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# Gas chromatographic–mass spectrometric method for the qualitative and quantitative determination of disaccharides and trisaccharides in honey

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**Abstract**

An improved method has been developed to identify and quantify honey disaccharides and trisaccharides by gas chromatography and mass spectrometry. The procedure, based on mass spectral and retention data ("retention time windows") determined on two capillary columns with different stationary phases allowed the identification and quantitation in honey of 16 disaccharides and 9 trisaccharides, some of which were not previously identified by GC. The reliability of the analytical results was considerably improved by the use of this procedure: several unidentified disaccharides and trisaccharides were detected, and their presence was taken into account in the quantification.


*Keywords:* Honey; Disaccharides; Trisaccharides; Gas chromatography–mass spectrometry

## 1. Introduction

The resolution of GC capillary columns affords chromatographic profiles which contain qualitative and quantitative information useful for characterization purposes, but the analytical study of oligosaccharides in complex mixtures, such as honey, presents difficult problems. Honey is made up of about 70% monosaccharides (glucose and fructose) and 10% oligosaccharides. These minor constituents are composed of several units (from two to six) of glucose and fructose with the glycosidic bond in different positions and configurations. Siddiqui [1] characterized up to 14 disaccharides and 11 trisaccharides in honey, and a critical review by Doner [2] indicates satisfactory evidence for about 10–13 disaccharides and 8–9 trisaccharides. The number of sugars identified or quantified by high-resolution chromatographic methods (sometimes coupled with MS) is presently lower [3–12]. The main problems arise from the lack of commercial standards, the coelution of compounds (even when using long, polar columns), the similar MS fragmentation pattern obtained for most isomers and the interference of other matrix compounds eluting along with saccharides.

TMS oximes and TMS ethers are probably the most popular derivatives for GC analysis of carbohydrates. Since TMS ethers give a different derivative for each anomeric form present in a solution, and up to five derivatives may be expected for every hexose [13], TMS sugar GC profiles can be very complex. Oximation of the free carbonyl groups prior to silylation suppresses the anomeric centre, and only two forms (*E* and *Z*) are obtained for every reducing sugar [13–15]. Disaccharides having glucose, galactose or mannose as their reducing moiety produce two well-resolved peaks, whose ratio varies between 3:1 and 10:1 [15]. Funcke and von Sonntag [16] found for aldohexoses that the major peak corresponds to the *E* (syn) isomer, while the minor peak is the *Z* (anti) isomer. Disaccharides having fructose as the reducing moiety give two peaks with an approximate 1:1 area ratio [14,16].

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Non-reducing saccharides (sucrose, trehaloses, melezitose, erlose and raffinose) produce only a peak corresponding to the octakis-TMS derivative [14,15].

Compound coelution frequently results in peak broadening, but it can go undetected if both compounds present the same retention. Chromatographic information can be enhanced by an alteration of sample characteristics; a double derivatization procedure has been employed to obtain two different chromatographic profiles (TMS oximes and TMS ethers) from the same sample and to confirm peak assignments from their differences or to detect possible coelutions [17]; nevertheless, this method did not resolve all the present sugars.

In this work, we have followed a different approach, which is less demanding in sample preparation and uses two capillary columns coated with different stationary phases. In this way, two different, and if possible complementary, chromatographic profiles are obtained for each sample in order to improve the qualitative and quantitative data obtained and to help in component identification and in the detection of unknown honey carbohydrates. The use of GC–MS made it possible to identify a disaccharide and two trisaccharides not previously identified by GC in honey.

