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HONEY QUALITY IN THE ALPINE ECOSYSTEM AREA

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I dedicate this work to my family,

Mariangela and Lorenzo.

ABSTRACT

This dissertation is focused on the characterization of honey samples with different botanical origins from valleys Trentino Alto-Adige.

The characterization of the honey was carried out using physicochemical parameters: pH, total soluble solids content (°Brix) and moisture content, and chemical parameters: carbohydrates, aromatic profile, and isotopic composition. The physicochemical factors are within the range of values reported in International and European regulations. The results show that these factors contribute to a lesser extent to the characterization of honey with different botanical origin. Carbohydrates, mainly disaccharides and trisaccharides, are related to the botanical origin and, their content is also influenced by honey aging processes. The statistical analysis applied to the results highlights that these allow characterizing honeys with different floral origins as well as inter-annual variability.

The isotopic composition of light elements is likely associated with the geographical origin. The comparison of the Trentino Alto-Adige honey samples with those from other geographical areas show differences in the isotopic composition.

The volatile organic components allow characterizing honey with different botanical origins, and highlight that the composition can be related to the vegetable source (nectar and/or honeydew) used by honeybees.

Statistical analysis applied to chemical parameters demonstrates that carbohydrates and volatile organic compounds are the factors that allow differentiating the honey based on botanical origin.

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Chapter 1. General introduction of honey

1.1 Definition of honey

According to the Council Directive 2001/110/EC relating to honey**,** the Council of European Union legally defines the honey as follows: "honey is the natural sweet substance produced by bees (*Apis mellifera)* from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in honeycombs to ripen and mature." Based on this above-mentioned directive, honey products can be considered as having both animal and vegetal origin. Honey is a sugar-based foodstuff which is produced only by honeybees, where nectar and honeydew are the main raw material normally used for the production of this food. Therefore, no additions of any other ingredients are permitted in natural honey. Eliminating pollen grains or other specific natural components of honey from the product is not allowed, exceptfor pollen which is removed during filtration to remove the foreign inorganic and organic matter. Additionally, as also reported in Codex Alimentaruis (2001), there are different types of honey which can be classified according to their origin, the method of production or the mode of presentation. According to the origin, honey can be differentiated into blossom or nectar honey, produced mainly from the nectar of plants, and honeydew honey is mainly manufactured from excretions of plant-sucking insects (*Hemiptera,* for instance) on the living part of plants or secretions of living parts of plants (Codex Alimentarius, 2001; EU Regulation, 2001). Honey may also be classified according to the processing and harvesting modes such as extracted honey, pressed honey, filtered honey, comb honey, strained honey, chunk honey or cut comb in honey, as well as its use such as industrial or bakers' honey (Alvarez-Suarez, et al., 2014; EU Regulation, 2001; Pavlova et al., 2018). Moreover, the different honeys may be supplemented by information referring to: the type of vegetable or floral origin, if the product comes wholly or mainly from the indicated source and possesses its organoleptic characteristics (Thrasyvoulou et al., 2018); the physicochemical and microscopic characteristics; the regional, territorial or topographical origin, if the product comes entirely from the designate area; the species quality criteria (EU Regulation, 2001; Thrasyvoulou et al., 2018).

1.2 Honey precursor: nectar and honeydew

Honeybees produce honey by collecting two main vegetable materials, nectar and honeydew (Doner, 1977). The nectar is a dilute solution of sugars secreted by particular glands (nectaries)

situated in the flowering plant (Al-Qassemi & Robinson, 2003). Carbohydrates are the most common compounds present innectar (DeGrandi-Hoffman et al., 2016). The sugars composition of honey consists of a solution with variable concentration, each sugars contributes from 5% to 80% of the total content. The sugars represent about 95% of the dry matter, while amino acids are approximately 0.05%, minerals in a range from 0.02 to 0.45%, and organic acids, vitamin and aroma compounds are also present in a small amounts (Bogdanov, 2011a). Although, fructose, glucose, and sucrose are the major sugars, each plant species has a typical sugar composition (Al-Qassemi & Robinson, 2003). For instance, fructose and glucose are predominantly in rape and dandelion nectar; whilst sucrose is mostly present in the nectar of *Fabiaceae* and *Labiateae* plants such as acacia, clover, sage, and lavender. However, the concentration of carbohydrates depends on different climatic factors such as temperature, soil, humidity and season (Bogdanov, 2011b). Honeydew is the liquid secretion of plant-sucking insects such as *Hemiptera*, frequently aphids. These insects ingestthe sap, the foliage or other external parts of the plant, and excrete the surplus as droplets, thereby forming the honeydew (Al-Qassemi & Robinson, 2003). Honeydew is a solution of sugars with different concentration range where, each sugar contributes from 5% to 60% of the total. The honeydewcontains mainly sucrose, and oligosaccharides. Some insects produce significant amounts of trisaccharides as melezitose. Smaller amounts of amino acids, proteins, minerals, organic acids, and vitamins are also present. Additionally, honeydew contains algae and fungi. Honeydew production depends on mainly the population of plant-sucking insects, as well as weather conditions during the time of honey production (Bogdanov, 2011b).

1.3 Types of honey: blossom or nectar honey (unifloral and multifloral) and honeydew honey

Honey can be classified as monofloral and multifloral (Di Bella et al., 2015; Pita-Calvo et al., 2017). Monofloral or unifloral honey is defined as the product originating predominantly from a single botanical source or a particular plant variety (Persano Oddo & Bogdanov, 2004; Pires et al., 2009). Generally, unifloral honeys are considered sweetener products with higher market value due to its limited production and as a consequence its reduced availability (Pita-Calvo et al., 2017). This type of honey often has higher prices than other honey types, such as blend honey. In some countries of Europe, for instance Italy, France, and Spain, as much as 30 to 50% of the commercialized honey sold is unifloral. In Europe, approximately one hundred botanical species are usually used by honeybees to make unifloral honeys (Persano Oddo & Bogdanov, 2004). A previous report by the same authors collected data about the production and properties of the main European unifloral honeys. These varieties are as follows: rape, heather, sweet chestnut, citrus, eucalyptus,

sunflower, lavender, rhododendron, robinia, rosemary, dandelion, thyme, lime, honeydew (Persano Oddo & Piro, 2004). Eucalyptus honey is one of the major unifloral honeys present in Atlantic and Mediterranean coastal areas, while in mountainous areas chestnut honey is the most abundant product (Escuredo et al., 2014). Central and Eastern Europe frequently produce unifloral honeys like acacia and lime honeys, which are considered to be important nectariferous botanical species (Dobre et al., 2011) However, in Northwest Spain, bramble honeys are an n appreciate honey. Rape and sunflower are the most commercialized European unifloral honeys (Escuredo et al., 2014). Although honey produced in different geographical areas can be characterized by identical floral species, the resulting canactually be different due to factors such as seasonal climatic variations or to some other geographical characteristics (Anklam, 1998).

Multifloral honeys, also known as polyfloral honeys, is honey produce with contributions from a wide range of different botanical sources, where no singlevegetable species is predominant, e.g., meadow blossom honey and forest honey (Alvarez-Suarez et al., 2014; Soares et al., 2017). In terms of pollen content multifloral honey contains contributions from several pollen grains derived from various plant species, and no single pollen type can be considered the principal source (Soares et al., 2017).

Honeydew honey, is produced by honeybees after gathering the so-called "honeydew", the liquid secretion associated with a wide variety of sucking insects, generally related to the genus *Rhynchota* (Alvarez-Suarez et al., 2014).

Honeydew honey, along with blossom honey, such as fir and pine, are especially appreciated in different countries of Europe, but they are less appreciated in other parts of the world (Bogdanov, 2011c). Honeydew can be manufactured by a wide variety of plants such as *Coniferae*, *Abies alba L.* and *Picea excelsa (Lam) Link.* produced in Central and Northern Europe; *A. cephalonica Loudon* and *Pinus halepensis Miller* and *P. brutia Ten.*, mainly from Greece; and *Latifoliae* produced in most countries of Europe, essentially from diverse *Quercus* species (Persano Oddo & Piro, 2004).

Therefore, all of the types of honey can be completely different from each other due to their possible sources that can contribute in different proportions. The associatednectar and/or honeydew can also differ as a result of deriving from a wide variety of vegetation (Persano Oddo & Bogdanov, 2004).

1.4 Chemical composition

Honey is a complex foods produced by nature (Ouchemoukh et al., 2010). Humans have consumed honey since ancient times, due to not only to its sweet flavor but also for its high nutritional importance and for health benefits (Alvarez-Suarez et al., 2014; Meo et al., 2017).

Honey is a variable and complex mixture of a variety of compounds that contains approximately more than 180 different components from floral source and/or they result from biochemical reactions that occur during the ripening of honey (Alvarez-Suarez et al., 2013; Anklam, 1998; Escuredo et al., 2013; Pita-Calvo et al., 2017). The composition of honey represents a significant nutritional source of macro and micronutrients (Alvarez-Suarez et al., 2013). Sugars are the most representative organic compounds present in honey (Anklam, 1998; de la Fuente et al., 2011; Miguel et al., 2017).

Besides carbohydrates, other minor components present include as amino acids, proteins, enzymes, organic acids, minerals, volatiles compounds, B vitamins, and antioxidant phenols (Ajibola, et al., 2012; Escuredo et al., 2014; Miguel et al., 2017; Pita-Calvo et al., 2017).

1.4.1 Sugars

Honey is a food in which carbohydrates are the predominantly compounds (de la Fuente et al., 2011; Miguel et al., 2017; Pita-Calvo & Vázquez, 2018; Puscas et al., 2013). Sugars account for approximately 80% (w/w) of the total soluble solids and about 95% of honey's dry weight (Anjos et al., 2015; Arias et al., 2003). The carbohydrates are responsible for multiple physical characteristics such as energy value, hygroscopicity, viscosity and crystallization phenomena (Da Silva et al., 2016).

Honey is a supersaturated solution of two main monosaccharides, glucose and fructose, which are present in a range from 65% to 85% of total soluble solids (Da Costa Leite et al., 2000; de la Fuente et al., 2011; Doner, 1977; Ruiz-Matute et al., 2010). The mean concentration of these monosaccharides is approximately 40% and 30% for fructose and glucose, respectively (Alvarez-Suarez et al., 2013; Pita-Calvo & Vázquez, 2018). The ratio between these simple sugars is a valuable indicator for the categorization of monofloral honeys (Da Silva et al., 2016). Fructose is normally present in a higher concentration than glucose, resulting in the extreme sweetness of honey (Anjos et al., 2015). In contrast, glucose is a dominant sugar only in some honey such as rape and dandelion (Escuredo et al., 2014). The proportion of fructose and glucose in honey is widely affected by the source of the nectar (Anklam, 1998).

According to the requirements of Codex Alimentarius (2001) and the EU regulation (EU Regulation, 2001), the total amount of fructose and glucose for nectar honey have to exceed to 60%, while for honeydew honey or in the case of the blend between honeydew and blossom honey these sugars have to be up to 45% (Thrasyvoulou et al., 2018).

Oligosaccharides, which are other minor carbohydrates, such as disaccharides, trisaccharides, and tetrasaccharides, found in most honey, although they are present in low concentration (Arias et al.,

2003; Da Costa Leite et al., 2000; Puscas et al., 2013; Ruiz-Matute et al., 2010). Disaccharides (sucrose, maltose, turanose, isomaltose, maltulose, trehalose, trehalose, nigerose, kojibiose, palatinose, laminaribiose, gentiobiose) and trisaccharides (maltotriose, isomaltotriose, erlose, panose, isopanose, kestose, raffinose, and melezitose) range from 10% to 15% of the carbohydrates in honey (Da Costa Leite et al., 2000; Miguel et al., 2017; Pita-Calvo & Vázquez, 2018; Ruiz-Matute et al., 2010). These oligosaccharides are mainly formed by glucose and fructose residues with the glycosidic bounds in diverse configurations and position (Pita-Calvo & Vázquez, 2018; Ruiz-Matute et al., 2010). Oligosaccharides are important components related to the geographical and botanic origin (Bogdanov et al., 2004; Escuredo et al., 2014) as well as for their high nutritional value such potential "prebiotic" properties (Al-Qassemi & Robinson, 2003; Ouchemoukh et al., 2010).

Currently, more than 20 oligosaccharides have been identified in different varieties of honey produced around the world (Arias et al., 2003; Da Costa Leite et al., 2000; Goodall et al., 1995; Jan Mei et al., 2010; Mateo & Bosch-Reig, 1997; Ouchemoukh et al., 2010; Ruiz-Matute et al., 2010). It is worth underlining that these oligosaccharides are not present in the nectar, but that they are due to the activities of the honeybees' enzymes during the ripening of honey (Alvarez-Suarez et al., 2013). In contrast, sucrose is present in the nectar and is enzymatically hydrolyzed into glucose and fructose, then transformed by the enzymes activity in different oligosaccharides (Alvarez-Suarez et al., 2013; Ruiz-Matute et al., 2010). Based on this finding, sucrose is an important sugar that is accounts for approximately 1% of the dry weight of honey (Anklam, 1998). However, in genuine honey, the amount do not exceeds the 5%, except for some types of honey such as robinia, eucalyptus, citrus, lavender (Pita-Calvo et al., 2017; Thrasyvoulou et al., 2018). Some honeydew honey also contains tetrasaccharides, pentasaccharides and hexasaccharide (Sanz et al., 2005).

A number of previous studies examine carbohydrates profile in honey types around the world with several chromatographic analytical techniques (Arias et al., 2003; Cotte et al, 2004; Da Costa Leite et al., 2000; de la Fuente et al., 2011; Escuredo et al., 2014; Gòmez Bàrez et al., 1999; Goodall et al., 1995; Kamal & Klein, 2011; Morales et al., 2008; Morales et al., 2006; Ouchemoukh et al., 2010; Rybak-Chmielewska, 2007; Sanz et al., 2005; Swallow & Low, 1990; Terrab et al., 2001; Weston & Brocklebank, 1999). However, while these multiple methods exist, the International Honey Commission (IHC) defined the official chromatographic methods (HPLC-RI, HPLC-PAD, and GC-FID) for the determination of sugars profile in honey (Bogdanov, 2009).

1.4.2 Water

Water, is the second most important component of honey, after sugars, and its concentration ranges from 10% to 20% (Pavlova et al., 2018; Pita-Calvo et al., 2017; Pita-Calvo & Vasquez, 2018). Honey moisture content depends on different variables, such as environmental and seasonal conditions, botanical origin, degree of maturity achieved in the hive, the manipulation and processing from beekeepers during the harvest period, as well as storage conditions of the honey (Conti, 2000; Da Silva et al., 2016; Karabagias et al., 2014b; Saxena et al., 2010; Terrab et al., 2004).

Some physical properties of honey like the crystallization process, viscosity and sensory characteristics such as color, flavor, taste, solubility, and conservation can also be influenced by the water content (Da Silva et al., 2016; Escuredo et al ., 2013). In some kinds of honey, a high amount of water content could lead to an acceleration of the crystallization process (Gomes et al., 2010). The water content is also correlated with the fermentation processes from yeast, and can possibility prevent the deterioration of honey (Pita-Calvo et al., 2017). The higher moisture content is responsible for undesirable honey fermentation activity during storage caused by the reactions with osmotic yeasts (Escuredo et al., 2013; Saxena et al., 2010). On the other hand, water content lower than 17.1% can be considered as a safety value where the fermentation processes is practically avoided (Conti, 2000). Based on this consideration, the maximum amount of water contained by honey is regulated, and in accordance with European regulations, the limit value is fixed to ≤20%, except for some honeys like Calluna honey which is permitted to have a water content higher (EU Regulation, 2001).

1.4.3 Nitrogen compound

The percentage of nitrogen compounds in honey is very low, with mean values around 0.04%. Protein, amino acids, and enzymes are the most representative nitrogen substances found in honey (Alvarez-suarez et al., 2013; Anklam, 1998).

Proteins content in honey can account for 0.5% of the total, and the most important proteins are globulin and albumin that derive directly from pharyngeal glands of honeybees (Anklam, 1998; Pita-Calvo et al., 2017).

According to the previous authors, most of the protein in honey come directly from salivary glands when the honeybees transform nectar and honeydew, while the remaining proteins derives from pollen, which is naturally rich in protein, and contains between 10% to 35% proteic material (Pavlova et al., 2018).

A small quantity of protein is constituted by enzymes, which originate from the glands of honeybee (Pita-Calvo et al., 2017). These enzymes are largely represented by α- and β-glucosidase (invertase or sucrose), α- and β-amylase (diastase), and β-fructosidase, which play an important role in the hydrolysis of sugars, generally sucrose, contained in the floral nectar (de la Fuente et al., 2011; Miguel et al., 2017; Pita-Calvo et al., 2017; Ruiz-Matute et al., 2010).

The total amount of amino acids in honey is about 1% (w/w) of the total nitrogen components and its origin is attributable not only to the honeybee but also to the floral source (Alvarez-Suarez et al., 2013; Boonchiangma et al., 2015; Rebane & Herodes, 2008). Pollen is the major source of amino acids, and their profile could be an important indicator of their botanical origin and geographical origin. Proline is the most abundant amino acids found in honey and range from 50 to 85% of the total amino acids present (Alvarez-Suarez et al., 2013; Anklam, 1998; Boonchiangma et al., 2015; Kečkeš et al., 2013). However, when other amino acids are present, the qualitative and quantitative composition differs, and can include glutamic acid, aspartic acid, glutamine, histidine, glycine, threonine, b-alanine, arginine, a-alanine, tyrosine, valine, methionine, cysteine, isoleucine, leucine, tryptophan, phenylalanine, ornithine, lysine, serine, asparagine and alanine (Da Silva et al., 2016; Hermosín et al., 2003; Sun et al., 2017). In recent years, numerous studies have focused on determination and quantification of amino acids in different honey around the world (Boonchiangma et al., 2015; Bouseta et al., 1996; Cotte et al., 2004; Hermosín et al., 2003; Iglesias et al., 2004; Kečkeš et al., 2013; Kivrak et al., 2017; Paramás et al., 2006; Pérez et al., 2007; Rebane & Herodes, 2008).

1.4.4 Organic acids

Honey is naturally acidic and its pH value is due to the presence of organic acids, with concentration of approximately 0.57% (Karabagias et al., 2014b; Khalil et al., 2012). The most representative compound is gluconic acid. However, another organic acids have been found in honey, although in small amounts, such as formic, acetic, citric, lactic, maleic, malic, oxalic, pyroglutamic, succinic, aspartic, butyric, fumaric, pyruvic, propionic, tartaric, oxalic, levulinic, galacturonic (Anklam, 1998; Bogdanov, 2011b; Da Silva et al., 2016; Miguel et al., 2017). These organic acids are produced from the sugars through the enzymes secreted by honeybees during the transformation of the nectar into honey. Gluconic acid is generated by the glucose oxidase enzyme, which is produced by the honeybees during the honey ripening (Da Silva et al., 2016; Karabagias et al., 2014a). Although organic acids are present in a low amount, organic acids may be used to differentiate the honeys according to their geographical and/or botanical origin. Some organic acids such

asgluconic and citric acid, are a valuable parameter to discriminate the floral honey and honeydew honey (Da Silva et al., 2016).

The organic acids are also associated with other chemical properties such as electrical conductivity, color and flavor. Generally, the pH value of nectar honeys can range from 3.3 to 4.6, (with one average value of 3.9). However, for only some types of honey such as chestnut, the pH value is relatively high and ranges from 5 to 6. Honeydew honeys have a higher pH value when compared with nectar honey, and its value range from 4.5 to 6.5, (with 5.2 as average value), (Ajibola et al., 2012; Bogdanov, 2011b). In honey, the pH value is a significant quality parameter related to stability and the shelf life, and also is useful to reveal microorganism contamination (Pita-Calvo et al., 2017). The determination of pH, along with other authenticity parameters, can also corroborate adulterations (Da Silva et al., 2016).

Despite their importance, little information exists in the literature about organic acids content in different kinds of honey (Keke & Cinkmanis, 2019; Mato et al., 2003; Nozal et al., 2003; Suárez-Luque et al., 2002).

1.4.5 Inorganic components

A wide variety of inorganic components, including both macro and microelements, exist in honey (Da Silva et al., 2016; Madejczyk & Baralkiewicz, 2008).

The elements content in honey range approximately from 0.04% to 0.2% of its dry weight, in light honeys and dark honeys, respectively (Alvarez-Suarez et al., 2013; Miguel et al., 2017). Although the amount of inorganic components is quite low, they contribute significantly to the biomedical and nutritional properties of honey, play an important role in several biological functions and are very important in the human diet (Alvarez-Suarez et al., 2013; Madejczyk & Baralkiewicz, 2008). The most representative element is potassium, with an average content in honey of 1500 mg kg^{-1} (Anklam, 1998). Other macro and microelements have been determined in various honey. The more abundant elements are magnesium, calcium, phosphorus, while sodium, lithium, cobalt, manganese, cadmium, iodine, zinc, copper, nickel, iron, barium, silver, chromium, selenium, and arsenic, are present a lower concentration (Ajibola et al., 2012; Anklam, 1998; Miguel et al., 2017). The element concentrations in honey are related to the vegetable source in which the honeybees gather the raw material. Therefore, the content of these elements depends on the type of soil where the plant and the corresponding nectar were grown. Thus, elemental composition in honey could be different according to the geographic origin, the type of soil, as well as floral origin (Alvarez-Suarez et al., 2013; Escuredo et al., 2013; Madejczyk & Baralkiewicz, 2008). The content of ele-

ments in honey could provide a valuable indication about the area visited by honeybees. We therefore may use elemental concentrations as a potential indicator of the geographical origin of honey, and as significant indicator of potential environmental pollution (Anklam, 1998; Da Silva et al., 2016; Madejczyk & Baralkiewicz, 2008). On the base of these considerations, in recent years, elemental composition of honey has been investigated and presented in a number of papers from different countries around the world (Caroli et al., 1999; Chua et al., 2012; Da Silva et al., 2016; Madejczyk & Baralkiewicz, 2008; Rodriguez-Otero et al., 1994; Vanhanen et al., 2011).

The quality of foodstuffs is of particular interest to consumers, industries, manufacturers. Therefore, it is important to increase the number of studies aimed at improving the techniques for monitoring the origin, authenticity and traceability of food products (Potočnik et al., 2020). Different fingerprinting techniques have been exploited for authentication and traceability of honey. The authenticity of honey can be defined considering the processes and activity characteristics performed by industries and beekeepers as well as considering their botanical and geographical origin (Bogdanov & Martin, 2002). Stable isotopes of the principal bioelements (i.e., $^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{34}S/^{32}S$) have been applied to discriminate the origin of various food products (Potočnik et al., 2020). Stable isotope analysis of light elements (C, H, N, and S) of bio-compounds has gained much interest for determining the geographical origin of many food products including honey (Camin et al., 2016; Ogrinc et al., 2003).)

Originally, the analysis of carbon isotope ratios was used to identify the adulteration of honey. The isotope ratios of carbon ($^{13}C/^{12}C$) in raw honey and in honey proteins can identify the presence of sugars from C-4 plants (AOAC, 1991). For authenticity, the difference of these two values needs to be lower than 1‰. Several studies about the analysis of stable isotope elements were conducted on honey from around the world to define the geographical origin and authenticity (Berriel, 2018; Bontempo et al., 2017; Kropf et al., 2010b; Zhou et al, 2018). Analyzing carbon stable isotopes in honey uses the method called carbon stable isotope ratio analysis (SCIRA), which was improved and named internal standard carbon stable isotope ratio analysis (ISCIRA) (AOAC, 1991).

1.4.6 Other components

There are several additional minor compounds found in honey such as vitamins, volatile organic compounds, antioxidant substances, phenolic, and methylglyoxal.

Essential bioactive compounds found in honey in a small amount include vitamins ‘‘A (retinol), vitamin E (tocopherol), vitamin C (ascorbic acid), vitamin K (antihaemorrhagic vitamin), panto-

thenic acid, and also a various vitamin belonging to B group such as vitamin B1 (thiamine), vitamin B2 (riboflavin), nicotinic acid (B3), riboflavin (B2), pyridoxine (B6), biotin (B8), folic acid (B9) (Ajibola et al., 2012; Meo et al., 2017; Miguel et al., 2017). Several other organic compounds have been detected in different kinds of honey, belonging to volatile components. According to the literature, approximately 600 volatile substances have been identified in various honey, belonging to different chemical classes such as aldehydes, ketones, acids, alcohols, esters, hydrocarbons, as well as sulfur, furan, and cyclic compounds (Karabagias et al., 2014b; Kaškonienė & Venskutonis, 2010).

Aroma compounds occur in honey at extremely low concentrations, constitute a variety of classes of substances with dissimilar chemical and physical properties (Plutowska et al, 2011).

Generally, these compounds derive from diverse biosynthetic pathways, plant sources, the transformation of the nectar through honeybee metabolism, the thermal, handling and storage processing of honey, as well as the environmental conditions and microbial activities (Jerković et al., 2006; Karabagias et al., 2014b).

The composition of aroma components is also strongly affected by factors, such as botanical and geographical origin, climate conditions, soil, storage procedures, honey handling and honeybee species (Makowicz et al., 2018).

Volatile substances are the major parameter responsible for the aroma of honey, which contribute to its flavor. Aroma profiles represents a fingerprint of the honey, so aromacould be used as specific chemical markers to determine the honey origin and characteristics (Baroni et al., 2006; Cuevas-Glory et al., 2007; de Lima Morais da Silva et al., 2017; Radovic, et al., 2001). This possibility has prompted several authors to analyze the volatile profile of honey (Alissandrakis et al., 2007; Bianchi et al., 2011; Castro-Vázquez et al., 2007; Karabagias et al., 2014a; Karabagias et al., 2014b; Patrignani et al, 2018; Senyuva et al., 2009). Honey also contains phenolic compounds, which are a chemically heterogeneous group and account for approximately 10,000 compounds. Based on their chemical structure, these molecules are grouped into diverse chemical classes, generally into non-flavonoids like phenolic acid and flavonoid such as flavones, flavonols, flavanones, flavanols (Da Silva et al., 2016).

These substances are the most important bioactive molecules present in honey, being closely related to its antioxidant activity. This group composition depends on the floral origin, weather and geographical conditions(Meo et al., 2017). Some bioactive compounds that are present in most of the types of honey or can be found only in specific varieties are: quercetin, kaempferol, myricetin, chrysin, hesperetin, pinocembrin, isorhamnetin, rosmarinic acid, gallic acid, vanillic acid, caffeic acid, syringic acid, þ-coumaric acid, and ferulic acid (Alvarez-Suarez et al., 2014; Anklam, 1998).

Additionally, it has been suggested that many beneficial properties can derive from a large number of other compounds found in honey. For example, it is reported that high antimicrobial activity derives from the hydrogen peroxide formed in many honey types and/or the methylglyoxal present in Manuka honey (Atrott et al., 2012; Can et al., 2015; Meo et al., 2017).

1.5 Physical characteristics of honey

Honey, as a natural food that does not need any further transformation, and its physical properties, such as color, electrical conductivity, crystallization and optical rotation, can vary noticeably. Such parameters are related to the botanical origin of honey, and are used to evaluate the quality of honey (Pita-Calvo et al., 2017).

1.5.1 Color

Color is an important attractive factor of honey, and it influences the price for the commercialization of honey. Color is a parameter that affects consumers from the point of view of the acceptability and preference of honey (Da Silva et al., 2016; Pereyra et al., 1999).

The honey color can vary significantly, and depends mainly on the botanical origin and its inorganic components. For instance, light-colored honeys are generally characterized by low elements concentrations, while dark-colored honeys usually presents a high amount of elements (Gomes et al., 2010; A. Iglesias et al., 2012). However, yellowish (acacia and sunflower honeys), reddish or greenish (rosemary honeys) are the most common color of honey (Bogdanov, 2011c; Da Silva et al., 2016). Generally, blossom honey is lighter than honeydew honey, except for some nectar honey such as chestnut and heather that can be darker-colored (Pita-Calvo et al., 2017).

Factors such as temperature, storage conditions, presence of flavonoids, and carotenoids may also influence the honey color (Pereyra et al., 1999; Pita-Calvo et al., 2017).

Normally, the color is measured by an optical comparison using a Pfund or Lovibond scale, but the color grading can also be determined using spectrophotometric methods (Cimpoiu et al., 2013; Pita-Calvo et al., 2017).

1.5.2 Electrical conductivity

Electrical conductivity is included in the international standard for honey and substitutes the ash analysis, according to the survey on several different honey from around the world (Bogdanov et al., 2004; Codex Alimentarius, 2001).This parameter is strongly associated with the elements concentration, organic acids, and protein and is related to the variability in the botanical origin (Iglesias et al., 2012; Moise et al., 2011; Terrab et al., 2004). Consequently, this physical factor is one

of the most important quality parameters to classify floral honeys (especially monofloral) and to differentiate blossom honeys from honeydew honeys (Bogdanov et al., 2004; Da Silva et al., 2016). The determination method of electrical conductivity in honey is reported in the International Honey Commission (IHC), which is based on the measurement of the electrical resistance (reciprocal of electrical conductivity) of a honey aqueous solution (Bogdanov, 2009).

The recommended limit for electrical conductivity in honey is not more than 0.8 mS cm^{-1} for blossom honey, except for some unifloral honeys i.e. strawberry tree (*Arbutus unedo)*, bell heather (*Erica*), Lime (*Tilia spp*), eucalyptus, and not less than 0.8 mS cm^{-1} for honeydew and chestnut honey (Codex Alimentarius, 2001).

1.5.3 Crystallization

Crystallization is affected by the value of the moisture content of honey (da Silva et al., 2016). The crystallization phenomenon transformation occurs naturally in honey and the process is related to the monosaccharide content in honey, especially fructose and glucose (Escuredo et al., 2014).

Generally, fructose is the abundant sugar in honey in comparison with glucose, but some types of honey present high glucose concentration, such as rape and dandelion honey (Da Silva et al., 2016). Consequently, according to the ratio of fructose and glucose the honey has different crystallization tendencies. In sunflower, rape, and dandelion honeys, the previous author reported that in this honeys the crystallization is rapid because the glucose amount is higher than the fructose content (Escuredo et al., 2014; Persano Oddo & Piro, 2004). On the other hand, chestnut honey is liquid for a longer time because the fructose concentration is high in comparison with a low glucose content (Persano Oddo, & Piro, 2004). Heather honeys have a moderate crystallization with respect to other different honeys (Da Silva et al., 2016). The fructose and glucose ratio is useful to explain the crystallization process because fructose is more water-soluble than glucose, so some types of honey have a quicker granulation than others. Therefore, the ratio can be used to predict the crystallization tendency (Laos et al., 2011).

1.5.4 Optical activity

This physical characteristic of honey is related to the sugars contained in honey. The carbohydrates present in honey have the property of rotating the plane of polarised light. For instance, fructose and glucose exhibit a negative and positive optical rotation, respectively. But in general, this parameter depends on the total concentration of carbohydrates in honey. Using this physical parameter is possible to differentiate blossom honey, with negative optical value, from honeydew honey

characterized by positive optical value. The measurement of optical rotation in honey is carried out using a polarimeter (Bogdanov et al., 2004).

1.6 Production of honey and extraction procedures of honey

The production of honey around the world is mainly based on the honey obtained from the species of *Apis mellifera* honeybee (Alvarez-Suarez et al., 2018). The world honey production in 2018 was about 1.86 million tons (Ismea, 2019). The major producers of honeys are located in Asia (China) which accounts for 49%, in Europe with 21%, and also in the America (United States, Mexico, and Argentina) with 18% (Alvarez-Suarez et al., 2018; Ismea, 2019; Meo et al., 2017). However, limited production of honeys obtained from different types of honeybees, principally stingless honeybees, are produced in different countries of the world, such as Australia, Africa, and South America (Alvarez-Suarez et al., 2018).

Italy, in Europe, represents the country with the fourth greatest number of hives (1.4 million), followed by Spain (2.9 million hives), Romania, and Poland (1.8 and 1.6 million hives, respectively) (Ismea, 2019). According to the report published by Ismea (2019) the Italian honey production accounts for approximately 8-23 thousand tons (Ismea, 2019). The Italian honey production, includes over 1.4 million hives, and is divided into 390 thousand and 556 thousand hives used for permanent and nomad beekeeping respectively. The remaining hives are mainly used for self-consumption. Geographically, the production is widespread in all regions of Italy. However, the alpine region is the most productive area. For instance, Piemonte region (northwestern Italy) accounted for more than 5,000 tons in 2018, followed by Lombardia (northwestern Italy), Veneto and Trentino Alto-Adige (northeastern Italy) regions that account for more than 5 thousand hives. Average data per hive showed that the national production accounts for approximately 30 kg/hive. In particular, this production is about 33 kg/hive for the northwestern and northeastern regions (alpine area), 35 kg/hive for the central regions and 22 kg/hive for the southern regions (Ismea, 2019). However, the geographical characteristics of the alpine area along with meteorological conditions and climatic changes can particularly compromise the beekeeping activity in these areas, influencing both production and economic losses (Ismea, 2019). Therefore, increasing alpine honey production is tied to increasing the value and the quality of the different honey characterize dby typical multifloral and unifloral honeys.

Except for the classification of honey according to the vegetation or floral source (blossom honey and honeydew honey), honey can also be classify based on the processing procedures such as centrifuged honey, pressed honey and drained honey (Pavlova et al., 2018).

In beekeeping the extraction of the honey represents an important step. The honey extraction from the combs involves different steps such as removal of the honeycombs from the beehives, the uncapping of the honeycombs, and then separating honey from combs as pure liquid (Maradun & Sanusi, 2013). Regarding the extraction procedures of honey, the three most important physical systems are centrifuging, pressing and draining (Codex Alimentarius, 2001; EU Regulation, 2001). The most common beekeeping practice of extraction is represented by centrifugation system, which is carry out through a stainless-steel centrifuge designed specifically for honey (Kadri et al., 2017). The advantage of centrifugation system consists in an increase in the efficiency of honey production and a saving of energy by the bees in rebuilding the honeycomb, in comparison with another types of physical extraction (Maradun & Sanusi, 2013). Although centrifugation is the process commonly used, in most African countries the extraction of honey is carried out with a pressure system (Bogdanov, 2011c). In the honey extraction by pressure system, pressure is applied to the uncapped and cut honey combs until that the honey is completely released (Maradun & Sanusi, 2013). Finally, draining process is another kind of physical extraction system that I also reported in major regulation (Codex Alimentarius, 2001; European Union, 2001). This process, although is the simplest, it is time consuming. After the cutting, the combs are put to drained for the time necessary to recovery all the honey (Maradun & Sanusi, 2013).

