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Contribution of non-enzymatic transglycosylation reactions to the honey oligosaccharides origin and diversity

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Abstract: Non-enzymatic transglycosylation reactions are known to occur under high sugar concentrations, high temperatures, low moisture environments and acidic conditions. Although honey is not a thermally processed food, its high sugars concentration under an acidic and low moisture environment for prolonged periods of time may also promote these reactions. To test this hypothesis six model solutions containing combinations of sucrose and glucose or fructose, prepared with water and diluted citric acid at pH 4.0 and 2.0, were incubated at 35 °C during up to 5 months, similar to the honey under hive conditions. Electrospray ionization mass spectrometry (ESI-MS) allowed to observe polymerization products soon after their incubation. After 5 months, a degree of polymerization of 6 was detected, similarly to the honey samples used for comparison. Maltose, isomaltose, inulobiose, sophorose, gentiobiose, 1-kestose and panose were detected in both model solutions and honey samples, showing that non-enzymatic transglycosylation reactions also contribute to oligosaccharides origin and diversity in honey.

Keywords: ESI-MS; honey ripening; ICS-29; inulobiose; 1-kestose; maltose; methylation analysis; oligosaccharides formation; orbitrap-mass spectrometry; reversion reactions.

Introduction

Non-enzymatic transglycosylation reactions consist of the transfer of glycosyl units to the hydroxyl groups of other glycosides, yielding new carbohydrates [1]. These reactions are known to occur during the dry thermal processing of food matrices containing oligo- and polysaccharides and with low moisture content, as reported to coffee [2, 3]. Following the same principle, carbohydrates polymerisation is also described in sucrose [4] and glucose [5] caramelisation, as well as during the production of invert syrup [6, 7], through acid-catalysed hydrolysis of sucrose at high temperatures. These are also known as reversion reactions.

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Therefore, non-enzymatic transglycosylation reactions are favoured by high temperatures, low water content, high sugar concentration and acidic environments [2–7].

Honey is mainly composed of carbohydrates, having a great diversity of oligosaccharides [8]. However, this diversity, and quantity in honey raw materials, such as nectar and pollen, are far from that observed in honey [9]. Consequently, there is a need for understanding the mechanisms behind oligosaccharides formation in this matrix. The research on this subject, mainly conducted in the past century, demonstrated the α-glucosidase activity of invertase, i.e. the capacity to transfer α-glucosyl residues to other carbohydrates, preferentially to other glucose moieties [10]. As this transglucosylation activity was reported both to bees' and honey's invertases [10–12], the action of this enzyme was proposed as the source for α-glucose linked sugars. Nonetheless, there is still no explanation for the origin of the remaining oligosaccharides, namely those with β-glucose residues and with more than one fructose moiety. The presence of osmophilic yeasts in honey that possess enzymes α-glucosidases and β-fructofuranosidases may justify these oligosaccharides [8]. Another possible explanation is the occurrence of non-enzymatic transglycosylation reactions which are chemically favoured in honey, due to its high carbohydrate concentration (95 % of honey dry weight), low moisture content (below 20 %) and low pH value (less than 4.7) [13]. Due to bees' thermoregulation processes, beehive temperatures average 35 °C in brood nests [14], which is a low temperature when compared to those employed during the thermal processing of foods and carbohydrates. However, the long ripening periods, allied with the high sugar concentration and the mildly acidic conditions, may contribute to the honey oligosaccharides diversity. These reactions are an explanation for the increase in the complexity of sugars during honey storage [15–17]. However, the naturally occurring enzymes and other substances in honey could also justify the sugars polymerisation, which do not allow to assure the occurrence of non-enzymatic transglycosylation reactions. Therefore, the relevance of non-enzymatic transglycosylation reactions in honey oligosaccharides origin should be ascertained and the mechanism responsible for the oligosaccharides formation should be discussed based on models with controlled parameters.

The present work aims at confirming the occurrence of non-enzymatic transglycosylation reactions contributes to honey oligosaccharides origin and diversity, through the evaluation of solutions mimicking honey during the ripening process. The solutions were composed of two of the main nectar sugars (sucrose plus fructose/glucose), with a moisture percentage of 20 % (w/w) and exposed to the average hive temperature (35 °C). The occurrence of polymerisation reactions was assessed by electrospray ionization mass spectrometry (ESI-MS) and by gas chromatography mass-spectrometry (GC-qMS) techniques.

Experimental

Carbohydrate samples and preparation of the mixtures

Sucrose (Prolab), fructose (Sigma-Aldrich) and glucose (Scharlau), with purity ≥98 %, were used to prepare the six mixtures: three composed of sucrose (Suc) plus glucose (Glc) (1:1 w/w) and another three of sucrose plus fructose (Fru) (1:1 w/w) with 80 % (w/w) of sugars. The three model solutions of each carbohydrate mixture differ in the dissolving solution: ultrapure water, diluted citric acid at pH 4.0 and diluted citric acid at pH 2.0. All solutions were homogenized on a vortex mixer and by sonication and stored in an oven at 35 °C. Samples were collected after 3 and 5 months of incubation, for further analyses.

