**Universidade de Aveiro** Departamento de Química

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# Origin of honey oligosaccharides: from model solutions to hive conditions

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Alimentar, realizada sob a orientação científica do Doutor Manuel António Coimbra Rodrigues da Silva, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro e da Doutora Elisabete Verde Martins Coelho, Investigadora de Pós-Doutoramento do Departamento de Química da Universidade de Aveiro.

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**Keywords**

Honey, oligosaccharides, model solutions, maturation conditions, transglycosylation, reversion reactions

**abstract**

Honey is essentially composed by carbohydrates, fructose (38 %) and glucose (31 %), mainly resultant of invertase activity. Furthermore, several oligosaccharides can be identified in honey, comprising 5 to 10 % of total carbohydrates. The origin of these oligosaccharides is still uncertain, as most of them are not present neither in nectar or in pollen. The research on this subject, mainly conducted in the past century, demonstrated the capability of invertase, namely the α-glucosidase activity, to transfer α-glucosyl residues to other carbohydrate moieties. As this transglucosylation activity was reported both to bees' and honey's invertases, it was proposed that the action of invertase was the source for α-glucose linked sugars. Nonetheless, there is still no explanation for the origin of the remaining oligosaccharides.

The present work hypothesizes that nonenzymatic reactions could also occur in honey promoting the formation of oligosaccharides. This can be supported by the fact that honey maturation conditions, such as high sugar concentrations in acidic media, induce condensation of carbohydrates, reactions also known as reversion reactions. In order to validate this hypothesis, six aqueous model solutions (moisture content of 20 %) containing sucrose plus glucose, and sucrose plus fructose were prepared using diluted citric acid at pH 4.0, pH 2.0, and with no acid addition. The model solutions were kept in an oven at 35 ºC, which is the normal temperature inside beehives with brood production. Besides the influence of honey maturation conditions on its oligosaccharides profile was assessed by analysis of honeys with different properties, particularly the duration and season of maturation.

Electrospray ionization mass spectrometry (ESI-MS) analysis allowed monitoring the changes occurring in each model solution along 5 months. This method revealed the occurrence of non-enzymatic oligosaccharide synthesis with a degree of polymerization (DP) up to 6 after 5 months. Ligand-exchange/size-exclusion chromatography (LEX-SEC) separation of the oligosaccharides formed in model solutions and present in honey and methylation analysis allowed to observe that the produced oligosaccharides had a glycosidic linkage composition similar to that obtained for honey oligosaccharides. In higher amounts, for most fractions, were terminally-linked glucose (Glc) residues together with lower amounts of (1→2)-, (1→4)- and (1→6)-Glc. Concerning fructose, terminally-linked fructose (Fru) residues were the most abundant and (2→1)- and (2→6)-Fru were in minor amounts. In addition, several branched residues were identified, being (1→2,3,4,6)-Glc the most abundant, and found predominantly in solutions prepared with citric acid. The structure and identity of the oligosaccharides were further elucidated by gas-chromatography coupled to mass-spectrometry (GC-MS) after derivatization to the alditol acetates derivatives.

**palavras-chave**

Mel, oligossacarídeos, soluções modelo, condições de maturação, transglicosilação, reações de reversão

**resumo**

O mel é composto essencialmente por hidratos de carbono, sendo a frutose (38 %) e a glucose (31 %) maioritariamente resultantes da atividade da invertase. Além disso, vários oligossacarídeos foram identificados no mel, constituindo 5 a 10 % do total dos açúcares. A origem destes oligossacarídeos ainda é incerta, uma vez que a maioria não é reportada nem no néctar nem no pólen. A investigação desta temática, maioritariamente conduzida no século passado, demonstraram a capacidade da invertase, designadamente a atividade de α-glucosidase, em transferir resíduos α-glucosyl para grupos funcionais de outros hidratos de carbono. Uma vez que esta atividade de transglucosilação foi reportada tanto para a invertase das abelhas como do mel, especulou-se que a ação desta enzima estivesse na origem dos açúcares com ligações de α-glucose. No entanto, ainda não existe uma explicação para a origem dos restantes oligossacarídeos.

O presente trabalho coloca a hipótese de que reações não enzimáticas possam ocorrer no mel, promovendo a formação de oligossacarídeos. Esta hipótese pode ser suportada pelo facto das condições de maturação do mel, como as concentrações elevadas de açúcar em meio ácido, induzirem à condensação dos hidratos de carbono, reações também designadas por reações de reversão. De forma a validar esta hipótese, seis soluções modelo aquosas (teor de humidade de 20 %) de sacarose com glucose e de sacarose com frutose foram preparadas com ácido cítrico diluído a pH 4.0, a pH 2.0 e sem adição de ácido. As soluções foram mantidas numa estufa a 35ºC, correspondente à temperatura média no interior da colmeia aquando da criação do ninho. Também se acedeu à influência das condições de maturação do mel no seu perfil de oligossacarídeos, através da análise de méis com diferentes propriedades, destacando-se a duração e o tempo de maturação.

As análises de ionização em electrospray acopladas a espectrometria de massa (ESI-MS) permitiram monitorizar as alterações que ocorreram nas soluções durante 5 meses. Este método revelou a ocorrência da síntese de oligossacarídeos não enzimática, com um DP máximo observado de 6, após 5 meses. A separação dos açúcares formados nas soluções modelo e presentes nos méis através de cromatografia de afinidade e de exclusão-molecular (LEX-SEC) e a análise de metilação permitiram observar que os oligossacarídeos produzidos tinham uma composição em ligações glicosídicas semelhante à dos oligossacarídeos do mel. A maioria das frações era composta principalmente por resíduos de glucose ligados pelo terminal e por resíduos de fructose ligada terminalmente e, em menor quantidade, por resíduos de glucose com ligações (1→2), (1→4) e (1→6) e por resíduos de frutose com ligações (2→1), (2→3) e (2→6). Também foram identificados resíduos correspondentes a ramificações, sendo o (1→2,3,4,6) -Glc o mais abundante e encontrado predominantemente em soluções elaboradas com ácido cítrico. A estrutura e a identidade dos oligossacarídeos anteriormente mencionados foram clarificados através da cromatografia gasosa acoplada à espectrometria de massa, após derivatização nos seus acetatos de alditol.

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**Acronyms**

**$a_w$** – water activity
**DP** – degree of polymerization
**ESI-MS** – electrospray ionization mass spectrometry
**FOS** – fructooligosaccharide
**GC-FID** – gas chromatography-flame ionization detection
**GC-qMS** – gas chromatography-quadropole mass spectrometry
**HMF** – hydroxymethylfurfural
**LEX/SEC** – ligand-exchange/size-exclusion chromatography
**LOD** – limit of detection
**PMAAs** – partially methylated alditol acetates
**SCFAs** – small-chain fatty acids
**TLC** – thin-layer chromatography
**VOCs** – volatile organic compounds

# Chapter I

Introduction

# I. Introduction

## I.1. Theoretical framework and objectives

Honey is a sweet and nutritious natural product with economic importance worldwide, being the most important primary product of beekeeping (1). It has been a highly valued food item since primitive times, on account of its sweetness and nutritional value (2). At present the annual world honey production is about 1.2 million tons, which is less than 1% of the total sugar production (3), and is worth at least 1.7 thousand million dollars (4). The major honey exporting countries are China, New Zealand and Argentina (5). The apiculture sector is an important part of the EU agriculture, not only because of pollination but also for the maintenance of the plants' genetic diversity and of ecological equilibrium (6). In the EU there are around 630 thousand beekeepers and 16 millions of hives, producing 234 thousand tons of honey per year (7), from which 17 thousand are Portuguese beekeepers with about 567 thousand hives (6). The consumption of honey differs strongly from country to country, being higher in the developed ones (3).

Honey is one of the oldest natural products used by humans, and yet, its biological properties and potential benefits to health are still far from being adequately assessed (8). In the past, most of the health benefits attributed to honey were based on mere observations or generalizations without any scientific support (9). However, in the last few years, there has been a renewed interest in research that investigates the potential health benefits of natural and unprocessed honey in the management of various diseases (10). This has resulted in findings that attribute several medicinal effects to honey, such as prebiotic activity. This effect is related to honey's oligosaccharides (11). The oligosaccharides profile is relatively well established, contrarily to its origin.

For this reason, the present work has the principal objective of inferring about the oligosaccharides origin, through the preparation of solutions composed by two of the main nectar carbohydrates (sucrose plus fructose/glucose), with the same sugar weight as honey (80% w/w). In addition, this study aims to assess the influence of honey's pH, ripening temperature and maturation time on its oligosaccharide content and profile, by evaluation of honeys with different geographical and botanical origin, whose maturation occurred in different seasons and over a determined period of time.

## I.2. Enterprise – More than Honey, Lda.

More than Honey, Lda. was created in 2014 and with it the Beesweet brand. This enterprise is located in Oliveira de Azeméis, Aveiro. Its activity is based on the purchasing of honey from beekeepers, aromatization and selling of the final product, both nationally and internationally.

Beesweet offers a range of products with seven different flavours. Aromatized honey flavours are citrus (Nº 1 Citrus), mint (Nº 5 Winter), seasalt (Nº 10 Seasalt), cinnamon (Nº 25 Christmas), chocolate (Nº 66 Beelove) and spicy (Nº 88 Fire). Besides, a rare honey originated from blueberry flowering is also part of the gamma.

# Chapter II

State-of-the-art

# II. State-of-the-art

## II.1. Honey

### II.1.1. History

Honey along with other bee products have been utilized by humans since prehistoric times in all societies world-wide (12). Humans apparently began hunting for honey at least 8,000 years ago, as evidenced by a cave painting at Cueva de la Arana in eastern Spain. The painting shows two honey-hunters collecting honey and honeycomb from a wild bee nest, while the bees themselves hover around the entrance. The figures are depicted carrying baskets or gourds, and using a rope to reach the wild nest (13).

Honey has had a valued place in traditional medicine for centuries (14). Most ancient civilizations, such as the Egyptians, Chinese, Greeks and Romans used honey not only as a natural sweetener but also for medicinal purposes, to treat wounds and diseases of the gut (15).

For a long time in human history honey was an important carbohydrate source, being the only largely available sweetener until the early 1700s, when industrial sugar production began to replace it. From this point sugar consumption rose inexorably, while honey consumption declined. Beekeeping ceased to be the general custom that it had been in former years, there was no longer a hive in every garden. On the other hand, from 1850 very significant advances were made in bee-keeping and hive technology and yields per hive rose. Nowadays, honey is a more expensive item compared to sugar, often eaten as a special treat, whilst sugar is the ubiquitous sweetener and everyday food (13). Furthermore, honey is utilized by the cosmetic industry, being incorporated into cosmetics in glycerol-honey gels and tanning cream products (16).

### II.1.2. Definition

Honey is, according to the Council of the European Union (2002), “the natural sweet substance, produced by *Apis mellifera* honeybees 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 honeycombs to ripen and mature”. Therefore, there

are, respectively, two types of honey according to its origin: blossom or flower honey, obtained from the nectar of plants, and honeydew honey, obtained from excretions of plant-sucking insects (*Hemiptera)* or from secretions of living parts of plants (17).

### II.1.3. Production

The transformation of nectar in honey starts immediately after the flower pollen, nectar and honeydew are collected and deposited in the bee's pouch, also known as honey sac. First, in this compartment, the mixture is enriched with some of the own bee's substances from their hypopharengeal glands to induce changes, which includes sucrose hydrolysis to its monomers by invertase enzymatic activity (2,18). The following step consists in the bees' return to the hive, where they regurgitate the content of their honey sac to the colony bees, in a process known as trophallaxis. These nurse bees pass it over to each other and finally fill the six-sided individual cells of the honeycomb, for storage and ripening (16,19). During this process the bees fan with their wings, thus lowering honey's humidity, filling the combs when the water contents reaches 30-40 %. At the same time, the bees add additional enzymes to the honey. The invertase transforms sucrose into fructose and glucose, while glucose oxidase oxidizes glucose to gluconic acid and hydrogen peroxide, the latter acting as an agent against bacterial spoilage. Furthermore, it occurs the absorption of proteins from plants and bees; the absorption of acids from the bee's body and, also, the assimilation of forage minerals, vitamins and aroma substances (16,20).

The warm colony temperature (35 º C) and more fanning lower further the honey humidity. Bees also suck out the honey and deposit it back into the combs, further lowering the water content of the honey. This transformation process takes place in 1 to 3 days. Generally, when honey reaches a humidity of less than 20 %, the bees close the cells with a wax lid, preventing absorption of moisture by honey (18). The ripening process continues, which is reflected by a continued hydrolysis of sucrose by the enzyme invertase and by the synthesis of new sugars (16).

Honey's harvest is performed when most of the honeycombs are capped. The hive frames are removed from the hive in order to be uncapped. This process can be done manually with an uncapping knife or with an automated uncapper machine. Next, the hive frames are placed in a honey extractor, where most of the honey is release by centrifugal

force. The honey can be clarified by filtration with a mesh size not greater than 0.2 mm, to remove big dimension residues like wax and bugs. Then, honey is transferred to stainless steel tanks where the decantation process takes place, and, finally, honey is homogenized. The ultimate step consists in the packaging of honey in appropriate containers (17).

### II.1.4. Classification

Honey can be designated according to its geographical and botanical origin, recovering time, production and or presentation style and, finally, to its use. Floral origin is closely related to honey's colour and flavour, and, according to it, it is possible to differentiate honey in single-flower and multi-flower. This classification is based on the percentage of flowers' pollen in the honey. Single-flower honey consists in honey with at least 45% of a species pollen, with an exception to rosemary (10% at least) and to chestnut (90%), while multi-flower honey contains nectar from multiple species. Pollen analyses can both infer about the botanical and geographical origin of honey (6,21).

Based on the recovery time, honey is characterized as: early, if collected until the end of May; main, if it is harvested between June and July; and late if it occurs in August or September.

Concerning honey production, it can be classified as: extracted honey, that is obtained by centrifugation of brood-free comb cells; pressed honey, which is collected by compressing the brood-free combs in a hydraulic press; and drained honey, which is obtained by draining the brood-free combs. According to the presentation style, honey may be designated as: normal honey which is in liquid or crystalline state or a mixture of the two; comb honey which is stored in the cells of freshly-built broodless combs and which is sold in sealed whole combs or sections of such combs; and chunk honey which is honey containing one or more pieces of comb honey (16,17).

Finally, based on its use, honey is distinguished as: honey for domestic use, being the highest quality product, and consumed and enjoyed in pure form, or baking honey, that is not of high quality and is used in place of sugar in the baking industry (16).

### II.1.5. Physical properties

Honey detains specific characteristics, which include colour, density, viscosity, hygroscopicity, electric conductivity and crystallization (22).

Colour is one of the parameters that varies most, and is mainly determined by its botanical origin. This feature is also dependent on ash content, maturation temperature and storage conditions (23), but transparency or clarity depends on the amount of suspended particles, such as pollen (24). The Codex Alimentarius Committee on Sugars (25) stipulates that the colour of honey should be nearly colourless to dark brown. Storage conditions, like temperature and duration time, must be considered, due to their influence in the occurrence of Maillard reactions, fructose caramelization and reactions of polyphenols, which result in the darkening of honey (26). Once crystallized, honey turns lighter in colour because the glucose crystals are white (24).

Honey density, at 20 º C, depends on the water content and may range from 1,4404 (14% water) to 1,3550 (21% water) (16).

Hygroscopicity is another property of honey and describes its ability to absorb and hold moisture from environment (16,27). This feature is problematic, once it causes difficulties in preservation and storage. Normal honey with water content of 18.8% or less will absorb moisture from air of a relative humidity of above 60%. The thermal conductivity of honey varies from 118 to $143x10^{-5}$ $Cal/cm^2/s/º$ C (28).

Viscosity is one of the most preponderant physical and sensory characteristics of honey, affecting the quality of the product as well as the design of honey-processing equipment, once it restrains all the stages involved in honey production (extraction, pumping, filtration, mixing and bottling) (29). This property is influenced by temperature, moisture content, as well as the presence of crystals and colloids in the product (30). It decreases with the increasing of temperature and water content (31).

Most honeys behave like Newtonian fluids (16), although there are reports in the literature for dilatant behaviour and thixotropic behaviour of some types of honey. The non-Newtonian behaviour has been attributed to the presence of colloids or high-molecular weight dextrans (29).

The surface tension of honey varies with the origin of the honey and is probably due to colloidal substances. Together with high viscosity, it is responsible for the foaming characteristics of honey (24).

### II.1.6. Composition

Honey as produced by honeybees from plant nectars is rather variable in its composition and properties, reflecting the impact of the botanical sources, geographical origin, climate and environmental conditions, as well as beekeeper skills in the final product (20,32). In addition, honeybees are in strict contact with the surroundings and, during their forage, they are easily exposed to potential pollutants that can change the honey composition and quality (33).

Honey is a food item that contains about 200 substances (34,35). This product is essentially a concentrated aqueous solution of inverted sugar, but it also contains a very complex mixture of other carbohydrates, water, and other substances such as enzymes, amino and organic acids, vitamins, minerals, pigments and aroma substances (36,37), and solid particles, mainly consisting of pollen, traces of wax and variable amounts of sugar-tolerant yeast (21).

Besides nectar, bees may collect honeydew which is, as previously mentioned, excretions of insects that feed in the phloem sap of plants. Honeybees process it as they do with nectar, resulting in honeydew honey. This product is different from floral honey once it presents lower values of glucose and fructose and higher levels of oligosaccharides, pH value, free and total acidity, ash and nitrogen (38,39).

A compositional comparison between flower honey and honeydew honey is provided in Table 1. The analytical data is based on 490 samples of flower honey and 14 samples of honeydew honey, and both are from the USA. Nevertheless, they basically represent the composition of honey from other countries (40). Also, pH values were obtained by analysis of 39 samples from nine Portuguese districts (41). The reducing disaccharides were analyzed collectively, and are normally reported as maltose. This fraction includes several carbohydrates, such as maltose, isomaltose, kojibiose, maltulose, palatinose, gentiobiose, cellobiose. Higher sugars' fraction constitutes the formerly designed honey dextrins, which includes tri- and higher oligosaccharides (42).

**Table 1 –** Composition of floral honey and of honeydew honey (%) (40,41)

| | Floral honey | | Honeydew honey | |
|---|---|---|---|---|
| **Constituent** | **Average value** | **Variation range** | **Average value** | **Variation range** |
| Moisture | 17.2 | 13.4 – 22.9 | 16.3 | 12.2 – 18.2 |
| Fructose | 38.2 | 27.3 – 44.3 | 31.8 | 23.9 – 38.1 |
| Glucose | 31.3 | 22.0 – 40.8 | 26.1 | 19.2 – 31.9 |
| Sucrose | 2.4 | 1.7 – 3.0 | 0.8 | 0.44 – 1.14 |
| Reducing disaccharides | 7.3 | 2.7 – 16.0 | 8.80 | 5.1 – 12.5 |
| Higher oligosaccharides | 1.5 | 0.1 – 8.5 | 4.70 | 1.3 – 11.5 |
| Nitrogen | 0.06 | 0.05 – 0.08 | 0.10 | 0.05 – 0.22 |
| Minerals (ash) | 0.22 | 0.20 – 0.24 | 0.74 | 0.21 – 1.18 |
| Free acids[a] | 22.0 | 6.8 – 47.2 | 49.1 | 30.3 – 66.0 |
| Lactones[a] | 7.1 | 0 – 18.8 | 5.8 | 0.36 – 14.1 |
| Total acids[a] | 29.1 | 8.7 – 59.5 | 54.9 | 34.6 – 76.5 |
| pH value | 3.8 | 3.0 – 4.7 | 4.4 | 3.9 – 4.9 |
| Diastase value | 20.8 | 2.1 – 61.2 | 31.9 | 6.7 – 48.4 |

[a] mequivalents of acid/kg of honey

The Codex Alimentarius Committee on Sugars (25) limits human intervention that could alter the composition of honey and thereby allows for the preservation of the natural character of honey. Similarly, it prohibits the removal of any constituent particular to honey, including pollen, unless such removal is unavoidable in the removal of foreign matter. Moreover, the previous Codex stipulates several composition criteria that honey must obey. These parameters are shown in Table 2.

**Table 2 –** Composition criteria of honey, adapted from (25)

| **Parameter** | **Value** |
|---|---|
| Sugars (fructose + glucose) | Min 60 g/100g |
| Sucrose | Max 5 g/100g |
| Moisture | Max 20% |
| Water insoluble solids | Max 0.1 g/100g |
| Electrical conductivity | Max 0.8 mS/cm |
| Free acididy | Max 50 meq/kg |
| Diastase activity | Min 8 or 3 (HMF≤ 15 mg/kg) |
| Hydroxymethylfurfural (HMF) | Max 40 mg/kg |

### II.1.6.1. Moisture and water activity ($a_w$)

Water is the second largest constituent of honey. It is one of the most important characteristics, influencing physical properties of honey such as viscosity and crystallization, as well as colour, flavour, taste, specific gravity, solubility and conservation (43).

