was poor for measurements of the di- and trisaccharides (mostly due to difficulty in getting a homogeneous honey sample and quantifying the small or poorly resolved peaks); and quantitation would be better achieved with the monosaccharides, glucose:fructose ratio and possibly the ratios of disaccharides to monosaccharides. This was attempted in the early stages of this research during the analysis of products supplied by MAF to prove that they contained honey (see Appendix A in Section 7.1)

This method is suitable for detection of the presence of honey in a product being investigated but might encounter problems when quantitation of the honey present at low levels of honey addition is required.

5 RECOMMENDATIONS FOR FUTURE WORK

- The analysis of the carbohydrates of honey with an on-column GC-FID allowed better detection of the small disaccharides of honey but performance of the GC system was gradually impaired by the amount of material injected onto the column. It would be preferable to remove the monosaccharide fraction from the samples prior to GC analysis, hence concentrating the oligosaccharide fraction. This would allow for greater resolution over time and a better limit of detection and quantitation but make the method more complex.

- Analysis by HPAE-PAD has been used elsewhere to quantify the sugars of honey and this method of analysis might be applicable for the quantitation of honey in products under investigation for the presence and amount of honey.

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7 APPENDICES

7.1 APPENDIX A: Preliminary Report on the Analysis of MAF Samples Delivered on 13th November 2009.

This preliminary report describes the analysis and preliminary results for the analysis of the following samples:

- Nin Jiom Pai Pa Koa cough syrup, batch #E080604HK2.
  Item description: Glass bottle (approximately 200mL) with silver label, new formulation which contains no honey according to manufacturer.
- Nin Jiom Pei Pa Koa cough syrup, batch #E090702HK1.
  Item description: Glass bottle (approximately 500mL) with red label and a price tag.
- Youn Yum pills, lot# LOT0904061.
  Item description: Black soft balls individually wrapped and contained in gold packaging.
- Nin Jiom Pei Pa Koa cough syrup, batch #E090305NZ1.
  Item description: Glass bottle (approximately 500mL) with red label and "Bao Ho" written in marker pen.
- Nin Jiom Pei Pa Koa cough syrup, batch #B090627HK3.
  Item description: Glass bottle (approximately 100mL) with red label and "Bao Ho" written in marker pen.

Preparation of samples and standards:

Xylitol was used as an internal standard. Raffinose was added as a recovery standard. All samples were reduced with $NaBH_4$ and trimethylsilylated for analysis by GC-MS.

Facts regarding honey drawn from the scientific literature (Note the highlighted points are important for the conclusions)

Composition:

- Water (approx. 17-19%)
- Ash, protein, lipid, organic, inorganic acids (traces)

- Complex mixture of carbohydrates (approx. 80%)

Complex mixture of carbohydrates is:

- Mostly glucose and fructose (85-95% of honey carbohydrates, with the **ratio of fructose to glucose being approximately 1.2**)
- **5% remaining carbohydrates are 15 disaccharides,** 11 trisaccharides and higher oligosaccharides (see Figure 1).

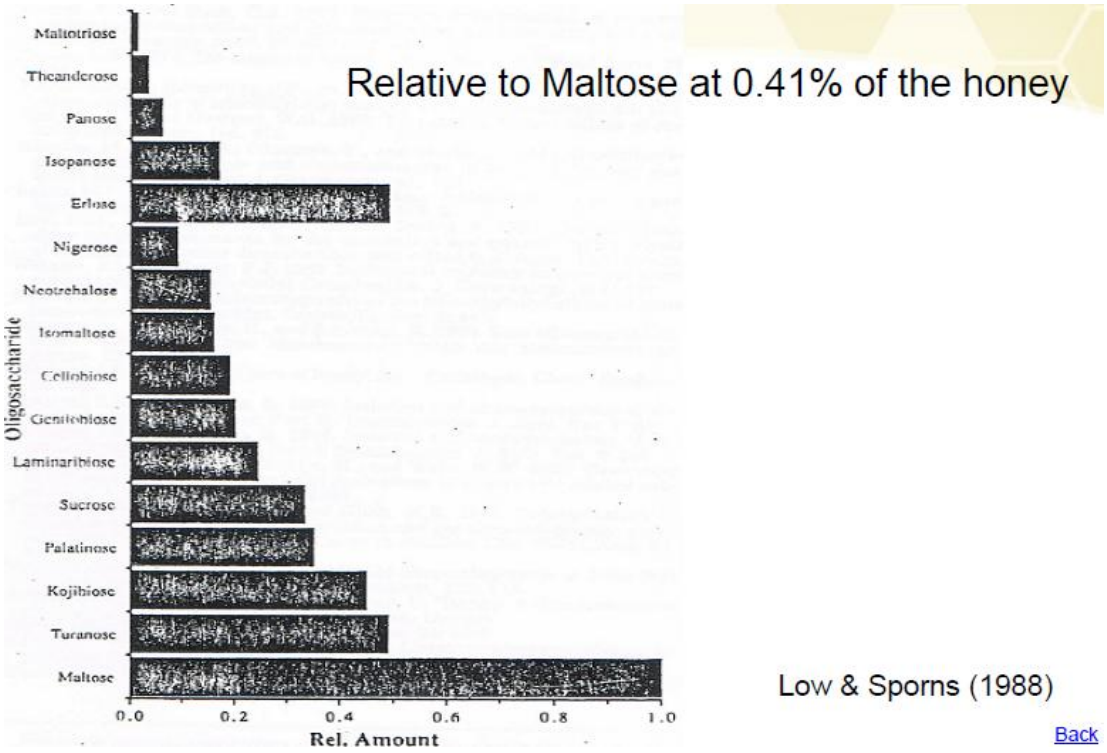

Figure 1: Disaccharides and Trisaccharides in honey (relative to maltose at 0.41% of the honey).

An average composition of honey from 490 samples (White et al., 1962) gave a fructose percentage in honey of 38.19% and for glucose of 31.28%.

