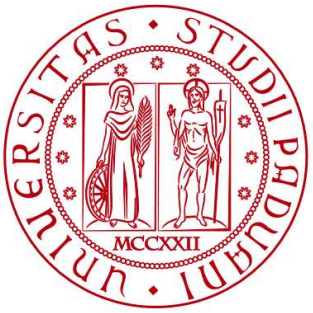

UNIVERSITÀ DEGLI STUDI DI PADOVA

Dip. di Agronomia Animali Alimenti Risorse Naturali e Ambiente
Dip. di Medicina Animale, Produzioni e Salute

Corso di Laurea Magistrale in SCIENZE E TECNOLOGIE ANIMALI

RAPID ASSESSMENT OF ITALIAN POLYFLORAL HONEY QUALITY BASED ON NON-DESTRUCTIVE SPECTRAL SENSORS

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"Tutto è possibile,
basta crederci, nonostante tutto ce l'ho fatta.
Questo mio traguardo lo voglio dedicare a me stessa,
ai miei genitori Gaetano e Michela perché nonostante l'anno passato avete sempre trovato la forza d'incoraggiarmi ad andare avanti e non mollare, grazie per il vostro amore e per aver tollerato i miei pianti e le mie crisi istriche,
a Michael perché senza il tuo amore, il tuo sostegno, il tuo appoggio, la tua presenza e la tua pazienza non sarebbe stata la stessa cosa, grazie di far parte della mia vita,
a mia sorella Greta per la quale vorrei essere fonte d'ispirazione,
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e infine, ai miei parenti più stretti, amici più cari e colleghi per avermi supportato e soprattutto sopportato in questo percorso."

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ABSTRACT

One of the main products of beekeeping is honey, a natural sweet substance produced by bees (*Apis mellifera*) from the nectar of plants or from the secretions of living parts of plants or from substances secreted by sucking insects found on the living parts of plants. The bees collect, transform, integrate with their specific substances, deposit, dehydrate, store, and allow to mature in the honeycombs of the hive, until they obtain "ripe" honey. The polyfloral honey is produced from the blend of nectars collected from various types of wildflowers; its unique aromas and flavours set it apart from other types of honey. In honey, 80% is made up of various sugars, mainly fructose and glucose, 17% represents the water content, and only 3% consists of other nutritional substances, such as nitrogenous compounds, mineral salts, and organic acids.

Originally, methods based on solvent extraction followed by laboratory analysis were used to assess the chemical quality of honey. These wet-chemistry methods were time-consuming, very expensive, and also destructive procedures.

To address the aforementioned issues, the quality of honey is assessed using chemical-physical analyses with official analytical methods, sensory analysis evaluating the perceivable attributes of the product through the five senses, and melissopalinological analysis, primarily used to verify the botanical origin of honeys, but also providing qualitative information and details regarding any processes that the honey may have undergone. Currently, reflectance spectroscopy in the near infrared (NIR) is increasingly being used, thanks to its advantages, as the sample preparation is very simple, quick, and eco-friendly, not using chemical solvents, and it allows for the simultaneous detection of multiple quality parameters.

The aim of this work was to assess whether the use of non-destructive spectral sensors, such as a VIS-based spectrophotometer and NIR-based devices, can assess different chemical, sensory, and instrumental colour quality traits of the polyfloral honey samples from three main ecological areas of Italy: SL = South Lowland, below 600 meters above sea level (asl); NM = North Mountain, above 600 meters above sea level; NL = North Lowland, below 600 m above sea level.

Moreover, the NIR data were used to build a PLS-DA model to assess a proposed overall merit quality class and the quality class of four sensorial traits.

The experimental design of my master's thesis (MSc degree) examined 215 samples of multifloral honey collected from various Italian beekeepers. The geographical and botanical origin, as well as the sensory test, were carried out by trained experts from the Italian "National Honey Observatory" at the end of the 2022 harvest season, during the "Three Drops of Gold - Great Honeys of Italy" competition. The sensory evaluation was conducted according to an official and certified protocol, and related data were kindly permitted to be used for this MSc dissertation.

After a slight thermal treatment (at 40 °C for approximately 30 minutes) of the honey samples, the spectral data were collected using a portable VIS spectrophotometer (operating in the 450-700 nm range) and a benchtop NIR device (operating in the 850-2500 nm range). The chemical traits were estimated using predictive equations from NIR spectroscopy performed in previous studies of UNIPD researchers. The chemical and organoleptic variables have been used to define a quality index useful for assessing the overall quality of honey as a quick tool to distinguish samples collected in different ecological areas. Furthermore, the degree of correlation between the instrumental chromatic coordinates and the sensory characteristics was assessed. Finally, a PLS-DA model was made via NIR spectroscopy data aimed at discriminating the honey overall merit quality class and the quality class of the four sensory traits. It follows from the results obtained that the chemical-physical characteristics are similar to other studies and in line with the limits defined and prescribed by the directive. Sensory analysis has found that there are minimal but significant differences in colour, odour, flavour, and texture; in fact, some honeys achieve a score very close to the maximum score. However, we must highlight that probably the samples investigated in our study came from a selection of high-quality honeys used for the competition. From the colour analysis, it emerged that some samples are brighter than others, along with some small colorimetric differences. The prediction from NIR spectroscopy has yielded good results, especially for certain chemical-physical characteristics such

as moisture and electrical conductivity, while it has been moderately accurate for sugars and sensory traits. The results of the correlations between instrumental colour and sensory characteristics highlighted the absence of any relationship between the physical and sensorial measurement of the organoleptic traits. In order to establish an overall chemical and sensory quality value of the honey samples, a quality index and three overall merit quality classes (1, low; 2, medium; 3, high) were proposed and statistical analysed according to the three ecological areas. The results showed very limited differences across the ecological areas, even though in the NM samples were recorded a lower value of the quality index. Through the creation of a PLS-DA model using the spectroscopy data, a rapid NIR prediction of the honey overall merit quality class and the quality class of the four sensory characteristics was also performed. The NIR was found to be a reliable rapid multi-analytical tool for a satisfactory classification performance in the case of the overall merit quality classes, while the prediction of the quality class of the four sensory traits (colour, odour, taste and toughness) did not achieve good results.

RASSUNTO

Uno dei principali prodotti dell'apicoltura è il miele sostanza dolce naturale prodotta dalle api (*Apis mellifera*) dal nettare delle piante o dalle secrezioni delle parti vive delle piante o dalle sostanze secrete dagli insetti succhiatori presenti sulle parti vive delle piante. Le api raccolgono, trasformano, integrano con le loro sostanze specifiche, depositano, disidratano, immagazzinano e lasciano maturare nei favi dell'alveare, fino ad ottenere il miele "maturo". Il miele millefiori è prodotto dalla miscela di nettari raccolti da diverse varietà di fiori selvatici; i suoi aromi e sapori unici lo distinguono da altri tipi di miele. Nel miele l'80% è costituito da vari zuccheri, principalmente fruttosio e glucosio, il 17% rappresenta il contenuto di acqua e solo il 3% è costituito da altre sostanze nutritive, come composti azotati, sali minerali e acidi organici.

Originariamente, per valutare la qualità chimica del miele venivano utilizzati metodi basati sull'estrazione con solvente seguita da analisi di laboratorio. Questi metodi di chimica umida erano procedure lunghe, molto costose e anche distruttive.

Per ovviare alle problematiche sopradescritte la qualità del miele viene valutata utilizzando l'analisi chimico-fisiche con metodi analitici ufficiali, l'analisi sensoriale valutando gli attributi percepibili del prodotto attraverso i cinque sensi e l'analisi melissopalinologica utilizzata principalmente per verificare l'origine botanica dei mieli, ma fornisce anche informazioni qualitative ed informazioni relative ad eventuali processi che il miele potrebbe aver subito. Attualmente, però, si sta sempre più utilizzando, grazie ai suoi vantaggi, la spettroscopia di riflettanza nel vicino infrarosso (NIR) in quanto la preparazione del campione è semplicissima, rapida ed ecologica, non utilizza solventi chimici, e permette di rilevare contemporaneamente più parametri di qualità.

L'obiettivo di questo lavoro era valutare se l'uso di sensori spettrali non distruttivi come uno spettrofotometro basato sul VIS e dispositivi basati sul NIR, potessero valutare diverse caratteristiche chimiche, sensoriali e strumentali della qualità del colore dei campioni di miele millefiori provenienti da tre principali aree ecologiche d'Italia: SL = South Lowland, pianura meridionale, al di sotto dei 600 m sul livello

del mare (slm); NM = North Mountain, montagna settentrionale, sopra i 600 m sul livello del mare; NL = North Lowland, pianura settentrionale, al di sotto dei 600 m sul livello del mare.

Inoltre, i dati NIR sono stati utilizzati per costruire un modello PLS-DA per valutare una classe di qualità complessiva proposta e la classe di qualità di quattro tratti sensoriali.

Il test sperimentale della mia tesi di laurea magistrale (laurea specialistica) ha esaminato 215 campioni di miele millefiori raccolti da diversi apicoltori italiani. L'origine geografica e botanica e il test sensoriale sono stati eseguiti da esperti addestrati *"dell'Osservatorio Nazionale del Miele"* italiano alla fine della stagione di raccolta 2022, durante il concorso *"Tre gocce d'oro-Grandi Mieli d'Italia"*, il giudizio sensoriale è stato dato secondo un protocollo ufficiale e certificato, e i dati correlati sono stati gentilmente autorizzati ad essere utilizzati per questa tesi di laurea magistrale.

Dopo un lieve trattamento termico (40 °C per circa 30 minuti) dei campioni di miele, i dati spettrali sono stati raccolti utilizzando uno spettrofotometro VIS portatile (operante nella regione 450-700 nm) e un dispositivo NIR da banco (operante nella regione 850-2500 nm). I tratti chimici sono stati stimati utilizzando equazioni predittive di spettroscopia NIR eseguite in studi precedenti dai ricercatori dell'UNIPD. Le variabili chimiche e organolettiche sono state utilizzate per definire un indice di qualità utile per valutare la qualità complessiva del miele come strumento rapido per distinguere i campioni raccolti in diverse aree ecologiche. Inoltre, è stato valutato il grado di correlazione tra le coordinate cromatiche strumentali e le caratteristiche sensoriali. Infine, è stato realizzato un modello PLS-DA, tramite dati di spettroscopia NIR finalizzato a discriminare la classe di qualità di merito complessiva del miele e la classe di qualità dei quattro tratti sensoriali. Dai risultati ottenuti si evince che le caratteristiche chimico-fisiche sono simili ad altri studi ed in linea con i limiti definiti e prescritti dalla direttiva. L'analisi sensoriale ha riscontrato che ci sono minime differenze ma significative di colore, odore, sapore e tenacità, infatti, alcuni mieli raggiungono un punteggio molto vicino al punteggio massimo. Tuttavia, dobbiamo sottolineare che

probabilmente i campioni esaminati nel nostro studio provenivano da una selezione di mieli di alta qualità utilizzati per la competizione. Dall'analisi del colore è emerso che alcuni campioni sono più luminosi di altri, insieme ad alcune piccole differenze colorimetriche. La previsione della spettroscopia NIR ha prodotto buoni risultati, soprattutto per alcune caratteristiche chimico-fisiche come l'umidità e la conducibilità elettrica, mentre è stata moderatamente accurata per zuccheri e tratti sensoriali. I risultati delle correlazioni tra colore strumentale e caratteristiche sensoriali hanno evidenziato l'assenza di qualsiasi relazione tra la misurazione fisica e sensoriale dei tratti organolettici. Al fine di stabilire un valore, di qualità chimica e sensoriale, complessivo dei campioni di miele, sono stati proposti un indice di qualità e tre classi di qualità di merito complessive (1, bassa; 2, media; 3, alta) e analizzati statisticamente in base alle tre aree ecologiche. I risultati hanno mostrato differenze molto limitate tra le aree ecologiche, anche se nei campioni NM è stato registrato un valore inferiore dell'indice di qualità. Attraverso la creazione di un modello PLS-DA utilizzando i dati della spettroscopia, è stata eseguita anche una rapida previsione NIR della classe di qualità di merito complessiva del miele e della classe di qualità delle quattro caratteristiche sensoriali. Il NIR si è rivelato uno strumento multi-analitico rapido e affidabile per una prestazione di classificazione soddisfacente nel caso delle classi di qualità di merito complessive, mentre la previsione della classe di qualità dei quattro tratti sensoriali (colore, odore, sapore e tenacità) non ha ottenuto buoni risultati.

Chapter 1

INTRODUCTION

1.1 THE BEE

The bees (Apis LINNAEUS, 1758) belong to the order of the Hymenoptera and are part of a genus of social insects in the family Apidae. It is the only genus in the Apidae family. Only two species can be managed by man: “Apis mellifera” and “Apis cerana”. Bees play a direct role in honey production but are also a biological indicator of the quality of the environment. The bee since ancient times is considered as the emblem of operosity, a symbolic insect in myths, legends and religions, known since prehistoric times for its usefulness. In ancient times, honey was the only source of sugar, which is why bees were highly regarded.

The evolution of bees has not been a simple process, over millions of years they have evolved, perfecting every aspect from the antennae to the tip of the abdomen, each distinctive feature the result of natural selection.

Evolution began about 270 million years ago with the "evolution of insects", it is assumed that bees, wasps, ants all evolved from a single ancestor, 130 million years ago the "first flowers" appeared, 100 million years ago there was the appearance of the "antiquarian wasps", it is thought that bees evolved from a species of wasp that feeds on insects covered in pollen, 90 million years ago the "corbiculate bees" appeared from which the bomb bees, honey bees and sweat bees evolved in common the pollen basket, only 55 million years ago there was the "development of social life" we observe different communities from the simplest to the most complex. (Dorling, 2016).