1.7 Multivariate statistical methods

In this work, multivariate statistical analysis approaches were used for statistical processing of the data obtained from the investigated parameters. Principal Component Analysis (PCA), Hierchical Cluster Analysis (HCA) were applied.

1.7.1Principal Component Analysis (PCA)

The Principal Component Analysis is the oldest technique of the multivariate analysis. The first approach was proposed by K. Pearson (1901) and then developed by H. Hotelling (1933).

PCA is widely used in different scientific fields due to a relatively simple method and non-parametric method that allows extracting relevant information from data set (Shlens, 2005).

In general, this technique is widely used to explore the data set and it uses laborious underlying mathematical principles to transform different correlated variables into a smaller number of variables, so-called principal components (PCs). The aim of Principal Component Analysis, using a vector space transform, is to reduce the dimensionality of a data set formed by a considerable number of interrelated variables, but maintaining as much as possible of the variability of the data set. This reduction is possible by changing to a new set of variables, the principal components (PCs), which are not correlated, and they are arranged so that the first few components maintain

the majority of the variability present in all of the original variables (Mishra et al., 2017).
The main goals of Principal Component Analysis are to extract the most relevant information from the data set; to evaluate the correlation between the variables and their relevance; to simplify the description of the data set and visualize the data on the orthogonal space; to compress the data set to reduce the dimensionality, but maintaining as much variability as possible of the original information (Jolliffe & Cadima, 2016; Mishra et al., 2017).
The Principal Component Analysis provides effective graphical performance that contains high information through the representation of single objects (scores plot), variables (loadings plot), and objects and variables simultaneously (biplot). The score plot representation allows analysing the behaviour of the single object in relation to the different principal components and their similarities. However, the loading plot provides information about the role of each variable, their correlations and their importance. Finally, the biplot shows the objects and variables simultaneously in the same graph and allows to assess of the relations between them (Rao, 1996).

1.7.2 Hierchical Cluster Analysis (HCA)

Cluster analysis is a type of statistical technique that classifies cases into groups that present relatively homogeneous characteristics within the group and relatively heterogeneous features between groups (Landau & Chis Ster, 2010; Norusis, 2010). Therefore, the main purpose of cluster analysis is to group cases into homogeneous clusters, but the algorithms and measures used to form the groups with specific properties into different clusters make this process quite complex (Yim & Ramdeen, 2015).
There are several methods to perform cluster analysis, and they can be divided by two main categories: hierarchical and non-hierarchical methods.
Non-hierarchical methods are based on so-called relocation techniques, in which data are initially separated and then are moved into the different clusters until the specific criterion is satisfied. Among other, the most common methods are Jarvis-Patrick and K-means.
Hierarchical methods are divided into two principal categories: divisive hierarchical and agglomerative hierarchical. About divisive hierarchical is not commonly used and the analysis starting from the original data set and the separation between samples is according to their major differences. In contrast, agglomerative hierarchical method is widely used and is based on to form a number of clusters equal to number of samples and then, according to the similarity, to form the clusters bigger than the original clusters. The agglomerative hierarchical methods involve for instance the single linkage, average linkage, complete linkage, centroid linkage, Ward method (Landau & Chis Ster, 2010). The representation of the clusters analysis is performed through the

dendrogram, which allows a visual examination with high information about the similarity of the studied objects.

1.8 Conclusion

Honey is a natural product used since ancient times as a sweetener, flavor agent and for its high nutritional importance due to the presence of several classes of different compounds, which provide beneficial properties for the human body. Based on chemical composition, honey is a complex combination of different chemical components, where sugars and water are the most abundant constituents, followed by proteins and amino acids, organic acids, elements, and additional essential components. In addition, the many honey types presents various physical characteristics such as color, electrical conductivity, optical activity and crystallization, which are important with respect to the quality, commercialization and consumers acceptability.

Honeybees use two main precursors to produce honey; blossoms or nectar and honeydew floral sources. Honey can be classified as blossom or nectar honey and honeydew honey depending on the vegetation origin. However, the classification of honey is also based on the extraction procedures of centrifugation, pressing or draining. Honey is well regulated by both International and European regulations, providing a classification of various honey types on the base of composition and the methods used to assess the quality.

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RESEARCH OBJECTIVES AND THESIS STRUCTURE

This dissertation presents results on the characterization of honey quality from the alpine ecosystem and in particular honey samples produced in the Trentino Alto-Adige region (northeastern Italy).
In the present work, the quality of honey was tested and assessed using different physicochemical and chemical parameters. Physicochemical measurements include pH, total soluble solids (°Brix index), and moisture content (%). Chemical parameters include the carbohydrate composition, volatile organic compounds and the ratio of stable isotope of light elements.

The study aims to enhance the production of honeys obtained from artisanal beekeepers situated in the alpine region, especially from Trentino Alto-Adige, using the above-mentioned analytical parameters. The obtained results were compared with the European and International legislation, and with data published in the literature. In addition, all data were treated using the chemometric approaches to find out which parameters can be important to describe the quality of honey samples and to attempt to differentiate the honeys according to botanical and geographical origins using chemical parameters.

This dissertation focuses on the following five main goals:
1) assessment of honeys by physicochemical parameters such as pH, total soluble solids and moisture content (chapter 2);
2) honey characterization of carbohydrate compositions, including the main sugars and the oligosaccharides, using one HPAEC-MS innovative analytical method (chapter 3);
3) application of stable isotopes of light elements in honey and proteins to verify the geographical origin of honey (chapter 4);
4) characterization of the botanical origin of honey using volatile organic compounds (chapter 5);
5) characterization of honey using statistical analysis of physicochemical and chemical parameters to identify their specific properties according to the geographical a botanical origin (chapter 6).

Chapter 2. Physicochemical characteristics of Italian honeys.

2.1 Introduction: Quality and physicochemical parameters of honey

The quality of honey is obtained by examining chemical and a physicochemical parameters, as well as sensorial and biochemical characteristics. Chemical and physicochemical quality criteria about honey are well described in both International legislation (Codex Alimentarius, 2001) and European regulation Directive 2001/110 (EU Regulation, 2001). To define the quality of honey the International Honey Commission (IHC) describes the analytical methods used for the determination of all parameters (Bogdanov, 2009). Italian legislation, follows the International and European regulation and recommends that the official methods of analysis be applied in order to determine and control the quality of honey (Decreto Ministeriale, 2003). The most important reported criteria to assess honey quality are moisture content, electrical conductivity, ash content, the concentration of reducing and non-reducing sugars, free acidity, diastase enzyme activity and hydroxymethylfurfural (HMF) content. However, none of these regulations specify the criteria of bacterial contamination and hygiene of the honeys (Gomes et al., 2010; Khalil et al., 2012).

The pH parameter is not mentioned among the honey quality factors in the current legislations. However, pH is a fundamental characteristic because it is related not only to the shelf life but also to the botanical origin of the honey (Pita-Calvo et al., 2017). Honey is naturally acidic, therefore its pH value is always lower than 7 (Bogdanov, 2011a). Normally, blossom honeys has the pH values ranging between 3.3 and 4.6. The mean blossom honey pH value is 3.9, only some varieties of honeys present higher values. For instance, chestnut honey has a pH value ranging from 5 to 6. In addition, honeydew honey has higher pH value ranging from 4.5 to 6.5, with one average value of 5.2 (Ajibola et al., 2012; Bogdanov, 2011a). The pH value in honey is a significant quality parameter related to the extraction and storage procedures because the pH influences the texture, stability shelf life and may reveal microorganism contamination (Khalil et al., 2012; Pita-Calvo et al., 2017; Terrab et al., 2004). In addition, the determination of pH is also used to corroborate possible adulterations of honey (Da Silva et al., 2016).

Besides determining specific components such as sugar, water content is also a valuable parameter to assess the quality of honeys. The moisture content is a significant factor of the honey standard composition criteria that beekeepers must be control to commercialize their product.
Moisture content of honey made by honeybees depends on many factors such as the floral or botanical source, beekeeping activities, degree of maturity, weather and climatic conditions in the

geographical area where this product was manufactured, as well as processing methods and storage conditions (Escuredo et al., 2014; Pita-Calvo et al., 2017; Thrasyvoulou et al., 2018). Besides carbohydrates, water is the second most important component in honey, and its content range from 15 to 23%. The content of water in honey, when is well-sealed in honeycomb by honeybees, is less than 18% (Thrasyvoulou et al., 2018). According to the Codex Alimentarius and European regulation the limits for moisture content is fixed at ≤20%. However, some honey types such as calluna, erica arborea, clover honey may exceed the limit (Codex Alimentarius, 2001; EU Regulation, 2001). Among other factors, this moisture amount is not constant and also depends on to the hygroscopicity of the honey, and the value may change according to the air humidity (Pavlova et al, 2018). The water content is associated with microbiological stability and the resistance to spoilage by yeast fermentation. Normally, honey with a high water content is more exposed to the growth of microbes microbial ing during storage (Pita-Calvo et al., 2017; Saxena et al., 2010). Fermentation processes can be avoided at value less than 17.1% water content (Conti, 2000). Additionally, moisture content can influence several physical characteristics of honey such as crystallization, viscosity, rheological behavior, and even flavor, specific gravity and weight, maturity, solubility, appearance, color, taste (Escuredo et al., 2014; Pavlova et al., 2018; Persano Oddo & Piro, 2004).

Traditionally, moisture content is commonly determined by refractometric methods (Bogdanov et al., 2004). However, more recently this parameter has determined by spectroscopic methods such as near-infrared (NIR) and mid-infrared (MIR), Fourier transform near-infrared (FT-NIR) and Fourier transform near-infrared with attenuated total reflectance (FTIR-ATR) (Pita-Calvo et al., 2017).

Most of the total soluble solids (TSS) in honey are carbohydrates (Krishnasree & Ukkuru, 2017). In many food products, total soluble solids are the main quality parameters which specify the sweetness of fresh and processed foodstuff. Total soluble solids are routinely determined using refractometer where the refractive index increases with solid content in food. The TSS data are reported as “degrees Brix” (°Brix). These data represents the percentage of the dry substance content of a pure aqueous solutions containing mainly sucrose (Magwaza & Opara, 2015).

Several surveys on honey have established that carbohydrates, mainly fructose and glucose, are the primarily constituent of the TSS. Generally, these values account for approximately 65% to 80% of solids by weight (Arias et al., 2003; Da Costa Leite et al., 2000; Doner, 1977). The general provision, in accordance with the compositional criteria of honey, require that the sum of fructose

and glucose content is more than 60% for blossom or nectar honey, and must exceed 45% for honeydew honey (EU Regulation, 2001).

Over recent years, the physicochemical characteristics of honey have been studied extensively. Several investigation on the use of parameters such as pH and moisture content, have been carried out in different honey produced in many countries around the world (Alves et al., 2013; Khalil et al., 2012; Meda et al., 2005; Nweze et al., 2017; Oroian et al., 2016; Tesfaye et al., 2016), for their characterization (Aazza et al., 2013; Di Rosa et al., 2019; Kivrak et al., 2017; Mondragón-Cortez et al., 2013), to assess the quality (Conti, 2000; Krishnasree & Ukkuru, 2017; Prica et al., 2014), and in association with other chemical or biochemical characteristic (Azeredo et al., 2003; Chua et al., 2012; de Sousa et al., 2016; Feás et al., 2010; Gomes et al., 2010; Saxena et al., 2010; Terrab et al., 2004; Tornuk et al., 2013). In a number of studies the total soluble solids were conducted on honeys from different countries (Khalil et al., 2012; Krishnasree & Ukkuru, 2017; Nyau et al., 2013).

The aim of this chapter is to explore the quality of different Italian honey types by measuring some of the physicochemical parameters related to its quality such as pH, moisture content, and total soluble solids (°Brix index). Honey samples with diverse botanical origin (floral nectar and honeydew), produced and harvested in Trentino Alto-Adige region (northeastern of Italy) were examined.

A statistical analysis was carried out to underline if there is any relation between physico-chemical parameters and the floral origin of the examined honeys.

2.2 Materials and methods

2.2.1 Honey samples

The study was conducted on forty-eight Italian honey samples of different botanical origin. The honeys were provided by local association of apiarists or from directly sampling on the farms of beekeepers. Samples come from different geographical areas of Trentino Alto-Adige (northeastern Italy), and were harvested between March 2017 and July 2018. The honey samples consisted of 23 multifloral (M), 4 acacia (A), 3 apple-dandelion (AD), 7 rhododendron (R), 4 honeydew (HD), and 7 chestnut (C). Table 2.1, reports all of characteristics of the investigated honeys such as sample code, floral and geographical origin, as well as the harvest year.

The sampling procedure ensures that the sample was representative of the honey lot and follows the recommendation of the International Honey Commission (IHC). In order to reduce the possible

external alteration and contamination the procedures of sampling, manipulation, and storage have been strictly observed. All honey samples were sampled in a hygenic and humidity-controlled laboratory. To avoid the alteration of the honey, the sampling was conducted using the homogenization of the honey and carefully stirred to reduce as much as possible the presence of air in the honey. All honey samples were collected in glass jars previously cleaned by immersion for about two hours using a laboratory detergent solution and then rinsed with ultra-pure water. The collected honey samples were then stored at +4 °C in a dark location until the analysis.

Table 2.1 Descriptive characteristics of honey samples analysed.

Multifloral honey				Monofloral and Honeydew			
Sample code	**Botanical origin**	**Geographical origin**	**Harvest year**	**Sample code**	**Botanical origin**	**Geographical origin**	**Harvest year**
M36	Multifloral	Val di Fiemme	2017	A1-18	Acacia	Valsugana	2018
M37	Multifloral	Val di Fiemme	2017	A11-18	Acacia	Valsugana	2018
M38	Multifloral	Val di Fiemme	2017	A22-18	Acacia	Val d'Adige	2018
M39	Multifloral	Val di Fiemme	2017	A28-18	Acacia	Val di Non	2018
M41	Multifloral	Val di Cembra	2017	AD43	Apple-dande-lion	Val di Non	2018
M42	Multifloral	Val di Fassa	2017	AD45	Apple-dande-lion	Val d'Adige	2017
M44	Multifloral	Val di Fiemme	2017	AD25-18	Apple-dande-lion	Val di Non	2018
M3-18	Multifloral	Val di Non	2018	R2-18	Rhododendron	Valsugana	2018
M5-18	Multifloral	Val di Non	2018	R4-18	Rhododendron	Val di Non	2018
M6-18	Multifloral	Val di Non	2018	R14-18	Rhododendron	Val di Fiemme	2018
M7-18	Multifloral	Valsugana	2018	R17-18	Rhododendron	Val di Fiemme	2018
M9-18	Multifloral	Valsugana	2018	R18-18	Rhododendron	Val di Fiemme	2018
M16-18	Multifloral	Val di Fiemme	2018	R24-18	Rhododendron	Valsugana	2018
M19-18	Multifloral	Val di Fiemme	2018	R27-18	Rhododendron	Val di Non	2018
M20-18	Multifloral	Val di Fiemme	2018	HD10-18	Honeydew	Val di Non	2018
M21-18	Multifloral	Val di Fiemme	2018	HD15-18	Honeydew	Val di Fiemme	2018
M23-18	Multifloral	Valsugana	2018	HD26-18	Honeydew	Val di Non	2018
M46-C	Multifloral	Val d'Adige	2018	HD29-18	Honeydew	Val di Non	2018
M46-D	Multifloral	Val d'Adige	2018	C8-18	Chestnut	Valsugana	2018
M46-P	Multifloral	Val d'Adige	2018	C12-18	Chestnut	Valsugana	2018
M47-C	Multifloral	Val d'Adige	2018	C13-18	Chestnut	Valsugana	2018
M47-D	Multifloral	Val d'Adige	2018	C40	Chestnut	Val di Fiemme	2017
M47-P	Multifloral	Val d'Adige	2018	C52-C	Chestnut	Val d'Adige	2018
				C52-D	Chestnut	Val d'Adige	2018
				C52-P	Chestnut	Val d'Adige	2018

2.2.2 Melissopalynological analysis

The botanical origin of the Italian honey samples was established using a melissopalynological analysis according to the method described by Louveaux et al., (1978).

Briefly, 10 grams of each honey was directly weighted into centrifuge tubes with conical ends, and then dissolved with 20 mL of hot ultrapure water with temperatures less than 40°C. The honey solution was centrifuged for 15 minutes at 3000 rpm. The supernatant was discarded, and the deposit was washed and centrifuged again with 10 mL of ultrapure water to remove the sugars. The washed deposit was spread and accurately distributed on a slide, and then dried at less than 40°C, and finally covered with solution of glycerin-gelatine. The obtained pollen grains were identified and counted using a microscope with a magnification of 320-450 X e 800-1000 X. Counting of the pollen grains was conducted on a square of 18x18 mm. The results were expressed according to the pollen grain frequencies, and as a percentage of the pollen present in each honey.

2.2.3 Physicochemical determination

Physicochemical parameters of honey samples, pH, moisture content (%), and total solid soluble (°Brix), were determined according to the recommendations based on sample preparation by the International Honey Commission (Bogdanov, 2009). Before analysis all liquid honey without extraneous matter was homogenized by stirring for at least three minutes. Crystallized honey was softened as needed by heating in a thermostatic bath at temperatures no more than 40°C, and then homogenized by stirring for at least three minutes. Each sample was measured in triplicate.

2.2.3.1 pH

The pH value of all honey samples was measured according to the AOAC Official Method 962.19, 1990 (AOAC, 1990) reported in previous work (Chua et al., 2012). Briefly, five grams of each sample were accurately weighed and then diluted with 20 mL of ultrapure water and, mixed well before measurements. The pH was determined using a digital pH meter which was calibrated before each session of analysis at a controlled temperature. Mass was determined using a Mettler Toledo MA235 (UK).

2.2.3.2 Moisture content and total soluble solids (°Brix)

The moisture content and the total soluble solids were determined by a refractometric method. One hand refractometer (RETK-73, Tecknoplus Ltd, China) equipped with automatic temperature compensation (ATC) was employed as a simple and fast instrument for the determination of the content of water and total soluble solids of each honey. The refractometer was appropriately calibrated with a calibration solution before each analyses session. A drop of the homogenized sample was placed on the surface of the refractometer prism and values were obtained directly from the instrument scale, which was expressed as a percentage.

2.3 Statistical analysis

Statistical analyses were performed using OrigionPro Statistical Software, Version 10 (OriginLab Corporation, Northampton, MA, U.S.A.).

Physicochemical parameter data were subject to a correlation analysis to highlight any possible relationship with the botanical origin and inter-annual variability. The Hierarchical Cluster Analysis was used to emphasize grouping in relation with sample botanical origin and inter-annual production. Ward's method and Euclidean were used as cluster method and distance type, respectively.

2.4 Results and discussion

2.4.1 Melissopalynological result

The botanical origin of the honey samples was established and confirmed by melissopalynological analyses according to the method proposed by Louveaux et al., (1978). Table 2.2, shown the results of melissopalynological analysis (performed by an expert) on the investigated honey samples.

The results of the melissopalynological analysis by this method estimates the percentage of the pollen frequencies and classifies pollen in the following classes for nectar honeys: the predominant pollen (>45% of the pollen grains counted), the accompanying and secondary pollen (between 16-45%), the important minor pollen elements (3-15%), and finally the minor pollen (<3%).

The ratio between "honeydew elements (HDE) and the total frequency of pollen from nectar (P)" classifies the honeydew honey. Thus, when the ratio (HDE/P) is more than 4.50 the product is classifies the honeydew honey. For our purpose, only the predominant pollen and secondary pollen were used to confirm and classify the botanical origin of the investigated honey sample.

Table 2.2 Characteristics of melissopalynological analysis on the investigated floral honey samples; principal and/or accompanying pollen, and important minor pollen types.

Sample	Floral type	Principal pollen (>45%) and/or Accompanying important pollen (15-45%)	Important minor pollen (3-15%)
M36	Multifloral	Ericaceae (42.5%) Salicaceae Salix (38.0%)	Hippocastanaceae Aesculus (5.7%) Rosaceae Malus/Pyrus (3.8%) Asteraceae T-Form (3.5%)
M37		Ericaceae (36.5%)	Trifolium repens (12.8%) Asteraceae T-Form (9.1%) Rosaceae Rubus (6.8%) Apiaceae (5.7%) Rosaceae (4.3%) Ranunculaceae Clematis (3.1%)
M38		Ericaceae (32.6%) Rosaceae Rubus (16.0%)	Asteraceae A-Form (9.6%) Buddleja (8.1%) Trifolium pretense (4.2%) Trifolium repens (3.7%) Asteraceae T-Form (3.2%) Lauraceae (3.2%) Rosaceae Malus/Pyrus (3.0%)
M39		Ericaceae (62.8%)	Fagaceae Castanea (12.1%) Apiaceae A/H-Form (4.0%) Ranunculaceae Clematis (3.5%) Boraginaceae Echium (3.3%) Trifolium repens (3.3%)
M41		Ericaceae (55.6%)	Apiaceae A/H-Form (8.8%) Asteraceae T-Form (3.8%) Rosaceae Rubus (3.8%) Salicaceae Salix (3.2%) Scrophulariaceae Rhinanthus (3.2%)
M42		Ericaceae (35.5%)	Apiaceae A/H-Form (10.9%) Rosaceae Rubus (8.1%) Trifolium repens (7.3%) Rosaceae (5.5%) Fabaceae Lotus (4.5%) Fagopyrum esculentum (3.3%) Scrophulariaceae Rhinanthus (3.1%)
M44		Fagaceae Castanea (81.1%)	Salicaceae Salix (10.2%) Rosaceae Malus/Pyrus (3.0%)
M3-18		Ericaceae (70.9%)	Rubuaceae (8.7%) Trifolium repens (4.8%)
M5-18		Fagaceae Castaneae (81.1%)	Vitaceae Parthenocissus (3.0%)
M6-18		Fagaceae Castaneae (81.1%)	Asteraceae A-Form (4.1%)
M7-18		Fagaceae Castaneae (78.4%)	Ericaceae (14.1%) Tiliaceae Tilia (3.1%)
M9-18		Fagaceae Castaneae (75.6%)	Tiliaceae Tilia (3.1%)
M16-18		Ericaceae (55.5%)	Rosaceae Rubus (5.7%) Rosaceae Fragaria-Potentilla (4.8%) Tiliaceae Tilia (4.4%) Ranunculaceae Clematis (3.5%) Scrophulariaceae Rhinanthus (3.5%)
M19-18		Ericaceae (25.3%)	Rosaceae Rubus (13.6%)

Sample	Floral type	Principal pollen (>45%) and/or Accompanying important pollen (15-45%)	Important minor pollen (3-15%)
			Fabaceae Onobrychis (9.6%) Salicaceae Salix (9.6%) Ranunculaceae Clematis (7.1%) Rosaceae Malus/Pyrus (5.1%) Apiaceae (4.0%)
M20-18		Ericaceae (80.4%)	Rosaceae Rubus (11.1%)
M21-18		Ericaceae (80.1%)	Rosaceae Rubus (5.7%) Tripholium Repens (4.1%)
M23-18		Fagaceae Castaneae (73.7%)	Tiliaceae Tilia (16.5%) Rosaceae Rubus (3.4%)
M46 C		Castanea Sativa (19.9%) Tripholium Repens (19.2%) Scrophulariaceae Verbascum (18.5%)	Rosaceae Rubus (11.1%) Rosaceae (4.5%) Clematis (4.2%) Rubiaceae (3.8%) Robinia (3.5%)
M46-P		Fagaceae Castaneae (19.3%) Scrophulariaceae Verbascum (17.9%) Tripholium Repens (15.2%)	Rosaceae Rubus (10.8%) Scrophulariaceae Rhinanthus (6.1%) Rosaceae (4.4%) Caesalpiniaceae Gleditsia (4.1%) Rubiaceae (3.7%) Fagaceae Robinia (3.4%)
M46-D		Rosaceae Rubus (21.3%) Fagaceae Castaneae (18.4%)	Scrophulariaceae Rhinanthus (12.5%) Tripholium Repens (11.4%) Ranunculaceae Clematis (8.1%) Fabaceae Robinia (4.4%) Cornaceae Cornus (4.0%) Rosaceae Malus/Pyrus (3.7%) Rubiaceae (3.7%) Salicaceae Salix (3.3%)
M47-C		Fagaceae Castaneae (41.2%)	Rosaceae Malus/Pyrus (11.1%) Salicaceae Salix (6.8%) Fabaceae Robinia (5.7%) Rosaceae Prunus (4.6%) Cruciferae (4.3%) Rubiaceae (4.1%) Rosaceae (3.8%) Asteraceae T-Form (3.3%)
M47-P		Fagaceae Castaneae (41.3%)	Rosaceae Prunus (8.6%) Rosaceae Malus/Pyrus (7.6%) Rosaceae Rubus (6.3%) Rhamnaceae (5.8%) Rubiaceae (4.6%) Cruciferae (3.5%) Asteraceae T-Form (3.3%)
M47-D		Fagaceae Castaneae (50.4%)	Rosaceae Malus/Pyrus (6.9%) Rhamnaceae (6.6%) Rosaceae Rubus (6.4%) Rosaceae Prunus (5.6%) Rubiaceae (3.7%) Fabaceae Robinia (3.4%)
A1-18	Acacia		Fabaceae Robinia (14.0%) Rosaceae Rubus (4.5%) Rosaceae (3.7%)

Sample	Floral type	Principal pollen (>45%) and/or Accompanying important pollen (15-45%)	Important minor pollen (3-15%)
A11-18		Fagaceae Castaneae (46.6%) Fabaceae Robinia (17.2%)	Rosaceae Rubus (7.8%) Scrophulariaceae Rhinanthus (5.0%) Rhamnaceae (3.8%) Tripholium Repens (3.4%)
A22-18		Fagaceae Castaneae (65.2%) Ericaceae (31.3%)	
A28-18		Fabaceae Robinia (19.8%) Rosaceae Rubus (19.4%) Rosaceae Malus/Pyrus (19.0%)	Fagaceae Castanea (14.1%) Ebenaceae Diospyros (4.6%)
AD43	Apple-Dande-lion	Fagaceae Castanea (32.2%) Asteraceae T-Form (23%) Salicaceae Salix (19.7%) Rosaceae Malus/Pyrus (18.1%)	Absent
AD45		Fagaceae Castanea (49.5%)	Asteraceae T-Form (14.0%) Rosaceae Malus/Pyrus (12.1%) Hydrophyllaceae Phacelia (3.8%) Simaroubaceae Ailanthus (3.3%) Vitaceae Parthenocissus (3.0%)
AD25-18		Rosaceae Malus/Pyrus (22.6%)	Vitaceae Parthenocissus (12.5%) Fabaceae Robinia (11.9%) Rosaceae Rubus (11.6%) Arecaceae Capmerops (9.8%) Hippocastanaceae Aesculus (4.2%)
R2-18	Rhododendron	Fagaceae Castanea (64.2%) Ericaceae (31.3%)	Absent
R4-18		Ericaceae (87.1%)	Fagaceae Castanea (6.5%)
R14-18		Ericaceae (84.6%)	Rosaceae Rubus (5.5%) Fagaceae Castanea (4.4%)
R17-18		Ericaceae (82.6%)	Rosaceae Rubus (3.7%)
R18-18		Ericaceae (91.0%)	Campanulaceae (3.9%)
R24-18		Ericaceae (91.1%)	Fagaceae Castanea (7.2%)
R27-18		Ericaceae (69.6%) Fagaceae Castanea (23.0%)	Absent
HD10-18	Honeydew	Honeydwe elements (HDE/P=77)*	Absent
HD15-18		Honeydwe elements (HDE/P=42)*	Absent
HD26-18		Honeydwe elements (HDE/P=450)*	Absent
HD29-18		Honeydwe elements (HDE/P=434)*	Absent
C40	Chestnut	Fagaceae Castanea (90.0%)	Absent
C8-18		Fagaceae Castanea (98.0%)	Absent
C12-18		Fagaceae Castanea (97.8%)	Absent
C13-18		Fagaceae Castanea (91.7%)	Absent

Sample	Floral type	Principal pollen (>45%) and/or Accompanying important pollen (15-45%)	Important minor pollen (3-15%)
C52-C		Fagaceae Castanea (97.0%)	Absent
C52-P		Fagaceae Castanea (96.4%)	Absent
C52-D		Fagaceae Castanea (95.1%)	Absent

Multifloral honey, which is normally produced by several nectar plants, shows a wide variability in pollen content. The pollen spectra reflects the principal plants and floral present in the studied Trentino Alto-Adige area. The monofloral samples were classified according to their floral origin in five monofloral honeys: acacia, apple-dandelion, rhododendron, honeydew and chestnut honey. In accordance with the requirements of the corresponding percentage of pollen, the percentage in monofloral nectar honey assume specific values. The pollen of *Robinia* is under-represented and the mean percentage can vary between 20% and 30%. In contrast, *Castanea* pollen is usually over-represented, so only honeys that contain at least 90% of *Castanea* pollen can be classified as chestnut honey. Rhododendron honeys require between 30% and 60% of *Ericaceae* pollen to be considered monofloral honey (Louveaux et al., 1978). Dandelion honeys is characterize a low percentage of pollen, from 5% and 15%. However Italian dandelion honey is often contaminated with *Salicaeae* (Persano Oddo et al., 1995).

The main typical range of pollen identified by melissopalynological analysis in our study was from 14.0% to 19.8% for *Robinia* (acacia honey), between 12.1% to 18.1% for *Malus/Pyrus*, from 14.0% to 23.0% for *Asteraceae* (apple-dandelion), from 31.3% to 91.1% for *Ericacea*e (rhododendron honey), and between 90.0% to 98.0% for *Castanea* (chestnut honey). All honeydew honey had HDE/P ratios higher than 4.50, and range between 42 and 450. Fig. 2.1 shows the photomicrographs of pollen grains found in the analysed honey samples.

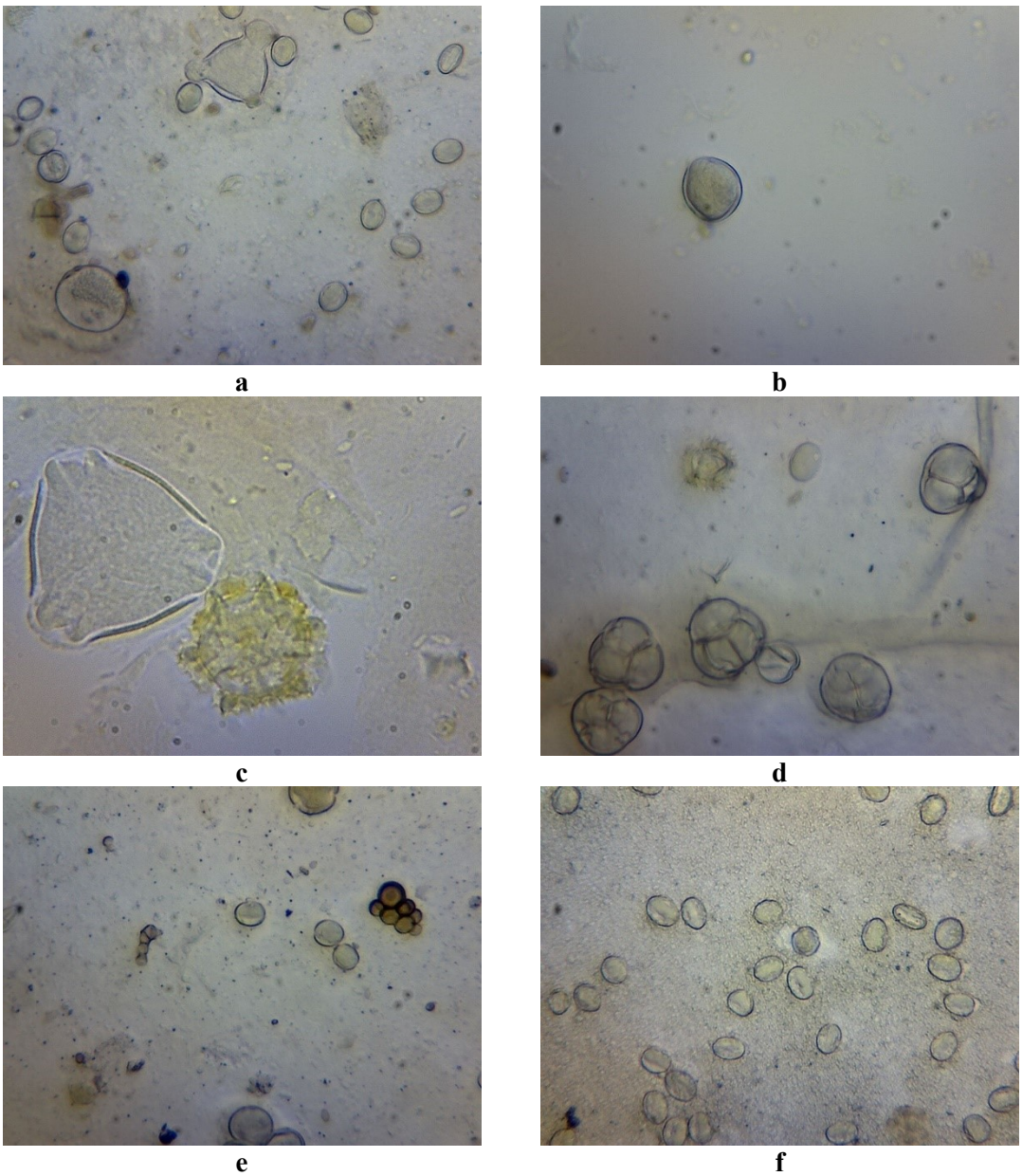

Fig. 2.1 Photomicrographs of pollen grains found in Italian honey samples. Multifloral (a), acacia (b), apple-dandelion (c), rhododendron (d), honeydew (e), and chestnut (f). Scale bars -10 μm.

2.4.2 Physicochemical results

Both European and International legislation (Directive 2001/110/EC and Codex Alimentarius, 2001) report the physicochemical criteria which describe the main quality parameters of honey (Codex Alimentarius, 2001; EU Regulation, 2001).

Table 2.3summarizes the data obtained for pH, moisture content (%) and total soluble solids (°Brix) for the forty-eight honey samples examined. The data reported as mean value and their associated standard deviation.

