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MASTER THESIS

TARGET SCREENING OF PHENOLIC COMPOUNDS IN HONEY SAMPLES IN ORDER TO DIFFERENTIATE THEM ACCORDING TO THEIR BOTANICAL AND GEOGRAPHICAL ORIGIN USING ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COMBINED WITH HIGH RESOLUTION MASS SPECTROMETRY «UHPLC-ESI-QTOF-MS»

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MASTER THESIS

Target screening of phenolic compounds in honey samples in order to differentiate them according to their botanical and geographical origin using ultra high performance liquid chromatography combined with high resolution mass spectrometry «UHPLC-ESI-QTOF-MS»

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Στοχευμένη σάρωση φαινολικών ενώσεων σε δείγματα μελιών με σκοπό τη βοτανική και γεωγραφική τους διαφοροποίηση με χρήση της τεχνικής υγροχρωματογραφίας υπερ-υψηλής απόδοσης συζευγμένη με φασματομετρία μάζας υψηλής διακριτικής ικανότητας

«UHPLC-ESI-QTOF MS»

ΠΑΝΑΓΙΩΤΑ ΚΑΤΣΙΑΝΟΥ

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ABSTRACT

Due to its economic importance, honey is subjected to various fraudulent practices like mislabeling (blossom sold as honeydew) or addition of illegal substances. Hence the evaluation and verification of authenticity is a task of great importance for the producers, consumers and regulatory bodies. Tremendous improvements in analytical methodologies have been carried out in order to provide an adequate answer to the detection of adulterants and to verify the botanical and geographical origin of honey.

In this thesis, the assessment of honey authenticity was based on the analysis of phenolic profile due to the fact that the concentration and the presence of these compounds are strictly connected to geographical and botanical origin. The determination of phenolic compounds was performed using an already in-house developed and validated UHPLC-ESI-QTOF MS method in order to find unique marker for discrimination of honey samples. A chemometric evaluation by statistical tools could be useful since honey is a complex mixture and the data obtained by fingerprinting techniques cannot easily be handled and interpreted.

The method was applied to 135 unifloral and polyfloral Greek honey samples derived from 5 different botanical origins. A database consisting of 25 phenolic compounds, encountered in honey, was utilized in order to identify and quantify each of these compounds in all samples. The target screening was performed using the appropriate internal standards for each analyte and it was based on certain identification criteria while the quantification was performed using the standard addition method. It has to be noticed that potential authenticity markers have been spotted via target screening approach. High average concentration of chrysin and pinocembrin has been measured in blossom honeys contrary to other unifloral honeys. Also another analytes such as pinobanksin, and galangin seems to differentiate blossom from thyme honey samples. In conclusion, the results obtained in the present work illustrate the importance of investigating polyphenols content in honey authenticity studies.

SUBJECT AREA: Food Authenticity, Adulteration

KEYWORDS: honey authenticity, honey adulteration, phenolic compounds, botanical origin, geographical origin, UHPLC-QToF-MS, authenticity markers, target screening

ΠΕΡΙΛΗΨΗ

Λόγω της οικονομικής του σημασίας το μέλι υπόκειται σε διάφορες τεχνικές παραπλάνησης όπως η λανθασμένη επισήμανση καθώς και η προσθήκη παράνομων προσμίξεων. Για αυτό το λόγο η εκτίμηση και η επιβεβαίωση της αυθεντικότητάς του είναι υψίστης σημασίας για τους καταναλωτές, τους παραγωγούς και τις κανονιστικές αρχές. Μεγάλη πρόοδος έχει σημειωθεί στις αναλυτικές μεθοδολογίες με σκοπό τον προσδιορισμό των προσμίξεων και την επαλήθευση της βοτανικής και γεωγραφικής προέλευσης του μελιού.

Στην παρούσα διπλωματική εκτιμάται η αυθεντικότητα του μελιού χρησιμοποιώντας το φαινολικό του προφίλ αφού σχετίζεται άμεσα με την γεωγραφική και βοτανική του προέλευση. Ο προσδιορισμός αυτών βασίστηκε σε μια ήδη ανεπτυγμένη και επικυρωμένη UHPLC-ESI-QTOF-MS μέθοδο με σκοπό να ευρεθούν νέοι δείκτες για τη διαφοροποίηση των δειγμάτων. Η χημειομετρική εκτίμηση των δεδομένων με χρήση στατιστικών εργαλείων είναι σημαντική διότι το μέλι αποτελεί μία σύνθετη μήτρα και ο όγκος των παραγόμενων δεδομένων δεν είναι έυκολα διαχειρίσιμος και ερμηνεύσιμος.

Η στοχευμένη σάρωση των ενώσεων πραγματοποιήθηκε με χρήση εσωτερικών προτύπων για κάθε αναλύτη και βασίστηκε σε συγκεκριμένα κριτήρια ταυτοποίησης ενώ η ποσοτικοποίηση έγινε με την μέθοδο προσθήκης γνωστής ποσότητας. Θα πρέπει να σημειωθεί ότι βρέθηκαν πιθανοί δείκτες αυθεντικότητας. Για παράδειγμα, υψηλότερη συγκέντρωση chrysin και pinocembrin βρέθηκε στα μέλια άνθεων σε σχέση με τα υπόλοιπα. Επίσης κάποιοι αναλύτες όπως το pinocembrin, το naringenin και το galangin βρέθηκαν να έχουν υψηλότερη μέση συγκέντρωση στα μέλια άνθεων σε σχέση με τα θυμαρίσια μέλια. Συμπερασματικά, τα αποτελέσματα της παρούσας διπλωματικής μεταπτυχιακής εργασίας αντανακλούν την σπουδαιότητα του φαινολικού περιεχομένου στις μελέτες αυθεντικότητας για το μέλι.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Αυθεντικότητα Τροφίμων , Νοθεία

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: αυθεντικότητα μελιού, νοθεία μελιού, φαινολικά συστατικά, βοτανική προέλευση, γεωγραφική προέλευση, UHPLC-QToF-MS, δείκτες αυθεντικότητας, στοχευμένη σάρωση.

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PREFACE

This master thesis was conceived and performed at the Laboratory of Analytical Chemistry (Department of Chemistry, National and Kapodistrian University of Athens, Greece) under the supervision of the Professor, Nikolaos Thomaidis.

First of all, I would like to thank my supervisor, Dr. Nikolaos Thomaidis, for giving me the opportunity to become a member of his research group as well as for the cooperation regarding this master thesis, the valuable professional and personal advice. I would also like to thank the other two members of the examination committee, Assistant Professor Ioannis Dotsikas and Assistant Professor Evagelos Gikas for their insightful comments and remarks.

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CHAPTER 1: Honey an overview

1.1 Definition

Honey is defined as a supersaturated aqueous solution of sugars to which the main contributors are fructose and glycose. It is produced by honeybees “Apis mellifera” from nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants. Apart from sugars, it incorporates a significant number of other minor components such as proteins, elements, aroma compounds, enzymes, organic acids, phenolic compounds, pigments, flavonoids, wax and vitamins which represent a detailed chemical fingerprint [1]. Some of the above mentioned constituents are added during the maturation process of honey while other originated either from honeybees or from plants. The composition of honey depends on many factors such as geographical and botanical origin which are the most representative. Other aspects, also affect the quality of honey, are weather conditions, honeybee population, processing, manipulation, packaging and storage time [2].

Figure 1. Production of honey in honeycombs, source:[3]

1.2 Types of honey

In accordance with Council Directive 2001/101/EC, there are two types of honey regarding its origin. The first type of honey obtained from nectar, especially from the carbohydrate exudates of the plants and is called blossom honey or nectar honey. The amount of nectar that is collected by the honeybees is related to either internal factors such as the nature of the plant or external factors like temperature, humidity, soil

conditions, the amount of wind and day length. The second type is the honeydew honey. It is produced by the secretion of living parts of certain trees or plants (genera Pinus, Abies, Castanea) or by the excretion of plant sucking insects such as aphids. These insects suck a part of tree sap which is rich in nutrients and after minerals and proteins being absorbed, the leftovers are excreted.

Then, honeybees consume nectar or honeydew, transform it by combining with enzymes and other compounds and finally regurgitate it on the honeycomb in order to dehydrated and store. The production of honeydew honey is greatly related to the plant species of a region, the climate and the local weather. Typical examples of blossom honeys are heather, blossom and citrus whereas common honeydew honeys derived from pine, fir and oak. Consumers have shown a different preference between these two types of honey. Indeed, in many European countries there is an increasing demand for honeydew honey [4]. Consequently, the differentiation of these two types of honey is of great importance in order to assure the quality of the product. The honeydew honey compared to blossom contains higher values of oligosaccharides, pH, acidity, ash content and electrical conductivity and lower values of monosaccharides (glucose and fructose).The most important reason which makes this honey more valuable is the antibacterial and antioxidant capacity because of the higher amount of phenolic compounds that contain.

The simplest way to verify the botanical origin is the determination of the electric conductivity. According to the Council Directive 2001/101/EC blossom honeys have electric conductivity values below 0.80 mS/cm, while the honeydew and the mix honeys exceed this value. However, there are many exceptions to this rule [5]. For this reason, certain physicochemical parameters and sugar composition are used as additional criteria to verify the botanical origin of honeys. In recent years some methods based on NIR and MIR spectroscopy have been developed for food authenticity and have been proved to have the capability to determine simultaneously several markers for honey quality control.

1.3 Honey in Greece

Greece has a long tradition and broad technical knowledge on bee-keeping. The favorable environmental and geographical attributes, the variety of Greek flora and the traditional methods of beekeeping have contributed to the production of a thick in consistency honey with a variety of tastes, scents and hues, makes it the most important sweetener. Greek honey has been declared a national product with prestige and reputation. It is rich in aromatic substances, vitamins, minerals and antioxidants and has

less humidity than others. For this reason it is widely accepted that Greek honey is a nutritional natural food which daily consumption enhances health.

Greece produces 13,000- 16,000 tons of honey annually which derived mainly from pine trees in Thasos, Halkidiki, Evia and Crete, fir trees in Peloponnese, thyme plants in Aegean and Ionian islands as well as Central Greece and oak trees found in several regions. Chestnut honey, cotton honey and orange honey are also encountered in Greece but to a lesser extent compared to the others. Each one of these honey matrices features exceptional characteristics which are comprehensively discussed in the following bullets [6].

- Thyme honey: this honey is the most preferable unifloral honey in Greece and its price ranges from two to three times higher than any other honey. It has been receiving great attention because bees which feed with these plants produce less honey, making it limited and thus more expensive. Thyme honey is light in color with intense aroma and tends to crystallize depending on storage time. Furthermore, it is characterized by antimicrobial and anti–estrogenic effect. The most famous Greek regions produced this type of honey are Crete and Kythera.
- Pine honey: It is the most abundant honey type in Greece since the 65% of the production is pine. It is rich in minerals like potassium, phosphorous, iron and sodium and antioxidants. Due to the fact that sugar content is low, the crystallization process is really slow and it is considered more suitable for patients with diabetes.
- Fir honey: The only PDO Greek honey found in Vytina, Peloponnese with no particular aroma but a good taste. It is the rarest type of honeydew honey, which never crystallizes and thanks to lower moisture content and higher pH value is not being degraded. Its color depends on the geographical origin and its appearance makes it unique.
- Oak honey: It is a dark amber honey and crystallizes firmly over a period of 1-2 years, forming large crystals. It has a spicy taste and it is not so sweet due to the low content of oligosaccharides. Moreover, oak tree honey has a series of health and nutritional benefits. According to recent researches, it is rich in antioxidants, thus being able to prevent a lot of diseases. Furthermore, it contains bee-glue which is antimicrobial and anti-inflammatory.

- Chestnut honey: Chestnut honeys possess a brownreddish color with a strong plant odour and aroma, and a bitter and slightly sour taste. In addition, it is fluid with a very slow crystallization rate. In European Union it is referred as blossom honey and share common characteristics with honeydew honey.
- Cotton honey: Cotton honeys are light to medium brown in color and crystallized in a few months after extraction (small and medium irregular crystals). It comes mainly from extrafloral nectaries (Molan 1998) and the total number of plant elements in cotton honeys is expected to be low. It possess the higher antibacteriostatic action.
- Citrus honey: This type of honey is very light to amber in colour. It is characterized by a delicate floral odour and aroma and crystallized soon after extraction (fine crystals), whereas it possess high content of zinc element. A characteristic example of citrus is the orange honey which contains variable amount of pollen from Citrus spp. (2.9–26.5%). In Greece citrus honey is mainly produced in three geographical areas (Argolida, Arta and Lakonia).
- Blossom honey: It is originated from wild flowers. It has a delicious aroma that is more delicate than other honeys and also can crystallize easily
  [7], [8], [9].

1.4 Chemical composition

Honey is a nutritional source consists of 200 macro and micro nutrients. As regards the former, sugars are the mainly constituents of honey representing 70% of its dry mass, whereas moisture accounts for approximately 20% of its content. Concerning the latter minerals, enzymes, proteins, free amino acids, organic acids, vitamins and phenolic compounds are encompassed. Essential oils, sterols, pigments and phospholipids are also present. The chemical structures and other properties of the above mentioned compounds will be described thoroughly in the next paragraphs. Prolonged storage period as well as thermal treatment of honey play significant role in either the final composition or the quality of honey. Special attention should be paid to phenolic compounds due to their beneficial properties in human health such as antioxidant activity even though their abundance is lower compared to other constituents [2].

1.4.1 Phenolic compounds

Phenolic compounds are secondary metabolites of plants, biosynthesized mainly for protection against oxidative stress and transferred via the nectar to the honey. They can appear with 8000 different structures depending on honey origin and therefore is expected to show different biological activities. They can be divided into two important groups, which are widely known in honey, namely phenolic acids and flavonoids as well as the derivatives of its category. They are low molecular weight compounds which can appear in conjugated forms with one or more sugars residues linked to their hydroxyl groups. Other compounds such as carboxyl and organic acids, amines and lipids can act in the same way as sugars [10].

To begin with, phenolic acids can be separated into two groups, the hydroxybenzoic acids derivatives and the hydroxycinnamic acids derivatives. The first group has a characteristic structure of C6-C1 and the most representative examples are vanillic acid and syringic acid. The last group has a characteristic C6-C3 structure and caffeic acid, ferulic acid and p coumaric acid belong to it.

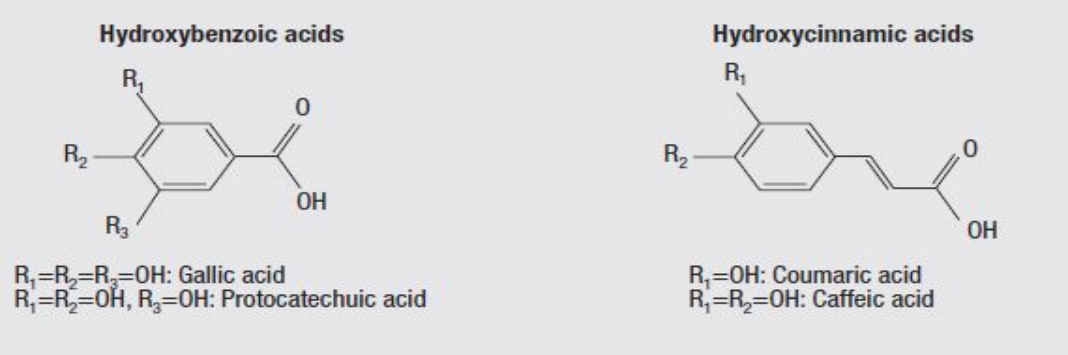

Figure 2. The structure of hyrdoxybenzoic and hydroxycinnamic acids, source: [11]

Flavonoids share a common carbon skeleton of diphenyl propanes, two benzene rings (ring A and B) joined by a linear three-carbon chain. The central three-carbon chain may form a closed pyran ring (ring C) with one of the benzene rings (figure 2).These compounds generally appears as glycosides with glycose as major sugar in order to form a flavonoid less reactive and more soluble to water which has a protective effect on plant.

Flavonoids represent one of the largest group of phenolic compounds. Occasionally they can be as free form but usually found as glycosides. They can be divided according to the degree of oxidation of the pyran ring C and the position of the secondary ring B into

several classes. The classes of flavonoids encountered in honey are the following: flavonols, flavones, flavanones, isoflavones, anthocyanidins and flavanols as shown in figure 3. More than 4000 flavonoids structures have been cited in literature and this number is constantly growing due to the occurrence of various substitution patterns in the basic skeletal structure and the tendency of polymerization that they possess. [12]

Flavonols have a double bond between C2 and C3, with a hydroxyl group in the C3-position and a ketone group in C4.The most common flavonol in foodstuffs is quercetin. It has to be mentioned that the presence of flavonols is stimulated by light. Flavones have a double bond between C2 and C3, only an oxygen atom in the C4 and are the less common flavonoids. Apigenin, luteolin and chrysin belong to this group. Flavanones are characterized by the presence of a saturated three-carbon chain and only an oxygen atom in the C4. Naringenin, hesperetin and eriodictyol are among the most common observed flavanones. Isoflavones contain an unsaturated chain between C_2 and C_3 carbons of the C ring. Also, they have hydroxyl groups at the positions 5 and 7of A-ring and at the position 4 of B-ring. They are also called phytoestrogens due to their structural similarity to estrogens. Anthocyanidins are found mainly as glycosides of their respective aglycones form, with the sugar moiety mainly attached at the 3-position on the C-ring or at the 5, 7-position on the A-ring. Anthocyanidins and isoflavones are prone to physicochemical degradation during thermal processing. Flavanols are the most abundant group in honey and contain a saturated three-carbon chain with a hydroxyl group in the C3. Unlike other classes of flavonoids, flavanols are not glycosylated in foods. The main representative flavanols in honey are catechin and epicatechin.

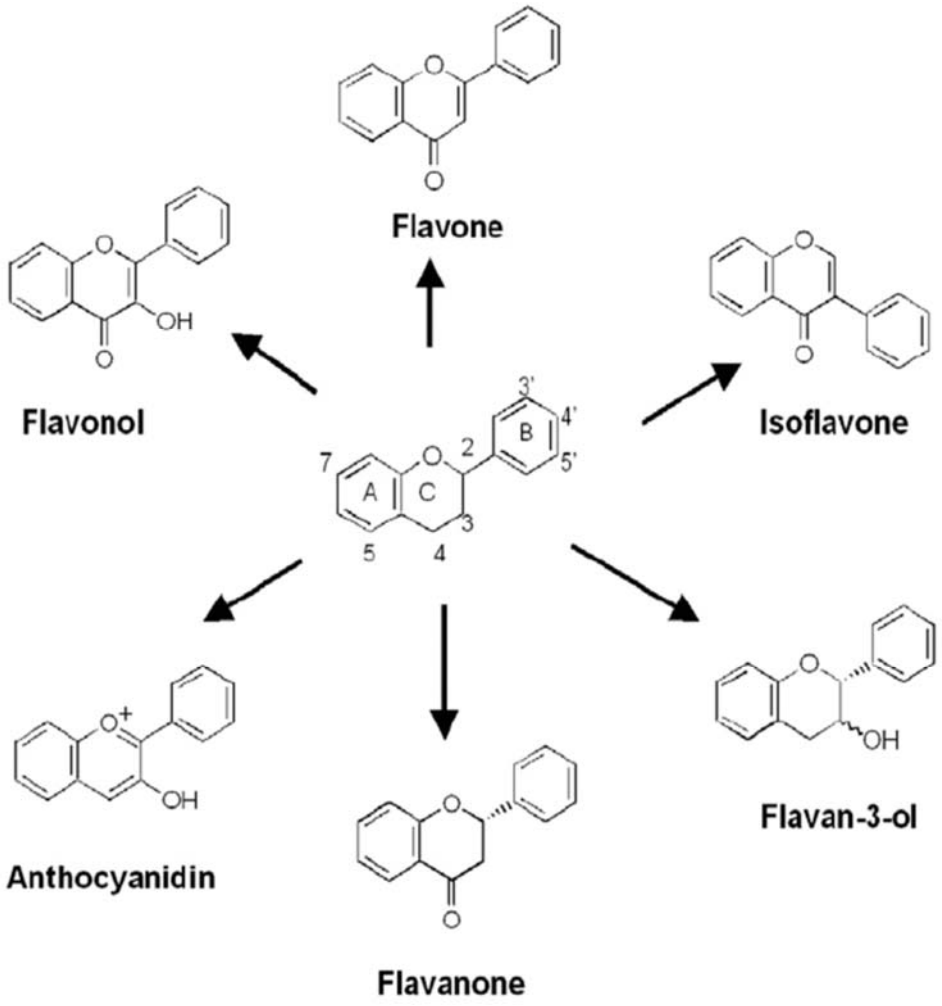

Figure 3. The structure of the more common flavonoids in honey,source: [11]

1.4.2 Sugars

Honey, as mentioned before, is a supersaturated sugar solution in which all kind of sugars may be found. Monosaccharides account for 75% of the sugar content while disaccharides are responsible for 10-15%. Small quantities of other sugars may also exist. Generally, fructose, glucose, sucrose, cellubiose, gentiobiose rhamnose, turanose, trehalose, nigerobiose, maltose, isomaltose, maltotriose, maltotetraose, maltulose, melezitose, nigerose, melibiose, raffinose, pelatinose, erlose and other carbohydrates have been found in several studies [14],[15],[16]. In the great majority of cases the main sugar fraction in honey is fructose except for some honey types like rape (Brasseica napus) and dandelion (Taraxaxacum officinale). Honeys in which the main sugar is glucose, undergo rapid crystallization. The concentration levels of fructose and glucose along with their ratio can be used as markers for the classification of monofloral honeys

[17]. Additionally, the trisaccharides melezitiose and raffinose can differentiate honeydew honey from blossom honey due to their absence from the second one. Disaccharides and trisaccharides, which are also present in honey, are hydrolyzed to monosaccharides.This hydrolysis may be taken place by enzymatic activity and the most typical example is the hydrolysis of sucrose to fructose and glucose by the enzyme invertase [18].

The profile of sugars is a powerful tool for assessment of honey quality since many attributes like viscosity, hygroscopicity and granulation are directly connected to the presence of sugars. Moreover sugar content is an indicator of authenticity since sugar composition can be affected by several factors such as botanical and geographical origin, processing and storage. Apart from these, nearly all of the energy value of honey is attributed to these compounds [2].

1.4.3 Organic acids

All honeys have a slight acidity because of the presence of a small quantity of organic acids approximately 0.5% of dry mass. These organic acids (OAs) are produced by enzymes which are secreted by honeybees during the transformation of nectar to honey. Attention should be paid to this group of compound of honey because they are not only used to assess the quality of honey and several properties but also to differentiate honeys according to geographical and botanical origin. OAs affect important properties of honey like color, flavor, electrical conductivity, potential of hydrogen (pH) and at the same time they are considered to be possible contributors to antioxidant and antibacterial activity. Also, free acid content is usually used for the determination of honey spoilage. The most common organic acid is the gluconic acid which originated from the enzymatic activity of glucose-oxidase on nectar glucose. The level of gluconic acid is correlated to the duration needed for the total transformation of nectar into honey. The longer the duration, the higher the concentration of glucose-oxidase added by the bees, and hence the larger the amount of gluconic acid synthesized .Furthermore, gluconic acid is produced by the metabolic activity of the bacteria belonging to the genus Gluconobacter spp. In fact, large quantities of gluconic acid are produced under aerobic conditions in the presence of high glucose concentrations. Other OAs found in honey are citric, levulinic and formic acids. These compounds may be originated as intermediates of the Krebs cycle or other enzymatic pathways [19]. They are many procedures to determine the organic acids of honey including enzymatic activity, chromatographic methods and a new developed method which is capillary electrophoresis. Enzymatic methods are characterized by

sensitivity, specificity and easy instrumentation, but are not used for a variety of organic acids. Liquid chromatography is a common technique to determine organic acids because it is characterized by sensitivity and reproducibility, whereas using gas chromatography required derivatization step because organic acids are not volatile compounds. Finally, capillary zone electrophoresis (CZE) is a simple technique characterizing by shorter times. It is a preferable technique for the separation of organic acids offering better resolution eliminating the matrix effects which are the disadvantages of chromatographic methods [20].

1.4.4 Proteins, amino acids & enzymes

Honey contains in a lesser extent proteins mainly enzymes and free amino acid. The amount of nitrogen is very low varied from 0.04 to 0.1%. The presence of proteins in honey is mainly attributed to pollen. However, honeybees also add proteins during the nectar secretion through salivary glands or pharynx. A great variety of amino acids may be found in honey. In detail, more than 20 different amino acids, like proline, glutamic acid, phenylalanine, alanine, leucine and tyrosine, have been reported in honey [21]. In fact, proline is the most dominant amino acid in honey and it is suggested as a criterion for the estimation of the maturation of honey and helps the detection of a possible adulteration with sugar. For this reason a minimum amount of at least 180 mg kg-1 of proline is defined as accepted value for pure honey. Blossom and honeydew honey may be discriminated upon their free amino acids contents. Furthermore, specific amino acids have been cited as identical for specific botanical origin. Typical examples of that is the case of tryptophan for acacia honey and proline and phenylalanine for lavender honey [22].

Enzymes as a fraction of the proteins are also presented in honey. They may originate from the bee, pollen, nectar and even from yeasts or micro-organisms present in honey. There are 3 main enzymes in honey which are invertase, a and b- glucosidase, diastase, glucose-oxidase, catalase and acid phosphatase. The properties of these enzymes are already known [20]. Diastase or amylase consists of α- and β-amylase which digest starch to simpler components. Diastase activity is a useful indicator of honey quality, because it is estimated that the higher the amount the higher the quality is. Regarding invertase, it is responsible for the hydrolysis of sucrose to a molecule of fructose and a molecule of glucoce and remains in honey active for some time. As for glucose-oxidase, it has been found that it converts glucose into δ-gluconolactone, which is then hydrolyzed to gluconic

acid, the most abundant organic acid of honey. Supplementary, glucose oxidase also produces hydrogen peroxide, which has proven to demonstrate anti-bacterial action [2].

Current methods for separation and quantification of proteins have comprehensively discussed in literature [23]. The common analytical choices, for the determination of the whole protein content, are Lowry and Bradford assays. However, these assays are time consuming and they are non-specific. Another widely used technique for peptide separation is gel electrophoresis like Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). A two dimensional gel electrophoresis is preferred in proteomic studies, where mass accuracy and resolution are better. Moreover, the importance of protein separation and identification by LC-MS/MS has to be highlighted. The strengthening of analytical capabilities brought revolutionary progress in the field mainly in biological samples. This improvement is strictly connected to the development of sub-2-μm stationary phase for UPLC systems, the soft ionization techniques such as matrix assisted laser desorption ionization (MALDI) as well as the the dominance of HRMS instruments like ToF. Finally, the investigation of honey proteome is a challenging field because of honey matrix, protein abundance, size and hydrophobicity and should be further studied.

1.4.5 Vitamins

Almost all of the vitamins in honey are water soluble, as expected, due to their aqueous nature containing a high quantity of sugars and an extremely low percentage of lipids. Especially, the B-complex vitamin such as thiamine (B1), riboflavin (B2), nicotinic acid (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B8 or H) and folic acid (B9) are the most abundant. Vitamin C also exists in honey. The vitamins are derived from the pollen grains and are preserved due to the low pH of honey. Their content is affected by several factors like storage condition, aging and processing. Particularly in honey, there are two factors that cause vitamin loss, the commercial filtration procedure and the presence of the enzyme glycose oxidase. The filtration contributes to the consumer's pleasure as honey can remain fluid for a longer period of time. However the filtration removes bee parts, wax and solids, including the majority of pollen that can hasten crystallization and dramatically reduces the content of vitamins. Concerning the second factor, this enzyme produces hydrogen peroxide which oxidize ascorbic acid (vitamin C) contributing to vitamin loss [21].

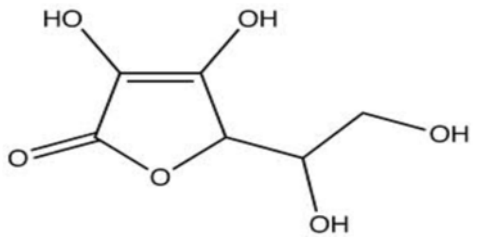

Figure 4. Vitamin C

Vitamins are essential compounds which enhance the nutritional value of honey. In fact, these nutrients do not penetrate into tissues and as a consequence there is no calorie income during their consumption. The development of analytical methods for the determination of vitamins has received increasing interest because of their health promoting benefits. Unfortunately, analytical choices, are limited due to their instability. Chromatographic method is the preferred technique in most of the cases because of the selectivity, sensitivity and utility (based usability). Recently, an UHPLC-MS/MS has developed for the determination of the B-group vitamins [24]. Moreover, several RP-HPLC methods have been proposed using UV-Vis, FLD and DAD detectors for the simultaneous determination of group-B [25]. Alternatively analytical options are microbiological assays, titrimetric procedures and the specific measurement of ascorbic acid which is based on near infrared spectroscopy (NIR) and flow injection analysis (FIA) system with amperometric detector [26]. It has to be noticed that the developed analytical methods can only measure one or few vitamins.

1.4.6 Minerals

Minerals are found in trace quantities in honey and their content ranges between 0.04% in light honeys to 0.2% in dark honeys. Macro- and trace elements such as potassium magnesium, calcium, iron, phosphorus, sodium, manganese, iodine, zinc, lithium, cobalt, nickel, cadmium, copper, barium, chromium, selenium, arsenic, and silver have been reported in various honeys. The most plentiful element is potassium corresponding the one third of the total content. These elements perform an essential function in biological systems improving psychological responses, strengthen the metabolism, influencing the circulatory system and act as catalysts in several biochemical reactions. Botanical origin interfere directly to its mineral content. It is estimated that dark honeys such as honeydew, chestnut or heather honey possess a greater amount of minerals than light blossom honeys. In addition, the content of trace elements depends also on the type of soil in

which the plants and nectar found. Taking the above mentioned facts into consideration, mineral content can be used in authenticity studies in order to discriminate the different types of honey according to geographical or botanical origin [2].

Moreover, honeybees and honey have been proposed to be bioindicators of the environmental status of an area, because in some cases residue levels of potentially toxic elements are observed in honey and its products. Arsenic, lead, mercury and cadmium, are toxic if the maximum limit is exceeded. The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) have jointly proposed the acceptable levels of these toxic elemets. The quantification of toxic mineral elemets is significant in the case of organically produced honey. (The production of organic honey implies organic beekeeping which is defined in European regulation EEC No 2092/91, Annex I.) The qualification of beekeeping products as being form organic origin is pertinent directly to both the characteristic of the hives treatments (e.g. application of veterinary drugs) and the quality of the environment. Organic honey must not be contaminated by veterinary drugs introduced by bee keepers which are generally the most important contaminants [27].

Aiming to determine minerals different techniques have been used including flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma –mass spectrometry(ICP-MS), total reflection x-ray fluorescence, potentiometric analysis and ion chromatography. Nevertheless, ICP-MS is the technique with the greatest potential. It features by extremely low detection limits down to ppt level and multielement analysis including rare earth elements (REEs) determination. The main drawbacks of ICP-MS in comparison to AAS techniques are the high cost for instrument and consumables and the limitations in use such as the low acidity of samples that may be necessary [28].

1.4.7 Volatile compounds

Volatile organic compounds (VOCs) comprise a group of compounds of high importance in honey. Volatiles are responsible for the aroma profile of a foodstuff which in concern with other factors such as taste and physical characteristics contributing to a characteristic flavor. There is a great amount of identified VOCs in honey, more than 600 compounds, which belong in many different chemical families. In detail, hydrocarbons, aldehydes, ketones, alcohols, acids, benzenes and its derivatives furans and pyrans, terpenes and its derivatives, C13 norisoprenoids and cyclic compounds are characteristic

volatiles. They are originated from various biochemical pathways such as transference from plants or from the conversion of some plant constituents to volatile compounds. Also, thermal treatment, microbiological and environmental contamination may affect the content of volatiles as this type of molecules are thermosensitive. The aroma of honey is strongly connected to the type of nectar or honeydew which collected by honeybees during the production of honey. Consequently, some of the volatile compounds can be used as indicators to differentiate honeys according to the botanical origin. For example, 3,9- epoxy-1p-mentadineno,cis rose have been proposed to be characteristics for lemon honey. Diketones, sulfur and alkanes are markers for lavender and hexanal or heptanal for eucalyptus. It has been proved that every unifloral honey exhibit a distinctive aroma profile compared to multifloral honey due to the presence of specific VOCs [29]. Furthermore, the geographical origin is a relevant factor that can influence its volatile content. The differences between honeys from different countries are derived from different compositions of nectar or pollen which affect the chemical composition. Except from the floral markers, VOCs may also be used as indicators of honey freshness. A typical example of that are alcohols with methyl groups like 2-methyl-2-buten-1-ol and 3-methyl-3-butene-1-ol.