The first fossils of the first "Honeybees" date back to 35 million years, only 250 thousand years ago there was the "meeting with man", man has had a strong impact on the world of bees sometimes not always positive.

Over millions of years, bees have spread throughout the world, adapting to the environment and diversifying among themselves. (Dorling, 2016).

Bees are more common in the Northern Hemisphere than the Southern Hemisphere, and in temperate or arid climates than in tropical climates. The highest biodiversity is found in the United States. Bees tend to stay away from the

poles, but some species are also found near the equator, it is easier to find bees in a desert than in a tropical forest because they prefer low plants and flowers to lush, slender trees. According to Michael Orr, when it rains in the desert there are mass flowers that cover the entire area, it creates a larger reserve in the wilderness and therefore there is a lot of potential for new species. (www.focus.it)[1].

There are at least 20,000 species of bees on earth, the most common being Apis mellifera (Figure 1.1) is one of the most managed species and used for the production of honey and the pollination of crops, which originated and was domesticated in Europe, when the Europeans colonized other parts of the world they brought it with it with the result that today the Apis mellifera is present on all continents except Antarctica. (Dorling, 2016).

The Apis mellifera contains numerous breeds within it that differ by their morphological characteristics due to their geographical spread, the main breeds are for example the "Apis mellifica mellifica" bee black that populates western and northern Europe, "Apis mellifera carnica" widespread mainly in the Central Eastern Alps, Austria and Slovenia, the "Apis mellifera caucasian" widespread in the Caucasus, the "Apis mellifica ligustica" or Italian bees is widespread in almost all the Italian territory from the north to Calabria in Sicily there is the "Apis melliffera sicula" of dark colour. (apicolturaonline.it)[2]

Figure 1.1 *Apis mellifera* (credit: Irene Sandonà)

[1]https://www.focus.it/ambiente/animali/mappa-mondiale api

[2]https://www.apicolturaonline.it/classif

1.2 HONEY

Honey is one of the main products of beekeeping as well as pollen, royal pepper, propolis and bee venom. (Waykar et al., 2016)

There are different ways to define honey, but according to Legislative Decree 21 May 2003, n. 179, which came into force on 21 July 2004, it is stated that: «"Hone" is the natural sweet substance produced by bees (Apis mellifera) from the nectar of plants or from the secretions of living parts of plants or from substances secreted by sucking insects present on living parts of plants which they collect, transform, combine with specific substances of their own, deposit, dehydrate, store and leave to mature in the honeycombs of the hive». (D.L 179, 2004).

Honey has a high energy value, provides the immediate replenishment of energy losses, it is estimated that 1 kg of honey contains 3350 calories, it can also be considered as a supplement and is known around the world for its healing power for example antibacterial, antiseptic, antiviral and is also used to treat wounds, also helps to fight hemoglobin in the blood, in fact it is used also as a laxative blood purifier, as a preventive against colds, cough and fever, honey is also effective in reducing heart disease, cancer, the decline of the immune system...this thanks to the antioxidant capacity of honey.

The recommended doses of intake per day of honey are: 10-15 g per child; 30-35 g for young people; 30-50 g for a healthy man; 20-30 g for the elderly. (Waykar et al., 2016)

1.2.1 Production and types of honey

Honey production begins with nectar, a watery mixture of sugars, amino acids, proteins, lipids, minerals, and other components produced by the nectar-secreting cells of plants. Bees are the main producers of honey.

Sucrose, glucose, and fructose are the most common sugars in nectar in extremely variable proportions. After collecting the nectar, which is mixed with the secretions from the salivary and hypopharyngeal glands of the bee, foraging bees return to the hive and unload it to the "house bees," who dry it and transform it into honey in two stages, the first stage involves drying it in the mouth of the bee, which

repeatedly expels and reabsorbs the droplet of honey in the making, during the second phase, the nearly ready honey is deposited into the cells of the honeycomb, which are filled only partially and left open to complete the evaporation. Once this phase is finished, the bees seal the cells with a cap, a layer of wax they secrete, to prevent moisture exchange and ensure the preservation of the "mature" product. The conversion from nectar to honey takes one to three days and results from the evaporation of excess water facilitated by the airflow generated by the bees' wings, along with the conversion of sucrose into glucose and fructose through enzymatic action. The honey will be harvested only from the honey supers, which are additional modules, added when the massive influx of honey begins to facilitate the storage of honey in a separate section of the hive. The honeycombs contain mature honey, recognizable by the cells sealed with the capping. The beekeeper, to remove the honey supers, must free them from the bees using a device known as a bee escape, which allows the bees to exit the super but prevents them from re-entering, or by using a blower. (Piana et al., 2020)

The honey extraction process is very simple. It begins with the "uncapping" (Figure 1.2.1.1) of the honeycomb frames from the honey super, which involves removing the layer of wax that seals the cells containing the honey. This is done manually with a special knife or mechanically with devices that cut or crush the capping. Subsequently, the frames are placed in the "honey extractor" (figure 1.2.1.2), a centrifugal extractor that extracts honey from the combs without damaging them through centrifugal force rotation. The honey is then filtered of impurities, particularly pieces of wax, using bag filters made of nylon or metal mesh. So, it is decanted and left to rest in an appropriate container known as a "maturation vessel", this also frees it from the air bubbles that were incorporated in the previous processes. Honey can be directly packaged in final containers or stored in barrels for preservation or wholesale marketing without the need for any additional treatment. Therefore, for the quality of honey, it is essential that storage takes place in cool environments and that the transfer and preparation for the market occur with limited use of heat. (Piana, 1994)

There are different types of honey, which depend on the sources of nectar and the type of crops grown in the area. (Waykar et al., 2016)

Different types of honey, such as unifloral or monofloral honeys, which are produced by bees primarily from the nectar or honeydew of a single plant, it is possible to obtain them when a bloom is sufficiently extensive and abundant and does not coincide with other blooms. The beekeeper must ensure to leave empty hives at the beginning of the flowering and remove them before the next bloom occurs, are for examples chestnut honey, acacia honey, and so on. Another type of honey is polyfloral honey, obtained from simultaneous blooms that cannot be separated at a later time. The polyfloral honey are an infinite number of possible combinations of plants, so a single name can refer to a myriad of different products. Polyfloral honeys are not inferior to monofloral honeys in quality and value. Furthermore, polyfloral honey are often a symbol of a combination of extremely refined tastes, aromas, colours, and textures. (Naldi et al., 2015).
Bees select plant species that contain more than 20% of the sugar content.

Figure 1.2.1.1 Representation "uncapping", removal of the wax layer, operculum, from mature honey. (Credit Irene Sandonà)

Figure 1.2.1.2 Representation of the honey extractor. (Credit Irene Sandonà)

1.3 WET CHEMISTRY AND HONEY QUALITY

Honey has a complex composition, on average, it is made up of about 80% various sugars, mainly fructose and glucose. 17% is the aqueous part and only 3% is made up of other substances, including nitrogenous substances, mineral salts, and organic acids. The differences that exist between one product and another are mainly due to the different nature of the nectar or honeydew of origin and consist of a different quantitative ratio between the main components (various sugars and water) and the minor components. This results in a wide range of products that differ greatly in appearance, texture, colour, smell, and taste, which can cater to very different uses and preferences. (Piana et al., 2020)

Honey is mainly composed of:

Carbohydrates:

Honey is primarily composed of carbohydrates, that is sugars (75-80%), such as glucose and fructose, simple sugars, monosaccharides, making up 90% of the total sugars. They are formed or derived directly from nectar thanks to the action of the enzyme invertase, which is an enzyme secreted by the bee that can split sucrose in the nectar or honeydew into glucose and fructose. Sugars are responsible for the numerous physical and nutritional properties of honey: the sweetening power, viscosity, hygroscopicity, physical state (liquid or crystallized), energy value. In fact, for example, due to its high solubility in water, fructose tends to make the final product sweeter or more bitter depending on the liquid content, while glucose has a lower sweetening power and a greater tendency to crystallize, which occurs due to time and storage temperatures. In addition to the aforementioned monosaccharides, there are also disaccharides such as maltose and sucrose, to which minor tri- and oligosaccharides are subsequently added, essential for determining the botanical origin of honey, even though they have no effect on its organoleptic properties, the average content of which is between 5 and 10% of the total sugars. There are also small amounts of complex sugars present. (Guida per conoscere il miele, 2023[1]; Zanotto S., 2024)

[1]https://conapi.it/wp-content/uploads/2023/03/Conapi-Guida-per-conoscere-il-miele.pdf

Water:

The quality is determined by the water content. The botanical origin, the atmospheric and environmental conditions before and after extraction, the intensity of the nectar flow, the production season, the beekeeper's techniques, and the storage conditions are all important factors. 17% is considered an ideal value. In fact, a higher content would cause fermentations, while workability would be altered at levels below a certain threshold.

Nitrogenous substances:

They are amino acids and proteins of different origins and are present in honey in small amounts, averaging 0.2-0.3%. Most of them have a botanical origin, but some, like proline, come from bee secretions and are found in all types of honey. These substances can be derived from pollen grains in honey or are already present in nectar and honeydew. (Guida per conoscere il miele, 2023[1])

Minerals:

Even though there are significant variations among the different types of honey, their content is generally low (from 0.02 to 1%). The main minerals present in honey include potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), chloride (Cl), sulphur (S), phosphorus (P), silicon (Si), iron (Fe), manganese (Mn), and copper. (Cu). Light honeys (acacia, citrus, linden, and rhododendron) are less rich in mineral substances compared to dark honeys (honeydew or forest honey) and chestnut honey. (Guida per conoscere il miele, 2023[1]; Zanotto S., 2024)

Vitamin:

Honey contains small but detectable amounts of vitamins; their concentrations in honey are typically measured in parts per million (ppm) and primarily come from nectar. The following have been highlighted: vitamin C, some B vitamins (B1, B2, B6), vitamin PP, vitamin K, pantothenic acid, and vitamin P. vitamins that belong to the group of water-soluble vitamins. (Guida per conoscere il miele, 2023[1]; Ball W. 2007)

[1]https://conapi.it/wp-content/uploads/2023/03/Conapi-Guida-per-conoscere-il-miele.pdf

Organic acids:

They are numerous, although their presence rate ranges from 0.10 to 1.5%. It is possible to find various acids such as acetic, butyric, citric, formic, fumaric, gluconic, and others... The main one is gluconic acid, which is produced by the action of the enzyme glucose oxidase secreted by the glands of bees. Organic acids are responsible for the acidic pH, which ranges from 3.5 to 4.5 for nectar honeys. Some organic acids are already present in the honeydew and nectar, while others are formed through the bee's intervention during the processing of honey. The acidity of honey, along with the high osmotic pressure due to the high concentration of sugars, helps ensure microbiological stability, which gives honey its famous preservative properties. (Guida per conoscere il miele, 2023[1]; Ball W. 2007)

Proteins and amino acids:

The finished honey has a low nitrogen content and is low in protein, generally containing between 0.2% and 1.6%; their amount also varies depending on the type of bee. Honey contains other free amino acids, the most prevalent being proline; most of the amino acid content comes from the bees, not from the nectar or pollen. The total amount of amino acid rarely exceeds 300 ppm. (Ball W. 2007; Zanotto S., 2024)

Various physical characteristics of honey are influenced by its chemical composition. Honey has a high refractive index (about 1.49) and high viscosity because it is a concentrated sugar solution. It also has a specific weight of about 1.4 and a specific heat that is approximately 40% lower than that of pure water. Sugars, acids, and other volatile components including aliphatic and aromatic acid esters, aldehydes, ketones, and alcohols contribute to the flavour and aroma of honey. Among the compounds that seem to have the smell and taste of honey is phenylacetaldehyde, but there are also several compounds typical of botanical origins. There are seven families of scents and aromas used to describe honey vegetal, animal, floral, fruity, warm, aromatic and chemical.

[1]https://conapi.it/wp-content/uploads/2023/03/Conapi-Guida-per-conoscere-il-miele.pdf

The colour of honey is undoubtedly a distinctive characteristic that varies significantly depending on the season, the source of nectar, and the processing. Honey can have a colour similar to a diluted caramel solution, that is, an amber tone that ranges from almost transparent to very dark. It is usually noticeable that the colour changes drastically from light to dark as one moves from the honeys of spring flowers to summer flowers and into autumn. With aging, it is physiological for honey to become darker, resulting in an increase in the percentage of glucose, a decrease in polysaccharides, and the degradation of fructose with the production of hydroxymethylfurfural. Sure, it's difficult to determine the type of honey with certainty based on colour because it is also influenced by the presence and size of any glucose crystals. They, being white, can reflect light and therefore change colour compared to a more liquid honey. It is not clear what exactly the colour of honey derives from; Some researchers suggest that the colour is due to the presence or absence of carotenoids; Others suggest that polyphenols are responsible. Another possibility is a chemical caramelization of the sugars in honey, catalysed by the acids present. Others suggest a Maillard reaction between sugars and amino acids. Whatever the source of the colour, it is generally true that the darker the honey, the more intense the flavour. (Ball W. 2007; Zanotto S., 2024)

To verify the quality of honey, the following methods are used:

Physical-chemical analyses, for which there are official analytical methods. In particular, the quantity of sugars is checked, the water content is measured using a refractometer, and the content of hydroxymethylfurfural (HMF) is assessed, substance that forms in honey due to aging and the subsequent degradation of fructose; it represents an index of freshness and the state of preservation of honey. The law sets the maximum allowable limit of HMF detectable in honey at 40 mg/kg. Checked the diastatic index is evaluated by measuring diastase, an enzyme naturally present in honey, which is used to determine whether the honey has undergone thermal treatments. Legislation stipulates that the diastatic index should generally not be less than 8 units per gram. The electrical conductivity is also measured, in milliSiemens per centimetres (mS/cm), and the legal limit is set at less than 0.8 mS/cm for honey in general, which is directly related to the soluble mineral component that may be present in varying concentrations in different

types of honey. Finally, the colour of the honey is assessed, which naturally varies from lighter shades to darker ones of amber, reaching almost black. There are also honeys with more yellow tones or with greenish or reddish reflections; generally, the colour is linked to the botanical origin. It is measured in mm on the Pfund scale, combined with optical comparators like the Lovibond (Table 1.3.1). The Lovinbond comparator is equipped with a container for the sample to be measured and a millimeter scale of reference, that of Pfund precisely, equipped with a pointer to indicate the position where the sample has the same graduation compared to the references slide. The reference-coloured slides are supported by a disk called Lovinbond.

