



Carbohydrate determination in honey samples by ion chromatography–mass spectrometry (HPAEC-MS)

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Abstract

Honey is a complex mixture of carbohydrates, in which the monosaccharides glucose and fructose are the most abundant compounds. Currently, more than 20 oligosaccharides have been identified in different varieties of honey normally at quite low concentration. A method was developed and validated using high-performance anion-exchange chromatography coupled to a mass spectrometry detector to investigate the composition of carbohydrates in honey samples. The method was tested for linearity range, trueness, instrumental and method detection and quantification limits, repeatability, and reproducibility. It was applied to determine seven monosaccharides, eight disaccharides, four trisaccharides, and one tetrasaccharide in various honey samples. The present work describes the composition of sugars in unifloral, multifloral, and some honeydew honey, which were produced and collected by beekeepers in the Trentino Alto-Adige region. Statistical techniques have been used to establish a relationship based on levels of carbohydrates among different Italian honey. The results emphasize that mono- and oligosaccharide profiles can be useful to discriminate different honeys according to their floral characteristics and inter-annual variability.

Keywords High-performance anion-exchange chromatography–mass spectrometry (HPAEC-MS) · Italian honey · Mono- and oligosaccharide profiles

Introduction

Honey is a natural sweetener and nutritional food that is produced by bees (*Apis mellifera*) from the nectar of flowers of plants or honeydew [1–3]. The composition of honey is rather variable and depends mainly on its floral nectar or honeydew source and others factors, such as environmental and seasonal conditions, and processes and transformations occurring in

bees [4, 5]. Honey is a complex mixture of approximately more than 180 compounds, of which carbohydrates account for about 80% (w/w) of the solids content [6]. Glucose and fructose are the major monosaccharides in honey and their content ranges from 65% to 85% of total soluble solids [1, 7, 8]. The remaining sugars are disaccharides, trisaccharides, and tetrasaccharides present, in the majority of honeys, at low concentration [9]. These oligosaccharides are mainly formed of glucose and fructose residues linked by glycosidic bond [8]. Oligosaccharides are important substances to determine both geographical and botanic origin of honey [10]. In honey, these compounds also contribute significantly to its high nutritional value as a potential “prebiotic” property, by growing and balancing the intestinal microflora in human and animal intestine, controlling the gastrointestinal peristalsis, and reducing the incidence of serious illness such as colon cancer and diarrhea [4, 11, 12]. Indeed, oligosaccharides are generally considered non-digestible compounds (raffinose and stachyose for instance), because they cannot be hydrolyzed by human gastrointestinal enzymes [4]. Moreover, they provide positive effects by protecting the performance of the gastrointestinal organs and also by stimulating the growth of some specific

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bacteria, especially bifidobacteria [12]. In vitro studies suggested that the oligosaccharides influence the growth of probiotic bacteria such as bifidobacteria and lactobacilli [8]. However, these components could have inhibitory activity against some pathogenic bacteria, like *Helicobacter* or *Staphylococcus*, probably because the oligosaccharides attach to the cell walls of these bacteria and prevent their adhesion to human tissues [13].

Previous works were carried out to determine carbohydrate profiles in various foods [14–16] including sugar components in honey [17–19]. The content of major sugars in honey, such as glucose, fructose, and sucrose, and the presence of minor compounds such as di- and trisaccharides have been intensively determined in recent years [1, 2, 9, 10]. Moreover, tetrasaccharides, pentasaccharides, and hexasaccharides have been also found in some honeydew [20].

Currently, more than 20 oligosaccharides have been identified in different varieties of honey produced in diverse countries around the world [1, 6, 8, 11, 17, 21–23]. In previous works, the oligosaccharides profile has been widely investigated in honey samples originating from Argentina [6], Brazil [1], Algerian [11], Spain [7], the UK [17], France [24], and Portugal [21].

Several analytical techniques have been employed to determine carbohydrates in honey samples. Indeed, these compounds are mainly determined by high-performance liquid chromatography (HPLC) coupled to different detectors [6, 11, 25, 26]. For example, Arias et al. [6] developed an analytical method based on HPLC with UV detection to determine some oligosaccharides. Moreover, sugars can also be analyzed using gas chromatography coupled to mass spectrometry (GC-MS) [27, 28] and gas chromatography with flame ionization detector (GC-FID) [20, 23, 24]. However, these methods require time-consuming sample treatments; for example, the method developed by Arias et al. [6] to determine oligosaccharides required solid-phase extraction procedures by porous graphitic carbon cartridge. The analytical method suggested by Da Costa Leite et al. [1] required the dissolution of honey in a mixture of acetonitrile and water followed by centrifugation of the mixture. The derivatization with different detection systems is mandatory for the gas chromatographic determination [20, 23, 24]. As reported also by other authors the derivatization can be arduous and laborious [2, 7].

The International Honey Commission (IHC) reports several chromatographic methods for sugar determination, of which high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPLC-PAD) and GC-FID are the common analytical techniques applied [29]. However, HPAEC-PAD is the most applied method for the determination of oligosaccharides in honey [10, 11, 30].

High-performance anion-exchange chromatography coupled to an integrated pulsed amperometric detector and on-line single quadrupole mass spectrometry (HPAEC-

IPAD-MS) was also applied to analyze some sugars in chicory coffee, beer, and honey [31].