Table 2.3 Physiochemical properties: pH, total soluble solids (°Brix %), and moisture content (%) in honey sam-ples analyzed with different floral origin. Data are based on three replicates of each sample (n=3) and are expressed as average ± SD.

Sample	Floral type	pH (mean ± SD)	Moisture content (%) (mean ± SD)	°Brix (%) (mean ± SD)
M36	Multifloral	4.38 ± 0.00	19.2 ± 0.3	79.0 ± 0.0
M37		4.26 ± 0.01	15.5 ± 0.0	83.0 ± 0.0
M38		4.22 ± 0.01	16.7 ± 0.3	81.5 ± 0.0
M39		4.50 ± 0.02	15.0 ± 0.0	83.5 ± 0.0
M41		4.48 ± 0.01	14.8 ± 0.3	84.0 ± 0.0
M42		4.46 ± 0.01	15.5 ± 0.0	83.0 ± 0.0
M44		4.24 ± 0.01	13.5 ± 0.0	85.0 ± 0.0
M3-18		3.94 ± 0.01	17.2 ± 0.3	81.0 ± 0.0
M5-18		4.43 ± 0.01	16.0 ± 0.0	82.2 ± 0.3
M6-18		4.70 ± 0.00	18.7 ± 0.1	79.7 ± 0.3
M7-18		3.93 ± 0.01	17.0 ± 0.0	81.5 ± 0.0
M9-18		3.72 ± 0.01	17.0 ± 0.0	81.0 ± 0.0
M16-18		4.17 ± 0.01	17.0 ± 0.0	81.5 ± 0.0
M19-18		4.06 ± 0.00	17.0 ± 0.0	81.1 ± 0.1
M20-18		3.84 ± 0.00	18.0 ± 0.0	80.5 ± 0.0
M21-18		4.02 ± 0.00	17.5 ± 0.0	81.0 ± 0.0
M23-18		4.16 ± 0.01	18.0 ± 0.0	80.5 ± 0.0
M46-C		3.41 ± 0.03	17.7 ± 0.3	80.8 ± 0.3
M46-P		3.39 ± 0.03	17.3 ± 0.3	80.7 ± 0.1
M46-D		3.51 ± 0.02	18.0 ± 0.0	80.3 ± 0.3
M47-C		3.70 ± 0.01	16.8 ± 0.3	82.5 ± 0.1
M47-P		3.71 ± 0.01	16.8 ± 0.6	82.3 ± 0.3
M47-D		3.80 ± 0.01	17.0 ± 0.5	82.3 ± 0.3
Mean value		***4.04 ± 0.01***	***16.8 ± 0.1***	***81.6 ± 0.1***
A1-18	Acacia	3.58 ± 0.00	16.8 ± 0.3	81.7 ± 0.3
A11-18		3.25 ± 0.00	19.0 ± 0.0	79.0 ± 0.0
A22-18		3.30 ± 0.00	17.0 ± 0.0	81.5 ± 0.0
H28-18		3.50 ± 0.00	17.0 ± 0.0	81.5 ± 0.0
Mean value		***3.50 ± 0.00***	***17.0 ± 0.0***	***81.5 ± 0.0***
AD43	Apple-Dandelion	3.97 ± 0.01	14.0 ± 0.0	84.5 ± 0.0
AD45		4.12 ± 0.01	12.0 ± 0.0	86.5 ± 0.0
AD25-18		3.81 ± 0.01	18.5 ± 0.0	80.0 ± 0.0
Mean value		***3.97 ± 0.01***	***14.8 ± 0.0***	***83.6 ± 0.0***
R2-18	Rhododendron	3.42 ± 0.01	16.5 ± 0.0	82.0 ± 0.0
R4-18		3.54 ± 0.01	17.0 ± 0.0	81.3 ± 0.3
R14-18		3.59 ± 0.01	17.5 ± 0.0	81.0 ± 0.0
R17-18		3.32 ± 0.01	15.8 ± 0.3	82.5 ± 0.0
R18-18		3.30 ± 0.01	18.0 ± 0.0	80.0 ± 0.0
R24-18		3.21 ± 0.00	18.8 ± 0.0	79.5 ± 0.0
R27-18		3.36 ± 0.00	15.5 ± 0.0	83.0 ± 0.0
Mean value		***3.39 ± 0.01***	***17.0 ± 0.0***	***81.3 ± 0.0***
HD10-18	Honeydew	4.31 ± 0.01	17.0 ± 0.0	81.0 ± 0.0
HD15-18		4.26 ± 0.01	17.0 ± 0.0	81.5 ± 0.0
HD26-18		5.08 ± 0.01	14.0 ± 0.0	84.5 ± 0.0
HD29-18		4.95 ± 0.00	17.0 ± 0.0	81.5 ± 0.0
Mean value		***4.65 ± 0.01***	***16.2 ± 0.0***	***82.1 ± 0.0***
C40	Chestnut	4.44 ± 0.01	15.0 ± 0.0	83.2 ± 0.3
C8-18		5.04 ± 0.01	18.5 ± 0.0	80.0 ± 0.0
C12-18		4.37 ± 0.01	16.5 ± 0.0	82.0 ± 0.0
C13-18		4.09 ± 0.01	16.5 ± 0.0	82.0 ± 0.0
C52-C		4.23 ± 0.01	16.5 ± 0.0	82.0 ± 0.0
C52-P		4.26 ± 0.01	16.5 ± 0.0	82.0 ± 0.0

Sample	Floral type	pH (mean ± SD)	Moisture content (%) (mean ± SD)	°Brix (%) (mean ± SD)
C52-D		4.16 ± 0.01	17.0 ± 0.0	81.5 ± 0.0
Mean value		***4.37 ± 0.01***	***16.6 ± 0.0***	***81.8 ± 0.0***

2.4.2.1 pH value

Honey is an acidic food matrix (Bogdanov, 2011a) whose acidity is naturally related to the presence of organic acids (Khalil et al., 2012). Gluconic acid is the main significant organic acid represented in honey samples. However, in honey produced in various regions around the world other organic acids are also found such as aspartic, butyric, citric, acetic, formic, fumaric, galacturonic, formic, glutamic, glutaric, butyric and some others (Da Silva et al., 2016). Generally, these organic acids derive from the enzymatic activity of glucose oxidase which the honeybees produce during ripening time(Karabagias et al., 2014a). Organic acids profile are useful tools to differentiate between the honeys according to their botanical and geographical origin. The acid composition, in addition to pH and electrical conductivity, is related to flavour and color (Mato et al., 2006). The pH of nectar or blossom honeys can range between 3.3 to 4.6, with an average value of 3.9 (Bogdanov, 2011b).

Table 2.3 reports the pH values where the mean values for blossom honey investigated in this work are 4.04 for multifloral, 3.50 for acacia, 3.97 for apple-dandelion, 3.39 for rhododendron, and 4.37 for chestnut. The value for honeydew honey was 4.65. In Fig. 2.2 reports the box and whisker plot of pH valuse of different floral honey.

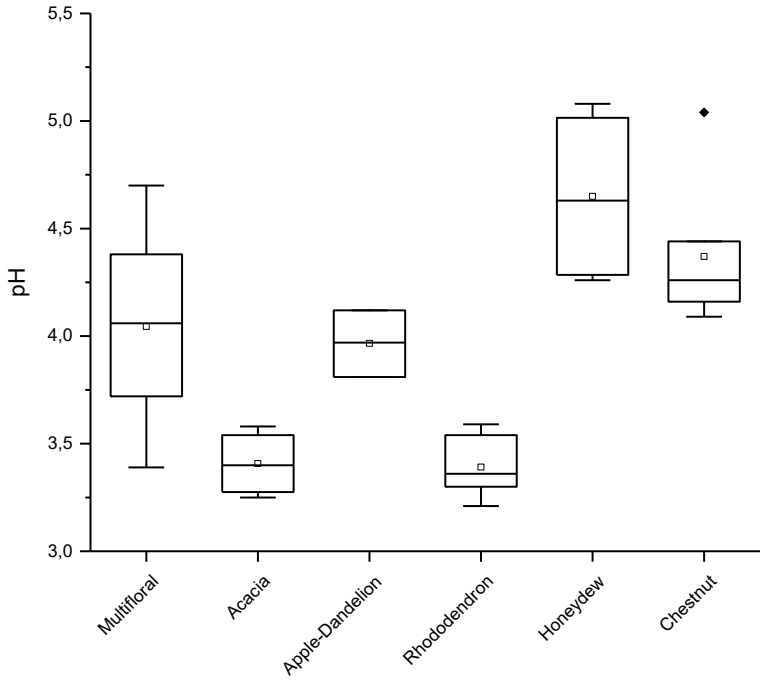

Fig. 2.2 pH value by floral honey.

The mean pH value for multifloral honey in this work (4.04) is in accordance with reported pH values for multifloral honey studied in European countries. In two regions of Portugal, the Luso region and northwest of Portugal, ranged between 3.55 and 4.34 and from 3.5 to 4.2, respectively (Feás et al., 2010; Silva et al., 2009). The pH values are also comparable to those obtained in polyfloral honey from northeastern Romania, where the mean value was 4.0 (Oroian et al., 2016). pH values observed in countries around the world have similar values to our results. For instance, multifloral honey from Brazil and from different regions of Burkina Faso, have mean values of 4.02 and 4.0 respectively (Meda et al., 2005; Oliveira et al., 2014). The results obtained from polifloral Cuban honeys were slightly higher than above mentioned results where the mean value was 4.76 (Alvarez-suarez et al., 2018).

The mean value of pH found for the acacia honey was 3.50, and is comparable to values reported in previous studies carried out on honey with the same floral origin from Malaysia (3.21). Different regions of Burkina Faso (3.6) and European acacia honey (3.7 to 4.1) have values slightly above our reported means (Chua et al., 2012; Meda et al., 2005; Rouff et al., 2007). One study conducted on acacia honey from Pakistan resports an average pH value of 4.37, which is moderately less than found in the present research (Sajid et al., 2020).

To the best of our knowledge, few literature data are available regarding honey produced by apple and dandelion floral source. However, our mean value (3.97) was close to the results published in a study conducted on apple honey from the Kashmir valley of India (4.35) (Ahmad et al., 2019). An Italian survey conducted on dandelion honeys by Persano Oddo et al., (1995) reported one higher pH value (4.5) than those obtained by our study.

The pH values of dandelion honeys from Central Europe (Switzerland, Germany, and Italy), was indicate to range between 4.2 and 5.0 (Rouff et al., 2007). These data are similar to our results, considering the limited statistical validity of our data set due to the reduced number of samples for this monofloral honey (3 samples). In general, the data of apple-dandelion honey in our work are in agreement with the pH values reported in the literature for the same monofloral honey samples (Ahmad et al., 2019; Persano Oddo et al., 1995).

The mean pH value obtained for rhododendron honey, one monofloral honey produced in the mountains, is the lowest (3.39) in comparison with the other unifloral honeys. Persano Oddo et al.'s, (1995) survey of rhododendron honeys from Italy also reports a mean pH value lower than other monofloral honeys, with a value of 3.9. A range of pH values with a minimum of 3.7 and a maximum of 4.6 for this type of floral honey was reported by Rouff et al., (2007).

Normally, chestnut honey has a relatively high pH value, between 5 to 6, and it is an exception compared with other monofloral honey (Bogdanov, 2011a). However, in our study, the pH value was slightly lower (4.37) than above-mentioned range. Other surveys carried out in Italy reported comparable pH values. A study of chestnut honey from Sicily (Italy) obtained a mean value of 4.89 (Di Rosa et al., 2019). The highest value was reported in an investigation of Italian chestnut honey (5.5), where some chestnut samples had a maximum value of 6.4 (Persano Oddo et al., 1995; Rouff et al., 2007).

The mean pH of honeydew honey was 4.65, higher than the nectar honeys. The pH values detected in our honeydew honey samples were very similar to those shown for honeydew honey studies, in samples from the Czech Republic (4.53), Morocco (4.17), and Poland (4.63) (Čelechovska & Vorlová, 2001; Díez et al., 2004; Ryback-Chmieliwskaet al., 2013). In one study conducted on Greek honeydew the pH ranged from 4.42 to 5.20 (Karabagias et al., 2014a).

The highest pH values in honeydew honeys with mean values of 5.16 and 5.3 were from Italian *Abies* honeydew and in honeydew from northeastern Romania, respectively (Oroian et al., 2016; Persano Oddo et al., 1995). Honeydew honey has an average pH value of 5.2, higher than nectar honeywith a general range from 4.5 to 6.5 (Ajibola et al., 2012; Bogdanov, 2011b).

Finally, the pH values reported in this study are also comparable with data obtained in diverse types of nectar honey from different countries around the world such as Brazil (3.56 to 4.00), Indian (3.7 to 4.4), Algeria (3.70 to 4.00) and Portugal (3.3 to 4.4) (Alves et al., 2013; Azeredo et al., 2003; Khalil et al., 2012; Saxena et al., 2010).

2.4.2.2 Moisture content

Water is the second most abundant compound present in honey and encompasses15 to 23% of the total (Pavlova et al., 2018).

The moisture content of honey can depend on different variable such as the geographical and botanical origin of nectar, climatic and environmental conditions, degree of maturity achieved in the hive, manipulation by beekeepers during the harvest, as well as storage conditions and processing techniques of the honey (Conti, 2000; Da Silva et al., 2016; De-melo et al., 2018; Ojeda de Rodríguez et al., 2004; Terrab et al., 2004). Additionally, moisture content is widely related to the harvest season (Karabagias et al., 2014b).

In some types of honey high amount of water may lead to an acceleration in the crystallization process (Gomes et al., 2010). The moisture content influences the commercial value of honey (Escuredo et al., 2013).

The water content can affect the fermentation processes that can be produced from yeasts, the capacity of honey to inhibit the growth of yeasts and prevent the deterioration of honey is reduced when there is an increase in water (Pita-Calvo et al., 2017). Moisture content higher than 18% could be responsible for undesirable fermentation activity that take place during honey storage, caused by the action of osmotic yeasts (Escuredo et al., 2013). The fermentation processes in honey is practically avoided if the moisture content value is lower than 17.1% (Conti, 2000). However, in some honey types the water content can exceeds 20%, for example heather, clover and strawberry honeys (Persano-Oddo et al., 1995; Thrasyvoulou et al., 2018). In the honey samples of the present study, moistures range between 12.0% to 19.2%, (Table 2.3).

Honey samples exhibit a mean percentage of water content depending on their floral origin: multifloral (16.8%); acacia and rhododendron (17.0%); apple-dandelion (14.8%); honeydew (16.2%); and chestnut (16.6%); as summarized in Table 2.3. Fig. 2.2 reports the box and whisker plot of the moisture content of different botanical Italian honey.

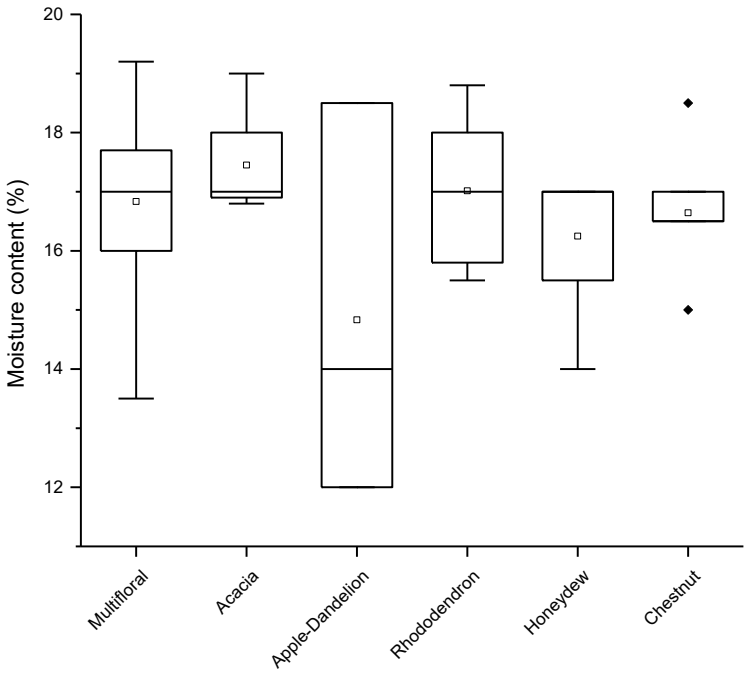

Fig. 2.3 Moisture content of the floral honey.

The mean water content in our multifloral honey samples (16.8%) is practically coincident to honey samples from northeastern Romania and in polyfloral samples from Cuba, that have mean values of 16.83% and 16.74%, respectively (Alvarez-Suarez et al., 2018; Oroian et al., 2016). The water content percentage is also in agreement with honey from different regions of Portugal. The moisture content ranged from 13.98% to 18.90%, central Portugal had a mean value of 17.5% and

the Atlantic coastal had a the mean value of 17.7% (Escuredo et al., 2013; Feás et al., 2010; Silva et al., 2009).

The values were also similar to those reported for polyfloral honey samples from Venezuela (17.80% to 20.40%) and those from different regions of Burkina Faso (16.7% to 20.1%). It should be emphasized that some samples contain a water content higher than the legal limit (Meda et al., 2005; Ojeda de Rodrìguez et al., 2004).

The mean moisture content in acacia honey obtained in our study (17.0%) was comparable to those found in the same floral honey from Eastern Europe (Romania) and honey produced in Saudi Arabia, which showed mean values of 17.28% and 17.32%, respectively (Alqarni et al., 2016; Oroian et al., 2016). A relatively lower value was determined in acacia honey from Serbia (Vojvodina region) which exhibited an average value of 16.3% (Prica et al., 2014).

A higher value is present in a study conducted on fresh and branded acacia honey from Pakistan (18%) (Sajid et al., 2020). Acacia honey from the west coast of Peninsular Malaysia and in Burkina Faso have higher values (20.62% and 21.9%), respectively (Chua et al., 2012; Meda et al., 2005).

The mean moisture value in rhododendron honey samples is similar that of acacia honey, 17.0%. Comparable mean values were found in previous investigations carried out on Italian and Turkish rhododendron honey, 16.6%, and 16.63%, respectively (Persano Oddo et al., 1995; Rasgele & Kekecoglu, 2013). A survey of the main European unifloral honeys showed a mean value of water content in rhododendron samples of about 16.6%, very close to those mentioned here (Persano Oddo and Piro, 2004).

Unfortunately, data from the literature for apple-dandelion honeys is limited, so our results are compared with available data for monofloral honey produced from apple trees or dandelion.

In the present work, apple-dandelion honey contains the lowest average value of moisture content (14.8%). Our results were lower than the percentage of moisture found in apple honeys from India (Kashmir valley), which has a value of 18.82% (Ahmad et al., 2019).

Results reported in previous surveys were similar, where European dandelion honey had an average water content of 16.2%, and Italian dandelion samples had mean water content values of 16.9% (Persano Oddo et al., 1995; Persano Oddo and Piro, 2004).

The honeydew honeys moisture values were similar to those of monofloral and multifloral samples investigated in this work, with a mean value of 16.2%.

The results are in accordance with several investigations carried out in European honeydew such those produced in Czech Republic, Romania, Italy, and Spain where the average values were respectively 15.6%, 16.60%, 16.1%, and 16.9% (Celechovská & Vorlová, 2001; Escuredo et al., 2013; Oroian et al., 2016; Persano Oddo et al., 1995).

According to Diez et al., (2004), honeydew honeys from Morocco showed relatively higher average values (17.20 to 21.01%), except for one group of samples which exhibited the mean value of 16.20%. One study carried out on honeydew from New Zealand reported a value similar to those of Moroccan honeydew, with a mean value of 17.9% (Vanhanen et al., 2011).

The moisture content in chestnut honey had an average value of 16.6%. This water percentage was in line with the value measured in the other investigated monofloral honeys. However, this value was moderately lower than those found in chestnut honey from different geographical areas around the world such as from Italy, different Turkish areas (Giresun, Kastamonu, Artvin, Trabzon and Zonguldak) the western Blacksea region of Turkey, and European Atlantic areas with mean values of 17.4%, 19.70%, 18.05%, and 18%, respectively (Can et al., 2015; Escuredo et al., 2013; Persano Oddo et al., 1995; Rasgele & Kekecoglu, 2013).

As observed, different kinds of honey from various botanical origin may have different moisture contents. For all honey floral types, the data suggested that the values are in accordance with the quality compositional criteria defined from the International regulation which require that moisture value must be ≤20% (EU Regulation, 2001).

According to the results is possible to conclude that the extraction time and procedure, and the maturity of the honey samples during the harvest were adequate. The examined floral honey can be considered stable with regard to fermentation upon storage and with adequate quality.

2.4.2.3 Total soluble solids (°Brix)

The values of Brix are strictly related to the sugar content. Therefore the total soluble solids can be used as a valuable index for testing the quality of honey, including verifying the authenticity or revealing the possible addition of artificial components and/or adulterants (Silva et al., 2009; Terrab et al., 2004). However, the honey quality is not only related to the total sugar content, it must also consider the moisture content, the presence of hydroxymethylfurfural (HMF), acidity, diastase activity, and water-insoluble solids (EU Regualtion, 2001).

The Brix scale is employed in the food industry to estimate the amount of total carbohydrates (Anguebes et al., 2016). Honey is a concentrated solution of carbohydrates, in which glucose and fructose are the most abundant. However, the carbohydrates content also includes numerous oligosaccharides (Escuredo et al., 2013). The main carbohydrates in honey usually represent between 65% to 80% of the total soluble solids (Pita-Calvo et al., 2017).

The results presented in Table 2.3 show that for all botanical types of honey the average total soluble sugars content was greater than 80%, where the minimum and maximum values were registered by rhododendron and apple-dandelion, with mean value of 81.3% and 83.6%, respectively.

Fig. 2.3 reports the box and whisker plot of °Brix index value of different floral types of honey.

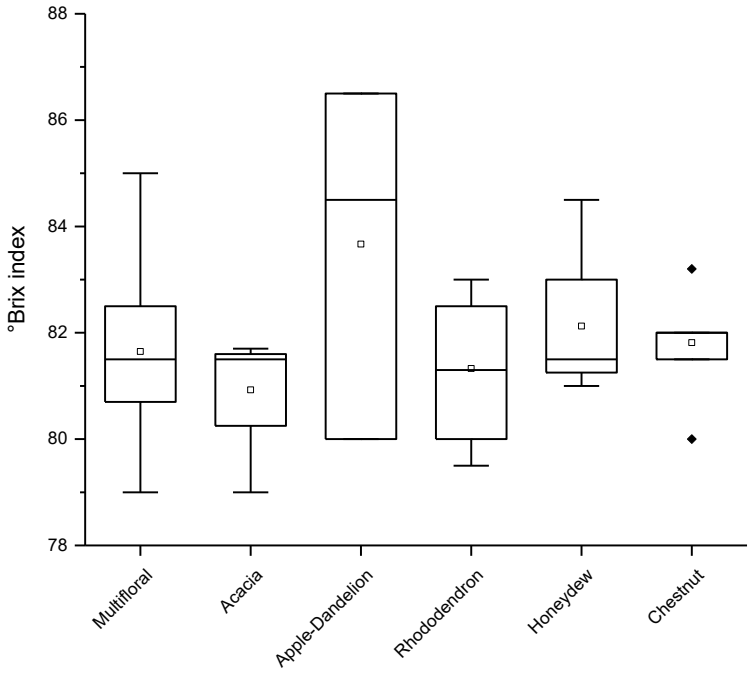

Fig. 2.4 °Brix index value of floral honey.

Data from the literature data on total soluble sugars are limited. However, we were able to compare our results to the few available results available. The average multifloral value obtained in the present work was 81.6%. This data is in agreement with the total soluble sugar found in multifloral honey harvested in eastern Europe (northeastern Romania) which showed a comparable mean value of 81.90% (Oroian et al., 2016). In addition, our total solublgar data were clearly in accordance with wildflower honey harvested in the central Italy, which ranged from 78.6 to 83.0% (Meli et al., 2016).

The mean total soluble solids in acacia honey was 81.5%. In previous investigation on acacia honey produced from individual beekeepers in central Italy (Marche) the authors reported a total soluble sugars of 80.6%, where similar results were produced in the same floral honey from Suceava county (Romania; 80.3%) (Meli et al., 2016; Oroian et al., 2016). According to Alqarni et al., (2016) the mean value of 80.0% was obtained for acacia honey samples, produced in Saudi Arabia.

In our study, apple-dandelion honeys showed the highest mean value, 83.6%, in comparison with other monofloral and multifloral honey. Ahmad et al., (2019) demonstrate that honey samples from apple nectar in the Kashmir valley of India displayed a much lower total sugar content with respect

to our results (78.45%). Unfortunately, no data was found for dandelion honey to compare with our results.

In the present work, the average of total soluble sugars found in rhododendron honeys was an unusual results with a mean of 81.3%. To the best of our knowledge, very few literature data are available about this specific physical parameter on this type of honey. However, it is possible to compare the results with a sample (monofloral, *Erica*) harvested in the Luso region of Portugal. The total sugar was comparable to that exhibited in our samples, with a mean value of 80.3% (Silva et al., 2009).

Honeydew honeys demonstrated a mean value of 82.1% and was the second-highest total sugars data of the present study. Quite similar values were present in samples from the northeastern Romania, mean percentage was 81.90% (Oroian et al., 2016). Honeydew collected by local beekeepers in Marche region (Central) reported a lower value (80.4%) than our study (Meli et al., 2016). However, another study on honeydew samples (mainly produced by *Abies alba*) reports lower total sugars values ranging from 71.6% to 77.9%, with an average value of 75.3% (Rybak-Chmielewska et al., 2013).

Chestnut honey showed a mean value of 81.8%. For the same botanical honey similar values were obtained from two different districts of Portugal, Braga and Bragança, with mean values of 83.07% and 81.50%, respectively (Karabagias et al., 2018).

Finally, all honeys presented in this study have very similar °Brix index values, so we can say that the divers honey has a comparable level of total of carbohydrates. These carbohydrates are mainly constituted from fructose and glucose, and normally account for approximately 65% to 80% of solids by weight. On the base of these observations, considering that European regulation requires that the total quantity of fructose and glucose for nectar honey and honeydew honey must account for more than 60% and 45%, respectively.The honey from our study complies with compositional criteria established from regulation.

2.5 Statistical elaboration

Hierarchical Cluster Analysis was used to explore the relationship among the physico-chemical parameters and floral origin characteristics of monofloral and multifloral Italian honeys, as well as any possible relationship due to the harvested year.

This chemometric approach demonstrates that the Italian honey was distributed and separated into three main clusters, as reported in a Figure 2.4.

The first main cluster (right section of the graph) is constituted by most of the honey samples produced and harvested during 2017 (M37, M39, C40, M41, M42, M44, AD43, AD45), except

for only three honeys harvested during 2018 (R17-18, R27-18, and HD26-18). These samples are characterized by the lowest moisture content amount, between 12.0% and 15.5%, and the highest °Brix index value from 83.0% to 86.5%.

The second and the third cluster were formed mainly by honey samples produced in the 2018 apicultural campaign. The second cluster (left section of the graph) was constituted by honey samples that are characterized by the highest values of moisture content, ranged from 18.0% to 19.2%, and the lowest values of °Brix index, between 79.0% to 80.5%.

The third cluster (the middle one) is represented by the most of Italian honey samples investigated in thisstudy, and comprises the major multifloral honey and monofloral samples, especially acacia, chestnut and honeydew honey. These samples have values ranging from 80.7% and 82.5% for °Brix index, and from 15.5% to 17.7% for moisture content.

According to these results, it is possible to assume that the physicochemical factors, especially moisture content and total soluble solids, seem to relate to the different botanical origins and inter-annual characteristics of the honey.

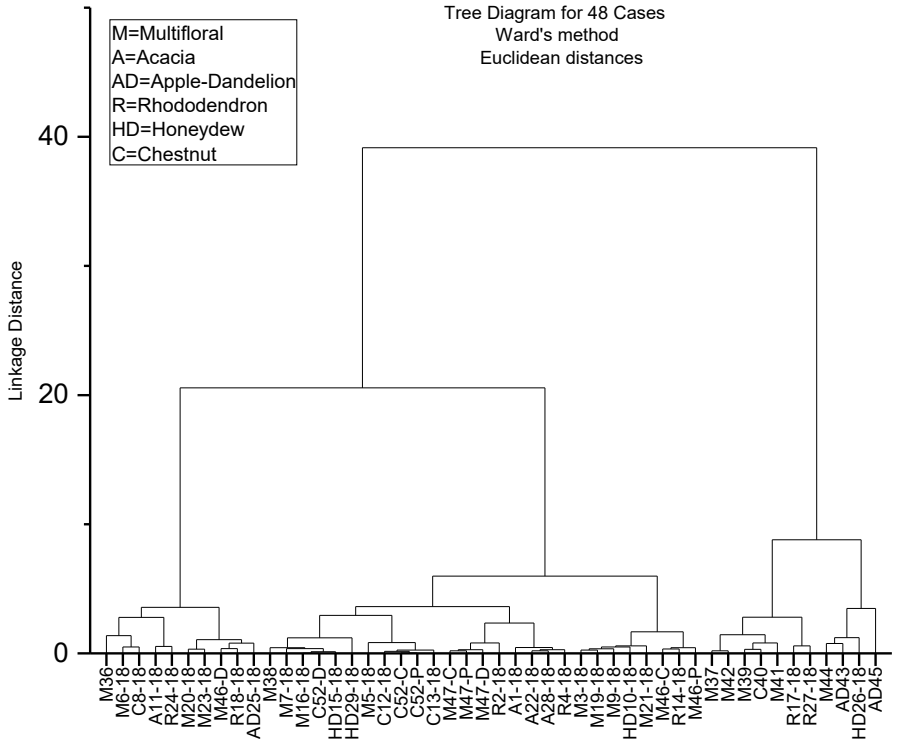

Fig. 2.5 Dendrogram of the Hierarchical Cluster Analysis obtained for 48 Italian honey samples using physicochemical parameters (pH, moisture content and °Brix index).

2.6 Conclusion

Honey is susceptible to several physical and chemical modifications over its time of preservation. Physicochemical parameters are one of the most factors used to assess the quality of honey. These parameters include moisture content, total soluble solids (TSS) or °Brix index, electrical conductivity, ash content, reducing and non-reducing sugars, free acidity, and diastase activity. Quality is defined internationally by the Codex Alimentarius, which establishes the essential quality requirements of honey intended for direct human consumption. Overall values of pH, moisture content, and total soluble solids were in accordance with the literature data and the law limits of European legislation, confirming that the analyzed honeys to comply with the quality requirements.

The statistical results provide information on a possible correlation between physicochemical parameters, such as moisture content and total soluble solids, and the floral origin and inter-annual features of honey samples.

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Chapter 3. Carbohydrate determination in honey samples by ion chromatography-mass spectrometry (HPAEC-MS).

Preface

This chapter summarizes the work of the doctoral activity to determine carbohydrate in honey, a part of which is already published on the journal "*Analytical and Bioanalytical Chemistry*", titled "Carbohydrate determination in honey samples by ion chromatography-mass spectrometry (HPAEC-MS)". The full article is available for more details at the end of this dissertation.

The authorization to use the article for this thesis dissertation has been granted by the journal. This chapter contains a part of the published manuscript (https://doi.org/10.1007/s00216-020-02732-3).

This chapter integrates the already published data set with additional samples of multifloral, chestnut honey, and rhododendron, which were sampled and analysed after the publication of the article. Therefore, the work about the composition of carbohydrates and the elaboration is an extension of the published paper including the whole data set.

3.1 Introduction

In honey, carbohydrates account for about 80% (w/w) of the solids content (Arias et al., 2003). The major monosaccharides in honey are glucose and fructose, where their content ranges from 65% to 85% of total soluble solids (Da Costa Leite et al., 2000; de la Fuente et al., 2011; Ruiz-Matute et al., 2010). The remaining sugars are disaccharides, trisaccharides and tetra-sasccharides present at low concentration, in the majority of honey (Doner, 1977). These oligosaccharides are mainlyformed of glucose and fructose residues linked by glycosidic bonds (Ruiz-Matute et al., 2010). Oligosaccharides are important substances to characterize honeys on the base of both geographical and botanic origin (Bogdanov et al., 2004; Escuredo et al., 2014). Oligosaccharides also significantly contribute to the high nutritional value of honey including as a potential "prebiotic" property (Al-Qassemi & Robinson, 2003; Ouchemoukh et al., 2010).

The content of major sugars in honey as glucose, fructose and sucrose along with the presence of the minor compounds such as di- and trisaccharides have been intensively determined (Escuredo et al., 2014; Da Costa-Leite et al., 2000; Doner, 1977; Pita-Calvo et al., 2017). Moreover, tetrasaccharides, pentasaccharides and hexasaccharides have been also found in some honeydew (Sanz et al., 2005).

Currently, more than 20 oligosaccharides have been identified in different varieties of honey produced in diverse countries around the world (Anjos et al., 2015; Arias et al., 2003; Da Costa Leite et al., 2000; Goodall et al., 1995; Jan Mei et al., 2010; Mateo & Bosch-Reig, 1997; Ouchemoukh et al., 2010; Ruiz-Matute et al., 2010). Oligosaccharides profile has been investigated in honey samples originating in Argentina (Arias et al., 2003), Brazil (Da Costa Leite et al., 2000), Algerian (Ouchemoukh et al., 2010), Spain (de la Fuente et al., 2011), the United Kingdom (Goodall et al., 1995), France (Cotte et al., 2003), and Portugal (Anjos et al., 2015).

Many analytical techniques have been used to determine sugars in honey samples. In literature, carbohydrates are mainly determined by high-performance liquid chromatography (HPLC) coupled with different detectors (Arias et al., 2003; Cano et al., 2006; Ouchemoukh et al., 2010; Weston & Brocklebank, 1999), by gas chromatography coupled with mass spectrometry (GC-MS) (Ruiz-Matute et al., 2007; Terrab et al., 2001) and flame ionization detector (GC-FID) (Cotte et al., 2003; Mateo & Bosch-Reig, 1997; Sanz et al., 2005). The International Honey Commission (IHC) reports HPLC-PAD and GC-FID as the common chromatographic methods for sugar determination (Bogdanov, 2009). However, high-performance anionic exchange chromatography (HPAEC) provides a valuable and powerful analytical tool for the separation of sugars. High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) is the most applied method for the determination of oligosaccharides in honey (Escuredo et al., 2014; Morales et al., 2006; Ouchemoukh et al., 2010). HPAEC-PAD coupled online with single quadrupole mass spectrometry (HPAEC-IPAD-MS) was also used to analyze sugars in chicory coffee, beer and honey (Bruggink et al., 2005).