## 2. Experimental

### 2.1. Standard substances

Analytical-standard cellobiose (4-*O*-β-D-glucopyranosyl-D-glucose), cellotriose (*O*-β-D-glucopyranosyl (1-4)-*O*-β-D-glucopyranosyl (1-4)-D-glucose), erlose (α-D-glucopyranosyl (1-4)-α-D-glucopyranosyl (1-2) β-D-fructose), β-gentiobiose (6-*O*-β-D-glucopyranosyl-β-D-glucose), isomaltose (6-*O*-α-D-glucopyranosyl-D-glucose), isomaltotriose (α-D-glucopyranosyl (1-6)-α-D-glucopyranosyl (1-6)-D-glucose), 1-kestose (β-D-fructofuranosyl (2-1)-β-D-fructofuranosyl (2-1)-α-D-glucose), kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose), laminaribiose (3-*O*-β-D-glucopyranosyl-β-D-glucose), maltose (4-*O*-α-D-glucopyranosyl-D-glucose), maltotriose (*O*-α-D-glucopyranosyl (1-4)-*O*-α-D-glucopyranosyl (1-4)-D-glucose), melezitose (α-D-glucopyranosyl (1-3) β-D-fructosyl (2-1)-α-D-glucose), nigerose (3-O-α-D-glucopyranosyl-D-glucose), panose (*O*-α-D-glucopyranosyl (1-6)-α-D-glucopyranosyl (1-4)-glucose), raffinose (*O*-α-D-galactopyranosyl (1-6)-α-D-glucopyranosyl (1-2)-D-fructose), sucrose (2-*O*-α-D-glucopyranosyl-β-D-fructofuranoside), were obtained from Sigma; leucrose (5-*O*-α-D-glucopyranosyl-D-fructose), melibiose (6-*O*-α-D-galactopyranosyl-D-glucose), palatinose (6-*O*-α-D-glucopyranosyl-D-fructose), α,α-trehalose (1-*O*-α-D-glucopyranosyl-α-D-glucopyranoside), α,β-trehalose (1-*O*-α-D-glucopyranosyl-β-D-glucopyranoside), β,β-trehalose (1-*O*-β-D-glucopyranosyl-β-D-glucopyranoside), turanose (3-*O*-α-D-glucopyranosyl-D-fructose) were obtained from Fluka (Madrid, Spain); maltulose (4-*O*-α-D-glucopyranosyl-D-fructose) was from Aldrich Chem. Co. (Milwaukee, WI); and sophorose (2-*O*-β-D-glucopyranosyl-D-glucose) was from Sarsynthèse (Merignac, France). Trehalulose (α-glucopyranosyl (1-1) fructose) was a gift from Dr. W. Wach of Südzucker AG, Mannheim.

### 2.2. Samples

Six artisanal honey samples were acquired directly from beekeepers of different regions of Spain. Two commercial nectar honeys and two commercial honeydew honeys were purchased at local markets.

### 2.3. Sample preparation

The formation of oximes before trimethylsilylation limits the possible tautomers of reducing sugars to two forms: *E* (*syn*) and *Z* (*anti*). Samples were prepared by diluting 0.5 g of honey to 25 mL with 80% ethanol; 1 mL of the solution was mixed with 1 mL of phenyl-β-D-glucoside (1 mg/mL) and evaporated under vacuum. Sugar oximes were formed using 2.5% hydroxylamine chloride in pyridine and heated to 75 °C for 30 min. After reaction, samples were persilylated using hexamethyldisilazane (HMDS) and trifluoroacetic acid (TFA) at 45 °C for 30 min [18] and centrifuged at 7000 × *g* for 5 min at 5 °C [19].

Reproducibility of retention time is the main requirement for a reliable characterization. A fitting procedure, using data from two standard added compounds (β-phenyl-glucoside and dotriacontane) and from the erlose present in all honey samples, which elute in different chromatographic zones, was used to reduce retention time deviations.

### 2.4. GC analysis

Stationary phases were chosen from among those having an operating temperature high enough to allow the elution of TMS oximes of trisaccharides. Methyl silicone was chosen as an apolar stationary phase that is very frequently used for medium–high temperature work [11,20,21], while phenylmethylsilicone (a medium-polarity phase) was selected, since it presents different elution characteristics for disaccharides, as shown in a preliminary study [15]. Column A was a 25 m × 0.25 mm i.d. × 0.1 μm film thickness fused silica column coated with Rtx-65 TG (Crossbond 35% dimethyl–65% diphenyl polysiloxane from Restek, Bellefonte, PA, USA). Column B was a 30 m × 0.25 mm i.d. × 0.25 μm film thickness fused silica column, coated with SPB-1 (crosslinked methyl silicone from Supelco, Bellefonte, PA, USA).