Regarding the analysis of VOCs in honey, GC-MS or GC-MS/MS is the ideal choice in order to quantitatively and qualitatively measure volatile molecules. Because of the low concentration of these molecules and the necessity to remove the matrix interferences such as sugars which are the main components of honey, several choices for the extraction of VOCs implied. Firstly, there are distillation methods like hydrodistillation which efficiently extract the VOCs. Nevertheless, these methods may lead to the formation of furan and pyran derivatives due to the effect of heat on sugar or amino acids LLE or ultra-sound extraction (USE) using non-polar extractants, like n-hexane, dichloromethane, diethyl ether, are also capable for selective extraction as they do not mixed with the polar substances of honey like sugars. The last analytical tries in order to reduce the use of expensive and toxic solvents and the generation of artifacts (pyrans and furans) due to heating are solid phase micro-extraction (SPME), solid phase extraction headspace (HS) methods. SPME has the advantage of being a flexible, simple method that is used for a wide range of analytes and a relatively economic extraction technique. On the other hand, HS is performed directly to the sample and no manipulation is required. In this way, valuable time is saved and the VOCs profile is obtained as it occurs in the matrix. The use of HS SPME is also mentioned as the appropriate method

because it is characterized by enhanced recovery efficiency compared to other techniques [30], [1].

1.5 Physical characteristics

The physical properties of honey influence decisively its quality. The most important characteristics are density, viscosity, hydroscopicity and granulation. The density is related to the water content of honey. In other words, a low amount of moisture results to a more viscous state. Viscosity of honey influences beekeepers during the production of the product. As the viscosity of honey increases, both the extraction procedure from the comb and the filtration become harder. However, the heating of the honeys is the solution for these kind of problems. Special attention should be paid on the heating temperature as honey includes various sensitive compounds like flavonoids and vitamins which strongly affect the nutritional value. On the other hand, a high moisture level may cause the fermentation of the honey. In detail, hydroscopicity is the capacity of honey to increase its moisture content when the humidity of the environment is higher than the products'. However, this is a reversible property as honey moisture may decrease if the environment is dry enough.

Granulation or crystallization of honey is of great importance. There is a misconception by the consumers that granulation of honey is linked to inferior quality. Absolutely different from what it is believed, honey crystallization is a natural process and happen due to the fact that it is a supersaturated solution. Thus, glucose tends to precipitate out of solution and the solution changes to the more stable saturated state. In fact, monohydrate glucose molecules are the initial points for the formation of crystals. There are several factors that pose impact on crystallization. Some batches of honey never crystallize, while others do so within a few days of extraction. It is generally accepted that honey removed with extractors and pumps is likely to crystallize faster than if it was left in the comb. Most liquid honey crystallizes within a few weeks of extraction. The tendency of honey to crystallize depends primarily on its glucose content and moisture level. Additionally, the stimulation of granulation is influenced by any small particle (dust, pollen, bits of wax or propolis, air bubbles) presented in honey. Moreover, storage conditions such as temperature and moisture may also influence the tendency of granulation. A noticeable fact is that honeys with less than 30% glucose resist to crystallization [31], [32]

1.6 Organoleptic characteristics

1.6.1 Color

Color is the first attractive sensory characteristic of honey. It is estimated as an important parameter in its acceptance by consumers which is related to increased profitability. Also, it is used as a crucial indicator for the assessment of honey's quality [29]. Honey's color is a controversial issue and there are many different opinions. To begin with, it is dependent on the nectar source, the time of storage, and on the season while various color pigments such as anthocyanins, carotenoids and flavonoids may be involved in the formation of the final color of an individually harvested honey. Also, caramelization of the saccharides catalyzed by the enzymes and the heat treatment during the production may play a key role in the darkening of the unifloral honey due to a non -enzymatic reaction like Maillard (that happening between the sugars and the amino acids of honey). Whereas during the crystallization process, honeys typically become lighter [33]. Whatever the source of the color, it is generally true that the darker the honey, the more intense the flavor is. Honey's color can vary from light tones to almost black amber tones, with the most common being bright yellow, reddish or greenish. Generally, honeydew honeys are darker than blossom honeys except from chestnut and heather which are characterized by dark colors [34]. Many scientists have found that honeys with darker colors, possess greater number of phenolic compounds which means increased antioxidant capacity. For these reasons darker honeys like honeydew are more preferable to the consumer society [6].

Color classification of honey is globally made using the Pfund color scale. This visual method is proposed by the United States Department of Agriculture (USDA) and it is based on the comparison of honey color to a standard colored glass. The color is measured using the Pfund colorimeter which has a simple instrumentation. The reference unit is the Pfund scale ranging from 0 to 140 mm. Initially the scale corresponds to very light-colored honey and increases up to the darkest honey (figure 3). Nevertheless, the method cannot distinguish small variations of color while the determination is time consuming, needs a great amount of sample and it is significantly influenced by the operator as each individual can observe in a different manner the hue of the color. In this way different approaches have been proposed in order to accurately assess honey color. Nowadays using spectrophotometric approaches have been found seven categories

according to hone's color which are the following: water white (means colorless), extra white (darker), white, extra light amber (ELA), light amber (LA), amber and dark amber.

An innovative approach has been focused to the measurement of the color intensity using Minolta equipment which is based on reflectance spectrometry. This color system is simple because any color is defined by a mixture of specific colors and also cannot be affected by environmental factors [35], [36]. To conclude, another new approach which has recently proposed by Marina A. Dominguez and María E. Centurión is based to digital image analysis combined with chemometric tools for determining honey color. This effort provides more reliable and faster determination compared to the reference method, while less sample is required [37]

Figure 5. The Pfund color scale, source:[38].

1.6.2 Flavor and aroma

Consumer preferences for a specific high quality product can be directly linked to its flavor and aroma. These factors show a great variation which is directly connected to the botanical origin and geographical origin of the product. Additionally, they can also be affected by processing conditions and storage time. In the case of unifloral honeys or PGI and PGO honeys, flavor and aroma can be considered as unique characteristics because of the presence of a distinctive profile offering a different organoleptic result. For this reason, these products command premium price on domestic and international markets [39].

To begin with, the dominant flavor of all honeys is sweetness due to the presence in great quantity of two major sugars (glucose and fructose). It is widely believed that honey with a high fructose content, like acacia unifloral honey, are sweeter compared to those with high glucose concentration such as rape unifloral honey. On the other hand, acids, and other volatile components like C1–C5 aldehydes and alcohols have a major impact on flavor and aroma.

Aroma is an important feature for the organoleptic character of a product and is mainly attributed to volatile compounds and to a lesser extent to non –volatile. Carboxylic acids are chemical compounds that have different aroma, ranging from spicy to rancid depending on the length of carbon chain. Short chain acids such as acetic acid, have a spicy aroma, while butanoic acid and hexanoic acid, which are usually found in butter, are linked to a rancid aroma [1]. Moreover, many phenylacetic esters have a honey-like taste and aroma while methyl and ethyl formate and the amino acid, proline have been identified in honey contributing to the flavor. Other factors that surprisingly affects organoleptic characteristics are the mineral content of the honey and heat treatment processing. About the first factor, it has been proved that high mineral content is proportional to a darker honey with a stronger flavor [40].Concerning the second factor, intense heat processing has an effect on the flavor of honey due to the loss of volatile components and other thermosensitive compounds which may contribute to the organoleptic character of honey [41].

1.7 Quality criteria

Nowadays, food safety is an unquestionable issue, especially in the western world. Attention should be paid on quality because consumers' standards are high. In fact, they are willing to spend a lot of money for qualitative foodstuffs even though their prices are higher. In EU, quality labels system known as Protected designation of origin (PDO) and Protected geographical indication (PGI) are defined in order to protect characteristic attributes of famous agricultural products which are vulnerable to unfair practices as well as guarantee the quality and authenticity of products consumed on daily basis by customers such as honey. Quality includes all the features that confirm a product's value increasing consumer's satisfaction [42]. This comprises negative attributes such as spoilage, contamination with filth, discoloration, off-odors and positive attributes such as the origin, color, flavor, texture, processing method of the food [43]. In the case of honey, specific standards are required in order to guarantee and protect it from possible adulterations [2]. Quality of honey depends on the plant source, the chemical composition of these plants as well, as on the climatic conditions and soil mineral [44].

Council Directive, 110/2001/EC governs all quality criteria of honey, while authenticity issues are defined internationally by the Codex Alimentarius. According to the legislation, the parameters that should be tested in order to assure quality of honey are sugars content, moisture content, water insoluble content, electrical conductivity, free acidity, 5-

HMF and diastase activity. The accepted values of each compound are well defined and if a product does not meet them, then it cannot be placed on the market. All these quality parameters are well discussed above while table 1 summarizes their acceptable values according to European directive 110/2001/EC

1.7.1 Sugars content

Storing and thermal processing of honey can affect the composition of sugars. Referring to the first one, it has been proved that sucrose concentration is slightly decreased when honey is stored at a temperature of 4 Celsius, while higher reduction has been found in higher temperatures. This is not the case for monosaccharides (glycose and fructose) which concentration is increased at higher temperatures. On the other hand, the thermal processing is responsible for a chain of reactions leading to a decrease in sugar content. The most common of these are Maillard reactions, non -enzymatic and caramelization reactions as well as degradation of sugars in acidic medium. In these cases furans like furfural, which is derived from pentoses, and 5-HMF, derived from hexoses (glucose and fructose) are formed. Consequently, these compounds have strongly connected to an undesired product as there is a degradation in quality. Except from these, other compounds such as 2 acetylfuran, isomaltol, 3,4 dihydroxy-2 methyl-5,6 diiodropyran-4one and maltol may alter the sensory and organoleptic characteristic of honey. These analytes are formed by acid degradation during heat processing in presence with amino acids [2], [33].

As already mentioned, honey is mainly constituted of sugars. In this way, legislation stipulates that fructose and sucrose should be over 60 g 100 g^{-1} and 45 g 100 g^{-1} for floral and honeydew honey respectively .It has to be mentioned that sugars composition is an important indicator of authenticity due to its dependence on the botanical and geographical origin. Therefore, if a different amount of reducing sugars is detected then a fraudulent practice should be considered possible [45].

1.7.2 5-(Hydroxymethyl)furfural

5-HMF is widely recognized as an indicator of honey freshness and overheating. It is formed by the Maillard reaction during heat treatment or by the decomposition of hexose in acidic media. Also it can be produced when honey is stored for a long time at low temperatures, however in this case the concentration of 5-HMF is lower in contrast to the first way of production. A proposed mechanism for the formation of 5-HMF in honey is

illustrated in Figure 6. The increase of this compound is directly related to a high heat treatment temperature and a long storage time. Consequently, old honeys contain higher concentrations of HMF than fresh honey. Beside these 2 factors, the concentration of 5 HMF depends on sugar composition, Ph, water activity and divalent cation concentrated to a media [46]. 5-HMF content is stipulated by the EU Directive 110/2001 in order to ensure the quality level of honey. In detail, the upper eligible concentration for this compound in honey is about 40 mg/kg. However, there are two occasions with different limits. The first case is honey produced from countries or regions with tropical temperatures. The reason is that high temperature increases 5-HMF concentration and consequently the limit is doubled (80 mg/kg). Moreover, honey with a low enzymatic level has an eligible 5-HMF content that should not exceed 15 mg/kg [4]. Finally, it is estimated that 5-HMF can be cytotoxic, mutagenic, carcinogenic and genotoxic. For this reason, analytical methods have been well established and described for the determination of this analyte in order its concentration to be monitored in food products like honey. Methods based on chromatographical principles with usually UV detectors or capillary electrophoresis are the most common analytical choices

Additionally, 5 HMF is used as an indicator of authenticity. In fact, a value of 200 mg/kg is considered as possible fraud with invert sugar syrup while a value of 500 mg/kg is a decisive factor of adulteration with this syrup [47]. Several studies, which are comprehensively reviewed by C.P. Calvo and M. Vazquez [7], indicate that there is a greater concentration of 5-HMF in honeydew honey than in blossom honey.

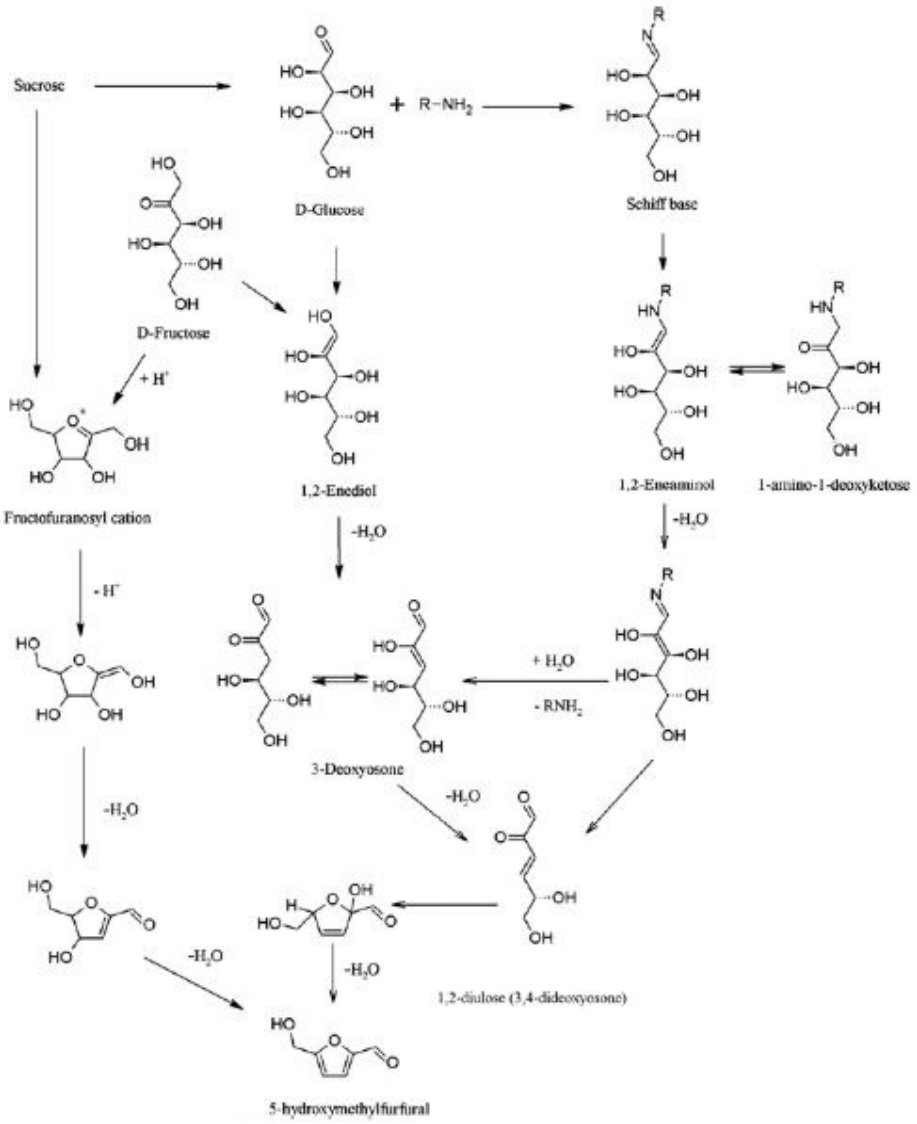

Figure 6. The formation of 5-HMF in honey, source: [48]

1.7.3 Moisture content and water activity

The importance of moisture content of honey should be underlined as it greatly affects the quality of the product because it determines the capability of honey to remain stable and resist spoilage by yeast fermentation. In addition, as already mentioned the water represents the second major constituent of honey which affect its psychochemical characteristics. According to the Council Directive 2001/101/EC in general the moisture content of honey should not exceed the 20% of the total product [4]. However, moisture content is not stable due to its dependence of several factors such as the level of maturity achieved in the hive, the processing techniques and storage conditions. These factors can affect physicochemical properties of honey like crystallization and granulation and solubility. In detail, the moisture content varies as honey shows a high hydroscopicity (capability of absorbance water under certain conditions). For example, under high relative humidity conditions, it is proved that there is an increase in moisture content. Also, moisture content changes alongside the environment that surrounds honey as well as during processing activities of the product. Indeed, storage of honey should be taken place under a dry environment in order to assure that there is no any spoilage by an emerging fermentation [2]. Nevertheless, it is not the moisture content which governs and controls the microbial growth but water activity. Water activity represents the water content which is “available” for the microbial growth. Water molecules are mainly binded to soluble solids. Consequently, microorganisms have not enough moisture in order to grow. Honey contains osmophilic and osmotolerant yeasts that can produce ethyl alcohol and carbon dioxide affecting the quality of the honey [49]. In the case of crystallization, the soluble solids are reduced and the water activity is increased so there is a greater chance for the degradation of quality. Honey usually has a water activity between 0.50 and 0.65. In Food Science a water activity values under 0.60 presents a stable environment where microorganism cannot survive. The determination of moisture, using a refractometer, is a routine procedure in honey analysis. It has to be noticed that this kind of measurement is not a precise one and in order to access accurately the moisture content a Karl-Fischer titration should be performed .It is a titration that exploit a selective chemical reaction based on the oxidation of sulfur dioxide by iodine in the presence of water with methanol as working solution [50], [51].

1.7.4 Diastase activity

As already mentioned, diastases include α- and β-amylase which are a group of starch-digesting enzymes. Alpha amylase degrades starch to a mixture of the disaccharide maltose, the trisaccharide maltotriose and oligosaccharides known as dextrins. Diastase activity comprises another option for the evaluation of honey quality. Alongside with 5-HMF content, a decrease diastase activity can indicate excessive aging or thermal processing in a temperature over 60 ^{o}C. EU Directive 110/2001 poses as a minimum of diastase activity equal to 8 diastase number (DN) in Schade units for the majority of the honeys. In detail, diastase activity is expressed as the DN in Schade units. Particularly, one diastase unit is related to enzymatic activity which can hydrolyze 0.01 g of starch in 1 h at 40 ^{o}C according to 1 g of honey. However, there is an exception for honeys with low enzymatic activity and the permissible value of DN units is over 3. The content of diastase activity varies depending on the age of the bees, the physiological period of the colony, the quantity of nectar and the sugar content. In fact, honey produced from young nectars in early spring is the reason for a lower enzymatic activity. At this time of the year the sugar content is higher as the bees are not enough enzymatic active and the quantity of nectar is lower [2]. Diastase activity can be determined using the classical Schade procedure or by the commercial Phadebas tablets. The Schade procedure is a method which uses a standard starch solution. The starch solution is treated with triiodide and produces a blue color. Then the starch solution is mixed with the honey, and the enzymatic activity of the sample reduces the intensity of the blue color. This reduction of the color is successively measured at 660 nm. This methology is based on direct potentiometric measurement of free triiodide ions released from the complex of starch - triiodide ions using a platinum redox electrode as a detector [52]. However, Schade procedure is characterized by many limitations. The absorbance range should be limited to a small absorbance area, the consecutive dilutions in the process can increase the associated error of the method and the variability of the activity of this enzyme causes invalid measurements. For this reason, there is a growing interest to establish more validate methods in order to obtain accurate results in shorter time [20].

1.7.5 Ash content and electrical conductivity

The ash content is a measure of quality because it is a decisive factor of the mineral content which comprise one of the minor components of honey. The mineral content can give information about the possible environmental pollution, discriminate different types

of honey according to botanical origin and contribute to its nutritional value. Although no standard values have been defined by Codex Alimentarius Committee, a number of studies have shown that the average ash content is 0.17% (w/w). More specifically, blossom honeys are characterized with a value of ash content ≤0.6% (w/w) whereas honeydew and mix of honeydew with blossom honeys or chestnut honey have an ash content ≤1.2% (w/w). Overall honeydew honeys are generally characterized by higher values of electrical conductivity, pH, acidity and ash content than blossom honey [4].Nowadays the electrical conductivity is determined in routine honey quality control replacing the measurement of ash content. There is a correlation between electrical conductivity, ash content and acidity indicating the presence of ions, organic acids and proteins. The higher the content of these constituents, the higher the electrical conductivity is. The maximum value that has been provided by Codex Alimentarius is 800.000 mS/cm [2].

1.7.6 Free acidity and pH

Free acidity is an important factor which is related to the degradation of honey. The presence of organic acids (phosphates, sulphates) which are in equilibrium with lactone and internal esters are responsible for the acidity of honey. It is common acceptable that the permitted value of free acidity is 50.00 meq/kg and consequently values greater than this limit are indicative of possible fermentation of sugars into organic acids. The acidity is not a stable parameter because it is affected by the harvest season, the presence of organic acids, the geographical origin and the time elapsed between the nectar collection by bees and the density of honey in honeycombs. Furthermore free acidity contributes to the inhibition of the grow of microorganisms, flavor and taste of honey, enhancement of some chemical reactions and antioxidant capacity [53]. The acidity can be measured by direct measurement of pH or by titration against sodium hydroxide equivalents. However the latter one, has a major drawback which is the drift in the equivalent point of titration due to lactone hydrolysis, leading to inaccurate values [45]. On the other hand, a level of pH between 3.2 to 4.5 has been defined by regulatory committees as suitable for the inhibition of the growth and proliferation of microorganisms as the optimum pH for most microorganisms is 7.2 to 7.4 [2].

1.7.7 Water insoluble solids

The measurement of water insoluble solids is an important way to detect honey's impurities that are higher than permitted values. Wax is the major source of this water insoluble contamination which include pollen, honey-comb debris, bees and filth particles. According to Codex and European standards the permitted values of water insoluble solids have been defined as 0.1 g/100 g honey. However, the measured values are lower enough in contrast to theoretical due to centrifugation and filtration techniques in which the honey is subjected nowadays [45]

Table 1. Quality criteria of honey according the EU Directive 110/2001/EC

quality criterion	type of honey	acceptable value according to110/2001/EC
fructose and glucose content	blossom	> 60 g/100 g
	honeydew blends of honeydew honey with blossom honey	>45 g/100 g
sucrose content	in general	> than 5 g/100 g
	false acacia, alfalfa etc	> 10 g/100 g
	lavender, borage	> 15 g/100 g
moisture content	in general	< 20 %
water-insoluble content	in general	< 0,1 g/100 g
	pressed honey	< 0,5 g/100 g
Electrical conductivity	honey not listed below, and blends of these honeys	<0,8 mS/cm
	honeydew and chestnut honey and blends of these except with	>0,8 mS/cm
	those listed below not more than 0,8 mS/cm	
	exceptions: strawberry tree, bell heather , eucaluptus	
	lime (Tilia spp.), ling heather, manuka, tea tree	
Free acidity	in general	< 50
	baker's honey	< 80
Diastase activity	in general, except baker's honey	>8
	in general, except baker's honey	>3
HMF	in general, except baker's honey	< 40 mg/kg
	honeys of declared origin from regions with tropical climate and blends of these honeys	< 80 mg/kg

1.8 Health benefits of honey

Honey has been an ingredient of traditional medicine from ancient times because of dietary and therapeutical properties. It is estimated to be effective in healing of wounds and burns, in gastrointestinal disorders, and as antimicrobial and anticancer agent. Many studies have shown that the important effects of honey on human digestion have been linked to oligosaccharides, capsaicin, and its capability to maintain to a stable level the non-protein sulfhydryl compounds in gastric tissues. The capacity to inhibit the growth of microorganisms is attributed to its low water activity, acidity and the enzyme glycose oxidase which produces the hydrogen peroxide [54]. Recent studies have shown the important role of honey in treating of cardiovascular diseases as well as shed light on its antioxidant properties. The antioxidant capacity of honey is emerged mainly from phytochemicals compounds like phenolic acids, flavonoids, carotenoids as well as to a lesser extent from proteins, enzymes and amino acids. Consequently the honey is estimated not only a high value foodstuff, but also a nutrient source of antioxidants [52]. A characteristic example of honey which possess an unparalleled value for health is Manuka honey known as healing honey [55], [56]. In this subchapter, the antioxidant capacity of honey is critically discussed while the main health beneficial properties of honey are briefly summarized.

1.9 Antioxidant capacity

The term “oxidative stress” describes the lack of equilibrium between the production of free radicals and the antioxidant protective activity in a given organism. Antioxidant capacity (AOC) or antioxidant activity is the ability and potential of honey to reduce oxidative reactions within the food systems resulting to beneficial effects on human health. Particularly, these oxidative reactions can cause harmful reactions in food products such as lipid oxidation, enzymatic browning, damage of proteins and nucleic acids leading to adverse health problems like chronic diseases and cancers. Honey is a foodstuff with a great variety of antioxidants. In detail, flavonoids, phenolic acids, enzymes such as glucose oxidase and catalase, ascorbic acid, organic acids, carotenoid-like substances, amino acids, Maillard reaction products, and proteins. Consequently, AOC is an indicator of the presence of beneficial bioactive compounds contributing to well - being [13]

The AOC varies greatly depending on the botanical origin of honey due to different content of plant secondary metabolites as polyphenolics as well as it is affected by environmental factors and processing. Moreover, it also associated with honey color. In fact the darker the color the greater the AOC of honey [57].

Radical scavenging capacity is another important antioxidant property of honey (figure 7). It has been proved that honey is capable of scavenging hydroxyl and superoxide radicals resulting to controlling of lipid peroxidation and to a following decrease of inflammatory diseases [58].

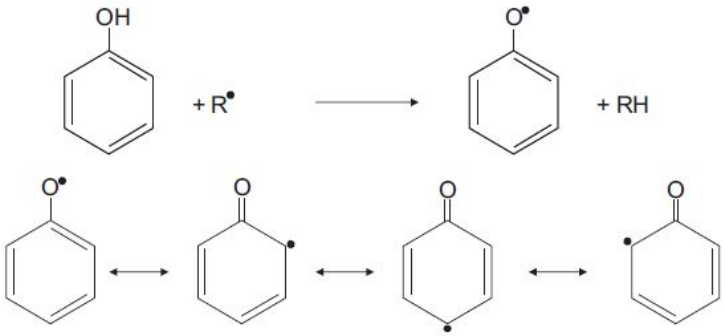

Figure 7. Radical scavenging mechanism of phenolic compounds, source: [13]

Phenolic antioxidants as terminators of free radicals and chelators of metal ions that are capable of catalyzing lipid peroxidation. Phenolic antioxidants interfere with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals, as illustrated in the following reactions,

$$ROO^* + PPH \rightarrow ROOH + PP^*$$

$$RO^* + PPH \rightarrow ROH + PP^*$$

Figure 8. Reaction of free radicals with phenolic compounds, source:[10]

It is generally accepted that phenolic compounds greatly affect the AOC in honey. This antioxidant capacity can be attributed to the fact that they act as metal chelators and excellent free radical scavengers as well as gene modulators. To begin with, gallic acid has the better antioxidant properties among the hydroxybenzoic acids. AOC is improved with the increased number of OH groups in aromatic ring. Consequently, benzoic acid (1 OH group) has a decreased AOC compared to gallic acid (3 OH groups). On the other hand, hydroxycinnamic acids feature a stronger free radical scavenging ability. This characteristic attribute appears to be linked to the inclusion of the unsaturated chain

bonded to the carboxylgroup as a distinctive structure which provides stability by resonance to phenoxyl radical, even offering additional sites for the attack of free radicals. Flavonoids have also impressive AOC. The presence of certain hydroxyl groups in the flavonoid rings increases antioxidant activity. Substitution patterns in the A ring and B ring, the 2,3-double bond (unsaturated) and 4-oxo group in the C ring also affect the antioxidant activity of flavonoids. Nevertheless, the glycosylation of flavonoids decreases their antioxidant activity when compared to the corresponding aglycones. In detail, hydroxyl groups on the B ring donate a hydrogen and an electron to radicals stabilizing them, whereas the heterocyclic character of flavonoids contributes to the antioxidant capacity by the 3-OH substitution of the B ring. A characteristic example is the flavonole quercetin which has a great capability to reduce efficiently the oxidative stress due to its individual structure which fulfills the requirements [13].

There is a great variety of analytical methods for the determination of antioxidant activity. These methods are cited both in many reviews and original articles [59], [60]. For this reason, there is no need for further discussion and the principles of these measurements are summarized in the following table 2, as is described by J. Alvarez-Suarez et al.,

Table 2. Assays for the determination of AOC, source: [61]

Principle	Assay	Principle of Measurement	Quantification
Electron transfer	TEAC	ABTS•+ radical cation is reduced by antioxidants, causing absorbance decrease at 734nm	Trolox equivalents (mM TE/ 100g of honey)
	DPPH	DPPH• radical is reduced by antioxidants, causing absorbance decrease at 515nm	EC_{50}, RSE, Trolox equivalents (mM), ascorbic acid equivalents (mg/100 g of honey)
	FRAP	The ferric 2,4,6-tripyridyl-*s*-triazine complex is reduced by antioxidants, causing absorbance increase at 593nm	Ferrous ions equivalents, Trolox equivalents (mM TE/ 100g of honey)
Hydrogen atom transfer	ORAC	Free radicals are produced by AAPH and the fluorescent indicator protein *β*-PE is subsequently oxidized. Fluorescence is measured with emission and excitation wavelengths of 565 and 540 nm	Trolox equivalents (μmol TE)/gram of honey

1.10 Benefits from the consumption of honey

Honey is a foodstuff that can play a decisive role for a balanced nutrition. This nutritive character originates from the various compounds of honey that have been already discussed in this thesis. The health benefits from the consumption are numerous .The mechanisms and the reasons for each beneficial property is out of the scope of this thesis

but in order to fully describe the state of the art the main health benefits from the consumption of honey are presented in the following bullets [62], [63], [64]

- Cardiovascular Diseases
    - Inhibition of inflammation
    - Improvement of endothelial function
    - Improvement of plasma lipid profile
    - Increase of low-density lipoprotein (LDL) resistance to oxidation

- Cancer
    - Antimutagenic capacity
    - Induction of apoptosis
    - Antiproliferative effect
    - Cytotoxic effect on several cancer cell lines
    - Antimetastatic effect
- Hypertension
    - Reduction of systolic blood pressure and malondialdehyde (MDA) levels
    - Ameliorament of susceptibility of kidneys to oxidative stress
- Diabetes
    - Reduction of glycaemia
    - Reduction of serum fructosamine
    - Reduction of glycosylated hemoglobin concentration
    - Attenuation of post-prandial glycemic response

CHAPTER 2: Honey Authenticity

2.1 Introduction of authenticity

The globalization of food markets and the resulting increase in the variety of food products have boost the desire of consumers to know the origin and the composition of the foodstuffs that they buy [65]. Nowadays, especially in the western world, food which is produced close to the nature, organic food and natural food like honey are more preferable by consumers due to the growing awareness of nature and the health benefits that they possess. In fact, they want to spend more money in order to purchase a product of high quality [66]. Labeling legislation dictate the necessary compounds and criteria that should be listed on the package. Thus, consumers are fully informed about the components and the additives of the foodstuffs. On the other hand, the regulatory bodies are responsible to develop the methodologies in order to assure that the label of a product comply with specifications.

Authenticity means the quality of being authentic or genuineness (www.dictionary.com, last accessed 03/2017). This word originates from the Greek word authentikós which means original, primary, at first hand and it is equivalent to authént (ēs) which means one who does things himself. Similar definitions which describe authenticity are genuineness and truthfulness of origins, attributions, commitments, sincerity, and intentions. Food authentication is the process that verifies that a food is in compliance with its label description. This may include, among others, the origin (species, geographical or genetic), production method (conventional, organic, traditional procedures, free range), or processing technologies (irradiation, freezing, microwave heating) [65]. In a broader sense it means fulfilling of chemical and physical characteristics which are defined by the proposed legislation [67]. Individuals like importers, exporters, consumers and organizations including the scientific community, law enforcement authorities, and food producers are significantly interested in food authentication. The interest for food authenticity reached unprecedented levels and continue to grow, so this field attracts high attention from authorities and media around the world. The assurance of existence of specific quality attributes in high-value products like honey, is a tasq of great interest since these products are susceptible to fraudulent techniques.