Recently, nuclear magnetic resonance (NMR) has been used for food analysis. NMR technique has been applied in honey samples analysis for different purposes, such as quantification of carbohydrates and other components (del Campo et al., 2016; Schievano et al., 2017), characterization of monofloral honey (i.e. *Coffea spp.)* (Schievano et al., 2015), determination of botanical and geographical origin (Schievano et al., 2010; Zhang et al., 2020), and differentiation of monofloral and polyflorar honeys through the metabolomic profile (Schievano et al., 2016).

The literature methods are normally applied for the determination of major sugars and few oligosaccharides, however, to better characterize honeys, the quantification of a larger number of oligosaccharides is necessary.

The objective of the present work was to develop and to apply the method to determine carbohydrates, including a significant number of oligosaccharides, in honey using high-performance anion exchange chromatography coupled with mass spectrometry (HPAEC-MS) with a simple pre-analytical procedure, to characterize samples with a different floral origin.

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

3.2 Materials and methods

3.2.1 Reagents and standards

All chemicals substances, sugar standards, and the reagents had a known purity (>98%). D(-)-arabinose, D(+)-glucose, D(-)-fructose, D(+)-xylose, D(+)-mannose, D(-)-ribose, D(+)-glucose(13C), D(+)-galactose, D(+)-lactose, 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, stachyose were supplied by Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Ammonium hydroxide was obtained from Fluka (Sigma Aldrich, Buchs, Switzerland). Ultrapure water (18 MΩ 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).

3.2.2 Sampling and sample preparation

Honey samples with different floral origin were produced on various geographical areas of the Trentino Alto-Adige region, and collected from local beekeepers. The samples were harvested between 2017 and 2018.

A total of 50 honey samples were analysed and encompass: 23 multifloral and 25 monofloral honeys including 4 acacia, 3 apple-dandelion, 7 rhododendron, and 4 honeydew, and 7 chestnut (details are reported in Table 2.1, Chapter 2); 2 Argentinian commercial multifloral honeys (MARG14 and MARG733) were added for comparison (for details see Table S1, the supplementary material of the published paper).

The collected samples were stored at +4°C until analysis. Before analysis, all honey samples were homogenized and weighed (50 mg) into a volumetric flask (50 ml), were subject to the internal standard ($^{13}C_6$-glucose) and finally diluted with ultrapure water.

3.3 Instrumental parameters

Qualitative and quantitative analysis of carbohydrates (monosaccharides and oligosaccharides)

was carried out using ion chromatography coupled to a single quadrupole mass spectrometer.
The chromatographic separations were achieved using CarboPac PA10™ column equipped with a CarboPac PA10™ guard column. Sodium hydroxide (NaOH) was used as an eluent and the separation was carried out in gradient mode (see the full article for details). The injected volume and the flow rate was 25 µL and 0.25 mL min^{-1}, respectively. The mass spectrometer source was an electrospray (ESI) in negative ionization mode, and the data for all sugars were collected in selected ion monitoring (SIM). The Chromeleon 6.8 software was used for the acquisition and elaboration data.

3.4 Statistical analysis

Multivariate statistical techniques were applied to the carbohydrates concentration data to establish possible relationships among the botanical origin or inter-annual variability and sugar composition.
Hierarchical Cluster Analysis and Principal Component Analysis were carried out using the STATISTICA 10.0 software (StatSoft, Inc., 2007, Tulsa, USA). Hierarchical Cluster Analysis used Ward's method and evaluated Squared Euclidean Distance. The Principal Component Analysis was performed to study the relation between carbohydrates profiles and botanical origins of honey

3.5 Result and discussion

3.5.1 Sample preparation and chromatographic optimization

The pre-analytical procedure developed was simple, fast and without the need for expensive steps such as purification or solvent extraction. Briefly, the samples were accurately weighed and diluted with ultrapure water (1:10,000). The proposed procedure is solvent-free as only ultrapure water is required thus reducing the time of sample preparation.
To evaluate the performance of chromatographic separation, some specific chromatographic parameters were calculated: retention time, peak width, asymmetry factor, number of theoretical plates, the height of theoretical plates, and resolution factor of each carbohydrate. Data are reported in Table S2 in the published article.

3.5.2 Quantitative performance of the method

A summary of the monitored and optimized mass parameters used for the quantification (the mass

to charge ratio, $[M-H]^-$), is reported in Table S3 (supplementary material of the published article). Details for the validation of analytical procedure are shown in Table 3.1 whichreports: instrumental precision, instrumental detection and quantification limits method detection and quantification limits, relative standard deviation (RSD %), and trueness (Error %). Details illustrating the chromatographic separation achieved by using one standard solution containing all of the carbohydrates as well as one monofloral honey sample (rhododendron) are available in the published article.

Table 3.1 Validation parameters of the analytical procedure for the carbohydrate quantification: instrumental limit of detection (LOD, mg L^{-1}), instrumental limit of quantification (LOQ, mg L^{-1}), instrumental precision as RSD %, method detection limit (MDL, mg g^{-1}), method quantification limit (MQL, mg g^{-1}) and trueness (Error %). Instrumental detection limits (LOD, mg L^{-1}) reported in the literature.

	This study						LOD (mg L^{-1}) previous studies		
Carbohydrate	**LOD (mg L^{-1})**	**LOQ (mg L^{-1})**	**RSD%**	**MDL (mg g^{-1})**	**MQL (mg g^{-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			
Isomaltotiose	0.02	0.06	8	0.19	0.62				
Erlose	0.06	0.2	10	0.6	2	25			
Stachyose	0.4	1	9	4	13	26			

[a]Tezcan et al., 2011;[b]Puscas et al., 2013; [c]Tůma et al., 2011

3.6. Carbohydrate determination

The developed HPAEC-MS method was applied to determine the carbohydrate composition in honeys with different floral origin (multifloral, monofloral and some honeydew honeys) and produced in different areas of the Trentino Alto-Adige (Italy). Seven monosaccharides (arabinose, fructose, glucose, galactose, mannose, ribose, and xylose), eight disaccharides (sucrose, lactose, lactulose, kojibiose, palatinose, turanose, melibiose, and nigerose), four trisaccharides (raffinose, melezitose, isomaltotriose and erlose) and one tetrasaccharide (stachyose) were analyzed.

The average concentration of each sugar of the honey samples are shown in Table 3.2. Arabinose, xylose, ribose, mannose, galactose and stachyose had concentrations below MDL in all analyzed samples, and therefore, were not used to characterize the honeys.

Table 3.2 Average concentration of carbohydrate in multifloral, acacia, apple-dandelion, rhododendron, honeydew and chestnut honeys. Concentration are expressed in mg kg^{-1}.

	Glucose	**Fructose**	**Sucrose**	**Melibiose**	**Lactose**	**Lactulose**	**Kojibose**	**Turanose**	**Palatinose**	**Nigerose**	**Melezitose**	**Raffinose**	**Isomaltotriose**	**Erlose**
M36	156462	355811	163	443	33750	15153	5125	27912	32838	7264	748	220	2974	621
M37	152603	333638	645	764	23170	7781	3695	16358	6823	5293	22045	4834	1638	7464
M38	140283	326943	869	553	21716	8268	6345	19243	9904	5985	17270	6384	1178	10726
M39	141141	308652	2338	633	17240	6919	3900	18246	6388	5174	14298	9299	647	28905
M41	125599	296125	1846	535	18682	6695	4105	14617	5651	4325	28418	9934	1091	18498
M42	130327	315952	1951	884	22670	6698	5545	19650	7901	5043	27733	9027	1354	22900
M44	152625	363453	812	394	29442	8161	9935	19541	16071	6690	19364	4990	1312	13147
M3-18	142386	356529	1636	291	18947	6670	9888	10967	2506	7068	51702	3151	757	14078
M5-18	111456	308560	1049	313	13183	4800	4547	7887	2379	4243	89841	4757	657	10999
M6-18	105756	291449	903	686	18256	6419	5948	6770	3193	4814	130263	6477	820	5546
M7-18	146359	356609	8941	277	13551	4610	5259	9254	1900	4509	1905	2902	755	20677
M9-18	132956	362067	1237	142	11908	4997	4546	9827	2866	4589	4894	230	682	9373
M16-18	157990	351825	3522	316	18201	5853	6191	13973	5172	4762	6099	5602	841	24245
M19-18	145468	330871	3052	407	14401	4372	4636	7134	2334	3841	2527	4132	602	22988
M20-18	159719	368122	5220	260	14906	5780	5603	16154	4252	4943	2805	2121	799	29246
M21-18	168823	369696	4918	393	16567	6143	4227	13953	4606	4779	5217	3896	903	29832
M23-18	165145	374027	1012	259	17256	5533	5970	12788	2883	5347	7525	372	723	6775
M46-C	147701	421917	33008	251	10331	3503	3379	10856	3612	3323	1928	128	640	16674
M46-P	149989	549749	38130	163	10463	3669	3538	10741	2751	3666	2501	176	346	17226
M46-D	151212	510658	30893	191	9799	3557	<LOD	9728	<LOD	3598	2754	245	347	15658
M47-C	160948	396874	18842	232	13697	4315	5359	15275	7214	4766	2692	357	686	14119
M47-P	152328	485322	16733	218	12727	4144	4205	12336	6705	4477	2415	296	394	14532
M47-D	158117	498609	16838	207	13767	4477	3677	13221	3029	4599	2699	326	391	13130
A1-18	137757	367408	2379	256	15481	5309	5159	10407	2855	5040	3399	1037	710	14241

A11-18	142142	381360	1037	174	12772	4687	5005	7207	2934	4388	842	166	610	9564
A22-18	155767	419521	4950	298	14272	4723	5225	14518	5497	4326	611	91	683	16045
A28-18	161626	399540	2683	226	12255	5692	6304	12807	4968	4584	3622	351	449	17925
AD43	186749	414952	706	333	23526	8350	7674	21849	14843	6468	4406	424	1066	6510
AD45	179847	385330	23791	262	19111	6083	5936	16347	6958	3862	1666	158	980	8171
AD25-18	178651	386746	980	217	14560	5153	4573	15882	8524	4328	3388	417	1096	9038
R4-18	141390	351310	2340	175	17544	5983	7913	10721	2753	6550	17148	1120	658	19144
R2-18	150873	342192	9793	183	14418	6219	6636	13236	1146	5457	1482	483	641	36050
R14-18	159102	349668	6888	258	15079	5931	5272	15676	3343	5546	1516	984	746	34084
R17-18	161747	366501	45383	140	12815	6841	3967	20569	3223	4433	1702	307	<LOD	53822
R18-18	152879	346089	28241	165	11216	5318	3833	11931	1221	4314	778	279	573	44273
R24-18	162749	375678	6991	295	14860	4639	4081	12311	2021	4023	856	236	910	31701
R27-18	156354	356956	21796	79	13379	7212	4807	15483	<LOD	5295	2386	138	543	53248
HD10-18	120186	319467	1178	357	14746	5987	5359	16890	7577	5307	71175	4506	622	10099
HD15-18	148602	321044	2043	403	18228	5373	4528	10330	5066	4659	7113	7274	617	19868
HD26-18	122182	292669	7757	272	23624	5701	5941	12181	6020	5303	83652	10969	790	30724
HD29-18	116900	296231	2347	364	22410	7206	6595	13785	4907	6372	127237	10599	938	15146
C40	125140	300994	2073	718	23738	7816	5564	22445	9203	6189	10169	8543	841	23368
C8-18	123232	346561	397	455	31705	11156	7957	21726	7840	8639	1860	124	1718	842
C12-18	131703	349013	835	447	22216	6486	6199	13314	5289	5592	5815	1582	965	5850
C13-18	154452	391394	1726	346	21609	7690	9558	19440	6341	8151	12372	989	855	13200
C52-C	146196	388348	2274	262	16257	5742	5898	13776	4901	5535	4210	1092	763	6134
C52-P	149131	519423	1995	272	15582	5663	4534	14053	4505	5319	4256	1080	446	6165
C52-D	167602	571552	2329	311	18265	6516	6369	15191	6900	6648	4752	1350	464	7462
MARG14	190003	398866	637	<LOD	4467	3557	2465	10507	14276	2261	269	<LOD	<LOD	636
MARG733	192606	388508	659	<LOD	4464	3381	2444	8412	11638	2499	305	<LOD	<LOD	516

In one study on samples from Spain, galactose had concentrations ranging between 0.0052 and 0.0151% (Val et al., 1998).

Figure 3.1 reports the mean concentrations and standard deviations of the carbohydrates in the forty-eight honey samples which are grouped by their floral origins.

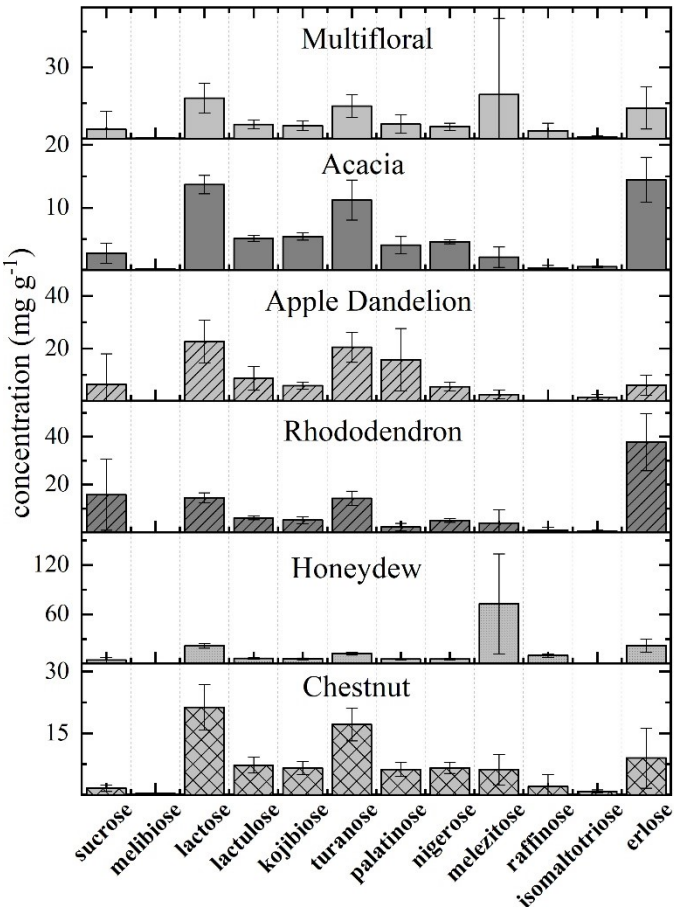

Fig. 3.1 Average concentration and standard deviation of oligosaccharides in multifloral, acacia, apple-dandelion, rhododendron and honeydew honeys.

Fructose and glucose are the main simple sugars found in all honey types, and they are not displayed in Figure 3.1. The sum of both sugars as a percentage (g/100g of honey) was approximately 44 % for honeydew, 51% for rhododendron, 52% for multifloral, 58% for apple-dandelion and 54% for acacia. These results are lower than the compositional criteria of honey, which requires that these two monosaccharides should exceed 60% for nectar honey and 45% for honeydew honey (Thrasyvoulou et al., 2018).

Fructose was the major sugar found in all honeys. The chestnut honey showed the highest level (41%), while honeydew reported the lowest content (31%). These data agree with literature (Escuredo et al., 2014; Terrab et al. 2001).

Glucose was the second major simple sugar found in honey, and apple-dandelion honey displayed the highest mean value (18%), while the mean content of glucose in honeydew honey was the lowest (13%). Previously studies carried out in different European honey and honeydew honey samples demonstrate that glucose was present at higher concentration than in our results, these higher mean values ranged between 26.1% and 38.7% (Escuredo et al., 2014; Mateo & Bosch-Reig, 1997). However, concentrations can vary, with a previous work characterizing chestnut and honeydew honeys, contains mean concentrations as low as was 19.2% and 20.3%, respectively (Schievano et al., 2017).

In general the concentration of glucose determined in this work are lower than those reported in the literature. However, some oligosacharideswere present at a higher concentrations in all samples than other studies in the literature. For instance, lactose and lactulose were present in our samples as the two main disaccharides in all types of floral honey. In all analysed honey samples, the mean percentages were 2% and 1% for lactose and lactulose respectivelyThese two disaccharides are normally present in honey at low concentrations. The literature data show that lactose can be present in honey, but its concentration is usually approximately 0.01% (Val et al., 1998), or in some cases, it is not detectable (Tůma et al., 2011). Unfortunately, no literature data are available about the presence of lactulose, so the concentrations found in our study can not be compared with previous investigations. According to these findings, we hypothesise that glucose could play an important role as the main sugar for the formation of new oligosaccharides, including lactose and lactulose. It is well-known that glucose, along with fructose, is the main sugar normally present in most the oligosaccharides in honey (Ruiz-Matute et al., 2010).

A low concentration of sucrose was found in all honey with a similar content of approximately 0.3% for acacia, honeydew and chestnut honeys and about 0.8% for multifloral and apple-dandelion, where only the rhododendron honey contained a higher concentration (2%). The amount of sucrose in genuine honey is normally accounted approximately 5% (Pita-Calvo et al., 2017).

Sucrose concentrations vary due to the different activity of enzymes (α- and β-glucosidase, α- and β-amylase and β-fructosidase) which hydrolyze sucrose into glucose and fructose (de La Fuente et al., 2011; Ruiz-Matute et al., 2010). As reported in previous studies, the main oligosaccharides found in honey of different botanical origins are maltulose, turanose, maltose, isomaltose, kojibiose, trehalose isomaltotriose, panose, melezitose, raffinose, stachyose (Anjos et al, 2018; Cotte et al., 2003; de La Fuente et al., 2011; Escuredo et al., 2014; Gómez Bárez et al., 1999; Ruiz-Matute et al., 2010). The present study extend to other carbohydrates that recent investigations identify as important sugars in the composition of honey, to examine if these sugars have a

relationship with the floral varieties or quality of honey.

The other main disaccharides found in honey were turanose, palatinose, kojibiose, nigerose and melibiose. The mean concentration of turanose was most elevated in apple-dandelion and chestnut honey, which accounted approximately for 2%. In other types of floral honey this disaccharide contains a mean percentage of nearly 1.5%. Da Costa Leite et al. (2000) report for turanose a range from 0.78% to 2.03% in different Brazilian honeys.

Melibiose contains the lowest concentration, with an average percentage of approximately 0.04%, where this concentration is in accordance with previous published work (Schievano et al., 2017).

The remaining disaccharides, kojibiose, palatinose and, nigerose, show similar percentages for all the types of honey, with mean concentration ranging from 0.4 to 0.7%. The results are comparable with those reported by other authors in Spanish unifloral honey (de la Fuente et al., 2011) and also in Italian honey (Schievano et al., 2017).

The present study is in accordance with the literature where the trisaccharides melezitose and raffinose were the most abundant in honeydew honey, with percentages greater than 7% and about 1% for the two oligosaccharides respectively (Escuredo et al., 2014; Da Costa Leite et al., 2000; Terrab et al., 2001). The prevalence of melezitose in honeydew honey is considered one of its characteristics (Doner, 1977). Melezitose and raffinose were found in high concentrations in French honeydew samples, and were 5.7% and 2.1%, respectively (Cotte et al., 2004). In this work, the concentration of melezitose and raffinose were sligtly higher in multifloral honey (2% and 0.3% rispectively) than unifloral honey such as acacia, where the mean values were 0.2 % and 0.04% and chestnut where the average concentration were 0.6% and 0.2% rispectively. Literature data presented similar mean values of melezitose and raffinose in acacia honey, withe mean concentrations of 0.10% and 0.03% respectively. However, lower values of 0.22% and 0.04% were detected in chestnut honey (Cotte et al., 2003). It was hypothesized that the presence of these carbohydrates in honey may be due to contamination of the floral honey from honeydew or may be naturally present in the nectar (Da Costa Leite et al., 2000).

Erlose had concentration of 4% in rhododendron samples, 2% in honeydew and multifloral honey, more than 1% in acacia, and about 1% in apple-dandelion and chestnut honey.

Similar mean content of erlose was previously detected in honeydew honey from France (2.1%) (Cotte et al., 2004). The presence of this trisaccharide was also detected in some European monofloral honey (Cotte et al., 2003; Mateo & Bosch-Reig, 1997). Erlose is produced from sucrose by the metabolism of bees and its concentration can be modified during storage by enzymatic activity (Mateo & Bosch-Reig, 1997).

The content of isomaltotriose was comparable in all samples, where the percentage ranged from

0.06% in rhododendron to 0.10% in apple-dandelion. In a previous study, a lower concentration was found in clover (0.028%) and alfalfa honey (0.038%) (Swallow & Low, 1990).

The carbohydrate composition in Italian honey of different botanical origin was compared with sugar profiles of two commercial multifloral honeys from Argentina. Theglucose and fructose content of the Argentinian honey was similar to Italian multifloral honey, namely 19% and 39%, respectively. However, the mean value of oligosaccharides showed differed. Melibiose, raffinose and isomaltotriose had mean concentration below MDL values in both of the Argentinian samples. Kojibiose, turanose and nigerose showed slightly lower concentration (0.2%, 0.9% and, 0.2% respectively) in comparison with the Italian multifloral samples. Low concentrations were also detected for trisaccharides such as melezitose (0.03%) and erlose (0.06%). On the other hand, the average content of palatinose was double (1.4%) than the mean concentration observed in the multifloral and unifloral Italian honey.

3.7. Statistical elaboration

Statistical techniques have been used to determine the relationship among different types of Italian honey using the oligosaccharide content. Hierarchical Cluster Analysis was performed using Ward's Method and evaluating Squared Euclidean Distance.

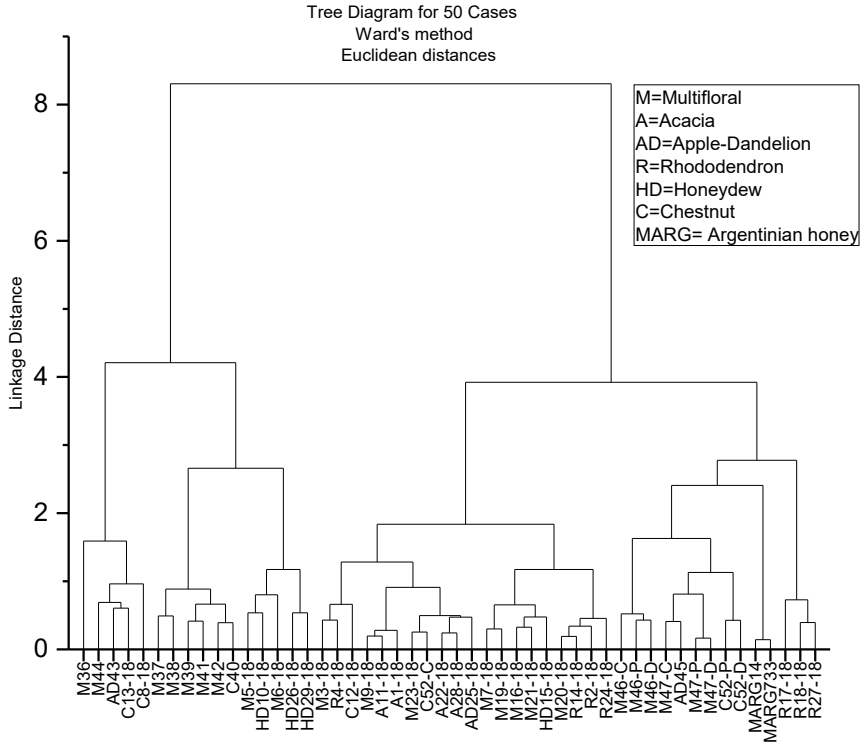

Fig. 3.2 Dendrogram of the Hierarchical Cluster Analysis obtained for the honey samples carbohydrate content.

The chemometric analysis produced a dendrogram which were divided into macro clusters. The results show two main groups of honeys sample, but divided in various subgroups, as shown in Figure 3.2.

The first main cluster was divided into four sub-groups, where the first three subgroups were harvested in 2017 (M36, M44, AD43, M37, M38, M39, M41, M42, C40, except for AD45 sample). Honey harvested in 2018 is clustered in the second main group (Figure 3.2).

The separation of samples from 2017 and 2018 could be explained considering the observed differences in the mean content of some oligosaccharides (Table 3.2). Sucrose is used by honeybees to form many sugars, and about 5% is generally contained in genuine honey (Pita-Calvo et al., 2017). Sucrose is hydrolysed in glucose and fructose by several enzymes (α- and glucosidase, β-amylase and β-fructosidase), which react to form other disaccharides, trisaccharides and tetrasaccharides (Ruiz-Matute et al., 2010). Sucrose content can be decreased during the storage of honey by invertase activity to form glucose and fructose (Al Somal et al., 1994; Pita-Calvo et al., 2017).

The sample AD45, collected in 2017, is included in the 2018 cluster, and contains a high sucrose concentration (23791 mg Kg^{-1}). High sucrose concentrations are usually found in honey harvestedearly in the season, in which an incomplete enzyme activity of invertase occurred (Azeredo et al., 2003).

Multifloral samples from Argentina were clustered into the group of honey harvested during 2018, although they form a small single group.

Principal Component Analysis was used to explore the relationship between variables and to emphasize possible relationships among the botanical origin or inter-annual variability and carbohydrate composition. Three principal components were obtained with eigenvalues >1, and they explained more than 71% of the total variance.

Figure 3.3a,b shows the biplot for the 50 object scores for mono- and oligosaccharides composition and the variable loadings in the space of the first threeprincipal components.

The first principal component, accounting for 41.1% of variance, differentiated the 2017 and 2018 samples. The variables with the highest loadings on the first principal component were lactose, lactulose, nigerose and isomaltotriose (see Figure 3a) and the larger part of di- and trisaccharides. Therefore, it is possible to hypothesize that this component is related to oligosaccharides concentration deriving from honey aging. These oligosaccharides derive from the reaction of glucose and fructose generated from sucrose hydrolysis (Pita-Calvo et al., 2017).

The second principal component, accounting for 19.9% of the total variance, differentiates the honeydew honey from nectar honey. The second principal component presents the highest loading for glucose, fructose, melezitose and raffinose. The first and second principal components differentiate the apple-dandelion honeys, where the variables with the highest loadings for these monofloral samples were turanose and palatinose.

The third principal component accounts for 10.6% of the total variance (see the biplot in Figure 3b). This component differentiates the rhododendron honey from multifloral honey and honeydew honey. The highest variable loadings were for sucrose and erlose, suggesting that these sugras are related to floral honey characteristics.

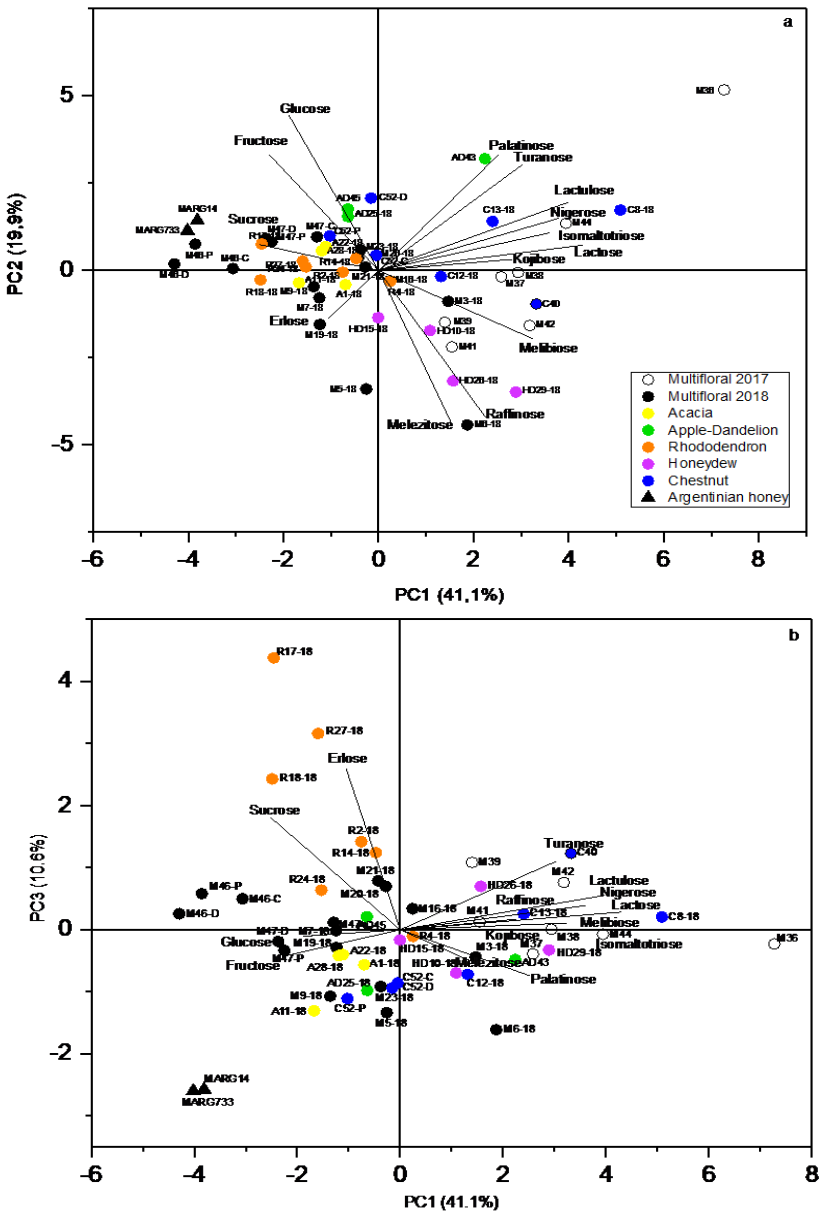

Fig. 3.3 Principal component analysis biplot relative to the honey sample compositions for the carbohydrate in the plane defined by the principal component1 and 2 (a) and the principal component 1 and 3 (b).

The first and third principal components differentiate the chestnut honey from other nectar honey, and the variables with the highest loadings were kojibiose, nigerose and fructose. These oligosaccharides can be characteristic of this floral honey. Figure 3.3b shows how the Argentinian samples were differentiated from the Italian samples. The results demonstrate that the Argentinian samples contain a lower content of oligosaccarides like turanose, nigerose, erlose and lactose and higher concentrations of glucose and fructose.

3.8. Conclusion

A high-performance anion-exchange chromatography method coupled with a mass spectrometry

detector was developed and used to determine the monosaccharides and oligosaccharides in Italian honey, with a diverse botanical and geographical origin, and for comparison, two Argentinean honeys. Monosaccharide and oligosaccharide profiles were useful to differentiate the honey according to their different botanical characteristics and inter-annual variability. Fructose and glucose were the main sugars found in all honey types, while di- and trisaccharides compositions showed that they are related to the aging and floral origin. Turanose and palatinose were representative of apple-dandelion honey, while sucrose and erlose were characteristic of rhododendron honey. Some oligosaccharides, mainly lactose, lactulose, nigerose and isomaltoriose were related to the aging of honey. Glucose, fructose, melezitose and raffinose can be useful to characterize honeydew samples, but melezitose also had higher concentrations in some honeys, probably due to the contamination or mixing with honeydew honey.

The chemometric approach was used to establish the relationship between the oligosaccharides profile and the botanical origin. The Hierarchical Cluster Analysis and Principal Component Analysis highlighted that the content of oligosaccharides could undergo modification during of the harvest period, given the separation of the honey collected in two different years. The fraction of minor oligosaccharides can be useful to establish the floral variety of honey and, in particular, the difference between disaccharides and trisaccharides composition can characterize honeys from different origins and aging processes.

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Chapter 4. Isotopic composition of light elements in Italian honey.

4.1 Introduction

Honey is one of the most complex food used as sweeteners and flavourful natural products (Miguel et al., 2017). An essential priority is to guarantee to the consumers the quality and the authenticity of the food, including the honey, by adequate control protocols. Honey, as various food products, is exposed to a large number of frauds. Honey are modified by changing the major components present through dilution or substitution of sugars and water. Between the more used fraudulent operations are the addition of sugar syrup (mainly High Fructose Corn Syrup (HFCS)) or artificial honey, and also mixing honeys produced by many floral sources (Cotte et al., 2004; Schellenberg et al., 2010).

Mislabelling regarding the botanical or geographical origin could be another adulteration practice, therefore, the honey is commercialized under counterfeit name (Anklam, 1998).

The authenticity of honey can be defined considering some important aspects, that is production (relating to the processes and activities by industries and the local beekeepers) as well as botanical and geographical origin (Bogdanov & Martin, 2002).

According to the European and International legislation, on the label of the honey must be reported the country or countries of origin where it is produced and it must be declared if the honey is a blend and, in case, the type of blend (e.g. “blend of EC honeys”, “blend of non-EC honeys” or both). Besides, this information might be associated with the type of plant species or vegetable resources (Codex Alimentarius, 2001; EU Regulation, 2001). In the above-mentioned regulations, no specific information are reported about the analytical methods to use for determination and verify the correct declaration on the label of honey.

However, some routine analyses based on chemical composition, physical parameters, pollen analysis (melissopalynological method) have been used to characterize both botanical and geographical origins (Anklam, 1998). The determination of geographical origin by melissopalynological analysis is based on observation of the whole pollen spectrum, which corresponds to the vegetation of a certain macro geographical area (Von Der Ohe et al., 2004). Unfortunately, this analytical method often is quite complicated to apply for geographical origin determination, for instance, when the honey is filtered (Schelleberg et al., 2010).

The geographical classification of honey samples results quite complex because in the European region missing the database about the characteristics of honey manufacturing from diverse countries belong to the European Union (Kropf et al., 2010a).

However, several studies have been carried out using different analytical approaches for

identification of geographical origin. In particular, for this purpose, in recent years, it is increasing the number of investigations based on the isotopic analysis.

Indeed, stable isotope analysis of light elements (C, H, N, and S) in different bio-compounds has gained much interest to determinate the geographical origin of many food products such as fish, fruit, meat, dairy products, as well as honey (Camin et al., 2016; Ogrinc et al., 2003; Schellenberg et al., 2010).