For carbohydrates analysis, high-performance anion-exchange chromatography (HPAEC) provides a valuable and powerful analytical tool for the separation of sugars. The reason can be explained because many carbohydrates present slight acidity at pK_a between 12 and 14. In alkaline conditions, the hydroxyl groups are converted into oxyanions, making it possible to selectively separate these species in anionic form. However, the separation can be strongly influenced by the number of hydroxyl groups present in the compound, by their position inside the sugar, and by the degree of polymerization [32].

Normally, the literature methods were applied for the determination of major sugars and few oligosaccharides to better characterize honeys, but the quantification of a larger number of oligosaccharides is necessary.

The objective of the present work was to develop a method to determine carbohydrates, including 13 oligosaccharides in honey, using high-pressure anion-exchange chromatography coupled to mass spectrometry (HPAEC-MS). The developed method is then used to characterize honey samples with a different floral origin. This is the first time that an HPAEC-MS method is developed to determine oligosaccharides in honey samples. High sensitivity and selectivity of the instrumental method coupled with the simple pre-analytical procedure are the two main advantages of this proposed method.

The carbohydrate profile was used to assess the composition of honey collected by beekeepers in the Trentino Alto-Adige region (Italy). A chemometric approach was applied to define the main relationship between the floral origins and inter-annual variability.

Materials and methods

Chemicals and reagents

All chemicals and reagents had a known purity (>98%). D(–)-Arabinose, D(+)-glucose, D(–)-fructose, D(+)-xylose, D(+)-mannose, D(–)-ribose, D(+)-glucose($^{13}C_6$), D(+)-galactose, D(+)-lactose, and D(+)-lactulose were obtained from Sigma Aldrich. D(+)-Sucrose was purchased by Fluka (Ronkonkoma, USA). D(+)-Turanose, D(+)-melibiose monohydrate, palatinose hydrate, kojibiose, nigerose, erlose, isomaltotriose, D(+)-raffinose pentahydrate, D(+)-melezitose, and stachyose were supplied by Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Ammonium hydroxide was obtained from Fluka (Sigma Aldrich, Buchs, Switzerland). Ultrapure water (18 M Ω cm, 0.01 TOC) was produced by a Purelab Ultra Sistem (Elga, High Wycombe, UK). Ultra-grade methanol was purchased from Romil LDT (Cambridge, UK).

Sample collection and processing/preparation

The honey samples of various botanical origins were directly collected from the apiarist's association and in the farms of beekeepers. The honeys were manufactured within different geographical areas of the Trentino Alto-Adige region (Italy) and harvested between 2017 and 2018; two commercial Argentinean samples were also analyzed for comparison.

In the present study, a total of 43 multifloral, unifloral, and honeydew honeys were analyzed (23 multifloral, of those MARG14 and MARG733 are Argentinean honeys), 4 acacia, 4 dandelion, 8 rhododendron, and 4 honeydew); all details are reported in Table S1 in the Electronic Supplementary Material (ESM). The collected samples were immediately stored at +4 °C until the analysis. To have a representative of the honey lot, the practical instructions according to the International Honey Commission (IHC) [29] were carefully followed. Furthermore, to reduce the possible external contamination and alteration, the operations of preparation, handling, and storage were strictly observed.

Before analysis, the liquid honey samples were mixed softly to guarantee homogenization, whereas the crystallized honeys were pre-softened by heating in a thermostatic bath at 40 °C. Each honey sample was directly weighted (50 mg) into a volumetric flask (50 mL) and spiked with $^{13}\text{C}_6$ -glucose, as internal standard, and then diluted with ultrapure water in a volumetric flask until a final concentration of 0.1 mg mL⁻¹. The final concentration of the internal standard in the samples was 1 mg L⁻¹.

Instrumental parameters

Qualitative and quantitative analyses of monosaccharides and oligosaccharides were carried out using an ion chromatograph (Thermo Scientific™ Dionex™ ICS-5000, Waltham, USA) coupled to a single quadrupole mass spectrometer (MSQ Plus™, Thermo Scientific™, Bremen, Germany).

The chromatographic separation was performed with a CarboPac PA10™ column (Thermo Scientific, 2 mm × 250 mm, 10 μm) equipped with a CarboPac PA10™ guard column (2 × 50 mm). The sodium hydroxide (NaOH) gradient generated by an eluent generator (Dionex ICS 5000EG, Thermo Scientific) was from 0 to 3 min at 1 mmol L⁻¹; gradient from 10 to 20 mmol L⁻¹ in 17 min; isocratic elution 20 mmol L⁻¹ from 20 to 30 min; then gradient from 20 to 100 mmol L⁻¹ in 15 min; column cleaning step with 100 mmol L⁻¹ for 5 min; equilibration at 1 mmol L⁻¹ from 50 to 65 min. The injection volume was 25 μL and the flow rate was 0.25 mL min⁻¹. NaOH was removed via suppressor (ASRS 500, 2 mm, Thermo Scientific) before introduction into the MS source.

Optimization of the mass spectrometer was performed to establish the best parameters and to maximize the intensity

of signal for each ion. Data for all carbohydrates were collected in selected ion monitoring (SIM), using $[\text{M}-\text{H}]^-$ ions according to their molecular weight because an electrospray (ESI) source was used in negative mode. A standard solution of sugars 1 mg L⁻¹ was used to select the best experimental parameters. In particular, cone voltage was tested from 40 to 100 V, needle spray voltage was evaluated for 2, 2.5, and 3 kV, while source temperature was changed from 200 to 400 °C. The most efficient ionization was obtained at an optimized temperature of 400 °C and a needle voltage of -3 kV. A summary of monitored and optimized parameters of each mass to charge ratio $[\text{M}-\text{H}]^-$ is reported in Table S3 (see ESM).