1. Analysis of sample E080604HK2.

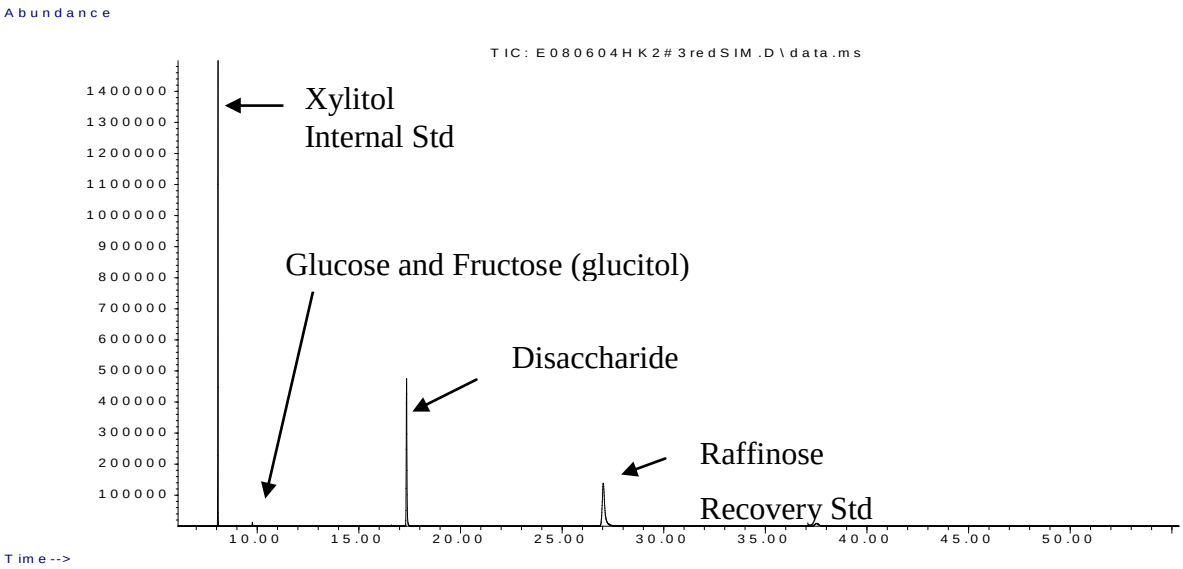

Figure 1: Ion Chromatogram (ions= 204,307, 361 and 451) of sample E080604HK2.

The amount of disaccharides compared to monosaccharides is totally unlike what occurs in honey. Therefore there is likely to be another disaccharide sweetener present.

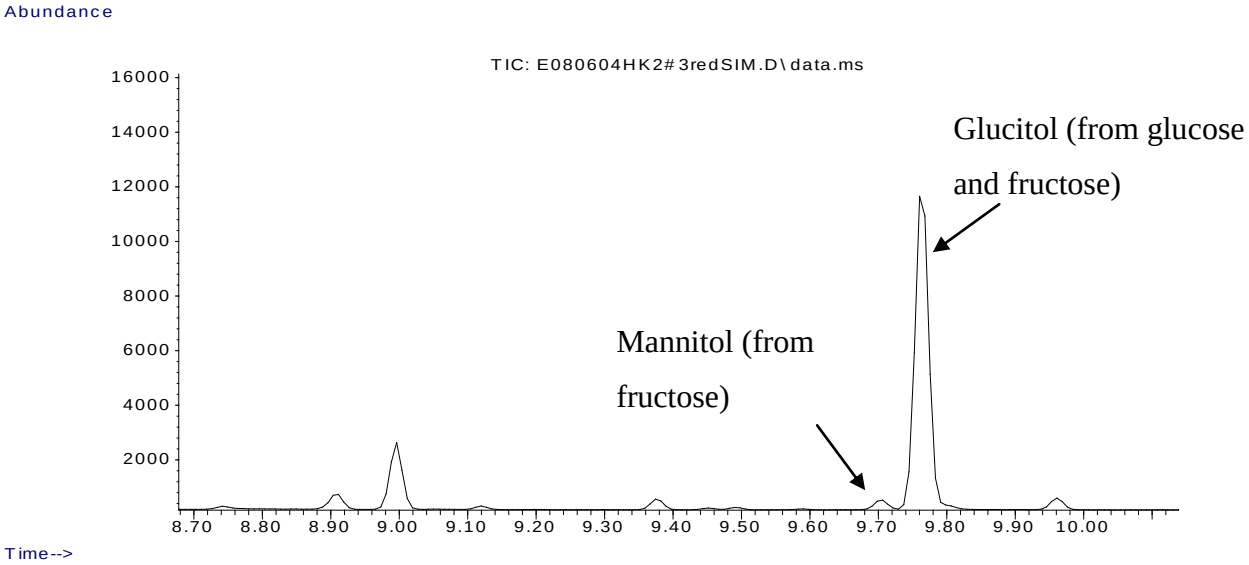

Figure 2: IC of sample E080604HK2 at the monosaccharide region.

Calculations of the ratio of fructose to glucose gave a ratio of 0.6 which is not consistent with a ratio found in honey. It would appear that there is more glucose present than occurs in honey.