As regards honey, the annual world production is about 1.2 million tons. In the European Union, which is both a major honey importer and a producer, the annual consumption per capita varies from medium (0.3–0.4 kg) in Italy, France, Great Britain, Denmark and Portugal to high (1–1.8 kg) in Germany, Austria, Switzerland, Portugal, Hungary and Greece, while in countries such as the USA, Canada and Australia the average per capita consumption is 0.6–0.8 kg/year [68]. Taking into consideration the above mentioned factors, honey adulteration is motivated by the profit.

Authenticity of honey can be divided into two separate aspects. The first one is referred to honey production type while the second one is referred to the designation namely geographical and botanical origin of honey. The first case is linked to the processing of honey during the production procedure. This processing include centrifugation, filtering and pasteurization. Centrifugation is used to extract the honey from the combs, while filtering is performed for the removal of pollen, wax or other undesired compounds. Particularly, filters with a mesh size not smaller than 0.2 mm have to be utilized. Nevertheless, a common fraudulent practice is the use of smaller filters in order to filter out various undesirable contaminants without the notice “filtered” on the label of the final product. Pasteurization, under standardized circumstances (7.5 minutes at 63 ^{o}C or for 1 minute at 69 ^{o}C) involving rapid heating and cooling, is needed for the deactivation of osmotic yeast. However, an extensive thermal handling will result to a decrease in honey’s quality because of the loss of VOCs and the reduction of the enzymatic activity. Consequently, all of these processing actions should be clearly mentioned on the package. Moreover, the addition of syrups or water are also deceitful actions focusing on the minimization of the cost production and increased profit [69].

The botanical and geographical origin that is mentioned on the label are two factors of great significance because of the fact that the final price is decisively influenced by them. According to the first one, consumers prefer honeys which come from a particular source due to their characteristic aroma and flavor [70] . A typical example of this is that in many European countries the honeydew honeys like fir honey are more expensive due to consumers’ preferences [69]. Furthermore, many of these honeys like Manuka and tupelo which are associated with particular floral sources have distinctive attributes and uses in several applications such as pharmaceutical. On the other hand a geographical origin misleading description is also an important authenticity task. PDO and PGI honeys are susceptible to adulteration techniques because of their high economic value and the

consequent growing demand of these products by the global market [51]. Many studies have shown that the imported honey from countries, especially China or South America usually features a lower price. In these countries, especially in China, various honey fraud incidents have emerged resulting in a bad reputation [69]. Differences in price exist between countries in Europe and even between geographical regions inside a country. To this end, the thesis aims to report an up to date view on the current issue of adulteration and the techniques used for the determination of the authenticity as well as the investigation of possible discrimination markers.

2.2 Adulteration

Food adulteration is the process in which the quality of food offered for sale is degraded either by the admixture or substitution of inferior substances or by the removal of some valuable ingredients. Also, biological and chemical contamination during the period of growth, storage, processing, transport and distribution of the foodstuff, contribute to the adulteration. According to Federal Food, Drug and Cosmetic Act (FFDCA) and the primary food safety law administered by the Food and Drug Administration (FDA), food can be declared adulterated if: a) a substance is added which is injurious to health, b) cheaper or inferior quality item added to food, c) any valuable constituent is extracted from main food ,d) the quality of food is lower that the standards, e) any substance has been added to increase bulk or weight or to make it more valuable.

Food adulteration has many risks because it is not only correlated to a decrease of quality but also is responsible for many diseases which are ranging from mild to life threatening for human health like vision, stomach disorders, skin diseases and abnormalities in liver function [71]. Many high value products like honey are susceptible to various adulteration techniques. The adulteration of food has progressed during years from being a simple means of fraud to a highly sophisticated and profitable business. Honey is a highly prized foodstuff which is used widely as a daily diet ingredient but also for its medical properties. It is estimated as the oldest natural sweeting agent with market value higher than the other commonly industrial sweeteners. According to legislation standards it is considered as a natural product that should not contain any additives or substances that could affect its quality. Nevertheless, honey is an obvious and profitable target of adulteration occurring in various ways [72]. Nowadays, because of the growing demand of honey's products in contrast to the decrease of honeybees' population an increase in adulteration processes has been noticed. The major adulteration is characterized as economically

motivated adulteration (EMA) which enhance the apparent value of the product, reducing at the same time the cost of production. It is important to notice that fraudulent practices not only affect the quality of the honey but also it is cheating the consumers who is expecting to get a natural product with characteristic organoleptic quantity and biological activity. At the end, adulteration might affect the production because the declining prices can reduce the bee keeping industry. This fact might has an impact on the whole ecosystem since bees are the only pollinators which maintain the biodiversity [73].

2.2.1 Direct and indirect adulteration

Direct adulteration is the adulteration in which a substance is added directly to honey. As already mentioned honey is a foodstuff with higher nutritional and economic value than other sweeteners like refined cane sugar, beet sugar and corn syrup and industrial syrups. These sweeteners are used as adulterants in order to reduce the cost of production and increase the quantity of honey in the market. Corn syrup (CS), high fructose corn syrup (HFCS), glucose syrup (GS), sucrose syrup (SS), inverted syrup (IS) and high fructose inulin syrup (HFIS) are common carbohydrates which are directly added to honey during the production. Recently a new sugar adulterant namely rice syrup is used and was derived from the partial hydrolysis of inulin and a following polymerization [72].

Beet sugar, rice syrups, high fructose corn syrups and industrial sugar syrups (glucose and fructose) are from C3 plants following a similar photosynthesis pathway namely Calvin cycle with plants from which the main monosaccharides come from. Consequently the detection of this adulteration is considered as a challenging task, due to their similar composition with the main monosaccharides of honey. On the other hand, cane sugar and sugars produced from the hydrolysis of maize starch come from the C4 plants. These plants fix CO_2 using Hatch-Slack cycle and they don't resemble to C3 plants which are more preferable for the production of honey. Consequently the detection of adulteration is more easily detectable [47].

Except from sugars, the extension of honeys might occurs with other low value honeys that are cheaper, resulting to degradation of pure honeys. Many instances have been observed with the most dominant being the acacia honey which has often adulterated with rape honey. Acacia has a mild taste and does not crystallize, whereas rape honey is sweet and easily crystallized. Since the color of rape honey is similar to acacia, the

alteration proves to be very challenging and the final foodstuff may possess different attributes in contrast to the original one.

The extension of honey by dilution with water is another fraudulent practice. The maximum content which is defined by Codex Alimentarius is 21% [70]. This limit can be raised in two instances mainly due to climatic and harvesting conditions. Studies have shown that harvesting of honey under humidity condition resulting to spoilage of honey. In addition, in some countries the nectar is harvested before the bees have had time to deposit, dehydrate, store and leave it in honey comb to ripen and mature. On the other hand, the intentional dilution of honey with additional water occurs with the aim to increase the honey's fluidity and prevent crystallization .In these cases the honey easily ferments and presents a decrease shelf time. Also it is characterized off taste flavor with high levels of dead yeast, glycerol, butanediol and ethanol [46], [69].

The indirect adulteration is originated by careless bee-keeping practices. In detail, feeding of bees with additional industrial sugars is a common practice used by apiarists in order to raise their honey's production in winter period when there is insufficient nectar to be collected by honeybees. The qualities of honey produced in this way include a sugar profile different from that of original honey, dilution of its nutritional components and also a pale color with weak flavor. These attributes are preferable by many consumers who dislike dark color honeys with strong flavor. Moreover, the honey produced by overfeeding of bees has similar composition with the original one except for a lack of minor components which normally derived from the plant sources of honey [70].

2.2.2 Label misdescription (False declaration)

The botanical and geographical origin are major adulteration practices which are used by many producers. This fact constitute a crucial threat for the bee-keeping industry and for the quality of honey. According to the botanical origin, many consumers prefer unifloral honeys which have distinctive aroma and flavor as well as other desired features. [70]. All these attributes are responsible for the high economic value of these honeys in the global market increasing consequently the producers' profit. For example, light honeys like orange blossom or acacia honey achieve higher prices than honey blends or other unifloral honeys, whereas in some other European countries like Germany and Austria honeydew honeys are more expensive than blossom. Nevertheless, in fact, honey is usually a mixture of different sources because bees can forage different plants making the unifloral honey a scarce commodity. Due to the aforementioned fact and the high

demand for unifloral honey, the misdescription of origin has become a dominant way of adulteration [69].

On the other hand, as a result of tariffs and other restrictions on international trade, many countries require the country of origin to be stated on its label. However, in many cases this does not happen because it is more profitable for a trader to misrepresent (masking) the country of origin. For instance, particular types of honey namely PDO and PGI which are related to specific regions and geographical areas present distinctive features. Studies have shown that are more susceptible to adulteration techniques due to their nutritional value and their high quality [70]

Nowadays consumers have a growing interest in the dangers associated with the presence of toxic substances in food, preferring those labelled as “organic”. Many instances have shown that even if the label indicates the designation ‘’organic’,’ this characterization is not real in many cases. According to legislation the production of organic honey should be based on an appropriate system friendly with the environment using natural resources, promoting environmental quality, animal welfare and human health. However, the production of a honey which is totally free from contaminants such as pesticides, heavy metals, pathogenic microorganisms, and GMO, is impossible [51].

2.3 Control of authenticity

The increasing number of adulterants and their consequent adverse effects in human health have grown the interest about food safety and authenticity of high value products. For this reason, tremendous improvements in analytical methodologies have been carried out in order to provide an adequate answer to global demands on food quality and detection of adulterants. Nowadays separation techniques used in a great extent for control authenticity of food, however there is a demand of replacing them with more sensitive techniques like spectroscopy. Among the spectroscopic techniques infrared-based techniques like NMR and MIR are preferred [71]. As already mentioned honey is high nutritional value foodstuff which is susceptible to various fraudulent practices. Honey adulteration is a major issue because increasingly sophisticated adulteration methods are constantly being developed.

2.3.1 Classical approaches

2.3.1.1 Mellisopalynology analysis

The melissopalynological method which was elaborated and proposed by the Intl. Commission for Bee Botany (ICBB) in 1970 (Louveaux and others 1970) and later revised and updated in 1978 (Louveaux and others 1978), is frequently used until now [74]. It is a traditional method for the discrimination of the botanical and geographical origin of honey. The identification and quantitation of pollen grains in honey by light microscopy aims to determining the plants that are visited by bees during honey production. Regardless of the simple and inexpensive instrumentation, this method has numerous limitations in its use. The long -time of analysis, the availability of a comprehensive collection of pollen grains, the instability of the pollen content due to seasonal factors and type of flora and at the end the necessity of experts with adequate skills to identify different types of pollen are some of the disadvantages of this method. Moreover, this method is not suitable for cases of inadequate honey filtration performed by beekeepers or adulteration by pollen addition. For these reasons, melissopalynology is commonly complemented by physico-chemical and sensory analysis in order to provide efficiently discrimination of origin [51].

2.3.1.2 Sensory analysis

Sensory analysis is an essential tool for the assessment of botanical origin. Higher-quality unifloral honey is honey which, with regard to the specific features like odor, taste, appearance, and tactile properties, is as close as possible to the hypothetical honey “standard”, obtained entirely from the specific plant species. The addition of foreign nectar with unusual and intense aroma can cause a serious defect on aroma and taste of adulterated honey in contrary to a standard one. The result of sensory analysis can indicate different forms of adulteration such as artificial honeys that are produced neither from nectar or honeydew, cases where adulterated honey is obtained by overfeeding of bees with industrial syrups or mixing pyre honeys with others produced by this way. The sensory analysis is a time consuming method which requires personnel with experience in carrying out sensory analysis [67].

2.3.2 Physicochemical parameters

Moisture, electrical conductivity, free acidity- pH value, specific rotation and hydroxymethylfurfural are the main physicochemical parameters of honey that are affected by its botanical origin. Various univariate and multivariate statistical methods employed in order to detect the most influential physicochemical factors for the assessment of authenticity. Both one way analysis of variance (ANOVA) and multivariate analysis such as PCA have indicated free acidity and electrical conductivity as factors with discriminating power. Afterwards, linear discriminant analysis (LDA) resulting in a reliable predictive model which present that these parameters can be used as a tool from modelling the botanical origin of honey. [67].

1. Free acidity: All honeys have a slight acidity due to 0.57% of organic acids that contain. The most characteristic acids are glyconic and citric acid. The concentration of these two acids contribute to differentiation of the two main types of honey namely blossom and honeydew honey. The acidity can be used to detect fraudulent practices. For instance, the addition of high fructose corn syrup aims to a significant increase of pH values [29].
2. Electrical conductivity: It can be used to discriminate the botanical origin of honeys. Studies have shown that blossom honeys as well as mixture of blossom with honeydew honeys have electrical conductivity less than 0.8 mS/cm. On the other hand honeydew and chestnut honeys should have more than 0.8 mS/cm [45] .The measurement of electrical conductivity is an easy and fast method which requires a simple instrumentation in order to assess the honey's quality [29].
3. Hydroxymethylfurfural: The measurement of this compound is a traditional method for identifying possible addition of invert syrup in pure honey. Although hydroxymethylfurfural is formed in pure honey, the level in adulterated honey is much higher. Adulteration with 5% of invert syrups results to an unpermitted value of this compound contrary to the limit of 80 μg/kg which is defined by Codex Alimentarius. However, high levels of hydroxymethylfurfural may be the result of excessive exposure of honey to heat or prolonged storage [70].

2.3.3 Analytical techniques

The assessment of authenticity encompass various analytical approaches based on spectrometric and spectroscopic data basically. Metabolomic approach in the field of authenticity has gained interest in recent years due to the higher level of sophistication of

the suspected fraud. Metabolomics describes the scientific study of small molecules, metabolites, on a biological system based on comprehensive chemical analysis with the aim to detect as many substances are possible. For this reason, various food fingerprinting techniques based on the profile of metabolites have been developed with the purpose of a differentiation of samples [75], [76].

The following section provides an insight in analytical techniques that have been used in recent decades for easier and more reliable evaluation of authenticity of this complex foodstuff like honey. These techniques include the determination of the mineral content, volatile profiles, phenolic profiles, carbohydrate profiles, amino acid composition and stable isotope ratios. The resulting data sets are very complex with one or two dimensions of thousand data points usually without assignment of signals to specific substances [77]. So the need of data handling and the higher demand for better classification of honey have resulted in the development of several chemometric tools such as principal component analysis (PCA), cluster analysis (CA) and linear discriminant analysis.

Metabolomics studies can be performed using target analysis, suspect analysis and non -target analysis

• Target analysis, a quantitative analytical approach using reference standards

• Suspect screening, a qualitative approach for candidate phenolic compounds without using reference standards

• Non-target screening, a qualitative approach for unknown polyphenols patterns.

The above mentioned approaches have been embellished below and are depicted step by step in the following figure. It has to be noticed that these approaches are firstly introduced in environmental analytical chemistry for the determination of the emerging pollutants [78], [79]. Afterwards, food analysis has also adopted these methodologies in order to elucidate the structure of important nutritive food constituents like polyphenols or harmful residues like pesticides. HR-MS is also a decisive factor for food authentication studies because a characteristic pattern of the selected analytes is obtained

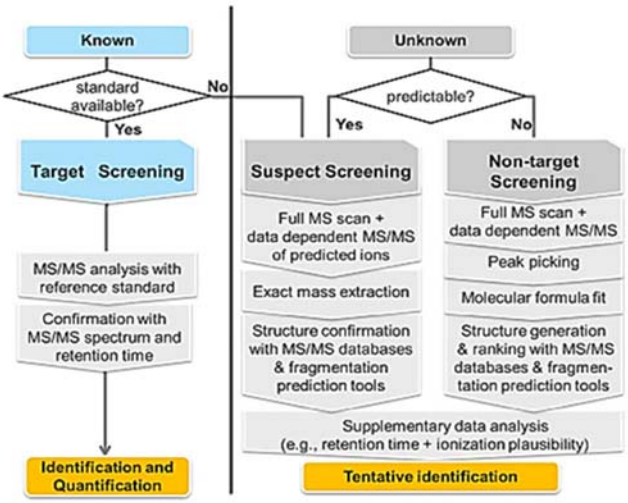

Figure 9. Flow chart of screening procedures. "Known" compounds have been confirmed or confidently identified before, other compounds are considered '"Unknown"', source: [79]

Target analysis: Target screening approach, as shown in figure referred to the detection and quantification of known compounds, which their reference standards are available and as a result their retention time and MS spectrum have already acquired. As a result, they can be included within a defined MS method and be monitored in routine analysis. LC combined with triple-quadrupole mass spectrometric detection (LC-QqQ MS/MS) is the prevailing technique for target analysis. The triple quadrupole (QqQ) analyzer permits application of MS/MS modes [e.g., production scan, precursor-ion scan, neutral-loss scan and selected reaction monitoring (SRM), which is the predominant]. The SRM mode provides several advantages and interesting characteristics for target analysis, such as increased selectivity, reduced interferences and high sensitivity, which allows robust quantification [80].

However, due to the fact that a predefined list of transitions have to be loaded in the MS method, only compounds from this list can be detected .The SRM limitations can be compensated by HR-MS target analysis. Practically, all compounds present in a sample that can be ionized in a specific ion source can be detected simultaneously with HR-MS instrument operating in full scan mode, making it unnecessary to select particular compounds and their transitions. Target compounds included in an accurate-mass database are screened in the sample based on retention time (tR), theoretical mass, isotopic pattern and MS/MS fragments [81]. Additionally, hybrid instruments have the option of data-dependent MS/MS acquisition, where MS/MS analysis is triggered if a

compound from a target-ion list is detected in the full scan. Due to their high mass resolving power, these instruments improve the identification of isobaric compounds and thus permit a more reliable identification process for target analytes [82].

Suspect screening: Contrary to target analysis, suspect screening approach does not depend on reference standards for confirmation. Despite the fact that a large number of polyphenols do not have reference standards available, compound-specific information for suspected molecules, such as molecular formula and structure can be used for the identification and confirmation process. The molecular formula allows the calculation of the exact m/z of the expected ion which is in turn extracted from the high resolution full-scan chromatogram. In case of positive findings, several confirmatory steps must be followed in order to reach structure-derived information [83]. The exact mass for each of the predicted analytes is extracted from the chromatogram and checked by comparing it with control samples. An intensity-threshold value is applied to cut off unclear spectra. The plausibility of the chromatographic tR, isotopic pattern, and ionization efficiency are used as further filters to narrow down the number of candidate peaks. Furthermore, using MS/MS or MSn, structures of suspected phenolic compounds are suggested based on the observed fragmentation pattern and diagnostic fragment ions. Depending on the above criteria, there are different confidence levels of identification in HR-MS analysis.

Non- target screening: Non-target screening is the analytical approach for investigation of analytes which can be detected in the samples but no previous information is available. It is usually performed after target analysis and suspect screening. Full identification of the non-target extracted m/z is a difficult task and for this reason HR-MS instrumentation is necessary in order to obtain high resolution data from full scan and MS/MS mode and elucidate reliably the detected m/z [84].

The assessment of massive quantities of data which offer HR instruments and finally the export of results require post-acquisition data-processing programs which offer rapid, accurate and efficient data mining. Thus a lot of open-source and commercial software exist, some of which are indicatively presented below:

- MZmine
- XCMS
- enviMass
- Bruker Metabolite Tools and Profile Analysis

- Waters MassLynx and MetaboLynx
- Thermo Scientific MetWorks.

The first and most critical step in non-target screening is peak peaking. This step gives the opportunity to exclude irrelevant peaks by the comparison of the sample with control or blank samples. Afterwards, the removal of noise peaks, mass recalibration, componentization of isotopes and adducts follow. The assignment of the molecular formula to the accurate mass of the peak is performed using heuristic filters such as the seven golden rules of Kind and Fiehn.

Exploration of online databases such as ChemSpider and PubChem or structure generation may lead to possible structures of the phenolic compounds. Also, information like molecular formula and substructures of the parent compound could be helpful for the purpose of the search restriction.

Even after filtering, strict criteria and thresholds, the number of peaks which correspond to non-target compounds is enormous and their interpretation would demand a great amount of effort and time. Therefore, the most intense peaks are chosen in order to be interpreted [85].

High mass accuracy coupled with high isotopic abundance accuracy is fundamental to elicit a reliable molecular formula generated by the software incorporated in the HR-MS instruments. The acceptable deviation of the experimental m/z from its corresponding theoretical of parent ions is usually defined at 5 ppm. This limit guarantees the correct prediction of their molecular formula. Higher errors, generally below 10 ppm, are acceptable in the workflow regarding their characteristic fragment ions. In spite of the fact that the accurate extrapolation of the elemental composition of a compound is essential, it is not sufficient to lead in a correct structure proposal. A process which is very helpful in structure investigation is the observation of the presence or absence of similar characteristic ions in the fragmentation pattern comparing the data obtained and online spectral libraries. In addition, information from experimental MS/MS spectra can be compared with in silico mass spectral fragmentation tools (e.g. MetFrag, MetFusion, Mass Frontier, MOLGEN-MS and ACD/MS Fragmenter) or with mass spectra in libraries (e.g. MassBank and MetLin). Nevertheless, the use of mass spectral libraries is restricted for LC/MS-MS data because they do not have a great amount of available data and mass spectra of different instruments are not so comparable [79].

Consequently, the HR-MS based identifications of the analytes differ among studies and compounds because it is not always possible to synthesize each compound and confirm it. In order to make easier the communication of identification confidence, Schymanski et al. [36] proposed a level system which is described in figure 5.

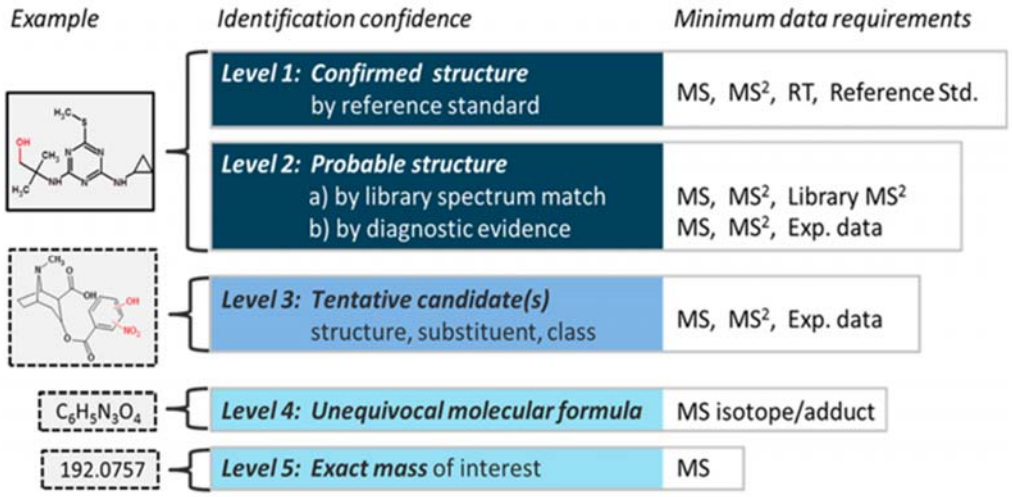

Figure 10. Proposed identification confidence levels in HR-MS analysis, source:[86]

- Level 1: Confirmed structure is the perfect situation where the candidate structure is confirmed by the measurement of a reference standard with MS, MS/MS and retention time matching.
- Level 2: Probable structure refers to a proposal for an exact structure based on different evidence.
    - Level 2a: Library which includes indisputable matching between literature or library spectrum data and experimental.
    - Level 2b: Diagnostic which refers in the case of no other structure fits in experimental data, but no standard or literature information is available.
- Level 3: Tentative candidate(s) is the situation where there is evidence for possible structure(s) but the experimental information is insufficient to the exact proposal.
- Level 4: Unequivocal molecular formula describes the case of an unambiguous formula which is assigned by the spectral information but there is no sufficient evidence to propose possible structures.

- Level 5: Exact mass (m/z) is detected in the sample but no experimental information exists in order to propose even a formula.

2.3.3.1 Stable carbon isotopic ratio analysis (SCIRA)

The C-isotope approach has become famous for the detection of adulterants in honey. It is based on δ13C value which reflects the carbon isotope ratio ($^{13}C/^{12}C$) differentiation between plant groups. This ratio results from the photosynthetic pathways in plants. As already remarked, the plants can be divided mainly to C3 plants which follow the Calvin and Benson cycle and to C4 plants which follow Hatch - Slack cycle. The standard values of δ13C are ranging between - 22‰ to - 33‰ for C3 plants and -10‰ to -20‰ for C4 plants.

Stable carbon isotope ratio analysis has been used to detect adulterated honey, and the results are expressed as $^{13}C/^{12}C = d^{13}C$ (‰). This analysis is more capable for the detection of adulteration with sugars from C4 plants such as cane sugar because the addition of these syrups results to a change in the ratio of $^{13}C/^{12}C$. On the other hand, the detection of adulteration with syrups from C3 plants such as beet sugar is a challenging task because of the fact that these plants are the main nectar sources of bees and do not alter the ratio of honey [72].

It has been defined that honey having $\delta^{13}C$ values above -23.5‰ can be expected to be adulterated with sugars from C4 plants. However due to the fact that $\delta^{13}C$ value is determined by plants used by the honeybees, it shows variability and cannot efficiently used for proofing possible adulteration. For this reason, this $\delta^{13}C$ value is determined in correlation with the δ13C value of the protein in honey. The protein value could be used as an internal standard because the isotope carbon ration doesn't be affected. Using the difference in stable carbon isotope ratio (SCIRA) between a honey and its protein fraction, an evaluation of honey adulteration with amounts of 7–20% and larger of corn or cane sugar can be carried out. The differences between the protein value and the δ13C value is acceptable at -1‰ providing the international limit of pure honeys. More negative differences indicate the addition of C4 plant sugars which are the common adulterants. The measurement of the ratio $^{13}C/^{12}C$ in the formed carbon dioxide is carried out with the isotope ratio mass spectrometer (IRMS) after combusting the samples in an elemental analyser to carbon dioxide, nitrogen and water [73].

2.3.3.2 Molecular spectroscopic techniques:

Spectroscopy in particular vibration spectroscopy is a fast, non-destructive and inexpensive method which is used in the assessment of authenticity of food products. In infrared spectroscopy (IR) samples absorb some of the incoming infrared radiation at specific frequencies producing a spectral fingerprint of the sample. Stretching, bending and rotating vibrations of the corresponding chemical groups in food products are responsible for the mid infrared fingerprints, whereas NIR spectra is connected with complex overtone and high frequency combinations of fundamental vibrations at shorter wavelengths [65].

IR spectroscopy, helps to identify a wide range of adulterants such as corn syrup, HFCS, inverted beet syrup, cane sugar syrup, partial invert cane syrup, beet sucrose, dextrose syrup. Its effectiveness for quantitative and qualitative analysis makes it a highly advantageous method contrary to other analytical techniques. In addition, it is characterized as environmental friendly due to the fact that it requires a small amount of sample with minimal or no preparation while the equipment which can be used is usually portable resulting to a fast assessment of authentication of honey. However, one major challenge is the requirement for advanced chemometric methods, such as PCA and PLS as compressing methods in order to develop predictive models and LDA for honey classification. Particularly, NIR spectra from fiber optic diffuse reflectance has been used to detect HFCS and invert sugar in honey. In addition, it was investigated for the qualitative and quantitave detection of beet sugar as well as for mixtures of fructose and glucose syrups in honey. Nowadays, Fourier transform infrared spectroscopy (FT-IR) has gained great attention in the field of authenticity due to its advantages such as higher acquisition of spectra and an improved signal to noise ratio. Many studies have shown that FT-IR and the subsequent use of appropriate statistics seems to be a powerful tool in the detection of fraudulent practices in honey [77].

Concerning Raman spectroscopy (RS) is another vibrational technique used in the control of authenticity and it is based on fundamental vibration modes that can be assigned to specific chemical functional groups within a sample molecule providing useful information for sample fingerprinting. It is used for the detection of adulteration with HFCS and maltose syrups because the addition of these syrups in authentic honey results to characteristic Raman shifts. The signals for authentic and adulterated honey show characteristic bands at given wave numbers. Also, qualitative identification is mostly done

because of high detection limits. As statistical tools partial least squares (PLS) and subsequent linear discriminant analysis (LDA) have been used with the aim to build a binary classification model. Fourier transform Raman spectroscopy is the most widely used analytical technique, providing useful food-fingerprinting. The principal behind this technique is the fact that the scattering of light from near infrared radiation is equivalent to the vibrational energy by molecules in the sample. It is more preferable than IR spectroscopy due to minimal fluorescence and water interference[72]. FT-RS in combination with statistical analysis is considered as a simple, cost-effective and non-destructive method which determine successfully the levels of glycose, fructose, maltose and sucrose in honey samples as the sugars differ not only in the carbon isotope ratio but also to the absorption energy between different isotopes. So pure honey can be easily distinguished from adulterated. In conclusion, an up to date version of Raman spectroscopy is Surface Enhanced Raman spectroscopy which provides lower detection limits than the original method allowing more applications in the detection of food adulterants [65].

Furthermore, another method which provide structural information in food systems with excellent repeatability and reproducibility is 1D and 2D Nuclear Magnetic Resonance (NMR). It possess several advantages such as it is a fast method which requires no calibration with internal standards, no sample preparation like extraction prior to analysis and has the capability to detect simultaneously and quantitatively all organic materials in the original state . The only drawback with the use of this technique is the high cost of instrumentation and the lower sensitivity contrary to others analytical techniques. In case of honey, NMR holds great potential for detection of a vast number of adulterants in shorter times. It has been widely used to identify the botanical origin of honey by specific fingerprints or specific markers which are helpful in the field of authenticity. This method achieves mostly quantitative analysis as all components in the spectrum are representative in proportions to relative concentrations. So a successful identification and assignments of signals to specific molecular structures have been succeeded with this technique. After construction of a sufficient database, the technique would allow the identification of the origin and the type of sugar syrup with high accuracy using its molecular fingerprint. Also, NMR spectroscopy in combination with multivariate statistical analysis has been used in the discrimination of geographical origin of honey samples as it enables a collection of comprehensive metabolic profiles that can be used in the discrimination of origin [72]. However very few studies have been reported on NMR based

detection of sugar adulterants in honey until now. Some of them emphasizes the great potential of NMR spectroscopy in fast detection of high fructose corn syrup and mainly C3 sugars in honey [77].

2.3.3.3 Atomic spectrometric methods

Advances in instrumentation and the growing development of high resolution methods in the field of atomic spectroscopy have provided a detailed mineral characterization of honey samples which has been used then for the identification of geographical and botanical origin of samples. Some minerals such as K and Na are indicators mostly of botanical origin because they are accumulated in plant cells while others are more associated with soil and environmental conditions.

Atomic absorption spectrometry is proved as the appropriate method for the assessment of mineral content in honey samples accompanied to mineral sample preparation. Nowadays inductively coupled plasma atomic emission spectrometry (ICP- AES) and inductively coupled plasma mass spectrometry (ICP-MS) have been developed and almost exclusively used for the determination of mineral content due to their ability of multielement measurement [65]. It has to be mentioned that a combination of 2 distinct techniques are used in order to analyze different minerals and trace elements separately (ICP-MS with FF-AAS). Also, the use of element profile coupled to isotope ratio analysis seems to be a very promising approach for the discrimination of honeys according to botanical and geographical origin. The qualitative and quantitative mineral profiles coupled to statistical analysis could be an essential tool for the discrimination of the botanical and geographical origin of honey samples as well as between organic and conventional types of honey [51].

2.3.3.4 Chromatographic methods

With the advent of modern chromatographic methods, there is a growing consideration of patterns of constituents that could serve as fingerprints for honeys from different botanical sources. This is very useful technique once more data are collected, showing the consistency of the patterns within types of honeys and the uniqueness of the fingerprint of each type

2.3.3.4.1 Carbohydrate profile

As already mentioned, sugars (saccharides) are the main components of honey and are considered useful parameters to assess honey adulteration by the addition of syrups.

Although their use as indicators of botanical and geographical discrimination, the amount and the ratio of particular carbohydrates such as fructose and glucose are suggested to use as markers to recognize unifloral honeys. For example, in pure honeys the actual proportion of fructose/glucose is defined 1.2:1. This ratio might be changed by the extra addition of sucrose. Whereas the same ratio will be stable when adulteration with high fructose corn syrup or invert syrup takes place. Also, the concentration of sucrose is approximately 1% of its dry weight. This percentage may be increased if the beekeeper overfeed the bees with industrial saccharose during the spring season. Different chromatographic methods are used to determine the carbohydrate profile of honey samples include Thin-layer chromatography (TLC), HPLC coupled to pulsed amperometric detector (HPLC-PAD), High performance anion exchange chromatography (HPAEC), Ultra high pressure liquid chromatography coupled to quadruple time of flight mass spectrometry (UHPLC-Q-TOF-MS), GC –MS based on two stationary phases, GC coupled to flame ionization detector and a combination of HPLC-PAD and (GC-FID).