To improve the ionization of carbohydrates, a solution of ammonium hydroxide in methanol (7%) was added post-column with a flow of 0.025 mL min⁻¹. The composition of this post-column solution was optimized, by evaluating different solvents (i.e., water, acetonitrile), and methanol afforded the best performance to improve the ionization, such as also reported in previous publications [33, 34].

The stability of the acquisition, related to ESI probe cleaning/dirtiness, was verified by 15 injections (three series of five injections) and the signal normalized for mass diluted did not show any drift. Acquisition and elaboration data were processed by Chromeleon 6.8 software.

Statistical analysis

Multivariate statistical techniques were applied to the sugars concentration data to establish possible relationships among the botanical origin or inter-annual variability and carbohydrate composition.

Hierarchical cluster analysis and factorial analysis were performed using STATISTICA 10.0 software (StatSoft, Inc., 2007, Tulsa, USA). Hierarchical cluster analysis was performed using Ward's method and evaluating squared Euclidean distance. Factorial analysis was performed using varimax rotation.

Results and discussion

Main advantages of proposed method

In comparison to other pre-analytical methods reported in the literature [6, 17, 35], the procedure developed is simple, fast, and without expensive steps, such as purification or solvent extraction. The honey samples are accurately weighed and diluted appropriately (1:10,000) with ultrapure water, following a procedure similar to that described by Bruggink et al. [31]. The proposed procedure is solvent-free as only ultrapure water is required, thereby also reducing the sample preparation time.

The main drawbacks to quantifying the content of oligosaccharides in honey are due to their low concentration in contrast with the high amount of monosaccharides (i.e., glucose and fructose) in the samples. The dilution allows one to reduce the glucose and fructose concentrations, while the high sensitivity of this developed IC-MS method permits one to determine trace concentrations of the other oligosaccharides. At the moment, another important disadvantage in the determination of oligosaccharides is the lack of standard reference materials [2, 30]. Besides, the separation of the oligosaccharides can be more difficult because of their similar structures [2]. In our method, mono-, di-, tri-, and tetrasaccharides are discriminated using mass spectrometry, reducing the number of peaks in each ion chromatogram. The chromatographic separation coupled to mass spectrometry only requires one to separate isobaric species, providing a better peak resolution in comparison to other detectors where all saccharides are determined in one single chromatogram [6, 11, 24, 25, 30, 35, 36].

Chromatographic optimization

To evaluate the performance of the chromatographic separation developed, some specific chromatographic parameters were calculated. Table S2 (see ESM) shows the retention time and the peak width of each sugar. Peak width varied between 0.3 min (fructose) and 1.4 min (kajibiose). For each peak, the asymmetry factor (A) is evaluated to define the column overload, the heterogeneity of column packing, and the heterogeneity of the stationary phase, as the chromatographic peaks may often show a tailing or fronting behavior. The peak asymmetry factor was estimated by the ratio, at 5% of the peak height, of the distance between the peak apex and the backside of the peak curve and the distance between the peak apex and the front side of the peak chromatographic, as follows: $A = (RW5\% + LW5\%)/(2 \times LW5\%)$, where RW and LW are the right and left part of the widths at 5% of the peak height. For ideal chromatographic peaks, the asymmetry is 1 [37]. An asymmetry factor around 1 is highly acceptable. In the developed chromatographic separation (ESM Table S2), a weak fronting occurs for turanose (0.7), melibiose (0.9), lactose (0.9), and stachyose (0.9). An asymmetry factor from 1.2 to 1.5 is considered satisfactory. The rest of the other sugars demonstrate tailing effects. This effect was always satisfactory because it ranged between 1.1 (nigerose, raffinose, isomaltotriose, and erlose) and 1.8 (xylose).

The chromatographic efficiency, as theoretical plate number, was estimated at different specific retention times [38] and ranged from 5230 (arabinose) to 70,388 (turanose).

The resolution factor (R_s) was calculated as the ratio of the difference between the retention time and the width at 50% of the height of the peak of two chromatographic peaks; a resolution factor of 1.0 is sufficient for a qualitative analysis, whilst a resolution of 1.5 or greater is optimal for an accurate

quantitative analysis [39]. The value obtained with the developed method ranged from 1.2 (turanose/palatinose) to 14.0 (raffinose/isomaltotriose), suggesting that the sugar peaks were well resolved and an accurate quantification can be carried out with this chromatographic run.

Quantitative performance of method

The chromatograms of standard solutions of sugars and one unifloral honey sample (rhododendron) are reported in Figs. 1 and 2, respectively. A weak shift in the retention time occurs in the sample chromatograms as a result of the matrix effect. The compound attribution is accurately performed by considering the difference in the retention time of internal standard between standard solution and sample. The same difference in the retention times between the oligosaccharide is observed; therefore, accurate identification of the compounds is maintained.

The analytical procedure was validated by determining the linear dynamic range, instrumental precision (as RSD %) in terms of repeatability and reproducibility, instrumental detection and quantification limits (LOD and LOQ), method detection and quantification limits (MDL and MQL), and trueness. All parameters are reported in Table 1.