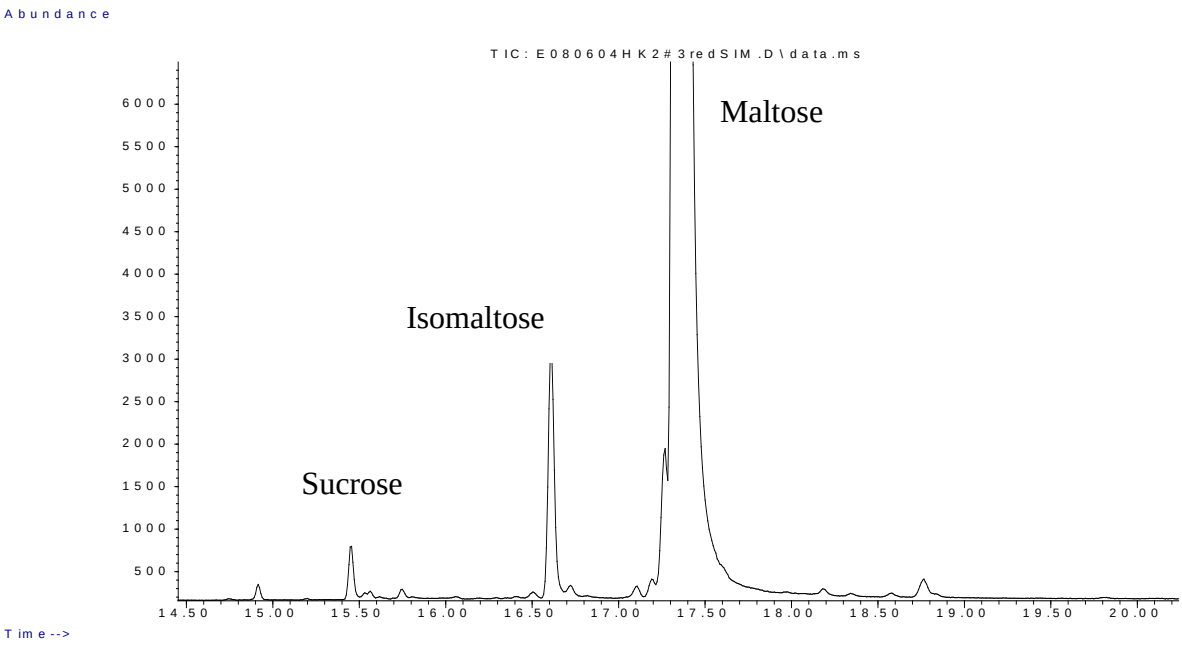

Figure 3: IC of sample E080604HK2 at the disaccharide region.

The chromatogram of the disaccharide region shows the presence of maltose as the dominant disaccharide, with traces of sucrose, and others. It is likely that the peak at about 16.6 is isomaltose which would occur in maltose syrups used as sweeteners. (These come from digestion of starch).

There are also small traces of other sugars that might come from honey. Based on the amount of fructose present (assuming that fructose is entirely from honey) the amount of honey present in the sample is ~1%.

2. Analysis of sample E090702HK1.

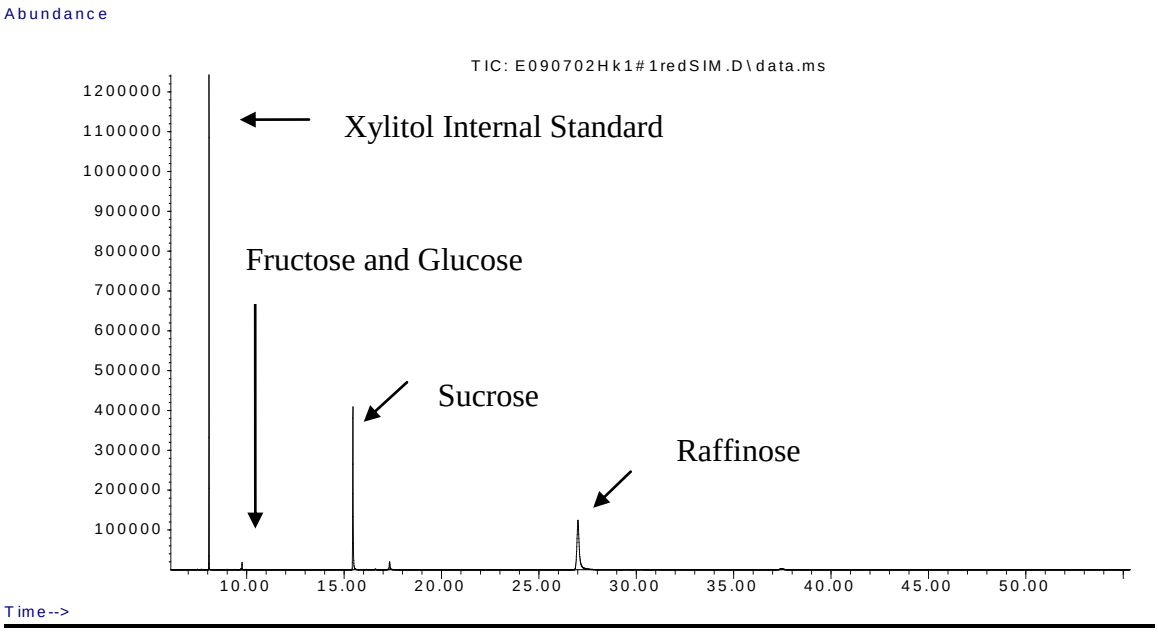

Figure 4: IC (ions = 204, 307, 361 and 451) of sample E090702HK1.

The amount of disaccharides compared to monosaccharides is totally unlike what occurs in honey. Therefore there is likely to be another disaccharide sweetener present.