The percentage of moisture in honey depends on its botanical origin, on the level of maturity achieved in the hive, as well as on the weather conditions during ripening; also extraction, processing and storage conditions can influence the water content, due to the hygroscopic character of honey (44,45). For these reasons the water content of honey varies greatly and it may range somewhere between 13 and 23 % (16).

Honey industry uses almost exclusively the moisture content as a criterion of microbial stability in honey (44). A higher water content of honey increases the probability of its fermentation and spoilage, so it should be less than 20 % (16). Nevertheless, the absolute water content is not responsible for the metabolism of the yeast but the amount of free water, described as water activity. The water activity of honey is within a range of 0.5–0.65, and $a_w$ values above 0.60 represent a critical threshold for microbial stability (46), as osmophilic yeasts are able to grow down to about an $a_{w=}$ 0.61-0.62 (44).

The water activity of honey depends mainly on the glucose content, once it has a direct effect on crystallization. Honey crystallization is known to be faster when having more glucose than 280-300 g/kg; a glucose/moisture ratio of 2.1 or higher; and a fructose/glucose ratio less than 1.14 (47). During crystallization of honey, glucose starts to crystallize first (48); and as fructose is more soluble it stays in solution for longer time (16). All the five hydroxyl groups of glucose interact with water molecules. So, after crystallization, glucose is found as glucose monohydrate, meaning that each glucose molecule fixes only one molecule of water. Therefore, less water is fixed in the crystallized state, which results in the increasing of $a_w$ in the liquid phase (46).

### II.1.6.2. Carbohydrates

Carbohydrates are the main constituents of honey, comprising about 95% of honey dry weight. Main sugars are the monosaccharides fructose and glucose, which are products

of the invertase activity towards the disaccharide sucrose. In almost all types of honey, fructose is the carbohydrate in the greatest proportion (16,40).

Besides, more than 20 different oligosaccharides have been identified (Table 3), representing 5 to 10% of the total carbohydrates (49). As shown in Table 4, the principal oligosaccharides in blossom honey are the disaccharides maltose and kojibiose, while erlose and theanderose are the mainly trisaccharides. In what concerns honeydew honey, the main oligosaccharides are melezitose and erlose (39).

**Table 3 –** Sugars identified in honey (49,50)

| Common name | Systematic name |
|---|---|
| Glucose | |
| Fructose | |
| Sucrose | α-D-glucopyranosyl-β-D-fructofuranoside |
| Maltose | *O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose |
| Isomaltose | *O*-α-D-glucopyranosyl-(1→6)-D-glucopyranose |
| Maltulose | *O*-α-D-glucopyranosyl-(1→4)-D-fructose |
| Palatinose | *O*-α-D-Glucopyranosyl-(1→6)-D-fructose |
| Nigerose | *O*-α-D-glucopyranosyl-(1→3)-D-glucopyranose |
| Turanose | *O*-α-D-glucopyranosyl-(1→3)-D-fructose |
| Kojibiose | *O*-α-D-glucopyranosyl-(1→2)-D-glucopyranose |
| Laminaribiose | *O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose |
| α,β-Trehalose | α-D-glucopyranosyl-β-D-glucopyranoside |
| Gentiobiose | *O*-β-D-glucopyranosyl-(1→6)-D-glucopyranose |
| Cellobiose | *O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranose |
| Inulobiose | O-β-D-fructofuranosyl-(2→1)-D-fructose |
| Raffinose | *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside |
| Melezitose | *O*-α-D-glucopyranosyl-(1→3)-*O*-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside |
| 3-α-Isomaltosylglucose | *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→3)-D-glucopyranose |
| Maltotriose | *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose |
| 1-Kestose | *O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside |
| 6-Kestose | *O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(6→2)-β-D-fructofuranoside |

| | |
|---|---|
| Neokestose | *O*-β-D-fructofuranosyl-(2→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside |
| Panose | *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose |
| Isomaltotriose | *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-D-glucopyranose |
| Erlose | *O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-β-D-fructofuranoside |
| Theanderose | *O*-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-β-D-fructofuranoside |
| Centose | *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→2)-D-glucopyranose |
| Isopanose | *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→6)-D-glucopyranose |
| Isomaltotetraose | *O*-α-D-glucopyranosyl-(1→6)-[*O*-α-D-glucopyranosyl-(1→6)]$_2$-D-glucopyranose |
| Isomaltopentaose | *O*-α-D-glucopyranosyl-(1→6)-[*O*-α-D-glucopyranosyl-(1→6)]$_3$-D-glucopyranose |

The disaccharides content depends largely on the plants from which the honey was derived, while geographical and seasonal effects are negligible. Furthermore, the content of sucrose varies appreciably with the honey ripening stage (16), once it depends on the extension of the invertase reaction. Many of these sugars are not found in nectar, as will be discussed later, but are formed during the ripening and storage.

**Table 4 –** Oligosaccharide composition of honey (49)

| **Sugar** | **Content[a] (%)** |
|---|---|
| *Disaccharides* | |
| Maltose | 29.4 |
| Kojibiose | 8.2 |
| Turanose | 4.7 |
| Isomaltose | 4.4 |
| Saccharose | 3.9 |
| Maltulose (and two unidentified ketoses) | 3.1 |
| Nigerose | 1.7 |
| α,β-Trehalose | 1.1 |
| Gentiobiose | 0.4 |
| Laminaribiose | 0.09 |

| | |
|---|---|
| *Trisaccharides* | |
| Erlose | 4.5 |
| Theanderose | 2.7 |
| Panose | 2.5 |
| Maltotriose | 1.9 |
| 1-Kestose | 0.9 |
| Isomaltotriose | 0.6 |
| Melezitose | 0.3 |
| Isopanose | 0.24 |
| Gentose | 0.05 |
| 3-α-Isomaltosylglucose | +[b] |
| *Higher Oligosaccharides* | |
| Isomaltotetraose | 0.33 |
| Isomaltopentaose | 0.16 |
| *Acidic fraction* | 6.51 |

[a]Values are based on oligosaccharide total content (= 100%) which in honey averages 3.65%. Only the most important sugars are presented. [b] Traces.

#### II.1.6.3. Proteins

Proteins are present in honey in very low amounts. These nutrients are essentially related to the presence of enzymes and free amino acids (43). Proteins and amino acids in honeys are attributable both to animal and vegetal sources, the major of these being pollen (27). The protein content of honey varies according to the species of the honeybees. *Apis cerana* honey contains from 0.1 to 3.3 % protein, while *Apis mellifera* honey contains between 0.2 to 1.6 % protein (51).

##### II.1.6.3.1. Amino Acids

Honey contains free amino acids at a level of 100 mg/100 g solids. It is possible to identify the geographical or regional origin of honeys, on the basis of several amino acid ratios (16). Honey contains almost all physiologically important amino acids (52), being proline the most abundant both for honey and pollen (53). In honey, proline represents a total of 50-85 % amino acids (16). This amino acid derives mainly from the salivary secretions of honeybees (36,53). Proline has been used as a criterion for the evaluation of the maturation

of honey and, in some cases, adulteration with sugar. A minimum value of 180 mg/kg is accepted as the limit value for authentic honey (54).

Besides proline, there are 26 amino acids in honeys (Table 5), being their relative proportions dependent on the honey origin (nectar or honeydew) (16). The most common are glutamic acid, alanine, phenylalanine, tyrosine, leucine and isoleucine (55).

**Table 5 –** Free amino acids in honey (16)

| **Amino acid** | **mg/ g honey (dry weight basis)** |
|---|---|
| Asp | 3.44 |
| Asn + Gln | 11.6 |
| Glu | 2.94 |
| Pro | 59.6 |
| Gly | 0.68 |
| Ala | 2.07 |
| Cys | 0.47 |
| Val | 2.00 |
| Met | 0.33 |
| Met-O | 1.74 |
| Ile | 1.12 |
| Leu | 1.03 |
| Arg | 1.72 |
| Tyr | 2.58 |
| Phe | 14.75 |
| β-Ala | 1.06 |
| γ-Abu | 2.15 |
| Lys | 0.99 |
| Orn | 0.26 |
| His | 3.84 |
| Trp | 3.84 |
| Unidentified AA's (6) | 24.5 |
| **Total** | **118.8** |

#### II.1.6.3.2. Enzymes

Enzymes are naturally present in honey, in small amounts, and play a vital role in the ripening of nectar into honey. The most prominent enzymes in honey are invertase, diastase and glucose oxidase. Others, including catalase and acid phosphatase can also occur.

Invertase, also known as α-glucosidase or saccharase, is originated from both nectar and bees, but the latter enzymes are more active (16,20). It has hydrolytic activity towards sucrose and other α-glucosides, which catalyse the exohydrolysis of α-glucosyl residues from the non-reducing terminal of a substrate, releasing α-glucose (56). Invertase is inactivated by heating and has a pH optimum between 5.8–6.5 (16,57).

Diastases are a group of amylolytic enzymes that include α- and β-amylases. The enzyme α-amylase hydrolyses starch chains in the α-D-(1→ 4) linkages, producing a variety of dextrins, and β-amylase release maltose from the end of the starch chain (58). These enzymes also originate from bees. Their pH optimum range is 5.0−5.3. Diastase activity is somewhat more thermally stable than invertase activity. Therefore, due to the sensitivity of invertase and diastase activities to heat, they are, together with the HMF content, of significance for assessing whether or not the honey was heated (16).

Another enzyme present in honey is glucose oxidase and is also derived from bees. It converts glucose into δ-gluconolactone, which is hydrolysed to gluconic acid. Besides, the enzymatic oxidation produces hydrogen peroxide, which is partly responsible for a bacteriostatic effect of nonheated honey (59). Its optimum pH is 6.1. Glucose oxidase is an active enzyme in nectar but is virtually inactive in honey. The enzyme may become active again if the honey is diluted. Therefore, the amount of gluconic acid in a honey should give some insight into the conditions of its ripening by the bees, since production essentially stops when full density is attained in the stored honey (60).

The enzyme catalase, which destroys hydrogen peroxide, also occurs in honey. Contrarily to the previous stated enzyme, it most probably originates from pollen which, unlike flower nectar, has a high activity of this enzyme (16). Consequently, the level of peroxide in a honey is effectively determined by its level of catalase and this will depend on how much pollen is collected by bees, the floral source of the pollen and also on the catalase activity of that pollen (10).

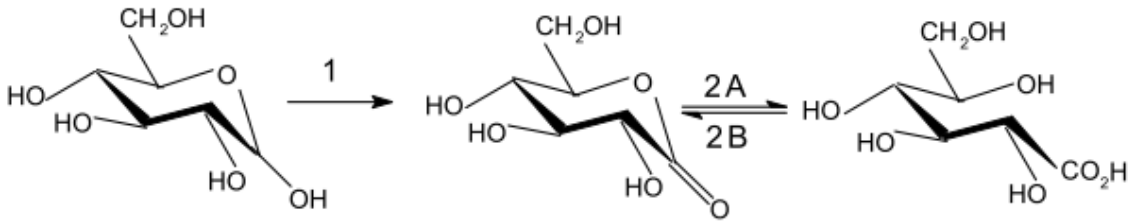
Acid phosphatase is an enzyme of honey whose values have been related to honey fermentation. Acid phosphatase is mainly present in pollen, although some activity comes from nectar. This is a lysosomal enzyme that hydrolyses organic phosphates at an acidic pH. Honeys that ferment more easily have shown higher acid phosphatase activities than unfermented honeys. The pH of honey has demonstrated to have a strong influence on the activity of acid phosphatase. A higher pH increases the acid phosphatase activity (61).

Another enzyme reported in honey is β-glucosidase (62), which is known to be ubiquitously in all the living kingdoms (63). Furthermore, this enzyme is part of a defence mechanism against herbivores and pathogens for both insects and plants (63–65). Concerning to honeybees (*Apis mellifera*), β-glucosidase activity was detected in the hypopharyngeal glands, hindgut, honey sac and ventriculus. This enzyme was reported to have activity in a pH range of 3.5-9.5 and in a temperature interval between 20-60 º C (66). However, it was shown that this enzyme, isolated and purified from both the honey sac and ventriculus of the honeybee, had no activity towards cellobiose. This result indicates that the role of β-glucosidase is not for the digestion of cellulosic material, having only aryl or alkyl β-glucosidase activity. Therefore, its function should concern the hydrolysis of glucoside toxins ingested by the honeybee that are similar to β-*p*-nitrophenyl-glucoside (β-PNPG), (66,67), or the activation of the honeybee defence mechanism' compounds like cyanogenic glucosides (63).

#### II.1.6.4. Organic Acids

All honeys have a slight acidity due to the presence of organic acids. These acids are related to honey's colour and flavour and, also, to its physical properties such as pH and electrical conductivity (68). Free acidity is an important parameter related to the deterioration of honey. The Codex Alimentarius Committee on Sugars (25) allows a maximum value of 50.00 meq of acid per kg of honey for free acidity. Higher values may be indicative of fermentation of sugars into organic acids. However, the presence of different organic acids, geographical origin and harvest season can affect the honeys' acidity (25,69). So, it is possible to find higher acidity values than the established limit, even in the absence of any sort of deterioration (41).

The principal organic acid in honey is gluconic acid that, as previously reported, results from glucose oxidase activity. The acid level is mostly dependent on the time elapsed between nectar collection by bees and achievement of the final honey density in honeycomb cells, once the glucose oxidase activity drops to a negligible level in ripened honey (16). There are other acids present in honey, but only in small amounts. Those are acetic, butyric, lactic, citric, succinic, formic, maleic, malic and oxalic acids (68). Besides, gluconic acid is in equilibrium with its lactone, namely δ-gluconolactone, which represents a potential reserve of acidity (lactonic acidity) when honey suffers alkalinisation (41). The reactions of interconversion between D-glucose, δ-gluconolactone and gluconic acid are schematized in Fig.1. Thus, total acidity is the sum of the free acidity and the lactonic acidity (59).




**D-Glucose** **Gluconolactone** **Gluconic Acid**

**Figure 1 -** D-Glucose oxidation catalysed by glucose oxidase and the equilibrium between gluconolactone and gluconic acid (59). 1: reaction catalysed by glucose oxidase; 2A: δ-gluconolactone hydrolysis; 2B: internal esterification of gluconic acid.

### II.1.6.5. Vitamins

Honey contains small and variable amounts of vitamins, being originated from the pollen grains in suspension. Vitamins found in honey are especially the vitamin B complex, which include thiamine (B1), riboflavin (B2), nicotinic acid (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B8) and folic acid (B9). Besides, ascorbic acid and vitamin K are also present. However, there is no doubt that the levels of these factors are nutritionally insignificant to humans (36,42).

The commercial filtration of honey may cause a reduction in vitamin content due to the almost complete removal of pollen. Another factor that causes loss of vitamins in honey is the oxidation of ascorbic acid by the hydrogen peroxide produced by glucose oxidase (70).

### II.1.6.6. Aroma substances

Honey volatiles are significantly responsible for the honey aroma and flavour (21). So, each monofloral honey possesses a specific aroma profile, once volatile substances influence remarkably the individual sensory characteristics (1).

More than 400 compounds have been identified as honey volatiles in different chemical families, originated from various biosynthetic pathways (16,27). The chemical families into which the volatile compounds in honey belong include: aldehyde; alcohol; ketone; acid; ester; benzene and its derivatives, furan and pyran; norisoprenoids; terpenes and its derivatives, sulphur; and cyclic compounds (1,16). β-Damascenone and phenylacetaldehyde are considered the most characteristic components because of their honey-like aroma (71). Generally, volatile organic compounds (VOCs) could be derived from the plant or nectar source, from the transformation of plant compounds by the metabolism of a bee, from heating or handling during honey processing and storage, or from microbial or environmental contamination (27,72). Once the composition of VOCs in honey is influenced by both nectar composition and floral origin, they are used as markers for the determination of honey's geographical and botanical origin (73–75).

### II.1.6.7. Minerals

Ash content is a measure of quality that evaluates the mineral content present in honey. The mineral content may be indicative of environmental pollution, when heavy metals are detected, and of geographical origin (21). It can be also used as a parameter to evaluate the nutritional value of honeys.

Usually, the major mineral content contribution is from potassium, followed by other minerals in lower quantities, such as sodium, phosphorus, magnesium, iron, calcium, zinc, and copper (3,43).

Mineral content is related to the colour of honey, with higher mineral content being paired with a darker colour (43,76). Further, these darker honeys normally have stronger flavours, which may partially be resultant of the high mineral composition, possibility acting for salting out. Actually, darker honeys have shown more content in sodium, potassium and sulphur (77), which are known to have a characteristic taste (77,78).

### II.1.6.8. Toxic Constituents

Honey, as any other food, can be contaminated or contain toxic compounds. Contaminations that result from the environment are pesticides, antibiotics, and heavy metals, per example (36). In the case of heavy metals, lead (Pb), arsenic (As) and mercury (Hg) can be found (79). Moreover, certain types of flower nectar have been reported to result in honey that is psychoactive and that can lead to toxicity, despite being innocuous to the bees and their larvae. This is the case of honeys produced by bees feeding on flowers of the *Ericaceae* and *Solanaceae* families. The toxicity is attributed to compounds of the flower nectar, such as glycoalkaloids and pyrrazolidine alkaloids (3,79). The symptoms encountered after honey poisoning are: vomiting, headache, stomach ache, unconsciousness, delirium, nausea, sight weakness (36).

Furthermore, some honey samples may also contain toxic compounds such as HMF, which is produced by the Maillard reaction during processing or storage, in an acidic environment (80).

### II.1.6.9. Microbiological composition

Microorganisms content is a parameter of concern, once it may influence quality and safety of honey. However, honey's anti-microbial properties combined with the control measures during its manufacture, results in minimal types and levels of microorganisms.

The primary sources of microbial contamination include pollen, the digestive tracts of honeybees, dust, air, earth and nectar, sources which are very difficult to control. The secondary sources correspond to air, food handlers, cross-contamination, equipment and buildings, which are in contact with honey after harvest. These contaminations are controlled by good manufacturing practices.

Most bacteria and other microbes cannot grow or reproduce in honey, due to the hostile conditions found in ripe honey. Therefore, bacteria, moulds and yeasts found in honey are expected to be present as spores, *i.e.* in the dormant form (24,81). Microorganisms that may be found in honey are specified in Table 6.

**Table 6 –** Microorganisms genus reported to be found in honey (81)

| **Bacteria** | **Fungi** | |
|---|---|---|
| | **Yeasts** | **Molds** |
| *Alcaligenes* | *Ascosphaera* | *Aspergillus* |
| *Bacillus* | *Debaryomyces* | *Atichia* |
| *Bacteridium* (sic) | *Hansenula* | *Bettsia alvei* |
| *Bacterium* (sic) | *Lipomyces* | *Cephalosporium* |
| *Clostridium* | *Nematospora* | *Chaetomium* |
| *Enterobacter* | *Oosporidium* | *Coniothecium* |
| *Flavobacterium* | *Pichia* | *Hormiscium* |
| *Klebsiella* | *Rhodotorula* | *Penicillium* |
| *Micrococcus* | *Saccharomyces* | *Peronsporaceae* |
| *Neisseria* | *Schizosaccharomyce* | *Peyronelia* |
| *Proteus* | *Schwanniomyces* | *Triposporium* |
| *Pseudomonas* | *Trichosporan* | *Urediancеae* |
| *Xanthomonas* | *Torula* | *Ustilaginaceae* |
| | *Torulopsis* | |
| | *Zygosaccharomyces* | |

### II.1.7. Health benefits

Honey's investigations about its beneficial effects reported several health benefits. These include gastroprotective (82), hepatoprotective (83), cardioprotective (84), hypoglycemic (85), antioxidant (35,86,87) and antihypertensive effects (88). Other effects such as antibacterial (43,89,90), anti-fungal (91,92), anti-viral (93), anti-inflammatory (94), and immunological (95) have also been documented and attributed to honey. Nonetheless, it is imperative to have well-designed, randomized controlled clinical trials that demonstrate these health-beneficial effects in humans.

### II.1.8. Dynamics of the beehive

#### II.1.8.1. Seasonality of honey production

Honey production is different during the year, in temperate zones. Brood rearing increases in the spring and reaches a maximum in early summer. So, during these seasons, honey production is reduced, because most of the collected nectar is consumed by the young non-foraging bees, and by the whole colony when the weather is no appropriate to nectar collection. After midsummer, colony's population decreases, and nectar that is surplus to the immediate requirements is transformed into honey and stored, so it can be used in winter.

It should be noted that, even during summer, the supply of nectar is not constant, being largely available during a "honey flow". This is when most of the colony's honey is produced and occurs when a great number of individual nectar-yielding flowers are open at the same time, having a duration of a few days or up to a few weeks (2).