The linearity of the calibration curve of each sugar was estimated using a series of standard solutions of the sugars at average concentrations of 0.05, 0.25, 0.5, 1, 2, and 4 mg L⁻¹ and a constant internal standard concentration (¹³C₆-glucose) of 1 mg L⁻¹. By considering the ratio between the peak area of

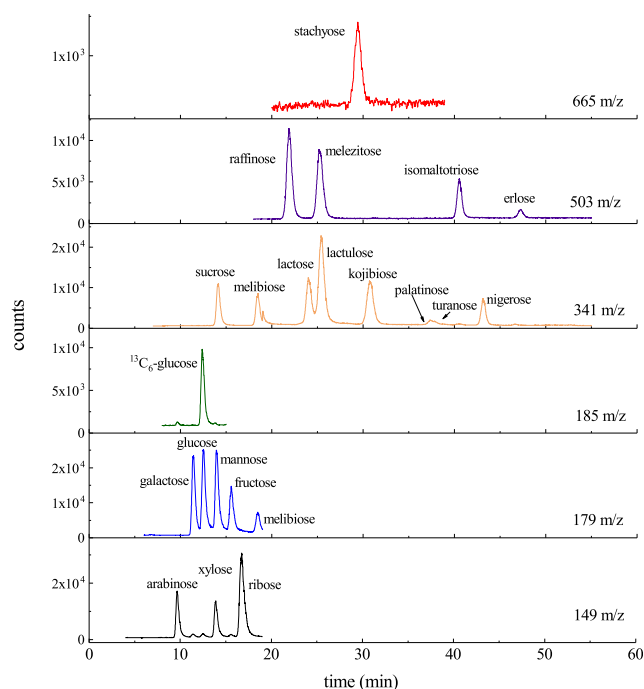


Fig. 1 HPAEC-MS chromatogram of standard solutions of carbohydrate. Column CarboPac PA10 (2 × 150 mm, 10 μm)

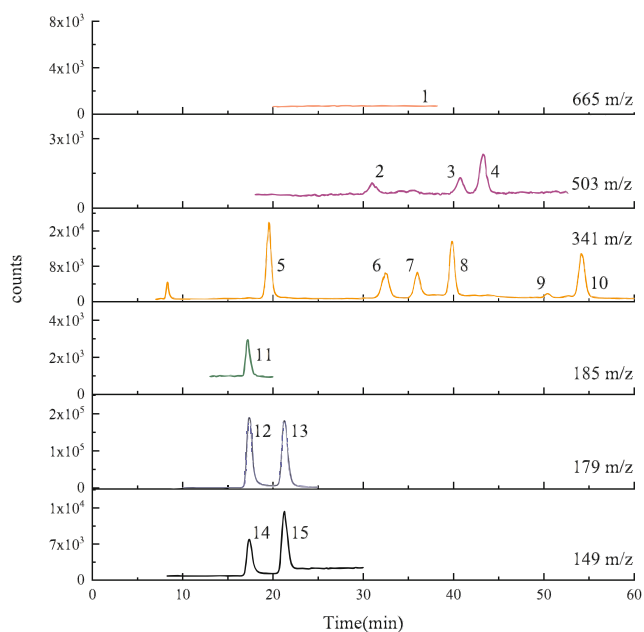


Fig. 2 HPAEC-MS chromatogram of one unifloral honey sample (rhododendron) using the developed method; the identified carbohydrate are 1 = stachyose (below MDL), 2 = melezitose, 3 = isomaltotriose 4 = erlose, 5 = sucrose, 6 = lactose, 7 = lactulose, 8 = kojibiose, 9 = palatinose, 10 = nigerose, 11 = $^{13}\text{C}_6$ -glucose (internal standard), 12 = glucose, 13 = fructose, 14 = xylose and 15 = ribose

saccharides and internal standard versus the concentration of the analytes, the R^2 value ranged from 0.990 (stachyose) to 0.999 (glucose, mannose, lactulose, kojibiose, and nigerose). The instrumental repeatability calculated as relative standard deviation (RSD %) was also estimated at the six concentration levels of standards ($n = 5$). The RSD values were always below 11%.

The instrumental limits of detection (LOD) and quantification (LOQ) were estimated as three and ten times the signal-to-noise ratio (S/N) of each carbohydrate, respectively (Table 1). The LOD values of monosaccharides were within the range from 0.006 mg L^{-1} (glucose and galactose) to 0.02 mg L^{-1} (fructose). The LOD values of disaccharides ranged from 0.005 mg L^{-1} (sucrose) and 0.1 mg L^{-1} (turannose); for trisaccharides, the LOD values ranged from 0.01 mg L^{-1} (melezitose and raffinose) to 0.06 mg L^{-1} (erlose). The LOD of stachyose was 0.4 mg L^{-1} .

The LOD values were lower than those reported in the literature in honey samples, see Table 1 [40, 42]. The LOD values found in this study for galactose, mannose, and ribose (0.006 , 0.007 , 0.08 mg L^{-1} , respectively) were 14–17 times lower than values established in a previous study (0.12 , 0.11 , 0.13 mg L^{-1} , respectively) by Tůma et al. [42]. To our knowledge, no reference data are available for stachyose.

In general, using this analytical technique, we obtained a reduction in the detection limits for all the sugars considered in this study. The details of the detection limits for each carbohydrate determined in this paper in comparison with literature are reported in Table 1.