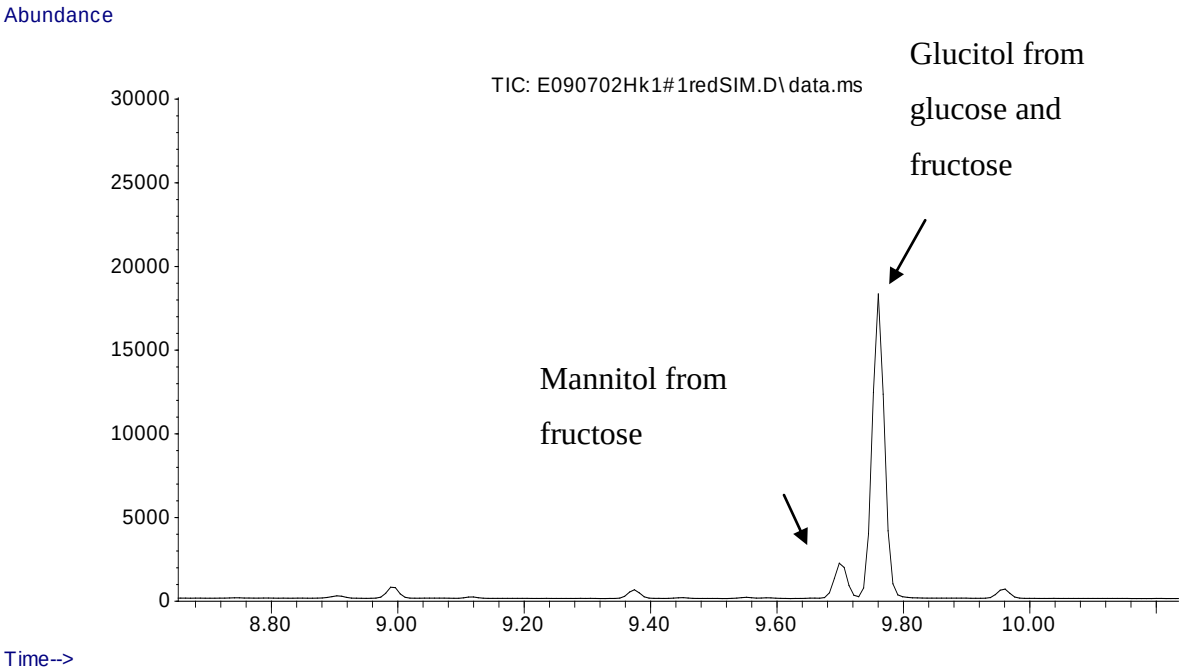

Figure 5: IC of sample E090702HK1 at the monosaccharide region.

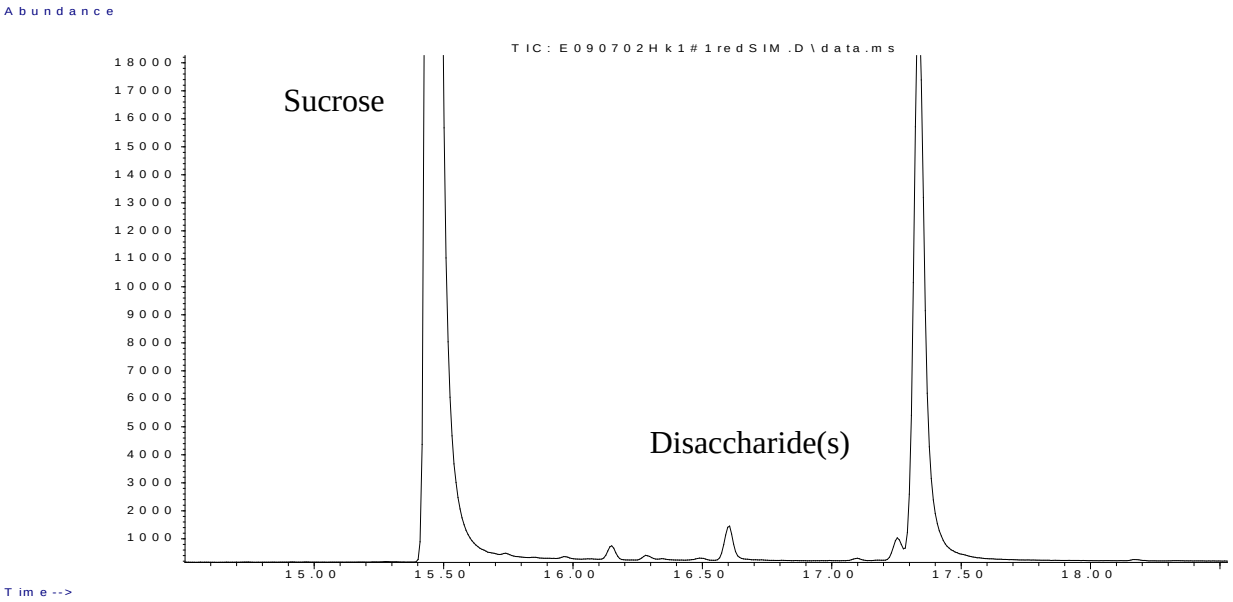

Figure 6: IC of sample E090702HK1 at the disaccharide region.

The analysis of sample E090702HK1 revealed the presence of the disaccharide sucrose at approximately 15.4 min and with traces of maltose at 17.3, and other disaccharides.

Calculations of the ratio of glucose to fructose gave a ratio of 0.8 which is not consistent with a ratio found in honey. Furthermore, the amount of sucrose is quite out of proportion. Maltose and possibly isomaltose are present but no other significant disaccharides and we conclude that this mixture has been sweetened using cane sugar, maltose syrup (trace) and glucose (trace).