HPLC has proven to be a widely accepted method to detect both C3 and C4 starch syrups, and it is also highly useful for the detection of a newly reported adulterant, rice syrup, that is harder to detect using other common methods. HPLC-PAD is the most appropriate method for the sugar analysis because it allows high sensitivity and does not require previously derivatization step which is time consuming. Also, HPLC-RID is suggested as a simple and easy method for detecting effectively sugars both from C3 and C4 plants. This is done by detecting the oligosaccharide peak as a syrup indicator.

The first official technique which was developed to detect adulteration with high fructose corn syrup was TLC. It has to be noticed that a specific column pretreatment is required in order to concentrate the trace of oligosaccharides. Pure honeys yield only 1 or 2 blue-grey or blue-brown spots at Rf values above 0.35. The adulterated samples show an additional series of spots or blue streaks. Nowadays a simple analytical method HPTLC which is based on the combination of TLC with the ratio of fructose to glucose ratio has been proposed for detection of adulterants but with a limited use despite of the invalid results.

The development of HPAEC has paved the way for a better control of authenticity of highly complex foodstuff like honey. It determines effectively the oligosaccharide's content even though honey contains large amounts of monosaccharides (60-70%) and disaccharides (10%). For instance, the detection of the adulteration with corn syrup and

high fructose corn syrup can be performed by HPAEC coupled to pulsed amperometric detector. The analysis is based on their different ionic chromatographic profiles of oligosaccharides and it is considered less expensive as earlier updated techniques.

GC coupled to several detectors is the method of choice for the detection of the adulteration of honey with high fructose corn syrup or inverted sugar syrups. The analysis is based on the measurement of Difructose anhydrates (DFAs) which are detected only on adulterated honey. The DFA content in food is dependent on food composition and processing and for this reason these compounds cannot be used as marker compounds for the presence of HFCS. Another drawback is the fact that honey samples require yeast treatment to concentrate the di- and trisaccharides, a process that is not essential in other advanced techniques. Many studies have shown that GC coupled to mass spectrometry is the only available method for the assessment of honey adulteration with HFIS. The detection is based on the determination of the specific marker inulotriose. GC-MS analysis appears to be useful in detection of indirect adulteration with HFCS. Another advanced technique that has been developed recently for the separation and identification of low molecular carbohydrates that possible be used as markers of botanical origin is the GCGC-TOF-MS which overcome the problem of coelution of anomeric structures [51].

A new technique which attracts a growing interest in recent years in the quality control and the field of authenticity is UHPLC-TOF-MS. This technique has the capability to detect several sugars ın adulterated honeys fast and simultaneously. It can operates either in a MS or MSMS modes providing accurate mass measurements of full product ions resulting to a subsequent exact determination of a variety of adulterants. This analytical technique could be used also for the discrimination of origin, based on the detection of specific marker compounds[72]

2.3.3.4.2 Volatile profile

Bearing in mind that aroma is one of the most noticeable characteristic of honey, aroma profile can be used potentially as a fingerprint for the classification of honey according to botanical and geographical origin. A small number of compounds have been reported as floral markers for assessing the origin and allows the discrimination of adulterated honey and the original one. It is well known that volatile composition can be affected by several factors such as floral origin, environmental condition, soil characteristics and processing conditions. Also, several authors have proposed the use of enantiomeric ratio of some

volatile constituents. For instance, the enantiomeric ratio of linalool and its oxides is used for the floral authentication of honey because it remains stable.

The aroma profile of honey is determined mainly using GC-FID and more commonly GC-MS. Although, these methods are characterized by highly efficiency and sensitivity, they have faced an important problem of coelution of compounds due to complexity of headspace aroma composition of honey. This problem can be prevented by using GC-GC-TOF-MS as analytical method of determination allowing rapid and comprehensive characterization of volatile profile due to increased chromatographic resolution and better spectral quality. This technique combined with several chemometric tools due to the large volume of the data can determine several marker compounds which are useful in the authentication of honey samples. For example, methyl anthranilate has long been used as marker for citrus honey. However, it suffers significant changes in concentration under varied environmental conditions and under different honey storage conditions. 3-aminoacetophenone has been found to be the main constituent of chestnut honey. Isophore and its derivatives have been reported to present in certain types of honey such as chestnut –tree honey, rape apple honey and thyme honey. In addition, 3,9 epoxy-1-p-mentadieno, t-8-p menthan-oxide-1,2-diol and cis-rose have been proposed as markers of lemon honey as well as diketones , sulfur compounds and alkanes are characteristic of eucalyptus honeys. Finally heptanal and hexanal are the main compounds of lavender honey [51] .These compounds facilitate the detection of the origin. Nevertheless the variability and the availability of these compounds which fraudulently added to honey samples resulting to inaccurate classification of honey based on these markers.

2.3.3.4.3 Amino acid and protein profile

An assessment of honey authenticity especially determination of botanical origin is based on the amino acid profile. It is usually combined with other parameters in order to provide a complete discrimination among honeys. In pure honeys, amino acids contribute to 1% (w/w) of the constituents of honey. The most abundant amino acid is proline which is used as a criterion for the detection of possible adulteration with industrial sugars. As proline originated by the salivary secretions of bees during the transformation of nectar to honey the minimum value of 180 mg/kg is defined for pure honeys. In addition, many studies have shown that tryptophan, arginine and cysteine are characteristic for certain floral types of honey. It has been suggested that certain enantiomeric rations between

concentration of various amino acids could be used to determine the geographical origin of a honey [67].

Amino acids are quantified by reversed- phase high performance liquid chromatography with fluorescence detector or by gas chromatography with the aim to gain knowledge about the possible use of amino acid patterns for classification. For example, vanile, alanine and a high content of tyrosine can be used as markers for the classification of lavender honey from rosemary and thyme honey. Also, high concentrations of aspartic and asparagine suggested as markers of raspberry and buckwheat honeys [51].

However, the results have shown that a single amino acid or groups of amino acids are not be useful for the characterization of certain types of honeys because there is a great variability, while the overall amino acid could be a useful indicator of the assessment of honey's authenticity. Both of these techniques are combined to chemometric tools such as PCA and LDA or CVA in order to evaluate the validity of the use of amino acid profile in honey's authenticity.

Except from amino acids, honey proteins originating from pollen or from enzymatic reaction between bee saliva and plant pollen can also be suggested as useful markers for honey botanical classification. (Baroni and others 2002). The determination of protein content has been performed using polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS-PAGE) or a new method called enzyme - linked immunoassay. The protein profile has been suggested from some authors for the geographical discrimination of honey samples with the development of MALDI -TOF MS. However, the analysis of amino acid profile is used in a greater extent than the analysis of protein content due to the fact that it is more representative factor for the determination of the botanical origin [87].

2.3.3.4.4 Phenolic profile

As already mentioned phenolic compounds are the main constituents of honey due to their antibacterial and antioxidant capability. They are considered as useful markers for honey characterization as well as their profiles can be used in the assessment of geographical and botanical origin. Analytical procedures used to determine polyphenols in honey include adequate extraction from the complex matrix, separation and quantification. Different profiling methods include thin layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis, colorimetric reactions and high performance liquid chromatography (HPLC) coupled to various detectors (diode array

detector (DAD), coulometric electrode array detector (CEAD) and mass spectrometer (MS)).

Among them, the most suitable method is liquid chromatography coupled to mass spectrometry because the content of phenolic compounds varies in a great extent. Also, few publications have emphasized as possible method for the determination of the flavonoid pattern the use of capillary electrophoresis. It combines short times and high separation efficiency of polyphenols in honey while it is characterized by a simple and a low cost instrumentation. All these attributes makes this technique more attractive for the development of improved methods. Besides LC chromatography, the analysis of various types of phenolic acids such as hydroxycinnamic acids and hydroxybenzoic acids in honey is usually performed by gas chromatography (GC) after a derivatization step (methylation).

In addition, a colorimetric assay of total phenolic content based on the reaction of these compounds with the reagent namely Folin Ciocalteu is widely used in the determination of total phenolic content of honey samples. The method is comprised by calibration with a pure phenolic compound, extraction of phenolics and the measurement of absorbance after reaction. The main drawback of the colorimetric assay is its low specificity, as the color reaction can occur with any oxidizable phenolic hydroxy group [88].

Currently, tandem mass spectrometry (MS/MS) has been explored for analysis of phenolic compounds due to its sensitivity and selectivity of detection. In LC/MS-MS which is the dominant technique, the identification of phenolic profile is based on the characteristic ion transitions determining certain target analytes. However, this technique is not suitable for full scan measurements and for complex matrices because it is not possible to analyze components for which their ion transitions have not been predefined. For this reason LC chromatography coupled to mass analyzer based on ion trap technology has been developed and commonly used for the analysis of a larger number of phenolic compounds. Nowadays the new standard methodologies are the use of ultra -high pressure liquid chromatography coupled to high resolution and accurate mass spectrometry and hybrid mass analyzers. The advantages of these modern techniques contrary to conventional one are the simultaneous determination of the phenolic content of honey, quantification and MS^n analysis based on high resolution and accurate mass measurement. In more detail, a noticeably shorter chromatography time with defined peaks will be achieved in comparison to tradition HPLC systems. Beside from the time of

analysis the detectors used play an important role. For example, the selectivity and sensitivity of TOF-MS detectors are a good choice for determination of low UV –sensitive substances like phenyllactic acid which are difficult to analyse in small amounts by DAD detectors. Their capability to determine accurate molecular masses facilitates the search of a molecular structure of an unknown phenolic compound which could help the assessment of honey authenticity. The identification and quantification of phenolic compounds in honey is performed either by available standards or is based on the search for the deprotonated molecule $[M-H]^-$ .The exact mass together with the interpretation of the fragmentation pattern provide sufficient structural information which is useful for the unknown compounds [89]. Lately, a different approach has been suggested by some authors based on 3D synchronous fluorescence spectroscopy (SFS). This spectra obtained from the honey phenolic fraction could serve as a good indicator of the botanical origin of honey.

From the analysis of phenolic profile, several marker compounds have been generated contributing to authentication issues. Speer and Montag have detected already in 1984 higher amount of phenylacetic and benzoic acids by GC –MS in heather honey. This has been confirmed later by Steeg and Montag (1988) who detected mandelic and phenyllactic acid in heather honey, hydroxycinnamic acid in rape honey, protocatechuic in honeydew honey and 4 hydroxybenzoic acid in buckwheat honey. In addition, to these studies, ellagic acid, abscisic acid, myricetin, tricetin, myricetin 3 methyl- ether were described as possible markers in heather honey (Soler et al., 1995; Ferreres and others 1996; Ku´s and others 2014). Using coulometry, Jorg and Sontag in 1992 have identified p -coumaric acid and ferulic as indicators for chestnut honey. Also, other compounds which have been found as possible markers for chestnut honey using HPLC-UV-Vis detection are 4 hydroxy benzoic acid, 4 hydroxyphenyllactic acid and phenylacetic acid (Dimitrova et al 2007). Hamdy and others (2009) have detected methyl anthranilate and hesperitin in citrus honey, as well as quercetin, p- hydroxy benzoic acid and cinnamic acid in monofloral honey. Also there are many other phenolic compounds which could serve as indicators of the botanical origin of honey samples and are mentioned in several studies.

Other instances which should be highlighted are the following: homogenistic acid for strawberry tree honey, (Tuberoso and others 2010), methyl syringate for rapessed honey (Ku´s and others 2014), 8-methoxykaempferol for rosemary honey (Ferreres and others 1994), cinnamic acid for acacia honey (Dimitrova et al., 2007). Moreover, benzoic acid

and its derivatives, both of the phenolic acids gallic and abscisic, and the flavonoids myricetin, tricetin luteolin and quercetin were proposed as possible markers for eucalyptus honey (Dimitrova et al.,2007; Yao et al.,2003,2004b,2005; Martos et al., 2000a,b). Also, the characteristic flavonoid quercetin has been identified as a marker compound for blossom honey (Soler et al., 1995; Ferreres et al., 1996). The presence of p-coumaric acid, ferulic and the absence of phenyllactic acids in blossom honeys is the deciding factor in HPLC chromatograms (Tomas- Barber´an and others 2001; Dimitrova et.al 2007). Except from these results, it has been found that rosmarinic acid seems to be characteristic for thyme honey while naringenin of lavender honey [73], [88], [89]. In conclusion, in many studies it was demonstrated that pinocembrin, pinobanksin, and chrysin are characteristic flavonoids found in propolis and in most European honey samples.

The analysis of phenolic profile is a crucial factor in the assessment of authenticity combining the identification of specific marker compounds with the use of chemometric analysis. Chemometric evaluation of the flavonoid data is necessary in order to perform a classification of the investigated honey samples. For example, PCA is used in order to establish a correlation between phenolic profile and botanical origin of honey.

2.4 Extraction of phenolic compounds

Due to the complexity of the honey's matrix the isolation and extraction of phenolic compounds is a crucial step prior to analysis because the qualitative and quantitative identification of phenolic compounds can be affected by interferences reducing the selectivity and sensitivity of the analytical method. So this step helps not only to the removal of interferences such as carbohydrates but also to an increased concentration of target analytes. Due to the wide range of polarities among the phenolic compounds, a suitable extraction procedure should be used in order to efficiently extract the compounds from the matrix. So, three different approaches have been utilized for the extraction of phenolic compounds since now.

Firstly, Amberlite XAD-2 resin has been one of the most popular adsorbent media for the extraction of phenolic compounds from honey. The honey samples are mixed with five parts of water (pH 2 with HCl) until completely fluid and then filtered through cotton to remove solid particles. The filtrate is then passed through a column of Amberlite XAD-2. Elution, accomplished first with aqueous HCl solution (pH = 2) and after with water, allows one to separate the phenolic fraction (retained on the column) from the polar interfering

substances like sugars. The phenolic fraction is then eluted with methanol and concentrated under reduced pressure at 40 Celsius in a rotary evaporator. In clean up step the residue after the evaporation is suspended in distilled water and extracted with diethyl ether or ethyl acetate. The extracts are combined and the diethyl ether is removed by flushing with nitrogen. The dried residue is then redissolved in methanol and filtered. This extraction procedure shows high sensitivity for the phenolic compounds with recovery rates over 95% [90]. Nevertheless, there are several drawbacks that have to be highlighted. A high amount of honey sample, 50g or even 100 g, needs to be dissolved in water at a solvent to sample ratio of 5:1. Thus, a large quantity of honey is needed for this extraction protocol and as a result only samples with adequate amount can be analyzed. Proportionally, high volumes of solvents are also needed. The extraction with Amberlite XAD-2 comprises many different steps and can be considered as time consuming pretreatment. Moreover, this resin retains selectively the phenolic compounds. So, this pretreatment is not suitable for metabolomics studies in which a comprehensive fingerprint of the sample need to be obtained and the extraction procedure should be generic.

The current analytical trend is to diminish the previously mentioned drawbacks, so various SPE methods have been proposed in order to overcome the problems of the previous extraction procedure. In this case, about 5 g of honey sample is required for the analysis. The phenolic compounds interacts with the adsorbent and retained by means of hydrophobic interactions [88]. Therefore, a great variety of sorbents have been used such as Bond Elut octadecyl C18, Oasis HLB and Strata-X. The cartridges are usually conditioned with methanol or acidified water. The polar substances are washed with water, while the polyphenols are commonly eluted with methanol. The methanolic extract is filtered and stored at -20 Celsius until analysis. The mechanism of retention with C18 sorbents depends on Van der Waals forces, hydrogen bonds or dipole–dipole interactions. On the other hand, polymeric sorbents such as OASIS and Strata have a broader Ph stability and a greater surface area due to selective π–π interactions with analytes containing aromatic rings. Also, OASIS cartridges exhibit both hydrophobic and hydrophilic retention characteristics resulting in a high analyte capacity. So the best recoveries are obtained using this sorbent especially for phenolic acids [91]. SPE combines the small amount of sample and reagents with high selectivity for phenolic compounds. Furthermore, the huge variety of sorbents give more choices for extraction while it is best fitted for methods with a target approach.

Liquid-liquid extraction (LLE) is a classic approach in order to extract compounds, which exhibit affinity to the solvent used for the extraction from the matrix. Ethyl acetate (EtAc) is used as extractant in the most of the cases [89], [92]. It has to be noticed that repeated extractions are performed in order to achieve better extraction efficiency. Furthermore, LLE is also used as a preliminary clean up step during SPE procedure indicating that LLE is a more generic extraction which permits metabolomics studies. Hence, the diluted-and-shoot approach has been recently proposed in order to obtain a fully representative polyphenols fingerprint [72], [76]. In this case, the honey sample analysed just after its solubilisation in water or in LC mobile phase.

Despite the growing interest in the application of advanced liquid extraction techniques only one example of accelerated solvent extraction (ASE) has been found in the literature during the last years. The extraction is performed dissolving the sample in acidified water with hydrochloric acid (pH = 2) at 25 °C by means of four different static cycles. Polyphenols are eluted with methanol, the solution is evaporated until dryness, and the residue is suspended in distilled water and extracted three times with diethyl ether. Extracts are again dried and dissolved in a methanol/water solution before the HPLC [94].

In recent years, some novel extraction methods have been developed including microwave assisted extraction (MAE) and ultrasonic extraction (US). These two techniques are characterized by reduced extraction times and an improvement of the flavonoid's yield. Nevertheless, the selectivity of MAE extraction is low with high amounts of non- phenolic compounds, while the exposure of honey in longer irridation times resulting to a decrease in the percentage of some extracted components such as chrysin due to degradation processes [95].

Also, another innovative effort which utilizes the evolution of nanotechnology is the multiwalled carbon nanotubes (MWCNTs) sorbents for phenolic compounds. MWCNTs are added to an acidified solution of honey, then the mixture is magnetically stirred in order to promote the retention of phenolic compounds onto the nanotubes. The main advantage of this approach lie in the possibility to simultaneously extract a really wide number of phenolic compounds with high recoveries and reproducibility [96].

2.5 Chemometric evaluation

Over the last decade rapid development has been shown regarding the use of multivariate analysis in the food authentication, classification and discrimination. Since honey is a

complex mixture and the data obtained by fingerprinting techniques cannot easily be handled, the need of better interpretation by statistical tools has increased. Data mining, data fusion and feature selecting are very important for the making sense of the huge data [65].

Principal component analysis (PCA), partial least squares (PLS) linear discriminant analysis (LDA), canonical variate analysis (CVA), cluster analysis, (CA), artificial neural networks (ANN) and k-nearest neighbors (KNN)) are the most commonly used multivariate analysis techniques in foods authentication. These multivariate techniques are divided into unsupervised and supervised approaches. According to the first one, these are used for pattern recognition within complex spectroscopic and spectrometric data resulting to identify clusters or trends among samples. A characteristic example is PCA which is estimated as a data compression method. It is carried out to reduce the dimensions of the original data to a smaller number of component sets by examining the relationship between measured parameters. This statistical procedure has the capability to transform a set of possibly correlated data of new variables, called principal components (PCs).The projection of the samples into new variables is carried out by linear combination of the original variables. Extracted information from the data is used to make predictions about unknown new samples. Since PCA visualizes the data structure, it is usually applied prior to real data analysis using discriminating techniques. On the other hand supervised techniques are based on prior creation of classification rules using a data set with objects of known class membership. So unknown objects can be classified to one of the existing classes [77]

The classification techniques that are most commonly used are class discriminating techniques and class modeling techniques. Class discriminating techniques are a family of methods used for treating multiple-group classification problems. Class discriminating techniques, which always attribute a new sample obligatory to one of the known classes used to build the model, include LDA, kNN, DPLS, support vector machine (SVM) and ANNs. Whereas classmodeling techniques such as soft independent modeling of class analogy (SIMCA), which do not always attribute a sample to a known class, have also been used to evaluate adulterants in honey. LDA is one of the most frequently used discriminating techniques in honey authentication and floral origin detection. It is a supervised pattern recognition technique that is based on discriminant canonicals in which the center of the matrix variance and covariance of each batch is calculated. In this method, the variance is maximized between categories and minimized within categories.

LDA is very helpful in determining the similarity or dissimilarity of the pattern of an unknown to those in calibration sets [97]

CHAPTER 3: Scope & Objectives

Honey is a foodstuff which is subjected to various frauds like addition of syrups or mislabeling due to a higher demand of honey products in contrast to the decrease of honey bee population. Particularly, unifloral honeys which represent a scarce commodity due to their characteristic organoleptic and biological features are possible targets of adulteration techniques like misdescription of botanical origin. Moreover, the honey obtained by specific plants such as Manuka is strictly connected to health beneficial properties like antioxidant activity. Another important factor influencing the final value of the product is the provenance in which it is produced. On this merit, the evaluation and verification of honey authenticity is a task of great importance for the producers, consumers and regulatory bodies.

Analytical chemistry plays a decisive role in the fight against the mislabeling of honey. Various analytical methods for the determination of botanical and geographical origin have been developed based on chromatographic, isotopic, elemental and spectroscopic principles. As already mentioned a great variety of analytes has been shown potential as origin markers. So the phenolic profile has been chosen to study because of their proven capability to assess the honey's authenticity.

So, the aim of this master thesis is the evaluation of the phenolic content of the most common greek honeys of various botanical origins using already developed method and the potential discrimination of the botanical and geographical origin based on the polyphenols content. Particularly, the objectives of this study are:

• The incorporation of new compounds in the already existed database with antioxidant compounds.

• The revalidation of a UPLC-QToF MS method for the determination of the phenolic compounds in order to find certain marker compounds for the assessment of authentication

• The target screening in representative number of phenolic compounds and assessment of phenolic profile.

•Classification of honey samples by multivariate approaches

• Possible suspect screening of phenolic compounds which have already detected in some of the honey samples.

Concluding, in the end of the present master thesis, future perspectives and work to be done are discussed.

CHAPTER 4: Material and methods

4.1 Reagents, standards and solvents

For the UHPLC-ESI-QToF system:

- Methanol (MeOH hypergrade for LC-MS, Sigma-Aldrich)
- Ultrapure water (18.2 MΩ cm^{-1}, produced by a Milli-Q water purification system)
- Ammonium acetate (Fluka, Sigma-Aldrich).
- Sodium formate (Sigma Aldrich)

For the experimental procedure:

- Syringic acid (purity 95 %), myricetin (purity 99%), eriodictyol (purity 99%) and taxifolin (purity 99%) were purchased from Extrasynthèse (Genay, France), 4-hydroxybenzoic acid (purity 99%), 3,4-dihydroxybenzoic acid (purity 97%), 2,5-dihydroxybenzoic acid (purity 99%), salicylic acid (purity 99%), vanillic acid (purity 97%), gallic acid (purity 98 %), ferulic acid (purity 98 %), epicatechin (purity 97 %), p-coumaric acid (4-hydroxycinnamic acid; purity 98 %), quercetin (purity 98 %) were obtained from Sigma-Aldrich (Steinheim, Germany), hydroxytyrosol (purity 98 %) and luteolin (purity 98 % was purchased from Santa Cruz Biotechnologies, caffeic acid (purity 99 %), vanillin (purity 99 %), ethyl vanillin (purity 98 %), apigenin (4,5,7-trihydroxyflavone; purity 97 %), and tyrosol [2-(4-hydroxyphenyl) ethanol, purity 98 %] were acquired from Alfa Aesar (Karlsruhe, Germany), cinnamic acid (purity 99%) was purchased from Merck (Hohenbrunn, Germany), genistein (purity 99%), galangin (3,5,7-Trihydroxyflavone; 97%) naringenin (4',5,7-Trihydroxyflavanone; 97%) were purchased form Alfa Aesar, pinobanksin (95%), pinocembrin (95%), hesperitin (3',5,7-Trihydroxy-4'-methoxyflavanone;97%), rosmarinic acid((R)-O-(3,4-Dihydroxycinnamoyl)-3-(3,4-dihydroxyphenyl)lactic acid;98%), chrysin(5,7-Dihydroxyflavone, 98%) were purchased from Sigma Aldrich.
- Stock solutions of 1000 mg L^{-1} were prepared for each analyte. 0.01g was weighed and diluted in MeOH in 10 mL volumetric flask. The solutions were stored at −20 °C in amber glass bottles to prevent photodegradation.
- Working solution of 25 mg L^{-1} was prepared. The working solution contained all the analytes. 40 µl of each analyte from stock solutions were transferred and

diluted in MeOH in a 25 ml volumetric flask. The solution was stored at −20 °C in amber glass bottle to prevent photodegradation.

- Ethyl Acetate (Sigma Aldrich)
- Sodium sulfate (Sigma Aldrich)
- Sodium chloride (Carlo Ebra reagents)
- Hydrochloric acid (Sigma Aldrich)

All the necessary dilutions were performed to standards and stock solutions according to the experimental requirements, in order to prepare the diluted standards. All working solutions were stored in the refrigerator.

4.2 Sampling and storage

135 honey samples were collected from various regions of Greece. This large number of samples consists of 114 unifloral honeys from 5 different botanical origins especially thyme, pine, blossom, fir and oak and 21 multifloral honeys namely fir-pine and pine-blossom. All samples were characterized according to their botanical type, based on their certified labeling. Samples were stored and preserved in a dark and cold room before analysis. Details considering geographical origin and botanical type of honey samples are presented in the following Table 3.

Table 3. Characterization of greek honey samples

a/a	code	Type	Region	Country
1	2016/oak/01	oak		**GREECE**
2	2016/oak/02	oak		**GREECE**
3	2016/oak/03	oak		**GREECE**
4	2016/oak/04	oak		**GREECE**
5	2016/oak/05	oak		**GREECE**
6	2016/oak/06	oak		**GREECE**
7	2016/oak/07	oak		**GREECE**
8	2016/oak/08	oak		**GREECE**
9	2016/oak/09	oak		**GREECE**
10	2016/oak/10	oak		**GREECE**
11	2016/oak/11	oak		**GREECE**
12	2016/oak/12	oak		**GREECE**
13	2016/oak/13	oak		**GREECE**
14	2016/oak/14	oak		**GREECE**
15	2016/oak/15	oak		**GREECE**
16	2016/fir/01	fir	Karpenisi	**GREECE**
17	2016/fir/02	fir	Karpenisi	**GREECE**

18	2016/fir/03	fir	Karpenisi	**GREECE**
19	2016/fir/04	fir	Karpenisi	**GREECE**
20	2016/fir/05	fir	Karpenisi	**GREECE**
21	2016/fir/06	fir	Karpenisi	**GREECE**
22	2016/fir/07	fir	Karpenisi	**GREECE**
23	2016/fir/08	fir	Karpenisi	**GREECE**
24	2016/fir/09	fir	Karpenisi	**GREECE**
25	2016/fir/10	fir	Karpenisi	**GREECE**
26	2016/pine/01	pine	Macedonia	**GREECE**
27	2016/pine/02	pine	Macedonia	**GREECE**
28	2016/pine/03	pine	Macedonia	**GREECE**
29	2016/pine/04	pine	Macedonia	**GREECE**
30	2016/pine/05	pine	Thasos	**GREECE**
31	2016/pine/06	pine	Thasos	**GREECE**
32	2016/pine/07	pine	Thasos	**GREECE**
33	2016/pine/08	pine	Thasos	**GREECE**
34	2016/pine/09	pine	Thasos	**GREECE**
35	2016/pine/10	pine	Thasos	**GREECE**
36	2016/pine/11	pine	Nikiti	**GREECE**
37	2016/pine/12	pine	Nikiti	**GREECE**
38	2016/pine/13	pine	Nikiti	**GREECE**
39	2016/pine/14	pine	Nikiti	**GREECE**
40	2016/pine/15	pine	Nikiti	**GREECE**
41	2016/pine/16	pine	Nikiti	**GREECE**
42	2016/pine/17	pine	Nikiti	**GREECE**
43	2016/pine/18	pine	Nikiti	**GREECE**
44	2016/pine/19	pine	Nikiti	**GREECE**
45	2016/pine/20	pine	Chalkidiki	**GREECE**
46	2016/pine/21	pine	Chalkidiki	**GREECE**
47	2016/pine/22	pine	Chalkidiki	**GREECE**
48	2016/pine/23	pine	Chalkidiki	**GREECE**
49	2016/pine/24	pine	Chalkidiki	**GREECE**
50	2016/pine/25	pine	Chalkidiki	**GREECE**
51	2016/pine/26	pine	Chalkidiki	**GREECE**
52	2016/pine/27	pine	Evia	**GREECE**
53	2016/pine/28	pine	Evia	**GREECE**
54	2016/pine/29	pine	Evia	**GREECE**
55	2016/pine/30	pine	Evia	**GREECE**
56	2016/pine/31	pine	Evia	**GREECE**
57	2016/pine/32	pine	Evia	**GREECE**
58	2016/pine/33	pine	Evia	**GREECE**
59	2016/pine/34	pine	Evia	**GREECE**
60	2016/pine/35	pine	Evia	**GREECE**
61	2016/pine/36	pine	Evia	**GREECE**
62	2016/blossom/01	blossom	Evros	**GREECE**

63	2016/blossom/02	blossom	Evros	**GREECE**
64	2016/blossom/03	blossom	Evros	**GREECE**
65	2016/blossom/04	blossom	Evros	**GREECE**
66	2016/blossom/05	blossom	Evros	**GREECE**
67	2016/blossom/06	blossom	Evros	**GREECE**
68	2016/blossom/07	blossom	Evros	**GREECE**
69	2016/blossom/08	blossom	Evros	**GREECE**
70	2016/blossom/09	blossom	Evros	**GREECE**
71	2016/blossom/10	blossom	Macedonia	**GREECE**
72	2016/blossom/11	blossom	Macedonia	**GREECE**
73	2016/blossom/12	blossom	Macedonia	**GREECE**
74	2016/blossom/13	blossom	Macedonia	**GREECE**
75	2016/blossom/14	blossom	Macedonia	**GREECE**
76	2016/blossom/15	blossom	Macedonia	**GREECE**
77	2016/blossom/16	blossom	Macedonia	**GREECE**
78	2016/blossom/17	blossom	Macedonia	**GREECE**
79	2016/blossom/18	blossom	Macedonia	**GREECE**
80	2016/blossom/19	blossom	Macedonia	**GREECE**
81	2016/blossom/20	blossom	Macedonia	**GREECE**
82	2016/blossom/21	blossom	Macedonia	**GREECE**
83	2016/blossom/22	blossom	Macedonia	**GREECE**
84	2016/blossom/23	blossom	Macedonia	**GREECE**
85	2016/blossom/24	blossom	Macedonia	**GREECE**
86	2016/blossom/25	blossom	Macedonia	**GREECE**
87	2016/blossom/26	blossom	Macedonia	**GREECE**
88	2016/blossom/27	blossom	Macedonia	**GREECE**
89	2016/blossom/28	blossom	Macedonia	**GREECE**
90	2016/blossom/29	blossom	Macedonia	**GREECE**
91	2016/blossom/30	blossom	Macedonia	**GREECE**
92	2016/blossom/31	blossom	Macedonia	**GREECE**
93	2016/blossom/32	blossom	Macedonia	**GREECE**
94	2016/blossom/33	blossom	Macedonia	**GREECE**
95	2016/blossom/34	blossom	Macedonia	**GREECE**
96	2016/blossom/35	blossom	Macedonia	**GREECE**
97	2016/thyme/01	thyme	Chania	**GREECE**
98	2016/thyme/02	thyme	Chania	**GREECE**
99	2016/thyme/03	thyme	Chania	**GREECE**
100	2016/thyme/04	thyme	Chania	**GREECE**
101	2016/thyme/05	thyme	Chania	**GREECE**
102	2016/thyme/06	thyme	Chania	**GREECE**
103	2016/thyme/07	thyme	Heraklion	**GREECE**
104	2016/thyme/08	thyme	Heraklion	**GREECE**
105	2016/thyme/09	thyme	Heraklion	**GREECE**
106	2016/thyme/10	thyme	Heraklion	**GREECE**
107	2016/thyme/11	thyme	Heraklion	**GREECE**

108	2016/thyme/12	thyme	Heraklion	**GREECE**
109	2016/thyme/13	thyme	Rethymno	**GREECE**
110	2016/thyme/14	thyme	Almyros Volos	**GREECE**
111	2016/thyme/15	thyme	Almyros Volos	**GREECE**
112	2016/thyme/16	thyme	Astypalaia	**GREECE**
113	2016/thyme/17	thyme	Astypalaia	**GREECE**
114	2016/thyme/18	thyme	Skyros	**GREECE**
115	2016/fir-pine/01	fir-pine		**GREECE**
116	2016/fir-pine/02	fir-pine		**GREECE**
117	2016/fir-pine/03	fir-pine		**GREECE**
118	2016/fir-pine/04	fir-pine		**GREECE**
119	2016/fir-pine/05	fir-pine		**GREECE**
120	2016/fir-pine/06	fir-pine		**GREECE**
121	2016/fir-pine/07	fir-pine		**GREECE**
122	2016/fir-pine/08	fir-pine		**GREECE**
123	2016/fir-pine/09	fir-pine		**GREECE**
124	2016/pine-blossom/01	pine-blossom		**GREECE**
125	2016/pine-blossom/02	pine-blossom		**GREECE**
126	2016/pine-blossom/03	pine-blossom		**GREECE**
127	2016/pine-blossom/04	pine-blossom		**GREECE**
128	2016/pine-blossom/05	pine-blossom		**GREECE**
129	2016/pine-blossom/06	pine-blossom		**GREECE**
130	2016/pine-blossom/07	pine-blossom		**GREECE**
131	2016/pine-blossom/08	pine-blossom		**GREECE**
132	2016/pine-blossom/09	pine-blossom		**GREECE**
133	2016/pine-blossom/10	pine-blossom		**GREECE**
134	2016/pine-blossom/11	pine-blossom		**GREECE**
135	2016/pine-blossom/12	pine-blossom		**GREECE**

4.3 Sample preparation

1,0 g of homogenized honey was diluted with 5 mL of acidified water (pH<2) with the addition of 2% sodium chloride. After being vortexed for 1 min, the diluted honey was extracted 3 times with 5 mL ethyl acetate (EtAc), respectively. Between each extraction the samples were centrifuged in order to achieve a better separation between the two phases. The combined organic phases were dried with sodium sulphate. Extracts were evaporated under a gentle nitrogen stream near to dryness and then reconstituted to 0.2 mL with a final proportion of MeOH: H2O (50:50). Finally, the extracts were filtered through a 0.2 µm RC syringe filter and were ready for injection in the RP chromatographic system.