#### II.1.8.2. Thermoregulation of the beehive

Temperatures of honeybee colonies fluctuate both daily and seasonally (96). The honeybee colony accomplishes very well the hive thermoregulation at high and low ambient temperatures. In hot weather, strategies as wide spacing among individuals in the hive, fanning and evaporative cooling are used. In cool weather, clustering reduces the exposed surface area, and the interior of the cluster is maintained at a relatively constant temperature. The cluster expands and contracts, as ambient temperatures rise and fall (97). In winter, as temperatures drop further, the bees draw closer together, conserving heat. The outer layer of bees becomes more tightly compacted, forming a definite shell (98). Furthermore, an active heat production is achieved with the increasing of the metabolic activity (96,99)

In order to understand the colony's thermoregulation, Fahrenholz (1989) developed a survey, over a period of 10 months (June 1985-March 1986), where measurements of temperature were made with reference to seasonal ambient temperatures and brood production. It was reported that, during the summer season, the temperature in the brood nest averaged 35 ºC with brief excursions up to 37.0 ºC and down to 33.8 ºC, while the ambient temperature changed between 15 ºC and 39 ºC. However, at the peripheral areas and at the hive entrance the temperatures may fluctuate widely with ambient temperature. Hives with broodless colonies showed lower temperatures than with breeding colonies.

During the period from October to March, the centre of an overwintering cluster it is maintained at an average of 21.3 º C, varying between 12 º C and 33.5 º C when the ambient temperature changed from -12 º C to 10 º C. The temperature at the cluster's periphery averaged 11.0 º C and constantly remained at a lower value than in the centre (100).

## II.2. Oligosaccharides origin

About 25 different oligosaccharides had been identified in honey (38). However, the origin of the majority is still a controversy, with the arising of multiple explanations. Most of the theories lay on the enzymatic activity of honey's enzymes, principally α-glucosidase. This chapter gathers all the available researches that attempt to give an answer to this problematic.

### II.2.1. Nectar composition

Floral nectars consist in a mixture of carbohydrates and of a wide variety of minor components, such as amino acids, proteins, enzymes, lipids, phenolics, glycosides, salts, alkaloids, vitamins, and other organic acids. Sucrose, glucose and fructose are the most abundant sugars, being their relative amounts determined by nectary invertase activity. Besides, other minor sugars are present in trace amounts in nectar. These may be monosaccharides (*e.g.* mannose, arabinose, xylose), disaccharides (maltose, melibiose) or, more rarely, oligosaccharides (raffinose, melezitose, stachyose) (19).

Percival (1961) (101) analysed 889 floral species and found three patterns of sugar composition: high sucrose nectar; about equal amounts of glucose, fructose, and sucrose; and high glucose and fructose. Honeybees showed to have preference to sucrose over fructose, and fructose over glucose, when fed with sugar solutions (102,103).

### II.2.2. Pollen composition

Pollen is the bees' main source of proteins, minerals, fats and vitamins, which are important for the normal development of a bee colony (104). As mentioned before, pollen contains enzymes, such as catalase (16) and acid phosphatase (61). Carbohydrates found in mature flower pollen comprise cell wall polysaccharides such as cellulose and pectin; starch;

disaccharides (*e.g.* sucrose and maltose); and also monosaccharides, such as glucose and fructose (105).

From the moment that pollen is collected by the bees, it acquires new characteristics (104), as well as different nutritional content (106), once bee pollen is a result of flower pollen mixed with nectar and bee secretions (107). A study conducted by Human and Nicolson in 2006 (106) showed that collection and storage of pollen by the bees resulted in a decrease of crude protein and in increased carbohydrate and moisture content.

### II.2.3. Honeydew composition

Honeydew is the excretory product of homopteran insects, such as aphids, whiteflies and scale insects, which feed by inserting their stylets into the phloem tissue of plants. Phloem sap is highly concentrated in carbohydrates and have relatively low concentration of other nutritional elements, such as minerals and amino acids. So, in order to obtain sufficient amounts of the minor components, insects must feed more or less continuously on phloem sap, being the excess excreted as honeydew (19,108). Therefore, honeydew represents an aqueous mixture of various sugars, which constitute more than 98% of the dry weight, together with amino acids and secondary plant compounds (109), namely volatile compounds (110). The sugar composition of honeydew depends on both the sap-sucking homopteran and its host plant. Most honeydews so far studied contained a mixture of monosaccharides (mainly fructose, glucose), disaccharides (sucrose, trehalose, maltose) and trisaccharides (melezitose, raffinose, erlose) (109,111).

Apart from the common origin of nectar and honeydew they are different, once the later passes through insects' digestive tract. This results in higher content of oligosaccharides, which are newly synthesized by the homopterans (38,109,112). Melezitose has been reported to be one of those oligosaccharides and represents, typically, 10% of honeydew (49). Another trisaccharide identified in honeydew was erlose (113,114).

### II.2.4. Transglycosylation reactions

Transglycosylation is a kinetically controlled reaction in which a glycosidase transfers a glycosidic residue from an activated donor to an acceptor, while retaining anomeric configuration (115). Thus, this type of reaction promotes the formation of

glycosidic linkages, or polymerization, and normally require high concentration of substrate (116).

### II.2.4.1. Melezitose origin

As previously reported, melezitose is absent from nectar or found in very small amounts, contrarily to honeydew. Therefore, its presence in floral honey requires explanation.

Bacon and Dickinson (1957) (114) reported the presence of the trisaccharide melezitose in honeydew, despite its absence in sap where the aphis and scale insects feed. Also, an enzyme preparation from aphids was incubated with sucrose and produced free fructose and glucose, melezitose and erlose. Therefore, this study hypothesizes the presence of an enzyme from aphids with a transglucosylase activity, capable of converting sucrose to melezitose.

Byrne and Miller (1990) (108) investigation support the previous stated. Their analyses of the phloem sap found no melezitose. However, honeydew produced from the same phloem sap by two scale insects had about 10 % of melezitose in its composition.

As seen in Table 4, floral honey shows small amounts of this trisaccharide, contrarily to honeydew honey, in which melezitose averages 4% (3). Its origin in the latter is related to considerable amounts of melezitose in honeydew. Concerning to floral honey, it can be suggested that the small quantity of this sugar may have originated as a result of honeydew collection by honeybees, beyond nectar collection (38,39).

### II.2.4.2. α-glucosidase from honey and honeybees

There are a few investigations that attribute the origin of oligosaccharides with α-glucosyl linkages to honey and honeybee invertase.

White and Maher (1953) (117) analysed the action of a honey invertase preparation on several sugars, such as sucrose, maltose, raffinose, melezitose, glucose, starch, melibiose, lactose and cellobiose. The results obtained with sucrose hydrolysis showed the production of six oligosaccharides, being the major one erlose. Besides, fructose, glucose, sucrose and maltose were also identified. The composition of honey invertase hydrolysate of maltose comprises 3 unidentified oligosaccharides, in addition to glucose and maltose. The invertase

reaction with glucose resulted in the formation of maltose and isomaltose. The sugars present after raffinose and melezitose hydrolysis with invertase were only the structural monosaccharides of both trisaccharides. Besides, raffinose hydrolysis also resulted in melibiose (*O*-α-D-galactopyranosil-(1→6)-*O*-α-D-glucopiranose), hence there is a small fructosidase activity in the honey invertase preparation. Also, starch degradation showed the presence of an amylase in the preparation. In the case of melibiose, lactose and cellobiose, no hydrolytic action took place. Therefore, it can be concluded that α-galactosidase, β-galactosidase and β-glucosidase where low or absent of the tested enzyme preparation. This study suggests that honey invertase possesses transglucosylation activity. However, it should be taken in consideration that these results were obtained with crude preparations, and not with highly purified enzyme preparations.

In 1988 Low (57) had also demonstrated the presence of an enzyme with transglucosylation activity in honey. A crude enzyme with invertase activity, isolated from honey, was prepared and incubated with a typical nectar-type solution of fructose, glucose and sucrose, and also with a solution of glucose and a solution of fructose. Incubation with the first solution resulted in detectable amounts of disaccharides (sucrose, neotrehalose, turanose, maltose, kojibiose, gentiobiose and isomaltose) and of only one trisaccharide (erlose). Glucose incubation with the crude enzyme preparation resulted in measurable amounts of maltose, gentiobiose, nigerose, isomaltose and kojibiose, but no trisaccharides. Fructose incubation did not form oligosaccharides. It can be concluded that the analysed crude enzyme has transglucosylation activity, especially with the nectar-type solution, but has no transfructosylation activity, once no oligosaccharides where form when only fructose was incubated.

As already mentioned, honeybees invertase is considered to be the main source of honey invertase (16,20). Therefore, it is imperative to evaluate if this enzyme activity matches the results obtained with the enzyme isolated from honey. A study conducted by Huber and Mathison in 1976 (118) with an α-glucosidase isolated from honeybees, that was purified to homogeneity, has also reported transglucosylation activity. The reaction of this enzyme with sucrose as substrate resulted in three bands, when separated by TLC (thin-layer chromatography). These correspond to glucose and fructose, sucrose (and possibly other disaccharides) and trisaccharides. However, the presence of trisaccharides was not further proved, once none of them were isolated and or identified.

### II.2.4.3. Enzymatic activity of microflora

Honey is a matrix with a varied microflora, as previously mentioned. Bacteria, yeast and mould may be found in honey (24). These organisms also possess hydrolytic enzymes, such as α-glucosidases and β-fructofuranosidases. It may be hypothesized that some oligosaccharides present in honey are originated by these enzymes (38).

Microbial β-fructofuranosidases are known to have the capability of catalyse the synthesis of short-chain fructooligosaccharides (FOS), apart from the sucrose hydrolysis. Depending on the enzyme source, one to three fructosyl moieties are linked to sucrose by different glycosidic bonds (119). Therefore, these enzymes may originate the honey oligosaccharides with more than one β-fructofuranosyl.

Below are presented a few works that demonstrate both transfructosylation and transglucosylation activity of enzymes from microorganisms that are found in honey.

#### II.2.4.3.1. Transfructosylation activity

A study isolated a total of 1752 strains of osmophilic yeasts from pollen and honey. Among them, only 409 strains had the capability to hydrolyse sucrose to fructose and glucose. Also, it was observed that 52 osmophilic yeasts produced extra and/or intracellular β-fructofuranosidase, which was capable of converting sucrose to fructooligosaccharides. The majority of the yeasts belonged to those isolated from pollen. The FOS obtained were 1-kestose, nystose, 6-kestose, neokestose and fructofuranosyl nystose. Only one strain, obtained from honey, could convert sucrose to 6-kestose and neokestose, and it was identified as a *Candida* sp. (120).

An investigation carried by Álvaro-Benito (2007) (121) was performed with *Schwanniomyces occidentalis,* a yeast known to be present in honey (38). The hydrolytic reaction of sucrose with yeast β-fructofuranosidase resulted in the identification of two trisaccharides, 1-kestose and 6-kestose. Accordingly, the amount of fructose quantified was considerably smaller than that quantified for glucose. Thus, it can be concluded that this β-fructofuranosidase hold transfructosylation activity.

Khandekar (2014) (122) also reported the transfructosylation activity for *Saccharomyces cerevisiae*. This investigation presented the synthesis of FOS from sucrose

using the yeast invertase. Apart from the sucrose monomers, the reaction resulted mainly in 1-kestose and small amounts of nystose.

In addition, several studies have been reported on the synthesis of FOS using β-fructofuranosidase from either bacterial sources or fungal sources, like *Aspergillus* (123–125), and *Bacillus* (126).

#### II.2.4.3.2. Transglucosylation activity

Several common microorganisms are known to have enzymes with transglucosylic action. The yeasts α-glucosidase is known to be capable of transfer α-glucosyl from substrate to D-glucose, D-xylose, D-mannose and also to D-fructose (127). For example, studies with an enzyme from brewer's yeast reported the synthesis of turanose, maltulose and isomaltulose during sucrose hydrolysis (127,128). Another study conducted with brewer's yeast α-glucosidase, using phenyl-α-glucoside as substrate and D-fructose as an acceptor, resulted in the formation of turanose and maltulose and another three unidentified sugars (127).

The osmophilic yeast (129), *Schizosaccharomyces pombe*¸ was also part of an investigation. It was demonstrated that disaccharides, namely nigerose, kojibiose and isomaltose, were synthesized from glucose with a yeast preparation (130).

Species of *Aspergillus* were also described in honey, and are part of the intestinal microflora of honeybees (81). Actually, an α-glucosidase isolated from *Aspergillus nidulans* was reported to have strong transglucosylation activity towards maltose. The transglucosylation reaction resulted, mostly, in panose, isomaltose and maltotriose, even with low concentration of substrate (131).

### II.2.5. Non-enzymatic reactions

Condensation of sugars is known to occur when an acid solution of sugar of high concentration is left at ambient temperature. Therefore, these conditions induce the formation of oligosaccharides in a process designed as reversion (128,132–136), which consists essentially of non-enzymatic transglycosylation reactions. Besides, anhydrosugars and furan derivatives can also be obtained in these conditions. Reversion products are undesirable in acid-catalysed hydrolysis of polysaccharides, as the objective of these

processes are the production of syrups and of fermentable substrates. Therefore, the kinetics of reversion reactions have been a subject of study, in order to optimize the monosaccharide production (135,137,138).

Between the three main sugars of nectar, glucose was the most reported to yield reversion products. Besides, Silberman (1961) (134) described the tendency of other aldoses (D-galactose and D-mannose) to form oligosaccharides. Also, the same study reported that none of the tested ketoses (D-fructose and D-sorbose) produced oligosaccharides, but were found to produce furan derivatives, namely 5-hydroxymethyl-2-furaldehyde. The predominant products of glucose reversion are those with (1→6)-linkages (134,139,140). This is likely due to steric interactions, being the two glucose rings kept farther apart than with the other linkages (141). Besides, the hydroxyl from the hexopyranoses' C6 belongs to the primary hydroxyl group, which is known to be more reactive than the remain secondary hydroxyl groups (142). Actually, glucose reversion presents at least 11 possible products, which are describe at Table 7. So, reversion could be the origin of honey's carbohydrates, especially those with β-glucopyranosyl, once β-glucosidase activity was not associated with these oligosaccharides. Nonetheless, it should be noted that all the above mentioned researches, concerning glucose reversion, were performed under high temperatures. Further, oligosaccharides were isolated from two different commercial invert syrups, which are obtained by acid or enzymatic hydrolysis of sucrose in high concentration. Between the identified carbohydrates are 6-kestose (*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(6→2)-β-D-fructofuranoside) and *O*-α-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl-α-D-glucopyranoside, from commercial beet medium invert syrup (143), and turanose (*O*-α-D-glucopyranosyl-(1→3)-D-fructose), *O*-β-D-fructofuranosyl-(2→6)-D-glucose and α,β-trehalose (α-D-glucopyranosyl-β-D-glucopyranoside), from commercial total invert sugar (135).

**Table 7 –** Glucose reversion products found in mildly acidic aqueous solutions (141)

| Common name | Systematic name |
|---|---|
| α,α-Trehalose | α-D-glucopyranosyl-α-D-glucopyranoside |
| α,β-Trehalose | α-D-glucopyranosyl-β-D-glucopyranoside |
| Isotrehalose | β-D-glucopyranosyl-β-D-glucopyranoside |
| Kojibiose | *O*-α-D-glucopyranosyl-(1→2)-D-glucopyranose |
| Sophorose | *O*-β-D-glucopyranosyl-(1→2)-D-glucopyranose |
| Nigerose | *O*-α-D-glucopyranosyl-(1→3)-D-glucopyranose |
| Laminaribiose | *O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose |
| Maltose | *O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose |
| Cellobiose | *O*-β-D-glucopyranosyl-(1→4)-D-glucopyranose |
| Isomaltose | *O*-α-D-glucopyranosyl-(1→6)-D-glucopyranose |
| Gentiobiose | *O*-β-D-glucopyranosyl-(1→6)-D-glucopyranose |

The reaction mechanisms for reversion reactions (Fig. 2) involve the formation of an intermediate carbocation at the C1 carbon atom of glucose, resultant of the loss of a water molecule under acidic medium. This hydroxyl group has the largest affinity for protons, and the resulting carbocation is stabilized by the oxonium ion resonance structure. The hydroxyl group of another sugar molecule can then add to the carbocation site to form a disaccharide. Due to the carbocation give essentially a planar structure, the hydroxyl group can add to either side and the stereochemistry of the anomeric C1 is twisted. Thus, addition of the other hydroxyl groups to the carbocation can form α and β isomers of 1,6-, 1,4-, 1,3- and 1,2-linked disaccharides, as seen in Table 7 (141).

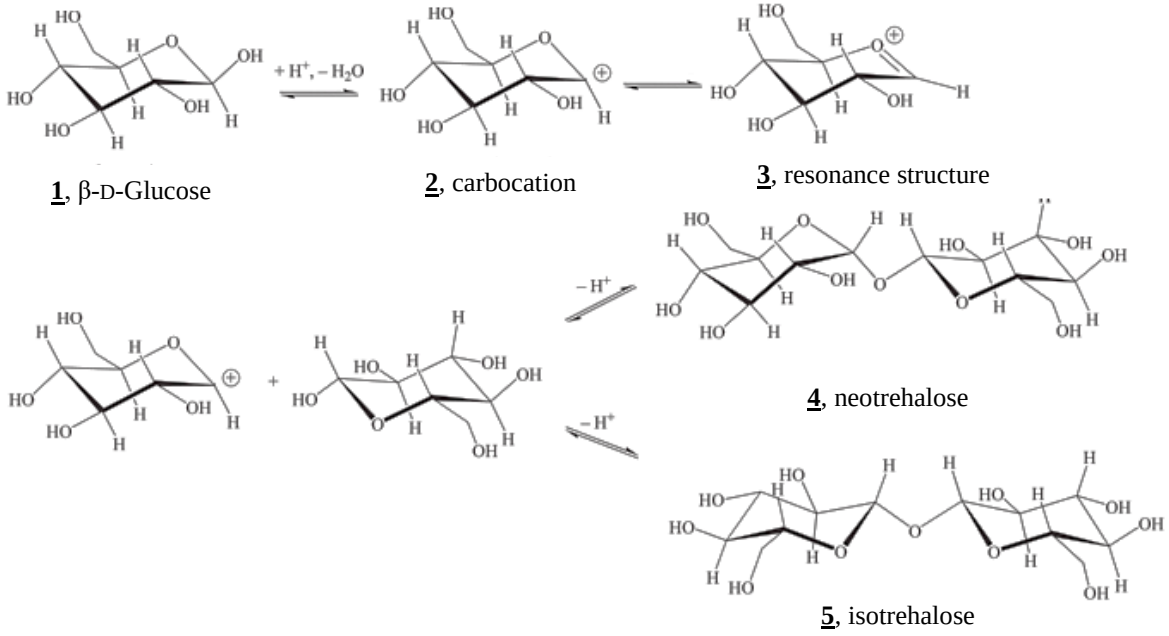



**Figure 2 –** Reversion mechanism for the formation of neotrehalose and isotrehalose (141).

It is hypothesized that the previous non-enzymatic reactions may be behind the origin of some honey oligosaccharides (49). Below are presented a few researches that support this hypothesis.

White (1961) (144) studied the effect of honey storage at room-temperature (26 ± 3 º C) during 22 months. Extensive changes in sugar composition had occurred, and the change was in the direction of increased complexity. Monosaccharide content decreased 18.5%, with glucose decreasing 13%, while fructose decreased 5.5%. The reduction of glucose in twice as much as fructose may be reflecting the specificity of honey invertase to glucose transference. Besides, the reducing disaccharide fraction and trisaccharide content increased to 68% and 13%, respectively, over their original levels. Content in sucrose had also increased. There are a few possible explanations for the reported changes: the transglucosylase activity of invertase; the presence of fungal or bacterial enzymes with transfructosylase activity; and reversion. As previously referred, a high sugar concentration and a considerable acidity, which are honey characteristics, over a period of time would promote condensation of monosaccharides. However, since honey enzymes were not inactivated, all of these theories can be assumed.

More recently, another study was conducted in order to infer about the changes undergoing during storage. This investigation consisted in the preparation of four samples

of the same nectar honey, in which two of them were subjected to stabilization treatment (in order to destroy the enzymes), through heating at 100 º C, during 15 minutes. From these two, one was stored at a temperature of 20 º C, and the other at 4 º C. The two remain samples were also stored at those different temperatures. The analysis, after 24 weeks of storage, showed that samples subjected to stabilization had less variations in their carbohydrates content. Nevertheless, variations still occurred, being equal for both storage temperatures. The most impacted values belonged to sucrose, with a reduction to half of its initial composition, and to melezitose and erlose (+250%), while the remaining disaccharides (turanose, maltose, isomaltose and trehalose) showed small reductions. After half-year, the nectar sample stored at 20 º C, without heat treatment, had no traces of sucrose, composition in monosaccharides suffered a small increase, with turanose content increasing 20% of its initial value. Moreover, the non-stabilized samples stored at 4 º C showed no significant variations to all sugars, except melizitose and erlose content that had a huge increasing, relatively to its initial value. Therefore, these results indicate the occurrence of nonenzymatic processes, since compositional variations took place in samples lacking of enzymatic activity (145).