This is possible because the isotopic effects of chemical transformation and physical processes that occur in natural biological cycles influence the natural abundance of the stable isotopes, which are involved to form the principal biocompounds. Therefore, the relative abundance of the stable isotope of food is useful to predict and confirm their origin (Anklam, 1998).

The analytical method of carbon stable isotope ratio was originally used to identify the adulteration of honey with the addition of C-4 syrup (Cordella et al., 2002; She et al., 2019). In particular, the isotope ratios of carbon ($^{13}C/^{12}C$) evaluated in raw honey in comparison with relative carbon ($^{13}C/^{12}C$) isolated from honey proteins have been used as the official method to identify the addition of the sugars from C-4 plants (AOAC, 1991). This is possible because honeybees collect nectar and pollen mainly from the blossom of the C-3 plants, in comparison with nectar from C-4 plants. The metabolic pathway synthesizing sugars related to the C-3 and C-4 plants produce a different $^{13}C/^{12}C$ ratio, so it is possible to distinguish which exogenous sugars were added (Zhou et al., 2018).

The C-3 (rice, wheat, and potato) and C-4 (maize, sorghum, and sugarcane) plants can be divided according to their photosynthetic metabolism. The principle is based on the different mechanisms of carbon (CO_2) fixation between two types of plants. In particular, the C-3 photosynthesis pathway occurs only in the mesophyll cells using the Calvin cycle process, while the C-4 plants photosynthetic process occurs among two anatomically and biochemically different cells (mesophyll and bundle sheath cells) using a Hatch-Slack pathway (Wang et al., 2012). Generally, C-3 plants produce a $\delta^{13}C$ value close to -25‰, while $\delta^{13}C$ value in C4 plants produce a is near to -10‰ (Anklam; 1998).

A survey conducted on honey from 20 European geographical areas showed that the stable isotope ratios of carbon, nitrogen, sulfur, and hydrogen were determined from the protein fraction (White et al., 1998). Recently, in Slovenia, monofloral honeys (black locust, lime, and chestnut) from four geographical macroregions was investigated for physicochemical parameters (moisture content, pH value, free acids) and elemental isotopic composition to highlight differences between honeys originating from diverse areas (Kropf et al., 2010a). A similar study was carried out in Uruguay, where stable isotope of light elements and physicochemical parameters (such as pH value,

moisture content, total sugar) were used to differentiate honeys produced in diverse geographical environments with monocultures, grassland and native forest (Berriel, 2018). One study conducted on commercial honey samples from Australia and other countries across five continents was carried out using a combination of the isotope ratios of carbon ($^{13}C/^{12}C$) and trace element concentrations to define the geographical origin and authenticity (Zhou et al., 2018).

In one study conducted on Italian honey samples, with wide botanical origin (multifloral, rhododendron, citrus, eucalyptus, chestnut, acacia, and honeydew), the stable isotope ratio of light elements and elemental composition were analysed to assess the relationship among these parameters and the honey geographical origin (Bontempo et al., 2017). In one previously published study only isotopic fingerprinting was used to discriminate both monofloral and multifloral produced in different geographical regions of Romania (Dinca et al., 2015).

Because the honey elemental composition is affected by different factors such as soils, rocks, climate and environmental conditions, many studies using the elemental profile was carried out to discriminate the geographical origin of honey (Baroni et al., 2015; Bogdanov et al., 2007; Kropf et al., 2010a; Rodríguez García et al., 2006).

Use of element isotopic compositions of light elements (C, H, N, S) to confirm the geographical provenance derives from factors affecting it. Indeed, carbon and hydrogen isotope are associated with climate and weather conditions as precipitation and groundwater occur in the production region, while sulfur isotope reflects the geology of the rocks (Kropf et al., 2010a; Kropf et al., 2010b). On the other hand, nitrogen isotopic ratio reflects different factors such as soil conditions, environment, but is also influenced by fertilizers (Kropf et al., 2010b; Schellenberg et al., 2010) (Schelleberg et al., 2010; Kropf et al., 2010b).

Regard to determination of carbon stable isotope ($^{13}C/^{12}C$) in raw honey and the carbon stable isotope ($^{13}C/^{12}C$) in protein of honey it can be done using a method called internal standard carbon stable isotope ratio analysis (ISCIRA) where the ratio of carbon stable isotope ($^{13}C/^{12}C$) in protein is used as internal standard (AOAC, 1991; White et al., 1998).

The instrument designed to measure small differences in the isotope abundances is one elemental analyser (EA), in continuous flow mode, hyphenated to one isotope ratio mass spectrometer (IRMS) (Anklam, 1998).

This chapter aims to evaluate if the isotopic data of light elements can be used to discriminate the honey produced in different valleys of the Trentino Alto-Adige, where each valley is characterized by different climatic conditions, a broad variety of floral and vegetation sources, and a wide geological characteristic.

The stable isotope ratios of light elements of 48 authentic Italian honeys, and for comparison 16

honey samples from different countries around the world, are presented and discussed. The $\delta^{13}C$ values ($^{13}C/^{12}C$) were determine in raw honey and in protein isolated from the honey, while $\delta^{15}N$ ($^{15}N/^{14}N$) and $\delta^{34}S$ ($^{34}S/^{32}S$) were determined only in the protein, since the amount of N and S in the raw honey is too low to obtain an accurate values.

The chemometric approach, using the isotopic profile, is used to establish if there are differences between groups of honeys belonging to the diverse geographical areas.

4.2. Materials and methods

4.2.1. Reagents and standards

High purity of sodium tungstate dihydrate (p.a. ≥99%) were purchased from Sigma-Aldrich, (Munich, Germany). Sulfuric acid (p.a. 98%,) was acquired from Merck (Darmstadt, Germany). As reference standard materials for carbon, nitrogen and sulfur stable isotopes were used the standards IAEA-600 (caffeine), IAEA-CH-6 (sucrose), IAEA-CH-3 (cellulose), and IAEA-S-3 (silver sulphide) were obtained from the International Atomic Energy Agency (IAEA), (Vienna, Austria); while USGS61 (caffeine n°1), USGS62 (caffeine n°2), USGS43 (Isotopic Reference Material, Indian Human Hair), were purchased from U.S. Geological Survey (Reston, USA). IAEA-CRP 2013 (casein standard) is laboratory working standard, it was obtained from IAEA.

Ultrapure water (18.2 MΩ) was produced by Milli-Q Millipore system from Merck (Darmstadt, Germany).

4.2.2 Honey samples

Honey sampling was conducted directly from local beekeepers and/or association of producers in different geographical areas within Trentino Alto-Adige region (Northeastern Italy). All honey samples were harvested during the 2017 and 2018 production season.

The botanical type of Trentino Alto-Adige honey was ascertained by melissopalynological analysis. In total 48 honey samples were investigated: 23 multifloral (produced and characterized by different floral nectar), 4 acacia samples *(Robinia sp.),* 3 apple-dandelion honey samples *(Asteraceae sp. and Rosaceae sp.)*, 7 rhododendron samples *(Ericaceae sp.),* 4 honeydew samples (with different ratio between honeydew elements and pollen grains), and 7 chestnut honeys *(Castanea sp.)*. See Table 2.2, Chapter 2.

Samples were produced in six valleys located in the central part of Trentino Alto-Adige region: Val di Non (11), Valsugana (10), Val di Fiemme (14), Val d'Adige (11), Val di Fassa (1), and Val

di Cembra (1). In table 4.1, are reported the number of samples originating in the six geographical areas, with the details about the number of samples of each honey type.

Table 4.1 Geographical area details for the honey samples from Trentino Alto-Adige.

Honey type	Geographical area					
	Val di Non	**Valsugana**	**Val di Fiemme**	**Val d'Adige**	**Val di Fassa**	**Val di Cembra**
Multifloral	3	3	9	6	1	1
Acacia	1	2	-	-	-	-
Apple-dandelion	2	-	-	1	-	-
Rhododendron	2	2	3	-	-	-
Honeydew	3	-	1	-	-	-
Chestnut	-	3	1	2	-	-
Total	11	10	14	11	1	1

In figure 4.1 are showed the valleys in the Trentino Alto Adige region, in which the apiaries were localized and the honey samples were produced and collected.

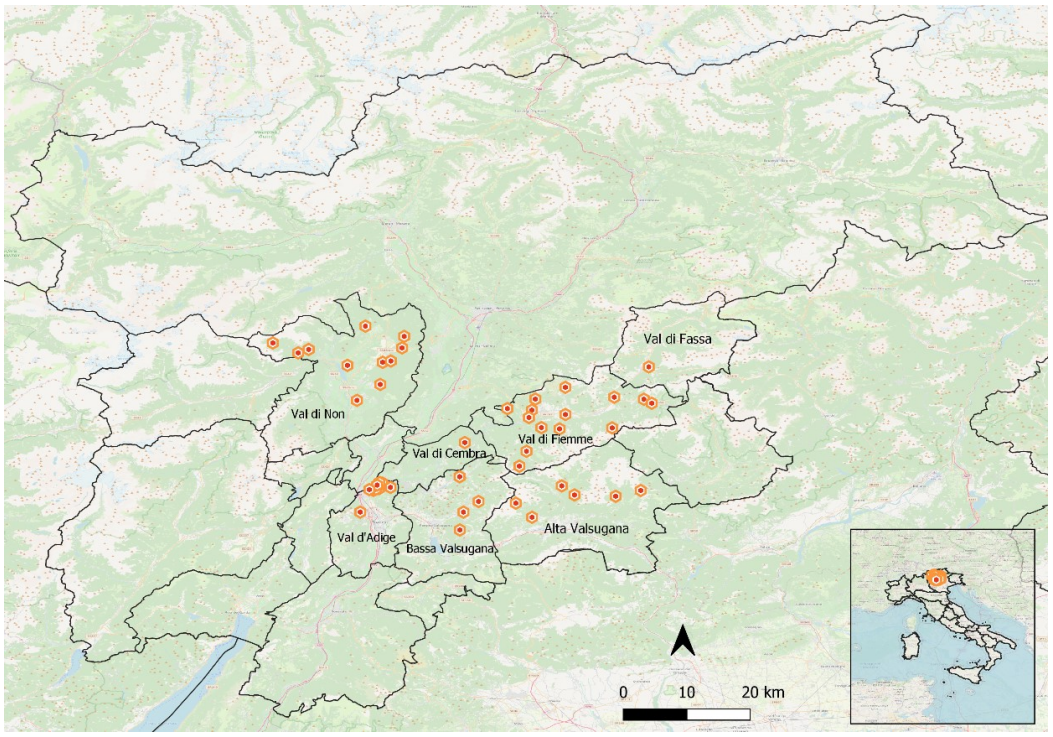

Fig. 4.1 Map of geographical areas of Trentino Alto-Adige region showing the distribution of the honey samples collected in the selected valleys.

In order to compare the results obtained on Trentino region samples, some honey samples from

different geographical areas around the world were also analysed. These honey samples were manufactured during the season 2017-2019. In total, 16 honey samples were collected, and they were divided into 11 multifloral, 4 monofloral (including ulmo, eucalyptus, acacia, and buckwheat), one honeydew, as reported in Table 4.2. The botanical origin of these samples were acquired from the honey labels.

Table 4.2 Honey from different countries around the world and produced in various geographical area during seasons 2017-2019.

Sample code	Honey type	Geographical area	Harvest year
M-FVG	Multifloral	Friuli Venezia Giulia region (Northeastern Italy)	2018
U-Chile	Monofloral (Ulmo)	Chile (Chiloè Island, South America)	2018
M-Sicily1	Multifloral	Sicily (South Italy)	2017
M-Sicily2	Multifloral	Sicily (South Italy)	2017
Euc-Sicily	Eucalyptus	Sicily (South Italy)	2017
M-Ethiopia	Multifloral	Ethiopia (Eastern Africa)	2018
M-Tanzania	Multifloral	Tanzania (Eastern Africa)	2017
M-Hun-Ucr	Multifloral	Hungary/Ukraine	2018
A-Hun	Acacia	Hungary	2018
M-Lazio	Multifloral	Lazio region (Center of Italy)	2018
HD-Serbia	Honeydew	Serbia and Montenegro	2019
M-Serbia	Multifloral	Serbia and Montenegro	2019
M-Greek	Multifloral	Greece	2019
Buck-Canada	Buckwheat	Canada (North America)	2018
M-ARG	Multifloral	Argentina (South America)	2018
M-TW	Multifloral	Taiwan (Eastern Asia)	2018

The samples just obtained were stored at +4 °C in a dark and fresh place until the analysis. To guarantee a representative honey lot, it was been strictly followed the protocol reported from the International Honey Commission (IHC) (Bogdanov, 2009). In addition, to reduce the possible external contamination and alteration, handling and storage, the preparation processes have been carefully observed. Before each analytical session, to achieve a better homogenization, the honey samples were softly mixed.

4.2.3 Sample preparation

To determine the $\delta^{13}C$ in the protein fraction this component was extracted in accordance with the AOAC Official Method 998.12 (AOAC, 1991). About 10-12 g of honey sample was weighed into the 50 mL centrifuge vials and 4 mL of ultrapure water was added, then it was mixed until complete

dissolution. A volume of 2.0 mL of sodium tungstate at 10% and 2.0 mL of sulphuric acid solutions 0.335 M were quickly mixed in a separate tube, then immediately added to the aqueous solution of honey. The mixture was energetically stirred and immersed in a water bath at approximately 80°C until when the flocs of proteins were observed. The sample tube was filled with ultrapure water, then was mixed and centrifuged for 10 minutes at 3000 rpm, and the supernatant was discarded. The protein precipitate obtained was washed, mixed, and centrifuged ten times with about 50 mL of ultrapure water. This step allows us to completely eliminate the carbohydrate components, which they could be contaminate the protein fraction.

The extracted protein was dried using the oven at 40°C. After drying, 2.5 mg of dried honey protein of each sample was accurately weighted in a tin capsule (SerCon, United Kingdom), and then accurately closed. The samples were loaded in the autosampler of instruments for measurement of $\delta^{13}C$ protein, $\delta^{15}N$ protein and $\delta^{34}S$ protein.

4.2.4 Stable isotopic analysis of light elements by EA-IRMS

The analysis was performed using an Iso-Prime-100 Isotope Ratio Mass Spectrometer (IRMS) with Vario Cube (OH/CNS) Pyrolizer/Elemental Analyzer (IsoPrime, Cheadle Hulme, UK).

The determination of $\delta^{13}C$ in raw honey samples (bulk isotopic ratio) was carried out using the SCIRA method, while the ISCIRA method was employed for the measurement of $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ in the honey protein (protein honey isotopic ratio), following the official method (AOAC 998.12; White et al., 1998). All the measurements were repeated in triplicate.

The $\delta^{13}C$ value in bulk honey is calculate by the equation $y={}^{13}C*k+n$; coefficients k and n are obtained by calibration using the IAEA-CH-6 and IAEA-CH-3 standards, the equation used was $y={}^{13}C$ *1.035 -47.423. The $\delta^{13}C$, $\delta^{15}N$ values in protein fraction is calculated by the equations $y={}^{13}C*k+n$ and $y={}^{15}N*k+n$; where coefficients k and n are obtained by calibration using the USGS61, USGS62 standards. The equations used were $y={}^{13}C$ *1.040 -47.131 and $y={}^{15}N$ *0.998 -3.442, rispectively. Finally, $\delta^{34}S$ value in honey protein is calculated by the equation $y={}^{34}S*k+n$; coefficients k and n are obtained by calibration using the USGS43 and IAEA-S-3 standards. The equation was $y={}^{34}S$ *1.043 -2.317. Therefore, given the ^{13}C bulk values, and ^{13}C, ^{15}N, ^{34}S values in honey protein, the $\delta^{13}C$ bulk honey, and $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ in the protein fraction of each sample were determined.

The ratios of ^{13}C /^{12}C, $^{15}N/^{14}N$, and $^{34}S/^{32}S$ were expressed as delta notation per mil (‰) in terms of departure from the international standards: Vienna Pee Dee Belemnite (VPDB) for carbon and AIR standard for nitrogen, and Vienna Canyon Diablo Troilite (VCDT) unit for sulphur, respectively. The accuracy of measurements were verified using international standards. Table 4.3

reports the certificated value and the experimental value found for each certified standard.

For the determination of bulk honey isotopic ratios the IRMS was calibrated using three certified reference standards: IAEA-600 which was employed as a normalization standard, while the IAEA-CH-6 and IAEA-CH-3 were used as a standard analysis. The evaluation of isotopic elements ratio from honey protein USGS61 and USGS62 standards were used. Generally, these two reference materials are designated for normalization of stable carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$) and hydrogen ($\delta^{2}H$) measurements.

Table 4.3 Certified and found values for each international standard material used for accuracy determination.

International Standard	Unit	Analyte	Certified value ± SD (‰)	Found value ± SD (‰)
IAEA-600 (caffeine)	‰ VPDB	$\delta^{13}C$	-27.771 ± 0.043	-27.75 ± 0.10
	‰ Air N_2	$\delta^{15}N$	+1.0 ± 0.2	/
IAEA-CH6 (sucrose)	‰ VPDB	$\delta^{13}C$	-10.449 ± 0.033	-10.53 ± 0.08
IAEA-CH3 (cellulose)	‰ VPDB	$\delta^{13}C$	-24.724 ± 0.041	-24.74 ± 0.05
IAEA-S-3 (Silver Sulphide)	‰VCDT	$\delta^{34}S$	-32.3 ± 0.2	-32.69 ± 1.07
USGS43 (Indian Human Hair)	‰ VPDB	$\delta^{13}C$	-21.28 ± 0.10	-21.26 ± 0.03
	‰ Air N_2	$\delta^{15}N$	+8.44 ± 0.10	+8.31 ± 0.04
	‰ VCDT	$\delta^{34}S$	+10.46 ± 0.22	+10.57 ± 0.32
USGS61 (caffeine n°1)	‰ VPDB	$\delta^{13}C$	-35.05 ± 0.04	-35.05 ± 0.03
	‰ Air N_2	$\delta^{15}N$	-2.87 ± 0.04	-2.89 ± 0.03
USGS62 (caffeine n°2)	‰ VPDB	$\delta^{13}C$	-14.79 ± 0.04	-14.85 ± 0.07
	‰ Air N_2	$\delta^{15}N$	+20.17 ± 0.06	+20.13 ± 0.02
IAEA-CRP 2013	‰ VPDB	$\delta^{13}C$	-20.30 ± 0.09	-20.44 ± 0.04
	‰ Air N_2	$\delta^{15}N$	+5.62 ± 0.19	/
	‰ VCDT	$\delta^{34}S$	+4.18 ± 0.74	4.43 ± 0.46

USGS43 standard material is essentially employed for calibration of stable hydrogen ($\delta^{2}H$) and oxygen ($\delta^{18}O$) measurements, but it also is appropriate for measurements of the stable isotopes of carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), and sulfur ($\delta^{34}S$). The standard IAEA-S-3 and casein standard (IAEA-CRP-2013) are often used for sulfur and carbon calibrations.

These reference standards were analyzed daily for the determination of the stable isotope of light elements of interest, as well as to correct the results for the drift that can be occur during the measurements, , and finally to evaluate the quality of the analyzed within each sequence of samples examined.

4.3 Statistical analysis

Multivariate statistical techniques were applied to the data to establish possible relationships among the botanical origin or geographical origin and the stable isotope compositions of light elements ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$).

The statistical elaboration was performed using OriginPro 10.0 software (Originlab Corporation,

USA). The hierarchical cluster analysis was used to find the possible differences in order to better classify the Italian honeys according to the geographical origin, using as the isotopic composition of light elements such as carbon bulk, and carbon, nitrogen, and sulfur isolated from protein. For comparison, different honeys from various countries around the world were added. Ward's method and Euclidean were used as cluster method and distance type, respectively.

4.4 Results and discussion

In the present study, a total of 48 Italian honey samples and for comparison 16 world's honey were investigated. The Italian honeys were from six different botanical origins, multifloral, acacia, apple-dandelion, rhododendron, honeydew, and chestnut. These honeys were sampled from seven different valleys located in the Trentino Alto-Adige region (Italy). (See Fig.1).

The carbon element ($\delta^{13}C$) values were determined in raw honey and in honey protein fractions, while the nitrogen and sulfur were quantified in the protein isolated from the honey . It is worth to underline that the nitrogen and sulfur isotopic composition cannot be measured directly in honey, because these chemical elements are present at too low concentration; therefore, the $\delta^{15}N$ and $\delta^{34}S$ can be quantitatively measured through precipitation and isolation of the protein (Schellenberg et al., 2010).

In Table 4.4, are summarized the mean values and standard deviation (expressed as delta notation) for carbon bulk, and carbon, nitrogen, and sulfur obtained from honey protein of samples collected in the valleys of Trentino Alto-Adige honeys. In Table 6.4 are reported the values of the isotopic composition of light elements for samples from other world areas.

Table 4.4 Average values of isotopes of carbon bulk, and carbon, nitrogen, and sulfur in honey protein obtained in honeys from different geographical areas of Trentino Alto-Adige.

Geographical area	$\delta^{13}C_{bulk}$ (‰)		$\delta^{13}C_{prot}$ (‰)		$\delta^{15}N_{prot}$ (‰)		$\delta^{34}S_{prot}$ (‰)	
	mean	U*	mean	U	mean	U	mean	U
VN	-25.72	± 0.08	-25.39	± 0.12	1.47	± 0.17	5.07	± 0.81
VS	-25.90	± 0.12	-25.46	± 0.10	0.54	± 0.23	4.66	± 0.34
VF	-25.32	± 0.09	-25.61	± 0.07	0.49	± 0.12	5.54	± 0.53
VA	-26.23	± 0.08	-25.52	± 0.07	2.40	± 0.08	3.99	± 0.59
VC	-25.25	± 0.07	-25.50	± 0.03	0.41	± 0.16	5.14	± 0.32
VFS	-26.02	± 0.13	-26.13	± 0.07	0.28	± 0.08	5.19	± 0.58

VN=Val di Non; VS=Valsugana; VF=Val di Fiemme; VA=Val dìAdige; VC=Val di Cembra; VFs=Val di Fassa; *Uncertainty of the value calculated on three replicates using 95% as confidence interval (α=0.05).

The mean value of carbon bulk honey and the carbon from protein were quite similar to all honey

samples analyzed, belonging to the different valleys of Trentino Alto-Adige region. In addition, the differences between carbon bulk and carbon protein were quite small, that is lower than 1.80%. This important information conferms that these Italian honeys samples were not adulterated by the addition of artificial sugar from external vegetation sources, such as C-4 plants. Indeed, the value of carbon bulk honey in comparison with the value of carbon from protein provides a measure of the presence of sugars come from C-4 plants. When the difference between these two values is greater than 7%, honey is considered adulterated with exogenous carbohydrates (AOAC, 1991). The value of isotopic composition of light elements for each honey samples from valleys from Trentino Alto-Adige region are reported in Table 4.5.

Table 4.5 The value of stable isotope of light elements for each honey samples from different geographical areas of the Trentino Alto-Adige.

Honey	Geographical area	$\delta^{13}C_{bulk}$ (‰) ± U*	$\delta^{13}C_{prot}$ (‰) ± U	$\delta^{13}N_{prot}$ (‰) ± U	$\delta^{13}S_{prot}$ (‰) ± U
M3-18	VN	-25.70 ± 0.09	-25.05 ± 0.12	-0.27 ± 0.08	4.59 ± 1.61
M5-18	VN	-25.65 ± 0.06	-25.56 ± 0.26	2.48 ± 0.29	5.60 ± 0.57
M6-18	VN	-25.44 ± 0.12	-25.77 ± 0.02	2.74 ± 0.09	5.18 ± 0.32
A28-18	VN	-25.46 ± 0.13	-24.59 ± 0.12	3.02 ± 0.04	4.42 ± 2.48
AD43	VN	-26.93 ± 0.05	-26.27 ± 0.04	4.34 ± 0.15	4.66 ± 0.83
AD25-18	VN	-26.58 ± 0.11	-25.42 ± 0.01	2.83 ± 0.25	5.88 ± 0.57
M4-18	VN	-25.73 ± 0.06	-25.26 ± 0.01	-0.05 ± 0.13	3.03 ± 0.75
R27-18	VN	-26.02 ± 0.07	-25.03 ± 0.21	-1.32 ± 0.26	5.17 ± 0.25
HD10-18	VN	-24.96 ± 0.15	-24.92 ± 0.05	0.85 ± 0.22	4.78 ± 0.33
HD26-18	VN	-25.16 ± 0.03	-25.65 ± 0.38	0.07 ± 0.33	6.64 ± 0.06
HD29-18	VN	-25.24 ± 0.04	-25.78 ± 0.08	1.45 ± 0.02	5.82 ± 1.11
M7-18	VS	-26.01 ± 0.08	-26.04 ± 0.12	0.16 ± 0.17	4.95 ± 0.81
M9-18	VS	-24.66 ± 0.11	-24.39 ± 0.02	1.09 ± 0.08	5.09 ± 0.32
A1-18	VS	-25.94 ± 0.19	-25.01 ± 0.10	1.47 ± 0.27	4.11 ± 0.49
A11-18	VS	-25.50 ± 0.17	-24.61 ± 0.07	1.81 ± 0.15	4.53 ± 0.25
M23-18	VS	-26.07 ± 0.09	-25.73 ± 0.06	0.33 ± 0.17	5.50 ± 0.13
R2-18	VS	-26.17 ± 0.14	-25.46 ± 0.14	-0.06 ± 0.32	3.71 ± 0.25
R24-18	VS	-26.50 ± 0.11	-26.19 ± 0.17	0.09 ± 0.11	3.74 ± 0.26
C8-18	VS	-26.24 ± 0.06	-26.37 ± 0.19	0.15 ± 1.10	4.55 ± 0.38
C12-18	VS	-26.22 ± 0.08	-25.51 ± 0.02	-0.93 ± 0.03	5.35 ± 0.17
C13-18	VS	n.a. ± n.a.	-25.32 ± 0.08	0.38 ± 0.06	4.68 ± 0.29
M36	VF	-25.83 ± 0.00	-25.77 ± 0.09	-1.33 ± 0.09	3.46 ± 0.37
M37	VF	-25.63 ± 0.12	-26.10 ± 0.10	1.25 ± 0.23	5.98 ± 0.34
M38	VF	-25.06 ± 0.09	-25.96 ± 0.06	1.47 ± 0.08	5.64 ± 0.02
M39	VF	-25.02 ± 0.06	-25.58 ± 0.12	0.67 ± 0.17	4.46 ± 0.54
M44	VF	-26.27 ± 0.13	-25.35 ± 0.06	3.12 ± 0.04	5.48 ± 0.58
M16-18	VF	-24.18 ± 0.07	-26.00 ± 0.02	1.44 ± 0.15	5.39 ± 0.41
M19-18	VF	-27.18 ± 0.03	-26.24 ± 0.06	2.67 ± 0.07	6.08 ± 2.20
M20-18	VF	-25.71 ± 0.14	-25.86 ± 0.07	1.58 ± 0.09	5.61 ± 0.93
M21-18	VF	-23.34 ± 0.06	-25.50 ± 0.07	1.03 ± 0.14	5.79 ± 0.35

Honey	Geographical area	$\delta^{13}C_{bulk}$ (‰) ± U*	$\delta^{13}C_{prot}$ (‰) ± U	$\delta^{13}N_{prot}$ (‰) ± U	$\delta^{13}S_{prot}$ (‰) ± U
R14-18	VF	-26.08 ± 0.06	-25.38 ± 0.07	-1.40 ± 0.29	5.52 ± 0.31
R17-18	VF	-26.10 ± 0.17	-24.89 ± 0.11	-2.36 ± 0.06	5.83 ± 0.33
R18-18	VF	-25.88 ± 0.11	-25.00 ± 0.09	-1.04 ± 0.09	6.12 ± 0.75
HD15-18	VF	-23.03 ± 0.03	-25.78 ± 0.03	0.51 ± 0.04	5.48 ± 0.17
C40	VF	-25.76 ± 0.07	-26.11 ± 0.09	-0.78 ± 0.22	5.21 ± 0.13
M46-C	VA	-25.32 ± 0.13	-24.76 ± 0.04	3.26 ± 0.09	5.38 ± 0.12
M46-D	VA	-25.31 ± 0.10	-24.72 ± 0.11	3.25 ± 0.06	4.68 ± 0.16
M46-P	VA	-25.32 ± 0.09	-24.66 ± 0.07	3.25 ± 0.12	4.48 ± 0.53
M47-C	VA	-26.75 ± 0.19	-25.50 ± 0.09	2.03 ± 0.04	2.97 ± 1.01
M47-D	VA	-26.72 ± 0.05	-25.55 ± 0.06	1.96 ± 0.05	3.29 ± 0.31
M47-P	VA	-26.67 ± 0.06	-25.43 ± 0.02	2.01 ± 0.08	3.99 ± 0.30
A22-18	VA	-25.79 ± 0.05	-24.80 ± 0.12	1.94 ± 0.19	5.33 ± 1.57
ADF45	VA	-27.92 ± 0.15	-28.28 ± 0.06	3.80 ± 0.01	1.41 ± 0.74
C52-C	VA	-26.19 ± 0.05	-25.62 ± 0.05	1.66 ± 0.03	4.39 ± 0.11
C52-D	VA	-26.24 ± 0.02	-25.67 ± 0.10	1.71 ± 0.22	3.95 ± 0.60
C52-P	VA	-26.30 ± 0.10	-25.78 ± 0.02	1.50 ± n.a.	4.07 ± 0.27
M41	VC	-25.25 ± 0.03	-25.50 ± 0.06	0.41 ± 0.05	5.14 ± 0.22
M42	VFs	-26.02 ± 0.15	-26.13 ± 0.04	0.28 ± 0.06	5.19 ± 1.38

VN=Val di Non; VS=Valsugana; VF=Val di Fiemme; VA=Val dìAdige; VC=Val di Cembra; VFs=Val di Fassa; n.a.=Not Available; * Uncertainty of the value calculated on three replicates using 95% as confidence interval (α=0.05).

The range value of $\delta^{13}C$ bulk (or honey) from different geographical areas were as follow; Val di Non was between -26.93‰ to -24.96‰; Valsugana was from -26.50‰ to -24.66‰; Val di Fiemme ranged from -27.18‰ to -23.03‰; Val d'Adige was from -27.92‰ to -25.31‰. However, in the case of Val di Cembra and Val di Fassa only one honey sample was analysed, and the value of $\delta^{13}C$ bulk honey was -25.25‰ and -26.02‰, respectively.

Data from literature, report for $\delta^{13}C$ bulk from Trentino's Italian honey a range between -26.2‰ to -23.7‰, with an average amount of -24.7‰, which are in accordance with the values found in the present work (White et al., 1998). A very similar value was also reported in a study conducted on other honey samples from Trentino, where a mean value of -24.8‰ was showed (Schellenberg et al., 2010).

The range values of $\delta^{13}C$ honey in the proteins of each valley were quite close to those measured in the honey bulk, and these were as follow; Val di Non ranged between -26.27‰ and -24.59‰; Valsugana was from -26.37‰ to -24.39‰; Val di Fiemme ranged from -26.24‰ to -24.89‰; Val d'Adige was from -28.28‰ to-24.66‰. For Val di Cembra and Val di Fassa only one sample was analyzed and the values of $\delta^{13}C$ protein were -25.50‰ and -26.13‰, for the two valleys, respectively.

In a survey conducted on Italian honey samples the mean value of $\delta^{13}C$ honey in proteins ranged between -25.5‰ and -23.4‰ (White et al., 1998).

The $\delta^{13}C$ values, for both bulk and protein carbon, are quite similar to each other. Effectively, if these values are obtained from the same origin, the two values of isotope composition should be similar (Cengiz et al., 2014). This can be explained because the honeybees feed on pollen and nectar, the components which are content are used to produce the honey proteins, mostly as enzymes, useful to ripen the nectar and produce honey (Anklam, 1998; White et al., 1998). As a result, a very close value of $\delta^{13}C$ of the whole honey and the proteins isolated from this food is obtained (White et al., 1998). However, during the honey production season, these two values can be subjected to a small variation, because the honeybees collect pollen and nectar, which can have values slightly different for the carbon isotopic composition (White et al., 1998). Also, the metabolism of the vegetation, mainly the plants and its related products, affects the carbon isotope profile (Schellenberg et al., 2010). On the other hand, carbon isotope profile is associated also to the climate conditions (temperatureand humidity environments), so it can be useful to assign the geographical origin of food (Baroni et al., 2015).

The nitrogen and sulfur, due to their very low concentration in honey, their isotopic composition was determined only on the isolated protein fraction. Regard to nitrogen isotope value, in all samples ranged from -2.36‰ to 4.34‰. The $\delta^{15}N$ value intervals obtained for the different valleys were as follow: Val di Non was between -1.32‰ and 4.34‰; Valsugana was from -0.93‰ to 1.47‰; Val di Fiemme ranged from -2.36‰ to 3.12‰; Val d'Adige was from 1.50‰ to 3.80‰, for Val di Cembra and Val di Fassa only one sample was investigated, the values of $\delta^{15}N$ were 0.41‰ and 0.28‰, respectively (See Table 4.5). As reported in Table 4.4, the lowest mean value was reported in Val di Fiemme, with 0.49‰, while the highest value was found in the honey sample produced in the Val d'Adige area, with an average value of 2.49‰. These data are quite comparable with those reported in one previous study carried out on honey samples from Trentino Alto Adige, where the mean value of $\delta^{15}N$ was 0.8‰ ± 1.2‰ (Schellenberg et al., 2010).

Generally, the value of $\delta^{15}N$ range from -10‰ to +15‰, but its amount depends on the types of vegetation, the characteristics of the soil, and climate and/or environmental conditions (Camin et al., 2016). The $\delta^{15}N$ values contained in plants are related to the nitrogen composition and content of ammonia and nitrates in the soil, originating from the atmospheric nitrogen. The nitrogen presents in the soil is transformed by physical processes and microbiological activities into the inorganic and organic compounds. Therefore, the nitrogen content in honey proteins could depend on the composition of the soil where the plants or the flowers were grown (Schellenberg et al., 2010).