Analyses on column A were carried out in a Perkin-Elmer Autosystem GC equipped with a flame ionisation detector (Perkin-Elmer, Norwalk, CT, USA). Injector and detector temperatures were 300 and 320 °C, respectively; oven temperature was held at 170 °C for 10 min, programmed to 215 °C at a heating rate of 15 °C min$^{-1}$, and then pro-

grammed to 240 °C at 1 °C min$^{-1}$ and finally programmed to 320 °C at 5 °C min$^{-1}$ and held for 20 min. Analyses on column B were carried out in a Fisons chromatograph with flame ionisation detector (HRGC Mega2 FISONS, Milan, Italy). The injector and detector temperature was 300 °C; oven temperature was held at 200 °C for 20 min, programmed to 270 °C at a heating rate of 15 °C min$^{-1}$, then programmed to 290 °C at 1 °C min$^{-1}$ and finally programmed to 300 °C at 15 °C min$^{-1}$ and held for 40 min. Chromatographic peaks were measured using a Chrom-Card 1.20 acquisition system (CE Instruments, Milan, Italy).

GC–MS analyses were carried out using the same capillary columns, installed in a HP-5890 chromatograph with a MD 5971 quadrupole mass detector (both from Hewlett–Packard, Palo Alto, CA, USA) working in EI mode at 70 eV. Helium was used as carrier gas, and injections were made in the split mode, with a split flow of 40 mL/min. Acquisition was done using HPChem Station software (Hewlett–Packard, Palo Alto, CA, USA).

## 3. Results and discussion

TMS derivatives (oximes and ethers) of 18 disaccharide and 8 trisaccharide standards were injected in two columns of different polarity. Retention behaviour was different for both columns, the difference being very marked for some compounds such as trehaloses. Retention indices of most standards are given elsewhere [15].

Non-reducing disaccharides (sucrose and trehaloses) gave TMS derivatives which eluted before TMS oximes on column B (methyl silicone) due to their lower molecular weight. GC retention indices were 2733 for sucrose, 2844 for α,α-trehalose, 2870 for α,β-trehalose and 2891 for β,β-trehalose. Sucrose also eluted first on column A (phenylmethylsilicone), but the elution of trehaloses was different (retention indices were: 2515 for sucrose, 2618 for α,α-trehalose, 2719 for α,β-trehalose and 2758 for β,β-trehalose) and some of them eluted in between TMS oximes. Disaccharides with a 1-6 link (palatinose, gentiobiose and isomaltose) eluted last on both columns and were easy to recognize, whereas the remaining disaccharides eluted with variable overlapping.

Most trisaccharide standards were resolved on both columns. Non-reducing compounds (erlose, melezitose and 1-kestose) eluted first, whereas isomaltotriose (with two 1-6 linkages) eluted last. Isomaltotriose did not elute at 300 °C on methyl silicone column (column B).

### 3.1. Mass fragmentation features

Although many saccharides produce similar mass spectra, by careful inspection, structural features such as linkage type and ring size can be correctly assigned in many cases. Details on the MS fragmentation of disaccharide TMS derivatives (TMS ethers [22,23] and TMS oximes [24]) have already been published. Some common aspects were observed in the above-listed trisaccharide standards: the ratio of intensities of fragments *m*/*z* 204/217 was close to 1 in raffinose, erlose and melezitose (trisaccharides with two glucose rings); a ratio for this value $\ll 1$ was observed in 1-kestose (trisaccharide with two fructose rings), while ratios close to 2 were found in maltotriose and panose (reducing trisaccharides with three glucose rings). Ratios of fragments 437/451 close to 1 were found in trisaccharides (reducing and non-reducing) with a terminal fructose unit, as in raffinose, erlose and 1-kestose; this ratio was smaller in melezitose, where the fructose ring, related to the ion at *m*/*z* 437, is linked to two glucose rings.