To simplify the identification of each model solution, these will be designated by the first letter of the two carbohydrates used in the mixture, followed by the character related to each dissolving solution (W for ultrapure water, 4 for diluted citric acid at pH 4.0, and 2 for diluted citric acid at pH 2.0). The Roman number, III or V, indicates the sampling month (3 and 5 months, respectively). Example: SG2_III corresponds to the model solution of sucrose plus glucose prepared with diluted citric acid at pH 2.0, with an incubation of 3 months (Table 1).

Table 1: pH Values of the model solutions, after 3 months (mean ± standard deviation).

	Sucrose:Glucose			Sucrose:Fructose		
Solution	Ultrapure water	Citric acid, pH 4.0	Citric acid, pH 2.0	Ultrapure water	Citric acid, pH 4.0	Citric acid, pH 2.0
Code	SGW_III	SG4_III	SG2_III	SFW_III	SF4_III	SF2_III
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

Honey samples

Three multiflora honey samples were used in this study, obtained from three different regions of Portugal, in July 2016, differing on the ripening periods (2; 4; and 12 months). Henceforth, these will be named according to their ripening period (e.g. H2 corresponds to honey with 2 months of ripening).

pH Determination

The pH was measured using a pH-meter (TitroMatic 1S) with a precision of ±0.02 pH units in solutions with a concentration of 10 % (w/v), according to the Harmonised methods for honey analysis [18]. All measurements were performed in triplicate.

Oligosaccharides fractionation by ligand-exchange/size-exclusion chromatography (LEX/SEC)

Honey samples and model solutions were fractionated by a 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 °C, the injected sample volume was 500 μL and ultrapure water (Milli-Q®) was used as eluent at a flow rate of 2.80 mL·min^{-1}. A refractive index detector (Knauer K-2401, Berlin, Germany) was used. The column calibration was carried out using glucooligosaccharides (DP 1–4). The fractions of acids and neutral compounds were separately collected, dried and kept in a desiccator at ambient temperature for further analysis.

Electropsray mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS^n) conditions

The occurrence of non-enzymatic transglycosylation reactions in model solutions throughout time, as well as its extension, was assessed by ESI-MS and electrospray ionization collision-induced dissociation tandem mass spectrometry (ESI-CID-MS^n) analyses. Each sample, previously dissolved in ultrapure water, was diluted in methanol/water (1:1, v/v) containing formic acid (1 %, v/v). 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, USA), and conditions were described in a previous work [2]. The analyses were carried out in positive ion mode because high sensitivity was achieved in positive than in negative ion mode. Oligosaccharides were easily ionized to form $[M+Na]^+$ ions. Data were acquired and analysed using Xcalibur software.

ESI-MS spectra were also acquired in the high resolution Q Exactive hybrid quadrupole – Orbitrap mass spectrometer (Thermo Firsher Scientific, Germany), interfaced with an H-ESI II ion source, that allowed accurate mass measurements of the new species assigned to the maximum degree of polymerisation (DP) of honey oligosaccharides, as well as for the analysis of the honey acidic fraction. The acquisition method was performed as described by Moreira et al. [2].

Oligosaccharides identification and quantification

Oligosaccharide fractions obtained from honey and model solutions were reduced with sodium borohydride and acetylated. The alditol acetates were analysed by gas chromatography-mass spectrometry (GC-qMS), as previously described [19]. Sucrose, trehalose, kojibiose, maltose, laminaribiose, nigerose, isomaltose, cellobiose, sophorose, gentiobiose, melezitose, erlose, panose, isomaltotriose, nigerotriose and cellotriose, which are reported in honey [20], were used as standards, along with samples in which sugar composition was determined. The amounts of di- and trisaccharides were depicted as equivalents of sucrose and melezitose, respectively (i.e. these were calculated with the response factors of sucrose and melezitose).

Glycosidic linkage analysis

The glycosidic linkage composition of di- and trisaccharides model solutions' fractions was determined by GC-qMS of the partially methylated alditol acetates (PMAAs), as described by Reis et al. [21]. The oven temperature was programmed as follows: 80–140 °C at 7.5 °C/min (hold 5 min at 140 °C), to 143.2 °C at 0.2 °C/min, to 200 °C at 12 °C/min and then to 250 °C at 50 °C/min. The helium carrier gas had a flow rate of 8.5 mL/min and the column head pressure was 124.1 kPa. Identification was achieved by comparing with the standard mass spectra (MS) and with other spectra available at the laboratory made database. Further, the retention time and fragmentation pattern correspondent to terminally-, $(2\rightarrow1)$- and $(2\rightarrow3)$-linked fructose residues, not available in the literature, were obtained by conversion of sucrose, 1-kestose and melezitose into PMAAs using the same procedure (Table S1).