The sulfur isotope composition ($\delta^{34}S$) for the analyzed samples collected in the various geographical areas were: Val di Non ranged from 4.42‰ to 6.64‰; Valsugana from 3.71‰ to

5.50‰; Val di Fiemme from 3.46‰ to 6.12‰; Val d'Adige from 1.41‰ to 5.38‰; the values of $\delta^{34}S$ found in the single samples from Val di Cembra and Val di Fassa were 5.14‰ and 5.19‰, respectively. In Table 4.4, are reported the average values of isotopic sulfur composition for each valley under investigation. Literature data, about $\delta^{34}S$ profile, obtained in samples from Trentino Alto Adige (Italy) reported a very similar mean value to those found in this study, 5.05‰ ± 0.5‰ (Schellenberg et al., 2010).

Many factors affect the level of sulfur in plants or vegetation such as quantities of the sulfides compounds present in the soils, local rocky layer under the soil, microbial activities in the soil, sulfate aerosol deposition on the vegetation in areas nearness the sea and the human activities by fertilization practices (Camin et al., 2016; Kropf et al., 2010b). Generally, the common values for plants range from –5‰ to +22‰, but the most frequent interval is between +2‰ and +6‰ (Camin et al., 2016).

The isotope composition of carbon, nitrogen, and sulfur for the samples object of this study were compared with some honeys originating in different countries from the world's, the results are reported in the Table 4.6. The botanical origin for these samples were those reported on the label (see Table 4.2). The samples of different geographical areas were five from Italy (North, Centre, and South); five from European countries (Hungary, Ukraine, Serbia-Montenegro, and Greece); two from the African continent (Tanzania and Ethiopia); three samples from American continent (one from Canada, one from Chile and one from Argentina); and finally one from Taiwan. The carbon isotopic composition of bulk and carbon protein were quite similar to the other honey samples investigated. The $\delta^{13}C$ values in the bulk ranged between -27.34‰ and -24.02‰ and, while the values protein carbon range between -27.90‰ and -24.65‰. Based on the obtained data, it is possible to state that these commercial honeys were authentic (the differences among the two carbon values was lower than 1.50‰).

The highest value of carbon bulk was observed in honey come from Canada, with a value of -27.34‰. This value was close to those reported in a previous work for Canadian honey samples, where the data ranged between -26.6‰ and -18.00‰ (White et al., 1998). The lowest values were in Sicily honeys, the average value was -24.02‰; the value agree with that reported in one previous survey carried out in samples from Sicily, the mean value was -24.20‰ (Schellenberg et al., 2010).

Table 4.6 Values of isotopes of carbon bulk, and carbon, nitrogen, and sulfur in honey protein obtained in honeys from different countries of the world.

		$\delta^{13}C_{bulk}$ (‰)		$\delta^{13}C_{protein}$ (‰)		$\delta^{15}N_{protein}$ (‰)		$\delta^{34}S_{protein}$ (‰)	
Sample code	**Geographical area**	**value**	**U***	**value**	**U**	**value**	**U**	**value**	**U**
M-FVG	Friuli V.G. (Italy)	-26.40	± 0.09	-25.32	± 0.17	0.27	± 0.00	5.47	± 1.15
M-Lazio	Lazio (Italy)	-25.11	± 0.02	-25.71	± 0.12	3.80	± 0.19	3.86	± 1.35
M-Sicily1	Sicily (Italy)	-24.95	± 0.06	-25.53	± 0.07	3.74	± 0.07	-6.32	± 0.52
M-Sicily2	Sicily (Italy)	-25.72	± 0.11	-25.83	± 0.04	2.58	± 0.02	5.62	± 0.61
Euc-Sicily	Sicily (Italy)	-24.02	± 0.09	-24.65	± 0.01	3.21	± 0.16	3.42	± 1.10
M-Hun-Ucr	Hungary/Ukraine	-25.97	± 0.04	-26.44	± 0.08	4.57	± 0.13	5.03	± 0.68
A-Hun	Hungary	-26.11	± 0.11	-26.50	± 0.16	3.19	± 0.10	6.21	± 0.16
HD-Serbia	Serbia-Montenegro	-25.99	± 0.01	-25.95	± 0.01	2.44	± 0.11	7.01	± 0.84
M-Serbia	Serbia-Montenegro	-26.23	± 0.01	-25.61	± 0.12	3.69	± 0.15	1.35	± 0.35
M-Greek	Greece	-25.35	± 0.06	-25.81	± 0.12	3.64	± 0.15	5.13	± 0.29
M-Ethiopia	Ethiopia (Africa)	-26.45	± 0.05	-27.90	± 0.11	3.92	± 0.04	9.05	± 0.28
M-Tanzania	Tanzania (Africa)	-25.56	± 0.04	-25.85	± 0.11	2.64	± 0.03	8.92	± 0.37
Buck-Canada	Canada	-27.34	± 0.05	-27.60	± 0.04	4.14	± 0.11	-3.60	± 0.31
U-Chile	Chile	-26.38	± 0.08	-26.70	± 0.05	0.03	± 0.05	14.85	± 0.16
M-ARG	Argentina	-25.94	± 0.16	-26.55	± 0.02	4.51	± 0.03	5.19	± 0.38
M-TW	Taiwan	-26.01	± 0.10	-25.59	± 0.15	3.07	± 0.04	6.29	± 0.41

* Uncertainty of the value calculated on three replicates using 95% as confidence interval (α=0.05).

The highest value on carbon in protein, -27.90‰, was registered in one sample produced in one African country (Ethiopia). The lowest protein carbon value was obtained in a honey sample from Sicily, having a value of -24.65‰. To the best of our knowledge, no literature data are available for protein carbon isotope in honeys from Africa and Sicily.

The data of carbon bulk from Greece honey obtained in this study, the value was comparable with those reported in a work conducted on honeys from the same country, where the mean value was -25.1‰ (Schellenberg et al., 2010). The data of mean values of $\delta^{13}C$ bulk and $\delta^{13}C$ protein in Greek honey samples reported in one other investigation were -24.31‰ and -25.67‰ respectively (Zhou et al., 2018).

The literature data for carbon bulk in Argentinian honeys range between -26.7‰ and -20.2‰ (the mean value was -25.5‰) (White et al., 1998). The analysis of carbon isotope determined in honeys from Uruguay ranged from -25.54‰ to -24.49‰ for carbon bulk and between -25.75‰ and -25.57‰ for carbon protein (Berriel et al., 2018). The values of $\delta^{13}C$ bulk and honey protein found in two honeys from south Italy (Sicily) were close to those reported in a previous work conducted on honeys from Italy with a mean value of -24.62‰ and -24.51‰, respectively (White et al., 1998), in comparison with honey samples from centre Italy (Lazio region) and north Italy (Friuli V.G. region), (see Table 4.6).

One previous study on honey from Hungary reported average values of -24.77‰ and -25.97‰ for $\delta^{13}C$ bulk and $\delta^{13}C$ in protein, respectively, which were quite similar to those obtained in our work (Zhou et al., 2018).

Our data obtained on honeys from Serbia-Montenegro were higher than those found in honey from the same geographical areas with respect to $\delta^{13}C$ bulk, the literature data are -17.37‰ (Zhou et al., 2018). To our knowledge, no data are available about isotope carbon composition for honey from Ukraine, Africa, Chile, and Taiwan countries.

The lowest value of nitrogen isotopic composition we obtained in the world's samples was in the Chilean honey, it was 0.03‰, the highest one was obtained in the sample coming from Hungary and Ukraine, it was 4.57‰. Considering the sulfur isotope profile, the minimum value was -6.32‰ obtained in one sample from Italy (Sicily) and the maximum value was 14.85‰ found in the Chilean honey. This latter absolute high value (Chilean honey), could be explained by the sea-spray effect due to the nearness of the Pacific ocean, that can influence the sulphate deposition on the plants and vegetation near the production area (Camin et al., 2016).

Unfortunately, not many research data are available for nitrogen and sulfur isotopic composition in honey samples. One study carried out on Greek honeys reported a mean value of $\delta^{15}N$ of 1.3‰, significantly lower than the data obtained in this study (3.64‰), while the sulfur isotope

composition account for 5.13‰, which was comparable to that reported in one previous work, 5.6‰ (Schellenberg et al., 2010).

One study on Italian honeys (Sicily) reported the mean value of isotopic nitrogen composition of 3.9‰, the data is close to the value obtained in our study (it range from 2.58‰ to 3.74‰), however, the same authors reported a mean value of 2.0‰ for $\delta^{34}S$, which was significantly different from our data (from -6.32‰ to 5.62‰) (Schellenberg et al., 2010).

4.5 Statistical elaboration

The Hierarchical Cluster Analysis was employed to attempt in order to separate and differentiate the Italian honeys, produced in different valleys of Trentino Alto-Adige, between honeys produced from diverse countries around the world, using the isotopic composition of light elements such as carbon bulk, and carbon, nitrogen, and sulfur in isolated honey proteins.

According to the obtained cluster diagram (Fig.4.2), it was observed that the profile of the light elements is influenced both from the geographical origin and from the floral component of the honey samples studied.

For the studied honeys, through the Hierarchical Cluster Analysis was possible to emphasize the distribution and separation of all honeys within two main clusters.

The first main cluster (left part of the graph) is constituted by two sub-clusters, where the most of the honey samples produced in the geographical area of Trentino Alto-Adige are present; in particular, in the first sub-cluster all the rhododendron monofloral honeys were grouped, while, in the second sub-cluster the acacia monofloral honeys and honeydew honeys were grouped.

The second main cluster showed a wide variability, but it is worth noting that all the honey samples from different countries of the world were grouped, with the exception of some honeys from Trentino Alto-Adige (Fig.4.2).

Respect to the multifloral honeys, having an extremely diversified floral composition, as a consequence they are distributed and grouped according to the different floral sources, which these honeys were produced. Indeed, the separation of multifloral honey was not evident.

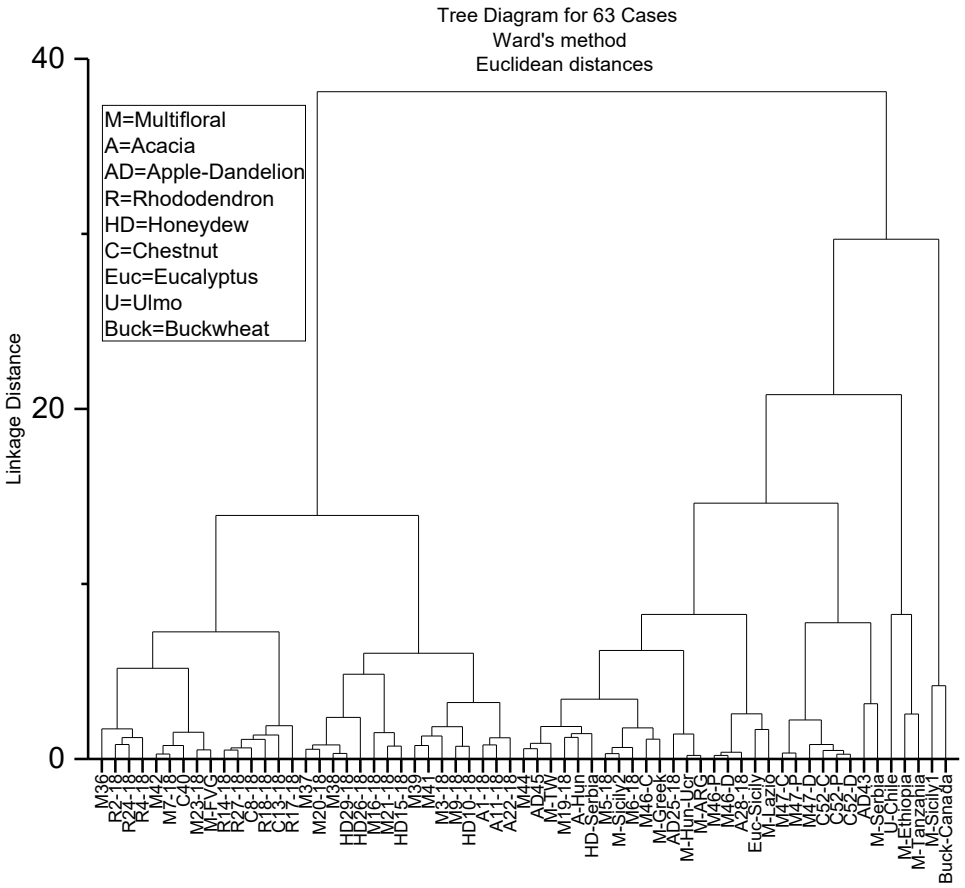

Fig. 4.2 Dendrogram of the Hierarchical Cluster Analysis obtained for the Trentino Alto-Adige and world's honey samples content of isotope composition.

4.6 Conclusion

In this chapter the composition of stable isotope of light elements of Italian honeys produced in the Trentino Alto-Adige region was studied by analysis of samples from different valleys. The results are compared with data available from honeys collected from regions around the world.

According to the obtained data about the isotope profile, the Hierarchical Cluster Analysis was employed to attempt the separation of the honey sample come from the different geographical origins. Therefore, to differentiate the honey samples from different valleys of the Trentino Alto-Adige region (Italy) among honey collected in different countries of the world according to the isotope composition of light elements.

Indeed, the cluster analysis showed how it was possible to identify two main clusters. The first main cluster included the most honey samples from Trentino Alto-Adige, while the second main cluster was constituted by all world's honeys, where were also included a small number of Italian honey samples. This aspect seems to confirm that the isotopic composition of light elements is a characteristic related to the geographical origin.

Indeed, using the composition of stable isotope light elements, carbon bulk, and carbon, nitrogen and sulphur isolated from protein, it was possible to separate honeys produced in different part of the world, including also honeys harvested in different part of Italy.

There are evidence that other statistical multivariate analysis can obtain a good classification of honey samples of different geographical origin, for instance by linear discriminant analysis (LDA) (Kropf et al., 2010b). However, it was not possible to apply this statistical method at our data set because the honey samples come from one single macro area, namely the Trentino Alto-Adige region, where they were gathered in six different valleys very close each other. Moreover, a reduced number of samples were available for each valley, in some cases single samples, and they were frequently different also for the botanical origin.

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Chapter 5. Determination of volatile organic compounds in honey

5.1 Introduction

Volatile organic compounds (VOCs) originated from biological systems are a complex mixture of compounds presenting different chemical characteristics; they represent substances that can be produced following diverse biosynthetic pathways specific of the biological system (Lubes & Goodarzi, 2017). Commonly, the nature of volatile molecules is lipophilic with low molecular weight, low boiling point, and high vapor pressure in natural conditions (Pichersky et al., 2006). Many volatile organic compounds are important because are involved in the defensive system of the organism and/or in the communication apparatus with the external environment by attracting pollinators (i.e. honeybee) or seeds disseminators (Lubes & Goodarzi, 2017; Reinhard et al., 2006). In plants, volatile organic compounds such as monoterpenes, sesquiterpenes, and more aromatic substancesare released from a specific site, where they are normally stored, or from characteristic glands or trichomes (Parè & Tumlinson, 1999). A complex mixture of these volatile organic compounds provides characteristic aroma and flavor to the plants, flower, and food such as fruits and vegetables, and which can be detected by the olfactory system (Lubes & Goodarzi, 2017). The analysis of volatiles organic compounds of foods is useful for quality control purpose or characterization, for instance, the aroma profile can be employed to determine frauds and/or origin identification of some food (Lubes & Goodarzi, 2017). During the last few years, attention has been focused on characterization of organic volatile components of honey, especially in authenticity and adulteration studies (Robotti et al., 2017).

Honey, as well as other products of biological origin, is characterized by a complex mixture of several volatile organic compounds with different chemical and physicochemical properties, which are normally are present in very low concentrations (Plutowska et al., 2011).

Currently, more than 600 volatile organic compounds have been identified in different types of honey samples. (Karabagias et al., 2014b). The major volatile organic compounds in honey are aldehydes, ketones, organic acids, alcohols, esters, hydrocarbons, terpenes, and cyclic compounds (Miguel et al., 2017).

Volatile and semivolatile organic compounds are mainly responsible for the honey fragrances (Rahman et al., 2017). Especially, terpenes and their derivatives, norisoprenoids, and benzene derivatives are the main categories represented in honey. Terpenes and their derivatives are well-known as organic molecules contributing to flavor, aromatic profile and for their biomedical activities. While, norisoprenoids, although are present in a very low concentration, are important

because strongly affect the aromatic profile. In addition, benzene derivatives can be used as chemical marker for the determination of the environmental pollution. However, one study conducted in New Zealand honeys some benzene derivatives are classified as the main volatile organic compounds with antibacterial properties (Pattamayutanon et al., 2017).

Volatile organic compounds such as aldehydes, alcohols, and fural derivatives are associated with the quality of honeys; these substances may be found in honey as consequence of heat exposure, microbial activities and storage conditions of honey. While, some linear aldehydes in some case are characteristic organic volatile compounds related to specific floral source (Kaškonienė & Venskutonis, 2010; Pérez et al., 2002).

Volatile organic compounds can be affected by many factors: vegetation or nectar source, honeybee metabolism through the elaboration of the plant substances, environmental sources (i.e. contamination or adsorption of odors from the air), bacterial activities, heating processing, handling procedures, and storage conditions (Karabagias et al., 2014b; Patrignani et al., 2018).

There are evidence that the composition of volatiles organc components in honey can vary significantly with botanical and geographical origin (Karabagias et al., 2014a); therefore, the characterization of the organic volatile profile in honey could be an effective method to evaluate the botanical and/or the geographical origin of honey (Miguel et al., 2017). Indeed, the volatile organic compounds represent a fingerprinting of honey from the different botanical area, because the amount and the type of these substances are related to the floral source (Bianchi et al., 2011). Furthermore, the presence of specific organic volatile compounds could be consider a chemical markers in monofloral honey and as concequence the volatile organic fraction can useful to verify the authenticity of the honey (de Lima Morais da Silva et al., 2017; Piasenzotto et al., 2003). The organic volatile compounds of honey produced by only a single floral source is practically unique, and distinct in comparison with multifloral honey (Rahman et al., 2017). Several studies have been conducted on the volatile organic compounds in different floral honeys from around the world to assess their quality (Piasenzotto et al., 2003), their characterization (Karabagias et al., 2014a; Karabagias et al., 2014b; Perez et al., 2002), for authenticity testing (Radovic et al., 2001; Senyuva et al., 2009) and to investigate their botanical and geographical origin (Guyot et al., 1998; Măda¸s et al., 2019; Panseri et al., 2013; Patrignani et al., 2018).

Honey is a complex substrate to analyze, indeed it presents a base-sugar matrix where are include, among other molecules, several volatile organic compounds, consequently to identify and quantify thesesubstances is quite complicated (Castro-Vázquez et al., 2003; Pérez et al., 2002). For this reason, it is necessary to eliminate the carbohydrates, the major constituents of honey, and then isolate the volatile organic compounds.

Many techniques are used to carry out the extraction of volatiles organic compounds such as hydrodistillation (HD), liquid-liquid extraction (LLE), simultaneous steam distillation extraction (SDE) or Likens-Nickerson simultaneous distillation extraction (LNSDE) and micro-simultaneous steam distillation-solvent extraction (MSDE), ultrasound solvent extraction (USE) (Alissandrakis et al., 2003; Manyi-Loh et al., 2011; Perez et al., 2002; Plutowska et al., 2011). However, these extraction methods require heat treatment, so temperature can lead to the formation of new compounds, decomposition, and oxidation of aroma molecules that are not naturally included in honey aroma (Manyi Loh et al., 2011).

Over recent years, the volatile organic compounds is performed using Headspace Solid-Phase Micro-Extraction (HS-SPME) as the main technique to extract the aroma compounds (Robotti et al., 2017). The advantage of this technique is solvent-free, easy-to-use extraction system because it does not need expensive equipments and can be used to isolate volatile organic compounds with different volatility and polarity (Bianchi et al., 2011). However, one disadvantage of SPME is its low repeatability in comparison with other extraction systems, therefore, it requires to optimize the adsorption process on the fiber (Robotti et al., 2017). Headspace Solid-Phase Micro-Extraction (HS-SPME) is usually hyphenated with gas chromatographic (GC) instrumental technique coupled with a mass spectrometer (MS) as detector (HS-SPME GC-MS) (Bianchi et al., 2011).

HS-SPME-GC-MS was commonly used in several studies focused on investigation of the volatile organic compounds of different honey types (Bayraktar & Onoğur, 2011; Beitlich et al., 2014; de Lima Morais da Silva et al., 2017; Jerković et al., 2009; Karabagias et al., 2014a; Karabagias et al., 2014b; Lušić et al., 2007; Pérez et al., 2002; Plutowska et al., 2011).

The aim of this chapter was to study the chemical composition of the volatile organic compounds of several floral types of Italian honeys, produced in Trentino Alto-Adige region. Relatively few works have been carried out on different floral honeys produced in this geographical area. Such an investigation will allow Italian honey producers and also local beekeepers to increase the knowledge about volatile organic fraction and to characterize honeys labelled as sourced from local single or multiple floral species. The volatile organic compounds were analyzed using headspace solid-phase micro-extraction coupled to gas chromatographic-mass spectrometry.

The volatile organic composition was analyzed by chemometric methods to compare honeys with different botanical origin.

5.2 Materials and methods

5.2.1 Origin of honey samples

The identification of the volatile organic compounds was carried out on 48 artisanal honey samples originally from different geographical areas in the Trentino Alto-Adige region (Italy). The honeys were purchased from local beekeepers and/or the apiarist's association and harvested for two years (2017 and 2018). Six different botanical varieties were analyzed, 23 multifloral, 4 acacia, 3 apple-dandelion, 7 rhododendron, 4 honeydew, and 7 chestnut (Details are reported in Table 2.1, chapter 2). The honey samples were stored in hermetically closed glass jars and were kept at +4 °C in dark conditions until analysis for volatile components.

5.2.2 Sample preparation and headspace solid phase microextraction conditions

The headspace solid-phase microextraction (HS-SPME) was performed using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber, 50/30 μm, (1 cm), Stableflex/SS, 23Ga (Supelco, Bellefonte, PA, USA). Before using, the fiber was preconditioned in the injector of the gas chromatograph at 270 °C for 30 min following the manufacturer's recommendations. The volatiles organic profile was recovered from a 10 ml clear glass vials (22.5mm×46mm) (Global Analytical Solution, Gerstel GmbH & Co. KG, German) with screw caps equipped with silicone/PTFE septa (Supelco, Bellefonte, PA, USA).

One gram of honeys was directly weighted in a 10 mL glass vials and mixed with 1 g of ultrapure water and 30% (w/w) of sodium chloride (NaCl), and then hermetically sealed, following the previous procedure (Bianchi et al., 2001; Karabagias et al 2014a; Robotti et al., 2017). The samples were automatically stirred and heated at 60 °C for 15 minutes to achieve the equilibration phase. The extraction step was performed introducing the fiber directly into the headspace of the vial at 60 °C and exposed for 90 min, the efficiency of the extraction process was improved by continuously stirring. The extracted volatile were thermally desorbed in the injector port of the gas chromatograph in splitless mode at 250 °C for 1 min. he optimization of the headspace solid-phase microextraction conditions was carried out on a multifloral honey.

5.2.3 Gas chromatography–mass spectrometry

The analyze was carried out by a gas chromatograph (model 7890B, Agilent Technologies, Palo Alto, CA, USA) coupled to a single quadrupole mass spectrometry (model 5977A inert MSD, Agilent Technologies, CA, USA), and equipped with autosampler (MPS, Gerstel, German).

The chromatographic separation was performed using a VF-WAXms column (30.0m length x 0.25mm I.D. x 0.25µm film thickness), (Agilent J&W GC column). The initial oven temperature was 60 °C, then heated until 140 ° at 3 °C min^{-1} C and held for 10 min, additional heating until 230 °C at 5 °C min^{-1} and held at this temperature for 2 min, and then ramped up to 250 °C, as previously reported (Bianchi et al., 2011; Perez et al., 2002; Piasenzotto et al., 2003; Plutowska et al., 2011). Helium was used as a carrier gas and the flow rate was set to1.0 mL min^{-1}. The mass spectrometer operated in the full scan acquisition mode with a range from 35 to 300 m/z and ion source temperature was set at 240 ºC. The splitless injector was set at 250 °C, the electron ionization system was employed at 70 eV. The total runtime was 67 min. Each sample was analyzed in three replicates.

The volatile organic compounds were identified by comparing the experimental mass spectra with those of the National Institute of Standard and Technologies library (NIST14).

5.3 Statistical analysis

The statistical analysis was performed using a OriginPro 10.0 software (Originlab Corporation, USA). Principal Component Analysis was used as tool to highlight a possible classification of Italian honeys according to their composition of the volatile organic fraction. For this purpose, the variables used were the 27 volatile organic compounds identified in these samples. Therefore, the data matrix used was, constituted by 48 honey samples, as cases, and 27 volatile organic compounds as variables.

5.4 Results and discussion

5.4.1 Volatile organic compounds in honey

The volatile organic compounds of 48 honey samples with the different botanical origins (multifloral, acacia, apple-dandelion, rhododendron, honeydew, and chestnut) and collected in north-eastern Italy (Trentino Alto Adige Region) was studied. Fig. 5.1 presents a representative chromatogram obtained for multifloral honey samples.

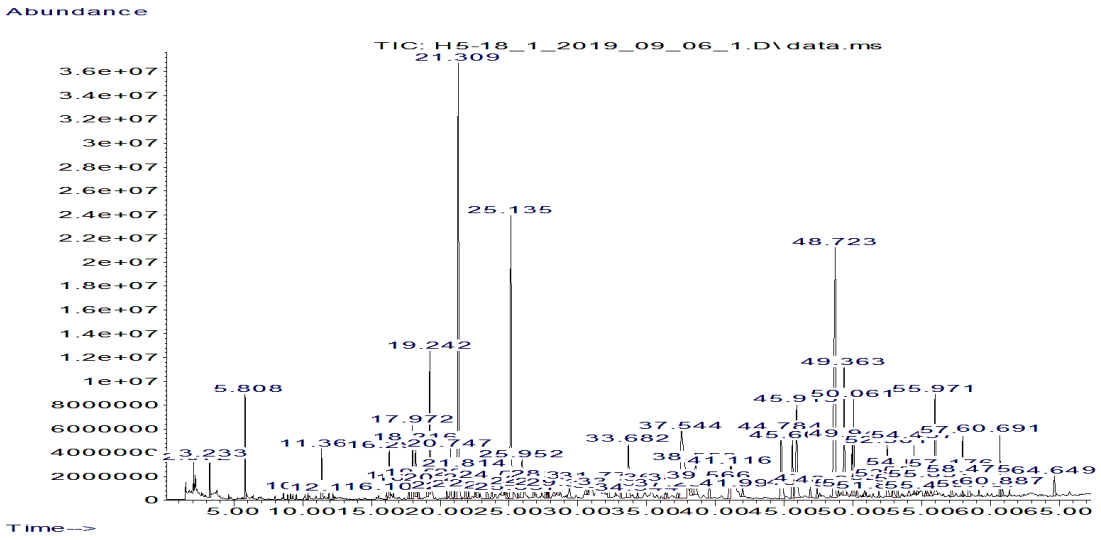

Fig. 5.1 Representative chromatogram obtained by headspace SPME GC-MS for multifloral honey samples (M5-18).

A total of eighty (80) volatile organic compounds, belonging to the main groups of components as carboxylic acids, aldehydes, alcohols were identified in the honey samples analyzed; twenty-seven volatiles (octane, γ-terpinene, octanal, 2-nonanone, nonanal, acetic acid, furfural, decanal, benzaldehyde, lilac aldehyde C, linalool, 1,5,7-Octatrien-3-ol, 3,7-dimethyl- (hotrienol), benzeneacetaldehyde (phenylacetaldeide), terpineol, heptanoic acid, phenylethyl alcohol, octanoic acid, caprolactam, nonanoic acid, thymol, n-decanoic acid, geranic acid, diethyl phthalate, benzoic acid, dodecanoic acid, dibutyl phthalate, and tetradecanoic acid) were found in most of the honey samples, while the remaining fifty-three volatile organic compounds were found in only between 2% and 50% of the total investigated honeys.

In Table 5.1, are listed all the eighty (80) volatile organic components found, where is reported the retention time and the molecular weight of each volatile substance.

Table 5.1 Volatile organic compounds identified in volatile fractions of different floral Italian honeys by HS-SPME-GC/MS.

Compound	Retetion time (min)	Molecular weight
Octane	2.1	114
Ethyl Acetate	2.6	88
Butanal, 3-methyl-	2.9	86
Ethanol	3.5	46
2,3-Butanedione	3.7	86
Pentanal, 3-methyl-	4.6	100
Propanal, 2-methyl-	6.8	72
Dodecane	8.6	170
D-Limonene	8.7	136
γ-Terpinene	10.4	136
o-Cymene	11.4	134
Octanal	12.2	128
2-Nonanone	16.1	142
1,3,8-p-Menthatriene	16.2	134
Nonanal	16.3	142
Tetradecane	16.6	198
p-Mentha-1,5,8-triene	17.4	134
trans-Linalool oxide (furanoid)	18.2	170
Acetic acid	19.1	60
Furfural	19.2	96
Decanal	20.5	156
Pentadecane	20.7	212
Benzaldehyde	21.3	106
Lilac aldehyde A	22.1	168
Lilac aldehyde C	22.5	168
Linalool	22.7	154
Lilac aldehyde B	22.9	168
Propanoic acid, 2-methyl-	23.6	88
Lilac aldehyde D	23.8	168
Terpinen-4-ol	24.5	154
Hexadecane	24.7	226
1,5,7-Octatrien-3-ol, 3,7-dimethyl- (Hotrienol)	25.1	152
Butanoic acid	25.8	88
Benzeneacetaldehyde	26.0	120
Butanoic acid, 2-methyl- and Butanoic acid, 3-methyl-	27.4	102
Terpineol	28.3	154
2-Furanmethanol	28.6	98
2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	30.6	134
1-Nonanol	30.8	144
Pentanoic acid, 3-methyl-	31.8	116
Hexanoic acid	33.7	116
1-Decanol	34.8	158
Nonadecane	35.8	268
Lilac alcohol A	36.8	170
Creosol	37.2	138
Benzylalcohol	37.6	108
Hexanoic acid, 2-ethyl-	38.1	144
Heptanoic acid	38.2	130
3,7-Octadiene-2,6-diol, 2,6-dimethyl-	38.6	170

Compound	Retetion time (min)	Molecular weight
Phenylethylalcohol	40.8	122
Cinnamaldehyde (E)	42.0	132
Octanoic acid	44.8	144
Heneicosane	46.9	296
m-Guaiacol	47.8	124
p-Cymene	47.9	134
Caprolactam	48.7	113
Eugenol	48.9	164
3-Phenylpropanol	49.1	136
Nonanoic acid	49.4	158
Thymol	50.1	150
n-Decanoic acid	52.5	172
Eicosane	53.2	282
Heptadecane	53.3	240
Octadecane	53.3	254
Geranic acid	54.1	168
Diethyl Phthalate	54.4	222
Benzoic acid	55.9	122
2,7-Octadiene-1,6-diol, 2,6-dimethyl-	56.2	170
Dodecanoic acid	57.2	200
Benzeneacetic acid	58.5	136
5-Hydroxymethylfurfural	58.6	126
Dibutyl phthalate	60.7	278
Tetradecanoic acid	60.9	228
trans-Cinnamic acid	63.7	148
n-Hexadecanoic acid	64.6	256

Of the 80 volatile organic compounds, 27 were selected because they featured in the larger part of the samples and of these was assessed the relative amount on the base of the chromatographic peak area. The area repeatability of the measurements was evaluated through the calculation of the percentage of the standard deviation (RSD%) on seven replicates using a multifloral honey, the most representative sample. The mean area values, standard deviation, and relative standard deviation of these selected volatile organic compounds are reported in Table 5.2, the relative standard deviation (RSD%) for all the volatile organic compounds was less than 18%.

Table 5.2 Data of mean area value, standard deviation, and relative standard deviation (RSD%) of identified volatile organic compounds calculated on seven replicates (n=7) of a multifloral honey.

Compound	Mean	Std Dev	RSD %
Octane	3753826	677092	18
γ-Terpinene	3168509	114746	4
Octanal	2392483	258763	11
2-Nonanone	2235022	143391	6
Nonanal	16689855	1406944	8
Acetic acid	7702118	344264	4
Furfural	57421079	3992440	7
Decanal	5206367	240636	5
Benzaldehyde	1,91E+08	15223791	8
Lilac aldehyde C	2618819	227678	9
Linalool	11761973	1201352	10
1,5,7-Octatrien-3-ol, 3,7-dimethyl- (hotrienol)	1,6E+08	9594495	6
Benzeneacetaldehyde	24809299	763505	3
Terpineol	9029434	698375	8
Heptanoic acid	7228056	598734	8
Phenylethyl alcohol	19807629	846223	4
Octanoic acid	49753568	2011451	4
Caprolactam	2,12E+08	13821363	7
Nonanoic acid	60961622	5071476	8
Thymol	35527182	1191042	3
n-Decanoic acid	20925199	757184	4
Geranic acid	11508485	343189	3
Diethyl Phthalate	15512064	1410201	9
Benzoic acid	37680359	1778475	5
Dodecanoic acid	8296098	918573	11
Dibutyl phthalate	14008593	875873	6
Tetradecanoic acid	2778336	441329	16

5.4.1.1 Multifloral

Based on the results, the most of multifloral honey samples demonstrated very similar volatile organic profile fingerprinting. The larger part of 27 volatile organic compounds were detected in all the multifloral honeys, only octane, terpineol, γ-terpinene, and 2-nonanone were found in a limited number of multifloral samples. The presence of octane in some honey, according to some authors, could be originated by beeswax (Patrignani et al., 2018).

The presence of the major volatile organic compounds found in multifloral honeys can be explained considering that these honeys are produced using different vegetation sources that honeybees process and transform in so-called multifloral honey. The aldehydes compounds, such as benzaldehyde, were commonly identified in different European honey, frequently in citrus honeys (Guyot et al., 1998), as well as in Brazilian orange honey (Bastos et al., 2002). Various volatile

organic compounds such as 3-butenenitrile were identified in commercial multiflower honey from Spain (Soria et al., 2008) or α-methylbenzyl alcohol and methyl-anthranilate which were found in multifloral honey samples from Turkish (Senyuva et al., 2009). The latter of these compounds were not detected in multifloral honeys analyzed in this work.