### 3.2. Qualitative features of honey analysis

Chromatograms of disaccharides from a typical nectar honey on both columns appear in Figs. 1 and 2. α,β-Trehalose and trehalulose were identified in all examined honey samples by coincidence of their retention time and mass spectra with those of pure standards. Both compounds were isolated from honey by Siddiqui [1]; α,β-trehalose was also reported by HPLC [25] in Alsike honey and has recently been found in honeys from Madrid [26]. α,α-Trehalose appeared as a minor component, while β,β-trehalose was not detected. Several peaks coeluted on both columns, but qualitative information was obtained from their retention indices, *E*/*Z* ratios and MS fragmentation patterns [14,23]

Figs. 1 and 2 (lower profiles) show the chromatogram of trisaccharides of honey eluted on both columns; the phenyl silicone column profile was richer in peaks. A number of unidentified compounds were found, some of which were probably compounds reported by Siddiqui [1]. Two peaks eluting near 1-kestose, with similar mass spectra (especially the fragment *m*/*z* ratios 217/204 and 437/451) tentatively assigned to 6-kestose and neokestose. The TMS derivatives of kestoses had a RT close to OV-17 (a 50% phenyl methyl silicone stationary phase roughly comparable to the one used here) [27], which supports this hypothesis. Column A was considered to be better for trisaccharide analysis, since more compounds could be detected and quantified, although coelution of 1-kestose and raffinose was observed. Several trisaccharides remained unidentified.

### 3.3. Quantitative features

Several honey sugar profiles were compared in order to select retention time intervals whose limits were free of chromatographic peaks for most honey samples. The selected "retention time windows" appear in the chromatograms in Figs. 1 and 2. Each window may include a peak from a single compound or, when they cannot be integrated independently, several coeluting peaks. In the case of real honey samples, where there are many oligosaccharides present for which standards are not available, these problems are greater, since such compounds can produce major quantitative errors when they coelute with other sugars. A quantitation procedure that