Results and discussion

Honey model solutions pH

The model solutions mimicking honey were prepared using mixtures of sucrose and fructose and sucrose and glucose, having 20 % of water from the dissolving solution (ultrapure water, diluted citric acid at pH 4.0 and diluted citric acid at pH 2.0). The pH values were monitored until the first sampling moment, after 3 months, by diluting the model solutions 10 times with water. It was observed a continuous decrease of the pH in the water dissolved mixtures, from a pH of 6.5 to 3.7 and 3.9 in SGW_III and SFW_III, respectively (Table 1). This can be justified by the greater affinity of sugars for H^+ than for water at high sugar concentrations, where the polyols act as solvents, increasing the solvation energy of the protons, which results in a greater acidity of the solution [22]. The solutions prepared with diluted citric acid at pH 4.0 showed a pH value of 4.8, when prepared, and a pH value of 3.7 for SG4_III and 4.4 for SF4_III after 3 months, showing the buffering effect of citric acid. Similarly, the solutions prepared with diluted citric acid at pH 2.0 had a final pH value of approximately 2.6 for both SG2_III and SF2_III.

The pH of the solutions prepared with water and diluted citric acid at pH 4.0 were within the range reported for honey [23], as well as for the honey samples analysed with pH ranging between 3.98 and 4.38.

Electrospray mass spectrometry analyses of model solution carbohydrates

The monitoring of changes occurring in model solutions composition was assessed by ESI-MS analysis. The oligosaccharides were mainly detected as sodium adduct ions ($[M+Na]^+$), which are characteristic of neutral oligosaccharides [3, 24].

To assure the purity of the standards, mixtures of Suc plus Glc and of Suc plus Fru, without any treatment or incubation period, were analysed. Ions at *m/z* 527, 689, 851 and 1013, corresponding to hexose oligosaccharides with a DP of 3, 4, 5 and 6, respectively, were absent or considered vestigial, as their relative abundance was inferior to 3 %. After incubation of model solutions, $[Hex_{3–6} + Na]^+$ ions relative abundance increased up to 34 % ($[Hex_3 + Na]^+$ ion for SF4_V) (Table 2). The assignment of these ions was supported on the basis of their fragmentation pattern under ESI-CID-MS^n conditions, that shows product ions resulting from glycosidic cleavages (ESI-CID-MS^2 spectra of $[Hex_n + Na]^+$ ions are provided in Fig. S1, as Supplementary material). Thus, the neutral loss of 162 and 180 Da correspond to the loss of a hexose residue ($-Hex_{res}$) and a hexose ($-Hex$), respectively. Also, it was noticed the appearance in the ESI-MS spectra of other new ions at *m/z* 347, 509, 671, 833, were assigned to the dehydrated oligosaccharide ions $[Hex_{2–5} - H_2O + Na]^+$. These ions also evidence the occurrence of non-enzymatic transglycosylation reactions in model solutions subjected to the temperature of 35 °C after 5 months. The acids promote the protonation of the hydroxyl group of the anomeric carbon at the reducing sugar end, leading to dehydration of the molecule. After dehydration, a carbocation is formed, which may react with the hydroxyl groups of the compounds present in the mixture [25–27]. This nucleophilic attack can occur at an intermolecular level, giving origin to new oligosaccharides ($[Hex_n + Na]^+$), or at an intramolecular level if the carbocation undergoes an internal ring formation, generating anhydrosugars ($[Hex_n - H_2O + Na]^+$) [27]. The solutions prepared with citric acid at pH 2.0, after 5 months of incubation (SG2_V and SF2_V), showed a high abundance of anhydro derivatives ($[Hex_n - H_2O + Na]^+$), more pronounced with fructose (Figs. S2 and S4). Concerning the new oligosaccharides formation, after only 15 days, the ion at *m/z* 527 ($[Hex_3 + Na]^+$) was observed in all solutions, with a relative abundance up to 30 %. Besides, the maximum DP observed for SG solutions was 4 ($[Hex_4 + Na]^+$), while for all SF solutions it was 5 ($[Hex_5 + Na]^+$). Thus, non-enzymatic transglycosylation reactions were shown to occur in model solutions soon after their incubation. An increase in the complexity of the synthesised oligosaccharides was observed over time and, after incubated for 5 months, the maximum DP increased for 5 in SG solutions and for 6 in SF solutions (Fig. 1). The presence of oligosaccharides with a DP of 5 and 6, but also of DP 7, were found in honey samples, confirmed through high resolution and high mass accuracy measurements using a hybrid quadrupole-Orbitrap mass spectrometer, due to the higher complexity of the honey matrices (Fig. 2).