Table 1.3.1 Pfund Scale for the colour Index of Honey (560 nm)

Colour name	Pfund scale
Water White	0 – 8 mm
Extra White	8 – 16 mm
White	16 – 34 mm
Extra light number	35 – 50 mm
Light amber	51 – 84 mm
Amber	85 – 114 mm
Dark amber	>114 mm

(Source: ResearchGate)[2]

The ***sensory analysis*** described in chapter 1.4.

The ***melissopalynological analysis,*** primarily used to verify the botanical origin of honeys, in conjunction with sensory analysis and physicochemical analyses, provides qualitative information, also related to any processes that the honey may have undergone, which result in the residual presence of particulate elements. For example, some types of adulteration are carried out by artificially adding pollens to support the botanical and geographical origin of the product. It is also possible to highlight the extraction method, the honey extraction from frames with brood present, contamination with dust, etc. (Guida per conoscere il miele, 2023[1])

[1]https://conapi.it/wp-content/uploads/2023/03/Conapi-Guida-per-conoscere-il-miele.pdf
[2]https://www.researchgate.net/figure/Scale-mm-Pfund-established-by-the-USDA-for-the-determination-of-the-color_tbl2_365874551

1.4 SENSORY QUALITY OF HONEY

Sensory analysis evaluates the perceivable attributes of a product through the five senses, such as colour, odour, taste, touch, texture, allowing for an understanding of how it is perceived by the consumer. Until the 1960s, it was based on personal experience, but it lacked reproducibility. In the twentieth century, new techniques were developed using panels of evaluators, protocols, and statistical methods for reproducible results. These methods are limited to research and development rather than routine use in quality control. For the honey, sensory analysis has been used to distinguish the botanical origin, identify defects, and define product standards. It is essential for understanding consumer preferences and revealing characteristics that cannot be identified through laboratory analysis. The training and standardization of evaluators are crucial to ensure the accuracy and reliability of the results. (Piana et al., 2004)

Italy is the only nation to have the most progress in the techniques of sensory analysis of honey. It is the only nation to have an official list of honey tastingers authorized by a ministerial decree. (Naldi et al., 2022)

The sensory analysis of honey focuses on improving quality and enhancing the product. The opinion of experts is not always representative of consumer preferences due to possible influences stemming from their profession. It is necessary to also consider the opinions of other trained individuals, by checking variables through repeated tests and statistical interpretation. The people involved must be trained in sensory aspects without excessive technical specialization.

The sensory analysis of honey requires in-depth knowledge of physiology, psychology, and sensory methodology, along with extensive sensory experience for accurate evaluation. The taster must have an olfactory and gustatory sensitivity above average, which depends more on training than on genetics, the perception of flavours can be influenced by health issues such as a cold or sinusitis. The taster must be motivated by more than just economic reasons and follow rules such as working under objective conditions and avoiding external influences during tastings. The honey tasting training course includes both a theoretical and practical component, with standardized 4-day courses for future tasters. Students learn to

evaluate sensory perceptions, to use specific vocabulary, to memorize the characteristics of honeys, and to quantify perceptions. The sensory analysis tests help participants become familiar with Italian honeys, improve their sensory skills, and express accurate perceptions. The setup of an optimal environment for sensory analysis aims to minimize the influence of external elements on tasting. The room should have controlled conditions for temperature, humidity, lighting, and odours, furnished for comfort during evaluations. The presentation of the honey sample is crucial: It is transferred to a glass for uniformity, but the visual characteristics in the original container are also examined. The tasting of honey involves three phases: observation, odour, and taste. Aspects such as physical appearance and colour are evaluated, it is sniffed moderately to perceive the smell, and it is tasted slowly to identify its flavour and aroma. It is important to focus on the chemical sensations at the first taste and evaluate the tactile characteristics afterward. Limiting the number of samples evaluated is essential for an accurate assessment and to avoid saturation. The objective of the honey taster is to provide a description of the product that accurately and consistently represents it. The visual evaluation of honey includes the presence of impurities such as foreign bodies, visible in the original jar. These impurities concentrate on the surface and can be obvious, such as fragments of insects or mineral particles. Liquid honey contains solid particles that give it a slight cloudiness, while crystallized honey can exhibit various intermediate forms. The presence of crystals and their cohesion can be assessed visually and through touch. The surface of honey can be shiny, wet, dry, or grainy.

The viscosity of liquid or creamy honey can be assessed through observation and touch, understanding the degree of fluidity by tipping the container or pressing it between the tongue and palate. Colour is essential for identifying the origin and aging of honey, ranging from almost colourless to black. Some display different shades such as bright yellow or greyish, with some showing greenish fluorescence. Colour can be measured with physical systems and optical comparators, influencing the classification of honey. In single-flower honeys, reference is made to the known limits for the designation, while for multifloral honeys, the coloration is described from colourless to black.

The quantitative and qualitative parameters are considered in the evaluation of the olfactory characteristics of honeys. The simplest description corresponds to the smell known for that type of honey. However, the communication of the subtle differences between products may be limited by sensory vocabulary. In the analysis of odours, it is advisable to assess the intensity, use specific references, evocations, and adjectives, avoiding generic non-descriptive terms.

The taste characteristics of honey are evaluated in the mouth, including tactile and somaesthetic sensations. The intensity of the four fundamental flavours provides little information about the quality of honey, with sweetness always present and acidity masked by sugar. The salty taste is rare, while the bitter one is perceived only at the moment of swallowing. Aromatic sensations are complex and can vary from olfactory perception. The persistence of sensations after swallowing is defined as aftertaste. The undifferentiated chemical component can give honey characteristics such as astringency, acidity, or freshness.

The tactile evaluations of honey focus on the mouthfeel and during the handling of the product. The liquid honey varies from fluid to very viscous, while the crystallized ones can be creamy or compact, with crystals of different sizes and shapes. Some crystals provide a refreshing sensation, while others can be irritating to the throat.

The sensory analysis of honey is more common than pure descriptive analysis for assessing its quality. The definition of quality varies depending on the context and the situation of application, with different systems proposed for the evaluation of honey. Quality standards include authenticity, healthiness, cleanliness, and freshness. For monofloral honeys, quality corresponds to botanical purity, while for polyfloral honeys, it varies according to consumer preferences. Evaluations based on consumer preferences are used for generic polyfloral honeys. The taster must put themselves in the consumer's shoes to assess the pleasantness, favouring light aromas in light honeys, more intense aromas in amber honeys, and strong aromas in dark honeys. The preferred tactile characteristics are fine crystallization and a pasty consistency. The various evaluation systems present worksheets that guide sensory analysis in areas such as visual, olfactory, gustatory, and tactile examination, with a final judgment on a seven-point scale. Some systems leave the

choice of parameters to the taster, with sheets that indicate defects with crosses and ask for a final judgment not mathematically related to the defects. This versatile method requires a preliminary discussion among the tasters for reproducible results. Broader scales do not always improve accuracy, as different scores do not always correspond to real qualitative differences. Some sheets guide the taster in evaluating the product's attributes, highlighting defects and providing rating scales. On the contrary, the National Association of Honey Tasters uses a detailed sheet with 14 attributes and a final score based on the sum of the partial scores. This card provides accuracy but may be limited in specific situations. (Piana, 1995)

1.5 NIR AND HONEY QUALITY

Originally, to determine the quality of honey and other food products, methods based on solvent extraction were used, followed by laboratory analysis and destructive procedures, which were time-consuming and required the use of chemical materials, in addition to being costly. These methods are not suitable for industries and the environment. To overcome these issues, numerous studies have been conducted to find a quick and non-destructive alternative method. Consequently, one of the most promising and non-destructive analysis methods in many fields, including agriculture, has emerged due to its advantages: a simple sample preparation, quick and environmentally friendly since no chemicals are used, is near-infrared reflectance spectroscopy, moreover, it has the potential to determine multiple quality parameters simultaneously. (Munawar A. A. et al., 2019)

The near-infrared (NIR) spectroscopy technique is an analytical method that exploits the interaction between matter and near-infrared radiation. In food science and technology, NIR is used because it is a precise, rapid, non-destructive, reliable, and cost-effective analytical technique. The ability of each chemical compound to absorb, transmit, or reflect light radiation is utilized. Spectroscopic techniques offer a rapid, high-throughput, and non-destructive method of analysis; these technologies require only a limited amount of training for operation, making them easy to use and accessible for field or production line applications. (McGrath T.F. et al., 2018)

NIR spectroscopy is based on the absorption of electromagnetic radiation at wavelengths between 780 and 2500 nm. It can be applied to qualitative or quantitative analysis of multiple components of a sample through a single measurement. It is used to quantify the compositional, functional, and sensory parameters of food throughout all stages of processed food production, from the analysis of raw materials to the verification of ingredients and inspection of the finished product. Near-infrared spectroscopy is a method that allows for real-time measurements. (Woodcock et al., 2007; Osborne B.G., et al., 1993)

In 1800, William Herschel discovered the region of spectroscopy in the electromagnetic spectrum in the near infrared. (da 780 a 2526 nm). Karl Norris

used it for the first time in the 1960s to analyse chemically complex samples. The spectral fingerprints of organic compounds can be obtained using NIR spectroscopy, which measures the combination and harmonics of the vibrations of the fundamental bonds O-H, C-H, and N-H. The application of NIR spectroscopy in fields such as the analysis of the composition of agricultural raw materials, the control of petrochemical processes, and non-invasive medical analysis only began in the 1990s. For the reasons mentioned above, NIR spectroscopy is now one of the most widely used analytical techniques.

In 1998, Ha et al. (1998) and Cho et al. (1998) determined the constituents of honey for the first time using NIRS spectroscopy, developing an NIR prediction method for the conventional quality parameters of Korean honeys using transflectance cuvettes with gold and aluminium back reflectors. They conducted a similar study to Qiu et al. (1999) of Hong Kong and Hou et al. (2007) began a research study in mainland China to assess the feasibility of using NIRS for honey quality analysis. (Chen et al., 2014; Bàzàr G., et al., 2015).

In the analysis of honey, NIR spectroscopy is used for five different aspects: components and properties, adulteration, recognition of botanical origin, verification of geographical origin, and brand identification. Most indicators, such as sucrose, maltose, fructose, glucose, moisture, ash content, and the ratio of fructose to glucose, can be quickly identified using NIR spectroscopic analysis. In fact, the authors García-Alvarez, Huidobro, Hermida, and Rodríguez-Otero (2000) examined the content of fructose, glucose, and moisture in honey, obtaining accurate results for calibration and independent validation for each of the three components using transmittance spectra. However, the forecast values for minor components such as free acid, lactone, some oligosaccharides, and hydroxymethylfurfural (HMF) are not very reliable. Moreover, the results regarding electrical conductivity and pH values are mostly unsatisfactory.

When we use NIRS to identify adulteration, we focus on distinguishing the most common type of adulteration, namely the addition of sugary materials such as high fructose corn syrup or malt syrup. Furthermore, the use of NIR spectroscopy for recognizing the botanical origin of honey represents a valuable, rapid, and non-

destructive tool for the authentication of certain honey varieties. For example, it has allowed for a reliable distinction between acacia honey, chestnut honey, and fir honeydew from other types of unifloral and polyfloral honey. (Chen et al., 2014; Bàzàr G., et al., 2015).

The honey spectra in NIRS spectroscopy at 1480, 1580, 1935, and 2100 nm show four intense absorption bands, these bands are associated with the first overtone of O–H, the first overtone of O–H stretching, the combination of O–H stretching and bending, and the first overtone of O–H bending, as well as the C–O stretching band, respectively. The C–H bonds are associated with absorption bands below 1202 and 2321 nm. (Latorre C. H. et al., 2013)

In spectroscopy, after the collection of spectra, the results are evaluated using chemometric models since the raw spectra are too complex to be processed visually. (McGrath T.F. et al., 2018)

1.6 CHEMOMETRICS

Chemometrics uses mathematical and statistical models to recognize patterns and relationships within highly complex data obtained from analytical techniques such as spectroscopy, chromatography, and mass spectrometry, and translates them into usable analytical parameters. These can be used to identify food samples based on geographical origin, species variety, and to highlight contamination and adulteration of a sample. Chemometric models extract important quantitative and qualitative information that distinguishes different clusters, ignoring redundant data and simplifying this process. (McGrath T.F. et al., 2018)

The chemometric approach is used to relate the physicochemical properties of the samples to be analysed with the absorption of radiation in the NIR wavelength range. This allows for predictions about their chemical origin.