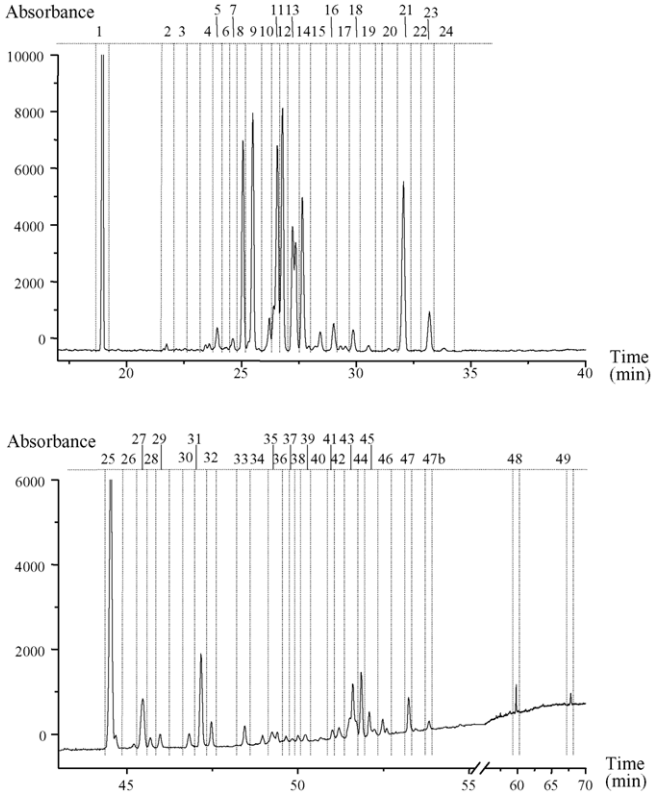


Fig. 1. Disaccharides (upper GC profile) and trisaccharides (lower GC profile) of a honey sample eluted using column A: (1) β-phenyl glucoside; (2) sucrose; (3–6) unknown; (7) α,α-trehalose; (8) maltulose (*E*); (9) cellobiose (*E*) + maltulose (*Z*); (10) cellobiose (*Z*) + laminaribiose (*E*) + leucrose 1; (11) turanose 1 + leucrose 2 + maltose (*E*); (12) nigerose (*E*) + turanose 2 + maltose (*Z*); (13) laminaribiose (*Z*) + kojibiose (*E*) + trehalulose 1 + unknown; (14) nigerose (*Z*) + trehalulose 2 + α,β-trehalose; (15) kojibiose (*Z*); (16) palatinose 1; (17) unknown; (18) palatinose 2; (19) gentiobiose (*E*); (20) gentiobiose (*Z*) + melibiose (*E*); (21) isomaltose (*E*); (22) melibiose (*Z*); (23) isomaltose (*Z*); (24) unknown; (25) C32 (internal standard); (26) unknown; (27) raffinose + 1-kestose; (28) 6-kestose; (29) neo-kestose; (30) erlose; (31) melezitose; (32–35) unknown; (36) maltotriose (*E*); (37) unknown; (38) maltotriose (*Z*); (39–43) unknown; (44) panose (*E*); (45) panose (*Z*); (46) unknown; (47) isomaltotriose (*E*); (47b) isomaltotriose (*Z*); (48, 49) unknown.

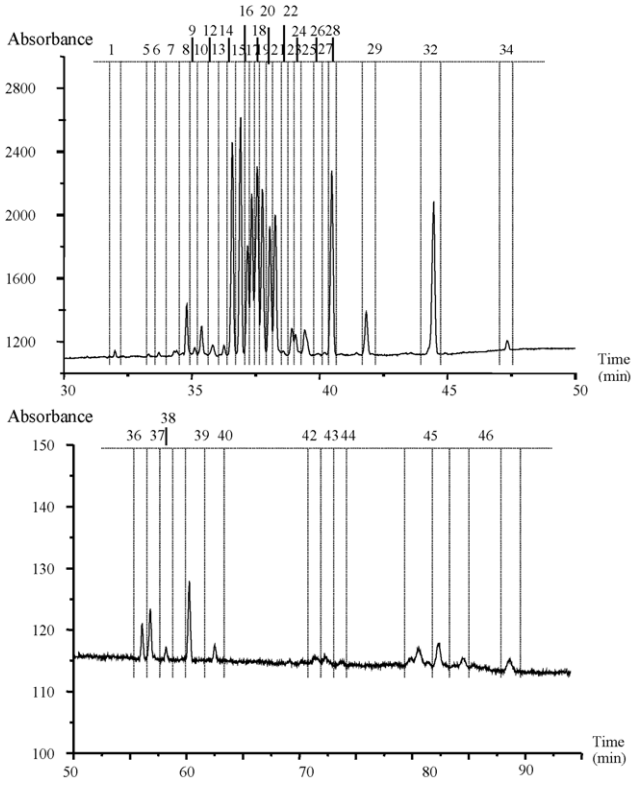


Fig. 2. Disaccharides (upper GC profile) and trisaccharides (lower GC profile) of a honey sample eluted using column B: (1) sucrose; (5, 6) unknown; (7) α,α-trehalose + unknown; (8) α,β-trehalose; (9, 10) unknown; (12) cellobiose (*E*); (13) unknown; (14) cellobiose (*Z*) + laminaribiose (*E*) + maltulose (*E*); (15) maltulose (*Z*); (16) nigerose (*E*) + leucrose 1 + unknown; (17) turanose 1 + leucrose 2; (18) laminaribiose (*Z*) + turanose 2 + maltose (*E*); (19) kojibiose (*E*); (20) maltose (*Z*) + trehalulose 1; (21) nigerose (*Z*) + trehalulose 2; (22) unknown; (23) palatinose 1 + gentiobiose (*E*); (24) kojibiose (*Z*); (25) palatinose (*Z*); (26) gentiobiose (*Z*); (27) melibiose (*E*); (28) isomaltose (*E*); (29) melibiose (*Z*) + isomaltose (*Z*); (30, 31) unknown; (32) C32 (internal standard); (33–35) unknown; (36) raffinose; (37) 1-kestose; (38) erlose; (39) melezitose; (40, 42) unknown; (43) maltotriose (*E*); (44) maltotriose (*Z*); (45) panose (*E*); (46) panose (*Z*).

takes account of the possible presence of unidentified sugar isomers was used to improve the quality of the quantitative data obtained.