Due to the presence of citric acid in the model solutions, new ions assigned as $[Hex_nCitA + Na]^+$ ions were also observed in the ESI-MS (Table 2, Fig. S3), evidencing the presence of oligosaccharides covalently linked with citric acid, especially in SG2_V and SF2_V (Fig. S5 in Supplementary material). Accordingly, as gluconic acid is the main acid in honey [28], the ion at *m/z* 397 in honey samples, can be assigned to two gluconic acids, $[HexonicA_2 + Na]^+$. Gluconic acid polymers were proposed in honey by Stinson et al. in [29]. However, no further evidence showing their occurrence has been reported, to the best of our knowledge.

Oligosaccharides identification and quantification

As the occurrence of non-enzymatic transglycosylation reactions in solutions mimicking honey properties was verified by mass spectrometry techniques, to further prove their contribution to the diversity of honey oligosaccharides, these were fractionated by LEX-SEC, derivatized to alditol acetates and the glycosidic linkages

Table 2: Non-enzymatic transglycosylation reaction products identified in model solutions by ESI-MS with respective *m/z* values of the $[M + Na]^+$ ions and proposed assignments.

Proposed assignment	No. (*n*) of hexose (Hex) units					
	1	2	3	4	5	6
$[Hex_n + Na]^+$	203	365	527	689	851	1013
$[Hex_n - H_2O + Na]^+$		347	509	671	833	
$[Hex_nCitA + Na]^+$	377	539	701			

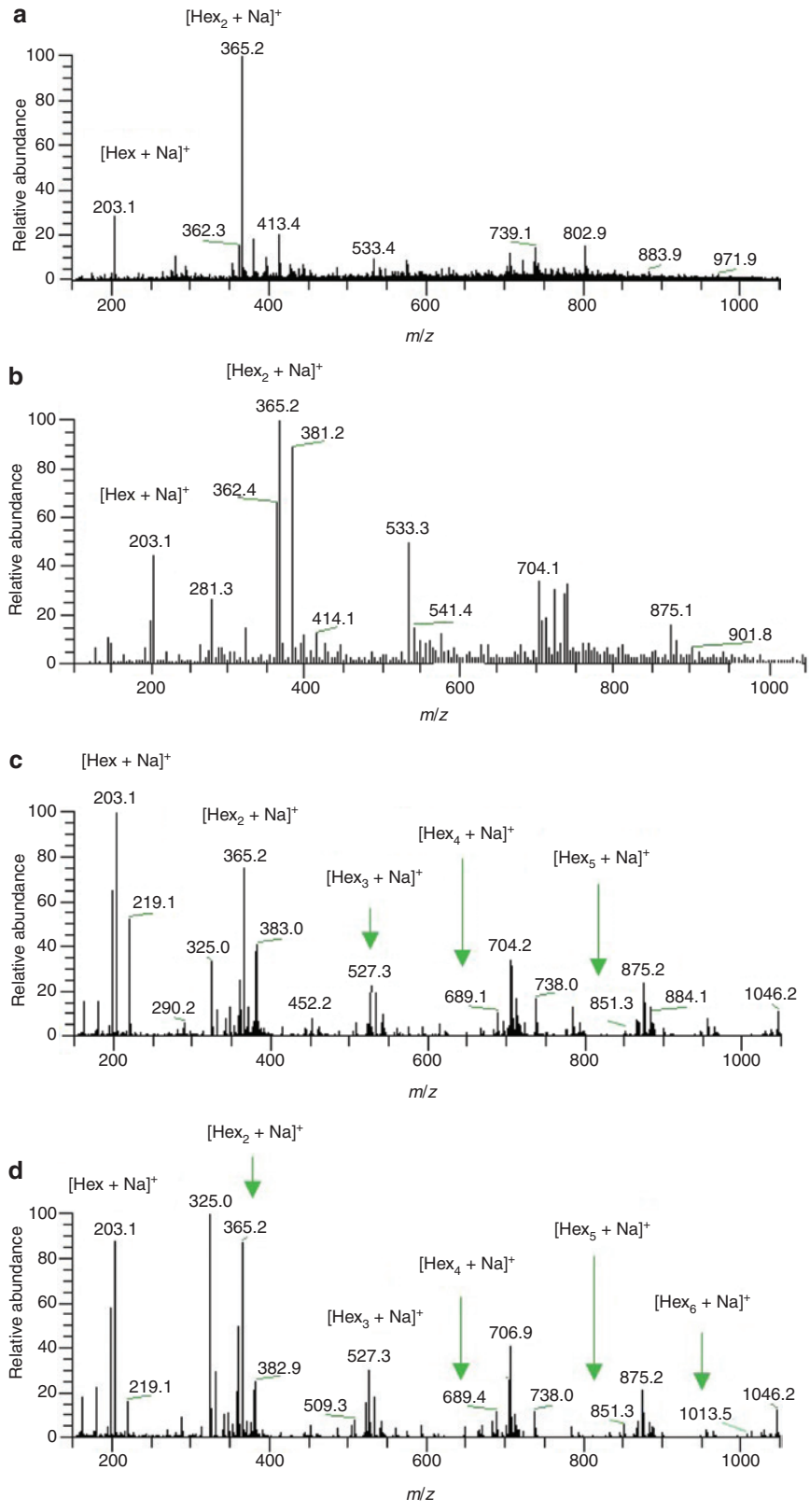

Fig. 1: ESI-MS spectra obtained for control mixtures of (a) SG and (b) SF and for the model solutions (c) SG4_V and (d) SF4_V.