3. Analysis of sample LOT0904061.

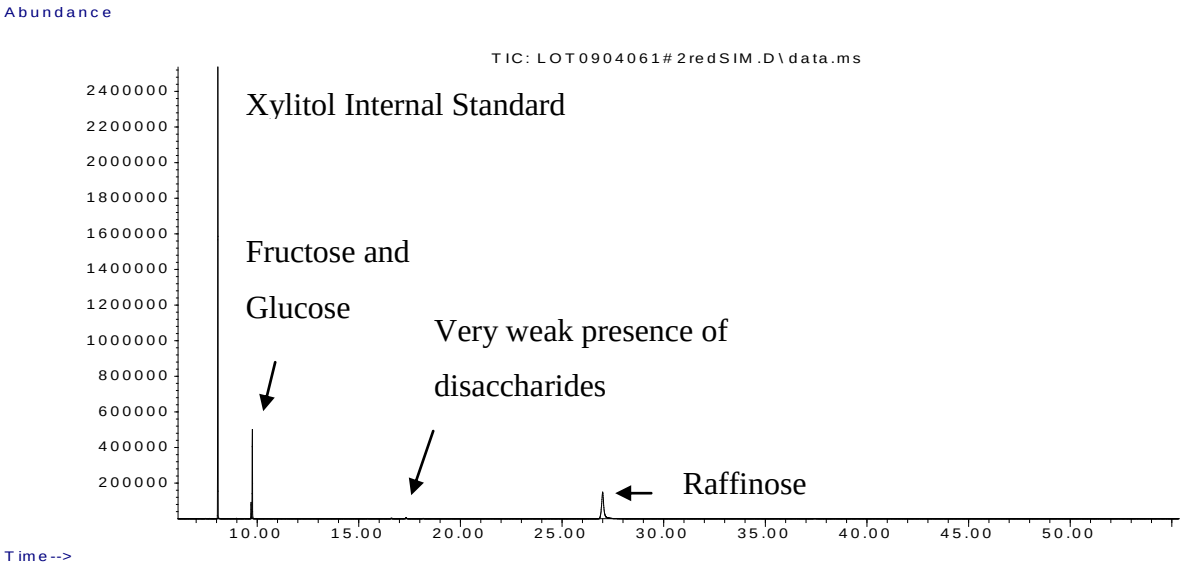

Figure 7: IC (ions = 204, 307, 361 and 451) of sample LOT0904061 .

The amount of disaccharides compared to monosaccharides is in this case more consistent with what occurs in honey.

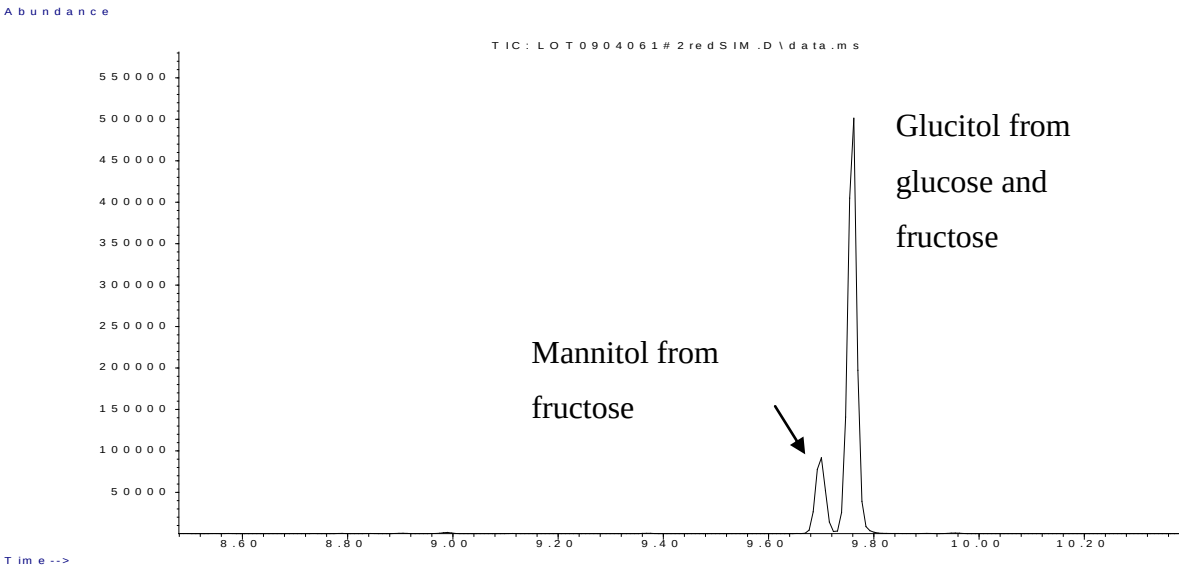

Figure 8: IC of sample LOT0904061 at the monosaccharide region.

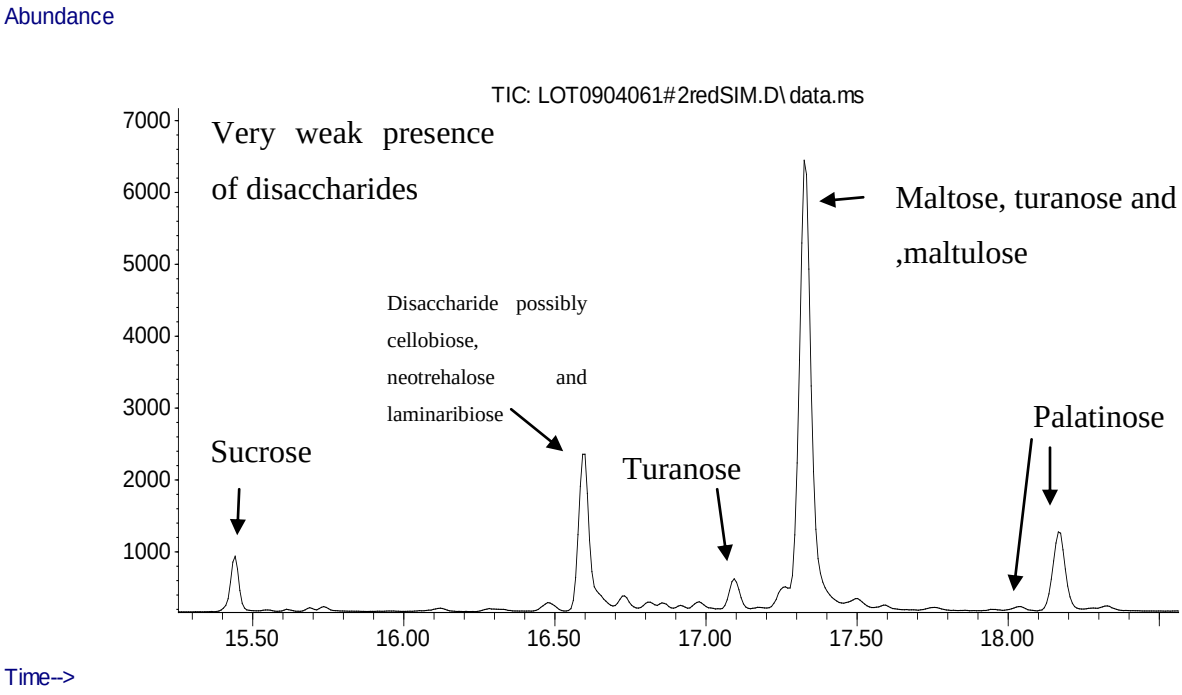

Figure 9: IC of sample LOT0904061 at the disaccharide region.