Chemometrics is based on some fundamental principles such as Principal Component Analysis (PCA), which is necessary to reduce the dimensionality and complexity of data while maintaining the most important information through the optimization of experimental conditions and analysis algorithms to extract the maximum information from the available data. To ensure their reliability, chemometric models must be rigorously validated. Furthermore, to guarantee accurate and reproducible results, the instruments must be calibrated. Chemometrics offers methods for multivariate calibration, such as Principal Component Regression (PCR) and Partial Least Squares Regression (PLS). Regression is a procedure that seeks to find a quantitative model that links a set of measured responses for numerous objects to a set of predictors that describe the objects. In particular, partial least squares discriminant analysis, PLS-DA, is a dimensionality reduction technique that finds a linear regression model by projecting the predicted variables and the observed variables into a new space, results from other studies show that PLS-DA identifies more homogeneous and better separated classes than other commonly used methods, such as nonparametric classifiers and other discriminant functions.

Chemometrics focuses on the analysis of complex relationships between different variables that are measured simultaneously using mathematical models. These

methods can be quantitative, predictions of chemical concentrations, or qualitative, discrimination of product categories. The first, known as exploratory methods or "data mining," are capable of finding trends or clusters in the data in an unsupervised manner. Secondly, supervised methods use input and output (the chemical composition of the sample to be predicted) to create models. (Barthès et al., 2019; Roussel et al., 2011; Melucci D., 2005-2006; Fordellone M. et al.[1])

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[1]https://www.dss.uniroma1.it/en/system/files/pubblicazioni/Fordellone.pdf

Chapter 2

OBJECTIVE OF THE DISSERTATION

The objective of the final MSc project was to assess the chemical, sensory and instrumental colour quality of a set of Italian polyfloral honey samples collected during the summer season of 2022 across three main ecological areas of Italy. The chemical traits were also estimated using near infrared (NIR) spectroscopy predictive equations performed in different previous research trials. Moreover, the chemical, instrumental colour and organoleptic traits were used to determine an index quality useful to determine a honey overall merit quality class useful to assess a comprehensive quality of the polyfloral honey samples collected in the different investigated geographical terroirs. The degree of the correlations between the instrumental colour coordinates and the sensorial traits were also estimated. Moreover, the NIR spectroscopy data were used to perform a PLS-DA model to discriminate among polyfloral honey samples both the overall merit quality classes and the quality class of the four sensory traits.

Chapter 3

MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

Two hundred and fifteen (n = 215) samples of Italian polyfloral honey collected in the spring and summer of 2022 by different beekeepers were considered for the study. Each honey was collected during the national competition called *"Tre Gocce d'oro-Grandi Mieli d'Italia"*, organized by the National Honey Observatory (Osservatorio Nazionale del Miele) in September 2022.

The botanical composition (polyfloral), geographical origin and sensory judgment were announced by qualified specialists of the National Honey Observatory. Experts have used the producers' statements, in the event of a shortage, have contacted the manufacturer to request admission to the competition.

Each sample was given a final score given by the sum of the chemical and sensorial qualities, with the aim of assigning an index quality to each sample, in order to carry out further comparisons on the index quality the honey samples were categorized into macro areas based on the altitude of the apiaries from which they came: South Lowland (SL) and North Lowland (NL) samples located at low altitudes (< 600 m above sea level), which included the possibility that the apiaries were both plain and hilly. These are samples from North Mountain (NM) apiaries located at high altitudes (> 600 m above sea level).

All samples were sent to the *Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe)* Experimental Chemistry Laboratory, where 200 g aliquots were sampled in conic tubes (Falcon 352098, 50 ml). Subsequently, the aliquots were sent to the LabCNX laboratory of the Department of Animal Medicine, Production and Health (MAPS) at Padova University (Campus of Agripolis).

The samples were maintained at 4 ± 2 °C until they were processed for analytical purposes after being recorded and recognized using the laboratory's progressive analysis codes. The subdivision of the samples by macro-areas is categorized in Tables 3.1 and Figure 3.1 For the subdivision, the recommendations of the National Honey Observatory were respected.

Table 3.1 Lists the three Italian macro areas together with the origin of the honey and the sample size. Honey samples from apiaries below 600 meters above sea level (asl) come from South Lowland (SL) and North Lowland (NL); samples from apiaries above 600 meters above sea level come from North Mountain (NM).

MACRO AREAS	SAMPLE NUMBER
SOUTH LOWLAND (SL) Abruzzo, Basilicata, Calabria, Campania, Lazio, Marche, Molise, Puglia, San Marino, Sardegna, Sicilia, Toscana, Umbria	130
NORTH MOUNTAIN (NM) Friuli-Venezia Giulia, Trentino-Alto Adige, Lombardia, Piemonte, Valle d'Aosta, Veneto	32
NORTH LOWLAND (NL) Emilia-Romagna, Friuli-Venezia Giulia, Trentino-Alto Adige, Liguria, Lombardia, Piemonte, Valle d'Aosta, Veneto	53

Figure 3.1 The regions of Italy divided into three macro areas: North Mountain (NM) in green; North Lowland (NL) in orange; South Lowland (SL) in yellow. Samples of lowland honey from apiaries under 600 meters above sea level (asl); samples of high mountain honey from apiaries over 600 meters above sea level (asl).

3.2 CHEMICAL ANALYSIS

The chemical analyses were as following: electrical conductivity, pH, free acidity, diastase index, maltose, glucose and fructose (Bisutti et al., 2019). The humidity of the honey samples was determined according to the AOAC method (2005). The pH and free acidity were detected using a Basic 20 Crison Instrument (Lainate, MI, Italy). To measure the electrical conductivity a Five Go F3 (Mettler Toledo, Novate Milanese, MI, Italy) was used. To calculate the diastase index a UV/Vis spectrophotometers from Jasco Europe (Rozzano, MI, Italy) and a PHADEBAS Honey Diastase Test kit (Magle, Sweden) were used. To quantify maltose, glucose and fructose were used the official analytical methods reported in the "Official Journal of the Italian Republic". (Gazzetta Ufficiale della Repubblica Italiana n 185 of the 11 agosto 2003), and specifically a Nexera x2 quaternary UHPLC system, equipped with a RID-20A refractive index detector (Shimadzu, Kyoto, Japan) was used. Moreover, these honey chemical traits were also predicted by using the NIR spectroscopy data.

3.3 SENSORY ANALYSIS

The sensory analysis was performed during the competition *"Tre Gocce d'oro-Grandi Mieli d'Italia"*, organized by the National Honey Observatory, from a large number of experienced tasting professionals registered at the register, with the collaboration of Ami (Ambassadors of Honey) association which includes most of the "tasting professionals" of honey. The evaluation usually takes place in two days, in the first day exercises are carried out to prepare the judgment criteria, in the second, each tastier taste a portion of the competing honey anonymously and independently, evaluating its tactile, olfactory and taste characteristics on a scale from 1 to 10. The final judgment was formed using the multiplier coefficients and the visual evaluation results carried out on the original anonymous vessels (the presentation of the honey was evaluated, i.e. presence of impurities and foam, homogeneity or clarity and colour). The calculation system used for sensory evaluation assigns a maximum of 100 points to the total characteristics: 25 points for visual features, 20 points for olfactory features, 40 points for taste features and 15 points for tactile features. Inherent characteristics of honey (smell and taste) are considered more than presentation characteristics (visual and consistency) as you want to promote the basic quality of honey compared to the aesthetic quality that could vary over time. (Naldi et al., 2022). The results of the evaluations were registered and kindly made available only for the scientific purpose of this MSc dissertation. Sensory data cannot be used neither for further purposes by readers of this dissertation nor for competitive comparisons among specific producers (data were given in an anonymous format).

3.4 NIR SPECTROSCOPIC ANALYSIS AND CALIBRATION

For this experimental test to analyse 215 samples of honey (n = 215) and record the spectral data, a monochromatic scanning spectroscopic instrument FOSS DS-2500, (FossNIR-System, Silver Spring, MD, USA) was used, this device covers a scanning range of 850 to 2500 nm at intervals of 0.5 nm in reflection mode. First of all, six samples at a time, were thermal treated, heated in a stove at a regulated T of 40 ± 2 °C for 30 ± 5 minutes. If a sample appeared un-homogeneous before analysis, was gently mixed with a spatula to make sure it was homogenous. Care was taken not to introduce air bubbles during the process. Within an hour of thermal treatment of the sample, the spectroscopic analysis was to be completed. For analysis, an aliquot of each sample (1 ± 0.5 g) was taken with a spatula and placed on a ring cell with a quartz window allowing the irradiation of approximately 12.6 cm^2. The sample was scanned using a gold reflector with an optical path of 0.5 mm.

A slight pressure was applied to ensure that the sample was evenly distributed across the entire quartz window. It was taken care not to add air bubbles during the process. Once preparation is completed, the cell was placed in the FOSS for sample reading, with the quartz window above the light source. After using hot water to wash and dry the ring cell a new aliquot was inserted for reading the next sample.

The spectra were collected as an average of three replies. The software WinISI 4 V4.10.0.15326 (FOSS Analytical A/S, Hillerød, Denmark) has been used to record spectral data as log(1/R), where R is the reflection of the sample. Prior to the statistical analysis, all spectral were mediated. Mathematical pre-treatments were not used. The WinISI 4 software V4.10.0.15326 (FOSS Analytical A/S, Slangerupgade 69, Hillerød, Denmark) was used to develop very robust calibration curve, whose specifications are listed in Table 4.3.1.

3.5 INSTRUMENTAL COLOUR

Subsequent to the NIR analysis, the instrumental colour of the 215 honey samples was immediately carried out using a Konica Minolta CD-600 portable visible spectrophotometer (Konica Minolta Sensing, Inc., Japan), which operates in a range of 450-700 nm, with an interval of 10 nm. For this analysis, a sample quantity (5 ± 1 g) was taken with a spatula and poured into a cylindrical 2.5-mL cuvette made of suprasil® quartz (Hellma Italia srl, Milano, Italy); as for the NIR analysis, if a honey sample appeared non-homogeneous before the analysis, it was gently mixed with a spatula to ensure it was homogeneous, taking care not to introduce air bubbles. The spectrophotometer was fixed in a vertical device and the cuvette was positioned above the light source performing the measurement in dark condition. The instrumental colour was taken using a D65 light source, an observation angle of 10°, and an area of 8 mm. After each scan, the cuvette was rotated by about 120° until three measurements were obtained for each sample. The cuvette and spatula were carefully washed with hot water and dried after the three measurements to avoid contamination between samples. Finally, the colour data were exported to the CIE-L*a*b* and HUNTER-L*a*b* systems, where L* indicates lightness, and a* a b* represent the redness and yellowness, respectively. The colour coordinates a* and b* were used to calculate C* (chroma) and H* (hue angle or the degree to which a colour stimulus can be described) according to the formulas:

$$h_{ab} = \arctan\left(\frac{b^*}{a^*}\right)$$

$$C^*_{ab} = \sqrt{\left(a^*\right)^2 + \left(b^*\right)^2}$$

3.6 QUALITY INDEX AND OVERALL MERIT QUALITY CLASSES

In order to establish the overall quality of each sample and therefore a "quality index", a dataset was created in Excel including all the samples under study, each of which was assigned the following chemical-physical analyses: humidity, electrical conductivity, pH, free acidity, as well as diastatic index, maltose, glucose, fructose predicted on the basis of analyses carried out previously as specified in paragraph 3.2. For each single chemical, instrumental colour coordinates and sensory traits investigated variables, tertiles were created using the percentile formula (=percentile(matrix;k)), that is, the sum of the results resulting from the analysis of each sample was first divided by 0.33, then by 0.66 and finally by 1, thus obtaining 3 results. These were assigned classes of quality from 1 to 3, 1 low quality, 2 medium quality and 3 high quality, referring to the standard values of the literature (Alvarez-Suarez et al., 2017; Bisutti et al., 2019; El Sohaimy S.A. et al., 2015; Filipe M.S., 2024; Segato et al., 2019). Once this operation was carried out, for each chemical-physical characteristic, each sample was assigned the class of merit to which it belonged. All this was replicated for the sensorial characteristics: sight, smell, taste and touch.

Finally, for each sample, the sum of the scores attributed according to the quality class of each chemical, instrumental colour coordinates and sensory traits investigated variables was made, thus obtaining a final total score (socalled quality index values). For this reason, the tertiles were also made in order to assign a definitive ***overall merit quality class*** to each sample on the basis of the socalled quality index value. The samples belonging to the overall merit class 1 (quality index values lower than 27 points) are considered low quality; those belonging to the overall merit class 2 (quality index values between 27 and 31) are considered medium quality; and finally, the samples belonging to the overall merit class 3 (quality index values higher than 31) are considered high quality.

3.7 STATISTICAL ANALYSIS

For the chemical, sensory and colour traits the assumption of normality and variance homogeneity was assessed using the Shapiro-Wlik Test. Then, the data were submitted to ANOVA adopting a linear model that considered the fixed factor ecological areas of honey collection (three levels: NM vs NL vs SL). Since the class quality variable is a categorical variable, a goodness of fit (Chi-square) test was used to compare the ecological areas. A set of correlations between the instrumental colour coordinates and the sensory traits was performed using the Pearson method.