4.4 UHPLC-HRMS/MS system and analysis

The analysis of honey samples was carried out using an UHPLC-QToF-MS system composed of:

- An UHPLC rapid separation pump system, Dionex UltiMate 3000 (Thermo Fisher Scientific)
- Autosampler
- QToF mass spectrometer, Maxis Impact (Bruker Daltonics)

Mass spectra acquisition and data analysis was processed with Data Analysis 4.4 and TASQ 1.4 (Bruker Daltonics, Bremen, Germany). The QToF-MS system is equipped with an ESI source, operating in negative ionization mode. The chromatographic separation was performed on a reversed-phase (RP) chromatographic system.

In RP mode, an Acclaim RSLC C18 column (2.1 × 100 mm, 2.2 µm) from Thermo Fisher Scientific, connected to an ACQUITY UPLC BEH C18 1.7 µm, VanGuard Pre-Column from Waters, and thermostated at 30 °C, was used.

Figure 11. The UHPLC-QToF-MS system

For negative ionization mode, the aqueous phase consisted of H_2O: MeOH 90:10 with 5 mM ammonium acetate and the organic phase comprised of MeOH with 5 mM ammonium acetate.

The elution gradient program started with 1% of organic phase (flow rate 0.2 mL min^{-1}) for one minute, increasing to 39 % by 3 min (flow rate 0.2 mL min^{-1}), and then to 99.9 % (flow rate 0.4 mL min^{-1}) in the following 11 min. These almost pure organic conditions were kept constant for 2 min (flow rate 0.48 mL min^{-1}) and then initial conditions were restored within 0.1 min, kept for 3 min and then the flow rate decreased to 0.2 mL min^{-1} for the last minute. The injection volume was set to 5 µL.

When RP chromatographic system was used, the operation parameters of ESI were the following: capillary voltage, 2500 V for positive and 3000 V for negative mode; end plate offset, 500 V; nebulizer pressure N_2); drying gas, 8 L min−1 (N_2); and drying temperature, 200 °C

All the samples were first analyzed in full scan mode. The QTOF-MS system was operating in broadband collision-induced dissociation (bbCID) acquisition mode and recorded spectra over the range m/z 50−1000 with a scan rate of 2 Hz. The Bruker bbCID mode provides MS and MS/MS spectra at the same time working at two different collision energies; at low collision energy (4 eV), MS spectra were acquired, where all of the ions

from the preselected mass range are heading towards the flight tube without isolation at the quadrupole and there is no collision-induced dissociation at the collision cell. At high collision energy (25 eV), isolation is taking place at the quadrupole, and the ions from the preselected mass range are fragmented at the collision cell.

For certain masses of interest, a second analysis including the list of the selected precursor ions was performed in AutoMS (data dependent acquisition) mode. The instrument provided a typical resolving power (full width at half maximum) between 36,000 and 40,000 at m/z 226.1593, 430.9137, and 702.8636.

A QTOF-MS external calibration was daily performed with a sodium formate solution, and a segment (0.1−0.25 min) in every chromatogram was used for internal calibration, using a calibrant injection at the beginning of each run. The sodium formate calibration mixture consists of 10 mM sodium formate in a mixture of water/isopropanol (1:1). The theoretical exact masses of calibration ions with formulas $HCOO(NaCOOH)_{1\text{-}14}$ in the range of 50−1000 Da were used for calibration.

CHAPTER 5: Results and discussion

5.1 Enlargement of natural products database

Incorporation of new compounds in an already existed database of natural products was carried out. Particularly, chrysin, genistein, galangin, hesperitin, pinobanksin, rosmarinic acid, pinocembrin and naringenin, which have been already referred in literature to exist in honey, were added. For this reason, standards of each compound were injected to ultra-high pressure liquid chromatography coupled to mass spectrometer (UHPLC-MS) equipped with an electrospray ionization source (ESI) in negative mode. This technique is very useful to produce mainly pseudomolecular ions, $[M-H]^-$ using electrospray in which high voltage is applied to liquid in order to create an aerosol. It is a soft ionization source which achieves very little fragmentation so the pseudomolecular ions are usually observed. The analysis of standards was performed using data dependent acquisition mode using an inclusion list, which contains the pseudomolecular ion masses of the analytes, in order to acquire an MSMS spectra for each compound. Processing of the acquired data was carried out by the software data analysis (4.4) using a customized automation script that involves the following steps. The following paragraphs demonstrate a characteristic example of galangin to elucidate the following procedure.

- **Internal calibration**

  The internal calibration of mass spectrum was performed with a calibration solution. It consists of clusters of ammonium formate and elutes always in the time interval of 0.1-0.25 min in every chromatogram. The calibration depends on the difference between the theoretical and experimental measured masses of the calibrant. In addition, HPC (high precision calibration) is the algorithm which is preferred mostly because it can reduce effectively the mass error of each chromatogram.

  The base peak chromatogram (BPC), which corresponds to the ion with the higher intensity every time, of a sample was depicted below. The chromatographic peak of the calibrant as well as its corresponding MS spectrum were shown in the next 2 figures. The mass spectrum with the experimental measured m/z is utilized to internally calibrate each injection in order to reduce the mass error.

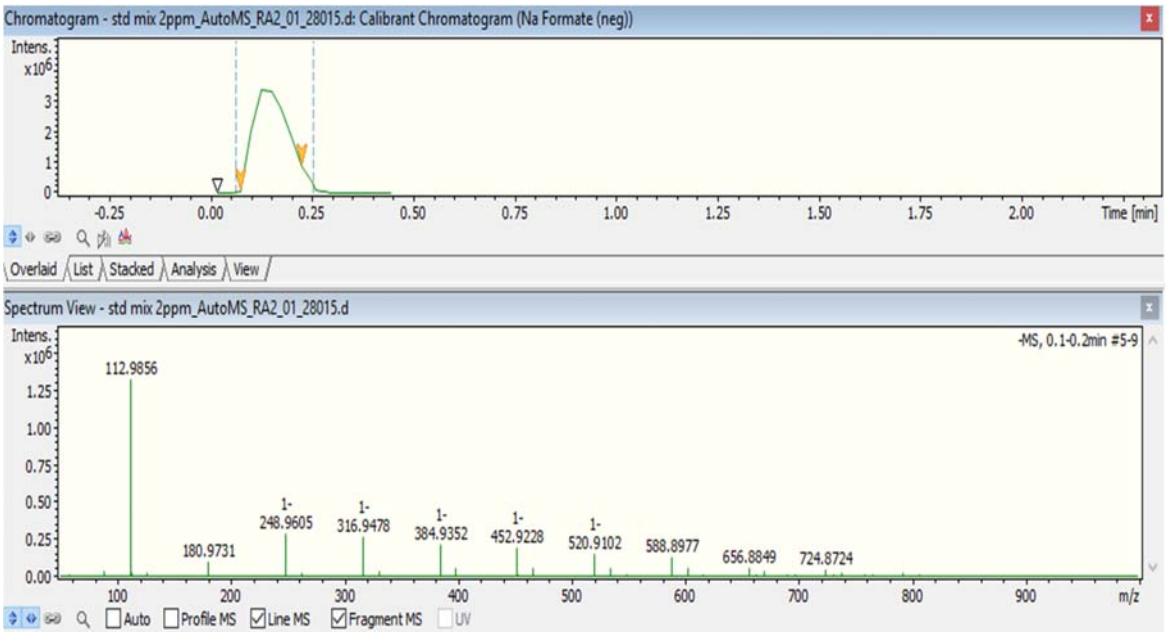

Figure 12. Base peak chromatogram and mass spectrum before calibration

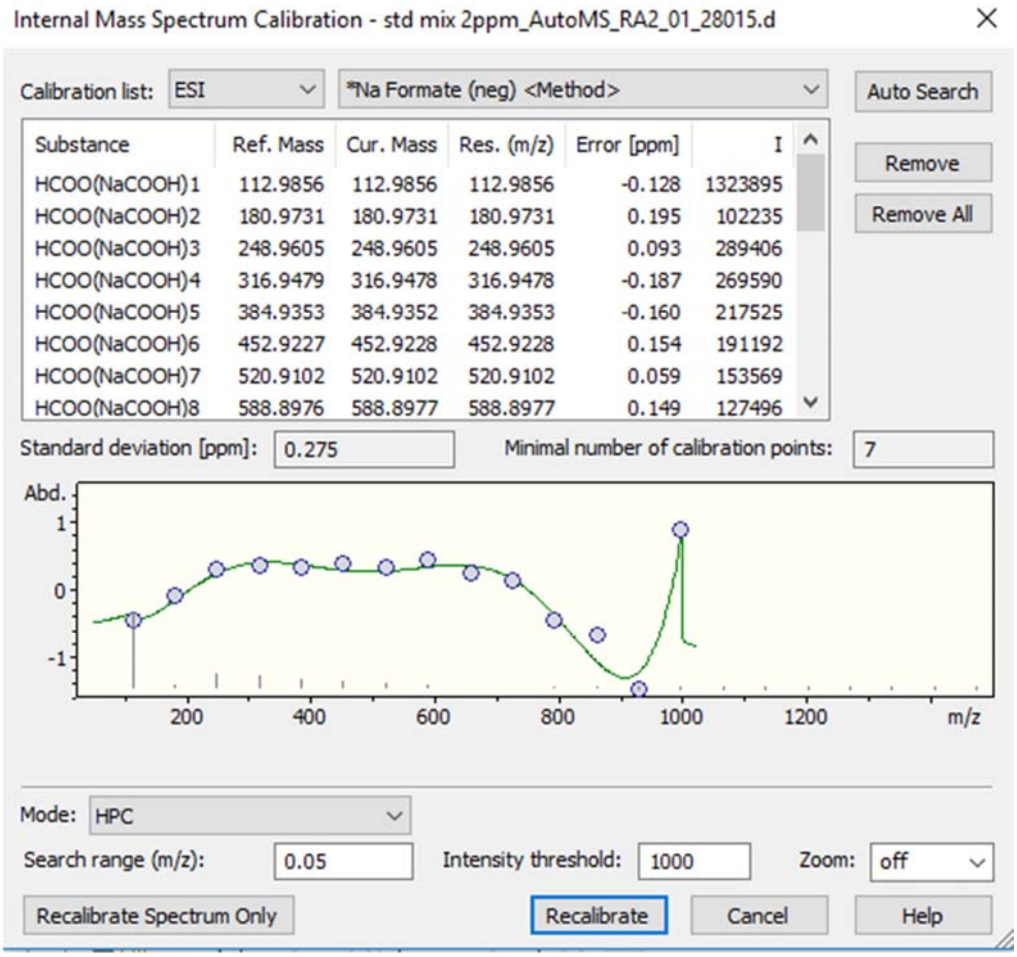

Figure 13. Internal calibration of mass spectrum.

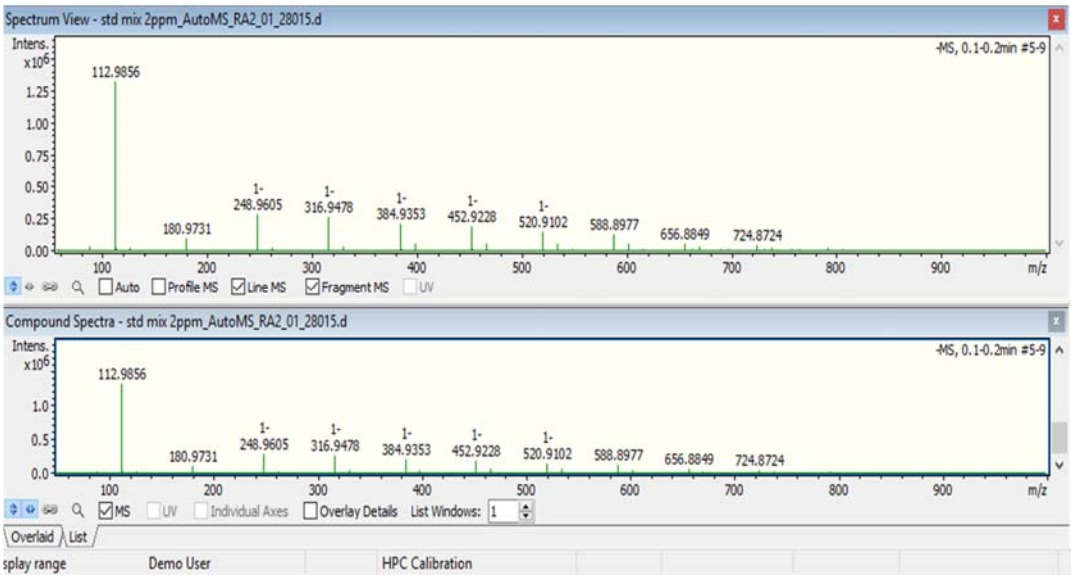

Figure 14. The mass spectrum of calibrant after calibration

- **Mass spectrum**

In the following figure the extracted ion chromatogram of the pseudomolecular ion of galangin is presented. As it is depicted three peaks are obtained if we extract this specific ion. This happen due to the fact that this pseudomolecular ion corresponds to three different compounds in our standard mix solution which are genistein, apigenin and galangin. Given that we already know the retention time of apigenin which have been incorporated in our database in previous work, we should identify which of the other two compounds correspond to which peak. Thus, we run the standards of galangin and genistein independently in order to identify them and we end up that the first peak is genistein while the third is galangin.

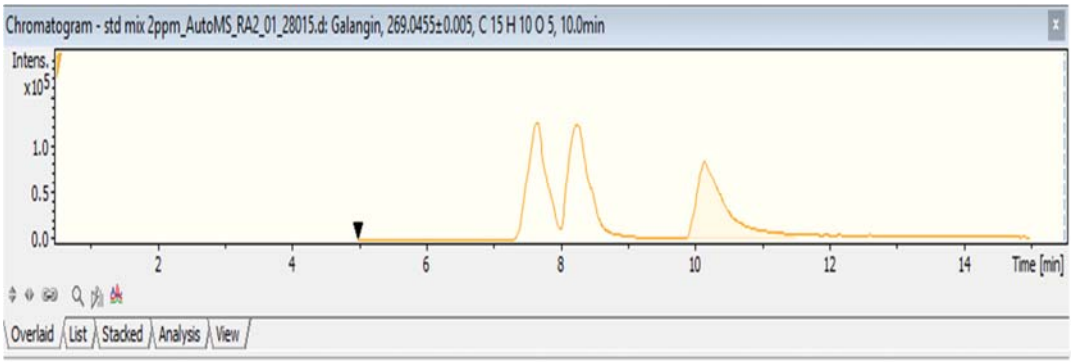

Figure 15. Extracted ion chromatogram of 269.0455

Subsequently, we thouroughly examined MS spectra in the time interval of 10.0-10.5 where galangin elutes in order to confirm that this peak corresponds to this compounds. Firstly, we should check the mass accuracy of the pseudomolecular ion as well as the isotopic fitting. Using Compass Isotope Pattern software, we can compare the theoretical isotope pattern of a formula with the experimental one in order to be sure that the correct elemental composition has been chosen. Then, the presence of other plausible ions which could be considered as in source fragments was investigated so as to be used as qualifier ions. In the pictures below is depicted the pseudomolecular ion with m/z 269.0455 as well as other abundant ions and the theoretical isotopic pattern of the proposed formula.

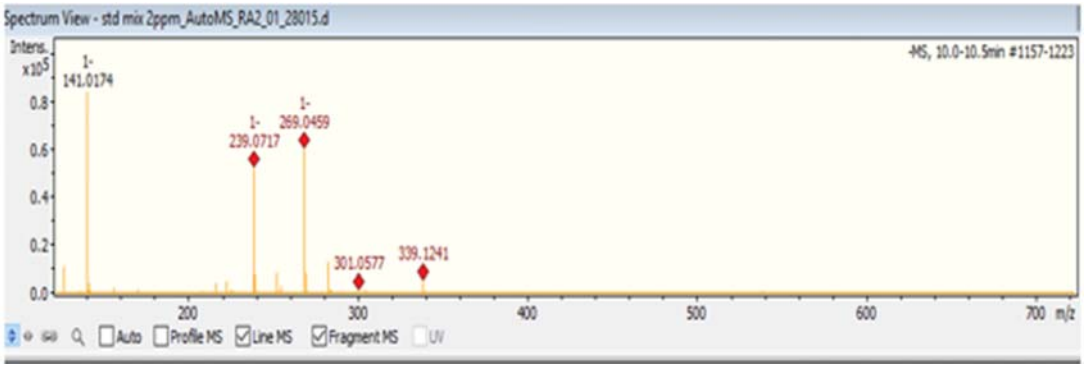

Figure 16. The MS spectra of galangin

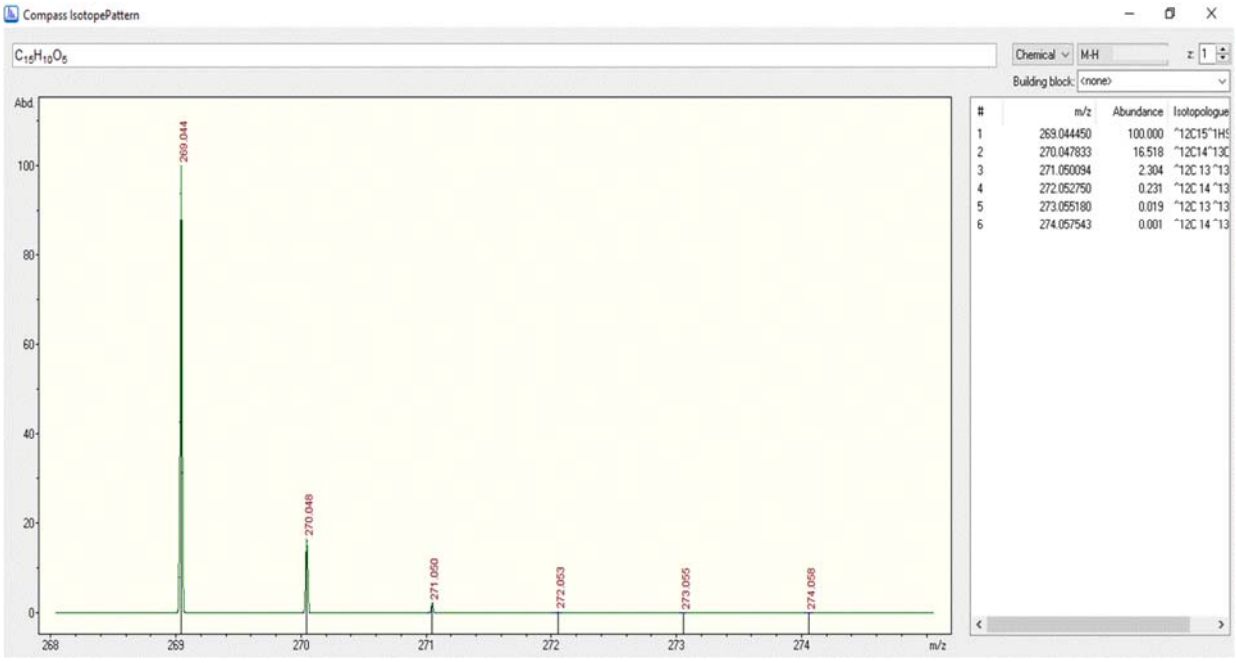

Figure 17. Isotope pattern

Finally, we inspected the MSMS spectrum of 269.0455 to find the most abundant fragments which could be used as qualifier ions for the identification of this compounds in the samples. As it is observed in the picture below the most abundant m/zs are 213.0548, 169.0657 and 197.0612.

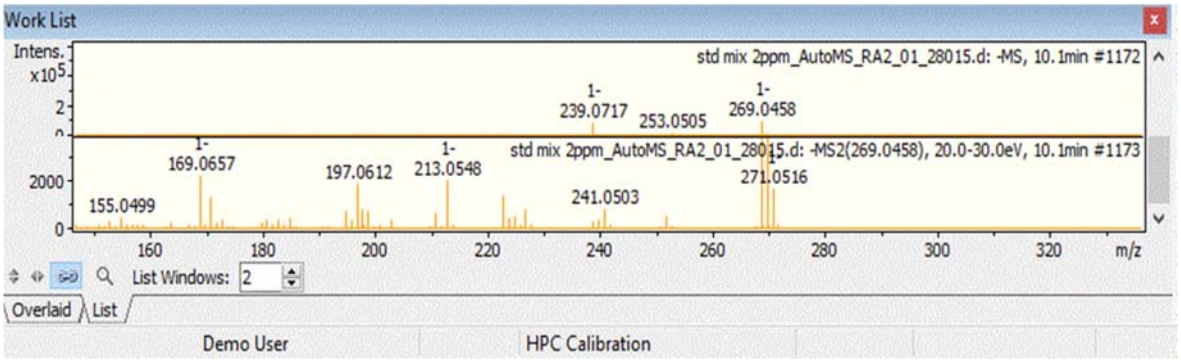

Figure 18. MS and MSMS spectrum of galangin

In conclusion, for the clarification of the molecular formula of these fragment ions "SmartFormula Manually" from Bruker was used. So possible formulas were attributed to ions according to user-definable settings such as the mass tolerance. For example, in our case the ion formula for the m/z 213.0568 was $C_{13}H_9O_3$.

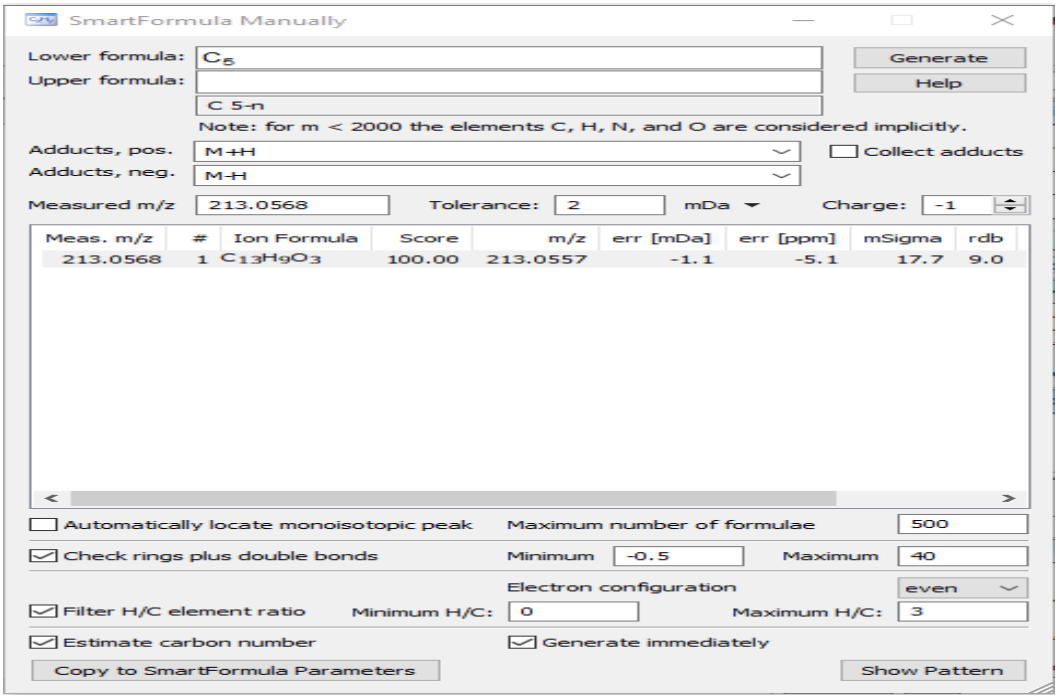

Figure 20. Assignment of possible elemental composition using"SmartFormula Manually" for one of the fragments

Following the same procedure for the rest of the standards, we managed to identify them so these compounds were added to our database. It has to be noticed that all of the antioxidant compounds which are included in this database have be found to exist in honeys according to literature.

Finally, a comma separated text file (csv) was created containing the following columns: compound name, retention time, molecular formula and m/z of the precursor ion of each compound of interest, the m/zs of the fragment ions as well as the exact molecular formula of each fragment ion.

5.2 Selection of the internal standards

Internal standards is used to improve the accuracy of quantitative analysis. Thus, we utilized internal standard to increase the quantitation confidence. We used 4 compounds as internal standards which are available in our lab. Three of them namely syringaldehyde, 8 prenylnaringenin and 2', 4'-Dihydroxychalcone are natural products occurring in other matrices like beer or plants while ethyl vanillin are synthetically produced. The proper internal standard for each analyte was selected comparing specific method performance criteria such as correlation coefficient, repeatability and recoveries. Three spiked samples containing all the analytes and internal standards were analysed the same day for the assessment of the repeatability. So the improvement of repeatability (RSDr %) has used as a criterion for the selection of the appropriate internal standard for each of the target compound. Furthermore, other indicator for the selection of the appropriate internal standard was the improvement of the correlation coefficient of the standard addition calibration curve using a sample spiked at 4 different levels. Finally, the last criterion was the improvement of analyte recovery using either the relative area of each target compound instead or the absolute analyte peak area. In the figures and tables below, it is presented the example of chysin and the selection of the appropriate internal standard for this flavonoid. In more detail, the internal standard 8 prenylnaringenin improved not only the correlation coefficient of the standard addition calibration curve but also the method repeatability and the analyte recovery.

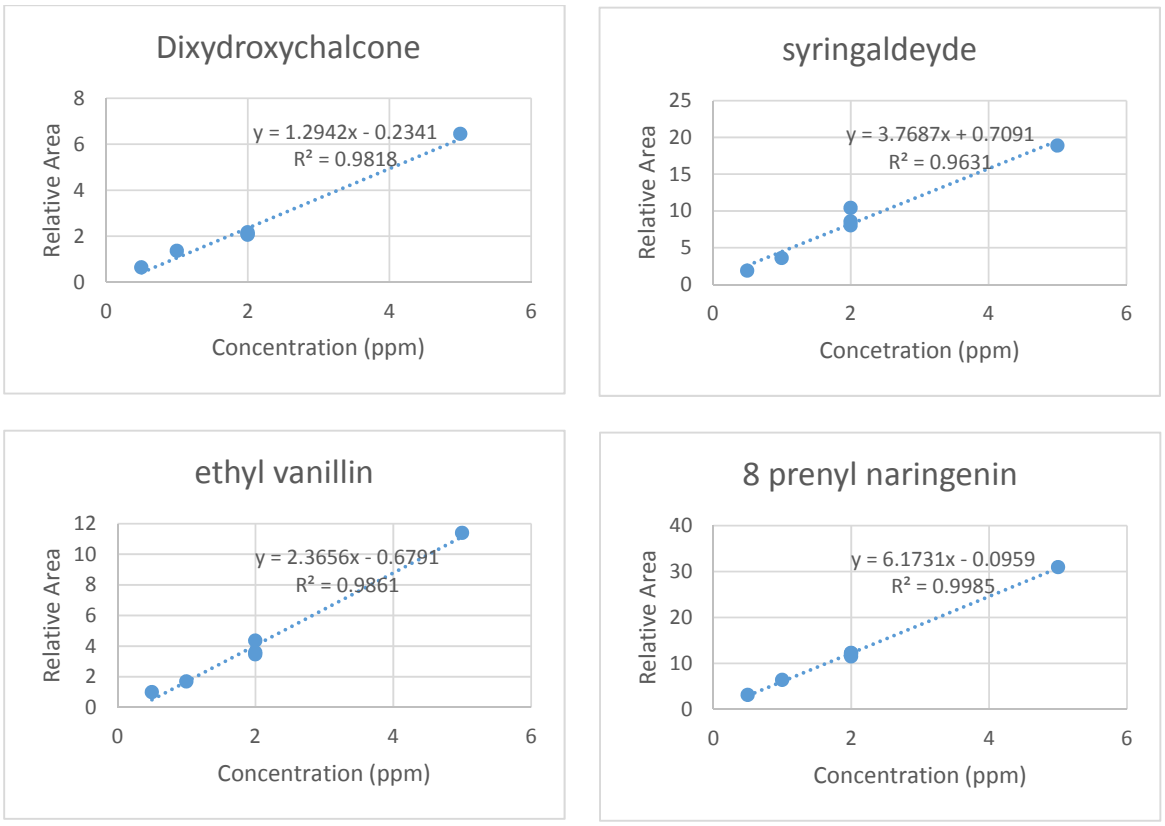

Figure 21. The selection of internal standards for chrysin based on the calibration curves.

Table 4. The recoveries of chyrin without any internal standard or using the appropriate internal standard (8 prenylnaringenin).

Thyme	Area	sample	spike	standard	R%
	Abs.Area	122396	2542941	3743113	65
	Rel.Area	1.190	12.551	12.858	88
Oak	Area	sample	spike	standard	R%
	Abs.Area	2885593	6815269	3743113	105
	Rel.Area	32.231	44.562	12.858	96
Pine	Area	sample	spike	standard	R%
	Abs.Area	2668510	3294056	3743113	17
	Rel.Area	19.646	29.938	12.858	80
Blossom	Area	sample	spike	standard	R%
	Abs.Area	1003809	4871483	3743113	103
	Rel.Area	83.938	96.234	12.858	96
Fir	Area	sample	spike	standard	R%
	Abs.Area	518410	1185412	3068672	22
	Rel.Area	16.568	22.707	6.635	93

Table 5. The selection of internal standard for chrysin based on method repeatability.