Besides, the precision, as repeatability (intra-day), and reproducibility (inter-day) were also estimated, as reported in Table S4 (see ESM). Repeatability was estimated by five injections at 0.5 mg L^{-1} of one honey sample and repeating the procedure three times on the same day, while reproducibility was assessed by analysis of five aliquots of the same sample and repeating the measurements for three different days. The results expressed as RSD% value were lower than 10% for all carbohydrates. The method detection limits (MDL) and the method quantification limits (MQL) were calculated for each sugar following the procedure reported by Bliesner [43]; the values range from 0.05 mg L^{-1} (galactose and glucose) to 4 mg L^{-1} (stachyose) and from 0.19 mg L^{-1} (galactose and glucose) to 13 mg L^{-1} (stachyose), respectively.

Trueness is one of the most important parameters for the method validation and it refers to the degree of closeness of the determined value to the known “true” value. The trueness was tested at lowest concentration of oligosaccharides. Five samples of honey were spiked with a solution containing all the sugars at a constant concentration comparable with normal amount detected in honey; the internal standard was also added at a concentration of 1 mg L^{-1} . The resultant values are reported as percentage errors in Table 1. High error values were observed for some analytes, especially turannose, palatinose, erlose, and stachyose, suggesting that reference standard material is mandatory to define this parameter.

Method application

The developed HPAEC-MS method was applied to determine the sugar composition in honey samples produced in different geographical areas of the Trentino Alto-Adige region (Italy). The honeys were directly collected from farms or the apiarist’s association, harvested during the 2017 and 2018, and two samples were commercial Argentinian honeys. A total of 43 honeys with different floral origin (multifloral, unifloral, and some honeydew honeys) were analyzed to determine seven monosaccharides (arabinose, fructose, glucose, galactose, mannose, ribose, and xylose), eight disaccharides (sucrose, lactose, lactulose, kojibiose, palatinose, turannose, melibiose, and nigerose), four trisaccharides (raffinose, melezitose, isomaltotriose, and erlose), and one tetrasaccharide (stachyose). According to the literature, carbohydrate composition in honey depends on different factors such as botanical and geographical origin, environmental and seasonal conditions, as well as storage and processing manipulation [10].

The descriptive characteristics and average concentration of each sugar in these samples are reported in Tables S1 and S5 (see ESM), respectively. Arabinose, xylose, ribose, mannose, galactose, and stachyose had concentrations below the MDL in all analyzed samples. Therefore, they were not used to characterize the honey samples in this study, although some

Table 1 Validation parameters of the analytical procedure for the carbohydrate quantification

Carbohydrate	This study						LOD (mg L ⁻¹) previous study		
	LOD (mg L ⁻¹)	LOQ (mg L ⁻¹)	RSD%	MDL (mg L ⁻¹)	MQL (mg L ⁻¹)	Trueness (Error %)	CE-DAD ^a	HPTLC ^b	CE-C ⁴ D ^c
Arabinose	0.01	0.04	7	0.1	0.4				
Xylose	0.01	0.04	3	0.1	0.4				
Ribose	0.008	0.03	4	0.08	0.27				0.13
Galactose	0.006	0.02	1	0.06	0.19				0.12
Glucose	0.006	0.02	3	0.06	0.19		29.2	14	0.11
Mannose	0.007	0.02	6	0.07	0.24				0.11
Fructose	0.02	0.06	3	0.2	0.6		29.8	31	0.13
Sucrose	0.005	0.02	2	0.05	0.2			22	
Melibiose	0.02	0.06	9	0.18	0.59	7			
Lactose	0.02	0.06	10	0.16	0.53				0.14
Lactulose	0.008	0.03	8	0.08	0.27				
Kojibiose	0.008	0.03	7	0.08	0.28	1			
Turanose	0.1	0.4	11	1.1	3.6	29			
Palatinose	0.09	0.3	11	0.9	3	21			
Nigerose	0.02	0.07	9	0.20	0.66	10			
Melezitose	0.01	0.04	6	0.11	0.36	10			
Raffinose	0.01	0.03	9	0.10	0.33	19			
Isomaltotriose	0.02	0.06	8	0.19	0.62				
Erllose	0.06	0.2	10	0.6	2	25			
Stachyose	0.4	1	9	4	13	26			

LOD instrumental limit of detection, LOQ instrumental limit of quantification, RSD relative standard deviation (instrumental precision), MDL method detection limit, MQL method quantification limit, LOD instrumental detection limits, CD-DAD capillary electrophoresis with diode array detection, CE-C⁴D capillary electrophoresis with contactless conductivity detection, HPTLC high-performance thin-layer chromatography

^a [40]; ^b [41]; ^c [42]

previous studies reported that some of these compounds had detectable concentrations, e.g., one study carried out on honey samples from Spain showed that galactose had concentrations ranging between 0.0052 and 0.0151% [44].

Figure 3 reports the mean concentration and standard deviation of the saccharides, which can be related to the floral origin, in the 43 honey samples (23 multiflora, 4 acacia, 4 dandelion, 8 rhododendron, and 4 honeydew).