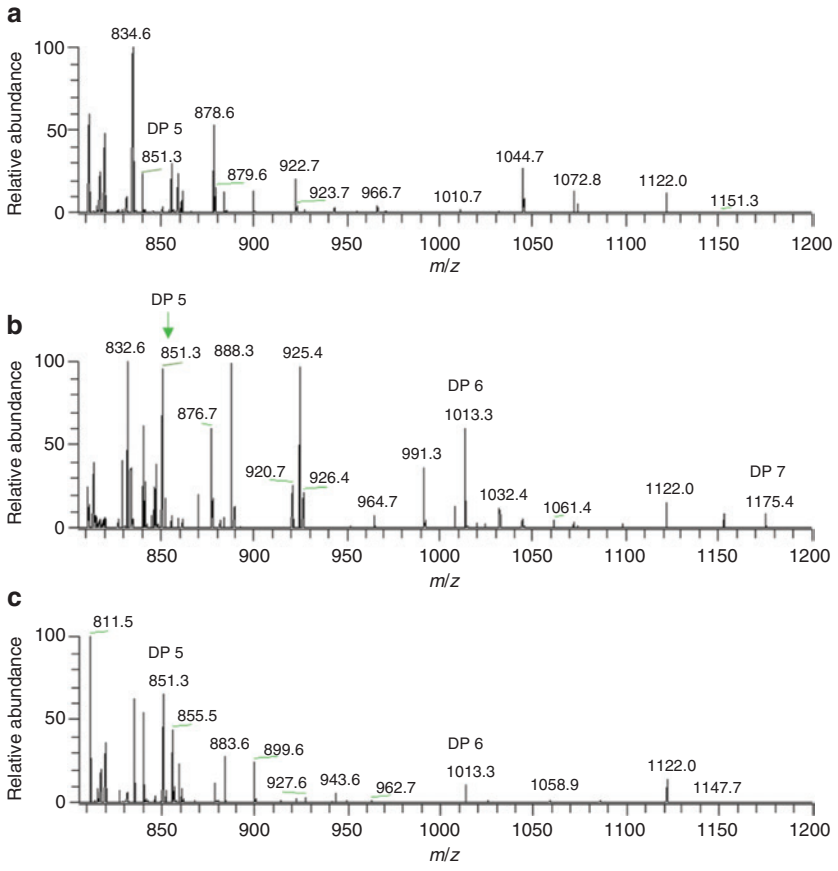

Fig. 2: ESI-MS spectra obtained for (a) H2, (b) H4 and (c) H12 using a high resolution Orbitrap-based mass spectrometer.

were identified by the formation of partially methylated alditol acetates (PMAAs, Table S2) and analysed by GC-qMS. Table 3 shows the tentative identification of the oligosaccharides based on this combined approach. Besides the compounds identified with standards, several peaks with MS characteristic of alditol acetate derivatives were also detected. It was possible to determine oligosaccharides polymerisation degree based on their retention time. The reducing sugars were distinguished from the non-reducing ones due to their fragmentation pattern differences (Table 3), as reducing sugars were converted by sodium borohydride into the respective alditols, while the non-reducing sugars were not modified. The subsequent acetylation of reducing sugars resulted in the formation of a pentaacetylated hexose residue with a mass of 375 Da. Therefore, the ion at *m/z* 375 is the diagnostic of reducing oligosaccharides. The glycosidic linkages of the reducing di- and trisaccharides produced on model solutions were (2→1-Fru), (2→3-Fru), (2→6-Fru), (1→2-Glc), (1→3-Glc), (1→4-Glc) and (1→6-Glc) (Table S2). These glycosidic linkages are in accordance with the presence of honey reducing disaccharides trehalulose, turanose and palatinose [30], the non-reducing disaccharide neotrehalose [31], the reducing trisaccharides maltotriose, centose, 3-α-isomaltosylglucose and isopanose, and the non-reducing trisaccharides neokestose, 6-kestose, theanderose, and [Fru(β2→6)Glc(α1↔α1)Glc] (Table 3).

Table 4 shows the quantification of the identified oligosaccharides based on the available standards comparison. The extension of the non-enzymatic transglycosylation reaction products increases over time, as observed by the increase of both di- and trisaccharides amount between 3 and 5 months of incubation. When observing the same incubation time and comparing model solutions with the same dissolving solution (water, diluted citric acid at pH 4.0 and at pH 2.0), in general, those prepared with fructose yielded higher amounts of di- and trisaccharides, with the exception of SGW_V which produced 10.7 mg of trisaccharides per gram of solution while SFW_V formed 9.1 mg/g. After 3 months, inulobiose [Fru(β2→1)Fru] and blastose [Fru(β2→6)Glc] were observed in all model solutions. After 5 months, inulobiose was preferably produced

Table 3: Compounds tentative identification based on retention time, fragmentation pattern and glycosidic linkage analyses.