To perform a rapid segregation of the overall merit quality classes and the quality class of the four sensory traits (colour, odour, taste and toughness) the NIR data were used to build a partial least square discriminant analysis (PLS-DA) by using the software WinISI 4 V4.10.0.15326 (FOSS Analytical A/S, Hillerød, Denmark). The goodness of classification of the three overall merit quality classes and the quality class of each sensory traits has been determined using a confusion matrix, which allows us to evaluate the classification performance within a specific categorical variable by comparing the actual values with the predicted values. The confusion matrix allows us to calculate: ***true positives (TP)*** which are the samples classified as belonging to the positive class and that actually belong to it; ***true negatives (TN)*** samples classified as belonging to the negative class and that actually belong to it; ***false positives (FP)*** identify samples that are classified as belonging to the positive class but actually belong to the negative one; and finally, the confusion matrix helps us identify ***false negatives (FN)*** samples classified as belonging to the negative class that actually belong to the positive class.

Example of a confusion matrix.

		Actual	Actual
		Positive	Negative
Predicted	Positive	**TP**	**FP**
Predicted	Negative	**FN**	**TN**

After calculating the true positive (TP), false negative (FN), true negative (TN), and false positive (FP), according to Bisutti et al., 2019, it is possible to evaluate for each analysis the sensitivity, that is, the percentage of true positive cases correctly identified, the specificity the percentage of true negative cases correctly identified, and the accuracy is the percentage of both true positive and true negative cases correctly identified. Finally, it is possible to assess the quality of the classifications through the Matthews correlation coefficient (MCC) as reported in Bisutti et al., 2019. The MCC varies between -1 and +1 and broadens its consideration to all values of the confusion matrix, i.e. it returns good results only if the result of all four values of the confusion matrix (TP, TN, FP, FN) is good, contrary to accuracy, we can say that this is the most reliable and truthful evaluation metric. (what-is-Matthews-correlation-coefficient-mcc)[1]

All of this through the following formulas:

$$Sensitivity = \frac{\mathrm{TP}}{(\mathrm{TP} + \mathrm{FN})}$$

$$Specifity = \frac{\mathrm{TN}}{(\mathrm{TN} + \mathrm{FP})}$$

$$Accuracy = 1 - \frac{(\mathrm{FP} + \mathrm{FN})}{(\mathrm{TP} + \mathrm{TN} + \mathrm{FP} + \mathrm{FN})}$$

$$MCC = \frac{\mathrm{TP} * \mathrm{TN} - \mathrm{FP} * \mathrm{FN}}{\sqrt{(\mathrm{TP} + \mathrm{FP})(\mathrm{TP} + \mathrm{FN})(\mathrm{TN} + \mathrm{FP})(\mathrm{TN} + \mathrm{FN})}}$$

[1] https://medium.com/@CuttiE_MarU/what-is-matthews-correlation-coefficient-mcc-bb07a94162ba

Chapter 4

RESULT AND DISCUSSION

4.1 CHEMICAL ANALYSIS

The results of the investigated honey chemical traits according to the ecological area of production is given in Table 4.1. Comparing the results obtained with other studies (Bisutti et al., 2019), we can say that they are similar and in line with the limits defined and prescribed by EU Directive 2001/110/EC, which states that honey must meet the following characteristics to be marketed: humidity level lower than 20%, electrical conductivity lower than 0.8 mS/cm, free acidity lower than 50 mEq/Kg, diastatic index to be determined after treatments must be no less than 8 (Schade scale), the content of fructose and glucose, considering the sum of the two, must not be less than 60g per 100g. (Directive 2001/110/EC). For this analysis, the samples under study were divided into three macro areas: NM (North Mountain) above 600 meters above sea level; NL (North lowland) and SL (South lowland) below 600 meters above sea level (asl). Taking into account the values prescribed by the directive, we can say that the physicochemical values for all groups have been met and are within the norm. The results of the statistical analysis (ANOVA) performed in order to test the effect of the ecological area of honey collection are reported in table 4.1. It can be observed that the values of humidity, conductivity, pH, and diastatic index in the three ecological areas are very similar. It is important to measure humidity as it affects the quality of honey and is closely related to environmental conditions, handling during the harvesting period, and storage as stated by Alvarez-Suarez J.M. et al., 2017, the humidity of all samples in the three macro-areas falls within the recommended international parameters. It is well known that honey has an acidic character, but to date, the pH has not yet been legislated; reference is made to other results such as Alvarez-Suarez J.M. et al., 2017 which study a comparison based on the physical-chemical parameters, chemical composition and biological properties of Cuban polyfloral honeys and we can say that our the pH values are very significant ($p < 0.05$). However, a decrease in free acidity, which is also important for quality and freshness, has been observed

in the NM area compared to the other two areas. It has also been observed that the diastatic index is higher in the SL area compared to the NM area, where it is lower; in all three areas, the honey samples fall within the recommended ranges (> 8 Schade units). Electrical conductivity did not show significant differences across the three macro areas, still complying with the parameters set by law. Furthermore, the percentage of glucose is significantly lower in the NL samples.

Table 4.1 Effect of the ecological area of production on the honey chemical traits.

	Areas			SEM	p-value
	NM	NL	SL		
Moisture (g/100g)	17.0[a]	16.6[b]	17.1[a]	0.14	0.019
Conductivity (mS/cm)	0.10	0.13	0.13	0.02	0.349
pH	3.70[a]	3.70[a]	3.46[b]	0.06	0.002
Free acidity (mEq/kg)	16.0[b]	26.2[a]	24.6[a]	1.15	0.001
Diastase index (Göthe units)	11.5[b]	13.6[ab]	14.4[a]	0.74	0.030
Maltose (g/100 g)	3.1	2.9	2.6	0.26	0.044
Glucose (g/100 g)	29.7[a]	28.4[b]	29.8[a]	0.31	0.001
Fructose (g/100 g)	42.6	42.7	43.1	0.21	0.163

NM, North Mountain; NL, North lowland; SL, South lowland. Superscript letters means differences at p-value < 0.05.

4.2 SENSORY ANALYSIS

The results of the investigated honey sensory traits according to the ecological area of production is given in Table 4.2.1. As reported the samples have been classified into the three macro areas, and by analysing the table, we can confirm that there are no substantial differences in colour, smell, taste, and toughness even though there is a significance effect for some sensory traits. However, it is important to underline that all the sensory analyses reach scores very close to the maximum score, which is 10. In particular, it can be noted that the colour view scores an average of 9.1 to 9.3 out of 10, with the lowest score in the NM (North Mountain) area, while the odour has a slightly lower score ranging from 7.9 to 8.4 out of 10, where the highest score was recorded in the NM area. The taste also scored between 8.0 and 8.3, with the highest score highlighted for honeys from the NM area. Finally, the sensory analysis toughness achieved an average score across the different areas ranging from 8.6 to 9.0, with no differences across the investigated groups.

Table 4.2.1 Effect of the ecological area of production on the honey sensory quality traits.

	Areas			SEM	p-value
	NM	NL	SL		
Colour view	9.1[b]	9.4[a]	9.3[a]	0.06	0.030
Odour	8.4[a]	8.0[b]	7.9[b]	0.06	0.001
Taste	8.3[a]	8.0[b]	8.1[b]	0.07	0.033
Toughness	8.7	9.0	8.6	0.16	0.189

Score is given as scale 1 (low) to 10 (high). NM, North Mountain; NL, North lowland; SL, South lowland. Superscript letters means differences at p-value < 0.05.

The data on sensory data highlighted that the investigated samples were a selection of high-quality honey collecting across very wide spatial ecological areas, thus the differences across these geographical and environmental terroirs are

limited. A better understanding of the effects of the terroir of honey collection might be achieved recording more details about the pedoclimatic conditions within sub-areas and monitoring also the effects of the different botanical clusters where the honey samples were collected. From the statistical analysis of the sensory data, it emerges that the differences, although limited, are significant despite the high variability within the territory. When averaging the results, they flatten out and appear similar, but from a statistical standpoint, the number of analysed samples is large, which leads to a very low SEM error, making even the smallest difference significant. This minimal difference can also be attributed to the fact that they are already selected honeys of high quality.

As still reported in the Materials and Methods chapter the data on sensory traits was recorded by the Italian National Honey Observatory, and made kindly available only for the didactic purpose of this master's degree dissertation and they cannot be used for any type of other purposes and not re-used without specific permission from the Italian National Honey Observatory.

Observing the results obtained in tables 4.2.2 and 4.2.3, both represent the effect of the ecological production area on the colour of honey, but the data export system varies. In fact, the first represents data exported using the Hunter-LAB system, while the second presents data exported in the CIE-LAB system. We can say that the samples contained in the SL macro area have significantly higher L* values in the data exported in the CIE-LAB system, so we can affirm that these samples are characterized by greater brightness, furthermore, the samples show higher values of yellow (b*), while we can observe lower values of red (a*) compared to the other two macro areas. Always considering the tables, particularly table 4.2.3, we can observe that the samples from the NL macro area show a low average brightness value (33.9) compared to the NM and SL macro areas, which are 37.1 and 38.9 respectively, but it has a high value of a*, which represents redness. We can also say that the comparison between the CIE-L*a*b* and Hunter-L*a*b* systems showed a similar trend; only small differences can be noted, with slightly higher values in the data exported using the CIE-LAB system.

According to El Sohaimy S.A. et al., 2015, changes in colour could be attributed to the beekeeper's interventions and the different ways of handling the honeycombs,

such as the use of old wax combs for honey production, mineral content, contamination from heavy metals, and exposure to high temperatures or light. Furthermore, according to Singh I. et al., 2018, colour represents one of the main parameters of quality degradation during honey storage and depends on moisture content along with the storage temperature of the honey.

Table 4.2.2 Effect of the ecological area of production on colour the honey with data exported in HUNTER-LAB L*a*b* system.

	Areas			SEM	p-value
	NM	NL	SL		
Hunter – L*	31.1[ab]	28.3[b]	32.2[a]	0.74	0.001
Hunter – a*	0.47	0.83	0.66	0.10	0.092
Hunter – b*	5.5[ab]	5.0[b]	6.5[a]	0.34	0.001
Hunter – C*	5.6[ab]	5.1[b]	6.6[a]	0.34	0.002
Hunter – H*	84.3[a]	79.8[b]	83.1[a]	0.84	0.002

Abbreviations are forms used to measure the colour of honey. L* is lightness, a* is redness, b* is yellowness, C* is chroma and H* is hue angle. NM, North Mountain; NL, North lowland; SL, South lowland. Superscript letters means differences at p-value < 0.05.

Table 4.2.3 Effect of the ecological area of production on colour the honey with data exported in CIE-LAB L*a*b* system.

	Areas			SEM	p-value
	NM	NL	SL		
CIE – L*	37.1[ab]	33.9[b]	38.3[a]	0.83	0.001
CIE – a*	0.66	1.22	0.94	0.14	0.055
CIE – b*	8.9[ab]	8.4[b]	10.6[a]	0.54	0.002
CIE – C*	9.0[ab]	8.5[b]	10.7[a]	0.54	0.003
CIE – H*	85.0[a]	81.0[b]	84.0[a]	0.75	0.001

NM, North Mountain; NL, North lowland; SL, South lowland.

Abbreviations are forms used to measure the colour of honey. L* is lightness, a* is redness, b* is yellowness, C* is chroma and H* is hue angle. NM, North Mountain; NL, North lowland; SL, South lowland. Superscript letters means differences at p-value < 0.05.

4.3 NIR SPECTROSCOPY PREDICTION

To obtain more reliable results from NIR data coupled with predictive algorithms, it is advisable to avoid heating preliminary samples to temperatures above 39 °C. This is because the intrinsic quality of honey has significantly altered at temperatures above this threshold, which would lead to distorted NIR prediction performance.

According to Escuredo O. et al., 2014, the degree to which the calibration best fits the dataset was measured using multiple correlation coefficients R^2_{CV} and the standard error of cross-validation SECV.

The results of the NIR prediction are given in Table 4.3.1. The performance of the calibration and equation seems to be accurate for moisture, electric conductivity and free acidity, as the R^2_V coefficient of determination from cross-validation is greater than 0.7, while they are moderately precise for the sugars. We can observe that the calibration determination coefficient R^2_{CV} developed high values for humidity and electrical conductivity, medium values for the other characteristics and low values for glucose. Similar values were obtained with the standard errors, SEC and SECV, showing a minimal difference between the values as in the study of Escuredo O. et al., 2014.

Table 4.3.1 Prediction of honey chemical traits by NIR.

Honey Calibration Statistics						
Constituent	Mean	SD	SEC	R^2_{CV}	SECV	R^2_V
Moisture (g/100 g)	17.6	1.1	0.20	0.97	0.22	0.96
Electric Conductivity (mS/cm)	0.19	0.12	0.02	0.97	0.02	0.95
pH	4.08	0.47	0.26	0.70	0.33	0.51
Free acidity (mEq/kg)	26.69	8.32	2.49	0.91	3.70	0.80
Diastase index (Goethe units)	11.2	4.6	1.99	0.81	2.92	0.59
Maltose (g/100 g)	7.96	1.99	1.00	0.75	1.56	0.38
Glucose (g/100 g)	28.73	2.28	2.00	0.33	2.25	0.32
Fructose (g/100 g)	41.33	2.90	2.45	0.42	2.71	0.41

SD, standard deviation; SEC, standard error of calibration; R^2_{CV}, coefficient of determination of calibration; SECV, standard error of cross-validation; R^2_V, coefficient of determination of cross-validation.