Method repeatability (Day 1)				
spiked sample	Dixydroxychalcone	8 prenyl naringenin	ethyl vanillin	syringaldeyde
thyme 08 spike 2 ppm(1i)	2.404	12.753	3.948	8.996
thyme 08 spike 2 ppm(1ii)	2.294	13.516	4.076	9.544
thyme 08 spike 2 ppm(1ii)	2.328	13.440	4.846	11.385
RSD%	2.4	3.2	11	13
Method repeatability (Day 2)				
thyme 08 spike 2 ppm(2i)	2.808	15.620	3.881	9.183
thyme 08 spike 2 ppm(2ii)	2.757	14.330	4.666	10.224
thyme 08 spike 2 ppm(2iii)	2.564	14.365	4.353	11.005
RSD%	4.8	5.0	9.2	9.0

5.3 Validation of the method

A developed and validated method from a previous master thesis was already existed, demonstrating the feasibility and the practicability of the present approach for the determination of phenolic compounds. Nevertheless, as a part of my master thesis, revalidation of this method was performed for learning purposes and comparing the current results with the previous one. The performance of the method was fully evaluated in terms of linearity, limits of detection (LODs) and limits of quantification (LOQs), precision and trueness and matrix effects. In detail, five honey samples from different botanical origins (fir, pine, blossom, oak and thyme) were fortified with a mixture of the analytes at the concentration level of 2 mg/kg. This mixture was composed by all analytes existed in the database. Also, a standard addition calibration curve for a certain thyme honey was constructed at 4 levels (0.5, 1, 2 and 5 mg/kg) from which the concentration level of 2 mg/kg was used for the assessment of the precision of the method. Together with control samples, the fortified samples were processed using the extraction procedure described above. Aliquots of the 5 samples extracts from these different botanical origins were used to prepare matrix matched calibration standards at the concentration level of 2 mg/L. The same calibration standards were also prepared in methanol: water (50:50). The results of validation are discussed in the following pages. Recoveries and ME% values at the level of 2 mg Kg^{-1} are presented in tables 9 and 10.

5.3.1 Linearity

According to ICH, the linearity is the ability of the analytical procedure to obtain test results that are directly proportional to the concentration of analyte in the sample. It was

determined from external calibration curves made by diluting standard solutions at 5 equally spaced concentration levels for each of the target analyte. These calibration curves were constructed automatically by the software TASQ 1.4 by Bruker. In almost all cases our data fitted very well in the linear model with correlation coefficients higher than 0.99 except for syringic acid which had correlation coefficient about 0.98. The calibration curve of p-coumaric acid is indicatively presented in figure 22.

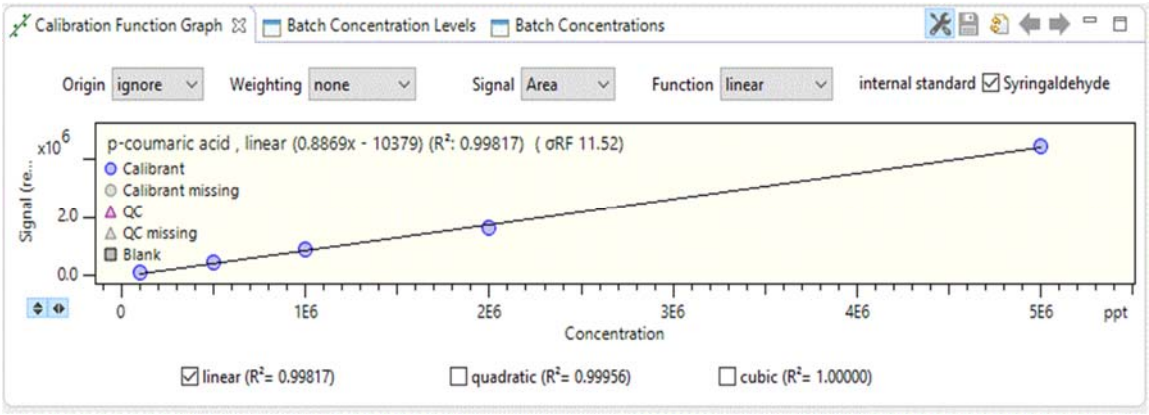

Figure 22. Calibration curve of p-coumaric acid

The results of the assessment of instrumental linearity are summarized briefly in the following table. The lowest concentration of 0.1 mg/L was excluded from the calibration curves of some target analytes (such as apigenin, hesperitin, eriodictyol, genistein, naringenin, pinobanksin, quercetin and vanillic acid) because it was not detectable.

The method linearity was assessed by spiking a sample with the analytes and internal standards in 4 concentration levels (0.5, 1, 2 and 5 mg/kg) as it has already mentioned in section 5.2. We utilized the proper internal standard for each analyte to compensate for extraction losses and matrix effects as thourhougly described in the previously mentioned section. The results of the assessment of method linearity are summarized briefly in the following table.

Table 6. The results of linearity for all the target analytes.

Analytes	Equation	R squared values
2,5 dihydroxybeznoic acid	y=0.49x+22643	0.998
3,4 dihydroxybenzoic acid	y=1.41x+343497	0.990
4 hydroxybezoic acid	y=0.71x+107235	0.998
apigenin	y=1.15x+696125	0.996
caffeic	y=3.07x+1109682	0.992
chrysin	y=7.42x-523614	0.998
cinnamic acid	y=0.86x-34340	0.998
eriodictyol	y=0.02x-2315	0.996
ferulic acid	y=0.79x-161544	0.991
galangin	y=2.06x-492638	0.994
gallic acid	y=1.18x+569726	0.992
genistein	y=2.75x+3280164	0.990
hesperitin	y=1.49x+912314	0.995
luteolin	y=2.00x-10965	0.998
naringenin	y=6.69x+5520278	0.995
p coumaric acid	y=0.89x-10379	0.998
pinobanksin	y=6.76x+5576570	0.996
pinocembrin	y=7.27x-364537	0.997
quercetin	y=0.48x+8817	0.998
rosmarinic acid	y=0.10x-28997	0.98
salicylic acid	y=2.21x+901021	0.995
syringic acid	y=0.54x-156192	0.98
taxifolin	y=1.38x-28664	0.997
vanillic acid	y=0.11x-21546	0.997
vanillin	y=1.53x+78229	0.998

Table 7. The results of method linearity for all the target analytes.

Analytes	Equation	R squared values
2,5 dihydroxybeznoic acid	y=0.37x-0.1362	0.993
3,4 dihydroxybenzoic acid	y=0.30x+0.717	0.990
4 hydroxybezoic acid	y=0.57x+0.0451	0.98
apigenin	y=1.50x-0.6463	0.990
caffeic	y=1.31x-0.0886	0.994
chrysin	y=6.17x-0.0959	0.9990
cinnamic acid	y=0.40x-0.0537	0.997
eriodictyol	y=0.08x-0.0434	0.997
ferulic acid	y=0.33x-0.0787	0.9990
galangin	y=0.89x-0.4653	0.993
gallic acid	y=0.27x+0.0092	0.994
genistein	y=3.26x-1.6986	0.991
hesperitin	y=0.94x+0.1616	0.995
luteolin	y=0.84x-0.5447	0.991
naringenin	y=6.79x+0.8383	0.997
p coumaric acid	y=0.61x-0.2207	0.96
pinobanksin	y=6.95x+0.6095	0.996
pinocembrin	y=9.33x-0.0603	0.992
quercetin	y=0.52x-0.4137	0.993
rosmarinic acid	y=0.09x-0.0298	0.990
salicylic acid	y=2.81x-3.015	0.86
syringic acid	y=0.55x-0.0553	0.991
taxifolin	y=1.14x-1.077	0.990
vanillic acid	y=0.14x+ 0.0132	0.98
vanillin	y=0.94x-0.1622	0.998

5.3.2 LODs and LOQs

LOD is considered to be the lowest concentration of analyte that is not necessarily quantifiable but is distinguishable from zero (signal /noise ratio≥ 3) whereas LOQ is the lowest concentration at which an acceptable precision could be achieved (signal /noise ratio≥10)[80].

In our case, LOQs and LODs were calculated from the standard addition calibration curves based on the following equations,

$$LOD = \frac{SDintercept}{slope}\ x\ 3.3 \qquad (1)$$

$$LOQ = \frac{SDintercept}{slope}\ x\ 10 \qquad (2)$$

Table 8. The LODs and LOQs for the target analytes

Analytes	LOD	LOQ
2,5 dihydroxybeznoic acid	0.070	0.21
3,4 dihydroxybenzoic acid	0.083	0.25
4 hydroxybezoic acid	0.098	0.23
apigenin	0.082	0.24
caffeic	0.065	0.19
chrysin	0.032	0.097
cinnamic acid	0.043	0.13
eriodictyol	0.048	0.14
ferulic acid	0.030	0.091
galangin	0.070	0.21
gallic acid	0.067	0.20
genistein	0.081	0.24
hesperitin	0.058	0.17
luteolin	0.079	0.24
naringenin	0.050	0.15
p coumaric acid	0.16	0.49
pinobanksin	0.055	0.16
pinocembrin	0.076	0.23
quercetin	0.067	0.20
rosmarinic acid	0.084	0.25
salicylic acid	0.33	0.99
syringic acid	0.081	0.24
taxifolin	0.084	0.25
vanillic acid	0.12	0.36
vanillin	0.037	0.11

LODs and LOQs were very good and the method is proved to be suitable for the detection of phenolic compounds in low concentrations. The values for LODs ranged between 0.030 mg /kg to 0.33 mg kg^{-1}, while for LOQs ranged between 0.091 to 0.99 mg Kg^{-1} respectively.

5.3.2 Precision – RSD_r %

Precision was attained from repeatability (intra-day precision) and reproducibility (inter-day precision). Regarding the first one, was obtained by analyzing three replicates of a

spiked sample in a certain concentration level (2 mg/kg) in the same day. However, reproducibility was tested over two different days by analyzing three replicates per day for a spiked sample in the same level. So a total of six replicates were performed. The obtained results for the repeatability (RSDr %) and the intermediate precison RSD_R % are demonstrated in the table below. Specifically, the vast majority of analytes showed RSD_r < 5% and RSD_R < 10% indicating the satisfactory precision which was achieved. For this reason the proposed method is acceptable and reliable for the determination of phenolic compounds [98].

Table 9. The repeatability and the intermediate precision of the method

Analytes	Precision (RSD%)	
	Repeatability	Intermediate precision
2,5 dihydroxybeznoic acid	5.0	7.5
3,4 dihydroxybenzoic acid	2.8	4.3
4 hydroxybezoic acid	3.3	8.2
apigenin	1.8	5.6
caffeic	6.2	8.0
chrysin	3.2	7.1
cinnamic acid	5.4	4.6
eriodictyol	3.4	9.6
ferulic acid	3.2	6.6
galangin	3.8	6.9
gallic acid	2.0	9.4
genistein	0.7	8.0
hesperitin	4.4	5.1
luteolin	11	8.7
naringenin	3.7	11
p - coumaric acid	1.5	8.0
pinobanksin	3.8	11
pinocembrin	7.0	7.9
quercetin	4.5	19
rosmarinic acid	4.5	9.7
salicylic acid	8.9	8.8
syringic acid	4.8	8.5
taxifolin	2.0	6.7
vanillic acid	3.8	4.4
vanillin	3.3	8.0

5.3.3 Matrix Effect % (ME %)

The measurement of matrix effect (ME) is an important issue as the selectivity of a proposed method is investigated. According to Truffeli et. al., Ion suppression or enhancement may be caused by sample matrix or interferences from metabolites. The mechanism and the origin of the matrix effect is not fully understood, but it may originates from the competition between an analyte and a co-eluting compound or undetected matrix components reacting with primary ions formed in the interface. Depending on the environment in which the ionization and ion evaporation processes take place, this competition may effectively decrease (ion suppression) or increase (ion enhancement) the efficiency of formation of the desired analyte ions present at the same concentrations in the interface. It is intuitively clear that the efficiency of formation of the desired ions is matrix-dependent due to the competition between the molecule of interest and a number of other undetected but co-eluting molecules present in the system that are capable of reacting with primary ions. This effect may reduce or increase the intensity of analyte ions and affect the reproducibility and accuracy of the assay [99].

To determine the presence of a matrix effect the following protocol was followed. Standard solution at one concentration level (2 mg/L) was injected in parallel with matrix standards (corresponding to aliquot of a sample from each one of the five different botanical origin classes fortified at the end of the sample preparation with the analytes) at the same level. The process is illustrated in figure 23 and the matrix effects was calculated using equation 3.

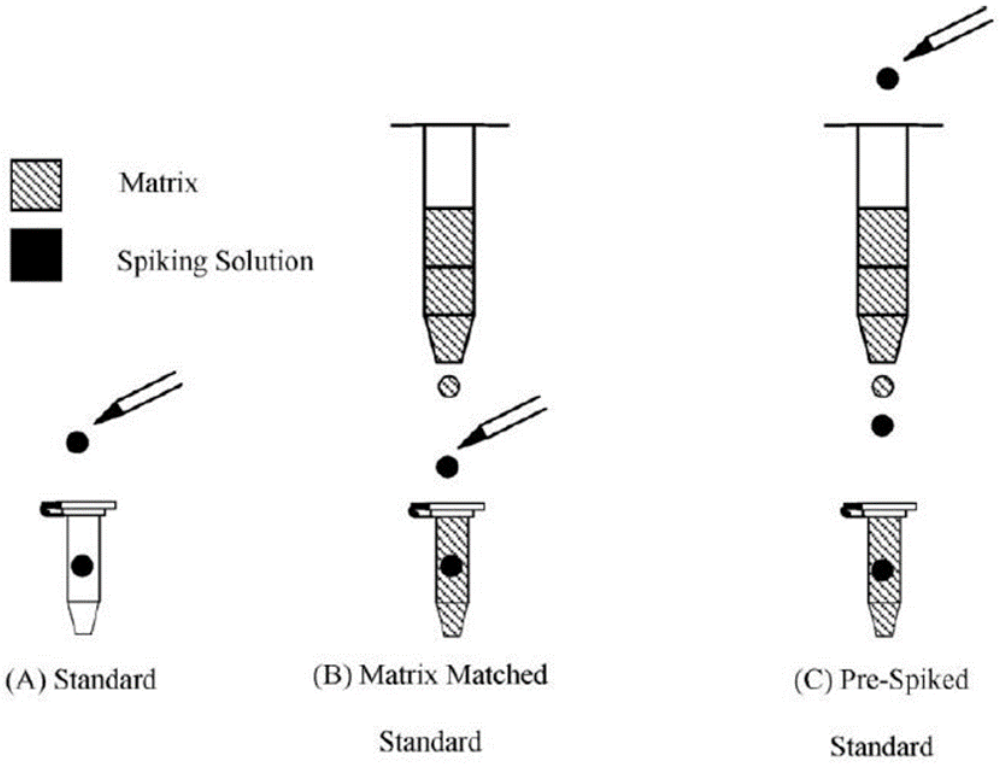

Figure 23. Preparation of solutions for the determination of recovery rate and matrix effect, source:[99]

In order to measure the ME, the matrix factor (MF) is necessary. MF and ME% are calculated based on the following equations

$$MF = \frac{Area\ matrix\ matched - Area\ sample}{Area\ standard} \quad (3)$$

$$ME\ \% = (1 - MF)\ x\ 100 \quad (4)$$

Ideally, the ME % is equal to zero meaning that neither ion suppression nor enhancement is taken place. It has to be noticed that positive values of ME% indicate ion suppression while negative enhancement. Thoroughly examine the ME% values of the method, we noticed that the signal of the most analytes was suppressed except from the signal of galangin which was slightly enhanced in all matrices (thyme, oak, pine and fir honey) as well as the signal of rosmarinic acid and quercetin which also enhanced but not in thyme honey. To sum up, the ME% values of the analytes in the different matrices ranged approximately between -20% to 30%.

Table 10. The matrix effect of target analytes in each of the 4 honey matrices (thyme, oak, pine, blossom, fir)

Analytes	Matrix effect (ME%)				
	Thyme 08	Oak 05	Pine 25	Blossom 19	Fir 01
2,5 dihydroxybeznoic acid	32	28	21	14	12
3,4 dihydroxybenzoic acid	32	20	22	31	33
4 hydroxybezoic acid	33	32	33	32	31
apigenin	31	9	9	4	1
caffeic	30	28	33	32	11
chrysin	17	26	33	28	17
cinnamic acid	28	5	12	-7	3
eriodictyol	11	-1	3	11	13
ferulic acid	34	24	25	6	9
galangin	-9	-6	-10	-7	-2
gallic acid	31	34	31	33	33
genistein	33	13	12	8	3
hesperitin	18	11	17	11	11
luteolin	30	-21	-9	2	-4
naringenin	25	33	32	32	14
p coumaric acid	31	33	29	6	1
pinobanksin	22	22	29	32	13
pinocembrin	8	11	22	19	-3
quercetin	6	-16	-12	-9	12
rosmarinic acid	12	-12	-9	-14	-7
salicylic acid	34	32	25	15	25
syringic acid	27	6	7	8	6
taxifolin	33	34	32	24	32
vanillic acid	30	19	18	18	10
vanillin	34	-1	10	-1	1

5.3.4 Trueness

Accuracy is one of the key parameter to be assessed for method validation and involves common systematic errors (bias). It is estimated through trueness and precision. Trueness is usually estimated using certified reference materials (CRM). In cases where this is not feasible, recovery of additions of known amounts of the analytes to a sample (blank matrix) can be utilized [100]. As already mentioned in the already validated method spiking experiments were conducted again at 4 spiking levels (0.5, 1, 2, 5 mg Kg^{-1}) for one botanical origin (thyme honey). Especially in one medium concentration level

(2mg/kg) 3 replicates were conducted. Trueness was assessed through the relative overall efficiency by the ratio of the relative area of the spiked sample substracting the blank to the relative area of the standard solution in this medium concentration level of 2 mg/kg. The recoveries were calculated using the equation $R\% = \frac{Area\frac{spike}{IS}}{Area\frac{MM}{IS}} x100$ (5). According to the following table, the majority of the analytes presented satisfactory recoveries ranging between 70% and 110% indicating the accurate determination of the method developed. Generally, mean recoveries of 70 -120% with relative standard deviations RSD <20% are acceptable when referring to validation experiments

Table 11. The recoveries of target analytes

Analytes	RECOVERIES (%)				
	Thyme 08	Oak 05	Pine 25	Blossom 19	Fir 01
2,5 dihydroxybeznoic acid	108	94	84	55	57
3,4 dihydroxybenzoic acid	109	74	71	83	70
4 hydroxybezoic acid	103	68	104	69	87
apigenin	76	94	59	44	46
caffeic	79	69	64	96	61
chrysin	88	96	80	96	93
cinnamic acid	85	101	71	101	73
eriodictyol	91	94	56	105	0
ferulic acid	74	98	79	96	72
galangin	90	102	56	60	45
gallic acid	66	66	62	47	45
genistein	76	104	102	93	102
hesperitin	100	101	89	86	100
luteolin	50	88	57	41	91
naringenin	106	85	68	59	99
p coumaric acid	82	65	83	108	62
pinobanksin	106	85	72	74	99
pinocembrin	108	106	102	66	84
quercetin	85	100	35	47	68
rosmarinic acid	107	94	82	72	61
salicylic acid	52	56	106	66	88
syringic acid	99	88	87	62	61
taxifolin	52	67	42	47	48
vanillic acid	95	78	101	86	101
vanillin	78	98	84	95	107

5.4 Application of target screening approach to honey samples

A total of 135 greek honey samples from 5 different botanical origins have been analyzed in order to measure their phenolic content. Quantitative analysis was performed in order to provide a comprehensive overview of the phenolic composition of honeys. The characterization of honey samples is extensively presented in section 4.2. In the next step, all samples were screened using the software TASQ CLIENT 1.4 from Bruker in order to confirm the presence or absence of the 25 target analytes. A TASQ method was created using the already mentioned database with target analytes. The screening of samples was based on some identification criteria such as retention time clossness, mass accuracy, isotopic fitting and qualifier ions presence. A score namely MRSQ that visualize the tolerance fit of all parameters can give a measure of the confidence of the identification based on the the quality of the retention time, m/z error, mSigma, and qualifier ion results. The parameters set for screening and scoring was

- RT Scoring – Narrow 0.2 min /Wide 0.4 min
- m/z Scoring – Narrow 2 mDa /Wide 5 mDa
- mSigma Scoring – Narrow 20 /Wide 50
- presence or absence of qualifier ions
- The ratio of S/N should be better than 3 and the intensity threshold better than 1000

Furthermore, the quantification of target analytes was performed using standard addition calibration curves. The concentration of each analyte was determined using the corresponding equation from the standard addition curve using relative areas, which has been corrected with the appropriate internal standard, instead of absolute. It has to be noticed that quantitave determination using absolute MS responses is difficult because they are subjected to significant day-to-day variation. So the internal standards are required to achieve reliable and accurate quantitave results controlling this variation and correcting the insufficiencies of the sample preparation or ion suppression phenomena.

In the following section it is demonstrated the procedure of the target screening of caffeic acid in the honey samples. The upper windows displays the identification points of caffeic acid which are useful to assess the presence or absence of this analyte to the samples. According to this, caffeic acid fulfills successfully the identification criteria exhibiting a good MRSQ score. In the second window we can see information about the pseudomolecular ion and the qualifier ions. Last but not least, the lower windows depict

the chromatogram of pseudomolecular and diagnostic ions and the isotopic pattern of caffeic acid in one sample (blossom 34)

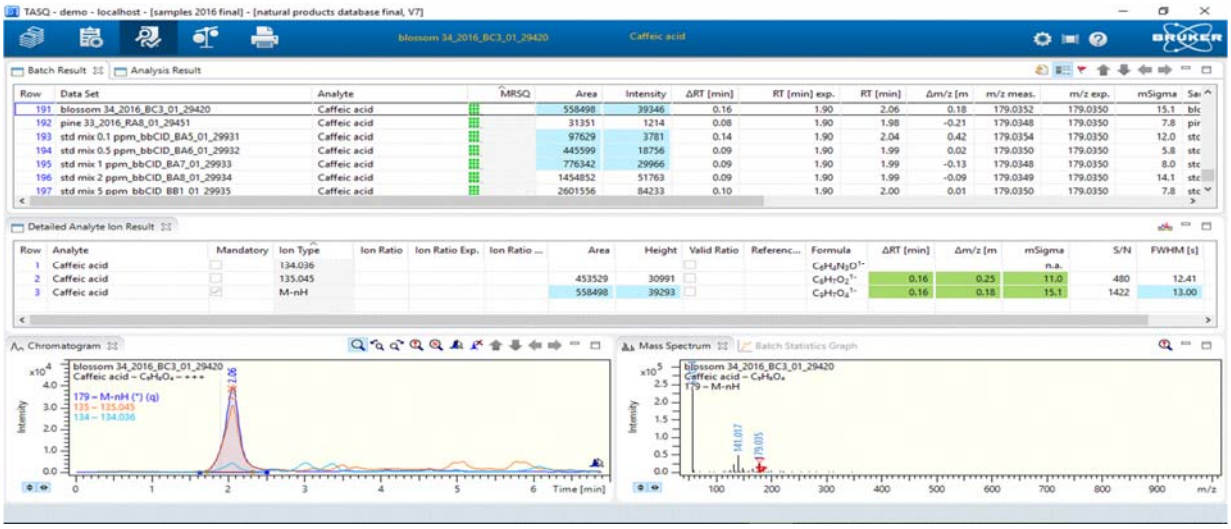

Figure 24. Target screening of samples for the determination of caffeic acid

Afterwards, a number of 25 analytes were determined in all samples namely 2,5 dihydroxybenzoic acid, 3,5 dihydroxybenzoic acid, 4 hydroxybenzoic acid, apigenin, caffeic acid, chrysin, cinnamic, eriodictyol, ferulic, galangin, gallic acid, genistein, hesperitin, luteolin, naringenin, p-coumaric acid, pinobanksin, pinoembrin, quercetin, rosmarinic acid, salycilic acid, syringic acid, taxifolin, vanillic acid and vanillin. The following table summarizes the average concentration of each compound along with its standard deviation in every different matrix. To begin with, according to the results obtained from the quantification, blossom honeys have shown a higher concentration of chrysin and pinocembrin comparing to the other unifloral honeys. The average concentrations of these compounds were about 3.6 ± 2.9 mg/kg and 2.1 ± 1.5 mg/kg respectively in blossom honeys whereas in other honey types these were determined in lower levels so they can be used as possible markers for blossom honeys. In addition, galangin and pinobanksin seems to differentiate blossom from thyme honeys in which these analytes have been found in trace quantities. However they cannot be used as possible markers for blossom honeys because they are existed also in satisfactory levels in other unifloral honeys. Moreover, thyme honey was the only unifloral honey with rosmarinic acid with average concentration 0.66 ± 1.12 mg/kg. It has to be stated that hesperitin has not been found in any measured honey. For this reason, 3 citrus honeys were analysed in order to confirm the presence of this analyte in these honey types. Many

studies have been already performed for the evaluation of the flavonoid hesperitin as possible marker for citrus honey. So, the results come to be added to the already existed literature data.

Table 12. The average concentration (mg/kg) of phenolic compounds in various honeys

Honey matrix	Blossom		Fir		Oak		Pine		Thyme		Fir-Pine		Pine-Blossom		Citrus		
	(N=35)		(N=10)		(N=15)		(N=36)		(N=18)		(N=9)		(N=12)		(N=3)		
Analytes	avg	SD	avg	SD	avg	SD	avg	SD	avg	SD	avg	SD	avg	SD	avg	SD	LOD
2,5 dihydroxybenzoic acid	0.50	0.28	1.4	1.3	0.97	0.30	1.2	0.63	0.28	0.17	0.74	0.36	0.64	0.23	0.13	0.03	0.070
3,4 dihydroxybenzoic acid	2.3	2.3	7.5	8.2	11	6.2	6.3	6.0	0.56	0.77	4.0	2.7	3.2	1.62	0.094	0.017	0.083
4 hydroxybenzoic acid	1.8	0.74	1.5	0.90	2.5	3.0	1.8	0.77	0.88	0.45	1.4	0.44	1.8	0.66	0.37	0.21	0.098
apigenin	0.19	0.075	0.12	0.046	0.13	0.022	0.13	0.034	0.28	0.21	0.13	0.043	0.24	0.12	0.11	0.02	0.082
caffeic acid	1.2	0.76	0.32	0.37	0.69	0.68	0.56	0.29	0.14	0.07	0.31	0.16	0.76	0.61	0.11	0.040	0.065
chrysin	3.6	2.9	0.55	0.48	0.78	0.32	0.95	0.78	0.15	0.32	0.69	0.52	2.9	2.7	0.25	0.14	0.032
cinnamic acid	0.21	0.13	0.17	0.078	0.21	0.12	0.16	0.14	<LOD	-	0.078	0.060	0.17	0.12	0.043	0.0010	0.043
eriodictyol	0.27	0.34	<LOD	-	<LOD	-	0.10	0.12	0.11	0.20	0.078	0.091	0.12	0.20	<LOD	-	0.048
ferulic acid	0.47	0.38	0.26	0.64	0.22	0.18	0.33	0.19	0.077	0.073	0.23	0.11	0.29	0.27	0.10	0.020	0.030
galangin	0.92	0.91	0.28	0.37	0.26	0.088	0.25	0.13	0.13	0.12	0.21	0.12	0.86	0.80	0.15	0.020	0.070
gallic acid	0.24	0.36	0.47	0.71	2.3	1.7	0.73	0.88	0.52	1.4	0.48	0.91	0.12	0.10	<LOD	-	0.067
genistein	0.083	0.0092	<LOD	-	<LOD	-	0.080	0.0050	0.15	0.13	<LOD	-	0.089	0.029	<LOD	-	0.081
hesperitin	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	0.068	0.010	0.058
luteolin	0.16	0.22	0.17	0.13	0.15	0.095	0.092	0.042	0.13	0.087	0.099	0.061	0.13	0.097	0.12	0.080	0.079
naringenin	1.9	1.1	0.56	1.0	1.2	1.5	0.65	0.29	0.12	0.075	0.45	0.29	1.2	0.79	0.43	0.43	0.050
p coumaric acid	1.3	0.68	0.73	0.53	1.6	1.9	1.1	0.67	0.44	0.18	0.65	0.18	1.1	0.34	0.28	0.080	0.16
pinobanksin	1.8	1.1	0.56	1.0	1.1	1.4	0.64	0.29	0.12	0.073	0.44	0.29	1.2	0.78	0.43	0.41	0.055
pinocembrin	2.1	1.5	0.22	0.20	0.45	0.22	0.52	0.36	0.10	0.056	0.35	0.32	1.4	1.3	0.17	0.080	0.076
quercetin	0.39	0.42	0.21	0.28	0.45	0.57	0.14	0.083	0.14	0.11	<LOD	-	0.45	0.42	0.23	0.040	0.067
rosmarinic acid	<LOD	-	<LOD	-	<LOD	-	<LOD	-	0.34	0.78	<LOD	-	<LOD	-	<LOD	-	0.084
salicylic acid	1.1	0.84	1.1	0.58	2.3	2.3	1.9	1.5	0.53	0.36	2.0	1.0	2.2	1.6	0.34	0.020	0.33
syringic acid	0.10	0.060	<LOD	-	0.15	0.13	0.11	0.076	0.09	0.021	0.11	0.081	<LOD	-	<LOD	-	0.081
taxifolin	0.15	0.11	0.19	0.12	0.21	0.18	0.45	0.27	0.36	0.30	0.40	0.20	0.61	0.32	0.22	0.020	0.084
vanillic acid	0.25	0.45	0.12	0.013	0.15	0.065	0.40	0.37	0.33	0.45	0.17	0.15	0.19	0.14	<LOD	-	0.12
vanillin	0.11	0.14	0.048	0.035	<LOD	-	0.071	0.046	0.11	0.092	0.088	0.052	0.078	0.065	0.040	0.0051	0.037

5.5 Statistical examination of data

ANOVA was developed by Sir Ronald A. Fisher and introduced in 1925. It is a test for the equality of means and aims to assess the differences between pairs of means. It is estimated as a powerful tool in comparison with the Student's t-test as it can be used for comparing the means of more than two groups.

In our case, analysis of variance was performed using the statistical software program Minitab (version 18). One way ANOVA was performed for all analytes in order to determine whether the means of the analytes differ among the five groups of samples (blossom, pine, thyme, oak and fir honeys).

Firstly, the characteristic example of chrysin is presented in the following figures in order to confirm the data about the use of this analyte as a possible marker in blossom honeys. The interval plot demonstrates that blossom shipping center has the highest mean concentration of chrysin (3.6 mg/kg) with a confidence interval (3.02 to 4.15). On the other hand, thyme shipping center has the lowest mean (0.15 mg/kg) of chrysin (-0.64 to 0.94). To determine statistical significance, an assessment of the confidence intervals for the differences of means is useful. The p value of chrysin is less than the significance level according to figure 25. So the null hypothesis is rejected and the conclusion was that some of the values have significant different means. In addition, according to the grouping information table, groups that are not share a letter are significant different. In our example the mean values of chrysin in blossom honeys significantly differ from all the other types of honey because blossom honeys do not share a letter with the other types of honey. In Tukey plot, if the confidence intervals do not contain zero, the corresponding means significantly differ. In our example the confidence intervals for the difference between the means of the following pair of groups (blossom- thyme, blossom-fir, blossom –oak, blossom-pine) do not include zero. Also, the table indicates that the individual confidence level is 99.34%. This result indicates that you can be 99.34% confident that each individual interval contains the true difference between a specific pair of means.

In order to determine whether the model meets the assumption of the analysis, we have to use the residual plot. According to the residual versus fit plot, the residuals are randomly distributed on both sides of 0 with no recognizable patterns in the points, while in the residual versus order the residuals are independent with no visual trends or patterns because they fall randomly around the center line. Last but not least, we are processing to check the normality to verify the assumption that the residuals are normally

distributed. If the sample size is greater than 15 or 20, the test performs very well with skewed and nonnormal distributions. If the sample size is less than 15 or 20, the results might be misleading with nonnormal distributions. In our case all groups have the adequate number of samples except for fir samples. Although, it is not important the normality plot for our case due to the fact that we have many samples in each group, the normality plot is given below.