Among alcohol substances, the hotrienol (1,5,7-Octatrien-3-ol, 3,7-dimethyl-) was detected in all multifloral honeys, this compound is quite common to find in most of honeys, as previously reported (de Lima Morais da Silva et al., 2017). Although, hotrienol can be naturally present in honey, its concentration can increase during the maturation of honey, depending on chemical and physical-chemical factors such as pH, temperature and enzymes (Jerković et al., 2010). Indeed, as previously reported, ripe honey contains higher amount of hotrienol in comparison with unripe honey (Rowland et al., 1995). Previous authors reported that hotrienol can be produced by thermal degradation of honey (Alissandrakis et al., 2003; Rowland et al., 1995).

According to the literature, some volatile orgnic compounds found in our samples, such as lilac aldehyde, nonanol, phenylethyl alcohol, benzenacetaldehyde, and linalool, were identified in multifloral honey samples produced in different areas in Turkey (Marmara, Aegean, Black Sea, Middle, Mediterranean, East Anatolian, and South-East Anatolian regions) (Senuya et al., 2009).

Regarding aliphatic acids, in all our multifloral samples were identified a relative short-chain acids, from acetic acid to dodecanoic acids. Among the identified volatile organic acids, benzoic acid was found in all multifloral honeys analyzed in our work, and it was also reported in Polish multifloral honey (Plutowska et al., 2011).

5.4.1.2 Acacia

The volatile organic fraction of the four acacia honey samples contained the most of 27 above-mentioned aroma compounds; the exception were thymol and therpineol, which they were detected in only one sample and geranic acid, octane, γ-terpinene and 2-nonanone which were not detected in any acacia samples.

Between the already mentioned substances characterizing the volatile organic compounds as nonanal and decanal, were identified in a previous study (Plutowska et al., 2011). The presence of heptanal in acacia honeys is of particular interest, indeed, in a previous study was reported as a characteristic compound of the volatile organic profile of this honey (Castro-Vázquez et al., 2014). The presence of these volatile compounds was confirmed in all the acacia honey samples here investigated. On the contrary, the phenylacetaldeide (benzeneacetaldehyde), which in this study was one of the most representative molecule found in headspace of acacia honey, did not was previously identified in this type of floral honey (Radovic et al., 2001). The benzeneacetaldehyde,

along with benzaldehyde, were previously identified in several other different honey types and they are molecules that can help to give an agreeable aroma to the "honey" (Karabagias et al., 2014b).

Besides of these above described compounds, 3,7-dimethyl-1,5,7-octatrien-3-ol (hotrienol) and acetic acid were also observed in the headspace analysis, analogous results were obtained on the analysis of the volatile organic compounds of Romanian acacia honey (Mădaş et al., 2019).

In some European acacia honey samples (France, German, and Italy) acetone, benzaldehyde, and furfural were detected as a principal component of volatile organicprofile. While the 3,7-dimethyl-1,6-octadien-3-ol (linalool) was one of the more representative compounds found in our acacia honeys, this was in accordance with the previous results reported for the analysis of italian acacia honeys (Radovic et al., 2001).

In contrast, the volatile organic profileregistered in acacia honey from Spain presented very low levels of volatile compounds, with some predominat components such as 2-phenylethanol, 2,3-pentanedione, as ethylphenylacetate, well as 2-phenylacetaldehyde (Serra Bonvehì & Ventura Coll, 2003) and this group of chemical substances were not detected in our samples, only the phenylethyl alchol and phenylacetaldehyde were detected also in our samples.

5.4.1.3 Apple-dandelion

Unfortunately, to the best of our knowledge no reference data are available about volatile organic compounds on apple-dandelion honeys. The volatile components of all three apple-dandelion honey investigated was characterized by the presence of the most of twenty-seven compounds. However, it is worth to note that benzenacetaldehyde, one of the main aldehyde component, was not detected in any of the apple-dandelion samples, also thymol, geranic acid, octane, terpineol, γ-terpinene, and 2-nonanone were undetectable in this monofloral honey. In addition, decanal and tetradecanoic acid were identified only in one sample in comparison with other floral types of honey, where they were detected in the most of samples.

In one study carried out to characterize the volatile organic fraction on dandelion Italian honeys was observed the presence of nitrile substances as the main components, as reported by the author (Piasenzotto et al., 2003). Furthemore, the volatile organic compounds of dandelion honey from Turkey was characterized by glyceraldehydes and benzamide, and also some nitrile molecules, the glyceraldehyde was also observed in high relative amount if compared to the other compounds (Özenirler et al., 2018). One study carried out on apple honey samples from Estonia revealed the presence of hexyl hexanoate and (E)-β-damascenone as typical volatileorganic components of this

monofloral honey (Seisonen et al., 2015). The compounds above-mentioned were not detected in our apple-dandelion honeys.

5.4.1.4 Rhododendron

Also the rhododendron samples contained the larger part of the 27 volatile organic compounds, only the phenylethyl alcohol was undetectable in all rhododendron samples. Furthermore, it is important to point out that geranic acid and terpineol were found in almost all rhododendron samples, unlike some other monofloral honeys (i.e. apple-dandelion) where these compounds were not detected or identified in only a few samples (i.e. acacia). Thymol and octane were identified only in few samples, while γ-terpinene and 2-nonanone were absent in all rhododendron honeys.

Senuya et al., (2009) reported that the typical volatile organic compounds of rhododendron honey from Turkey were n-decane, lilac aldehyde, 2-aminoacetophenone, benzenedicarboxylic, nonanal, isobutylphthalate, and damascenone and they indicated these components as possible floral indicators for this type of honey.

On the other hand, Tasdemir et al., (2003) in a previous work reported a different volatile organic profile in rhododendron honeys produced from five Turkish rhododendron plant species, and their major volatile organic constituents ranged from ethyl acetate, 6-methyl-5-hepten-2-one, 2-ethyl-hexanol, and α-terpineol for some types of rhododendron plants, to benzyl alcohol, limonene, and p-cymene for other kind of rhododendron species. Apart from some aldehydes constituents (nonanal and lilac aldehyde), the remain components were not detected in our rhododendron honeys.

However, a similar volatile organic compounds to our honeys of rhododendron was found in the same other honey types from Italy (Valtellina, Lombardia region), indeed, they presented high relative amount of ethanol, acetaldehyde, benzaldehyde, furfural, acetic, formic and butanoic acids, and linalool (Panseri et al., 2013).

5.4.1.5 Honeydew

This study was conducted also on volatile organic constituents of honeydew honeys. The volatile organic profile showed the presence of the major components already registered for blossom honeys. However, the volatile organiccompounds revealed some differences from nectar honeys. In particular, lilac aldehyde C, linalool, and hotrienol were found in only few honeydew samples, in comparison with other samples where these compounds were always present, both multifloral and monofloral honey samples.

In none honeydew honey was identified the γ-terpinene and, analogously to some nectar honeys, thymol, geranic acid, octane, terpineol, and 2-nonanone were found only in one or two samples.

In one study conducted on Turkish honeydew honeys, the main volatile organic compounds identified were n-decane, nonanal, α-α-dimethylphenyl acetate, nonanol and 2-methyl heptanoic acid (Senuya et al., 2009). The volatiles organic substances of pine honey (honeydew honey) samples produced in different areas on Turkey (Marmaris, Datça, and Fethiye) showed eight common volatile organic compounds as nonanal, nonanol, decanal, octanal, 16-oxosalutaridine, dodecanal, nonadecane, and pentadecane, the main contributors of aroma were aldehydic and alcoholic substances as nonanal, decanal, octanal, and nonanol (Bayraktar & Onoğur, 2011).

On the other hand, the presence of two main volatile organic compounds that are erythro- and threo-2,3-butanediol were reported for artisanal honeydew honeys from Madrid province (Spain) (Soria et al., 2005), while one study conducted on commercial honeydew honeys from Spain, among others, exhibited considerable content of dimethylsulfide (Soria et al., 2008).

Acetic acid was reported as reference volatile organic compound present in honeydew honey from Brazil (Campos et al., 2000; Plutowska et al., 2011), and in our honeydew samples this component was found in all analyzed samples. About acetic acid, along with butyric acids could be produced by honeybee metabolism (Mădaş et al., 2019).

Many volatile organic compounds were identified in Greek honeydew honeys (pine honeys) from different areas of Greece (Halkidiki, Evia, Thassos, and Samos regions). Aldehydes, ketones, esters, and alcohols, alkanes and alkenes were also found in the most of these pine samples. In particular, esters included ethyl derivatives of cyclic and aliphatic organic acids such as hexanoic, heptanoic, octanoic, nonanoic, decanoic, and benzoic acid. Alcohols such as 2-ethyl-1-hexanol, 1-octanol, and 1-nonanol and aldehydes as benzaldehyde, benzeneacetaldehyde were also identified in these honeydew honeys (Karabagias et al., 2014a). Most of the above-mentioned volatile organic compounds were also observed in our honeydew samples.

5.4.1.6 Chestnut

The volatile organic substances of chestnut honeys in this work revealed the presence of the major 27 selected volatile organic compounds. However, some substances as lilac alcdehyde, phenylethyl alcohol, tetradecanoic acid, and thymol were detected in only few chestnut honeys. The phenylethyl alcohol, it can be present in two different isomers, 1-phenylethanol and 2-phenylethanol, were described in the literature as characteristic compounds of the chestnut honeys from France and Italy (Guyot et al., 1998; Piasenzotto et al., 2003).

Octane and γ-terpinene were not found in any chestnut samples, terpineol, and 2-nonanone were detected in only one honey sample. While some monoterpenes such as γ-terpinene was found in

various type of monofloral honeys included chestnut, thyme and citrus (Guyot et at.,1998; Karabagias et al., 2014b; Plutowska et al., 2011).

It is interesting that, in our study, geranic acid was found in almost all the chestnut honeys, in contrast, was observed that in other monofloral samples such as acacia and apple-dandelion, this organic acid was absolutely absent. In addition, all the chestnut honeys here investigated contained also other carboxilyc acids such as acetic acid, octanoic and nonanoic acids, benzoic acid.

According with previous authors, the volatile organic profile obtained for chestnut honeys reported the presence of some characteristic volatile molecules such as benzaldehyde and nonanal (Serra Bonvehí & Ventura Coll, 2003; Verzera et al., 2001).

Although according to the reference data, the aminoacetophenone was another specific volatile organic molecules that was identified and isolated in this type of honey (Piasenzotto et al., 2003; Radovic et al., 2001), and p-anisaldehyde was a common volatile organic component found in Turkish chestnut honey, mainly associated with woody flavor (Senyuva et al., 2009), these compounds were not detected in our chestnut honey samples.

Volatile organic compounds furan-derivated such as furfural were detected in Italian honey samples, and the data are in accordance with the previous work conducted on chestnut honey from Europe (Germany, France and Italy) (Radovic et al., 2001).

5.4.1.7 Exogenous organic compounds

Many compounds belonging to the volatile organic compounds of honey can also be formed by mechanisms not directly related to the metabolism of honeybees, from the nectar of plants, or to the honeydew usually used for the production of this food. In fact, some components derive from processes of heating, conservation, storage, microbiological transformations, as well as from contamination of the environment (Karabagias et al., 2014b).

Many of these exogenous compounds such as furfural and its derivatives (i.e. 5-methylfurfural, furfuryl alcohol, dihydro-5-methyl-2(3H)-furanone, dihydro-2-methyl-3(2H)-furanone and 1-(2-furanyl)-ethanone, 5-hydroxymethylfurfural, furfuryl-n-butyrate) were detected in different kinds of honey as reported in several previous works (Bianchi et al., 2011; Karabagias et al., 2014b; Perez et al., 2002; Piasenzotto et al., 2003; Radovic et al., 2001; Soria et al., 2008). Furfural and related compounds are frequently associated to heating processes and preservation conditions of honey, therefore, they normally cannot be used as good floral markers (Castro-Vázquez et al., 2007; Guyot et al., 1998).

Some alcoholic compounds (for instance 3-methyl-1-butanol, 3-methyl-3-buten-1-ol) are formed by Maillard transformation (non-enzymatic browning reaction) during honey storage processes

(Serra Bonvehí & Ventura Coll, 2003). On the other hand, ethanol is produced by microbiological activities and is an indicator of the fermentation process occurring in honey (Piasenzotto et al., 2003).

The acidity of honey can lead to the migration of plastic additives such as diethyl phthalate and dibutyl phthalate (Koo et al., 2017). In general, phthalates are ubiquitary chemicals used in the plastics industry, and they are present together with other common contaminants in the environment (Warner & Flaws, 2018).

Some previous works have been carried out in order to determine plasticizer residues, especially phthalates, in nectar honey samples and also in royal jelly (Notardonato et al., 2020a, Notardonato et al., 2020b; Zhou et al., 2014). This literature evidence confirms what was observed in our honey samples, indeed, the main presence of diethyl phthalate, dibutyl phthalate, and furfural was observed in all the nectar and analysed honeydew honeys.

Besides these volatile organic components, caprolactam was found in all our honey samples. To the best of our knowledge, no literature data were found about the presence of caprolactam in honey and related products. Caprolactam is a precursor used in the food industry for the production of food packaging materials, indeed, this compound in contact with foodstuff can migrate into it, as reported in one study conducted on animal origin food (Bomfim et al., 2011). Therefore, this could be one of its possible origin in our samples. However, one study conducted on honey produced in Brazil by stingless bees also highlighted the presence of caprolactam between many bioactive compounds identified (Ávila et al., 2018).

5.5 Statistical elaboration

Results were processed using Principal Component Analysis (PCA). Principal Component Analysis was used to reduce the dimensional space of variables and to emphasize the relationship among the botanical origin and volatile organic compounds n, therefore, to highlight a possible differentiation of honeys according to their volatile organic composition.

Five principal components were extracted, with eigenvalues greater than 1, which explain more than 50% (56.0%) of the total variance. In details, the variances explained by each single principal components were 14.1%, 13.0%, 12.0%, 9.0%, and 8.0%, from PC1 to PC5, respectively.

Figure 5.2 show the score plot obtained for the 48 italian honey samples for volatile organic compounds investigated, while the loadings values of all variables of the first five principal component are shown in the Table 5.3.

According to the graph PC1 vs PC2 (Fig. 5.2a), it was possible to note that the second principal component (PC2) allowed to differentiate the rhododendron, chestnut, and also honeydew honeys

from other monofloral honey samples (acacia and apple-dandelion). The variables with the highest loading that allowed to separate the rhododendron samples from other honeys were the presence of two organic acids, that are dodecanoic and tetradecanoic acids, and one cyclic alcohol, the terpineol.

The variables with the highest loading that better characterize the chestnut honey samples were one aldehydic substance, the furfural, and one alcohol, the hotrienol.

About honeydew honeys, the variables that contribute to their separation from other honeys were aliphatic organic acids, so, decanoic, octanoic, dodecanoic and tetradecanoic, one aldehyde, that is octanal, and one alcoholic compound, the terpineol.

On the graph PC1 vs PC3 (Fig. 5.2b), the first principal component permitted to differentiate the apple-dandelion honey between the rest of monofloral honeys. In this case, the variables with higher weight were one cyclic organic acid, the benzoic acid, and two cyclic aldehydic molecules, the benzaldehyde and lilac aldehyde C.

In Fig. 5.2c is plotted the graph PC1 vs PC5, the fifth principal component was the component that characterize the acacia honey sample. Indeed, the variables with the highest loadings were organic acid, acetic acid and geranic acids.

The multifloral honeys, their distribution was influenced according to the percentage of pollen and/or nectar of the plants and flowers collected by honeybees, and used to produce these types of honeys. Therefore, some samples of multifloral honey have distributed close to the monofloral groups. For instance, the multifloral honeys M36, M44, M46-C, M46-P, M46-D, M47-C, M47-P, and M47-D were distributed near to apple-dandelion honey, because they contain significant percentage of *Malys/Pyrus*, *Asteraceae*, and *Robinia* as secondary important pollen. While, M3-18 contains a significant amount of *Ericacea*, so this sample was located close to the group of rhododendron honeys, (see Fig 5.2a). In addition, some multifloral honeys, mainly M23-18, M5-18, M6-18, and M7-18 are located more towards the group of chestnut honey because they contain a important percentage of *Castanea* pollen, so this nectar has influenced its composition (see Fig 5.2a). However, using only the volatile organiccompounds is not sufficient to obtain a very clear differentiation among monofloral honey groups. Therefore, the volatile organic composition cannot be used, as a unique variable, to describe and characterize the different types of monofloral honeys. Indeed, not for all classes of monofloral honeys was possible to identify both a clear group of each type of honey and a specific group of volatile organic substances.

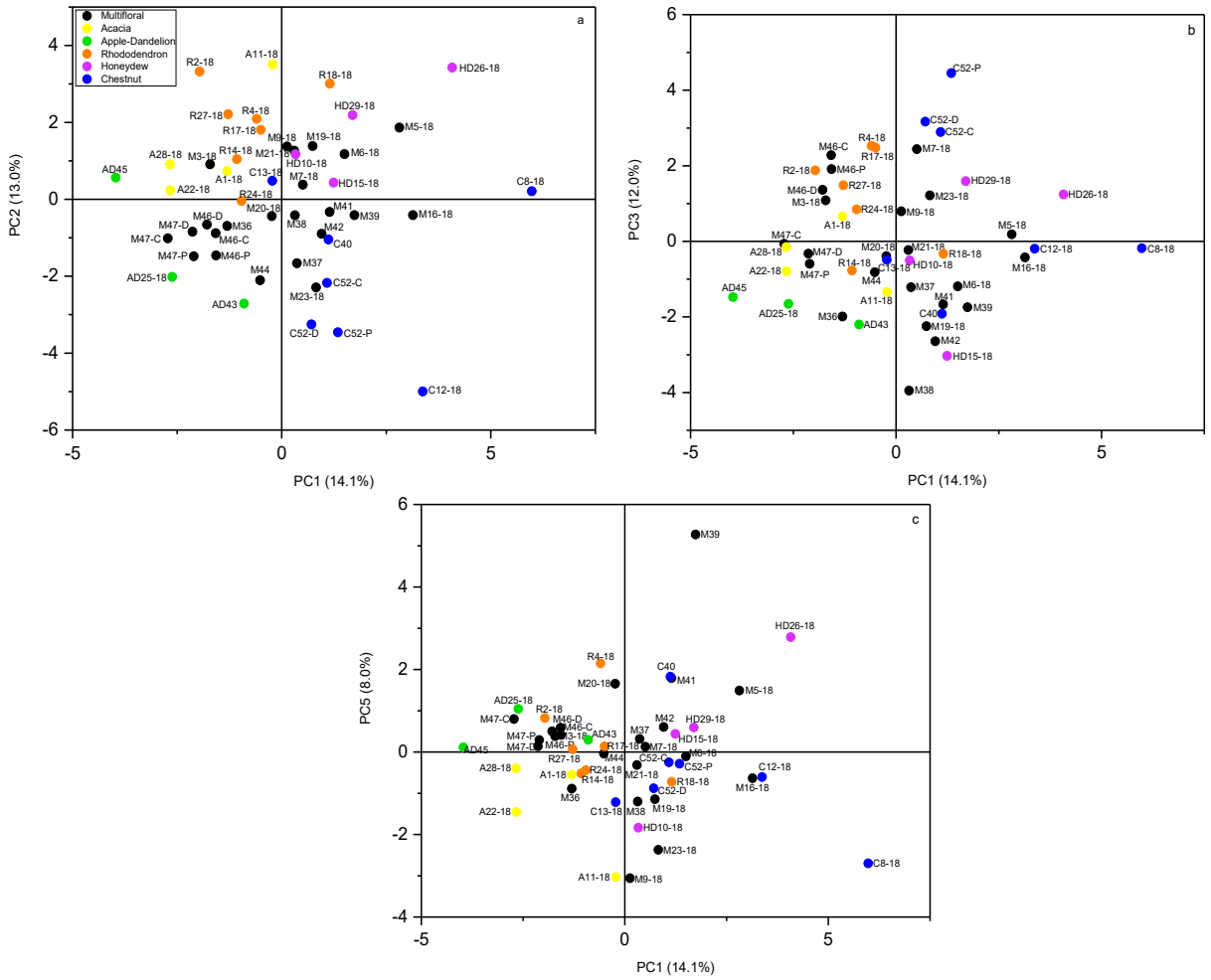

Fig. 5.2 Score plot obtained from principal component analysis about honey sample compositions for the content of carbohydrates and volatile organic compounds in the plane defined by the PC1 and PC2 (a), the PC1 and PC3 (b), PC1 and PC5 (c).

Table 5.3 Loading values of the first fifth components (PC1, PC12, PC3, PC4, and PC5) of 27 volatile organic compounds using principal component analysis.

Variable	PC1	PC2	PC3	PC4	PC5
Nonanal	-0,0094	-0,0288	0,4951	-0,1202	0,1173
Acetic acid	0,1208	0,1832	-0,0944	-0,1620	-0,3486
Furfural	0,1986	-0,2181	0,0963	0,2585	0,0002
Decanal	0,0051	-0,0526	0,4617	0,0528	0,0907
Benzaldehyde	-0,2961	-0,0573	-0,1686	0,2354	0,1129
Lilac aldehyde C	-0,2541	0,1029	0,0586	0,2731	0,2779
Linalool	-0,1597	-0,0226	0,1404	0,1078	0,3008
Hotrienol	-0,1098	-0,1825	0,2749	-0,0437	-0,1527
Benzeneacetaldehyde	0,0694	-0,0216	-0,1160	0,0793	-0,0329
Phenylethyl Alcohol	-0,0907	0,1160	-0,2090	-0,0871	-0,1651
Octanoic acid	0,3010	0,2163	-0,1908	0,0278	0,2275
Caprolactam	0,0538	0,2909	0,1249	0,1666	-0,2872
Nonanoic acid	0,3965	-0,1159	0,1121	0,0529	-0,0587
n-Decanoic acid	0,1962	0,2483	-0,0001	0,2302	-0,0609
Diethyl Phthalate	-0,1990	0,3775	0,1066	0,1455	-0,0228
Benzoic acid	-0,3431	0,0074	0,0170	0,3301	-0,0067
Dodecanoic acid	-0,0762	0,2730	-0,0172	0,2621	-0,0014
Dibutyl phthalate	-0,1078	0,3429	0,1193	0,0785	-0,2403
Tetradecanoic acid	0,0003	0,3895	-0,0422	-0,1530	0,0456
Thymol	0,0545	0,0005	-0,2498	-0,0444	-0,0529
Geranic acid	0,2656	-0,1174	0,1410	0,2973	-0,2658
Octane	0,2015	0,1475	-0,0172	-0,0646	0,4158
Terpineol	0,0205	0,2233	0,2104	-0,3382	0,1295
g-Terpinene	0,2802	0,0415	-0,0132	0,4069	0,0312
2-Nonanone	0,1661	-0,0318	-0,2237	0,1420	0,3396
Heptanoic acid	0,1881	0,1367	0,2362	0,0927	0,0279
Octanal	0,1521	0,2326	0,1179	-0,1279	0,2059

5.6. Conclusion

In this chapter, the volatile organic compounds of honeys harvested in Trentino Alto-Adige were analysed. A total of 80 volatile organic molecules were identified, of these 27 substances were used in order to characterize the honey samples with different floral characteristics.

The composition of volatile organic compound was useful to characterize honey samples according to their different botanical origin, although the separation between different groups of honeys was not complete. This multivariate analysis of data was carried out by one chemometric approach to establish the relationship between the profile of the volatile organic components and botanical origin of honey samples.

In general, a separation was obtained for monofloral honeys, the multifloral honeys weredistributed close to the monofloral groups, in accordance with their composition of pollens, which explain their distribution in the multivariate space.

However, the results evidentiated that only volatile organic composition was not sufficient in order to univocally characterize monofloral honeys.

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Chapter 6. Characterization and differentiation of honey samples using physicochemical and chemical parameters.

6.1 Introduction

The aim of this chapter was to evaluate the characterization of honeys using all parameters described and analyzed separately in the previous chapters: the physicochemical parameters (pH, °Brix index, and moisture content) and chemical composition (carbohydrates, volatile substances, and isotopic composition). Therefore, the main purpose is to differentiate and to classify the honey samples object of this study harvested in different valley of Trentino Alto-Adige with different floral origins.

The approach was chemometric using Principal Component Analysis in order to reduce the dimensionality of this multivariate study, by the combination of more variables to found out the relationship between botanical origin and the parameters determined.

The data used for statistical analysis was treated by autoscaling. Therefore, the mean and variance of each variable were zero and one, respectively.

6.2 Results

6.2.1 Statistical elaboration

The Principal Component Analysis was used to investigate the relationships among the floral origin of Italian honeys, both multifloral and monofloral samples, according to the composition of carbohydrate mixture and the volatile organic components. In the first step, were also included the physicochemical parameters (pH, °Brix index, and moisture content) and isotope composition (C bulk, C protein, N protein, and sulfur protein) but the loadings of these variables had minor influence in comparison with sugars and volatile organic compounds, as emphasized in previous chapters, these variables seem more related to the geographical origin than botanical origin.

Five principal components were identified with eigenvalues greater than one, which explain more than 50% (54.3%) of the total variance. In particular, the explained variance by principal components were as following 18.5% for PC1, 12.5% for PC2, 8.7% for PC3, 8.0% for PC4, and 6.6% for PC5.

Figure 6.1 shows the score plot obtained for the 48 honey samples using the investigated variables, the loadings values for the variables of the first five principal components are reported in the Table 6.1.

The information about descriptive characteristic, sample code and floral origin are reported in

Table 2.1, Chapter 2. While, the pollen percentage of all honey samples are reported in Table 2.2, Chapter 2.

The results obtained for the first two components is presented in the graph PC1 vs PC2 (Fig 6.1a), it can be observed that the first principal component (PC1) differentiates, one group containing both the acacia and rhododendron honey samples from the other monofloral honey types, however this component don't allow the separation among these two groups of honeys.

The first two principal components differentiated honeydew honey samples from the other monofloral honeys. The variables with the highest loading that characterize these samples are the presence of some carbohydrates, especially melezitose, raffinose, and erlose, and the presence of volatile organic components, mainly represented by aliphatic organic acids, that are octanoic acid, tetradecanoic acid, and heptanoic acid, and one aldehyde compound, the octanal (see Table 6.1).

In addition, the second principal component (PC2) also separates the apple-dandelion samples, the variables which contribute to differentiate these honeys are the concentration of some carbohydrate compounds than those of honeydews, in particular a monosaccharide, glucose, and two disaccharides such as palatinose and turanose. On the other hand, these samples seem not to be characterized or represented by the presence of volatile organic compounds, only the benzaldehyde content seem to contribute at the characterization of this honey type (see Fig 6.1a and Table 6.1).

The fourth principal component (PC4) differentiates the most of chestnut honey samples, with the exception of only two samples that are distributed outside this group. The variables with the highest loading are some volatile organic compounds that characterize these monofloral honeys, especially aldehydes, such as nonanal, decanal, furfural, one alcohol represented by hotrienol, and lastly two organic acids, nonanoic and geranic acid (see Fig 6.1b).

According to the graph with the first and fifth principal components (PC1 vs PC5), (see Fig 6.1c) it was possible to observe that the fifth principal component (PC5) differentiates and separates the acacia honeys and the rhododendron samples.

According to the obtained results, the variables with the highest loadings values, and therefore characterize the acacia samples, are represented by volatile organic compounds, mainly phenylethyl alcohol and benzeneacetaldehyde, while the variables which do not contribute to differentiate the acacia from rhododendron honeys are sugars such as fructose and sucrose.

Regarding rhododendron honeys, the compounds that characterize these samples are represented by the variable with the highest values such as carbohydrates, principally erlose, nigerose and kojibiose. While, the volatile organic compounds that better characterize these samples were aldehyde as lilac aldehyde C, octanal, and nonanal, and one organic acid, the dodecanoic acid, and one monoterpenic alcohol, the terpineol.

The multifloral honeys are naturally produced by several nectars and/or pollen plants and flowers, therefore, they are distributed according to the percentage of nectar and/or pollen. Observing their distribution, they are positioned close to the various groups of monofloral honeys. Some multifloral honey, as M23-18, M7-18 and M9-18, were near to the chestnut honeys group because they present a significant percentage of chestnut pollen *(Castanea)*; whereas the M19-18 sample, another multifloral honey, was classified from the Principal Component Analysis close to the group of apple-dandelion samples, indeed this sample contain a high percentage of apple tree pollen *(Malus/Pyrus)*. The samples M20-18 and M21-18 contain an important amount of rhododendron pollen *(Ericaceae),* and M46-C and M46-D samples is significantly affected from acacia pollen *(Robinia)*, therefore were located close to the rhododendron and acacia honey, respectively (see Fig. 6.1b).

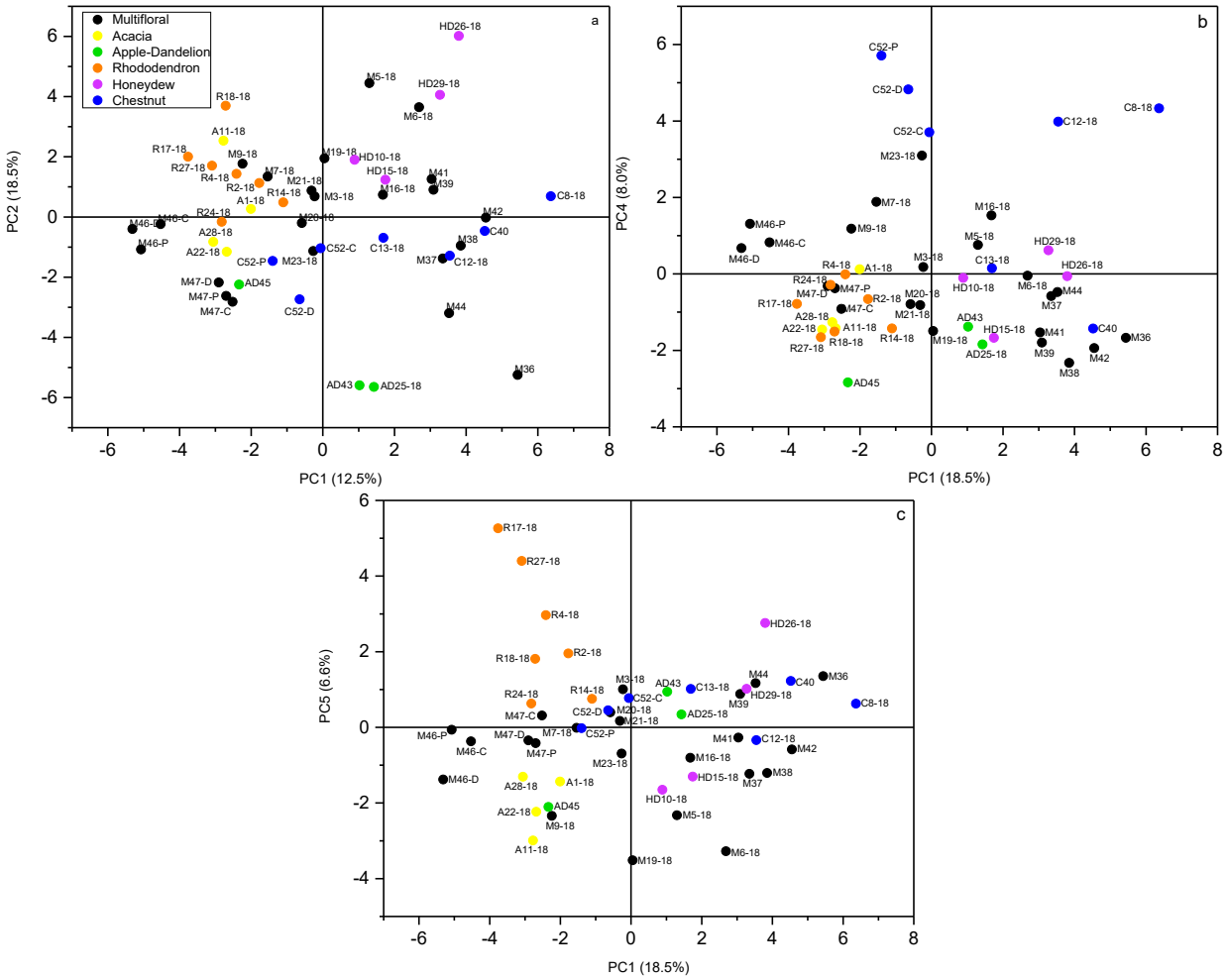

Fig. 6.1 Score plot obtained from principal component analysis about honey sample compositions for the content of carbohydrates and volatile compounds in the plane defined by the PC1 and PC2 (a), the PC1 and PC4 (b), PC1 and PC5 (c).

Table 6.1 Loading values of the first fifth components (PC1, PC12, PC3, PC4, and PC5) of different variables obtained from statistical analysis using principal component analysis.