The averaged spectra of all the samples analysed in this paper are visible in figure 4.3.1. These spectra were obtained using NIR spectroscopy. As stated by Bisutti et al., 2019, NIR spectroscopy allows us to compare honey samples based on their absorbance differences in the spectral range from 850 to 2500 nm. The honey samples were slightly heated, mixed, and then analysed with NIR instrument, as indicated in the Materials and Methods chapter. The informational spectra of NIR data could be influenced by the presence of sugar crystals, which can hinder the diffusion of light; for this reason, a preliminary heating treatment was carried out. In fact, According to Segato et al. (2019), moderate heating (39 °C for 30 min) does not significantly alter the spectral information, making this treatment suitable for analytical pre-processing purposes as it helps to homogenize honeys. However, this treatment should not alter the physicochemical properties of honeys.

The absorbance value (log (1/R)) of the honey samples is represented on the vertical axis; this value is a measure of the amount of light absorbed by the sample at a specific wavelength. The usable spectral range, which extends from visible light to the near-infrared (NIR) region, is shown on the horizontal axis. These wavelength values are expressed nm and cover a range from 850 to 2500 nm in intervals of 0.5 nm.

The spectra follow a similar profile with a continuous overlap between the samples. The shapes of the NIR spectra resemble those observed in Woodcock et al., 2007 and Bisutti et al., 2019, and our spectra also confirm the presence of dominant bands. In fact, according to Woodcock et al., 2007, the main characteristics of these spectra are the peaks from 1420 to 1470 nm and from 1900 to 1930 nm, both corresponding to deformations of O–H, C–H, and C–H2, and from 2050 to 2150 nm, which correspond to combinations of C–H. According to Bisutti et al., 2019, the wavelength from 2400 to 2500 nm is related to the deformation bands of O–H, N–H, and C–H. defining that these absorbance peaks are typically associated with the presence of an aqueous solution of fructose and glucose. Indeed, in figure 4.3.1, one can observe all these peaks corresponding to the dominant bands defined in the studies by Woodcock et al., 2007 and Bisutti et al., 2019. However, there are also visible minor absorption bands that could be related to the elongation vibrations of C-H and C-H2 in fructose and glucose compounds. The presence and

composition of specific molecules in different samples may be responsible for the variations in absorption observed. (Woodcock et al., 2007; Bisutti et al., 2019).

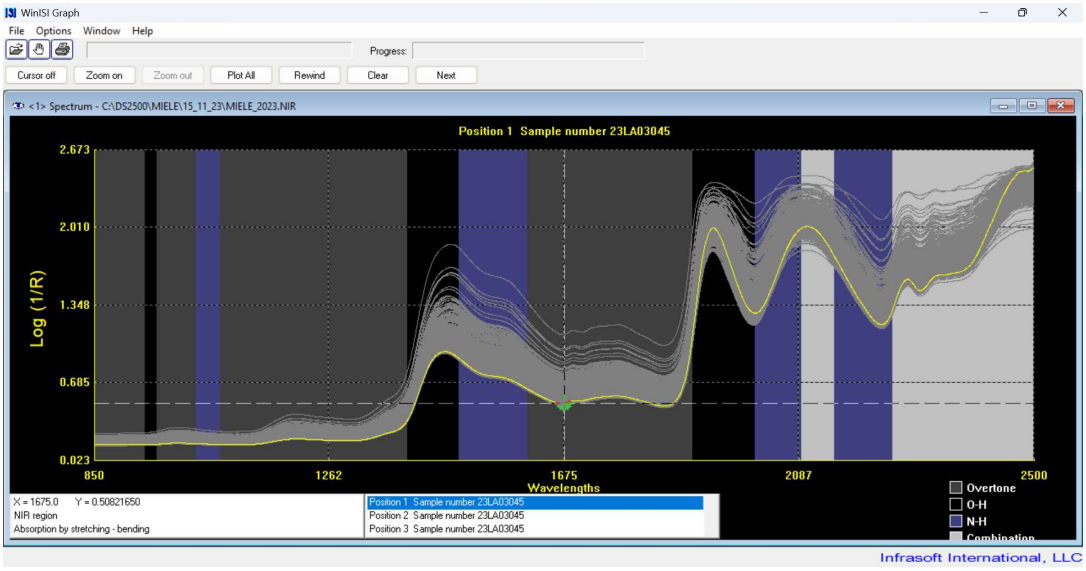


Figure 4.3.1 Representation of the spectra following the application of NIR spectroscopy.

4.4 QUALITY INDEX AND OVERALL MERIT QUALITY CLASS CLASSIFICATION

The effects of the ecological area on the quality index are illustrated on Table 4.4.1.

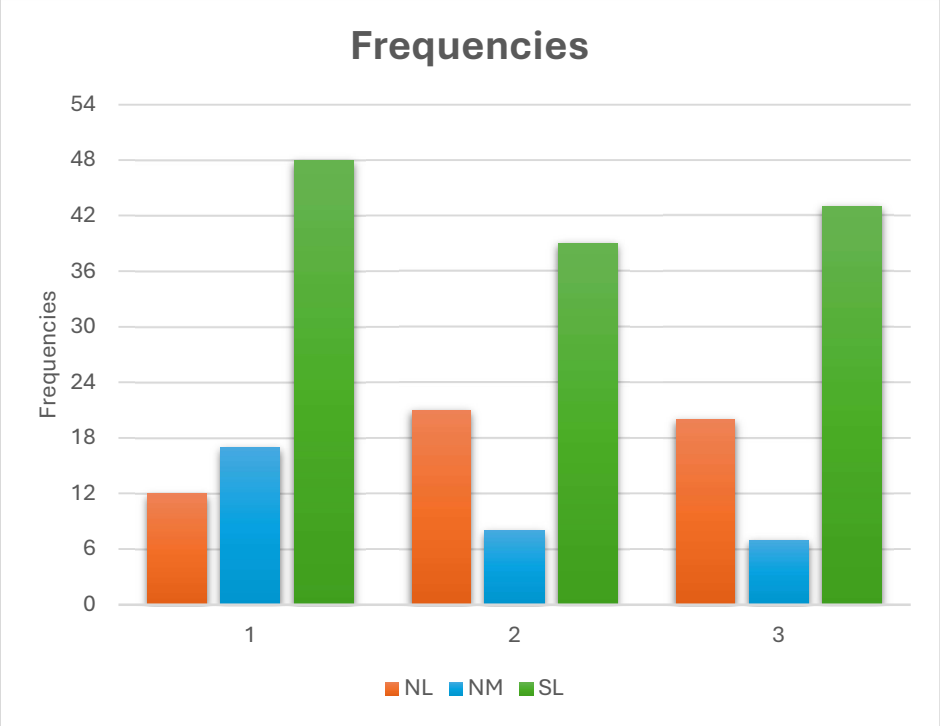
Table 4.4.1 Effect of the ecological area of production on the honey quality index.

	Areas			SEM	p-value
	NM	NL	SL		
Quality index (points)	28.0[b]	30.3[a]	29.4[ab]	0.53	0.048

NM, North Mountain; NL, North lowland; SL, South lowland. Superscript letters means differences at p-value < 0.05.

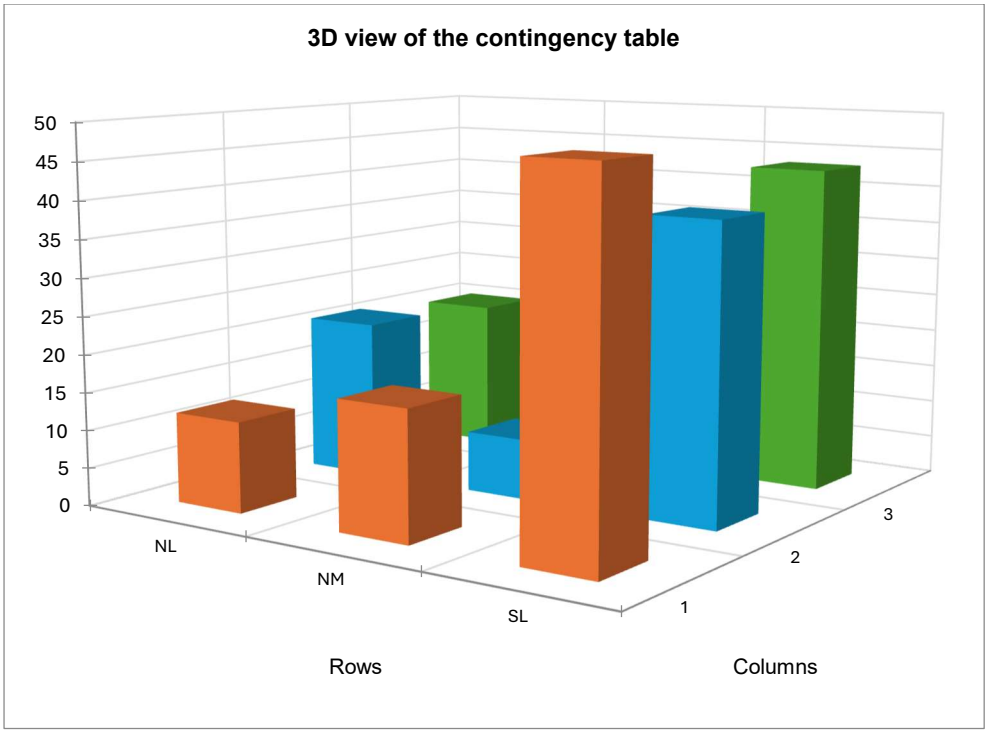
The histogram graphic 4.4.1 and the 3D graph 4.4.2 provides a graphical representation of the frequency of the overall merit quality classes [class 1 (quality index values <27); class 2 (27 ≤ quality index values ≤ 31); class 3 (quality index value > 31)] within the three ecological areas. In particular, it can be observed that the frequency of the NL area represented in red in class 1 is 12%, in class 2 it is 20%, and in class 3 it is 20%, while the frequency in the NM area shown in blue highlights a decrease in frequency from class 1 to class 3; specifically, class 1 is 17%, class 2 is 8%, and class 3 is 7%. Finally, it can be observed that the highest frequency occurred in the SL area, represented in green; in fact, the class 1 it shows 48%, class 2 it is 39%, and finally class 3 it is 43%.

Graphic 4.4.1 Representation of honey overall merit quality class frequency (number of samples within class) according to the ecological areas.



NM, North Mountain; NL, North lowland; SL, South lowland. 1, class 1; 2, class 2; 3, class 3. Classification according to the quality index (points): <27, class 1; 27 ≤ class 2 ≤ 31; > 31, class 3. ***Be careful the order is NL (orange), NM (blue) and SL (green) that is different from the order of presentation in the tables.***

Graphic 4.4.2 3D representation of honey overall merit quality class frequency (number of samples within class) according to the ecological areas.



NM, North Mountain; NL, North lowland; SL, South lowland. 1, class 1; 2, class 2; 3, class 3. Classification according to the quality index (points): <27, class 1; 27 ≤ class 2 ≤ 31; > 31, class 3. **Be careful the order is NL (orange), NM (blue) and SL (green) that is different from the order of presentation in the tables.*

In table 4.4.2, a statistical summary of graphs 4.4.1 and 4.4.2 can be observed, where we can see that in the NL area, the frequency of class 1 is lower than expected with a medium level of significance (p-value < 0.05), while always for class 1, it has been noted that the frequency for the NM area is higher than expected, also with a medium significance (p-value < 0.05).

Table 4.4.2 The computation of the confusion matrix for the honey overall merit quality classes (clusters) form k-means cluster analysis concerning the ecological areas.

	Areas		
	NL	NM	SL
Class 1 (n. samples)	12 (<,**)	17 (>,**)	48 (>, n.s.)
Class 2 (n. samples)	21 (>, n.s.)	8 (<, n.s.)	39 (<, n.s.)
Class 3 (n. samples)	20 (>, n.s.)	7 (<, n.s.)	43 (>, n.s.)

NM, North Mountain; NL, North lowland; South lowland (SL). Classification according to the quality index (points): <27, class 1; 27 ≤ class 2 ≤ 31; > 31, class 3. In parentheses it is indicated whether the observed value is lower (<) or higher (>) than the expected, along with the significance from Fisher's exact test.
n.s. p ≥ 0.10; * p<0.10; ** p<0.05; *** p<0.001

4.5 CORRELATIONS BETWEEN INSTRUMENTAL COLOUR AND SENSORY TRAITS

In table 4.5.1 is reported the correlations between the colour coordinates and the sensory variables, and in table 4.5.2 their significance according to the p-value. Observing tables 4.5.1 and 4.5.2, we can say that there is no strong correlation between the colour coordinates and sensory variables, as confirmed by the significance values (p-value > 0.10). On the contrary, there is a strong correlation between CIE-L*a*b* and HUNTER-L*a*b* systems, which is also confirmed by the significance values (p-value < 0.001).

Table 4.5.1 Correlation matrix between colour coordinates and sensory variables.