One-way ANOVA: chrysin versus SAMPLE

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
SAMPLE	5	blossom, fir, oak, pine, thyme

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
SAMPLE	4	212.0	53.010	18.67	0.000
Error	109	309.4	2.839		
Total	113	521.5			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.68491	40.66%	38.48%	37.01%

Means

SAMPLE	N	Mean	StDev	95% CI
blossom	35	3.583	2.886	(3.019, 4.148)

fir	10	0.547	0.479	(-0.509, 1.603)
oak	15	0.7847	0.3210	(-0.0776, 1.6469)
pine	36	0.945	0.776	(0.389, 1.502)
thyme	18	0.1478	0.3209	(-0.6394, 0.9349)

Pooled StDev = 1.68491

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

SAMPLE	N	Mean	Grouping	
blossom	35	3.583	A	
pine	36	0.945		B
oak	15	0.7847		B
fir	10	0.547		B
thyme	18	0.1478		B

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
fir - blossom	-3.036	0.604	(-4.711, -1.362)	-5.03	0.000
oak - blossom	-2.799	0.520	(-4.240, -1.357)	-5.38	0.000
pine - blossom	-2.638	0.400	(-3.746, -1.529)	-6.60	0.000
thyme - blossom	-3.436	0.489	(-4.790, -2.081)	-7.03	0.000
oak - fir	0.238	0.688	(-1.669, 2.144)	0.35	0.997
pine - fir	0.399	0.602	(-1.271, 2.068)	0.66	0.964
thyme - fir	-0.399	0.665	(-2.241, 1.443)	-0.60	0.975
pine - oak	0.161	0.518	(-1.274, 1.596)	0.31	0.998
thyme - oak	-0.637	0.589	(-2.270, 0.996)	-1.08	0.816
thyme - pine	-0.798	0.486	(-2.146, 0.550)	-1.64	0.475

Individual confidence level = 99.34%

Figure 25. One way analysis of chrysin

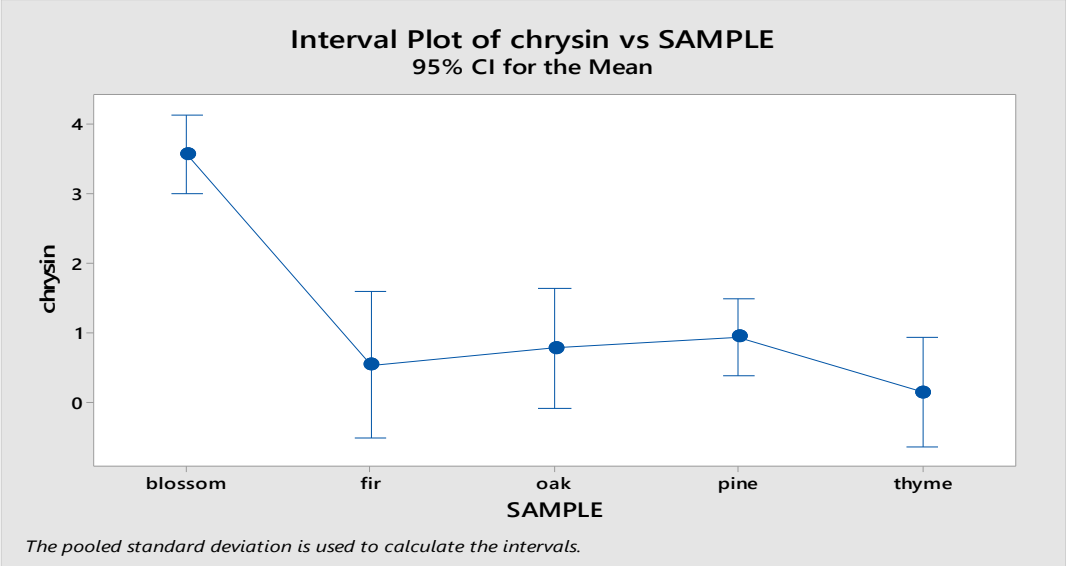

Figure 26. The interval plot of chrysin

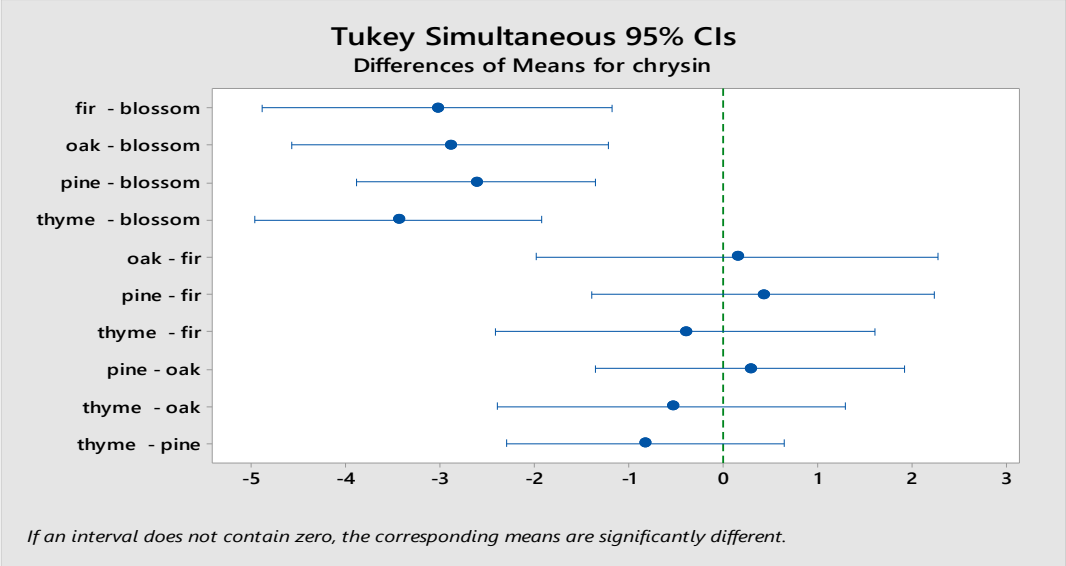

Figure 27. The Tukey plot of chrysin

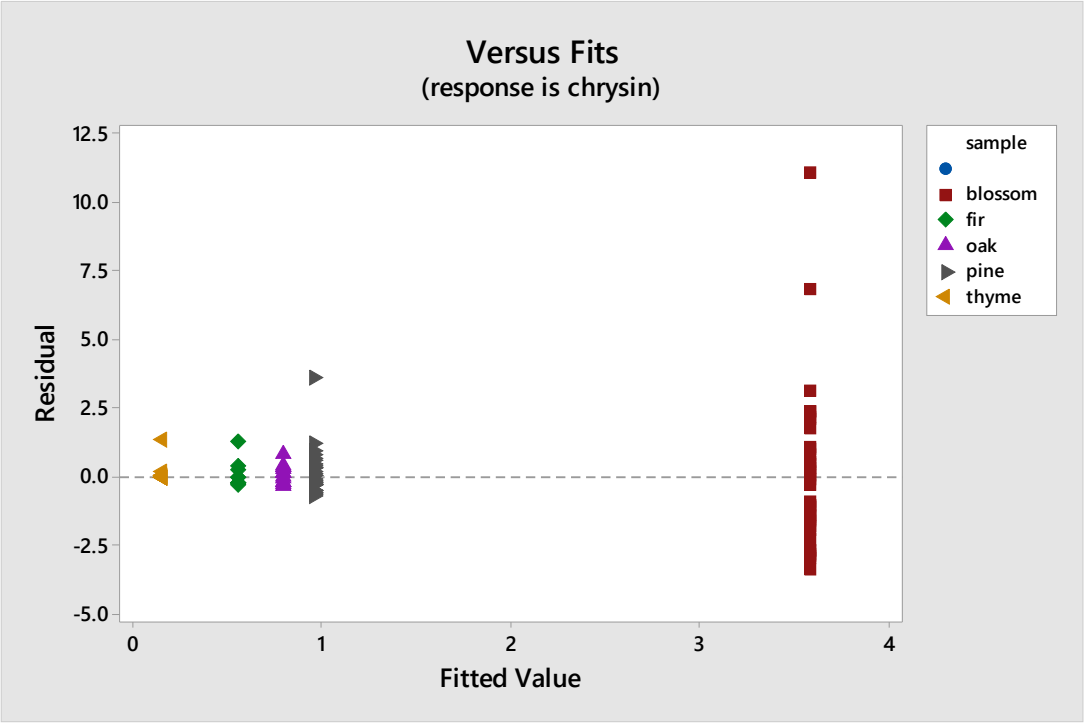

Figure 28. Versus fit plot

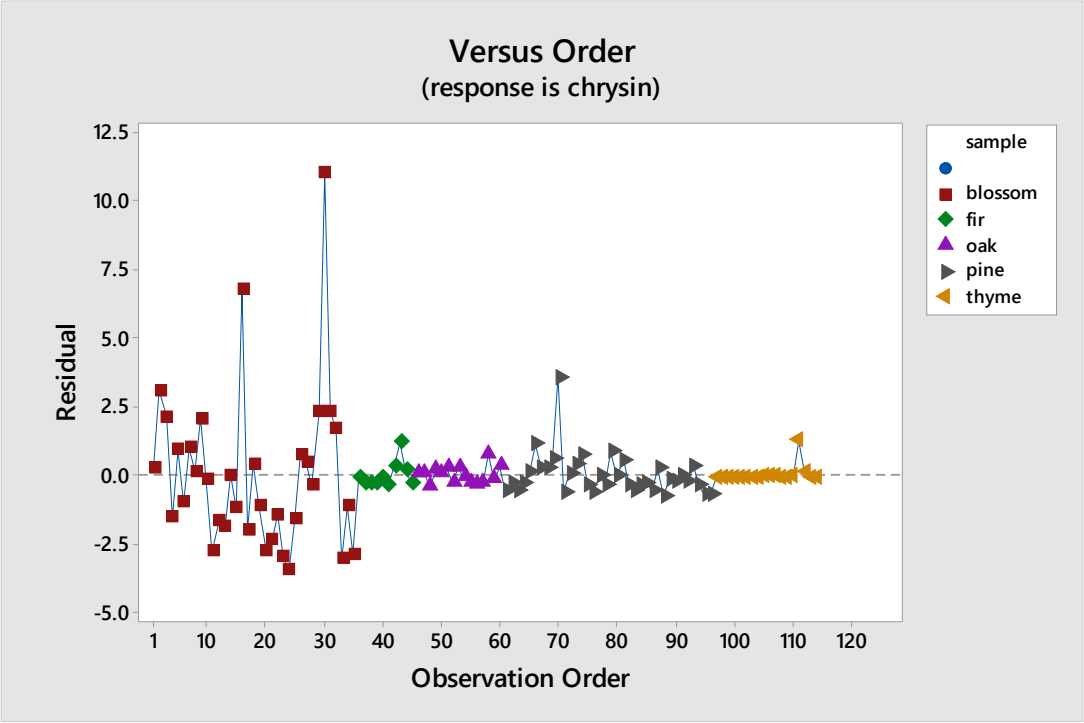

Figure 29. Versus order plot

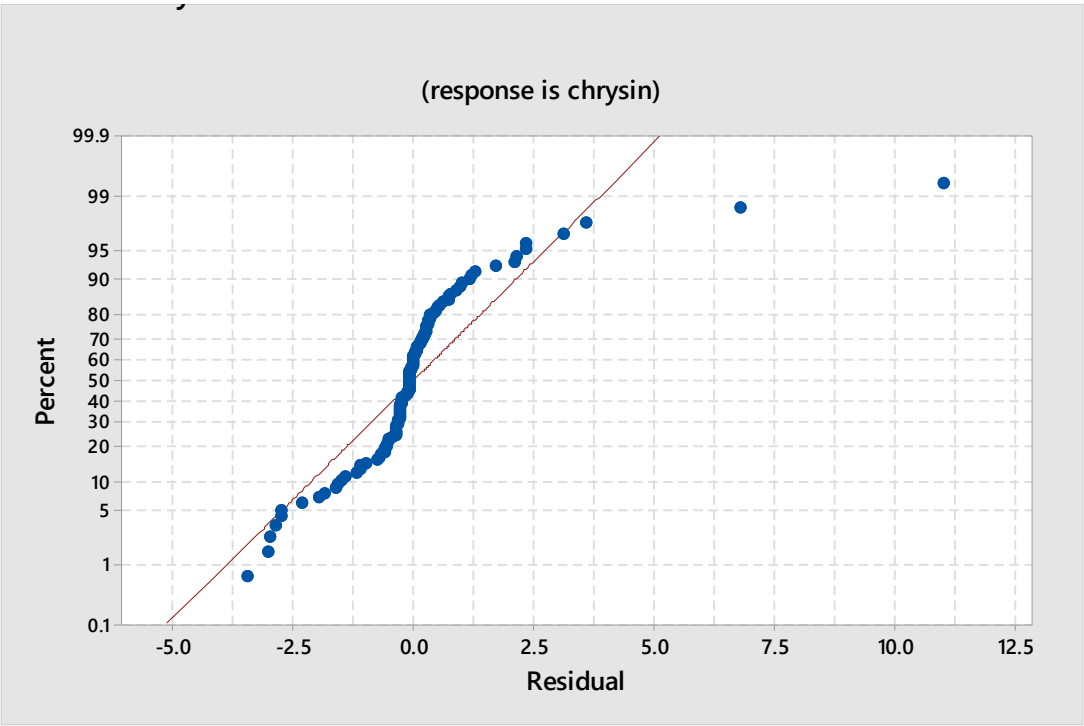

Figure 30. Normplot for residuals

Furthermore, ANOVA was performed in the same way for pinocembrin. This analyte has been found in higher concentration in blossom honeys contrary to other types of honey as already mentioned. The interval plot demonstrates that blossom shipping center has the highest mean concentration of pinocembrin (2.05 mg/kg) with a confidence interval (1.76 to 2.34). On the other hand, thyme shipping center has the lowest average concentration of pinocembrin (0.099 mg/kg) with a confidence interval (-0.30 to 0.5). In addition, the p value of pinocembrin is less than the significance level. So the null hypothesis is rejected as in case of chrysin. The grouping information table and the Tukey plot show that the mean values of pinocembrin in blossom honeys significantly differ from the other unifloral honeys.

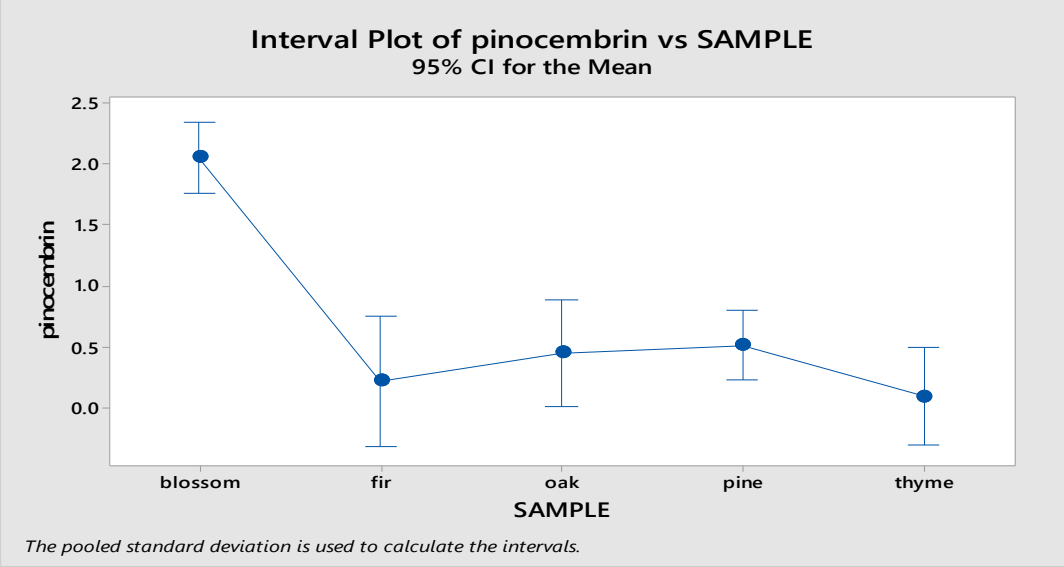

Figure 31. The interval plot of pinocembrin

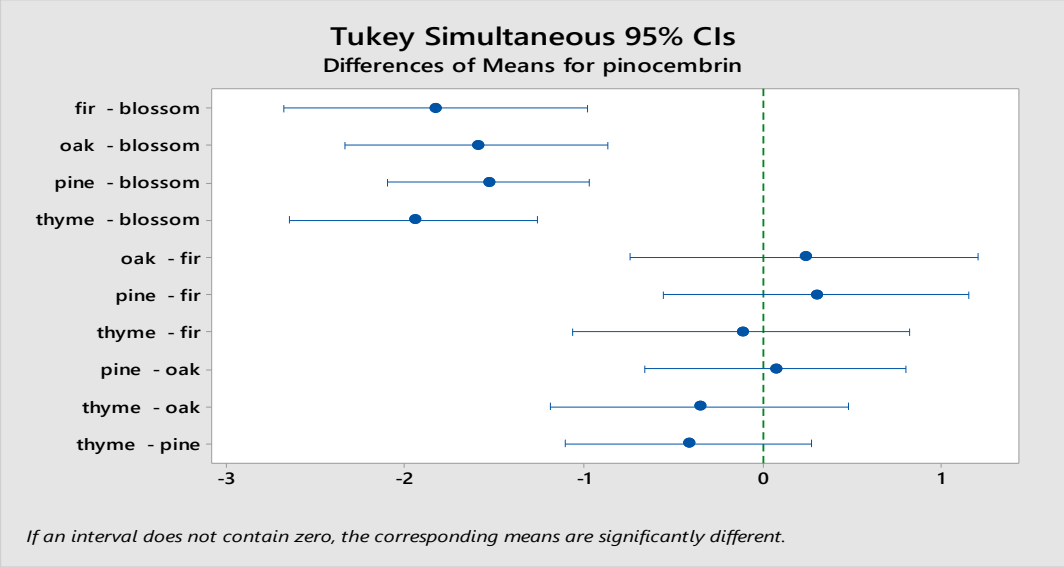

Figure 32. The Tukey plot of pinocembrin

Furthermore, Principal Components Analysis was employed using the statistical software program Minitab (version18) as a final step with the aim of compressing the already existing data and classifying honey samples in clusters based on botanical origin. This unsupervised multivariate analysis is used to identify a smaller number of uncorrelated variables, called "principal components", from a large set of data. With this analysis, you create new variables (principal components) that are linear combinations of the observed variables. The goal of principal components analysis is to explain the maximum amount of variance with the fewest number of principal components.

The selection of principal components is performed via the Kaiser criterion as well as with the interpretation of the scree plot. According to this criterion the principal components with eigenvalues greater than 1 can only be used as it is presented in the figure 30. While in the scree plot the principal components which are selected are those before the first point that start the line trend in the steep curve.

In our case the first three components have eigenvalues greater than 1. These three components explain 82.2% of the variance in the data set. The scree plot shows that the eigenvalues start to form a straight line after the third component.

Eigenanalysis of the Covariance Matrix

Eigenvalue	7.9150	4.1514	1.2133	0.9853	0.6154	0.4171	0.2290	0.1493	0.0960	0.0813
Proportion	0.490	0.257	0.075	0.061	0.038	0.026	0.014	0.009	0.006	0.005
Cumulative	0.490	0.747	0.822	0.884	0.922	0.947	0.962	0.971	0.977	0.982
Eigenvalue	0.0721	0.0555	0.0471	0.0335	0.0248	0.0180	0.0155	0.0087	0.0064	0.0044
Proportion	0.004	0.003	0.003	0.002	0.002	0.001	0.001	0.001	0.000	0.000
Cumulative	0.986	0.990	0.993	0.995	0.996	0.997	0.998	0.999	0.999	1.000
Eigenvalue	0.0039	0.0027	0.0000							
Proportion	0.000	0.000	0.000							
Cumulative	1.000	1.000	1.000							

113 cases used, 1 cases contain missing values

Figure 33. The Kaiser criterion for the selection of PC

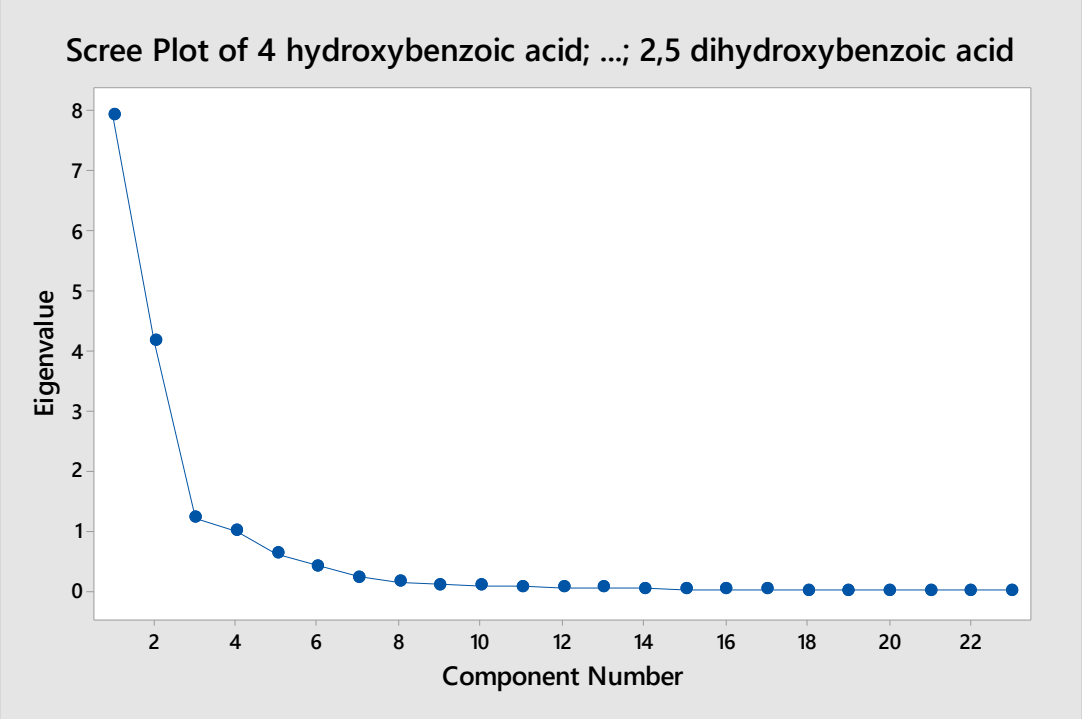

Figure 34. The scree plot for the selection of the principal components

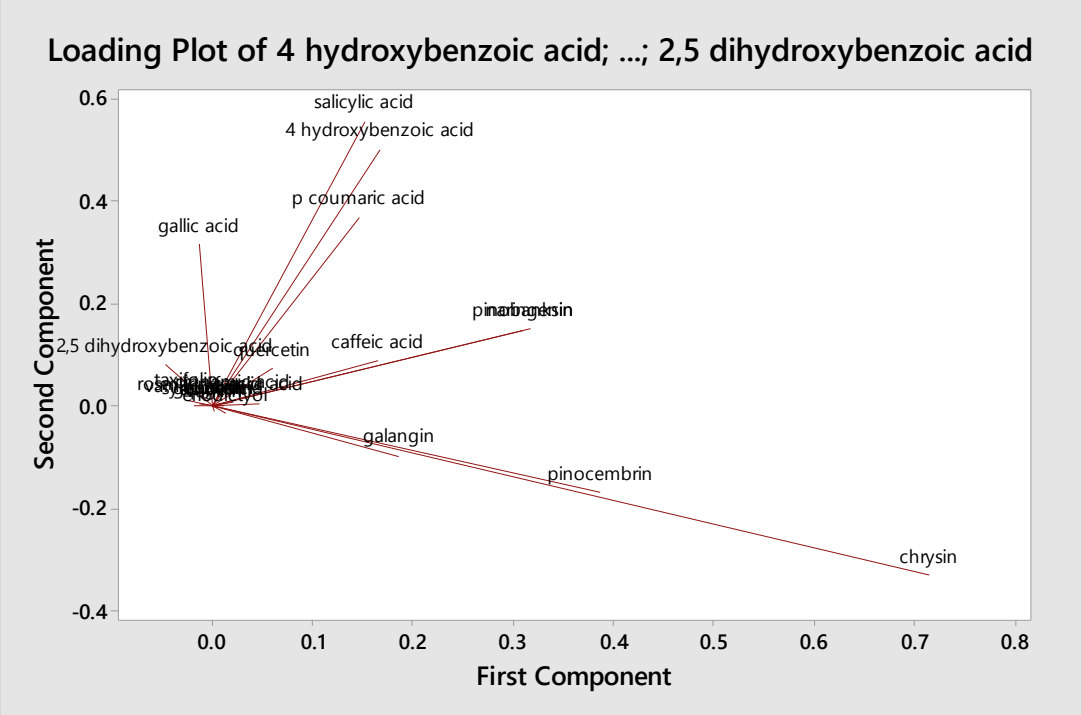

Figure 35. The loading plot of the variables

Coefficients, by which the original variables are multiplied to obtain the PCs, are represented in the loading plot which summarize and give information about the variables.

According to this plot the variable that positively correlate the most with the first principal component is chrysin and to a lesser extent pinocembrin and galangin.

Eigenvectors

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
4 hydroxybenzoic acid	0.167	0.498	0.350	0.194	-0.672	-0.040	0.300	0.032
apigenin	0.005	-0.005	-0.011	0.022	-0.011	-0.032	-0.044	0.035
caffeic acid	0.165	0.085	-0.032	0.290	0.017	0.139	-0.414	0.127
chrysin	0.715	-0.333	0.066	-0.348	-0.183	-0.028	0.005	-0.020
cinnamic acid	0.019	0.008	-0.011	-0.037	0.017	0.037	-0.017	-0.043
eriodictyol	0.014	-0.017	-0.021	0.039	-0.043	-0.015	-0.233	0.108
ferulic acid	0.047	0.003	-0.002	0.131	-0.095	0.127	-0.255	0.075
galangin	0.186	-0.099	0.003	-0.120	-0.124	-0.043	-0.091	-0.029
gallic acid	-0.015	0.313	-0.816	-0.330	-0.290	-0.049	-0.019	0.047
genistein	-0.003	-0.002	0.003	0.000	0.007	-0.013	-0.001	0.003
luteolin	0.011	-0.003	-0.009	-0.029	-0.028	-0.042	0.056	-0.000
naringenin	0.316	0.149	-0.224	0.375	0.277	0.072	0.273	0.030
p coumaric acid	0.146	0.367	0.049	0.143	0.025	-0.114	-0.641	-0.345
pinobanksin	0.309	0.146	-0.220	0.367	0.271	0.073	0.265	0.028
pinocembrin	0.387	-0.171	-0.037	-0.002	0.018	0.087	-0.101	0.033
quercetin	0.059	0.071	-0.079	0.034	0.071	-0.061	0.049	0.059
rosmarinic acid	-0.013	0.004	-0.112	-0.041	-0.109	-0.099	-0.063	0.096
salicylic acid	0.151	0.554	0.273	-0.538	0.477	-0.049	0.028	0.142
syringic acid	0.001	-0.005	-0.001	-0.011	-0.014	0.003	-0.028	-0.014
taxifolin	-0.026	0.011	0.084	-0.006	-0.031	0.036	0.043	0.093
vanillin	0.001	-0.011	0.011	0.010	-0.008	-0.016	-0.038	0.129
vanillic acid	-0.020	-0.000	0.041	0.039	-0.040	0.061	-0.169	0.876
2,5 dihydroxybenzoic acid	-0.048	0.079	0.014	-0.153	-0.066	0.949	-0.002	-0.108

Variable	PC9	PC10	PC11	PC12	PC13	PC14	PC15	PC16
4 hydroxybenzoic acid	0.050	0.020	0.015	0.078	0.039	0.078	0.031	0.023
apigenin	0.055	0.047	-0.029	0.008	-0.128	-0.049	0.110	-0.209
caffeic acid	0.421	-0.295	-0.035	0.367	0.198	-0.179	-0.333	-0.234
chrysin	-0.118	0.017	-0.042	-0.047	0.096	-0.361	-0.158	0.165
cinnamic acid	-0.014	-0.001	0.117	0.002	0.207	0.116	-0.028	-0.254
eriodictyol	0.201	0.099	-0.204	0.035	-0.079	0.402	-0.340	0.606
ferulic acid	0.118	-0.511	0.244	-0.509	0.150	-0.046	0.430	0.277
galangin	-0.188	-0.328	-0.182	-0.297	-0.124	0.614	-0.236	-0.408

gallic acid	-0.018	-0.072	0.136	0.102	-0.038	-0.024	-0.016	0.024
genistein	-0.004	-0.005	-0.027	0.006	-0.006	0.016	0.043	-0.012
luteolin	0.034	-0.119	-0.231	-0.010	0.152	-0.172	0.080	-0.333
naringenin	-0.061	0.047	0.012	-0.153	-0.014	0.023	-0.084	0.018
p coumaric acid	-0.376	0.275	-0.037	-0.091	-0.151	-0.119	0.065	-0.043
pinobanksin	-0.059	0.045	0.016	-0.155	-0.013	0.028	-0.084	0.014
pinocembrin	0.270	0.228	0.221	0.335	-0.228	0.368	0.533	-0.104
quercetin	-0.101	-0.285	-0.752	0.224	-0.137	-0.059	0.381	0.136
rosmarinic acid	0.528	0.460	-0.339	-0.512	0.060	-0.085	0.058	-0.168
salicylic acid	0.180	-0.090	0.052	-0.033	0.059	0.066	0.005	0.021
syringic acid	-0.018	-0.031	0.015	0.005	-0.042	-0.014	0.025	-0.049
taxifolin	0.173	-0.181	0.115	-0.116	-0.848	-0.279	-0.169	-0.072
vanillin	-0.076	0.030	-0.022	-0.027	-0.017	-0.011	-0.063	0.100
vanillic acid	-0.359	0.175	0.028	-0.015	0.015	-0.031	0.040	-0.098
2,5 dihydroxybenzoic acid	-0.069	0.118	-0.163	-0.046	-0.022	-0.004	-0.014	-0.012

Variable	PC17	PC18	PC19	PC20	PC21	PC22	PC23
4 hydroxybenzoic acid	0.007	0.009	-0.019	-0.007	0.003	0.005	-0.000
apigenin	0.020	0.920	-0.089	-0.228	0.037	-0.076	0.005
caffeic acid	0.182	-0.028	0.023	0.036	0.000	0.016	-0.002
chrysin	0.011	0.044	-0.038	-0.047	-0.054	0.006	-0.002
cinnamic acid	-0.338	-0.088	-0.799	-0.092	-0.294	0.061	-0.004
eriodictyol	-0.388	0.118	-0.009	-0.146	-0.009	-0.011	0.005
ferulic acid	-0.055	0.044	-0.017	-0.007	-0.007	-0.006	0.003
galangin	0.152	-0.011	0.100	0.056	0.022	-0.014	0.003
gallic acid	-0.004	0.007	0.017	0.015	0.006	0.008	0.001
genistein	0.006	0.062	0.128	-0.107	-0.142	0.972	0.007
luteolin	-0.769	-0.022	0.308	0.067	0.252	-0.002	0.012
naringenin	-0.025	0.011	0.002	-0.027	0.011	-0.001	0.700
p coumaric acid	-0.065	-0.049	0.020	0.027	-0.018	-0.002	0.001
pinobanksin	-0.041	0.022	0.015	-0.023	0.016	0.008	-0.713
pinocembrin	-0.071	-0.103	0.054	0.093	0.072	-0.004	0.002
quercetin	0.114	-0.067	-0.212	-0.008	-0.114	-0.026	-0.008
rosmarinic acid	0.151	-0.117	-0.057	0.017	-0.028	0.006	-0.002
salicylic acid	0.020	0.030	0.030	-0.004	0.030	-0.004	0.001
syringic acid	0.091	-0.244	-0.121	-0.773	0.558	0.026	-0.004
taxifolin	-0.155	-0.119	-0.116	0.031	-0.083	0.025	-0.001

vanillin	0.063	0.104	-0.382	0.530	0.689	0.205	0.001
vanillic acid	-0.026	-0.062	0.055	-0.066	-0.101	-0.029	-0.001
2,5 dihydroxybenzoic acid	-0.002	0.033	0.019	0.007	0.022	0.009	0.001

Figure 36. The coefficients of the variables

Afterwards, the visual inspection of the score plot follows which is a summary of the relationships among the observations (samples). In other words, samples that are highly similar, classified in one group. The following PCA plot shows that it is a difficult task to differentiate the groups of honey samples in clusters using only the data obtained for the target screening approach. For this reason non target screening will be more helpful in a better classification of honey samples based on botanical or geographical origin. The only discrimination could be accomplished is between blossom honey samples and thyme honey samples because the variables that affect mostly the first principal component don't exist in thyme honey samples.