Assignment	t_R[c]	Fragmentation pattern[d]	Glycosidic linkages
Sucrose[a]	26.52	169 (100); 211 (54.3); 109 (52.8); 127 (13.16); 331 (12.9); 271 (5.7)	*t*-Fru; *t*-Glc
Trehalose[a]	27.22	169 (100); 109 (52.0); 127 (14.3); 331 (9.0); 271 (7.8); 211 (6.3)	*t*-Glc; *t*-Glc
Kojibiose[a]	28.10	169 (100); 109 (57.0); 153 (51.5); 375 (21.7); 361 (16.0); 331 (13.4); 211 (8.2)	*t*-Glc; 2-Glc
Maltose[a]	28.97	169 (100); 109 (42.5); 153 (31.2); 331 (15.8); 375 (13.0); 361 (12.2); 211 (6.9)	*t*-Glc; 4-Glc
Nigerose[a]	28.98	169 (100); 109 (43.1); 153 (21.8); 361 (19.5); 331 (18.9); 375 (8.4); 211 (6.6)	*t*-Glc; 3-Glc
Inulobiose[b]	28.85	153 (100); 375 (70.6); 211 (54.3); 169 (24.9); 128 (22.7); 109 (20.3); 433 (18.4)	*t*-Fru; 1-Fru
	29.59	153 (100); 375 (69.2); 211 (49.3); 169 (21.8); 128 (27.0); 433 (20.6); 109 (12.1)	
Blastose[b]	29.13	153 (100); 375 (61.9); 211 (47.9); 169 (25.2); 128 (24.6); 109 (14.5); 433 (19.3)	*t*-Fru; 6-Glc
Isomaltose[a]	29.67	169 (100); 153 (74.7); 375 (53.9); 109 (44.3); 361 (32.8); 331 (13.2); 211 (9.0)	*t*-Glc; 6-Glc
Sophorose[a]	30.52	169 (100); 153 (63.4); 109 (50.6); 375 (40.9); 361 (21.5); 331 (15.4); 211 (6.8)	*t*-Glc; 2-Glc
Gentiobiose[a]	31.21	169 (100); 153 (78.0); 375 (65.3); 109 (45.3); 361 (31.0); 331 (15.5); 211 (8.4)	*t*-Glc; 6-Glc
1-Kestose[a]	40.70	169 (100); 211 (40.1); 109 (38.7); 331 (27.5); 127 (10.1); 271 (9.2)	*t*-Glc; 1-Fru; *t*-Fru
Melezitose[a]	40.67	169 (100); 109 (44.0); 331 (18.7); 271 (13.7); 127 (11.2); 211 (10.4)	*t*-Glc; 3-Fru; *t*-Glc
Erlose[a]	41.53	169 (100); 211 (74.2); 109 (53.2); 331 (35.4); 127 (12.4); 271 (5.9)	*t*-Fru; 4-Glc; *t*-Glc
Panose[a]	41.62	169 (100); 109 (38.0); 331 (36.1); 375 (20.8); 153 (20.3); 211 (9.7); 361 (5.9)	*t*-Glc; 6-Glc; 4-Glc
Inulotriose[b]	41.23	375 (100); 211 (75.0); 153 (60.3); 169 (47.1); 109 (26.0); 331 (25.0); 128 (15.8)	*t*-Fru; 1-Fru; 1-Fru
	41.45	375 (100); 211 (72.2); 153 (53.7); 169 (41.0); 331 (23.3); 109 (23.0); 128 (13.8)	
R[e] Disacchar.	27.15–32.33	169/153/211 (100); 331; 375; 361[g]	NR term.: *t*-Fru/*t*-Glc; R term.: 3-Fru[h]/6-Fru/2-Glc/3-Glc/4-Glc/6-Glc
NR[f] Disacchar.	30.14	169 (100); 109; 331; 271; 211	*t*-Fru; *t*-Glc
R Trisacchar.	41.12–42.39	169/153/211 (100); 331; 375; 361[g]	NR term.: *t*-Fru/*t*-Glc; Chain: 3-Fru[i]/6-Fru/2-Glc/3-Glc[j]/4-Glc/6-Glc
NR Trisacchar.	40.45–41.34	169/211 (100); 109; 331; 271	NR term.: *t*-Fru/*t*-Glc; Chain: 3-Fru[i]/6-Fru/2-Glc/3-Glc[j]/4-Glc/6-Glc