The very weak presence of a number of disaccharides in sample LOT0904061 as seen in Figure 9 is most probably from honey. As can be seen from the ion chromatogram, this product is mostly sweetened by fructose and glucose which is typical of honey.

Calculations of the ratio of glucose to fructose gave a ratio of 1.2 which is also consistent with a ratio found in honey.

Assuming the glucose and fructose only come from honey, the amounts of these monosaccharides would indicate a content of honey in the sample of between 57-99%. This result is based on White's range from ~85% to ~49 % for glucose and fructose combined.

4. Analysis of sample E090305NZ1.

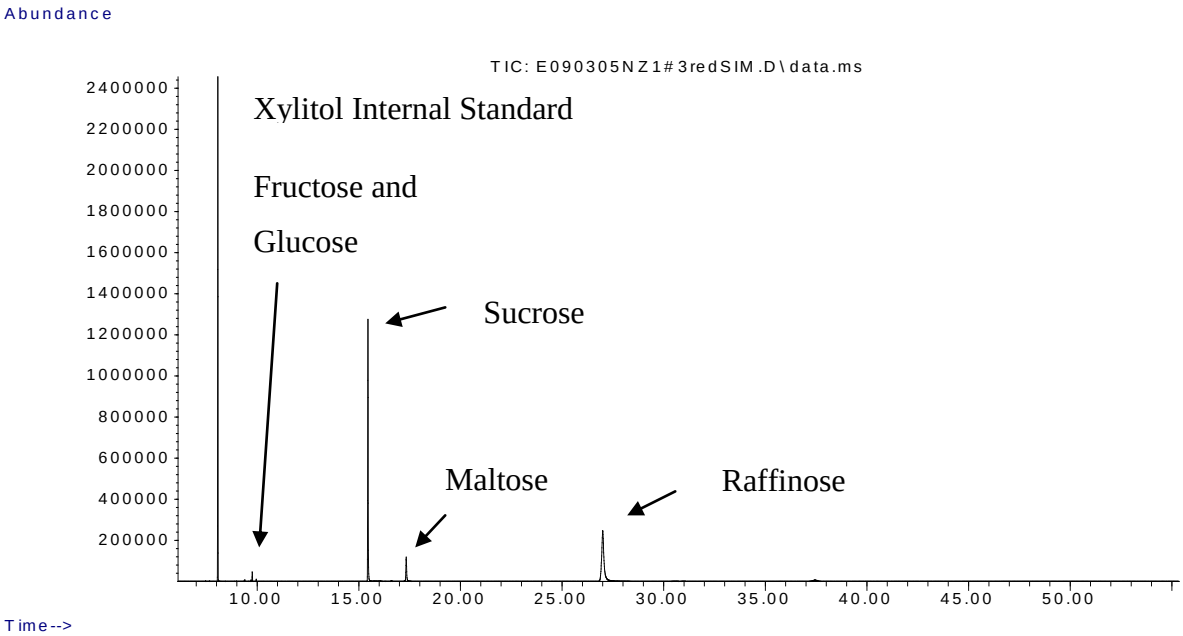

Figure 10: IC (ions = 204, 307, 361 and 451) of sample E090305NZ1.

The amount of disaccharides compared to monosaccharides is totally unlike what occurs in honey. Therefore there is likely to be another disaccharide sweetener present.

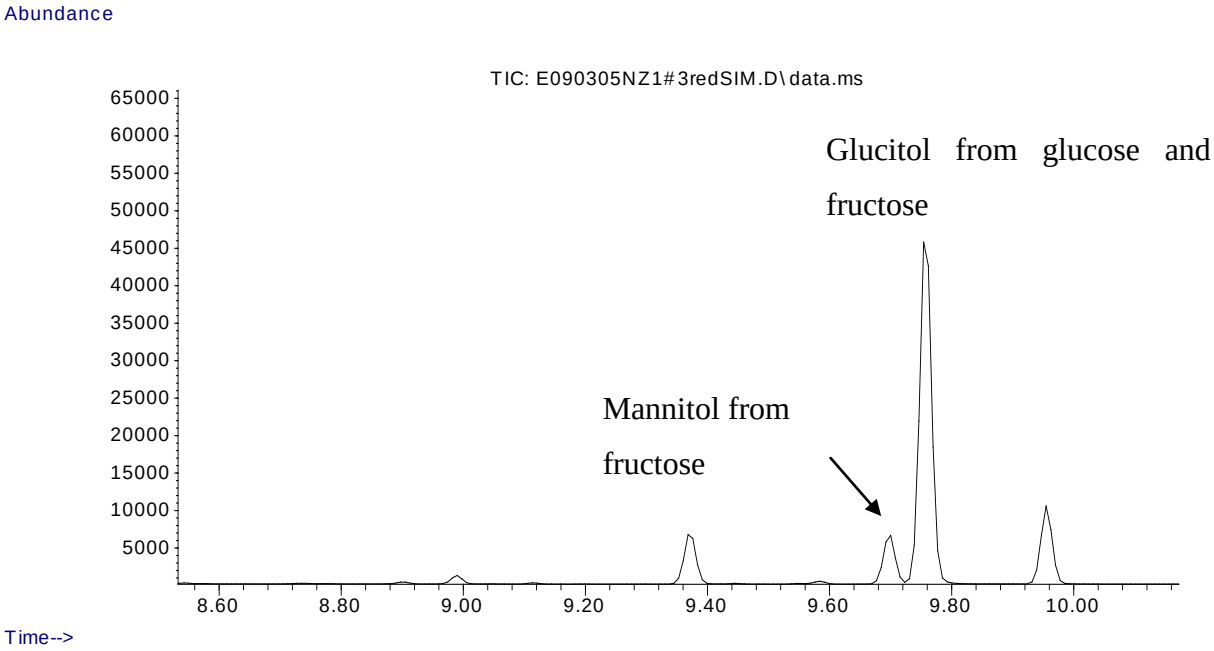

Figure 11: IC of sample E090305NZ1 at the monosaccharide region.