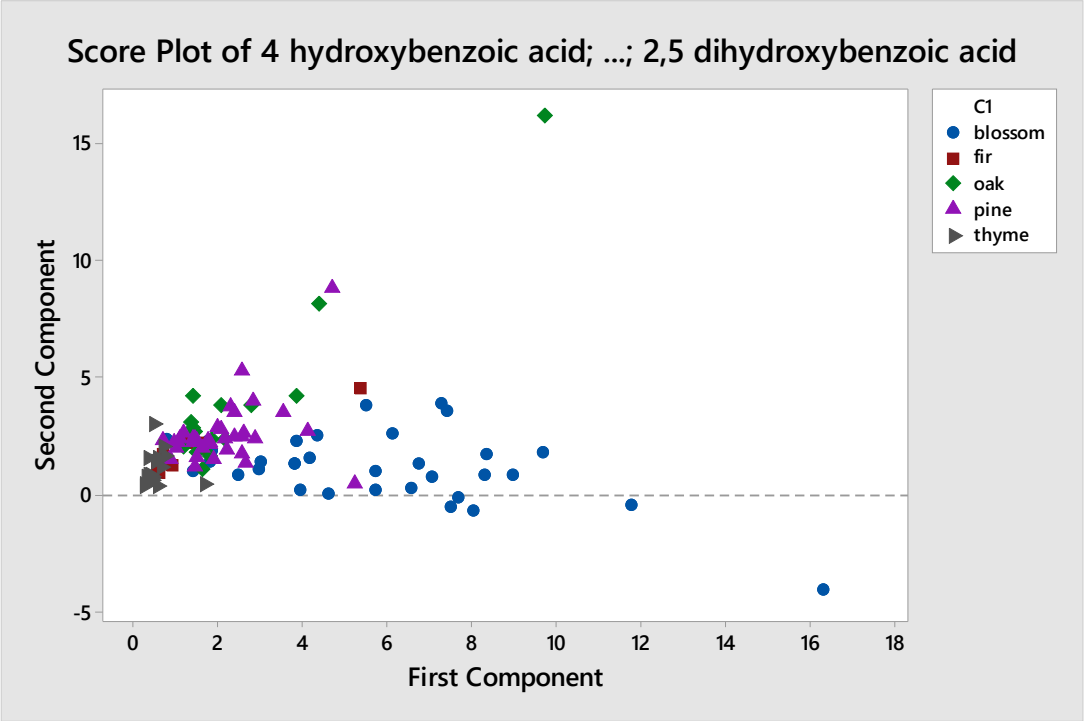

Figure 37. PCA plot for the classification of honey samples

CHAPTER 6: Conclusions

Honey authenticity is an issue which increasingly draws the attention of scientific community. Phenolic compounds, flavonoids and phenolic acids, are the most abundant antioxidant nutritive constituents and proved to be beneficial to human health. To this end, incorporation of new phenolic compounds in the already database of natural products has been performed by analyzing standards using a data dependent acquisition mode with inclusion list. Moreover, a method has been revalidated due to learning purposes for the simultaneous determination of 25 phenolic compounds and the results have indicated a good performance of the method. In more detail, linearity was very good in most cases with correlation coefficients higher than 0.99 whereas LODs and LOQs raged between 0.030-0.33 mg/kg and 0.091-0.99 mg/kg respectively. Also, the vast majority of analytes have shown $RSD_r\% < 5$ and $RSD_R\% < 10$ whereas the recoveries ranged between 70 and 110 % proving the acceptable trueness of the method. Regarding the matrix effects were ranged from -20% to 30%.

Additionally, target screening of 135 greek honey samples consisting from 114 unifloral honeys by different botanical origins and 21 multifloral honeys was performed after an appropriate selection of internal standards of each analyte. The quantification was performed using standard addition calibration curves and the phenolic content of these various honey matrices was measured as average concentration. The average concentration of determined polyphenols ranged from 0.0050 to 11 mg/Kg. The unifloral honey with the greater phenolic content was blossom. This type of honey is characterized by a higher concentration in 2 analytes namely chrysin and pinocembrin which can be used as floral makers. Also, a high concentration of pinobanksin and galangin have been found in blossom honey. These 4 analytes could be good indicators for the separation of blossom honeys from thyme honeys because of the lower average concentration in the second one.

It has to be highlighted that rosmarinic acid and hesperitin have been found only in thyme and citrus honeys respectively. So they can be used as potential markers for the botanical origin, although the number of samples was not so representative. To conclude, chemometric tools should be used in order to reveal different phenolic compounds patterns which would result to more efficient and reliable discrimination.

CHAPTER 7: Future Perspectives

The evaluation of the results of this thesis demonstrates the insufficient separation of phenolic compounds with Reversed Phase Chromatography which are weakly retained in this stationary phase. For this reason many of phenolic compounds elute simultaneously making the detection of analytes a difficult choice. Hillic Chromatography can be used as a complementary method for the more accurate determination of phenolic acids because the separation efficiency with Hillic Chromatography is better contrary to RP method.

In addition, in order to expand the literature data of phenolic compounds as biomarkers, GC –APCI-TOF-MS technique can be performed for the analysis of volatile compounds in honey which is very promising for the authentication and characterization of botanical origin.

To conclude, the results obtained in the present work illustrate the importance of investigating polyphenols content in honey authenticity studies. It has to be highlighted that suspect and non-target screening should be performed for all the available samples. In this way, all the information obtained by the analysis will be utilized and authenticity markers alongside with discrimination patterns will be revealed.

ABBREVIATIONS AND ACRONYMS

Table 13. Abbreviations and acronyms

5-HMF	5-hydroxymethylfurfural
ACN	Acetonitrile
AAS	Atomic Absorption Spectroscopy
AOC	Antioxidant capacity
avg	Average Value
bbCID	broadband Collision-Induced Dissociation
CRM	Certified Reference Materials
DFAs	Difructose Anhydrides
DN	Diastase Number
EIC	Extracted Ion Chromatogram
ESI	ElectroSpray Ionization
EtAC	Ethyl Acetate
FIA	Flow Injection Analysis
FT-ICR	Fourier Transform-Ion Cyclotron Resonance
GC	Gas Chromatography
GC-MS	Gas Chromatography coupled to Mass Spectrometry
GIs	Geographical Indications
HFCS	High Fructose Corn Syrup
HPAEC	High Performance Anion Exchange Chromatography
HPLC	High-Performance Liquid Chromatography
HR-MS	High Resolution Mass Spectrometry
HS	HeadSpace
IC	Ion Chromatography
ICP-MS	Inductively Coupled Plasma – Mass Spectrometry
ICP-OES	Inductively Coupled Plasma – Optical Emission Spectroscopy
PDO	Protected Designation of Origin
PGI	Protected Geographical Indicator
GMO	Genetically Modified Organisms
LC	Liquid Chromatography
LC-HRMS	Liquid Chromatography coupled to High Resolution Mass Spectrometry
LC-MS/MS	Liquid Chromatography tandem Mass Spectrometry
LC-QqQ MS/MS	Liquid Chromatography coupled with triple-Quadrupole Mass Spectrometry
LC-QTOF-MS	Liquid Chromatography–Quadrupole-Time-Of-Flight-Mass Spectrometry
LDL	Low-Density Lipoprotein
LLE	Liquid-Liquid Extraction
LODs	Limits Of Detection
LOQs	Limits Of Quantification
MALDI	Matrix Assisted Laser Desorption Ionization

ME	Matrix Effect
MeOH	Methanol
MF	Matrix Factor
MS	Mass Spectrometry
MWCNTs	Multiwalled Carbon Nanotubes
ND	Non Detected
NIR	Near Infrared Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
MIR	Mid Infrared Spectroscopy
NKUA	National and Kapodistrian University of Athens
FFDCA	Federal Food, Drug and Cosmetic Act
USDA	United States Department Of Agriculture
OAs	Organic Acids
PCA	principal component analysis
PDO	Protected Designation of Origin
PGI	Protected Geographical Indication
pH	potential of Hydrogen
PLS	Partial Least Squares
QqQ	TripleQuadrupole
QToF	quadrapole time of flight
RDI	Refractive Detector Index
CEAD	Coulometric Electrode Array Detectror
CZE	Capillary Zone Electrophoresid
TIC	Thin Layer Chromatography
REEs	Rare Earth Elements
RP	Reversed Phase
RPLC-QTOF-MS	Reversed Phase Liquid Chromatography-Quadrupole-Time-Of-Flight Mass Spectrometry
RSD	Relative Standard Deviation
SCIRA	Stable Carbon Isotopic Ratio Analysis
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SPE	Solid-Phase Extraction
SPME	Solid-Phase Micro-Extraction
SRM	Selected Reaction Monitoring
TOF	Time-Of-Flight
FLD	Fluorescence Detector
DAD	Diode Array Detector
SFS	Synchronous Fluorescence Spectroscopy
t_R	retention time
UHPLC	UltraHigh-Performance Liquid Chromatography
USE	Ultra-Sound Extraction
UV	Ultra Violet
UV-Vis	Ultra Violet - Visible
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
FAAS	Flame Atomic Absorption Spectrometry
VOCs	Volatile Organic Compounds
IS	Invert Syrup

HFCS	High Fructose Corn Syrup
GS	Glucose Syrup
PCA	Principal Component Analysis
CVA	Canonical Variate Analysis
CA	Cluster Analysis
ANN	Artificial Neural Networks
k-NN	K Nearest Networks
SVM	Support Vector Machine
SRM	Selected Reaction Monitoring
EMA	Economically Motivated Adulteration

References

[1] C. E. Manyi-Loh, R. N. Ndip, and A. M. Clarke, Volatile compounds in honey: A review on their involvement in aroma, botanical origin determination and potential biomedical activities, *Int. J. Mol. Sci.*, vol. 12, no. 12, 2011, pp. 9514–9532.

[2] P. M. Da Silva, C. Gauche, L. V. Gonzaga, A. C. O. Costa, and R. Fett, Honey: Chemical composition, stability and authenticity, *Food Chem.*, vol. 196, 2016, pp. 309–323.

[3] Ρ. Βελώνη, Όσο υπάρχουν μέλισσες, http://fractalart.gr/melisses/, last visit: 29/07/2018.

[4] C. Pita-Calvo and M. Vázquez, Differences between honeydew and blossom honeys: A review, *Trends Food Sci. Technol.*, vol. 59, 2017, pp. 79–87.

[5] A. Bentabol Manzanares, Z. H. García, B. R. Galdón, E. R. Rodríguez, and C. D. Romero, Differentiation of blossom and honeydew honeys using multivariate analysis on the physicochemical parameters and sugar composition, *Food Chem.*, vol. 126, no. 2, 2011, pp. 664–672.

[6] I. K. Karabagias, M. V. Vavoura, C. Nikolaou, A. V. Badeka, S. Kontakos, and M. G. Kontominas, Floral authentication of Greek unifloral honeys based on the combination of phenolic compounds, physicochemical parameters and chemometrics, *Food Res. Int.*, vol. 62, no. August, 2014, pp. 753–760.

[7] GreekCityTimes, Why Greek honey is considered best in the world, https://greekcitytimes.com, last visit: 10/06/2018.

[8] *Το ελληνικό μέλι, τα είδη του, τα χαρακτηριστικά και οι ιδιότητές τους*, επίσκεψη:30/07/2018.

[9] A. Tsigouri, M. Passaloglou-Katrali, and O. Sabatakou, Palynological characteristics of different unifloral honeys from Greece, *Grana*, vol. 43, no. 2, 2004, pp. 122–128.

[10] L. Bravo, Polyphenols: Chemistry, Dietary Sources, Metabolism, and Nutritional Significance, *Nutr. Rev.*, vol. 56, no. 11, 2009, pp. 317–333.

[11] M. Abbas *et al.*, Natural polyphenols: An overview, *Int. J. Food Prop.*, vol. 20, no.

8, 2017, pp. 1689–1699.

[12] U. M. Gašić, D. M. Milojković-Opsenica, and Ž. L. Tešić, Polyphenols as possible markers of botanical origin of honey, *J. AOAC Int.*, vol. 100, no. 4, 2017, pp. 852–861.

[13] J. M. Alvarez-suarez and F. Giampieri, Honey as a Source of Dietary Antioxidants : Structures , Bioavailability and Evidence of Protective Effects Against Human Chronic Diseases Honey as a Source of Dietary Antioxidants : Structures, Bioavailability and Evidence, *Curr. Med. Chem.*, vol. 20, no. 5, 2013, pp. 621–638.

[14] E. De La Fuente, A. I. Ruiz-Matute, R. M. Valencia-Barrera, J. Sanz, and I. Martínez Castro, Carbohydrate composition of Spanish unifloral honeys, *Food Chem.*, vol. 129, no. 4, 2011, pp. 1483–1489.

[15] M. J. Nozal, J. L. Bernal, L. Toribio, M. Alamo, J. C. Diego, and J. Tapia, The use of carbohydrate profiles and chemometrics in the characterization of natural honeys of identical geographical origin, *J. Agric. Food Chem.*, vol. 53, no. 8, 2005, pp. 3095–3100.

[16] L. Primorac, I. Flanjak, D. Kenjerić, G. Drabubalo, and Z. Topolnjak, “Specific rotation and carbohydrate profile of Croatian unifloral honeys,” *Czech J. Food Sci.*, vol. 29, no. 5, 2011, pp. 515–519.

[17] S. P. Kek, N. L. Chin, S. W. Tan, Y. A. Yusof, and L. S. Chua, Classification of Honey from Its Bee Origin via Chemical Profiles and Mineral Content, *Food Anal. Methods*, vol. 10, no. 1, 2017, pp. 19–30.

[18] B. Lichtenberg-kraag, Evidence for correlation between invertase activity and sucrose content during the ripening process of honey, *J. Apic. Res.*, vol. 53, no. 3, 2014, pp. 364–373

[19] I. Mato, J. F. Huidobro, J. Simal-Lozano, and M. T. Sancho, Significance of nonaromatic organic acids in honey, *J. Food Prot.*, vol. 66, no. 12, 2003, pp. 2371–2376.

[20] N. Sakaˇ and M. Sak-bosnar, Talanta A rapid method for the determination of honey diastase activity, vol. 93, 2012, pp. 135–138.

[21] J. Kečkeš, J. Trifković, F. Andrić, M. Jovetić, Ž. Tešić, and D. Milojković-Opsenica, Amino acids profile of Serbian unifloral honeys, *J. Sci. Food Agric.*, vol. 93, no. 13,

2013, pp. 3368–3376.

[22] S. Silici and K. Karaman, Chemometric approaches for the characterization of turkish rhododendron and honeydew honeys depending on amino acid composition, *J. Liq. Chromatogr. Relat. Technol.*, vol. 37, no. 6, 2014,pp. 864–877.

[23] L. S. Chua, J. Y. Lee, and G. F. Chan, Honey protein extraction and determination by mass spectrometry, 2013, pp. 3063–3074.

[24] S. Kivrak, Determination of B-group vitamins in Turkish honey using ultra-performance liquid chromatography with electrospray ionization coupled to tandem mass spectrometry, *J. Liq. Chromatogr. Relat. Technol.*, vol. 39, no. 19–20, 2016, pp. 847–851.

[25] E. E. Abano, R. G. Dadzie, and R. G. Abano, E. E., Dadzie, Simultaneous detection of water-soluble vitamins using the High Performance Liquid Chromatography (HPLC) - a review, *Croat. J. Food Sci. Technol.*, vol. 6, no. 2, 2014, pp. 116–123.

[26] V. L. Da Silva, M. R. F. Cerqueira, D. Lowinsohn, M. A. C. Matos, and R. C. Matos, Amperometric detection of ascorbic acid in honey using ascorbate oxidase immobilised on amberlite IRA-743, *Food Chem.*, vol. 133, no. 3, 2012, pp. 1050–1054.

[27] R. J. Paxton, J. Klee, S. Korpela, and I. Fries, Nosema ceranae has infected Apis mellifera in Europe since at least 1998 and may be more virulent than Nosema apis, *Apidologie*, vol. 38, no. 6, 2007, pp. 558–565.

[28] A. B. P. Leme, S. R. Bianchi, R. L. Carneiro, and A. R. A. Nogueira, Optimization of Sample Preparation in the Determination of Minerals and Trace Elements in Honey by ICP-MS, *Food Anal. Methods*, vol. 7, no. 5, 2014, pp. 1009–1015.

[29] P. M. Da Silva, C. Gauche, L. V. Gonzaga, A. C. O. Costa, and R. Fett, Honey: Chemical composition, stability and authenticity, *Food Chem.*, vol. 196, no. April, 2016, pp. 309–323.

[30] L. F. Cuevas-Glory, J. A. Pino, L. S. Santiago, and E. Sauri-Duch, A review of volatile analytical methods for determining the botanical origin of honey, *Food Chem.*, vol. 103, no. 3, 2007, pp. 1032–1043.

[31] A. Grégrová, V. Kružik, E. Vrácovská, A. Rajchl, and H. Čižková, Evaluation of factors affecting crystallization of disparate set of multi-flower honey samples,

Agron. Res., vol. 13, no. 5, 2015, pp. 1215–1226.

[32] Y. Ma *et al.*, Chemical and molecular dynamics analysis of crystallization properties of honey, *Int. J. Food Prop.*, vol. 20, no. 4, 2017, pp. 725–733.

[33] D. W. Ball, The Chemical Composition of Honey, *J. Chem. Educ.*, vol. 84, no. 10, 2007, p. 1643.

[34] Z. Can, O. Yildiz, H. Sahin, E. Akyuz Turumtay, S. Silici, and S. Kolayli, An investigation of Turkish honeys: Their physico-chemical properties, antioxidant capacities and phenolic profiles, *Food Chem.*, vol. 180, 2015, pp. 133–141.

[35] R. T. Szabó, M. Mézes, T. Szalai, E. Zajácz, and M. Weber, Colour identification of honey and methodical development of its instrumental measuring, *Columella J. Agric. Environ. Sci.*, vol. 3, no. 1, 2016.

[36] United States Deparment of Agriculture, United States Standards for Grades of Extracted Honey, *Fed. Regist.*, vol. 50FR15861, 1985, p. 12.

[37] M. A. Dominguez and M. E. Centurión, Application of digital images to determine color in honey samples from Argentina, *Microchem. J.*, vol. 118, 2015, pp.110–114.

[38] Pfund Colour Grading S ystem, https://wedderspoon.ca/manuka-honey-science/pfund/, επίσκεψη : 18-Jun-2018].

[39] L. Castro-Vázquez, M. C. Díaz-Maroto, and M. S. Pérez-Coello, Aroma composition and new chemical markers of Spanish citrus honeys, *Food Chem.*, vol. 103, no. 2, 2007, pp. 601–606.

[40] I. K. Karabagias, A. Badeka, S. Kontakos, S. Karabournioti, and M. G. Kontominas, Characterisation and classification of Greek pine honeys according to their geographical origin based on volatiles, physicochemical parameters and chemometrics, *Food Chem.*, vol. 146, 2014, pp. 548–557.

[41] L. W. Doner, Honey, *Encycl. Food Sci. Nutr, Second Ed.*, 2003, pp. 3125–3130,.

[42] Š. Velčovská and T. Sadílek, The system of the geographical indication - important component of the politics of the consumers' protection in European Union, *Amfiteatru Econ.*, vol. 16, no. 35, 2014, pp. 228–242.

[43] Y. Picó, Mass Spectrometry in Food Quality and Safety : An Overview of the Current Status, *Bs:Coac*, vol. 68, no. 1, 2015, pp. 3–76.

[44] L. A. Marghitas, D. S. Dezmirean, C. B. Pocol, M. Ilea, O. Bobis, and I. Gergen, The development of a biochemical profile of acacia honey by identifying biochemical determinants of its quality, *Not. Bot. Horti Agrobot. Cluj-Napoca*, vol. 38, no. 2, 2010, pp. 84–90.

[45] S. Bogdanov *et al.*, Honey quality and international regulatory standards: review by the International Honey Commission, *Bee World*, vol. 80, no. 2, 1999, pp. 61–69.

[46] F. C. Biluca *et al.*, 5-HMF and carbohydrates content in stingless bee honey by CE before and after thermal treatment, *Food Chem.*, vol. 159, 2014, pp. 244–249.

[47] B. Zábrodská and L. Vorlová, Adulteration of honey and available methods for detection – a review, *Acta Vet. Brno*, vol. 83, 2014, pp. S85–S102.

[48] E. Capuano and V. Fogliano, LWT - Food Science and Technology Acrylamide and 5-hydroxymethylfurfural (HMF):A review on metabolism , toxicity , occurrence in food and mitigation strategies, *LWT - Food Sci. Technol.*, vol. 44, no. 4, 2011, pp. 793–810.

[49] M. C. Zamora, J. Chirife, and D. Roldán, On the nature of the relationship between water activity and % moisture in honey, *Food Control*, vol. 17, no. 8, 2006, pp. 642–647.

[50] A. Gallina, N. Stocco, and F. Mutinelli, Karl Fischer Titration to determine moisture in honey: A new simplified approach, *Food Control*, vol. 21, no. 6, 2010, pp. 942–944.

[51] S. Soares, J. S. Amaral, M. B. P. P. Oliveira, and I. Mafra, A Comprehensive Review on the Main Honey Authentication Issues: Production and Origin, *Compr. Rev. Food Sci. Food Saf.*, vol. 16, no. 5, 2017, pp. 1072–1100.

[52] N. Sakač and M. Sak-Bosnar, A rapid method for the determination of honey diastase activity, *Talanta*, vol. 93, 2012, pp. 135–138.

[53] M. Pereira da Costa and C. A. Conte-Junior, Chromatographic Methods for the Determination of Carbohydrates and Organic Acids in Foods of Animal Origin, *Compr. Rev. Food Sci. Food Saf.*, vol. 14, no. 5, 2015, pp. 586–600.

[54] Campone, Determination of phenolic compounds in honey using dispersive liquid-liquid microextraction, *J. Chromatogr. A*, vol. 1334, 2014.

[55] G. Beretta, P. Granata, M. Ferrero, M. Orioli, and R. Maffei, Standardization of

antioxidant properties of honey by a combination of spectrophotometric / fluorimetric assays and chemometrics, vol. 533, 2005, pp. 185–191.

[56] S. P. Atel and S. C. Ichello, Manuka honey : an emerging natural food with medicinal use, 2013.

[57] R. LIANDA, L. SANT' ANA, A. ECHEVARRIA, and R. CASTRO, Antioxidant Activity and Phenolic Composition of Brazilian Honey and their Extracts, *J. Brazilian Chem. Soc.*, vol. 23, no. 4, 2012, pp. 618–627.

[58] J. M. Alvarez-Suarez, F. Giampieri, and M. Battino, Honey as a source of dietary antioxidants: structures, bioavailability and evidence of protective effects against human chronic diseases, *Curr. Med. Chem.*, vol. 20, no. 5, 2013, pp. 621–38.

[59] H. A. Alzahrani *et al.*, Evaluation of the Antioxidant Activity of Three Varieties of Honey from Different Botanical and Geographical Origins, *Glob. J. Health Sci.*, vol. 4, no. 6, 2012.

[60] L. Estevinho, A. P. Pereira, L. Moreira, L. G. Dias, and E. Pereira, Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey, *Food Chem. Toxicol.*, vol. 46, no. 12, 2008, pp. 3774–3779.

[61] J. Alvarez-Suarez, S. Tulipani, S. Romandini, A. Vidal, and M. Battino, Methodological Aspects about Determination of Phenolic Compounds and In Vitro Evaluation of Antioxidant Capacity in the Honey: A Review, *Curr. Anal. Chem.*, vol. 5, no. 4, 2009, pp. 293–302.

[62] M. Viuda-Martos, Y. Ruiz-Navajas, J. Fernández-López, and J. A. Pérez-Álvarez, Functional properties of honey, propolis, and royal jelly, *J. Food Sci.*, vol. 73, no. 9, 2008, pp. 117–124.

[63] S. Bogdanov, T. Jurendic, R. Sieber, and P. Gallmann, Honey for nutrition and health: A review, *J. Am. Coll. Nutr.*, vol. 27, no. 6, 2008, pp. 677–689.

[64] N. S. Al-Waili, K. Salom, and A. A. Al-Ghamdi, Honey for wound healing, ulcers, and burns; data supporting its use in clinical practice, *ScientificWorldJournal.*, vol. 11, 2011, pp. 766–787.

[65] G. P. Danezis, A. S. Tsagkaris, F. Camin, V. Brusic, and C. A. Georgiou, Food authentication: Techniques, trends & emerging approaches, *TrAC - Trends Anal. Chem.*, vol. 85, 2016, pp. 123–132.

[66] P. C. Molan, Authenticity of honey, *Food Authentication*, 1996, pp. 259–303.

[67] D. M. Opsenica, D. Luší, and Ž. Teší, Modern analytical techniques in the assessment of the authenticity of Serbian honey, *Arh. Hig. Rada Toksikol.*, vol. 66, no. 4, 2015, pp. 233–241.

[68] J. M. Alvarez-Suarez, S. Tulipani, S. Romandini, E. Bertoli, and M. Battino, Contribution of honey in nutrition and human health: A review, *Med. J. Nutrition Metab.*, vol. 3, no. 1, 2010, pp. 15–23.

[69] S. Bogdanov and P. Martin, Honey authenticity: a review, *Mitt. Leb. Hyg*, no. August, 2002, pp. 1–20.

[70] C. Alamprese, *Food Authentication*, First. London: Blackie Academic and Professional,an imprint of Chapman and Hall, 2018.

[71] S. Bansal, A. Singh, M. Mangal, A. K. Mangal, and S. Kumar, Food adulteration: Sources, health risks, and detection methods, *Crit. Rev. Food Sci. Nutr.*, vol. 57, no. 6, 2017, pp. 1174–1189.

[72] L. Wu *et al.*, Recent advancements in detecting sugar-based adulterants in honey – A challenge,*TrAC - Trends Anal. Chem.*, vol. 86, 2017,pp. 25–38.

[73] S. E. Strayer, K. Everstine, and S. Kennedy, Economically motivated adulteration of honey: Quality control vulnerabilities in the international Money Market, *Food Prot. Trends*, vol. 34, no. 1, 2014, pp. 8–14.

[74] V. Kaškoniene and P. R. Venskutonis, Floral Markers in Honey of Various Botanical and Geographic Origins: A Review, *Compr. Rev. Food Sci. Food Saf.*, vol. 9, no. 6, 2010, pp. 620–634.

[75] G. P. Danezis, A. S. Tsagkaris, V. Brusic, and C. A. Georgiou, Food authentication: state of the art and prospects, *Curr. Opin. Food Sci.*, vol. 10, no. July 2016, 2016, pp. 22–31.

[76] S. Esslinger, J. Riedl, and C. Fauhl-Hassek, Potential and limitations of non-targeted fingerprinting for authentication of food in official control, *Food Res. Int.*, vol. 60, 2014, pp. 189–204.

[77] S. Esslinger, J. Riedl, and C. Fauhl-Hassek, Potential and limitations of non-targeted fingerprinting for authentication of food in official control, *Food Res. Int.*, vol. 60, 2014, pp. 189–204.

[78] M. Krauss, H. Singer, and J. Hollender, LC-high resolution MS in environmental analysis: From target screening to the identification of unknowns, *Anal. Bioanal. Chem.*, vol. 397, no. 3, 2010, pp. 943–951.

[79] A. A. Bletsou, J. Jeon, J. Hollender, E. Archontaki, and N. S. Thomaidis, Targeted and non-targeted liquid chromatography-mass spectrometric workflows for identification of transformation products of emerging pollutants in the aquatic environment, *TrAC - Trends Anal. Chem.*, vol. 66, 2015, pp. 32–44.

[80] S. K. T. Seraglio *et al.*, Development and validation of a LC-ESI-MS/MS method for the determination of phenolic compounds in honeydew honeys with the diluted-and-shoot approach, *Food Res. Int.*, vol. 87, 2016, pp. 60–67.

[81] N. P. Kalogiouri, N. A. Alygizakis, R. Aalizadeh, and N. S. Thomaidis, Olive oil authenticity studies by target and nontarget LC–QTOF-MS combined with advanced chemometric techniques, *Anal. Bioanal. Chem.*, vol. 408, no. 28, 2016, pp. 7955–7970.

[82] Y. Sun *et al.*, Qualitative and quantitative analysis of phenolics in Tetrastigma hemsleyanum and their antioxidant and antiproliferative activities, *J. Agric. Food Chem.*, vol. 61, no. 44, 2013, pp. 10507–10515.

[83] P. Gago-Ferrero, E. L. Schymanski, A. A. Bletsou, R. Aalizadeh, J. Hollender, and N. S. Thomaidis, Extended Suspect and Non-Target Strategies to Characterize Emerging Polar Organic Contaminants in Raw Wastewater with LC-HRMS/MS, *Environ. Sci. Technol.*, vol. 49, no. 20, 2015, pp. 12333–12341.

[84] K. S. R. Raju, N. Kadian, I. Taneja, and M. Wahajuddin, Phytochemical analysis of isoflavonoids using liquid chromatography coupled with tandem mass spectrometry, *Phytochem. Rev.*, vol. 14, no. 3, 2015, pp. 469–498.

[85] E. L. Schymanski *et al.*, Strategies to characterize polar organic contamination in wastewater: Exploring the capability of high resolution mass spectrometry, *Environ. Sci. Technol.*, vol. 48, no. 3, 2014, pp. 1811–1818.

[86] E. L. Schymanski *et al.*, Identifying small molecules via high resolution mass spectrometry: Communicating confidence, *Environ. Sci. Technol.*, vol. 48, no. 4, 2014, pp. 2097–2098.

[87] E. Anklam, A review of the analytical methods to determine the geographical and botanical origin of honey, vol. 63, no. 4, 1998.

[88] A. M. Gómez-Caravaca, M. Gómez-Romero, D. Arráez-Román, A. Segura-Carretero, and A. Fernández-Gutiérrez, Advances in the analysis of phenolic compounds in products derived from bees, *J. Pharm. Biomed. Anal.*, vol. 41, no. 4, 2006, pp. 1220–1234.

[89] S. Trautvetter, I. Koelling-Speer, and K. Speer, Confirmation of phenolic acids and flavonoids in honeys by UPLC-MS*, *Apidologie*, vol. 40, 2009, pp. 140–150.

[90] I. A. A. da Silva *et al.*, Phenolic profile, antioxidant activity and palynological analysis of stingless bee honey from Amazonas, Northern Brazil., *Food Chem.*, vol. 141, no. 4, 2013, pp. 3552–3558.

[91] A. Michalkiewicz, M. Biesaga, and K. Pyrzynska, Solid-phase extraction procedure for determination of phenolic acids and some flavonols in honey, *J. Chromatogr. A*, vol. 1187, no. 1–2, 2008, pp. 18–24.

[92] S. Kečkeš, U. Gašić, T. Ć. Veličković, D. Milojković-Opsenica, M. Natić, and Ž. Tešić, The determination of phenolic profiles of Serbian unifloral honeys using ultra-high-performance liquid chromatography/high resolution accurate mass spectrometry, *Food Chem.*, vol. 138, no. 1, 2013, pp. 32–40.

[93] K. McComb and R. Frew, *Using new analytical approaches to verify the origin of honey*. Woodhead Publishing Limited, 2013.

[94] M. Ciulu, N. Spano, M. I. Pilo, and G. Sanna, Recent Advances in the Analysis of Phenolic Compounds in Unifloral Honeys, *Molecules*, vol. 21, no. 4, 2016.

[95] K. Pyrzynska and M. Biesaga, Analysis of phenolic acids and flavonoids in honey, *TrAC - Trends Anal. Chem.*, vol. 28, no. 7, 2009, pp. 893–902.

[96] A. Y. Badjah Hadj Ahmed, M. S. Obbed, S. M. Wabaidur, Z. A. Alothman, and N. H. Al-Shaalan, High-performance liquid chromatography analysis of phenolic acid, flavonoid, and phenol contents in various natural yemeni honeys using multi-walled carbon nanotubes as a solid-phase extraction adsorbent, *J. Agric. Food Chem.*, vol. 62, no. 24, 2014, pp. 5443–5450.

[97] S. Amiry, M. Esmaiili, and M. Alizadeh, Classification of adulterated honeys by multivariate analysis, *Food Chem.*, vol. 224, 2017, pp. 390–397.

[98] B. Dimitrova, R. Gevrenova, and E. Anklam, Analysis of phenolic acids in honeys of different floral origin by solid-phase extraction and high-performance liquid

chromatography, *Phytochem. Anal.*, vol. 18, no. 1, 2007, pp. 24–32.

[99] P. K. Sarkar, P. K. Prajapati, V. J. Shukla, B. Ravishankar, and A. K. Choudhary, Toxicity and recovery studies of two ayurvedic preparations of iron, *Indian J. Exp. Biol.*, vol. 47, no. 12, 2009, pp. 987–992.

[100] A. Malachová, M. Sulyok, E. Beltrán, F. Berthiller, and R. Krska, Optimization and validation of a quantitative liquid chromatography-tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices, *J. Chromatogr. A*, vol. 1362, 2014, pp. 145–156.