Fructose and glucose represent the main simple carbohydrates in all types of honey; therefore, they are not presented in Fig. 3. Their content, in agreement with other research [1, 2, 40], can vary from 65% to 85%. The percentage of fructose and glucose was 73% for honeydew, 83% for rhododendron, 84% for multiflora, 86% for dandelion, and 89% for acacia. These results are in agreement with the compositional criteria of honey; the total percentage of these two monosaccharides is more than 60% and 45% for blossom honey and honeydew honey, respectively [3]. Fructose was the major sugar found in all honeys, especially acacia honey where levels were higher than other samples. In contrast, the content of fructose in honeydew honey was lower than in other types of honey; this data

is in accordance with the literature [2, 10, 26]. Glucose was the second major simple carbohydrate found in honeys investigated in this work. The content of glucose in dandelion honey was higher than other samples. In contrast, in honeydew the mean content of glucose (21%) was lower than those in uniflora and multiflora honeys; other authors have reported a concentration of glucose in honeydew of 23.2% [10].

In general, a low concentration of sucrose was found in all honeys, although its level was higher in rhododendron honey (3%) than other samples, where similar sucrose contents were observed (1%). Sucrose undergoes transformation by specific enzymes such as α - and β -glucosidase, α - and β -amylase, and β -fructosidase, which hydrolyzes this sugar into glucose and fructose [7, 8].

Besides glucose and fructose, previous studies reported that the main carbohydrates (oligosaccharides) determined and found in honey samples of different botanical origins are maltulose, turanose, maltose, isomaltose, kojibiose, trehalose, isomaltotriose, panose, melezitose, raffinose, and stachyose [7, 8, 10, 24, 45, 46]. In this research, the study was also extended to other carbohydrates that recent investigations

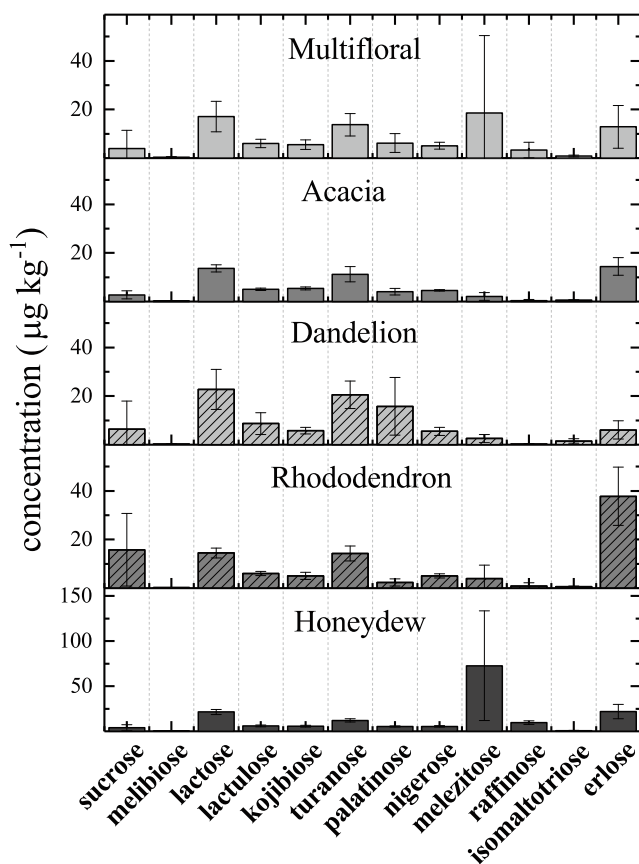


Fig. 3 Average concentration and standard deviation of oligosaccharides in multifloral, acacia, dandelion, rhododendron, and honeydew honeys

identify as important sugars entering the honey composition, to examine if these sugars have a relationship with the floral varieties or quality of honeys.

Lactose and lactulose were found in all honey samples. As reported in Table S5 (see ESM) and Fig. 3, lactose has been found as one of the main disaccharides in all the honey samples analyzed.

The percentage of lactose was 4% in dandelion, 3% in multifloral and honeydew, and 2% in acacia and rhododendron honeys. Literature data show that lactose should be present in honeys only at very low concentration, approximately 0.01% [44]; in another previous work, lactose was monitored in honey samples by a different technique, but the authors did not provide data about the presence of this disaccharide in honey [42]. Among other carbohydrates, lactose and galactose are important compounds in honey because they might be useful for its characterization, although these sugars can be present at low concentration [44].

Although to our knowledge no literature data are available about the presence of lactulose in honey, this compound was present in all honey samples we investigated at a percentage of 1%. The content of other minor sugars, mainly disaccharides and trisaccharides, has been quantified in this study. These oligosaccharides are formed by units of glucose and fructose

with diverse glucosidic bonds [8, 20]. Furthermore, the wide variety of these compounds in honey is due to the activity of certain enzymes, mainly α -D-glucosidase, which transfers α -D-glucopyranosyl groups from sucrose to an acceptor sugar [1]. In all analyzed honey samples, the main disaccharides, in addition to sucrose and lactose, were turanose, palatinose, kojibiose, nigerose, and melibiose. The mean levels of turanose and palatinose were most elevated in dandelion honey, which accounted for 3% and 2%, respectively. In acacia, multifloral, rhododendron, and honeydew, the percentage levels were 2% and 1% for turanose and palatinose, respectively. However, the mean percentage content of other disaccharides was quite similar between different types of honey (1%). The results are comparable with those reported by other authors for Spanish unifloral honeys [7] and in New Zealand honey (manuka honey) [26].