Concentrations of disaccharides and trisaccharides were calculated from the GC chromatographic profiles from columns A and B. Allowance has to be made for the possible presence of unidentified compounds producing higher values in a given column, and therefore, Table 1 shows, for each carbohydrate, the lowest of the concentrations (mg/100 g of honey) obtained using columns A and B. Average differences between these concentrations, also shown in Table 1, can be used to estimate the concentration of unidentified components.

Table 2 shows the total experimental and calculated values obtained for total disaccharides and trisaccharides on both columns for each honey. As expected, experimental values were higher than calculated values due to the presence of unknown compounds in real samples. Experimental and calculated values were closer when column B was used; nevertheless, quantitative results for trisaccharides seemed better on column A.

Mass ions corresponding to leucrose were not detected in the honeys by GC–MS analysis using either methyl or methyl phenyl silicone columns, and so leucrose concentration was assumed to be zero; the presence of this sugar in honey is doubtful [2]. Concentration values for maltose, trehalulose and 1-kestose were determined from column B data, since these compounds overlapped in column A. 6-Kestose, neokestose and isomaltotriose were determined only on column A, since they were not detected on column B. Trehalulose was found to be a main disaccharide along with maltulose, turanose, kojibiose, maltose and isomaltose, its concentration varying from 0.56 to 2.43 g/100 g. α,β-Trehalose was the more abundant isomer of this non-

Table 1
Carbohydrate values (g/100 g of honey) found for 10 honey samples and the average differences between columns A and B

| | H1 | H2 | H3 | H4 | H5 | H6 | H7 | H8 | H9 | H10 | Average differences |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sucrose | 0.09 | 0.04 | 0.08 | 0.29 | 0.08 | 0.04 | 1.38 | 0.24 | 0.19 | 0.48 | 0.06 |
| α,α-Trehalose | 0.04 | 0.11 | 0.00 | 0.00 | 0.03 | 0.05 | 0.00 | 0.32 | 0.05 | 0.00 | 0.06 |
| α,β-Trehalose | 0.44 | 0.33 | 0.48 | 0.57 | 0.27 | 0.22 | 0.17 | 0.65 | 0.48 | 0.25 | 0.12 |
| Cellobiose | 0.07 | 0.27 | 0.28 | 0.06 | 0.06 | 0.09 | 0.11 | 0.14 | 0.10 | 0.11 | 0.11 |
| Laminaribiose | 0.16 | 0.20 | 0.14 | 0.13 | 0.06 | 0.14 | 0.16 | 0.09 | 0.21 | 0.13 | 0.08 |
| Maltulose | 1.39 | 3.37 | 3.52 | 1.81 | 1.37 | 2.58 | 0.66 | 2.44 | 1.24 | 1.30 | 0.26 |
| Nigerose | 0.56 | 0.69 | 0.39 | 0.81 | 0.37 | 0.35 | 0.46 | 0.90 | 0.75 | 0.52 | 0.30 |
| Turanose | 2.04 | 2.61 | 2.26 | 2.41 | 1.28 | 2.01 | 0.72 | 2.87 | 1.75 | 1.62 | 0.32 |
| Maltose[a] | 1.35 | 1.26 | 1.32 | 1.77 | 0.77 | 0.56 | 1.60 | 1.98 | 1.80 | 1.55 | 0.00 |
| Kojibiose | 1.46 | 1.54 | 2.19 | 1.53 | 0.82 | 1.00 | 0.86 | 2.09 | 1.61 | 1.20 | 0.34 |
| Trehalulose[a] | 0.90 | 2.43 | 2.39 | 0.97 | 0.56 | 1.55 | 0.65 | 1.37 | 0.65 | 1.22 | 0.00 |
| Palatinose | 0.17 | 0.45 | 0.62 | 0.22 | 0.12 | 0.32 | 0.12 | 0.28 | 0.12 | 0.20 | 0.07 |
| Gentiobiose | 0.05 | −0.01 | −0.01 | 0.01 | 0.00 | −0.04 | 0.04 | 0.05 | 0.03 | −0.03 | 0.06 |
| Melibiose | −0.01 | −0.02 | 0.00 | 0.00 | −0.01 | 0.00 | −0.01 | −0.04 | 0.00 | −0.01 | 0.03 |
| Isomaltose | 1.03 | 2.59 | 3.07 | 0.98 | 0.71 | 1.62 | 0.64 | 1.60 | 0.82 | 1.20 | 0.27 |
| Raffinose | −0.06 | −0.07 | 0.10 | −0.04 | −0.05 | 0.05 | −0.06 | 0.73 | −0.03 | −0.05 | 0.08 |
| 1-Kestose[a] | 0.14 | 0.21 | 0.48 | 0.07 | 0.18 | 0.19 | 0.16 | 0.20 | 0.08 | 0.19 | 0.00 |
| 6-Kestose [b] | 0.01 | 0.04 | 0.16 | 0.00 | 0.03 | 0.03 | 0.03 | 0.03 | 0.02 | 0.02 | 0.00 |
| Neokestose[b] | 0.02 | 0.05 | 0.13 | 0.01 | 0.05 | 0.04 | 0.05 | 0.04 | 0.02 | 0.03 | 0.00 |
| Erlose | 0.48 | 0.13 | 0.31 | 2.47 | 0.39 | 0.04 | 0.30 | 0.65 | 0.34 | 0.79 | 0.07 |
| Melezitose | 0.10 | 0.11 | 0.10 | 0.16 | 0.13 | 0.08 | 0.00 | 6.57 | 0.03 | 0.09 | 0.09 |
| Maltotriose | 0.03 | 0.00 | 0.00 | 0.02 | 0.00 | 0.03 | 0.00 | 0.03 | 0.02 | 0.00 | 0.08 |
| Panose | 0.20 | 0.00 | 0.00 | 0.00 | 0.00 | 0.24 | 0.00 | 0.27 | 0.11 | 0.20 | 0.12 |
| Isomaltotriose[b] | 0.01 | 0.14 | 0.05 | 0.03 | 0.03 | 0.10 | 0.03 | 0.06 | 0.02 | 0.07 | 0.00 |