Furthermore, Castro-Vásquez et al. (2008) (146) had also conducted a research in order to analyse the effect of storage conditions in honey. A fresh citrus honey was stored at three different temperatures, 10, 20, and 40 º C, for 12 months. Monosaccharides presented very important losses during storage which amounted from 13.5 to 25.2%, being linear with the increasing of temperature. Disaccharide concentrations showed a general increasing trend during storage, with changes being more marked at 40 º C. This behaviour is common to the main disaccharides (nigerose, turanose, maltulose, isomaltose, and kojibiose). The most important change during storage corresponded to maltose, present initially at 2.5 mg/g and becoming one of the major disaccharides in the sample (23.2 mg/g) after 1 year at 40 º C. Changes in trisaccharide composition during storage appeared to depend on the individual component being considered. In conclusion, this study reports the increasing of complexity of honey's carbohydrates along with storage.

## II.3. Prebiotics

### II.3.1. Definition

Prebiotics are described as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, resulting in the improvement of host health (147). Further, a prebiotic can be considered as a growth substrate that fortifies the beneficial intestinal microflora (148). Thus, it does not promote potential pathogens such as toxin-producing clostridia, proteolytic bacteroides and toxigenic *Escherichia coli*. In this manner, bifidobacteria and/or lactobacilli become the predominant microorganisms in the intestine, which activity may promote health beneficial effects (149).

The prebiotic effect has been attributed to many food ingredients, particularly oligosaccharides and polysaccharides (including dietary fibre). Nevertheless, not all dietary carbohydrates are prebiotics (147). So, in order to establish a certain substrate as a prebiotic it needs to obey to the following criteria: (1) be neither hydrolysed nor absorbed in the upper part of the gastrointestinal tract (GIT); (2) be selectively fermenteded by commensal beneficial bacteria in the colon and (3) induce luminal or systemic effects that are beneficial to the hosts' health (147–149). Examples of oligomers suggested to have prebiotic potential are: lactulose; FOS; galacto-oligosaccharides; lactosucrose; isomalto-oligosaccharides; gluco-oligosaccharides; xylo-oligosaccharides; and palatinose (149).

### II.3.2. Health benefits

A number of benefits can be ascribed to prebiotic intake. These include protection against development of colon cancer and irritable bowel disease; increased mineral absortion; improved bowel habits; controlled serum lipids and cholesterol (149,150). These effects can be attributed to the end products of prebiotics' fermentation by colonic bacteria, which are hydrogen, methane, carbon dioxide, lactate and short-chain fatty acids (SCFAs), mainly acetate, propionate and butyrate. Butyrate is the preferencial energy source of colonocytes, being determinant to the metabolic activity and growth of these cells (150). It was shown to induce apoptosis in colonic cancer cell lines (151), thus is considered a protective factor against colonic disorders. Besides, acetate is metabolized by the brain,

muscles and tissues, whereas propionate is used by the liver and may interfer with the cholesterol' synthesis, lowering its production.

Further, fermentation and SCFA production also inhibit the growth of pathogenic organisms by reducing luminal and fecal pH. Low pH reduces peptide degradation and the resultant formation of toxic compounds such as ammonia, amines, and phenolic compounds, and decreases the activity of undesirable bacterial enzymes, which increase the incidence of bowel cancer. Moreover, low pH increases minerals solubility, increasing its absortion (149,150,152).

### II.3.3. Honey components with prebiotic effect

Several studies report a potential prebiotic effect of honey's oligosaccharides, both *in vitro* and *in vivo* (11,153,154). These studies describe that the presence of honey causes the enhancement of lactobacilli counts *in vitro* and in rats, when comparing to sucrose (153), and also the increasing of bifidobacteria's population, in pure culture (154). In addition, in 2005 Sanz (11) isolated the oligosaccharides from honey to assay their potential prebiotic activity. These oligosaccharides were seen to increase the populations of bifidobacteria and lactobacilli, but not the levels seen with commercial FOS.

Knowing the composition of the oligosaccharides fraction of honey, the assessed prebiotic activity could be mainly attributed to the occurring FOS, such as 1-kestose, 6-kestose and neokestose (11). FOS are well-established prebiotics (155,156), which have proven numerous health benefits (157). These carbohydrates consist of a chain of fructose unit linked to glucose (158,159), in which the fructosyl-glucose linkage is α(2→1) and the fructosyl-fructose linkages are β(2→1) (160). Depending on the degree of polymerization (DP), *i.e.* on the number of fructosyl residues, FOS exist in several forms such as 1-kestose (DP 3), nystose (DP 4) and 1-fructofuranosyl nystose (DP 5) (122). Besides, other honey sugars had been identified as non-digestible oligosaccharides with bifidogenic functions, such as raffinose, palatinose (161,162) and panose (163).

# Chapter III

Materials and Methods

# III. Materials and Methods

## III.1. Honey Samples

Honey under analysis was obtained from three distinct apiaries and with the ripening process differs in season and in duration. This allowed accessing the influence of the maturation conditions in honey composition, particularly in the carbohydrates profile and quantity. Table 8 describes the characteristics of honey under study, namely the botanical and geographical origin, as well as the period and duration of the maturation process.

**Table 8 –** Production characteristics of the honey samples.

| Sample | Geographical origin | Floral origin[a] | Period inside the hive | Length of maturation (months) |
|---|---|---|---|---|
| **H2** | Vila Real | Multiflora | May 16' – July 16' | 2 |
| **H4** | Oliveira de Azeméis | Chestnut, heather, blackberry[b] | March 16' – July 16' | 4 |
| **H8** | Oliveira de Azeméis | Eucalyptus | July 15' – March 16' | 8 |
| **H12** | Guarda | Multiflora | July 15' – July 16' | 12 |

[a]Information provided by the beekeeper. [b]The beekeeper grows these plant species in the field surrounding the hive.

## III.2. Model solutions

A total of six model solutions were prepared with the principal sugars present in nectar, *i.e.* sucrose, glucose and fructose. It was prepared three solutions of sucrose (Suc) plus glucose (Glc) and another three of sucrose plus fructose (Fru) with 80 % (w/w) of sugars. For these solutions, it was weighed 4 g of each carbohydrate, which were dissolved in 2 mL of ultrapure water, in 2 mL of a citric acid solution with pH 4.0 and in 2 mL of a citric acid solution with pH 2.0. All solutions were homogenized on a vortex mixer and by sonication and stored in an oven at 35 ºC, over 5 months.

To make easy the designation of these solutions, codes will be used henceforth. The solutions will be designated with the first letters of the two carbohydrates (SG and SF, for Suc plus Glc and for Suc plus Fru, respectively), followed by a number between 1 and 3 concerning the solvent used. Number 1 is for ultrapure water, number 2 is for the diluted citric acid at pH 4.0 and number 3 for the diluted citric acid at pH 2.0. Example: SG 3 corresponds to the model solution of sucrose plus glucose prepared with diluted citric acid at pH 2.0.

### III.3. Water activity (aw) determination

Honey water activity was determined at 23 ºC (± 0.7) using a Novasina Thermoconstanter electric hygrometer (Novasina – AG, Zurich, Switzerland).

### III.4. Water loss determination at 105 º C

The water loss was determined using an oven-drying method (164). Honey samples (1 g) were dried in an air-oven at 105 º C until a constant weight was achieved. The water loss at 105 º C estimates the moisture content of honeys.

### III.5. pH determination

The pH of honey and of model solutions was measured using a pH-meter (TitroMatic 1S) with a precision of ± 0.02 pH units in a solution of 10 % (w/v) of honey dissolved in ultrapure water, according to the Harmonised methods (165). All measurements were performed in triplicate.

### III.6. Oligosaccharides fractionation

The four different honeys and the six model solutions, with 3 and 5 months of incubation, were fractionated by semi-preparative ligand-exchange/size-exclusion chromatography (LEX/SEC) on a high-performance liquid chromatograph equipped with a Shodex sugar KS 2002 column (300 mm of length and 20 mm of internal diameter) from Showa Denko K. K. (Tokyo, Japan). The column was maintained at 30 º C, the injected

sample volume was 500 μL and ultrapure water was used as eluent at a flow rate of 2.80 mL/min. A refractive index detector (Knauer K-2401, Berlin, Germany) was used. All the collected fractions were dried and kept in a desiccator at ambient temperature, for further analysis. To obtain the retention time corresponding to the different degrees of polymerization, a standard solution containing fructose, glucose, sucrose and melezitose (20 mg/mL) was injected, using the same chromatographic conditions used for the samples separation.

### III.7. Electrospray ionization mass spectrometry (ESI-MS and ESI-CID-MSⁿ)

The occurrence of non-enzymatic transglycosylation reactions in model solutions throughout time, as well as its extension, was assessed by electrospray ionization mass spectrometry (ESI-MS) and electrospray ionization collision-induced dissociation tandem mass spectrometry (ESI-CID-$MS^n$) analysis. Each sample, previously dissolved in ultrapure water, was diluted in methanol/water (1:1, v/v) containing formic acid (1%, v/v). Samples were introduced into the mass spectrometer using a flow rate of 8 μL/min. Positive ion ESI-MS and ESI-$MS^n$ spectra were acquired using a LXQ linear ion trap (LIT) mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Positive mode was preferred because better signals were obtained in positive than in negative mode. This is due to the easier facility of sugars to form $[M+Na]^+$ ions. Typical ESI conditions were as follows: electrospray voltage, 5 kV; capillary temperature, 275 °C; capillary voltage, 1 V; and tube lens voltage, 40 V. Nitrogen was used as nebulizing and drying gas. ESI-MS spectra were acquired over the range *m/z* 100−1500. ESI-CID-$MS^n$ spectra were acquired with the energy collision set between 19 and 29 (arbitrary units). Data were acquired and analysed using Xcalibur software (166,167).

The Q Exactive hybrid quadrupole − Orbitrap mass spectrometer (Thermo Firsher Scientific, Germany), interfaced with an H-ESI II ion source, was employed for accurate mass measurements of the LEX-SEC fractions (F1 and F2) obtained from honey samples. Before MS analysis, the acidic fraction (F1) was incubated with cation exchange resin for 20 min at room temperature (168). The acquisition method was set with a full scan and 140,000 resolution (relative to *m/z* 200) in positive mode. The method parameters were as follows: AGC, 3e6; IT, 100 ms; scan range, 100− 1500; spray voltage, 3.0 kV; sheath gas,

5; aux gas, 1; capillary temperature, 250 º C; S-lens RF level, 50; probe heater temperature, 50 º C; and flow rate, 5 μL/min. The Q Exactive system was tuned and calibrated in positive mode using peaks of known mass from a calibration solution (Thermo Scientific) to achieve a mass accuracy of <0.5 ppm RMS. The data were processed with Xcalibur 3.0.63 software (166).

### III.8. Linkage analysis

The glycosidic linkages established between the monosaccharides of honey and of model solutions with an incubation of 5 months were identified by methylation analysis, using the same procedure as Simões et al. (2013) (169). This method consists in the methylation of the free hydroxyl groups, followed by the hydrolysis of polysaccharides. The resultant monosaccharides are reduced and the hydroxyl groups, which were involved in the glycosidic linkages or in the ring formation, are acetylated. The final products are partially methylated alditol acetates (PMAAs), which are analysed by gas chromatography-quadropole mass spectrometry (GC-qMS).

The di- and trisaccharides dried fractions of honey and of model solutions (1-2 mg), obtained from LEX-SEC, were placed in a vacuum oven during 2h with the presence of $P_2O_5$ and were dissolved in 1 mL of anhydrous dimethylsulfoxide (DMSO), stirring for 2h at ambient temperature. NaOH pellets (40 mg) powdered under argon were added to each solution, standing for 30 min in a magnetic stirrer at ambient temperature. Then, samples were methylated with $CH_3I$ (80 μL) during 20 min with stirring, followed by a second addition of $CH_3I$ (80 μL) and stirring for another 20 min. Distilled water (2 mL) and dichloromethane (3 mL), were then added, and dichloromethane phase was washed three times by addition of distilled water (2 mL). The organic phase was evaporated to dryness and remethylated to achieve a complete methylation of all free OH groups. The methylated material was hydrolysed with TFA 2 M at 121 º C for 1 h, cooled, and evaporated to dryness. The partially methylated sugars were then reduced with 0.3 mL of 2 M $NH_3$ and 20 mg of $NaBD_4$, during 1h at 30 º C, and the excess of the reducing agent was destroyed by the addition of 0.1 mL of glacial acetic acid. The acetylation was subsequently performed with acetic anhydride (3 mL) in the presence of 1-methylimidazole (450 μL) during 30 min at 30 º C. This solution was treated with water (3 mL) to decompose the excess of acetic

anhydride, and the partially methylated alditol acetates (PMAAs) were extracted with dichloromethane (2.5 mL). The dichloromethane phase was washed two times with water (3 mL) and evaporated to dryness. The PMAAs were separated and analysed by gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies 6890N Network. The GC was equipped with a DB-1 (J & W Scientific, Folsom, CA, USA) capillary column (30 m length, 0.25 mm of internal diameter and 0.10 μm of film thickness). The samples were dissolved in anhydrous acetone (20 – 30 μL) were injected in split mode with the injector operating at 250 º C, during 5 min. The temperature program used was as follow: initial temperature was 80 º C, with a linear increase of 10 º C/min up to 140 º C, and standing for 5 min at this temperature, followed by linear increase of 0.2 °C/min until 150 °C, followed by linear increase of 60 º C/min up to 250 º C, with further 2 min at this temperature. The helium carrier gas had a flow rate of 1.84 mL/min and a column head pressure of 124.1 kPa. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 50–700 in a 1 s cycle in a full scan mode acquisition. Further, the retention time and spectrum correspondent to terminally-, (2→1)- and (2→3)-linked fructose residues were obtained by conversion of sucrose, 1-kestose and melezitose into PMAAs, using the procedure mentioned above.

### III.9. Oligosaccharides identification and quantification

Oligosaccharides present in the model solutions, after 5 months of incubation, and in honey were quantified as alditol acetates derivatives with gas chromatography-flame ionization detection (GC-FID), using a modified version of the method of Blakeney et al. (1983) (170). The fractions containing di- and trisaccharides, obtained from LEX-SEC were derivatised by adding 200 μL of a sodium borohydride solution (15 % (m/v) in $NH_3$ 3M) and incubated at 30 º C for 60 min. After, the excess of the reducing agent was destroyed by the addition of glacial acetic acid (0.1 mL). The acetylation of the alditols was performed by adding 1-methylimidazole (0.45mL) and acetic anhydride (3mL) and allowed to react for 30 min at 30 º C. This solution was treated with water (3 mL) to decompose the excess of acetic anhydride, and the alditol acetates were extracted with dichloromethane (5 mL). The dichloromethane phase was washed two times with water (3 mL) and evaporated to dryness.

The alditol acetates were dissolved in anhydrous acetone (10 – 50 µL) and analysed by GC–FID equipped with a 400-5HT column (Quadrex, New Haven, CT, USA) with 25 m length and i.d. and film thickness of 0.25 mm and 0.05 µm, respectively. The oven temperature program used was: initial temperature of 100 º C, a rise in temperature at a rate of 10 º C/min until 200 º C, standing for 2 min, followed by a rate of 1.0 º C/min until 250 º C and maintaining this temperature 2 min, with a final linear increase of 15 º C /min until 400 º C, standing for 1 min. The injector and detector temperatures were, respectively, 300 and 400 º C. The flow rate of the carrier gas ($H_2$) was set at 1.7 mL/min. Derivatization of all model solutions' samples was performed in duplicate. The total sugars content was achieved by the sum of the individual contribution of each sugar residue to the total mass introduced into the LEX-SEC column.

Further, to attempt the identification of the oligosaccharides, the alditol acetates derivatives obtained from the disaccharides fractions were dissolved in anhydrous acetone (50 µL) and analysed by GC–MS, with the same column used for the linkage analysis. The samples were injected in split mode (split ratio of 33), with the injector operating at 250 º C, using the following temperature program: initial temperature of 140 º C followed by a linear increase of 5 º C/min until 180 º C, and standing 1 min at this temperature, followed by a linear increase of 5 º C/min until 250 º C, maintaining this temperature 10 min, with further linear increase of 10 º C/min until 325 º C, standing 3 min at this temperature. Linear velocity of the carrier gas (He) was set at 35 cm/s at 200 º C, with a solvent delay of 2 min. MS scans were performed for GC–MS between 700 and 33 *m/z* at 70 eV ionization energy.

### III.10. Statistical analysis

Significant differences ($p < 0.05$) in honeys' physical properties were assessed by one-way (ANOVA) using PRISM® GraphPad Software, Inc. (GraphPAd Software 7.03; GraphPAd Software, Inc., La Jolla, CA).

# Chapter IV

Results and Discussion

# IV. Results and Discussion

## IV.1. Physical properties of honeys

The physical properties, namely water activity, moisture content and pH, of the four honeys under study are presented in Table 9. All honey samples obey to the composition criteria established by The Codex Alimentarius Committee on Sugars (25), with $a_w$ values inferior to 0.60 and moisture percentages lower than 20 %. Also, the pH values are within the pH range of floral honey (3.0-4.7) (40). Among the four samples, no statistically significant differences were found in moisture content ($p > 0.05$), but statistically significant differences were found in $a_w$, and pH ($p < 0.05$).

**Table 9** – Physical properties of the four different honeys (Mean ± Standard deviation)

| Parameter | H2 | H4 | H8 | H12 |
|---|---|---|---|---|
| **Water activity ($a_w$)** | 0.530 | 0.557 | 0.564 | 0.533 |
| **Moisture content (%)*** | 15.58 ± 0.59 | 16.59 ± 0.64 | 17.10 ± 0.20 | 15.54 ± 0.53 |
| **pH** | 4.38 ± 0.03 | 4.19 ± 0.02 | 4.18 ± 0.08 | 3.96 ± 0.04 |

*Determined as water loss at 105 ° C.

## IV.2. pH values of Model Solutions

The pH values obtained for the six model solutions, after 3 months of incubation, are displayed in the following table (Table 10). Similar values are observed for solutions prepared both with water and with diluted citric acid at pH 4.0. Water solutions of highly concentrated sugars were reported with acid character, due to being amenable to be deprotonated and introduce acidity to the solution. Further, a study showed sugars to have a greater affinity for $H^+$ than water and proposed that, at high sugar concentrations, these polyols act as solvents, increasing the solvation energy of the protons, which results in a greater acidity of the solution (171). Moreover, solutions prepared with diluted citric acid at pH 2.0 had a final pH value of 2.6, approximately, but no explanation was found for this phenomenon.

**Table 10** – pH values of the model solutions, after 3 months (Mean ± Standard deviation)

| | SG | | | SF | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| **pH** | 3.67 ± 0.10 | 3.73 ± 0.09 | 2.56 ± 0.07 | 3.85 ± 0.03 | 4.41 ± 0.06 | 2.63 ± 0.01 |

## IV.3. Fractionation of carbohydrates by LEX/SEC

### IV.3.1. Honey

The LEX/SEC chromatograms obtained for the four honeys (20 mg/mL) are shown in the Figure 3. According to results of previous studies (172,173), the peak with the lowest elution time (8-10 min; F1) was assigned to the acidic fraction, which in honey represents essentially gluconic acid (16). Also, considering the elution time of the different carbohydrates from the standard solution, the fraction eluted at 13-15 min (F3) was mainly assigned to neutral trisaccharides, while the fraction eluted at 15-17 min (F4) was mainly assigned to neutral disaccharides. The fractions eluted at 17-19 min (F5) and 19-21 min (F6) were attributed to glucose and fructose, respectively. Besides, the fraction between the acidic and the trisaccharides' fraction (10-13 min; F2) was assigned to higher oligosaccharides, despite the absence of a peak.

As previously demonstrated, honeys under study have different ripening conditions. The chromatograms obtained by LEX/SEC for honeys which maturation had a length of 2 and 12 months and for honeys which maturation occurred during summer and winter were overlaid. Besides the refraction index being higher for fraction F3 of H4 when compared to that of H8, no significant differences are observed between either the two sets. Nonetheless, more accurate methods are necessary to understand the compositional differences between them, and thus the impact of the ripening conditions.