Variables	PC1	PC2	PC3	PC4	PC5
pH	0,2900	0,0523	-0,1033	0,1230	-0,0288
°Brix	0,0990	-0,1050	-0,3186	-0,0742	0,2073
Moisture	-0,1075	0,0848	0,3121	0,0798	-0,1963
13Cprotein	-0,1622	0,1433	0,1893	0,0194	-0,0337
15Nprotein	-0,1053	-0,1230	-0,2065	-0,0505	-0,2271
32Sprotein	0,0839	0,2156	0,0274	-0,0311	-0,1760
13C bulk	0,0358	0,0254	0,1273	0,0081	0,0467
Glucose	-0,1399	-0,2733	0,0391	-0,0803	0,0825
Fructose	-0,2061	-0,1980	-0,0346	0,1939	-0,0407
Sucrose	-0,2034	-0,0256	-0,1335	-0,0303	0,0891
Melibiose	0,2526	-0,0142	-0,1040	-0,0821	-0,1657
Lactose	0,2935	-0,1166	0,0972	0,0280	0,0920
Lactulose	0,2462	-0,1252	0,2080	-0,0113	0,1237
Kojibose	0,1438	-0,0487	0,1557	0,0634	0,2259
Turanose	0,1912	-0,2354	0,0906	-0,0695	0,1428
Palatinose	0,1817	-0,2353	0,1221	-0,0888	0,0069
Nigerose	0,1984	-0,0725	0,2345	0,1302	0,2381
Melezitose	0,1275	0,2212	-0,0901	-0,0004	-0,0108
Raffinose	0,2177	0,1752	-0,2092	-0,1090	-0,0104
Isomaltotriose	0,2254	-0,1394	0,1845	-0,0655	-0,0076
Erlose	-0,0834	0,1758	-0,0589	-0,1910	0,2667
Nonanal	-0,1428	0,0646	-0,1212	0,2905	0,1923
Acetic acid	0,0111	0,1560	0,1966	-0,0019	-0,1861
Furfural	0,0783	-0,0090	-0,0912	0,2611	-0,0762
Decanal	-0,1153	0,0564	-0,0949	0,3126	0,1548
Benzaldehyde	-0,0895	-0,1742	-0,0568	-0,2189	-0,0814
Lilac aldehyde C	-0,1285	-0,0273	0,0089	-0,1498	0,2577
Linalool	-0,0914	-0,0408	-0,0219	-0,0210	0,1677
Hotrienol	-0,1706	-0,0670	-0,0688	0,2314	-0,1464
Benzeneacetaldehyde	0,0580	0,0376	-0,0440	-0,0465	-0,2080
Phenylethyl Alcohol	-0,0052	0,0536	0,0818	-0,1753	-0,3076
Octanoic acid	0,1269	0,2536	-0,1087	-0,1193	-0,0103
Caprolactam	-0,0655	0,2064	0,2761	0,0925	-0,0298
Nonanoic acid	0,1504	0,0754	-0,0235	0,3235	-0,0314
n-Decanoic acid	0,0388	0,1801	0,0662	0,0070	-0,0909
Diethyl Phthalate	-0,1665	0,1510	0,1376	-0,1709	0,0903
Benzoic acid	-0,1786	-0,1344	0,0506	-0,1227	0,0244

Variables	PC1	PC2	PC3	PC4	PC5
Dodecanoic acid	0,0052	0,0591	0,2355	-0,1103	0,2309
Dibutyl phthalate	-0,1198	0,1506	0,2488	-0,0833	0,0513
Tetradecanoic acid	-0,0111	0,2501	0,0555	-0,2183	0,0520
Thymol	0,0970	-0,0099	0,0664	-0,1100	-0,1314
Geranic acid	0,0647	-0,0022	0,1105	0,3223	-0,0584
Octane	0,0920	0,1959	-0,1941	-0,0462	0,0921
Terpineol	-0,0734	0,1704	-0,0353	-0,0268	0,1945
γ-Terpinene	0,0973	0,0821	0,0458	0,1224	-0,0030
2-Nonanone	0,1342	0,0524	-0,1772	-0,1213	-0,0178
Heptanoic acid	-0,0090	0,1920	0,0028	0,1878	0,1339
Octanal	0,0702	0,2053	-0,0861	0,0173	0,1999

6.3 Conclusion

According to the results, it was possible to note that the chemical parameters, as carbohydrates and volatile organic compounds, allow defining and differentiating the honey samples of our study with different botanical/floral origin.

Indeed, using Principal Component Analysis, it was possible assumed that honeys with different floral origin present a characteristic composition in organic components, while the physicochemical parameters and the isotope composition of light elements do not contribute to the differentiation of honeys on the base of their botanical origin.

This can be explained because the volatile organic compounds and sugar components are strongly associated with the raw material used by honeybees for production of honey, so mainly nectar and honeydew sources. On the other hand, the physicochemical factors and the isotope composition of light elements are related to external factors, such as the characteristics of geographical origin in terms of climatic and/or environmental conditions, treatment procedures, handling, storage of honey samples.

Chapter 7. Final consideration

This dissertation was focused on the characterization of the quality of honey samples of different botanical/floral origin, obtained in the Alpine ecosystem area of the Trentino Alto-Adige.
The quality parameters investigated were both physicochemical, such as pH, the °Brix index and the moisture content, and chemical, such as carbohydrates (monosaccharides and oligosaccharides), volatile organic compounds, and the stable isotope of lights elements (bulk carbon, and carbon, nitrogen and sulfur from protein).

Honey is susceptible to several modifications, for instance during its production and in particular over its storage time, which affect their physicochemical characteristics. The values of these parameters in the studied honeys, pH, moisture content, and the total soluble solid (°Brix index) were in agreement with those found in literature and in accordance with International and European regulations. However, the statistical analysis showed that these parameters did not provide a correlation with botanical origin. Therefore, it was assumed that these physicochemical factors, although are useful to define the merceological quality, they do not seem useful as variables for a possible differentiation of honeys according to the floral origin.

A new analytical method based on high-performance anion-exchange chromatography coupled with a mass spectrometry detector (HPAEC-MS) was developed to determine sugars. This innovative method allowed investigating the monosaccharides and one extended group of oligosaccharides in honey samples, and the reduction time of analysis with a simple and fast pre-analytical procedure, based on honey sample dilution with ultrapure water
Oligosaccharide were identified in honey samples, together with fructose and glucose, the most abundant carbohydrates found in honey. The composition of disaccharides and trisaccharides showed they are associated with the floral origin and the aging of honey, as showed by statistical elaboration. Indeed, disaccharides, as turanose and palatinose, were indicative especially in dandelion honey, while sucrose and erlose were representative of rhododendron honey. While, glucose, fructose, and more melezitose, and raffinose can be useful to characterize honeydew honey. Finally, an important group of oligosaccharides, such as lactose, lactulose, nigerose, and isomaltotriose were related to the storage and the aging of honey. Indeed, the statistical elaboration confirmed the relationship between the profile of the carbohydrates and the botanical origin. In particular, hierarchical cluster analysis and Principal Component Analysis suggested that sugars could undergo modification during the harvest period, so the separation of the honeys collected

during two different years was observed. In addition, these statistical techniques highlighted that the fraction of minor sugars, as oligosaccharides can be useful to differentiate honey samples from various botanical origin. Therefore, only sugars composition was very useful to define and differentiate the samples of honey according to their different botanical characteristics, as well as inter-annual variability.

The isotopic composition of light elements was used as a variable to discriminate the honey samples according to both geographical origin and floral origin. In this case, to assess and emphasize the differentiation of Italian honeys of different geographical origin were compared with some honeys from different countries. Based on results of stable isotopes of light elements, the hierarchical cluster analysis showed that is possible to separate honey samples from Trentino Alto-Adige from those harvested in other world's region. Indeed, the isotopic composition of Trentino Alto-Adige honey grouped the samples in a single cluster; the samples of Trentino were also separated from other Italian honey samples produced in other regions, such as Lazio and Sicily. Unfortunately, considering the closeness of the different valleys of origin, it was not possible to discriminate the samples according to their valley origin. Therefore, the stable isotope of light elements can represent a usefull proxy to differentiate the geographical provenance of honey. However, by a larger data set other statistical technique could be useful to achieve complete differentiation of honeys according to the geographical origin.

Volatile organic components were analysed in the honey samples, and the composition of volatile substances was useful to discriminate the honey samples based on their different floral origin.
Although, the principal classes of volatile organic compounds, i.e. organic acids, aldehyde, and alcohol contributed to differentiate the honey samples, the statistical analysis showed the separation with the different botanical origin was not as evident as desired. Therefore, it was assumed that need to exted to obtain a good differentiation between honeys of different floral type. However, the study of the composition of the volatile organic compounds improve the characterization of the alpine honey produced in the Trentino Alto-Adige and to assess the quality of the local production.

Multivariate statistical analysis emphasized that carbohydrate composition and volatile organic compounds together allowed the discrimination and differentiation of honey according to the botanical origin. This can be explained because volatile organic compounds and carbohydrates are strongly related to the raw material (nectars and honeydew) used by honeybees to produce honey.

On the other hand, the physicochemical factors and the isotope composition showed a minor contribution to differentiate the samples. The physicochemical parameters and the isotopic of light elements seem mainly related to external factors, such as climatic conditions, geographical characteristics, as well as treatment, extraction, handling, and storage conditions of honey.

Investigation were carried out to test different extraction procedures, such as centrifugation, pressing and draining. For this purpose, three honey samples were extracted applying these different extraction procedures, the centrifugation, the pressing and the draining. The same samples were differentiate as M46-C, M46-P, M46-D; M47-C, M47-P, M47-D; C52-C, C52-P, and C52-D. Preliminary results showed that no significant variations were observed on chemical composition and physicochemical parameters of the investigated honeys.
Therefore, seem that the extraction procedures do not affect both chemical composition and physicochemical parameters. However, small variations on the composition could be covered by other factors can not be excluded. Further investigation on this topic are need.

In the next future, studies should be exteded to other important chemical compounds of nutritional or contamination importance, as well trace elements and heavy metals.
In addition, further investigation could be extended to the beehive products for instance propolis, pollen, wax.

APPENDIX A: SAMPLE CODE USED IN THIS DISSERTATION AND SAMPLE CODE EMPLOYED IN THE PUBLISHED ARTICLE.

The Table shows the sample code for honeys used in this dissertation and the sample code for honeys employed in the published article.

Sample code in this dissertation	Sample code in the published article	Sample code in this dissertation	Sample code in the published article
M36	/	A1-18	A1
M37	M37	A11-18	A11
M38	M38	A22-18	A22
M39	M39	A28-18	A28
M41	M41	AD43	MT43
M42	M42	AD45	MT45
M44	M44	AD25-18	MT25
M3-18	M3	R2-18	R2
M5-18	M5	R4-18	R4
M6-18	M6	R14-18	R14
M7-18	M7	R17-18	R17
M9-18	M9	R18-18	R18
M16-18	M16	R24-18	R24
M19-18	M19	R27-18	/
M20-18	MM20	HD10-18	B10
M21-18	/	HD15-18	MA15
M23-18	M23	HD26-18	MB26
M46-C	A46	HD29-18	MA29
M46-D	/	C8-18	
M46-P	/	C12-18	
M47-C	A47	C13-18	
M47-D	/	C40	
M47-P	/	C52-C	
MARG14	MARG14	C52-D	
MARG733	MARG733	C52-P	

Note:
Samples such as T36, M40, M48, M49, R2, and TC52, due to the small amount, were used only for the determination of carbohydrates composition. Therefore, they were included only in the published article.

Sample as M36, M21-18, M46-D, M46-P, M47-D, M47-P, C8-18, C12-18, C13-18, C40, C52-C, C52-P, C52-D, and R27-18 were sampled and analysed after the publication of the article. Consequently, these samples were involved in the present dissertation.

APPENDIX B: PUBLISHED ARTICLE

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

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RESEARCH PAPER

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 MΩ 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 $^{13}C_6$-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 $[M-H]^-$ 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 $[M-H]^-$ 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 (kojibiose). 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: $A = (\mathrm{RW5\%} + \mathrm{LW5\%})/(2 \times \mathrm{LW5\%})$, 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 (Rs) 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 ($^{13}C_6$-glucose) of 1 mg L^{-1}. By considering the ratio between the peak area of

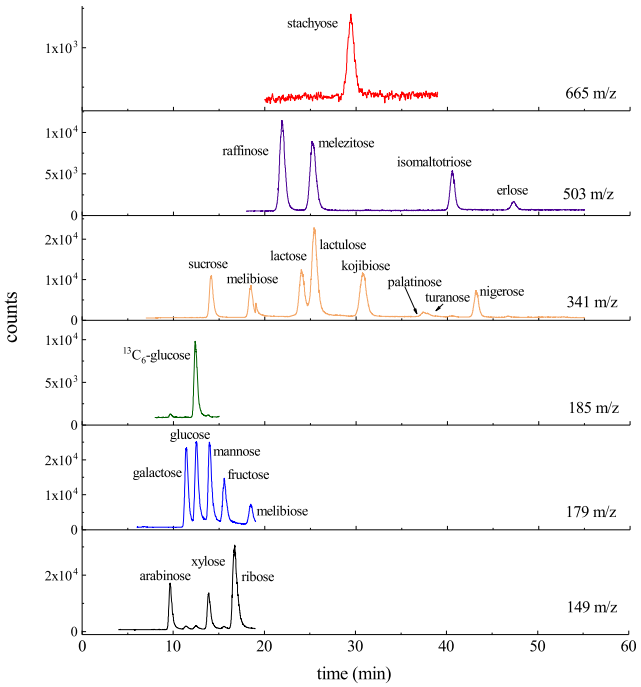

Fig. 1 HPAEC-MS chromatogram of standard solutions of carbohydrate. Column CarboPac PA10 (2 × 150 mm, 10 μm)

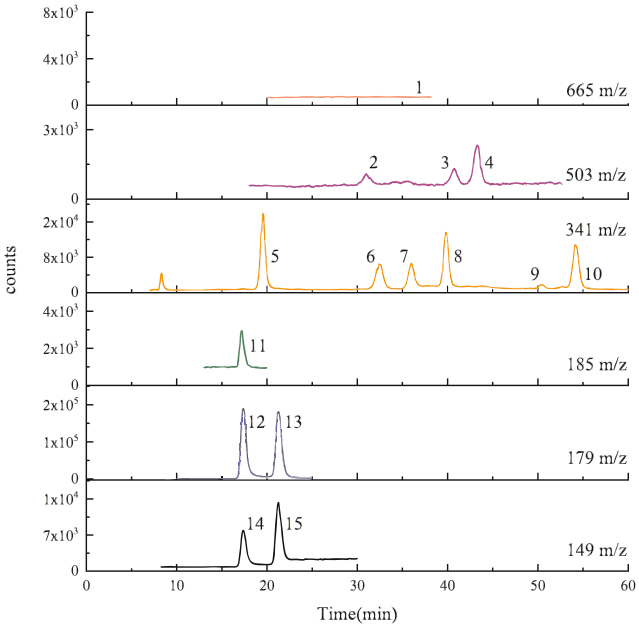

Fig. 2 HPAEC-MS chromatogram of one unifloral honey sample (rhododendron) using the developed method; the identified carbohydrate are 1 = stachyose (below MDL), 2 = melezitose, 3 = isomaltotriose 4 = erlose, 5 = sucrose, 6 = lactose, 7 = lactulose, 8 = kojibiose, 9 = palatinose, 10 = nigerose, 11 = $^{13}C_6$-glucose (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}(turanose); 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 turanose, 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, turanose, 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

This study							LOD (mg L^{-1}) previous study		
Carbohydrate	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^4D[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				
Erlose	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-C^4D* 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 multifloral, 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 multifloral, 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 unifloral and multifloral 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

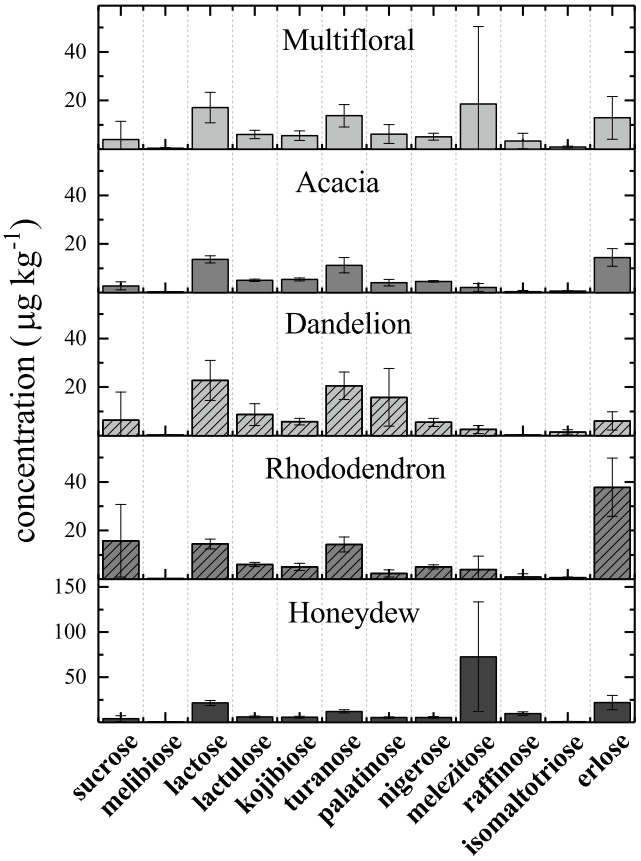

Fig. 3 Average concentration and standard deviation of oligosaccharides in multifloral, acacia, dandelion, rhododendron, and honeydew honeys

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 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 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.

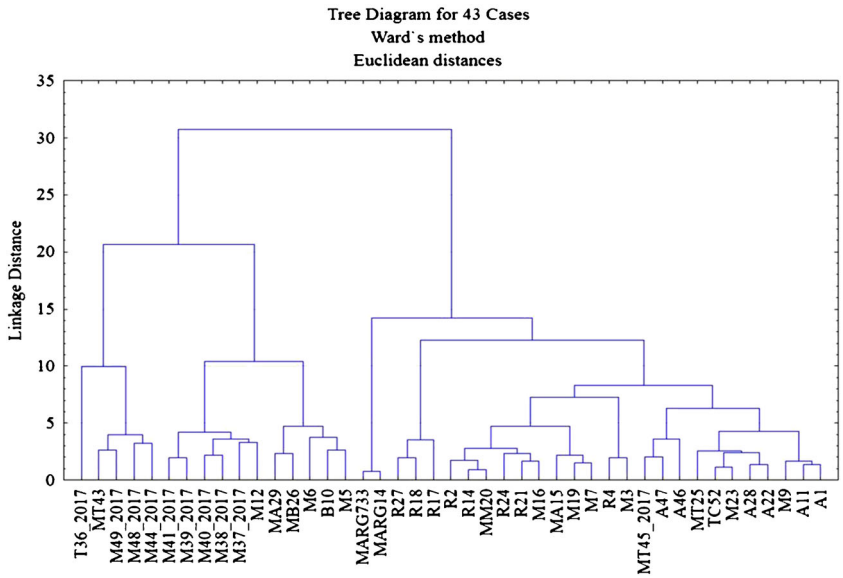

Fig. 4 Dendrogram of the hierarchical cluster analysis obtained for the honey sample content of carbohydrates

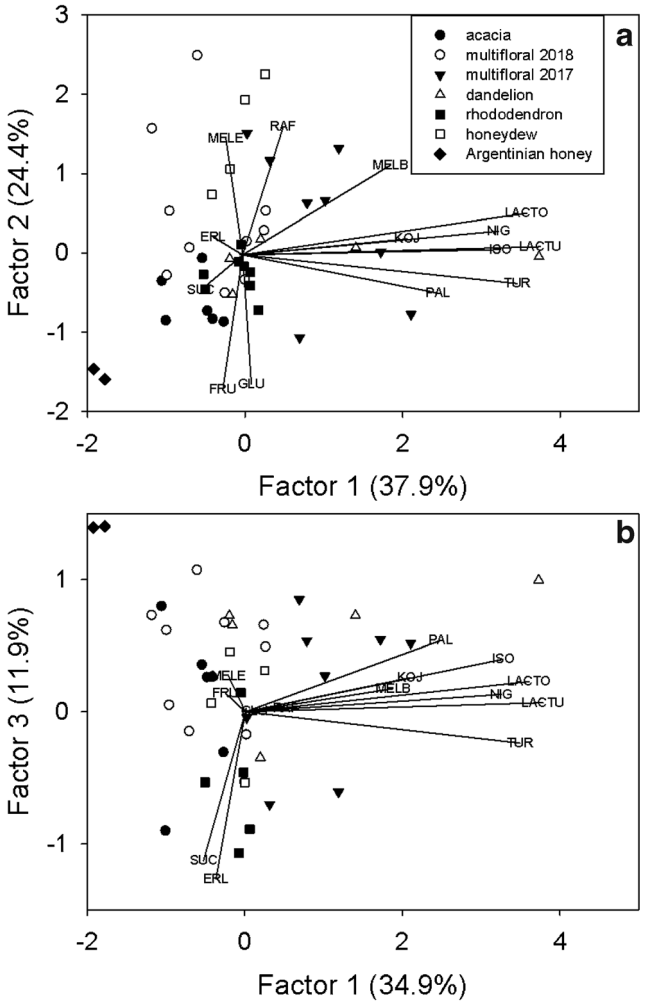

Fig. 5 Factor analysis biplot relative to the honey sample compositions for the content of carbohydrates in the plane defined by the factors 1 and 2 (**a**) and the factors 1 and 3 (**b**); PAL palatinose, RAF raffinose, MELE melezitose, KOJ kojibiose, ISO isomaltotriose, MELB melibiose, LACTO lactose, 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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Supplementary materials

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

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Supplementary Table

Table S1. Descriptive characteristics of honey samples analyzed in this work.

Multifloral honey				Monofloral and honeydew honey			
key	**Honey type**	**Geographical area**	**Harvest year**	**Key**	**Honey type**	**Geographical area**	**Harvest year**
M3	Multifloral	Val di Non	2018	A1	Acacia	Valsugana	2018
M5	Multifloral	Val di Non	2018	A11	Acacia	Valsugana	2018
M6	Multifloral	Val di Non	2018	A22	Acacia	Val d'Adige	2018
M7	Multifloral	Valsugana	2018	A28	Acacia	Val di Non	2018
M9	Multifloral	Valsugana	2018	MT25	Dandelion	Val d'Adige	2018
M12	Multifloral	Valsugana	2018	T36	Dandelion	Val di Fiemme	2017
M16	Multifloral	Val di Fiemme	2018	MT43	Dandelion	Val di Non	2018
M19	Multifloral	Val di Fiemme	2018	MT45	Dandelion	Valsugana	2017
MM20	Multifloral	Val di Fiemme	2018	R2	Rhododendron	Valsugana	2018
M23	Multifloral	Valsugana	2018	R4	Rhododendron	Val di Non	2018
M37	Multifloral	Val di Fiemme	2017	R14	Rhododendron	Val di Fiemme	2018
M38	Multifloral	Val di Fiemme	2017	R17	Rhododendron	Val di Fiemme	2018
M39	Multifloral	Val di Fiemme	2017	R18	Rhododendron	Val di Fiemme	2018
M40	Multifloral	Val di Fiemme	2017	R21	Rhododendron	Val di Fiemme	2018
M41	Multifloral	Val di Cembra	2017	R24	Rhododendron	Valsugana	2018
M44	Multifloral	Val d'Adige	2017	R27	Rhododendron	Val di Non	2018
A46	Multifloral	Val d'Adige	2018	B10	Honeydew	Val di Non	2018
A47	Multifloral	Val d'Adige	2018	MA15	Honeydew	Val di Fiemme	2018
M48	Multifloral	n.a.	2017	MB26	Honeydew	Val di Non	2018
M49	Multifloral	n.a.	2017	MA29	Honeydew	Val di Non	2018
TC52	Multifloral	Val di Fiemme	2018				

Multifloral honey				Monofloral and honeydew honey			
key	**Honey type**	**Geographical area**	**Harvest year**	**Key**	**Honey type**	**Geographical area**	**Harvest year**
MARG14	Multifloral	n.a.	2018				
MARG733	Multifloral	n.a.	2018				

Table S2. Chromatographic parameters of oligosaccharides separation by CarboPac PA10 of the standard solution: retention time (tR), peak width, number of theoretical plates (N) and height of theoretical plates (H), asymmetry (A) and resolution factor (Rs) . Resolution is calculated between each peak and that immediately preceding.

Compounds	tR (min)	Peak width (min)	Asymmetry factor (A)	N° of theoretical plates (N)	Height of theoretical plates (H) (µm)	Resolution factor (Rs)
Arabinose	9.3	0.5	1.4	5230	48	
Xylose	13.3	0.6	1.8	8593	29	6.6
Ribose	16.0	0.8	1.6	6947	36	3.3
Galactose	11.2	0.4	1.6	14981	17	
Glucose	12.4	0.6	1.3	8156	31	1.9
Mannose	13.8	0.6	1.5	9358	27	2.3
Fructose	15.5	0.3	1.3	37708	7	2.5
Sucrose	14.2	0.7	1.3	7311	34	
Melibiose	18.6	0.6	0.9	15177	16	6.0
Lactose	24.2	0.8	0.9	16500	15	7.1
Lactulose	25.5	1.0	1.3	10899	23	1.4
Kojibiose	30.9	1.4	1.2	8089	31	4.4
Turanose	37.5	0.6	0.7	70388	4	4.4
Palatinose	37.9	0.8	1.2	39149	6	1.2
Nigerose	43.2	0.7	1.1	61042	4	6.6
melezitose	22.0	1.0	1.2	7478	33	
Raffinose	25.5	0.8	1.1	14480	17	3.0
Isomaltotriose	40.7	0.9	1.1	33149	8	14.0
Erlose	47.4	1.0	1.1	34843	7	6.7
Stachyose	29.7	0.7	0.9	29979	8	

Table S3. Mass to charge ratio (m/z) of $[M-H]^-$ used for the quantification, cone voltage (V) and time range acquisition (min) for each compound determined.

Carbohydrates	**$[M-H]^-$**	**Cone Voltage (V)**	**Time range acquisition (min)**
Arabinose, xylose, ribose	149	40	4-19
Galactose, glucose, fructose, mannose	179	40	6-19
Sucrose, palatinose, turanose, melibiose, kojibiose, nigerose, lactose, lactulose	341	40	7-55
Raffinose, melezitose, isomaltotriose, erlose	503	60	18-55
Stachyose	665	70	20-55
$^{13}C_6$-glucose	185	40	8-15

Table S4. Repeatability and reproducibility. Data were expressed as mean concentration of carbohydrates (mg kg^{-1}) and standard deviation (SD).

	Repeatability	**Reproducibility**
Carbohydrate	**Mean ± SD (mg kg^{-1})**	**Mean ± SD (mg kg^{-1})**
Glucose	144150 ± 3303	146919 ± 12212
Fructose	351015 ± 11975	351975 ± 24211
Sucrose	19201 ± 157	19707 ± 1538
Lactose	8267 ± 87	8376 ± 685
Lactulose	3640 ± 202	3676 ± 280
Kojibiose	2767 ± 233	2740 ± 457
Turanose	7307 ± 650	7170 ± 871
Nigerose	3303 ± 190	3356 ± 345
Melezitose	608 ± 38	634 ± 77
Raffinose	266 ± 10	256 ± 25
Isomaltotriose	212 ± 5	224 ± 19
Erlose	286597 ± 12625	294439 ± 22812

Table S5. Average concentration of carbohydrate in multifloral, acacia, dandelion, rhododendron and honeydew honeys. Data were expressed in mg kg^{-1}.

	Carbohydrate (mg kg-1)													
key	**Glucose**	**Fructose**	**Sucrose**	**Melibiose**	**Lactose**	**Lactulose**	**Kojibiose**	**Turanose**	**Palatinose**	**Nigerose**	**Melezitose**	**Raffinose**	**Isomaltotriose**	**Erlose**
A1	137757	367408	2379	256	15481	5309	5159	10407	2855	5040	3399	1037	710	14241
A11	142142	381360	1037	174	12772	4687	5005	7207	2934	4388	842	166	610	9564
A22	155767	419521	4950	298	14272	4723	5225	14518	5497	4326	611	91	683	16045
A28	161626	399540	2683	226	12255	5692	6304	12807	4968	4584	3622	351	449	17925
M3	142386	356529	1636	291	18947	6670	9888	10967	2506	7068	51702	3151	757	14078
M5	111456	308560	1049	313	13183	4800	4547	7887	2379	4243	89841	4757	657	10999
M6	105756	291449	903	686	18256	6419	5948	6770	3193	4814	130263	6477	820	5546
M7	146359	356609	8941	277	13551	4610	5259	9254	1900	4509	1905	2902	755	20677
M9	132956	362067	1237	142	11908	4997	4546	9827	2866	4589	4894	230	682	9373
M12	131703	349013	835	447	22216	6486	6199	13314	5289	5592	5815	1582	965	5850
M16	157990	351825	3522	316	18201	5853	6191	13973	5172	4762	6099	5602	841	24245
M19	145468	330871	3052	407	14401	4372	4636	7134	2334	3841	2527	4132	602	22988
MM20	159719	368122	5220	260	14906	5780	5603	16154	4252	4943	2805	2121	799	29246
M23	165145	374027	1012	259	17256	5533	5970	12788	2883	5347	7525	372	723	6775
M37	152603	333638	645	764	23170	7781	3695	16358	6823	5293	22045	4834	1638	7464
M38	140283	326943	869	553	21716	8268	6345	19243	9904	5985	17270	6384	1178	10726
M39	141141	308652	2338	633	17240	6919	3900	18246	6388	5174	14298	9299	647	28905
M40	125140	300994	2073	718	23738	7816	5564	22445	9203	6189	10169	8543	841	23368
M41	125599	296125	1846	535	18682	6695	4105	14617	5651	4325	28418	9934	1091	18498
M44	152625	363453	812	394	29442	8161	9935	19541	16071	6690	19364	4990	1312	13147
A46	147701	421917	33008	251	10331	3503	3379	10856	3612	3323	1928	128	640	16674
A47	160948	396874	18842	232	13697	4315	5359	15275	7214	4766	2692	357	686	14119
M48	165086	402131	262	388	27639	10148	8849	22512	8704	8743	2989	900	1334	3709
M49	177084	400760	398	199	19604	7434	6892	15920	5253	6929	251	< LOD	1302	2574

Carbohydrate (mg kg-1)														
key	**Glucose**	**Fructose**	**Sucrose**	**Melibiose**	**Lactose**	**Lactulose**	**Kojibiose**	**Turanose**	**Palatinose**	**Nigerose**	**Melezitose**	**Raffinose**	**Isomaltotriose**	**Erlose**
TC52	146196	388348	2274	262	16257	5742	5898	13776	4901	5535	4210	1092	763	6134
MARG14	190003	398866	637	< LOD	4467	3557	2465	10507	14276	2261	269	< LOD	< LOD	636
MARG733	192606	388508	659	< LOD	4464	3381	2444	8412	11638	2499	305	< LOD	< LOD	516
B10	120186	319467	1178	357	14746	5987	5359	16890	7577	5307	71175	4506	622	10099
MA15	148602	321044	2043	403	18228	5373	4528	10330	5066	4659	7113	7274	617	19868
MB26	122182	292669	7757	272	23624	5701	5941	12181	6020	5303	83652	10969	790	30724
MA29	116900	296231	2347	364	22410	7206	6595	13785	4907	6372	127237	10599	938	15146
MT25	178651	386746	980	217	14560	5153	4573	15882	8524	4328	3388	417	1096	9038
T36	156462	355811	163	443	33750	15153	5125	27912	32838	7264	748	220	2974	621
MT43	186749	414952	706	333	23526	8350	7674	21849	14843	6468	4406	424	1066	6510
MT45	179847	385330	23791	262	19111	6083	5936	16347	6958	3862	1666	158	980	8171
R2	150873	342192	9793	183	14418	6219	6636	13236	1146	5457	1482	483	641	36050
R4	141390	351310	2340	175	17544	5983	7913	10721	2753	6550	17148	1120	658	19144
R14	159102	349668	6888	258	15079	5931	5272	15676	3343	5546	1516	984	746	34084
R17	161747	366501	45383	140	12815	6841	3967	20569	3223	4433	1702	307	< LOD	53822
R18	152879	346089	28241	165	11216	5318	3833	11931	1221	4314	778	279	573	44273
R21	168823	369696	4918	393	16567	6143	4227	13953	4606	4779	5217	3896	903	29832
R24	162749	375678	6991	295	14860	4639	4081	12311	2021	4023	856	236	910	31701
R27	156354	356956	21796	79	13379	7212	4807	15483	< LOD	5295	2386	138	543	53248

Table S6. Factor Scores (Factor 1, Factor 2 and Factor 3) of honey samples obtained by factorial analysis using Varimax rotation.

Key	Factor1	Factor 2	Factor 3
A1	-0,54231	-0,06545	0,35652
A11	-1,05545	-0,35208	0,79917
A22	-0,40968	-0,83510	0,26475
A28	-0,47701	-0,73376	0,26278
M3	0,26438	0,52912	0,49160
M5	-1,18127	1,56923	0,73108
M6	-0,60588	2,49070	1,07340
M7	-0,70429	0,06523	-0,14708
M9	-0,99319	-0,28115	0,61840
M12	0,24074	0,28271	0,65824
M16	0,02123	0,14658	-0,17293
M19	-0,95960	0,52793	0,05143
MM20	-0,00152	-0,33782	-0,53074
M23	-0,25674	-0,50573	0,67593
M37_2017	0,78809	0,62869	0,53759
M38_2017	1,01927	0,66160	0,27283
M39_2017	0,31692	1,16429	-0,70150
M40_2017	1,19173	1,31981	-0,60635
M41_2017	0,02650	1,50704	-0,04346
M44_2017	1,72270	0,01376	0,54674
A46	-1,00473	-0,85492	-0,89986
A47	-0,27016	-0,87018	-0,30747
M48_2017	2,10771	-0,77651	0,51882
M49_2017	0,69328	-1,07031	0,84975
TC52	-0,15686	-0,41738	0,65499
MARG14	-1,77560	-1,59438	1,40504
MARG733	-1,91772	-1,46508	1,39524

Key	Factor1	Factor 2	Factor 3
B10	-0,18884	1,05867	0,45364
MA15	-0,42336	0,73248	0,06496
MB26	0,00124	1,93351	-0,53587
MA29	0,25439	2,25355	0,31269
MT25	-0,19406	-1,02266	0,72693
T36_2017	3,73047	-1,01487	0,99673
MT43	1,40621	-1,33791	0,72944
MT45_2017	0,19806	-1,06484	-0,34789
R2	-0,07426	-0,11153	-1,06907
R4	-0,04956	0,10144	0,14396
R14	0,06495	-0,24392	-0,88714
R17	0,16958	-0,72083	-3,71117
R18	-0,52736	-0,27195	-2,16899
R21	-0,01189	-0,17189	-0,45988
R24	-0,50291	-0,45640	-0,53474
R27	0,06680	-0,40967	-2,46850

Table S7. Factor loadings (Factor 1, Factor 2 and Factor 3) of carbohydrates obtained by factorial analysis using Varimax rotation.

Key	Factor 1	Factor 2	Factor 3
Glucose	0,027418	-0,881271	0,005315
Fructose	-0,063138	-0,916578	0,103731
Sucrose	-0,133245	-0,241189	-0,826375
Melibiose	0,470153	0,610546	0,128491
Lactose	0,903794	0,283560	0,167999
Lactulose	0,947325	0,055227	0,050734
Kojibiose	0,523792	0,108548	0,189085
Turanose	0,873819	-0,194881	-0,173872
Palatinose	0,622477	-0,261869	0,396044
Nigerose	0,814669	0,161516	0,096871
Melezitose	-0,053649	0,791456	0,197338
Raffinose	0,129398	0,873721	0,022162
Isomaltoriose	0,820730	0,035727	0,292533
erlose	-0,093337	0,120669	-0,927980
Expl.Var	4,748816	3,681201	1,959367
Prp.Totl	0,339201	0,262943	0,139955