[a] Calculated using only column B.
[b] Calculated using only column A.

Table 2
Experimental and calculated values (g/100 g of honey) for total disaccharides and trisaccharides in both columns (samples H1–H10)

| | H1 | H2 | H3 | H4 | H5 | H6 | H7 | H8 | H9 | H10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Column A | | | | | | | | | | |
| Experimental | 11.87 | 19.77 | 25.70 | 17.22 | 8.46 | 13.88 | 9.39 | 26.22 | 12.30 | 12.71 |
| Calculated | 10.89 | 17.20 | 22.76 | 15.41 | 7.85 | 12.50 | 8.38 | 24.23 | 11.10 | 11.52 |
| Column B | | | | | | | | | | |
| Experimental | 12.79 | 20.82 | 19.46 | 16.79 | 8.42 | 12.55 | 10.63 | 27.71 | 12.10 | 14.14 |
| Calculated | 12.21 | 19.54 | 18.57 | 15.98 | 7.93 | 12.22 | 9.28 | 26.74 | 11.27 | 13.53 |

reducing disaccharide, α,α-trehalose being either lower or not detectable in all cases. A high melezitose concentration (6.57 mg/100 g honey) was observed in one honey sample (H8), and erlose concentration was also high (2.47 mg/100 g) in another sample (H4); both sugars are usually indicative of honeydew honey.

## 4. Conclusions

From these results, column A seems to be better suited to trisaccharide analysis, whereas column B could be better suited to disaccharides. Their combined use made it possible to identify several compounds. It is difficult to determine all the sugars present in honey samples, but the reliability of the analytical results is considerably improved by use of the procedure described: this procedure takes into account retention data from both columns and mass spectral data, making it possible to identify several disaccharides and trisaccharides and to obtain qualitative and quantitative data for compounds not available as standards.

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