The trisaccharides melezitose and raffinose were most abundant in honeydew honey (12% and 2%, respectively); the results agree with previous studies [1, 10, 28]. The prevalence of melezitose in honeydew honey is considered one of its characteristics [9]. Indeed, melezitose, raffinose, and erlose were found in high quantities in honeydew samples from France [24]. In this work, multifloral honey presented higher concentrations of melezitose (3%) and raffinose (1%) than unifloral honeys; this can be due to contamination of the floral honeys with honeydew or they can be naturally present in the nectar [1].

Erlose was detected in all honey samples. In rhododendron honey samples, erlose was present at 6%, while it accounted for 3% in honeydew, 2% in acacia, and 1% in multifloral and dandelion honey. This oligosaccharide was also quantified in different Spanish unifloral honey types, such as rosemary honey (2.1%) and eucalyptus honey (0.12–0.51%) [23]. Considerable content was found in acacia (1.88%) and lavender (1.40%), while lower amounts were found in chestnut (0.24%) [24]. Erlose is produced from sucrose by the metabolism of honeybees, and in honey its concentration generally undergoes a modification during storage through α -glucosidase enzymatic activity [23].

The content of isomaltotriose was relatively comparable in all honey samples where it was observed at a percentage ranging from 0.009% in rhododendron to 0.083% in dandelion. In a previous study, this oligosaccharide was found in unifloral honey such as clover (0.028%) and alfalfa (0.038%) [19].

Statistical analysis

Statistical techniques were used to determine the relationship among different types of Italian honey (43 samples as cases) using the oligosaccharide content (14 oligosaccharides as variables). To eliminate the different effects of the variable's amount and their diverse

variance, the data was normalized. Hierarchical cluster analysis was performed using Ward's method and evaluating squared Euclidean distance. The chemometric analysis produced a tree diagram whose cases, such as the samples, were divided into macro clusters. The squared Euclidean distance was used as a distance measure to obtain the similarity among the samples. The results obtained show two main groups of honeys sample, as shown in Fig. 4. Besides, these two principal groups were divided into various subgroups.

The first main group was divided into four subgroups, the first three subgroups of which had been harvested in 2017; the samples are as follows T36, MT43, M49, M48, M44, M41, M39, M40, M38, and M37, except for MT45 sample. In the second main group all of the samples were harvested during 2018, see Fig. 4.

The reason for the separation of sample 2017 and 2018 could be explained considering the differences observed in the mean content of some oligosaccharides (ESM Table S3). Many carbohydrates in honey are produced by honeybees from sucrose contained in the nectar; in genuine honey, sucrose represents about 5% of the total [2]. Indeed, monosaccharide residues, obtained after sucrose hydrolysis, react to form others disaccharides, trisaccharides, and tetrasaccharides [8]. As reported in the literature, sucrose content could be reduced during the storage of honey because the enzyme invertase acts on this sugar and the hydrolysis produces simple sugars, glucose, and fructose [2, 13].

The sample MT45, collected in 2017, is included in the 2018 cluster; indeed it presents a high content of sucrose ($23,791 \text{ mg kg}^{-1}$). High levels of this disaccharide are usually found in early harvested honeys, in which an incomplete

hydrolysis process of the sucrose into glucose and fructose by enzymes invertase occurs [47].

Factor analysis was used to explore the relationship between variables using a varimax rotation procedure to maximize the explained variance to emphasize possible relationships among the botanical origin or inter-annual variability and carbohydrate composition. Three factors were obtained with eigenvalues greater than 1, and they explained more than 74% of the total variance.

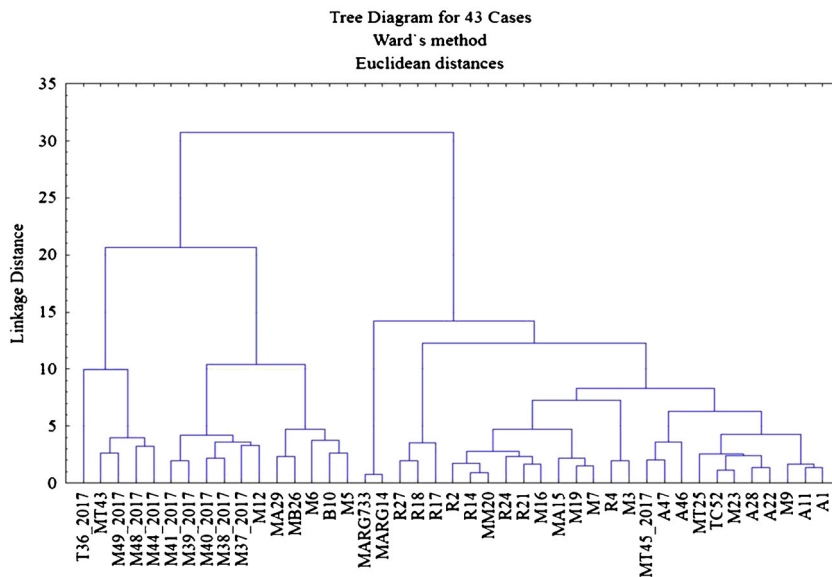
Figure 5 shows the biplot for the 43 object scores for mono- and oligosaccharide compositions and the variable loadings in the space of the first three factors.

The first factor differentiated the 2017 and 2018 samples. The variables with highest loadings on the first factor were lactose, lactulose, nigerose, and isomaltotriose (see Fig. 5a) and larger part of di- and trisaccharides. We hypothesize that this component is related to oligosaccharide concentrations deriving from honey aging; indeed, these derive from reaction of glucose and fructose generated from sucrose hydrolysis [2].