[a]Compound identified by standard comparison using GC-qMS retention time and mass spectrum; [b]Compound identified by comparison with a fructooligosaccharide mixture with known composition [19]; [c]Retention time (minutes) in the DB-1 column; [d]Values in parentheses are the relative intensities of the fragments. [e]R, Reducing; [f]NR, non-reducing; [g]not always observed; [h]except for SG2_V and SF2_V; [i]only for SG2_V and SF2_V; [j]except for SG4_V and SF2_V.

in SF solutions (10.6 mg/g and 14.3 mg/g for SFW_V and SF2_V, respectively), while blastose was mostly produced in SGW_V with an amount of 5.7 mg/g. Inulobiose was also detected in the honey samples, although in small amounts (up to 0.02 mg/g), while blastose was not detected. In fact, blastose is not recognized as a honey disaccharide. As this compound is formed in all model solutions but not in honey, it may be a candidate for tracing sucrose addition to honey.

All glucose solutions showed the presence of maltose and isomaltose, after the maximum incubation period, with SGW_V producing the higher amount of both reducing disaccharides (5.1 mg/g of maltose and 3.0 mg/g of isomaltose). Maltose was also produced in SF4_V (1.2 mg/g) and isomaltose was quantified in

Table 4: Quantification of honey oligosaccharides and non-enzymatic transglycosylation reaction products of model solutions with 3 and 5 months of incubation (mg of compound/g of sample).

Assignment	mg/g												
	Honey samples	SGW	SG4	SG2	SFW	SF4	SF2	SGW	SG4	SG2	SFW	SF4	SF2
		3 Months						5 Months					
DP 2													
Sucrose	1.0–2.8	NQ	NQ	0.3	NQ	NQ	0.5	NQ	NQ	0.8	NQ	NQ	0.9
Trehalose	0.1–2.9												
Kojibiose	ND–0.9												
Maltose	44.3–85.5	0.2						5.1	1.3	2.1		1.2	
Nigerose	ND–1.2												
Inulobiose	ND–0.02	2.8	1.2	0.6	10.3	2.1	1.1	4.9	4.4	4.9	10.6	4.9	14.3
Blastose	ND	0.3	0.5	0.1	3.1	0.5	1.0	5.7	0.8	0.6	4.2	0.1	4.5
Isomaltose	ND–8.2		1.1	0.4				3.0	1.6	2.1			0.1
Sophorose	2.8–11.8									0.3			
Gentiobiose	ND–0.2			0.1			tr			0.4			0.1
R	16.4–41.8	1.9	4.6	5.6	9.2	6.4	16.7	13.5	3.7	7.8	39.0	11.6	10.8
NR	0.2–14.2												
Dehydrated	0.1–1.9			1.7			7.0		7.2	4.6	16.8	9.2	39.3
DP 3													
1-Kestose	ND–0.4		0.1		0.6	0.2	0.1	3.9	0.7		3.6	4.7	0.1
Melezitose	1.4–9.6												
Erlose	ND–4.2												
Panose	ND–6.4							0.4					
Inulotriose	ND						tr						0.3
R	0.5–9.9						0.3				1.1	0.5	1.2
NR	1.7–13.2				2.6	0.4	0.2	6.4			4.4	21.2	0.2
Dehydrated	ND						0.3						1.3
Total	74.8–153.6	5.2	7.4	8.9	25.7	9.5	27.3	42.9	19.8	23.6	79.8	53.5	73.0

ND, Not detected; NQ, not quantified; R, reducing; NR, non-reducing; tr, trace amounts (<0.1 mg/g); Relative standard deviation (RSD%) <32 %.

SF2_V, but in small amounts (0.1 mg/g). Maltose is reported as the most abundant honey oligosaccharide [20], and was also the major compound from the honey samples analysed, amounting from 44.3 mg/g to 85.5 mg/g. Its presence is usually justified by the α-glucosidase activity of honeybees [10, 11] and diastase activity towards starch [32]. Isomaltose, also an α-glucosyl disaccharide reported to derive from invertase activity [10, 11], was quantified in honey samples (up to 8.2 mg/g). However, both maltose and isomaltose were produced non-enzymatically, mainly in glucose containing model solutions, which indicate another mechanism contributing to the presence of these disaccharides in honey.

Gentiobiose [Glc(β1 → 6)Glc] was produced in the most acidic solutions, with 0.1 mg/g and 0.4 mg/g for SF2_V and SG2_V, respectively. It was also found in small amounts in honey samples (maximum of 0.2 mg/g). Despite having a β-glucosyl moiety, this disaccharide was also reported as a product of honey crude enzymatic activity [10]. Sophorose [Glc(β1 → 2)Glc] was also formed in the glucose solution with the lowest pH (0.3 mg/g). This disaccharide was also present in honey samples, in concentrations between 2.8 and 11.8 mg/g. Sophorose, along with maltose, isomaltose and gentiobiose were also reported to be produced during the thermal treatment of glucose [5, 27]. Thus, the non-enzymatic production of these disaccharides already evidenced on acidic and extreme temperature conditions, was now demonstrated to occur at 35 °C, with high sugar concentration, acidic conditions and long reaction periods.