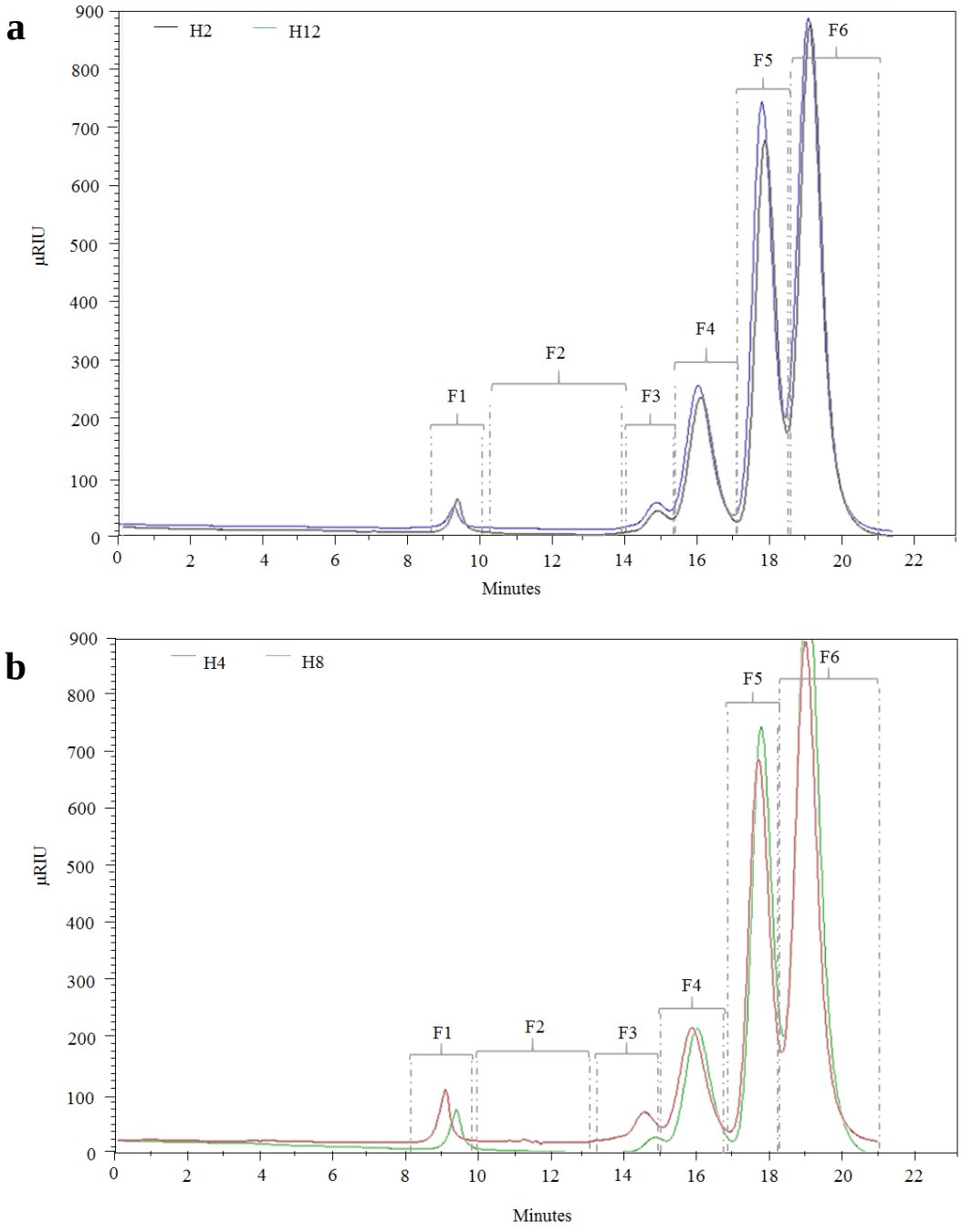


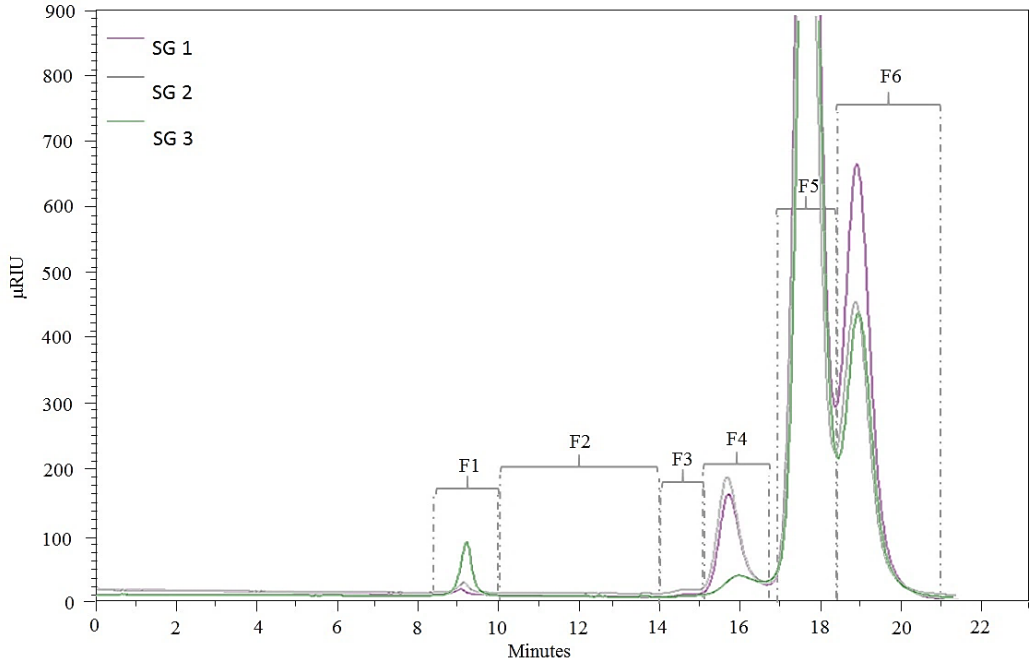

**Figure 3 –** LEX/SEC chromatograms of: (a) honey with 2 (H2) and 12 (H12) months of maturation; (b) honey maturated during summer (H4) and winter (H8).

### IV.3.2. Model solutions

Model solutions with 3, 4 and 5 months of incubation were separated by LEX/SEC in 6 different fractions, as previously described for honey. Over the 5 months' incubation

period, modifications on the fractions' refractive index are observed for both types of solutions (see Appendix A). As observed in Figure 4, the most evident differences are in fraction F4 (disaccharides), which suffered a decrease throughout time, presumably, as a result of the hydrolysis of sucrose. This can be supported by the appearance of the fructose fraction (F6) on glucose prepared solutions and of the glucose fraction (F5) on the fructose model solutions. The higher extent of this reaction, or the lowest intensity of fraction F4, was seen for the solutions with the lowest pH values (SG 3 and SF 3). This was expected, as hydrolysis of sucrose is acid-catalysed (174). That is in accordance with the increase in fructose peak from the model solution where it was absent (SG 3) and the increase in glucose peak also from SF 3.

**a**



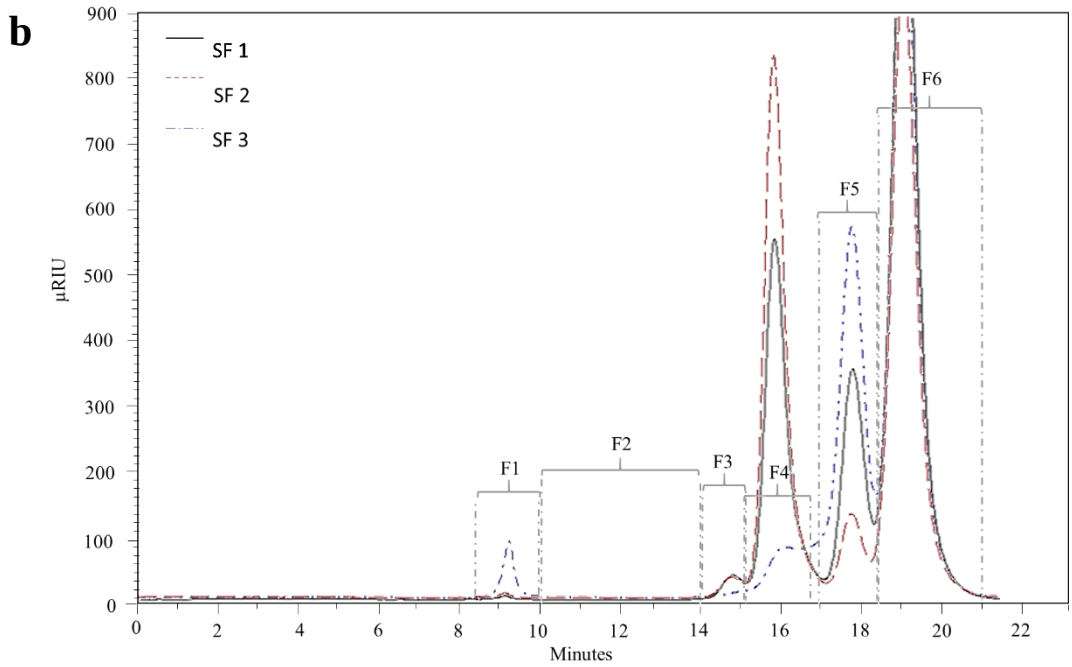



**Figure 4 –** LEX-SEC chromatograms of model solutions with 5 months of incubation of: (a) SG and (b) SF.

## IV.4. ESI-MS analysis

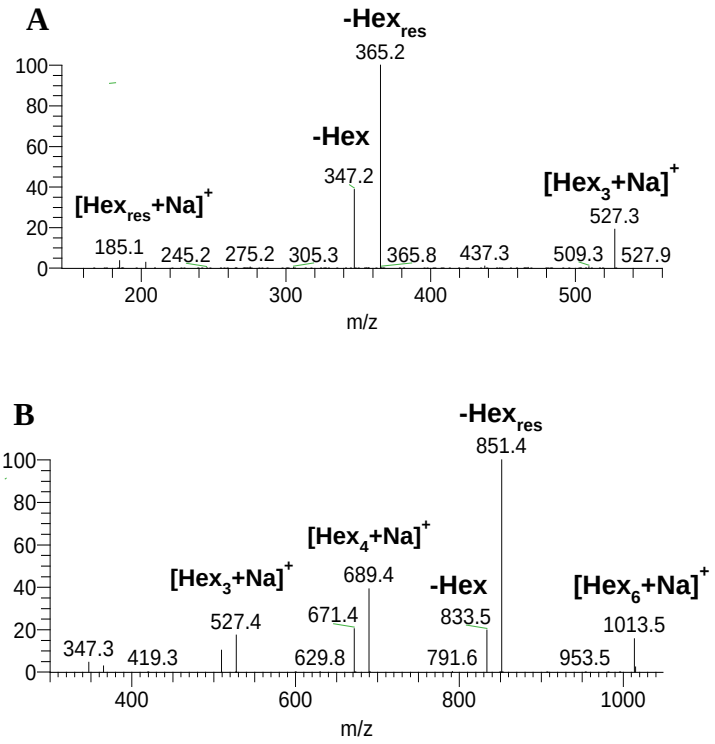
### IV.4.1. Model solutions

The monitoring of changes occurring in model solutions composition was also assessed by ESI-MS analysis. Initially, mixtures of Suc plus Glc and of Suc plus Fru, without any treatment or incubation period, were analysed to evaluate the presence of ions at *m/z* correspondent to a DP higher than 2. Ions at *m/z* 527, 689, 851 and 1013, corresponding to a DP of 3, 4, 5 and 6, respectively, were observed with a relative abundance up to 3%. Therefore, to avoid misinterpretations, only ions with a relative abundance ≥ 3 % in ESI-MS spectra acquired from incubated model solutions were considered. The $[M + Na]^+$ ions identified in the latter ESI-MS spectra are summarized in Table 10. The assignment of these ions was supported on the basis of their fragmentation pattern under ESI-CID-$MS^n$ conditions (Figure 5 and Figure 6), in which the neutral losses with 162 and 180 Da correspond, respectively, to a loss of a hexose residue (-$Hex_{res}$) and a hexose (-Hex).

**Table 10 –** Non-enzymatic transglycosylation products identified in model solutions by ESI-MS with respective *m/z* values of the $[M + Na]^+$ ions and proposed assignments

| Proposed assignment[a] | no. (*n*) of hexose (Hex) units | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** |
| $[Hex_n + Na]^+$ | 203 | 365 | 527 | 689 | 851 |
| $[Hex_n - H_2O + Na]^+$ | | 347 | 509 | 671 | 833 |
| $[Hex_n + CitA + Na]^+$ | 377 | 539 | 701 | | |

[a] "CitA" stands for citric acid.



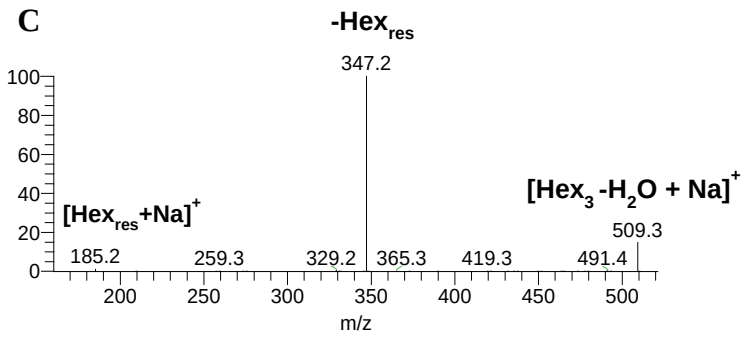



**Figure 5** – ESI-CID-MS$^2$ spectra acquired of the ions at *m/z* (A) 527 ([Hex$_3$ + Na]$^+$), (B) 1013 ([Hex$_6$ + Na]$^+$) and (C) 509 ([Hex$_3$ - H$_2$O +Na]$^+$) from the SF 2 solution after 5 months of incubation.

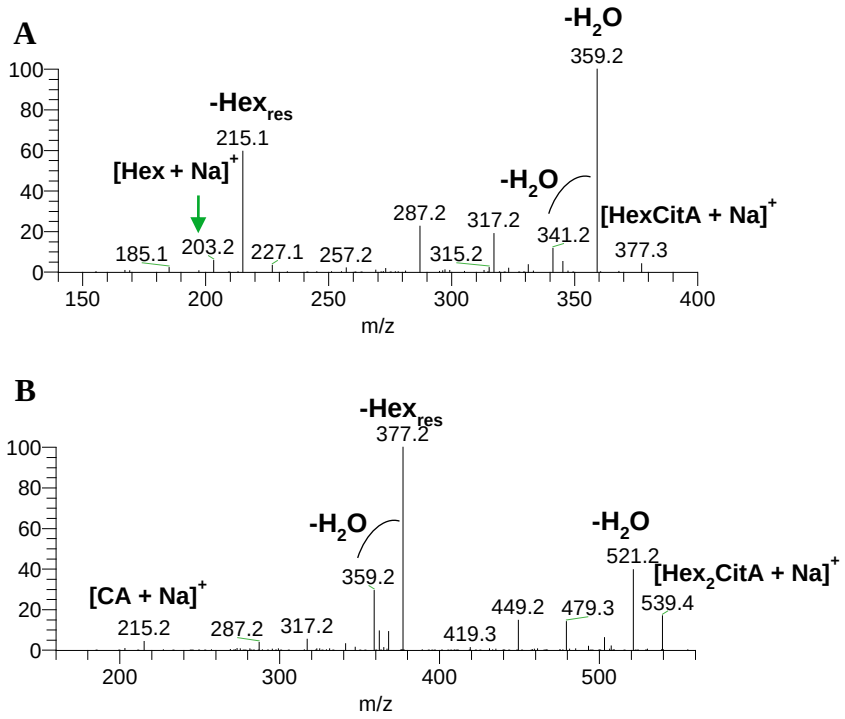



**Figure 6 -** ESI-CID-MS$^2$ spectra acquired from the SF 3 solution after 5 months for the ions at *m/z* (A) 377 ([HexCitA + Na]$^+$), and (B) 539 ([Hex$_2$CitA + Na]$^+$).

Non-enzymatic transglycosylation reactions were seen to occur in model solutions soon after their incubation, even in SG solutions without acid addition. As highlighted in Figure 7, after a period of 15 days at 35 º C, ions at *m/z* 527 ([Hex$_3$ + Na]$^+$) and 689 ([Hex$_4$+Na]$^+$) were observed in SG 1 and SF 1. The previous ions were also observed for the

remaining SF solutions with the same incubation time, but not for the remaining SG solutions (SG 2 and SG 3). Further, the ion at *m/z* corresponding to DP 5 was also observed in the SF solutions (see Appendix B).

An increase in the complexity of the synthesised oligosaccharides was observed over time, with a DP up to 6 for fructose solutions and a DP up to 4 for glucose solutions, after being incubated for 5 months. These results are consistent with the reactivity of fructose being much higher than that of glucose (81,137). The relative abundance of the $[Hex_n + Na]^+$ ions to the abundance of $[Hex + Na]^+$ for the six solutions are shown in Figure 8.

Besides oligosaccharides formation, ESI-MS spectra evidenced the existence of dehydrated derivatives ($[Hex_n - H_2O + Na]^+$), mainly in solutions at pH 2.0 (SG 3 and SF 3). This can be justified by the reported action of acids to promote the protonation of the hydroxyl group of the anomeric carbon at the reducing sugar end, leading to dehydration of the molecule (141). Nonetheless, as observed in Appendix C, hydroxyl protonation also occurs in the absence of acid, principally, in the C2' hydroxyl of β-fructofuranose, which exhibits high values of proton affinity (171). After dehydration, a carbocation is formed, which may react with the hydroxyl groups of the compounds present in the mixture (141,175,176). This nucleophilic attack occurs at intermolecular level, giving origin to the transglycosylation or reversion products, or at intramolecular level, with the oxygen of the primary hydroxyl group of the reducing sugar end with formation of a terminal anhydro (141,177).

Finally, $[Hex_n + CitA + Na]^+$ ions were identified, evidencing the presence of oligosaccharides esterified with citric acid, mainly in SG 3 and SF 3 (see Appendix D). Actually, citric acid is a chosen catalyst for polysaccharides synthesis trough sucrose polymerization (175,176) and trough monosaccharides polymerization (178). Therefore, the resultant polymer may be covalently linked to moieties of this acid, through Fischer esterification (179).