The second factor, accounting for 24% of the total variance, differentiates some of the honeydew from honey and presents the highest loading for glucose, fructose, melezitose, and raffinose.

The third factor, accounting for 12% of the total variance (see the biplot in Fig. 5b), differentiates the rhododendron honey from multifloral honey and honeydew. The highest variable loadings were for sucrose and erlose; therefore, we can hypothesize that the third factor is related to floral honey characteristics. Tables S6 and S7 (see ESM) reported the factor scores and the factor loadings related to the three factors obtained by factorial analysis.

Fig. 4 Dendrogram of the hierarchical cluster analysis obtained for the honey sample content of carbohydrates



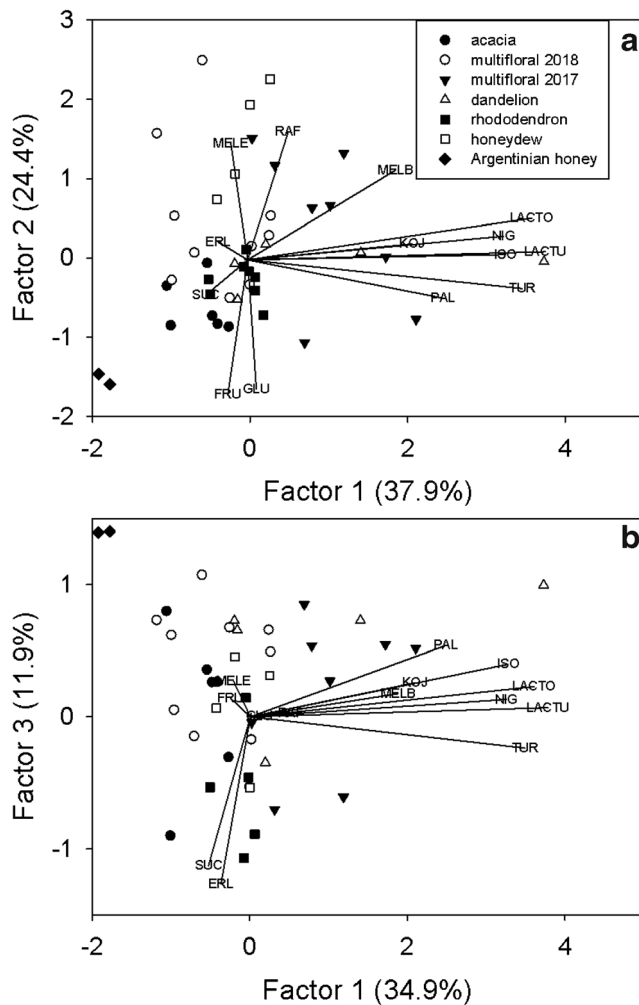


Fig. 5 Factor analysis biplot relative to the honey sample compositions for the content of carbohydrates in the plane defined by the factors 1 and 2 (a) and the factors 1 and 3 (b); PAL palatinose, RAF raffinose, MELE melezitose, KOJ kojibiose, ISO isomaltotriose, MELB melibiose, LACTO lactose, NIG nigerose, LACTU lactulose, TUR turanose, ERL erlose, SUC sucrose, GLU glucose, FRU fructose

Conclusion

An instrumental technique based on a high-performance anion-exchange chromatography method coupled with a mass spectrometer (HPAEC-MS) was developed to investigate the monosaccharides and one extended group of oligosaccharides in honey samples. The coupling of ion-exchange chromatography with mass spectrometry allowed the reduction of the sample preparation before analysis. The procedure requires a simple and fast pre-analytical procedure based on dilution with ultrapure water. The method was validated by testing the linearity, instrumental precision in terms of precision repeatability (intra-day) and reproducibility (inter-day), LOD, LOQ, MDL, MQL, and trueness.

Monosaccharides, disaccharides, trisaccharides, and tetrasaccharides were determined in Italian honey samples

with a diverse botanical and geographical origin within the Trentino Alto-Adige region and, for comparison, two Argentinian honeys. The monosaccharide and oligosaccharide profile of analyzed honeys was useful to define and differentiate the sample according to their different floral characteristics and inter-annual variability. Fructose and glucose were the most abundant carbohydrates in all types of analyzed honey in agreement with literature data, while the di- and trisaccharide composition showed they are related to the aging and floral origin. The contents of some disaccharides, such as turanose and palatinose, were representative especially in dandelion honey; sucrose and erlose were representative of rhododendron honey; a larger group of oligosaccharides, in particular lactose, lactulose nigerose, and isomaltotriose, were related to the aging of honey. The content of glucose, fructose, melezitose, and raffinose can be useful to characterize honeydew, and melezitose had higher concentrations also in some monofloral honey, probably due to possible contamination or mixing with honeydew honey.

The chemometric approach was used to establish the relationship between the profile of the oligosaccharides and the botanical origin; the multivariate statistical methods (hierarchical cluster analysis and factor analysis) highlight that the content of oligosaccharides could undergo modification during the harvest period, given the separation of the samples collected in two different years.

We can conclude that the fraction of minor oligosaccharides can be useful to establish the floral variety of honey samples and, in particular, the difference between disaccharides and trisaccharides can differentiate honey samples from different origins and according to aging. Furthermore, the result of the oligosaccharide content in honey could be important to carry out future investigations, especially considering their additional characteristics as important nutritional components, such as the prebiotic activity and the inhibitory action against microorganisms.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interests.

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