The trisaccharide 1-kestose [Fru(β2 → 1)Fru(β2 ↔ 1α)Glc] was produced in all model solutions, except in SG2_V, with similar amounts for the water prepared solutions (approximately 4.0 mg/g) and a higher

production for SF4_V (4.7 mg/g). These values were ten times higher than the amount found in honey (0.4 mg/g). This trisaccharide has been reported to be produced by pollen invertase [33]. Besides, 1-kestose was also shown to be produced by yeast enzymes present in honey [34, 35]. Nonetheless, this trisaccharide seems to have its origin also in non-enzymatic reactions. Similarly, the reducing trisaccharide panose [Glc(α1 → 6)Glc(α1 → 4)Glc] was detected in SGW_V (0.4 mg/g). It was also quantified in honey with concentrations up to 6.4 mg/g. It was reported to be a product resultant from maltose enzymatic activity of yeast present in honey [36].

SF2_V was the only solution where inulotriose [Fru(β2 → 1)Fru(β2 → 1)Fru] was quantified (0.3 mg/g). Accordingly, inulotriose was only produced in fructose solution with a pH value approximately 2.0 (Table 4), which is the same pH conditions employed during the production of syrups [6, 37]. Inulotriose was not detected in honey samples neither it is reported in honey. In fact, it was proposed a marker for honey adulteration with high fructose inulin syrups [38].

In addition to the identified compounds, a great quantity and diversity of di- and trisaccharides was quantified for both model solutions and honey samples. Reducing disaccharides were produced in the tested conditions in contents of up to 13.5 mg/g and 39.0 for SGW_V and SFW_V, respectively, reaching values close to those observed for honey samples (16.4 mg/g–41.8 mg/g). Furthermore, non-reducing trisaccharides were quantified in model solutions, especially in SGW (6.4 mg/g) and SF4 (21.2 mg/g), while reducing trisaccharides were only detected in fructose solutions, in identical amounts for SFW and SF2. In honey, both reducing and non-reducing trisaccharides were found within a similar range, with up to 1 g/100 g of honey (Table 4).

The identification of dehydrated compounds in model solutions is in accordance with the ESI-MS results, as well as their substantial amount in SF2_V, in which these structures yield 54 % of the produced compounds. This may be explained by the bond cleavage during the acid-catalysed hydrolysis of sucrose occurring at the fructosyl-oxygen bond. Thus, it yields D-glucose and a fructose carboxonium ion, and the latter can react with the other matrix components or with its own hydroxyl groups, as well as with another fructose carboxonium ion to form difructose dianhydrides [39]. The presence of dehydrated compounds in honey samples were vestigial when compared with those found in model solutions, especially in the extremely acidic fructose solutions. Therefore, when an exacerbated presence of these compounds occurs in honey, it may be indicative of its adulteration. The presence of dehydrate structures, namely difructose dianhydrides, was proposed as a strategy for the detection of honey adulteration with high fructose inulin syrups [38].

From all the tested conditions, solutions initially prepared with sucrose plus glucose had a higher tendency in producing oligosaccharides with the same structure as those reported for honey. This was in accordance with all honey oligosaccharides (except inulobiose), which have at least a glucose residue [20]. Besides, the mimetic glucose solution prepared with water produced a wider variety and quantity of oligosaccharides, when compared with the citric acid glucose solutions, possibly because the presence of carboxylic acids promotes a higher extent of oligosaccharides dehydration. Accordingly, acid-catalysed hydrolysis of sucrose was promoted in model solutions with citric acid at pH 2.0, where the final concentration of sucrose was lower than 1 mg/g for SG2 after 3 and 5 months of incubation (Table 4).

Conclusions

Model solutions of SG and SF, mimicking honey during the ripening process, increased their complexity in oligosaccharides with time, producing compounds with up to five and six polymerized monosaccharides, for SG and SF, respectively. Similar levels of complexity were observed for the analysed honey samples.

After 5 months of incubation, the mimetic solutions produced a wide variety of oligosaccharides, from which maltose, inulobiose, isomaltose, sophorose, gentiobiose, 1-kestose, and panose were found in honey samples. SGW_V performed the most mimetic solution, producing a wider variety of honey oligosaccharides as well as a higher amount of them.

Blastose was produced in all model solutions but not in honey, showing that it can be a candidate for tracing sucrose addition to honey. Similarly, the extension of dehydrated oligosaccharides can be used also as sugar addition markers.

Although these experiments were performed with mixtures of sugars, it is expected that similar reactions occur during the honey ripening process, allowing to explain the complexity of oligosaccharides identified in this matrix. Therefore, these results provide further evidence for the hypothesis of the occurrence of non-enzymatic transglycosylation reactions in honey during the ripening process, as well as further supports studies showing an increase of oligosaccharide complexity during storage.

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