Variables	HUNTER-l	HUNTER-a	HUNTER-b	HUNTER-C	HUNTER-h	CIE-l	CIE-a	CIE-b	CIE-C	CIE-h	Color view	Odour	Taste	Toughness
HUNTER-l	**1**	-0,003	**0,863**	**0,852**	**0,364**	**1,000**	-0,045	**0,805**	**0,795**	**0,388**	-0,015	-0,036	0,013	0,081
HUNTER-a	-0,003	**1**	**0,350**	**0,385**	**-0,744**	0,003	**0,998**	**0,414**	**0,441**	**-0,704**	0,018	-0,013	**-0,158**	0,119
HUNTER-b	**0,863**	**0,350**	**1**	**0,999**	**0,158**	**0,868**	**0,307**	**0,994**	**0,992**	**0,199**	0,021	-0,087	-0,088	0,096
HUNTER-C	**0,852**	**0,385**	**0,999**	**1**	0,118	**0,857**	**0,343**	**0,995**	**0,994**	**0,161**	0,022	-0,086	-0,094	0,101
HUNTER-h	**0,364**	**-0,744**	**0,158**	0,118	**1**	**0,366**	**-0,777**	0,110	0,078	**0,998**	-0,006	-0,019	**0,152**	**-0,154**
CIE-l	**1,000**	0,003	**0,868**	**0,857**	**0,366**	**1**	-0,039	**0,811**	**0,801**	**0,391**	-0,015	-0,034	0,015	0,075
CIE-a	-0,045	**0,998**	**0,307**	**0,343**	**-0,777**	-0,039	**1**	**0,372**	**0,400**	**-0,740**	0,021	-0,013	**-0,163**	0,120
CIE-b	**0,805**	**0,414**	**0,994**	**0,995**	0,110	**0,811**	**0,372**	**1**	**0,999**	**0,154**	0,028	-0,092	-0,101	0,093
CIE-C	**0,795**	**0,441**	**0,992**	**0,994**	0,078	**0,801**	**0,400**	**0,999**	**1**	0,123	0,029	-0,092	-0,106	0,097
CIE-h	**0,388**	**-0,704**	**0,199**	**0,161**	**0,998**	**0,391**	**-0,740**	**0,154**	0,123	**1**	-0,003	-0,020	**0,150**	**-0,154**
Color view	-0,015	0,018	0,021	0,022	-0,006	-0,015	0,021	0,028	0,029	-0,003	**1**	-0,098	-0,080	-0,064
Odour	-0,036	-0,013	-0,087	-0,086	-0,019	-0,034	-0,013	-0,092	-0,092	-0,020	-0,098	**1**	**0,481**	**-0,140**
Taste	0,013	**-0,158**	-0,088	-0,094	**0,152**	0,015	**-0,163**	-0,101	-0,106	**0,150**	-0,080	**0,481**	**1**	**-0,179**
Toughness	0,081	0,119	0,096	0,101	**-0,154**	0,075	0,120	0,093	0,097	**-0,154**	-0,064	**-0,140**	**-0,179**	**1**

Table 4.5.2 Significance table with respect to the correlation matrix between colour coordinates and sensory variables.

Variables	HUNTER-l	HUNTER-a	HUNTER-b	HUNTER-C	HUNTER-h	CIE-l	CIE-a	CIE-b	CIE-C	CIE-h	Color view	Odour	Taste	Toughness
HUNTER-l	**0**	0,959	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0,516	**<0.0001**	**<0.0001**	**<0.0001**	0,821	0,604	0,848	0,236
HUNTER-a	0,959	**0**	**<0.0001**	**<0.0001**	**<0.0001**	0,966	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0,789	0,851	**0,020**	0,082
HUNTER-b	**<0.0001**	**<0.0001**	**0**	**<0.0001**	**0,021**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0,003**	0,760	0,202	0,197	0,159
HUNTER-C	**<0.0001**	**<0.0001**	**<0.0001**	**0**	0,083	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0,018**	0,746	0,207	0,168	0,139
HUNTER-h	**<0.0001**	**<0.0001**	**0,021**	0,083	**0**	**<0.0001**	**<0.0001**	0,107	0,254	**<0.0001**	0,932	0,786	**0,026**	**0,024**
CIE-l	**<0.0001**	0,966	**<0.0001**	**<0.0001**	**<0.0001**	**0**	0,573	**<0.0001**	**<0.0001**	**<0.0001**	0,827	0,617	0,828	0,276
CIE-a	0,516	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0,573	**0**	**<0.0001**	**<0.0001**	**<0.0001**	0,762	0,845	**0,017**	0,078
CIE-b	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0,107	**<0.0001**	**<0.0001**	**0**	**<0.0001**	**0,023**	0,683	0,178	0,139	0,175
CIE-C	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0,254	**<0.0001**	**<0.0001**	**<0.0001**	**0**	0,072	0,673	0,181	0,120	0,157
CIE-h	**<0.0001**	**<0.0001**	**0,003**	**0,018**	**<0.0001**	**<0.0001**	**<0.0001**	**0,023**	0,072	**0**	0,964	0,770	**0,028**	**0,024**
Color view	0,821	0,789	0,760	0,746	0,932	0,827	0,762	0,683	0,673	0,964	**0**	0,150	0,240	0,354
Odour	0,604	0,851	0,202	0,207	0,786	0,617	0,845	0,178	0,181	0,770	0,150	**0**	**<0.0001**	**0,041**
Taste	0,848	**0,020**	0,197	0,168	**0,026**	0,828	**0,017**	0,139	0,120	**0,028**	0,240	**<0.0001**	**0**	**0,009**
Toughness	0,236	0,082	0,159	0,139	**0,024**	0,276	0,078	0,175	0,157	**0,024**	0,354	**0,041**	**0,009**	**0**

4.6 PLS-DA MODEL BASED ON NIR DATA

Tables 4.6.1 and 4.6.2 represent the performance of the PLS-DA classification model according to the results of the relative confusion matrices. Table 4.6.1 pertains to the assignment of the overall quality merit classes for honey samples, while Table 4.6.2 relates to the assignment of the quality class of each of the four sensory characteristics.

In general, we can say that the confusion matrix is a useful visual representation for evaluating the performance of the PLS-DA classification model, showing how class instances have been classified correctly or incorrectly.

In both tables, the classes predicted by the model that have been assigned to the honey samples are shown in rows and columns. The actual classes of honey samples are shown in columns.

Analysing table 4.6.1, we can say that all three overall merit quality classes exhibit high accuracy, with 0.81 for class 1, 0.79 for class 2, and 0.82 for class 3. Meanwhile, in table 4.6.2, we can observe that the accuracy values for the quality classes of the sensory characteristics are similar and less satisfactory. However, it is worth noting that the lowest accuracy value is found in class 1 for the sensory characteristic of odour, at 0.52.

In general, we can say that the classification performance of the overall quality merit class assignment is satisfactory.

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*Note: For the classification performance, accuracy has been considered: It represents the proportion of honey samples classified correctly, the sensitivity: It represents the proportion of true positive honey samples (TP) to the number of true positive honey samples and false negatives (FN) the specificity: It represents the proportion of true negative honey samples (TN) compared to the number of true negative honey samples and false positive samples (FP) and the Matthews correlation coefficient (MCC).

Table 4.6.1 Classification performance of confusion matrix in assigning the overall merit class of honey samples based on a PLS-DA model performed on NIR spectroscopy data.

Class			Actual		
Predicted	Sensitivity	Specificity	Class 1	Class 2	Class 3
Class 1	0.72	0.88	**61 (72%)**	9 (16%)	7 (9%)
Class 2	0.71	0.82	14 (16%)	**39 (71%)**	15 (20%)
Class 3	0.71	0.88	10 (12%)	7 (13%)	**53 (71%)**
Total (n=215)			85	55	75
Accuracy			0.81	0.79	0.82
MCC			0.61	0.50	0.60

Bold values represent the samples classified correctly. The percentages of assignment by class are expressed into parentheses.

Accuracy is the percentage of both true positive and true negative cases correctly identified; MCC (Matthew's correlation coefficient) it is an additional method to evaluate the quality of the classifications.

Classification of merit quality class according to the quality index (points) values: <27, class 1; $27 \leq$ class $2 \leq 31$; > 31, class 3.

Table 4.6.2 Classification performance of confusion matrices in assigning sensorial traits (colour view, odour, taste, toughness) within their specific quality class (based on tertiles) of honey samples based on a PLS-DA model performed on NIR spectroscopy data.

Colour view			Actual		
Predicted	Sensitivity	Specificity	Class 1	Class 2	Class 3
Class 1	0.92	0.17	**118 (92%)**	58 (83%)	14 (83%)
Class 2	0.17	0.92	9 (7%)	**12 (17%)**	2 (11%)
Class 3	0.06	0.99	1 (1%)	0 (0%)	**1 (6%)**
Total (n=215)			128	70	17
Accuracy			0.61	0.68	0.92
MCC			0.14	0.14	0.15

Odour			Actual		
Predicted	Sensitivity	Specificity	Class 1	Class 2	Class 3
Class 1	0.41	0.58	**34 (41%)**	29 (45%)	28 (40%)
Class 2	0.31	0.70	26 (33%)	**20 (31%)**	18 (26%)
Class 3	0.34	0.75	20 (26%)	16 (24%)	**24 (34%)**
Total (n=215)			80	65	70
Accuracy			0.52	0.59	0.62
MCC			-0.01	0.01	0.09

Taste			Actual		
Predicted	Sensitivity	Specificity	Class 1	Class 2	Class 3
Class 1	0.50	0.72	**37 (50%)**	20 (29%)	20 (28%)
Class 2	0.36	0.73	17 (24%)	**25 (36%)**	21 (29%)
Class 3	0.43	0.69	19 (26%)	25 (35%)	**31 (43%)**
Total (n=215)			73	70	72
Accuracy			0.64	0.61	0.61
MCC			0.22	0.09	0.12

Toughness				Actual	
Predicted	Sensitivity	Specificity	Class 1	Class 2	Class 3
Class 1	0.87	0.75	**74 (87%)**	30 (49%)	3 (4%)
Class 2	0.21	0.92	6 (7%)	**13 (21%)**	5 (8%)
Class 3	0.87	0.84	5 (6%)	17 (30%)	**62 (88%)**
Total (n=215)			85	60	70
Accuracy			0.80	0.72	0.85
MCC			0.61	0.19	0.69

Bold values represent the samples classified correctly. The percentages of assignment by class are expressed into parentheses.

Accuracy is the percentage of both true positive and true negative cases correctly identified; MCC (Matthew's correlation coefficient) it is an additional method to evaluate the quality of the classifications.

Chapter 5
CONCLUSIONS

The main outcome of this MSC dissertation is that the analysed chemical, colorimetric, and sensory quality variables of the investigated Italian polyfloral honey samples are similar to other studies and in line with the limits defined and prescribed by the EU directive 2001/110/EC. The sensory analysis detected minimal differences in terms of colour, odour, taste, and toughness across the three ecological areas where the honey samples were collected. The average values of the sensory characteristics are very high; this may be due to the fact that the analysed dataset consisted of a selection of high-quality honeys as they were intended for a national competition.

The prediction of NIR spectroscopy has shown good results, achieving a coefficient of determination (R^2_{CV}) above 0.70, especially for certain physicochemical characteristics, particularly for moisture (0.96) and electrical conductivity (0.95), while the accuracy for pH, free acidity, and diastatic index was moderately accurate. Referring to the sugars like maltose (0.38), glucose (0.32), and fructose (0.41), the NIR prediction performance were not accurate, but this is probably due to some difficulties to achieve a good repeatability in the wet chemistry determination. From the evaluation of the correlations between instrumental colour and sensory characteristics, it emerged that there is no significant relationship. In order to analyse the overall quality of the samples, a quality index was proposed based on the scores assigned to the chemical and sensory variables, resulting in slightly lower value for the NM-samples (North Mountain) compared to the NL (North Lowland) and SL (South Lowland) ones.

By creating a PLS-DA model with the NIR data, the prediction of the overall merit quality classes achieved good results, while the NIR model was not able enough reliable to discriminate the class of the specific sensory trait.

In conclusion, the use of non-destructive tools such as a VIS-based spectrophotometer and NIR devices leads to a satisfactory prediction of the chemical-physical characteristics and the proposed quality index, while they are

not reliable for sensory characteristics. Despite the quality of multifloral honey being influenced by many factors such as nectar composition, climate, geographical source, harvesting techniques, and storage conditions, this study reveals small but significant differences that are likely moderate, probably due to the fact that this dataset was a selection of the best high-quality multifloral honeys from Italy.

Chapter 6

APPENDIX

6.1 GRAPHICAL ABSTRACT AND EXPERIMENTAL PICTURES

Figure 6.1.1 The image shows the VIS CM-600d spectrophotometer, produced by Konica Minolta Ramsey Sensing, Inc., positioned in a vertical device with the cuvette above the light source. Each scan was conducted in a dark room, and the cuvette was rotated by about 120°. (Credit: Irene Sandonà)

Figure 6.1.2 The image shows the FOSS DS-2500 spectroscopic instrument (FossNIR-System, Silver Spring, MD, USA) whit ring cell with a quartz window allowing the irradiation of approximately 12.6 cm^2. This device covers a scanning range from 850 to 2500 nm at intervals of 0.5 nm in reflection mode. (Credit: Irene Sandonà)

Figure 6.1.3.a, 6.1.3b, 6.1.3.c These images represent some honey samples in chromatic order after thermal pretreatment, ready for analysis. Subsequently, the information was exported to the HUNTER L*a*b* system. where L* indicates brightness, a* the red color, and b* the yellow color. (Credit: Irene Sandonà)

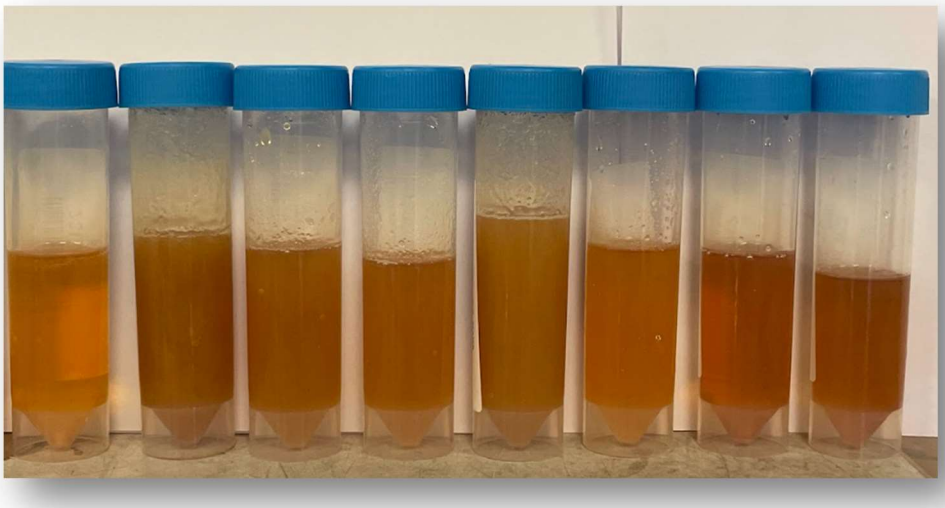

Figure 6.1.3.a The samples had on average these values:
L* 28.9, a* 0.6, b* 4.3.