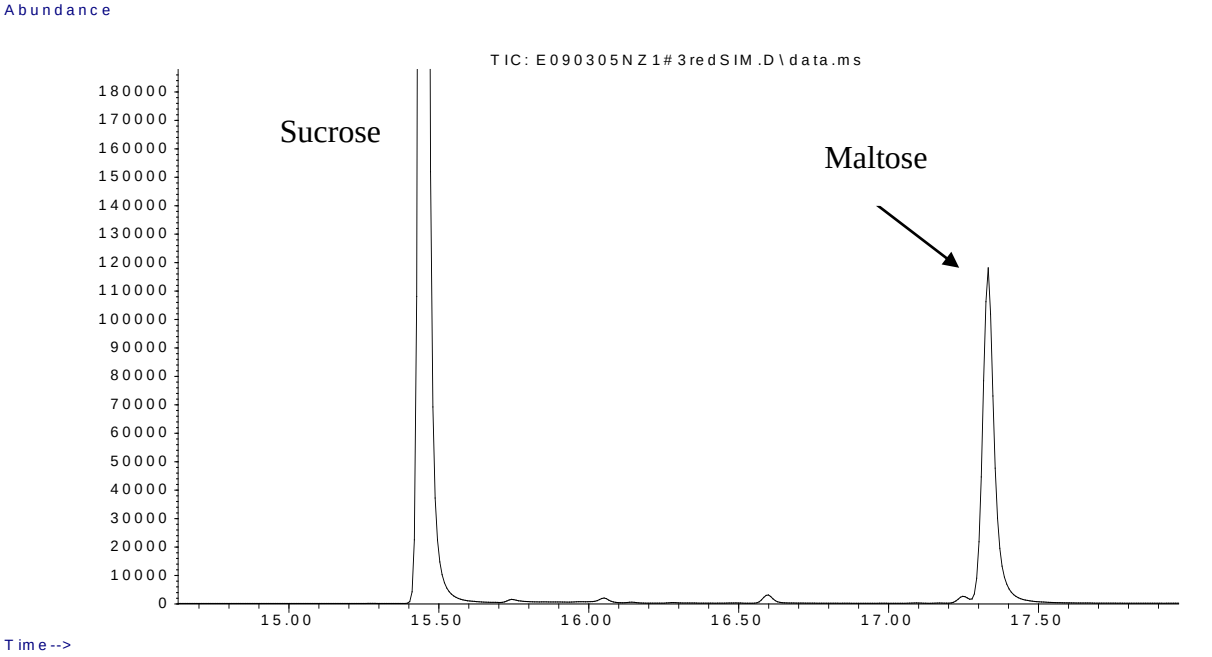

Figure 12: IC of sample E090305NZ1 at the disaccharide region.

The analysis of sample E090305NZ1 revealed the presence of the disaccharide sucrose at approximately 15.4 min and disaccharides at approximately 16.6 min (isomaltose?), 17.25 min (unknown), and 17.33 min (maltose).

Calculations of the ratio of glucose to fructose gave a ratio of 0.9 which is not consistent with a ratio found in honey. Sucrose was probably added as the main sweetener and possibly maltose syrup.

5. Analysis of sample B090627HK3.

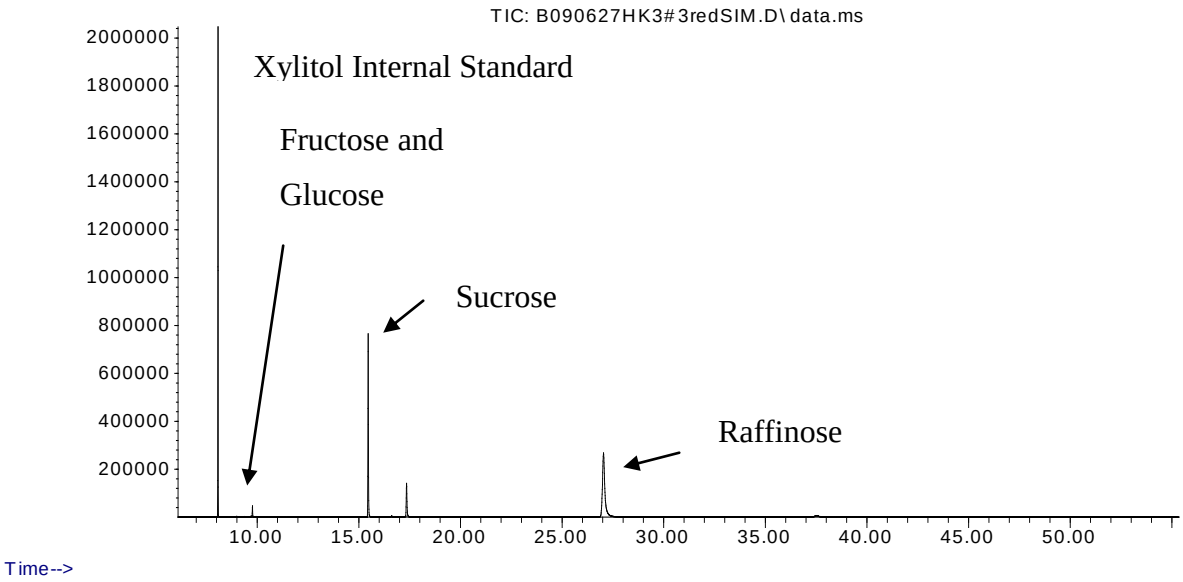

Figure 13: IC (ions = 204, 307, 361 and 451) of sample B090627HK3.