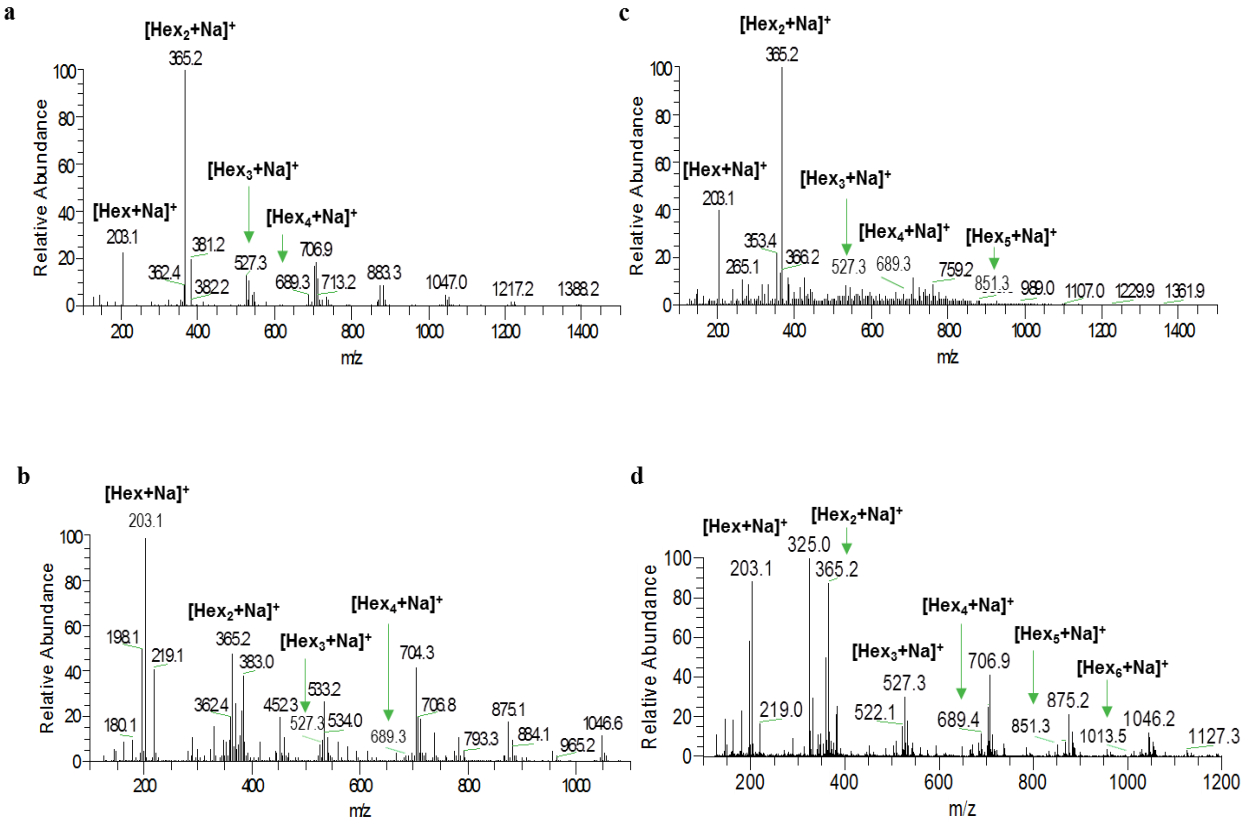
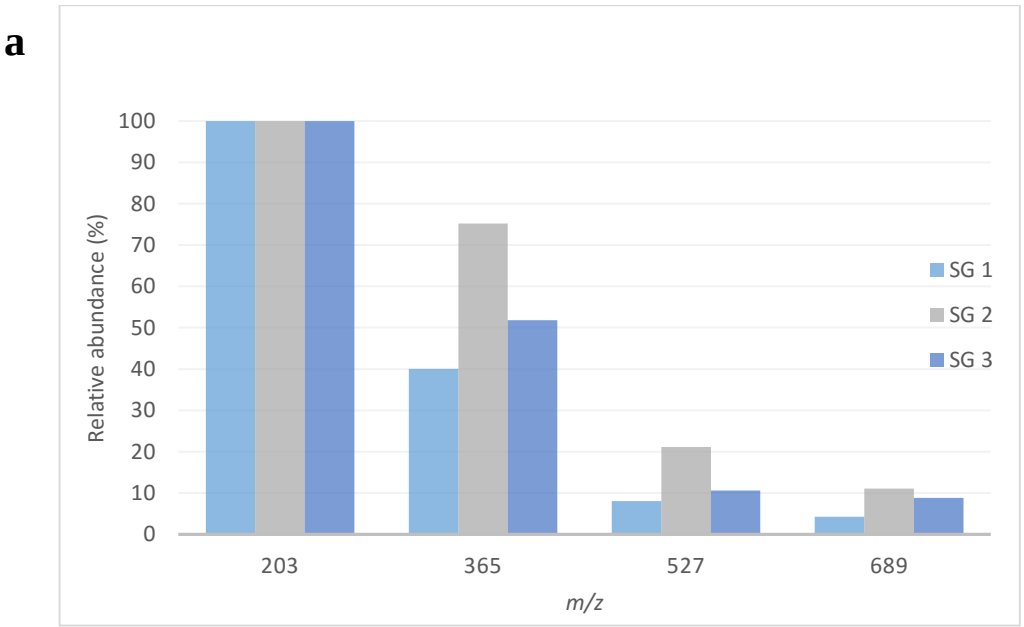



**Figure 7** –ESI-LIT-MS spectrum of a SG 1 sample, after (a) 15 days and (b) 5 months of incubation and of a SF 1 sample after (c) 15 days and (d) 5 months' incubation.



b

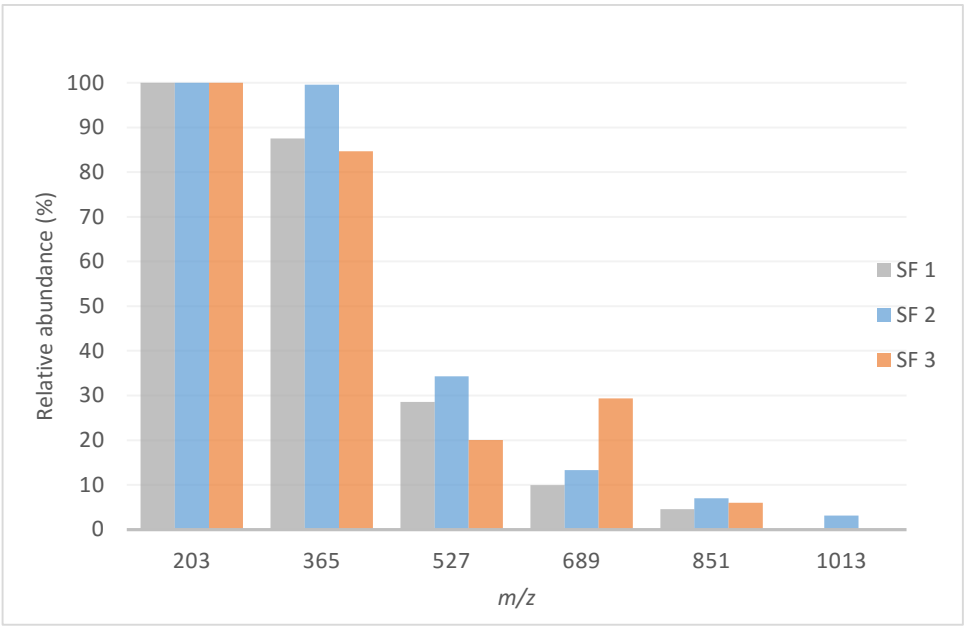



**Figure 8 –** Graphical presentation of $[Hex_n + Na]^+$ ions relative abundance for (a) SG model solutions and for (b) SF model solutions, after 5 months of incubation.

### IV.4.2. Honey

A hybrid quadrupole-Orbitrap mass spectrometer was used to analyse honeys' fractions (F1 and F2) obtained by LEX/SEC, which enabled the acquisition of high-resolution spectra and high mass accuracy measurements.

The maximum DP of each honey was accessed by analysis of fractions F2 (Figure 9). The major complexity was observed for H4, being composed by oligosaccharides with up to seven monomers, while H2 had oligosaccharides with the least DP. Considering $[Hex_n + Na]^+$ ions intensity, together with the LEX/SEC chromatograms, H4 appears as the honey with the greatest amount of carbohydrates, followed by H12. Nevertheless, qualitative and quantitative results will give a further insight into the compositional differences between the four samples.

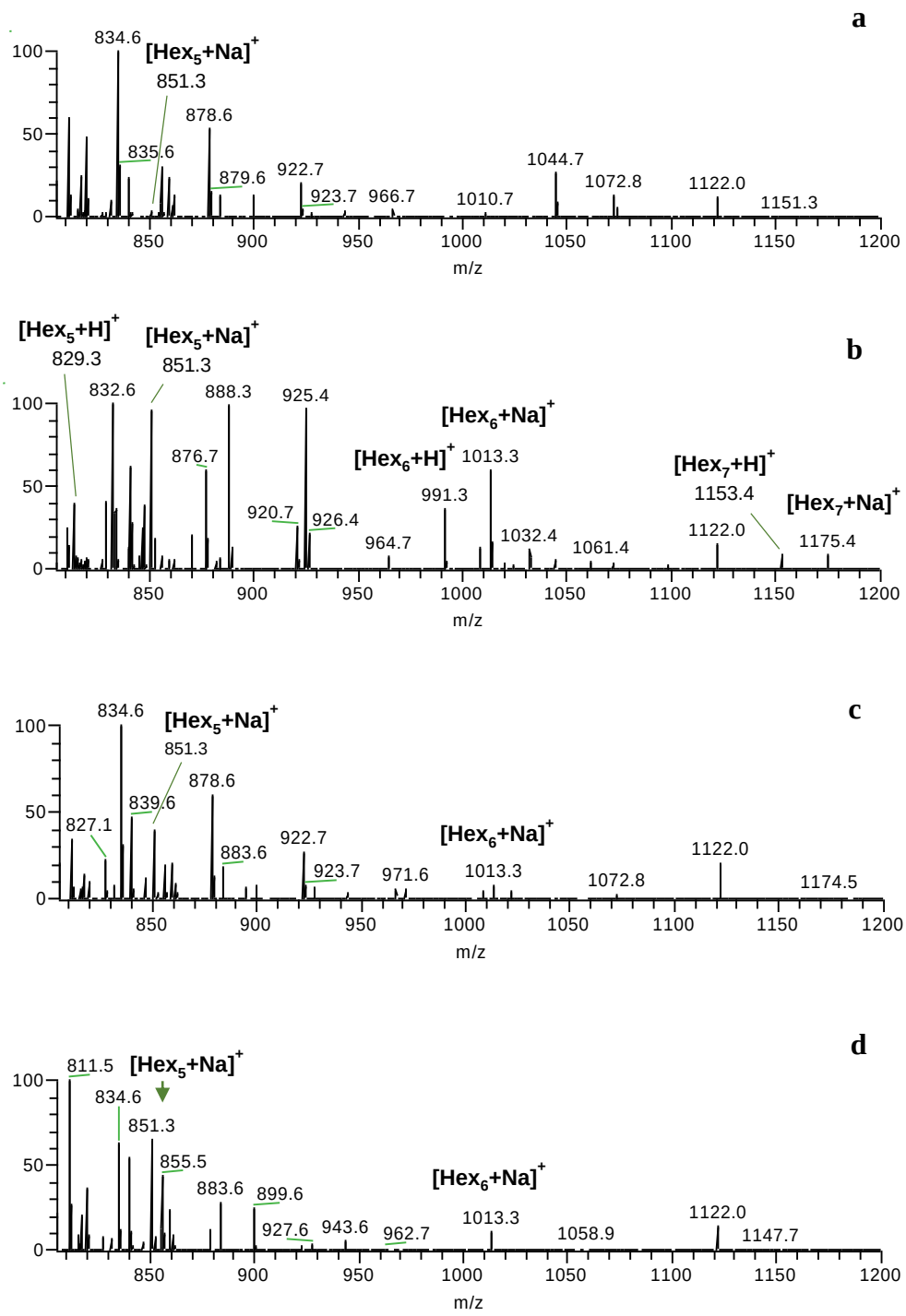



**Figure 9 –** ESI-MS spectra of the fraction F2 of (a) H2, (b) H4, (c) H8 and (d) H12, obtained by Q Exactive Orbitrap.

The assignment of the previous $[Hex_n + Na]^+$ ions was validated by high-resolution $MS^2$ spectra, and their fragmentation pattern is presented in the Figure 10. The fragmentation pattern with high abundant product ions (at *m/z* 851, 689, 527, 365) from the ion at *m/z* 1013 (Figure 10B), corresponding to the loss of one, two, three and four hexose residues ($Hex_{res}$), may indicate the occurrence of branched oligosaccharides in honey, namely in H4. The fragmentation pattern of the linear oligosaccharides produce an abundant glycosidic ion resulting from the loss of the residue located at the non-reducing end, followed by lower abundant fragments corresponding to the neutral loss of the following hexose residues (Figure 10A) (180). This may be further corroborated by the $MS^2$ spectra of different standards, namely, the linear trisaccharide α-(1→5)-arabinotriose and the branched tretrasaccharide $6^1$-α-D-Galactosyl-β-1,4-mannotriose, obtained by Q Exactive Orbitrap (see Appendix E).

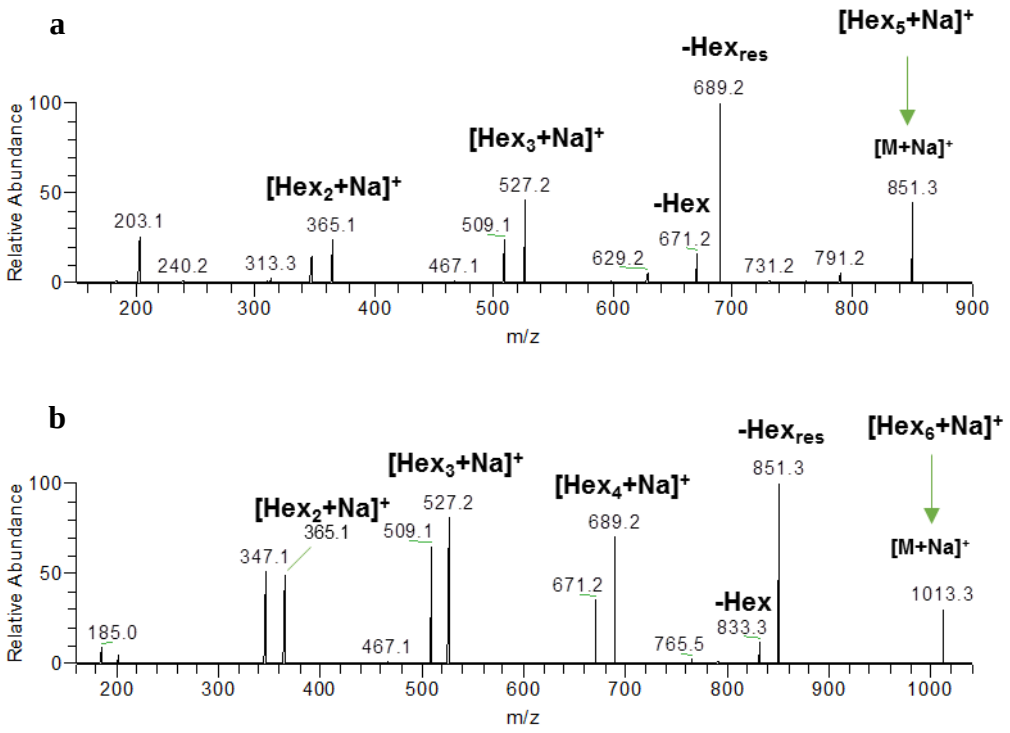



**Figure 10** – $MS^2$ spectra acquired of the ions at *m/z* (a) 851 ($[Hex_5 + Na]^+$) from H2, and (b) 1013 ($[Hex_6 + Na]^+$) from H4.

The acidic fractions (F1) were also evaluated, to attempt the identification of the acids comprising the honeys under study. As displayed in Table 11, the major compound found was gluconic acid, that was proved by high mass accuracy measurements obtained with Orbitrap-based mass spectrometer (*m/z* 219). Further, other species containing gluconic acid were also seen, such as the acid linked to a hexose (*m/z* 381) and another specie at *m/z* 397. The latter has high probability to have the formula $C_{12}H_{22}O_{13}Na$ and, once it was isolated in the acidic fraction, it can correspond two linked gluconic acids possibly by an ester linkage. Nonetheless, the presence of gluconic acid linked to hexoses or linked between them is not reported in the literature.

**Table 11 -** Accurate masses found by Q Exactive Orbitrap for the ions identified in F1 fractions of honey samples

| **Experimental mass *(m/z)*** | **Theoretical mass (*m/z*)** | **Mass error (ppm)** | **RDB equiv.** | **Composition** | **Proposed assignment(s)[a]** |
|---|---|---|---|---|---|
| 219.0473 | 219.0481 | -3.66 | 0.5 | $C_6H_{12}O_7Na$ | $[HexonicA + Na]^+$ |
| 381.0993 | 381.1009 | -4.29 | 1.5 | $C_{12}H_{22}O_{12}Na$ | $[HexHexonicA + Na]^+$ |
| 397.0941 | 397.0958 | -4.23 | 1.5 | $C_{12}H_{22}O_{13}Na$ | $[HexonicA_2 + Na]^+$ |

[a] "HexonicA" stands for hexonic acid

## IV.5. Glycosidic linkage analysis

The partially methylated alditol acetates were separated by GC-MS on retention time and spectrum, and then it was related to the established glycosidic linkage and quantified in molar percent from the chromatographic area.

The spectra of fructose PMAAs were attained by derivatisation of sucrose, 1-kestose and melezitose. The resulting fragmentation pattern is presented in Table 12.

**Table 12 –** Partially methylated alditol acetates identified from sucrose, 1-kestose and melezitose

| $t_R$[a] | Derivative compound | Linkage type | Fragmentation pattern[b] |
|---|---|---|---|
| 10.3 | 2,5-di-*O*-acetyl-(2-deuterio)-1,3,4,6-tetra-*O*-methyl-D-mannitol | *t*-*β*-D-Fru*f* | 129 (100), 162 (46.0), 161 (30.7), 87 (25.5), 102 (19.9), 101 (18.8), 145 (10.3), 118 (6.9) |
| 10.6 | 2,5-di-*O*-acetyl-(2-deuterio)-1,3,4,6-tetra-*O*-methyl-D-glucitol | *t*-*β*-D-Fru*f* | 129 (100), 162 (41.3), 161 (36.0), 87 (27.1), 102 (25.6), 101 (22.9), 118 (9.8), 145 (8.9) |
| 13.9 | 2,3,5-tri-*O*-acetyl-(2-deuterio)-1,4,6-tri-*O*-methyl-D-mannitol | (2→3)-*β*-D-Fru*f* | 129 (100), 161 (60.5), 101 (57.2), 221 (54.8), 147 (38.6), 87 (32.0), 207 (28.5), 234 (24.3) |
| 14.2 | 2,3,5-tri-*O*-acetyl-(2-deuterio)-1,4,6-tri-*O*-methyl-D-glucitol | (2→3)-*β*-D-Fru*f* | 129 (100), 161 (66.5), 101 (46.2), 147 (38.6), 114 (30.2), 87 (29.9), 234 (16.2), 174 (14.4) |
| 15.0 | 1,2,5-tri-*O*-acetyl-(2-deuterio)-3,4,6-tri-*O*-methyl-D-mannitol | (2→1)-*β*-D-Fru*f* | 129 (100), 87 (32.2), 161 (22.3), 190 (17.0), 101 (13.0), 113 (4.8), 118 (4.1), 234 (1.0) |

[a] Retention time (minutes) in DB-1 column. [b] Values in parentheses are the relative intensities of the fragments.

### IV.5.1. Honey

The glycosidic linkages of the oligosaccharides present in the F4 (disaccharides) and F3 (trisaccharides) fractions, obtained for the different honey samples, are presented in Table

13. In terms of fructose linkages, the terminally-linked fructose residues were the most abundant for all fractions, except for the disaccharide fraction of H4 (2.2 %) and of H12 (2.3 %). The second residue mainly observed was (2→1)-linked fructose residue, which was the most abundant for the previously mentioned fractions. This type of linkage is only present in the disaccharide inulobiose and in the trisaccharide 1-kestose. Another two residues were identified for the majority of fractions, (2→3)- and (2→6)-linked fructose residues. However, (2→3)-Fru was absent in the fraction F4 of H2 and of H8 and in the fraction F3 of H12, thus turanose cannot be found in H2 and H8 and melezitose is not present in H12. Also, (2→6)-Fru was not identified in the trisaccharide fraction of H12, meaning the neokestose is also absent in this honey. The fraction F3 of H8 (37.2 %) and the fraction F4 of H4 (10.3 %) exhibit the highest and the lowest value of the total amount of fructose residues, respectively. Furthermore, all the trisaccharides fractions showed higher amount of fructose residues than the disaccharide fractions, when compared within the same honey sample.

Concerning glucose linkages, the terminally-linked glucose residue was the most abundant for all fractions, being also the residue with the highest proportion among all the residues identified. This was expected, as this linkage occurs in most of the oligosaccharides reported in honey (49,50). In addition, (1→2)-, (1→4)- and (1→6)-linked glucose residues were observed with variable amounts for each fraction. By observation of the disaccharide fraction, (1→2)-Glc was higher for H4 (9.7 %) and lower for H8 (8.3 %), meaning kojibiose is more abundant in H4. Further, regarding the fraction F3, the same linkage is only found for the trisaccharide centose and it can be postulated that its abundance is higher for H8 (7.7 %) and lower for H12 (5.4 %). The glycosydic linkage (1→4)-Glc, which occurs in maltose and cellobiose disaccharides and in the trisaccharides maltotriose, erlose and centose was found predominantly in the disaccharide fraction of H12 (7.2 %) and in F3 of H2 (17.7 %), while its lower values were observed for fractions F4 and F3 of H4 (1.1 % and 4.5 %, respectively). Finally, (1→6)-Glc is found in isomaltose and gentiobiose (disaccharides) and in the trisaccharides neokestose, panose, isomaltotriose, theanderose and isopanose. Both fractions of H12 had considerable amounts of this linkage, along with F3 of H8 (14.5 %), while disaccharide fractions of H2 and H8 had the smallest abundances (1.9 % for both).

Moreover, a terminally-linked galactose residue was found for all the trisaccharide honey fractions, except for H12. This suggests the presence of raffinose (*O*-α-D-

galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) in H2, H4 and H8, once it is the only trisaccharide reported in honey with a galactosyl residue (49,50).

The results showed that the carbohydrates have a linear structure, being in accordance to what is reported in the literature (49,50). Apart from (2→4)-linked fructose residue, present in maltulose, and from (1→3)-linked glucose residue, occurring in laminaribiose and in nigerose, all the residues corresponding to the glycosidic linkages present in honey oligosaccharides were detected. Further, despite a few exceptions, all samples showed the same partially methylated alditol acetates, which suggests a similar oligosaccharides profile for the four honey samples. Nonetheless, the differences in the residues proportions indicate a variable amount of these oligosaccharides among the samples. For example, by the presence of terminally linked-Fru molar percentages, it can be assumed that sucrose occurs in lower proportions in H4 and H12 and in similar quantities in the remaining samples, since it is the only disaccharide with a terminal-fructose linkage.