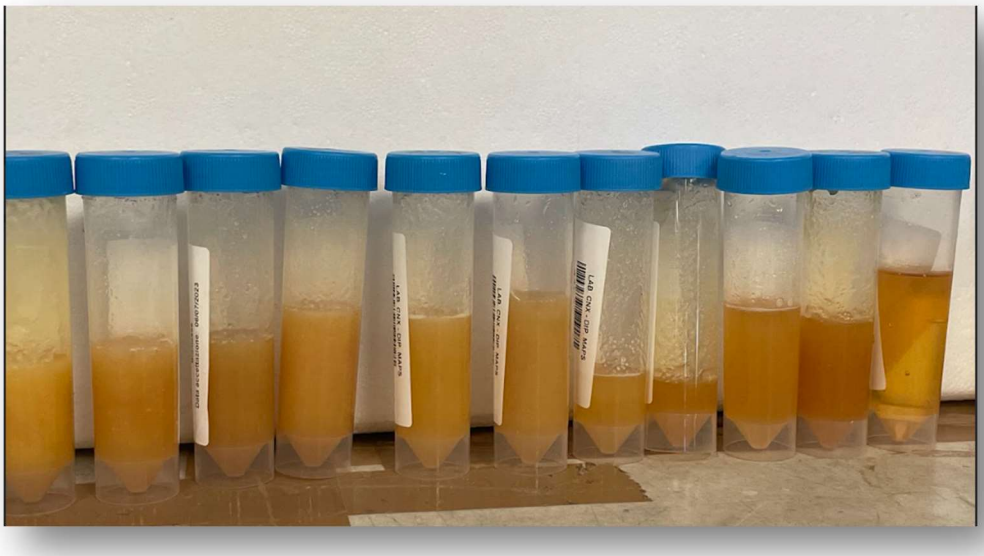

Figure 6.1.3.b The samples above had on average these values:
L* 28.2, a* 1.1, b* 6.5.

Figure 6.1.3.c The samples had on average these values:

L* 45.1, a* 0.4, b* 9.2.

6.2 ANATOMY OF BEES

The *Apis mellifera* belongs to the class "Insecta", the body is covered with a protective layer, provided with bristles and body hairs and is composed of three body segments: head, chest and abdomen (Figure 6.2.1).

The head formed by: *Eyes* -> have 5 eyes, 2 of which are composed of thousands of small elements that allow the shape of the image of the surrounding environment and 3 small, simple arranged at the top of the head used to see the light. Bees see in the ultraviolet spectrum, they cannot see the colours that we see, which is why flowers have evolved to have petals with often patterns in the UV spectrum to attract them increasing the possibility of pollination. *Antennas* -> are cylindrical shape, flexible used to smell and feel. (Honeybee research centre)[1]

Mouth apparatus -> formed by the upper lip, two mandibles used for biting, grasping food and working the wax, two maxillae used to grasp enemy insects, for defence and finally by the lower lip formed by various articles including the "ligula" is a kind of proboscis used in collecting nectar, the bee inserted into the floral calyx the ligula, touches and sucks the nectar. In the queen and drones the mouth apparatus is less developed. (apicolturaonline.it)[2]

The chest, the central segment, is covered with body hair that masks the segmentation is formed by muscles for motor function.

The chest serves as a base for the attachment of the 6 legs and the two pairs of wings. The *legs* are used both for walking and for collecting pollen and for cleaning the body. The front legs are shorter and have comb-like hairs used to clean their body from pollen; the middle legs are more robust and have the spur that bees use to detach pollen from the baskets; worker bees also have baskets on their hind legs to hold the pollen firmly. The *wings* are membranous and are made up of two thin plates; they twist and form a figure eight pattern which allows the bees to fly; if the wings only moved back and forth, they would not be able to fly due to their weight. (apicolturaonline.it)[2] The abdomen is morphologically composed of 10 segments and very important organs: *The wax glands*, the wax is produced only by worker bees between the thirteenth and eighteenth day of life, in small pieces cut into slices thanks to the presence of glands on the belly, they are able to remove it and use it

to build the honeycomb, initially the wax is white in colour, I assume the yellow or brown colour when the bees walk on it with dirty paws. *Honey stomach*, in addition to the normal stomach bees have a stomach called the "honey stomach" to store the nectar before it is returned to the hive, this stomach contains bacterial enzymes that are introduced into the honey, this is where it gets its health benefits.

The *sting* is in the last segment of the abdomen except for the kelp that is lacking. The sting is composed of 3 parts and is connected to the venomous apparatus, only the worker bees and the queen they have it, worker bees can only use it once because it is barbed formed by backward-faced teeth, while that of the Queen bee it is smooth and can sting several times. (Honeybee research centre)[1]

Bee venom is recognized as having a therapeutic function in cases of rheumatic conditions. (apicolturaonline.it)[2]

The *respiratory system*, bees do not have lungs, but small holes called spiracles along the sides of the abdomen for air exchange, are connected to the trachea that supply oxygen to the rest of the body. (Honeybee research centre)[1]

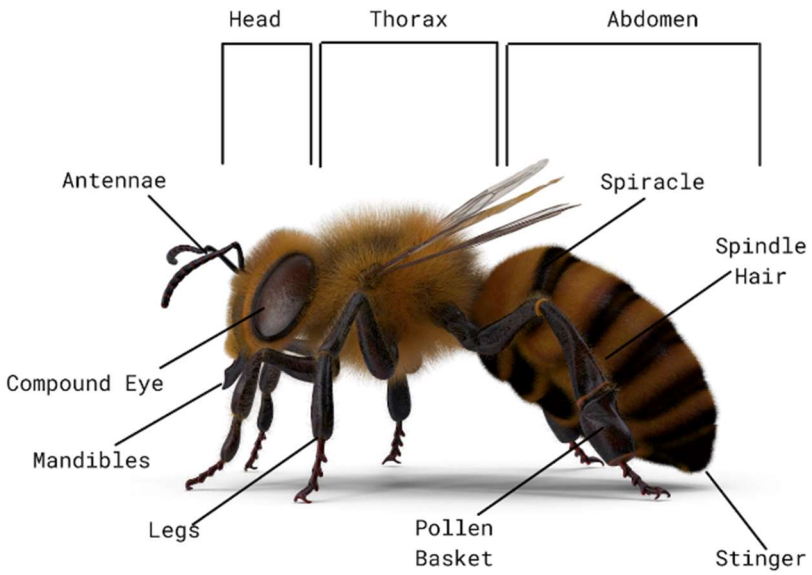

Figure 6.2.1: Graphical representation of the anatomical subdivision of the Mellifera Apis. (Source: Honeybee Research Centre)

[1]https://honeybee research centre, the university of Guelph

[2]https://www.apicolturaonline.it/morfolo.htm

6.3 EVOLUTION AND SOCIAL STRUCTURE

Bees generally have four stages of growth: egg, larva, pup and finally adult. The queen lays one egg (figure 6.3.1) per cell throughout the nest generally in the center, they are very small and look like a grain of rice, worker bees are laid in smaller cells generally in the center while kelp eggs are laid in larger cells tending to be at the end of the frame. After 3 days, the egg becomes a larva, (figure 6.3.2) it is the worker bees that take care of the growth of the larvae, when they are large enough the larvae are covered by the worker bee with a wax cap (figure 6.3.3), once covered the larva will wrap a cocoon around itself and will develop into a pupa (figure 6.3.4), here the eyes, legs, wings and other body parts develop. Finally, when the pup has finished its growth, it transforms into an adult (figure 6.3.5), when it is ready it will start to chew the cocoon and the wax casing and will emerge into the hive. Egg-laying queens take 16 days to reach maturity, worker bees 21 days, and kelp 24 days. (Honeybee research centre)[1]

Figure 6.3.1: Representation of the stages of development: egg
(Source: http://www.apicolturaveneroni.it/)

[1]https://honeybee research centre, the university of Guelph

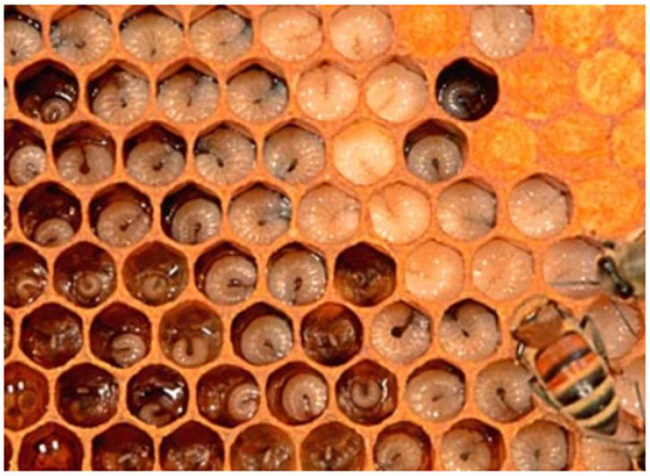

Figure 6.3.2: Representation of the stages of development: larva (Source: http://www.apicolturaveneroni.it/)

Figure 6.3.3: Representation of the stages of development: larvae are covered by the worker bee with a wax cap. (Credit: Irene Sandonà)

Figure 6.3.4: Representation of the stages of development: pupa (Source: http://www.apicolturaveneroni.it/)

Figure 6.3.5: Representation of the stages of development: adult (Credit: Irene Sandonà)

6.4 THE SOCIAL STRUCTURE OF BEES

Bees are social insects that form a colony within the hive in which there are three different types of individuals, each with its own function and different work: The "*queen bee*" (Figure 6.4.1) is similar to worker bees but is distinguished by its larger, more elongated abdomen and lacks the pollen-gathering apparatus and wax glands. To become a queen bee, a larva will be fed by worker bees with a royal palate (a substance secreted by the hypopharyngeal glands on the worker bee's head) and placed in a special longer cell. She is the only fertile female in the colony that can reproduce. She is fertilized by kelps and lays fertile eggs in cells that will hatch into worker bees and sterile eggs that will develop into kelps through a process called parthenogenesis. The queen usually begins laying eggs in the spring and can lay up to 2.000 eggs per day. The queen will make her "nuptial flight" between the tenth and fourteenth day after becoming an adult; she will be fertilized by the kelps and will be able to store the sperm in her spermatheca and use it to fertilize her eggs for her entire life, which lasts on average 3 years.

The queen rarely leaves the hive; she only leaves it for the "nuptial flight" or during swarming to form a new colony. When the queen gets old or no longer lays eggs as before, the worker bees will replace her, choosing among the larvae and feeding them with royal sage; the first queen to emerge will be the new one that will find the other queens and sting them to prevent them from being born; the old queen will also be stung. (Honeybee research centre- entomologia.it)[1-2]

"*Worker bees*" (Figure 6.4.2) are females that do not have the reproductive system developed like that of the queen, they can lay eggs in some rare cases in the absence of the director, the eggs are not fertile, so they only originate foxes; they constitute the majority of the colony; they do all the work in the hive, responsible for collecting food and water, protecting the harvester, and cleaning the queen. Bees born in late autumn are referred to as "winter bees" and live for four to six months while the colony overwinters. In general, the Worker bees have a maximum lifespan of six weeks. Within the hive, worker bees are organized into groups and assigned specific tasks. (Honeybee research centre- entomologia.it)[1-2]

The "*kelps"* (Figure 6.4.3) are the males of the colony; they are much larger than working bees; they are born from unfertilized eggs; therefore, they have half the genetic structure of the species; they live up to 3 months and are produced from late spring to mid-summer; they do not collect pollen or nectar; their function is to fertilize the queen; once the coupling with the queen is finalized, the kelp dies;
if in the year there are no "nuptial flight" in the colony in which they live, they participate in the "nuptial flight" of the queen of some other colony, contributing to the family's genetic wealth. The kelps are devoid of sting.
At the end of the summer, the kelps are chased by the working bees because there is not enough food for them in autumn and winter. (Honeybee research centre-entomologia.it)[1-2]

[1]https://honeybee research centre, the university of Guelph

[2]https://entomologia.it/le-api-apis-mellifera

Figure 6.4.1: Representation of the "*queen bee*" (Credit: Irene Sandonà)

Figure 6.4.2: Representation of the "*worker bee*" (Credit: Irene Sandonà)

Figure 6.4.3: Representation of the "*Kelps*" (Credit: Irene Sandonà)

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