The amount of disaccharides compared to monosaccharides is unlike what occurs in honey. Therefore there is likely to be another disaccharide sweetener present.

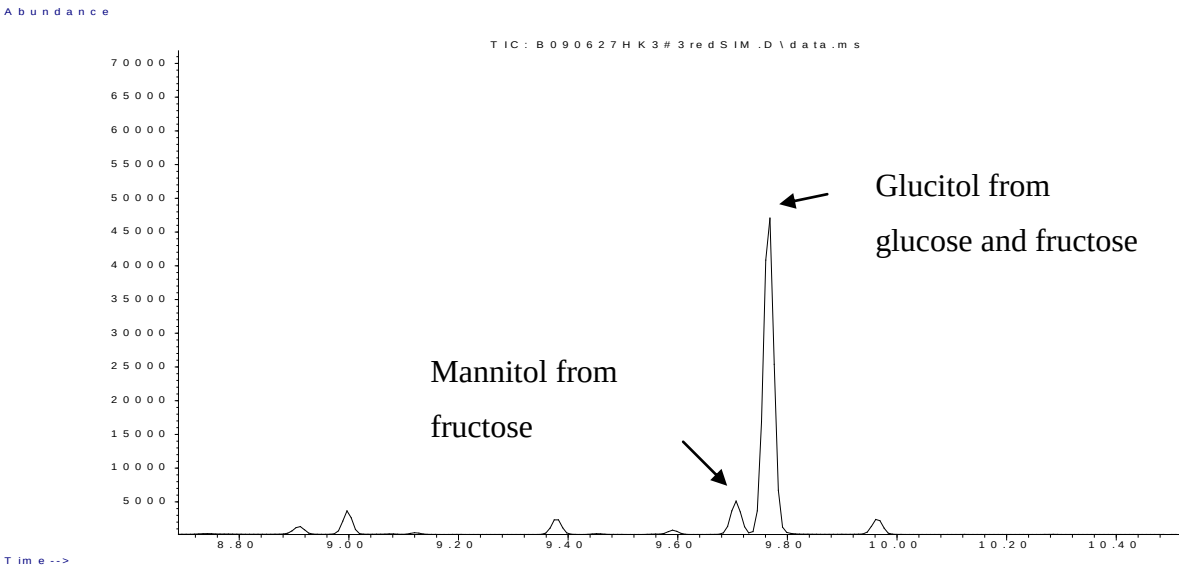

Figure 14: IC of sample B090627HK3 at the monosaccharide region.

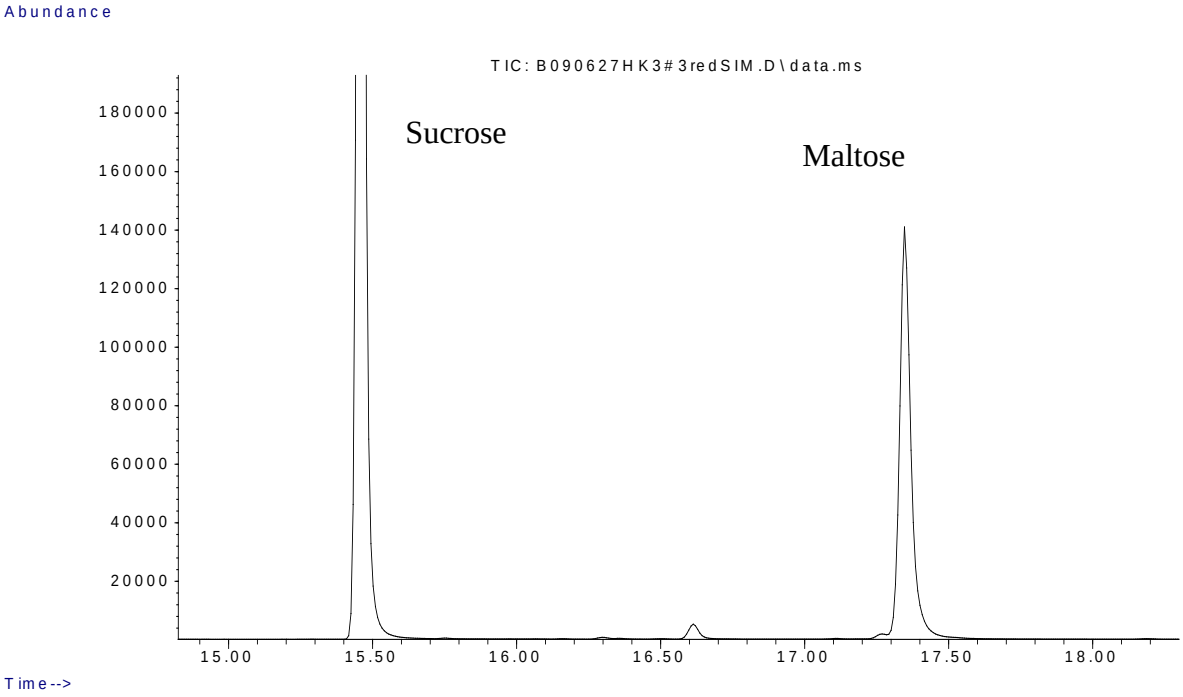

Figure 15: IC of sample B090627HK3 at the disaccharide region.

The chromatogram for sample B090627HK3 was very similar to the chromatogram for sample E090305NZ1. Presence of the disaccharide sucrose at approximately 15.4

min and disaccharides at approximately 16.6 min (isomaltose?), 17.25 min (unknown), and 17.33 min (maltose) is visible.

Calculations of the ratio of glucose to fructose gave a ratio of 0.8 which is not consistent with a ratio found in honey. Sucrose was probably added as the main sweetener and likely maltose syrup.

7.2 APPENDIX B: Supplementary data disc.