**Table 13 –** Glycosidic linkage composition (percentage area) of di- and trisaccharides fractions obtained for the honey samples

| **Glycosidic linkage** | **Fraction (% mol)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | H2 | | H4 | | H8 | | H12 | |
| | F4 | F3 | F4 | F3 | F4 | F3 | F4 | F3 |
| **t-Fru** | 15.7 | 18.5 | 2.2 | 13.9 | 16.7 | 26.6 | 2.3 | 12.9 |
| **1-Fru** | 10.9 | 5.5 | 4.3 | 7.3 | 12.7 | 7.9 | 10.0 | 3.2 |
| **3-Fru** | - | 2.8 | 1.7 | 3.0 | - | 0.8 | 2.1 | - |
| **6-Fru** | 0.9 | 2.3 | 2.0 | 0.9 | 0.2 | 2.0 | 0.7 | - |
| **Total** | 27.5 | 29.1 | 10.3 | 25.1 | 29.6 | 37.2 | 15.1 | 16.0 |
| **t-Glc** | 57.0 | 37.0 | 73.3 | 57.3 | 56.5 | 27.0 | 56.0 | 48.2 |
| **2-Glc** | 8.8 | 6.8 | 9.7 | 6.4 | 8.3 | 7.7 | 8.4 | 5.4 |
| **4-Glc** | 4.8 | 17.7 | 1.1 | 4.5 | 3.7 | 13.4 | 7.2 | 17.3 |
| **6-Glc** | 1.9 | 7.3 | 5.7 | 5.6 | 1.9 | 14.5 | 13.3 | 13.2 |
| **Total** | 72.5 | 68.8 | 89.8 | 73.8 | 70.4 | 62.6 | 84.9 | 84.0 |
| **t-Gal** | - | 2.1 | - | 1.1 | - | 0.2 | - | - |
| **Total** | - | 2.1 | - | 1.1 | - | 0.2 | - | - |

### IV.5.2. Model solutions

The glycosidic linkage composition of model solutions is disposed in Table 14. Similar to honey results, terminally-, (2→1)-, (2→3)- and (2→6)-linked fructose residues and terminally-, (1→2)-, (1→4)- and (1→6)-linked glucose residues were detected in most fractions. In addition, from all the identified residues, terminally-linked glucose residue was also the most abundant for all fractions. However, several branched residues were identified, being (1→2,3,4,6)-Glc the most abundant, followed by (1→2,3,4,6)-Mannose (Man), which were found predominantly in solutions prepared with citric acid. The remaining residues corresponding to branches are found in small amounts and in few fractions. These ramifications could be a result of glycosidic linkages with other sugar moieties or with citric acid. The latter can be supported by the results obtained with ESI-MS, in which $[Hex_nCitA + Na]^+$ ions were detected, being postulated that oligosaccharides are esterified with citric acid. Also, in the studies previously mentioned on the synthesis of polysaccharides through polymerization of disaccharides, catalysed with citric acid, linkage analyses revealed a highly branched character of the resultant polysaccharides (176,179). Furthermore, it should be noted that (1→3,4)-Man, (1→4,6)-Man, (1→2,3)-Glc, (1→2,4)-Glc, (1→3,4)-Glc and (1→4,6)-Glc were mainly detected in the disaccharide fractions, which is only possible in trisaccharides or more polymerized structures. This can be explained by the fractionation on LEX/SEC resulting in enriched fractions rather than pure fractions, meaning the presence of oligosaccharides with another DP than the one expected is possible. Besides, as citric acid is a tricarboxylic acid and only one molecule of acid was found to be linked to the hexoses, it is possible that one hexose is esterified with two or three carboxylic groups of the same acid molecule. However, when citric acid is absent these branched linkages were also found.

After the terminally linked residues, when focusing on the linear residues, (2→1)-Fru, (2→6)-Fru, (1→2)-Glc and (1→6)-Glc were the most abundant for the majority of fractions. The prevalence of these linkages can be further explained by the C6' hydroxyl of Glc and Fru and the C1' hydroxyl of Fru being more reactive, since they belong to the primary hydroxyl group (142).

As previously mentioned, the reported reversion products of glucose are linked by α/β,α/β(1→1), α/β(1→2), α/β(1→3), α/β(1→4) and α/β(1→6) bonds (141). All the residues corresponding to these linkages are observed in linkage analysis, except the (1→3)-glucose

linked residue, meaning nigerose and laminaribiose were not formed in the model solutions. Therefore, disaccharides like threalose, kojibiose, maltose, cellobiose, isomaltose and gentiobiose, which are also reported in honey (49), could have been formed in model solutions. Besides, other oligosaccharides identified in commercial syrups included α,β-trehalose, *O*-β-D-fructofuranosyl-(2→6)-D-glucose (135) and 6-kestose, O-α-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl-α-D-glucopyranoside and *O*-α-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl-α-D-glucopyranoside (143). The residues corresponding to the linkages of the previous sugars are, as well, present in Table 14, meaning the presence of these oligosaccharides in the model solutions is also possible.

**Table 14 –** Glycosidic linkage composition of di- and trisaccharides fractions obtained for model solutions samples

| **Glycosidic linkage** | **Fraction (% mol)** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **SG** | | | | | | **SF** | | | | | |
| | **1** | | **2** | | **3** | | **1** | | **2** | | **3** | |
| | F4 | F3 | F4 | F3 | F4 | F3 | F4 | F3 | F4 | F3 | F4 | F3 |
| **t-Fru** | 23.0 | 21.2 | 34.3 | 9.0 | 15.5 | 27.3 | 41.4 | 25.4 | 51.2 | 24.8 | 3.8 | 27.0 |
| **1-Fru** | 0.4 | 2.0 | 1.0 | 3.3 | 12.0 | 9.3 | 3.9 | 4.0 | 2.1 | 3.1 | 14.9 | 14.3 |
| **3-Fru** | - | - | - | - | - | 0.8 | 0.1 | - | 0.1 | - | - | - |
| **6-Fru** | 2.6 | 4.1 | 1.3 | 0.5 | - | 1.5 | 0.4 | 15.1 | 0.7 | 2.5 | - | 2.9 |
| **3,4-Fru** | - | - | - | - | - | - | 0.1 | - | 0.1 | - | 0.6 | - |
| **4,6-Fru** | - | - | - | - | 0.7 | 1.4 | - | - | - | - | - | - |
| **1,3,4,6-Fru** | 0.2 | - | - | 13.9 | 6.1 | - | - | - | - | 5.7 | 1.2 | 7.1 |
| **Total** | 26.2 | 27.3 | 36.6 | 26.7 | 34.2 | 40.3 | 45.9 | 44.4 | 54.3 | 36.1 | 20.5 | 51.4 |
| **t-Glc** | 63.9 | 38.8 | 56.4 | 34.9 | 36.1 | 27.3 | 47.2 | 28.4 | 37.8 | 37.0 | 77.9 | 22.8 |
| **2-Glc** | 0.7 | 10.0 | 2.9 | 3.9 | 1.9 | 15.5 | 2.8 | 11.4 | 1.7 | 0.6 | - | 12.8 |
| **4-Glc** | 3.4 | 4.1 | 2.2 | 5.6 | 1.6 | 2.4 | 1.6 | 1.0 | 2.4 | 1.5 | - | 0.4 |
| **6-Glc** | 4.1 | 14.9 | 2.0 | 3.2 | 5.4 | 12.6 | 2.3 | 14.3 | 3.6 | 13.2 | - | - |
| **2,3-Glc** | 0.3 | - | - | - | 1.3 | - | 0.1 | 0.2 | - | - | 0.5 | 1.0 |
| **2,4-Glc** | 0.1 | - | - | - | - | - | - | - | 0.1 | - | - | - |
| **3,4-Glc** | 0.1 | - | - | - | 0.2 | - | - | - | - | - | - | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **4,6-Glc** | - | - | - | - | - | - | 0.1 | - | 0.1 | - | - | - |
| **3,4,6-Glc** | - | - | - | - | - | - | 0.03 | - | - | - | - | - |
| **2,3,4,6-Glc** | 1.3 | 4.9 | - | 25.7 | 19.3 | 2.1 | - | 0.4 | 0.1 | 11.7 | 1.2 | 11.7 |
| **Total** | 73.8 | 72.7 | 63.4 | 73.3 | 65.8 | 59.7 | 54.1 | 55.6 | 45.7 | 63.9 | 79.6 | 48.6 |

## IV.6. Oligosaccharides identification

### IV.6.1. Honey

The chromatographic profiles obtained for disaccharides derivatives (alditol acetates) with GC-MS, as well as the MS fragmentation patterns were compared in order to establish compositional similarities between the four samples. Retention time and fragmentation patterns of the detected derivatives are depicted in Table 15. As fructose reduction yields glucitol and mannitol and, from all the disaccharides reported in honey, inulobiose is the only reducing sugar with fructose as the reducing end (49,181), inulobiose derivative is the only giving rise to two different peaks. Therefore, to facilitate the interpretation, each peak was considered to represent one disaccharide. Several peaks were found, but only sucrose, maltose, trehalose and cellobiose were identified with assurance, as those were the only standards available. From the eleven different disaccharides determined, six were present in all types of honey and with maltose and cellobiose being two of them. In addition, sucrose was visible in all honeys spectra unless in H12 spectra, which can be justified by its long permanence inside the hive and, thus, by a longer actuation period of the invertase enzyme over this carbohydrate.

To attempt the profiling of the different samples, data obtained by the diverse methodology was assembled. The possible structures comprising the honeys' disaccharides and trisaccharides fractions is compiled in Table 16. The trisaccharides were only proposed on the basis of the linkage analysis results.

**Table 15 –** Retention time and fragmentation pattern of oligosaccharides derivatives from the honeys fractions F4, obtained with GC-MS.

| | Assignment | $T_R$ [a] | | | | Fragmentation pattern [b] |
|---|---|---|---|---|---|---|
| | | H2 | H4 | H8 | H12 | |
| **Di 1** | Sucrose | 26.6 | 26.5 | 26.6 | | 169 (100); 211 (60.2); 109 (52.3) |
| **Di 2** | Trehalose | 27.3 | 27.2 | | | 169 (100); 109 (51.5); 211 (17.0) |
| **Di 3** | | 28.7 | 28.5 | 28.7 | 28.6 | 169 (59.2); 109 (26.6); 153 (24.8) |
| **Di 4** | | | 28.8 | | 28.7 | 169 (65.8); 109 (29.5); 153 (19.1) |
| **Di 5** | Maltose | 29.7 | 29.0 | 29.5 | 29.3 | 169 (74.6); 109 (34.9); 153 (21.6) |
| **Di 6** | | 30.0 | | 29.7 | | 169 (37.1); 153 (31.0); 109 (19.2) |
| **Di 7** | Cellobiose | 30.3 | 29.6 | 30.1 | 29.8 | 169 (35.6); 153 (26.1); 375 (22.6) |
| **Di 8** | | 30.7 | 29.9 | 30.4 | 30.2 | 169 (41.8); 153 (24.2); 375 (20.3) |
| **Di 9** | | | 30.1 | | | 169 (71.4); 153 (35.3); 109 (28.9) |
| **Di 10** | | 30.9 | 30.3 | 30.7 | 30.4 | 169 (51.3); 109 (24.1); 153 (21.3) |
| **Di 11** | | 31.2 | 30.4 | 30.9 | 30.6 | 169 (100); 109 (53.7); 127 (15.0) |

[a] Retention time (minutes) in DB-1 column. [b] Values in parentheses are the relative intensities of the fragments.

**Table 16 –** Proposed oligosaccharides composition of the four honey samples [a]

| | **H2** | **H4** | **H8** | **H12** |
|---|---|---|---|---|
| ***Disaccharides*** | | | | |
| Sucrose | X | X | X | - |
| Trehalose | X | X | - | - |
| Kojibiose | X | X | X | X |
| Maltose | X | X | X | X |
| Isomaltose | X | X | X | X |
| Cellobiose | X | X | X | X |
| Gentiobiose | X | X | X | X |
| Inulobiose | X | X | X | X |
| Turanose | - | X | - | X |
| Palatinose | X | X | X | X |
| ***Trisaccharides*** | | | | |
| Melezitose | X | X | X | - |
| Maltotriose | X | X | X | X |
| 1-Kestose | X | X | X | X |
| 6-Kestose | X | X | X | - |
| Neokestose | X | X | X | X |
| Panose | X | X | X | X |
| Isomaltotriose | X | X | X | X |
| Erlose | X | X | X | X |
| Theanderose | X | X | X | X |
| Centose | X | X | X | X |
| Isopanose | X | X | X | X |
| Raffinose | X | X | X | - |

[a] The compounds marked with an X are proposed to be found in the designated sample.

### IV.6.2. Model solutions

Several model solutions oligosaccharides were detected as their alditol acetates with GC-FID and GC-MS. As previously mentioned, fructose reduction yields mannitol and glucitol, and thus the same reducing sugar (with fructose as the reducing sugar end) will give rise to two different chromatographic peaks. Therefore, the number of peaks is not equivalent to the number of carbohydrates. Nevertheless, in order to give a general notion of the model solutions' composition, the number of peaks obtained with each equipment is presented in Table 17. It should be noted that sucrose was accounted in all the F4 fractions.

Despite the unavailability of standards, MS fragmentation patterns and the retention time allowed to establish compositional similarities between the samples. Concerning the disaccharides fraction, all the peaks identified for SG 2 were similar with seven peaks detected for SG 1. Also, all the peaks identified by SG 1 analysis were detected for SG 3 sample. This means that all the sugars formed in SG 2 were also formed in SG 1 and that the carbohydrates found in the latter were also present in SG 3, beyond others. In the case of model solutions prepared with Fru, similarities were also found, with 14 out of 16 peaks of SF 2 being identical to peaks detected for SF 1, while SF 3 had 14 equivalent peaks with SF 1. In addition, resemblances were found between the disaccharides fractions of Glc and Fru model solutions.

Regarding the trisaccharides fraction, no peaks were identified for SG 3 solution, despite the identification of ions at *m/z* 527 and 689 (DP 3 and DP 4, respectively) by ESI-MS. A possible explanation is the very low limit of detection (LOD) of ESI-MS, when compared to GC-MS and GC-FID. For the remaining solutions, common peaks were detected, being the seven peaks found in SG 1 similar for the remaining samples, and the additional peak found for SG 2 was the same as that for SF 2. Furthermore, 8 peaks were additionally detected for SF 3.

**Table 17 –** Number of peaks obtained for the different samples (SGs and SFs) with GC-FID and GC-MS

| Sample / No. peaks | SG 1 | | SG 2 | | SG 3 | | SF 1 | | SF 2 | | SF 3 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F4 | F3 | F4 | F3 | F4 | F3 | F4 | F3 | F4 | F3 | F4 | F3 |
| **GC-FID** | 8 | 7 | 6 | - | 8 | - | 18 | 3 | 11 | 8 | 19 | - |
| **GC-MS** | 14 | 7 | 7 | 8 | 22 | - | 20 | 7 | 16 | 8 | 21 | 16 |

In order to perceive if the oligosaccharides produced in the model solutions are found in honey, retention times and fragmentation patterns of oligosaccharides derivatives from both model solutions and honeys were compared (Table 18). Concerning fructose model solutions, besides sucrose, similarities were only seen for SF 2, with one spectrum being analogous to that of maltose. Already for the model solutions prepared with Glc, quite a few mass patterns resembled those of the disaccharides found in honey. Besides maltose, SG 2 had another peak similar with one of honey, SG 1 had another two, and SG 3 another four. Therefore, solutions initially prepared with sucrose plus glucose have a higher tendency in producing oligosaccharides with the same structure as those reported for honey. This is expected, because the majority of honey oligosaccharides have only glucose as their monomers (49).

**Table 18 –** Peaks identified for honey and for model solutions [a]

| | H2 | H4 | H8 | H12 | SG 1 | SG 2 | SG 3 | SF 1 | SF 2 | SF 3 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Sucrose** | X | X | X | | X | X | X | X | X | X |
| **Trehalose** | X | X | | | | | | | | |
| **Di 3** | X | X | X | X | | | | | | |
| **Di 4** | | X | | X | | | | | | |
| **Maltose** | X | X | X | X | X | X | X | | X | |
| **Di 6** | X | | X | | | | | | | |
| **Cellobiose** | X | X | X | X | | | | | | |
| **Di 8** | X | X | X | X | | X | X | | | |
| **Di 9** | | X | | | X | | X | | | |
| **Di 10** | X | X | X | X | | | X | | | |
| **Di 11** | X | X | X | X | X | | X | | | |

[a] "X" is used to mark the disaccharides that are present.

## IV.7. Oligosaccharides quantification

### IV.7.1. Honey

Several disaccharides and trisaccharides, along with a few tetrasaccharides, were detected; however, as previously reported, only a few were identified with accuracy. Concentrations of these sugars were calculated from the GC-FID chromatographic profiles by using response factors calculated from the carbohydrate standards (maltose for disaccharides and maltotriose for tri- and tetrasaccharides), which are displayed in Table 19.

Sucrose concentration is seen to be lower in H4 and H12, as previously indicated, and higher in the honey with the lowest ripening time (H2) and in honey which maturation occurred at low temperatures (H8). This was expected, as H2 had a smaller contact period with invertase. Further, despite the longer action of invertase, this enzyme activity was reported to be negatively influenced by the temperature (182). Moreover, as sucrose content depends on honey ripening stage (16), it can be postulated that this process was limited in those two honeys. Among the disaccharides, maltose appears as the predominant, being in

conformity with the literature (16,49). In accordance with ESI-MS analysis results, the H4 and H12 honeys shown oligosaccharides content with higher degree of polymerization, having considerable values for tri- and tetrasaccharides. Therefore, temperature is established has an important factor on the oligosaccharides synthesis extension. The length of maturation may also play a role on these reactions, but its impact is only noticed if this parameter is extremely low, as for the case of H2. Nonetheless, the influence of these parameters seems to be resultant of their synergy.

**Table 19 –** Carbohydrate values (mg/g of honey) found for the four honey samples (H2-H12)

| | mg/g | | | | | |
|---|---|---|---|---|---|---|
| | Suc | Mal [a] | Other di- [b] | Tri- [c] | Tetra- [d] | Total oligo- [e] |
| H2 | 11 | 155 | 255 | 3.8 | - | z425 |
| H4 | 2.5 | 53 | 189 | 85 | 2.3 | 332 |
| H8 | 12 | 354 | 474 | tr [f] | - | 840 |
| H12 | 1.9 | 63 | 129 | 41 | 3.2 | 238 |

[a] Maltose. [b] Other disaccharides. [c] Disaccharides. [d] Tetrasaccharides. [e] Total oligosaccharides. [f]Traces.

### IV.7.2. Model solutions

The carbohydrates produced in each model solution were quantified likewise, to give a further insight into the reactions that occurred, as well as the compositional differences between each other (Table 20). In accordance with the LEX/SEC chromatograms, SG 1 and SG 2 show a similar quantity of sucrose, while most of it was hydrolysed in SG 3. The content of the produced disaccharides in glucose solutions is also consistent with the number of peaks identified by GC-MS analysis (Table 17), with 6, 13 and 21 peaks identified for SG 2, SG 1 and SG 3, respectively, which are correspondent to the concentrations of 8, 12 and 21 mg/g. Regarding trisaccharides, quantifiable amounts were only detected for SG 1 (6 mg/g).

Concerning fructose model solutions, the sucrose hydrolysis tendency was maintained, with minimal amounts of sucrose in the SF 3 solution (6 mg/g) and with similar amounts in the two remaining model solutions, which is expected due to their similar pH value. The amount of the produced disaccharides was higher for SF 3 (171 mg/g), followed by SF 1 (72 mg/g) and, finally, by SF 2 (29 mg/g). Further, the trisaccharides abundance was higher for SF 2 (47 mg/g), proceeded by SF 1 (8 mg/g) and, lastly, by SF 3 in which no quantifiable amounts were detected.

By observing the disaccharides concentration in both glucose and fructose model solutions, a pattern was evidenced in terms of conditions with higher tendency in producing disaccharides. Model solutions prepared with an aqueous solution of citric acid at pH 2.0 were shown to produced more disaccharides both in terms of quantity and diversity, the latter proved by the variety of GC-MS peaks, whereas the solutions prepared with diluted citric acid at pH 4.0 showed the lowest tendency in producing disaccharides. Besides, none of the most acidic solutions (SG 3 and SF 3) produced quantifiable amounts of trisaccharides. Thus, citric acid seems to be acting both as a catalyst and as an obstacle, with the promotion of disaccharides formation and simultaneously inhibiting the trisaccharides production. A possible explanation is that, at higher concentrations of citric acid, the esterification of the disaccharides by citric acid may be hindering their further polymerization.

Furthermore, it should be noted that, when comparing the total quantity of oligosaccharides between SG and SF solutions, greater values were found for fructose solutions, being in agreement with the fructose reactivity (79,136) and with the results of GC-MS analysis.

**Table 20 –** Carbohydrate values (mg/g of solution) found for the six model solutions, after a 5-month period of incubation.

| | mg/g | | | |
|---|---|---|---|---|
| | Sucrose | Other Disaccharides | Trisaccharides | Total oligosaccharides[a] |
| SG 1 | 164 | 12 | 6 | 18 |
| SG 2 | 167 | 8 | tr[b] | 8 |
| SG 3 | 2 | 21 | - | 21 |
| SF 1 | 742 | 72 | 8 | 80 |
| SF 2 | 757 | 29 | 47 | 76 |
| SF 3 | 6 | 171 | tr[b] | 171 |

[a] Total oligosaccharides do not include sucrose concentration. [b] Traces.

# Chapter V

Concluding remarks

## V. Concluding remarks

The present work aimed to clarify the occurrence of nonenzymatic reactions during honey maturation and their role on the oligosaccharides production, through preparation and analysis of model solutions. Furthermore, four honeys with different maturation time and season were studied.

The carbohydrates identified in the four honey samples were almost linear, with mainly terminally-linked glucose residues and terminally-linked fructose residues, and minor proportions of (1→2)-, (1→4)- and (1→6)-linked glucose residues, and of (2→1)-, (2→3)- and (2→6)-linked fructose residues. The disaccharides maltose and cellobiose were identified in all honey samples; sucrose was not detected in H12, and threalose was only present in H2 and H4. It was seen that the maturation season has an impact on the amount of oligosaccharides, as honey which maturation occurred during summer (H4) had the higher amount of trisaccharides. Besides, honey with a ripening process of 12 months exhibited the second highest content of disaccharides and the highest content of tetrasaccharides, evidencing an effect of the ripening duration on the oligosaccharides synthesis.

Regarding model solutions, it was seen an increase of DP throughout time, with a maximum of DP 6, after a period of incubation of 5 months. After the same period, branched oligosaccharides were found, being the branched residue found in higher proportion the (1→2,3,4,6)-Glc, for all the six solutions. Nevertheless, overall the higher proportions of carbohydrates were composed by terminally-linked glucose residues and terminally-linked fructose residues, as seen for honey samples. The fructose solutions produced oligosaccharides in higher amounts and with higher DP, when compared to those synthesised in solutions prepared with glucose. However, SG solutions presented more disaccharides common to those found in honey, than SF solutions.

Thus, non-enzymatic transglycosylation reactions are confirmed to take place under maturation conditions. However, branched oligosaccharides may be present in honey, but in much smaller amounts, meaning that these reactions are occurring but in competition with other polymerization reactions, presumably mediated by invertase. Therefore, the future work on this subject should incorporate the invertase enzyme on the model solutions, to further validate these results, and should not include citric acid, as it was reported to be a catalyst of these reactions.

# Chapter VI

Appendix

# VI. Appendix

## VI.1. Appendix A

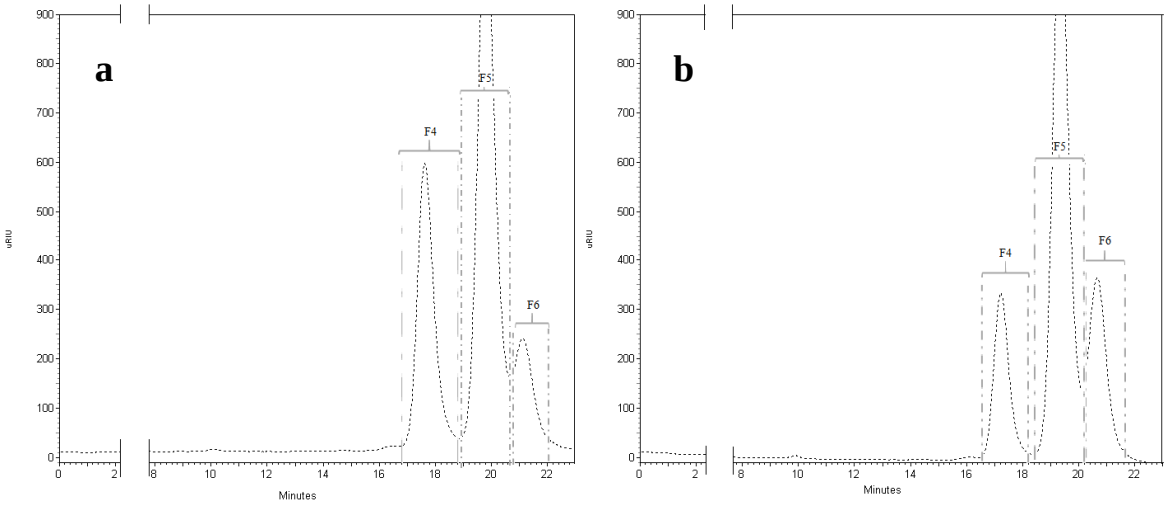



**Figure 11 –** LEX/SEC chromatograms of SG 1 after (a) 3 and (b) 4 months of incubation.

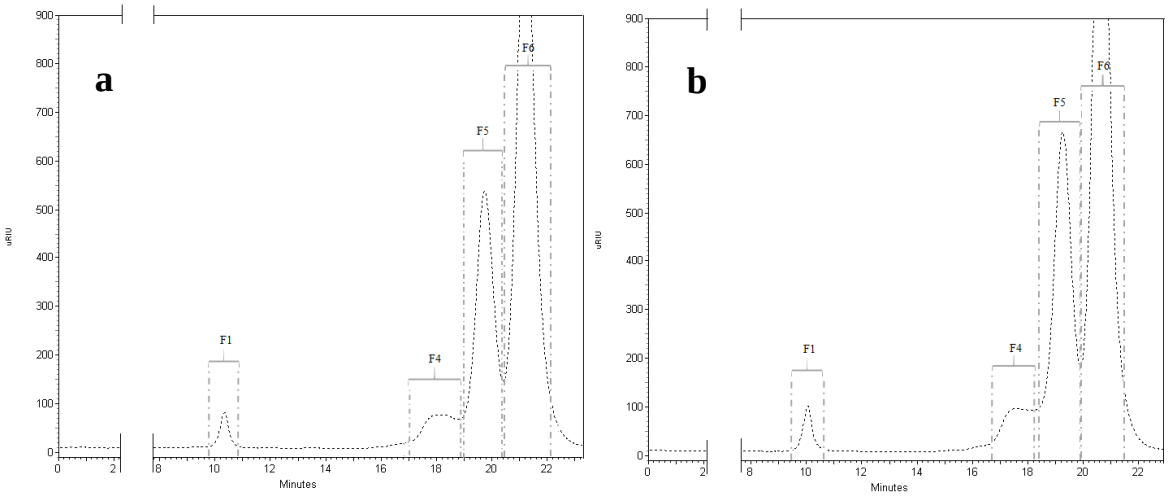
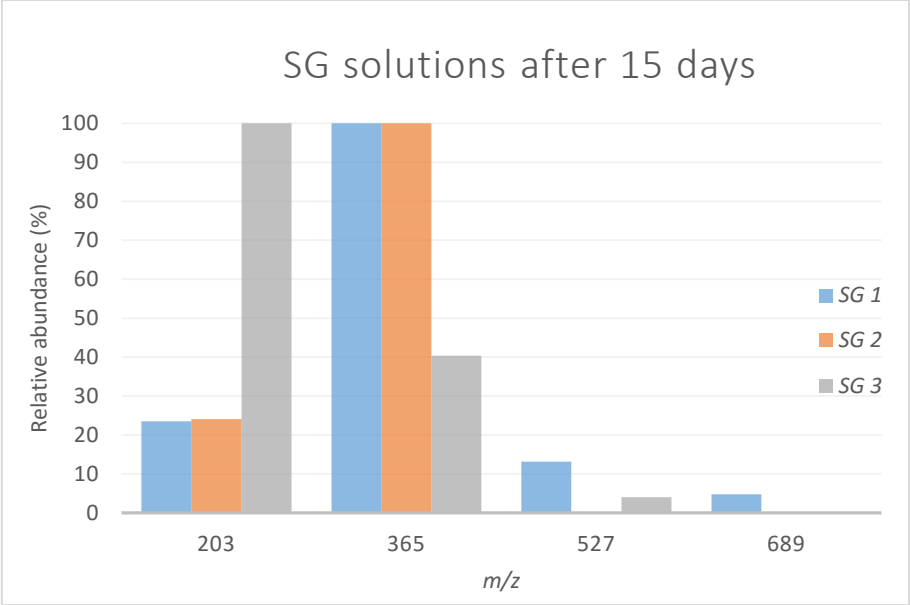



**Figure 12 –** LEX/SEC chromatograms of SF 3 after (a) 3 and (b) 4 months of incubation.

## VI.2. Appendix B

**a**




**b**

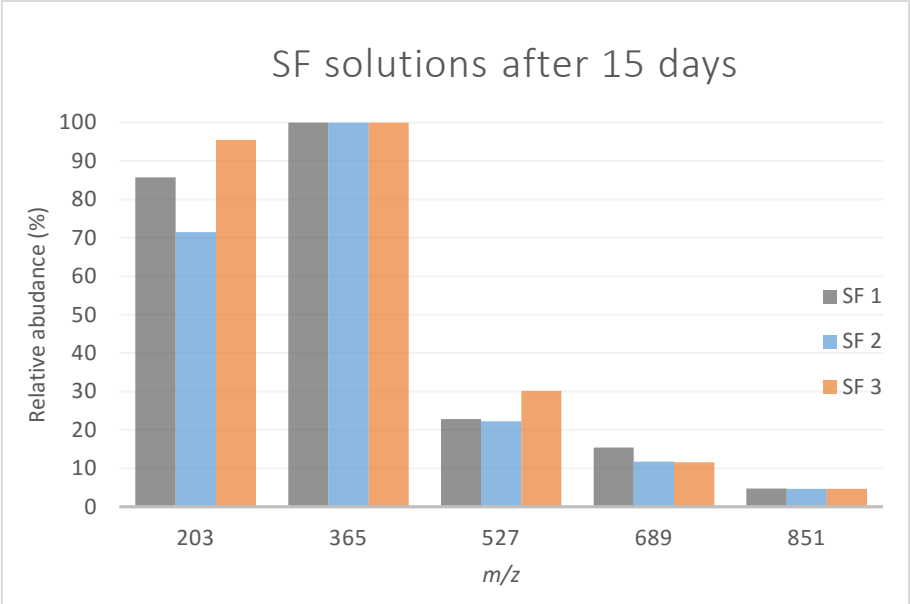
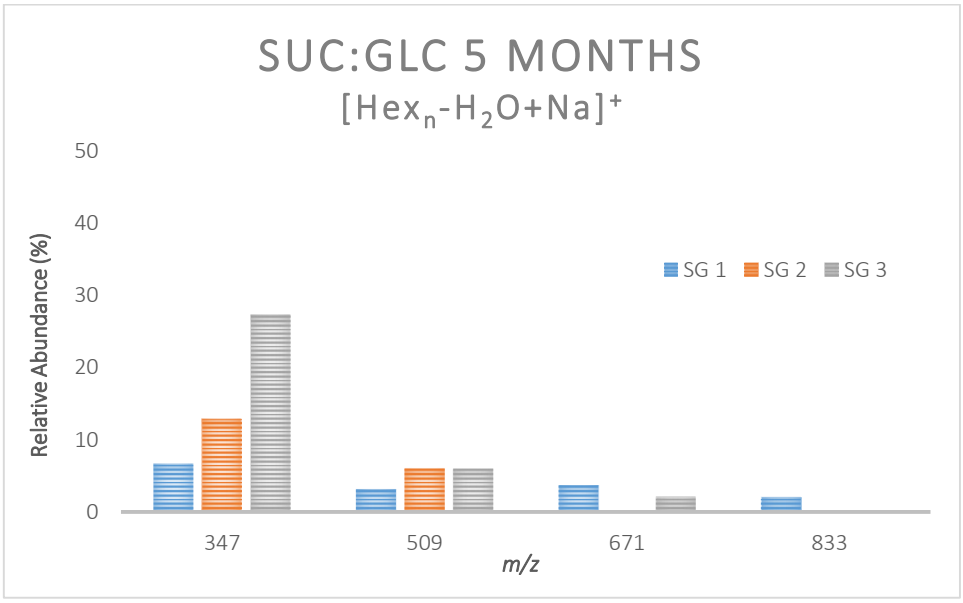



**Figure 13 -** Graphical presentation of $[Hex_n + Na]^+$ ions relative abundance for (a) SG model solutions and for (b) SF model solutions, after 15 days of incubation.

## VI.3. Appendix C

**a**




**b**

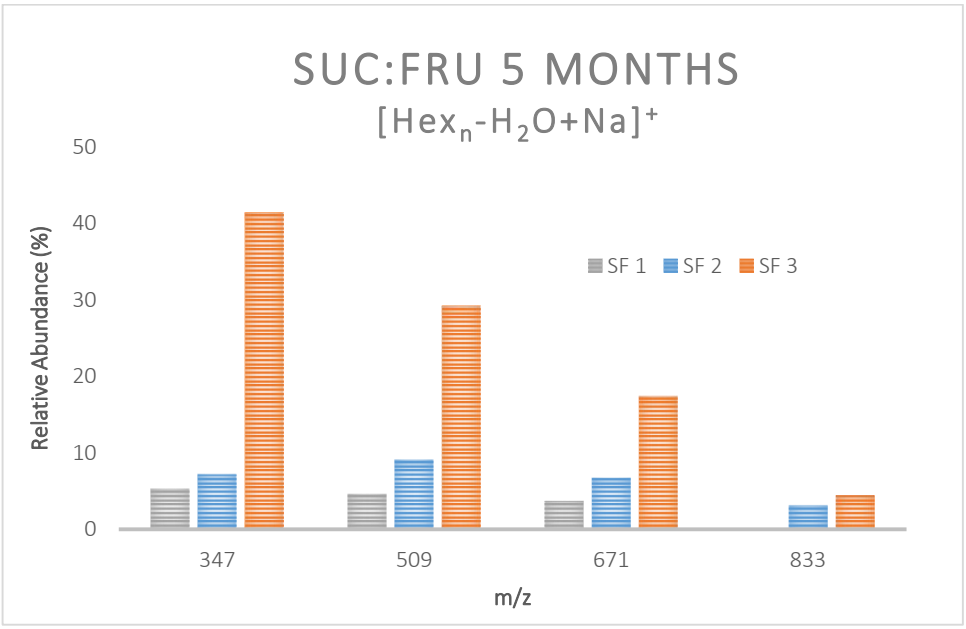



**Figure 14 -** Graphical presentation of $[Hex_n - H_2O + Na]^+$ ions relative abundance for (a) SG model solutions and for (b) SF model solutions, after 5 months of incubation.

.

## VI.4. Appendix D

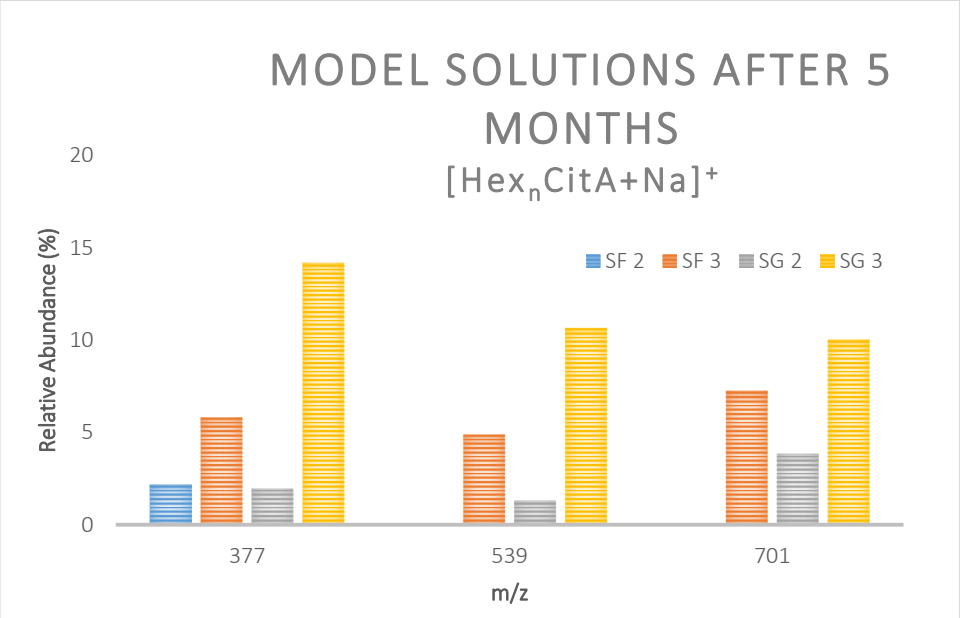



**Figure 15** - Graphical presentation of $[Hex_nCitA + Na]^+$ ions relative abundance for SG and SF model solutions, after 5 months of incubation.

## VI.5. Appendix E

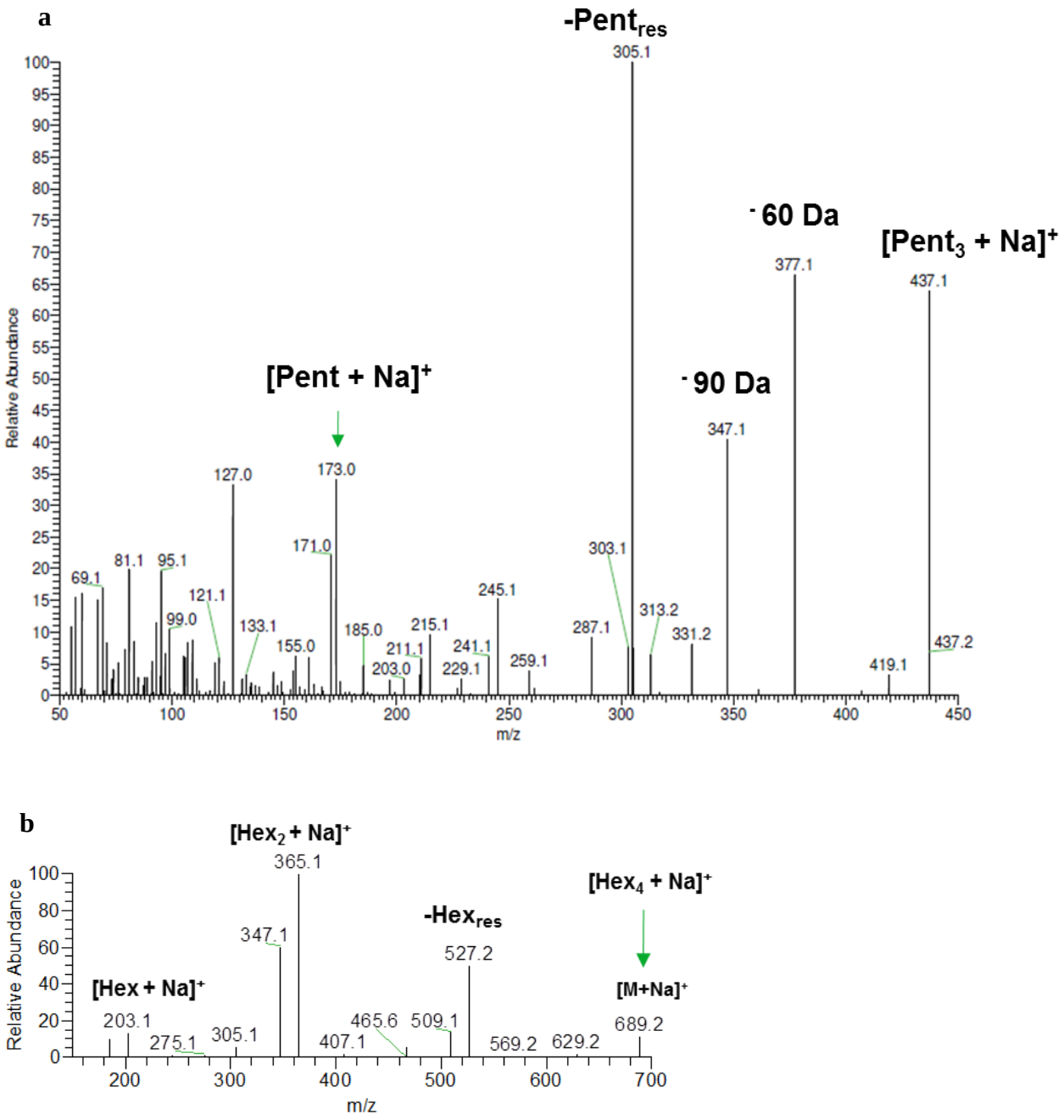



**Figure 16** – ESI-MS$^2$ spectra of the (a) trisaccharide α-(1→5)-arabinotriose and of the (b) tetrasaccharide 6$^1$-α-D-Galactosyl-β-1,4-mannotriose, obtained by Q Exactive Orbitrap.

# Chapter VII

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## VII. References


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