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Greek Honey Authentication: Botanical Approach

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Definition: Honey is a functional, honeybee product with a useful role in human nutrition and several health benefits. Greece is a Mediterranean region with several types of monofloral honey. Today, Greek honey has acquired an important position in national and international markets. Due to this increased industrialization and globalization, quality control is a necessity. Mislabeling constitutes one of the most notable types of fraudulence, while most consumers are looking for authentic honey. Moreover, producers and suppliers are searching for rapid and analytical methodologies to secure Greek honey in a competitive environment. In this context, we aimed to describe the classical (melissopalynological, physicochemical) and analytical (chromatographic, spectrometric, and spectroscopic) methods for the standardization of the botanical origin of Greek honey.

Keywords: Greek honey; authentication; melissopalynology; physicochemical; chromatography; spectroscopy



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1. Introduction and Research Field

Honeybees are an important group of insect pollinators; while they produce various bee products, honey is the most well-known. Since ancient times, honey constitutes the only sweetening product that can be stored and used exactly as produced in nature, a fact that makes it very important in terms of its authenticity. Practically, all types of honey are authentic and only human activity can affect them.

From a legal viewpoint, the European council directive (2001/110/EC) [1] defines honey as, “the natural sweet substance produced by *Apis mellifera* L. bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature”. Additionally, composition criteria including physicochemical characteristics according to main types of origin (blossom or honeydew), production, and/or presentation (comb, chunk, drained, extracted, pressed, filtered, and baker’s honey).

According to the literature during 1963–2017, in countries around the Mediterranean Basin, a total of 336 species of wild bees and honeybees and 54 beekeeping plants families were approximately estimated [2]. Greece is mainly inhabited by four common *Apis mellifera* L. subspecies namely *A.m. cecropia* in central and southern Greek mainland, *A.m. carnica* in Ionian Islands, *A.m. adami* in Crete and southern Aegean, and *A.m. macedonica* in Macedonia, Thrace, and parts of Thessaly and Epirus (Figure 1) [3].

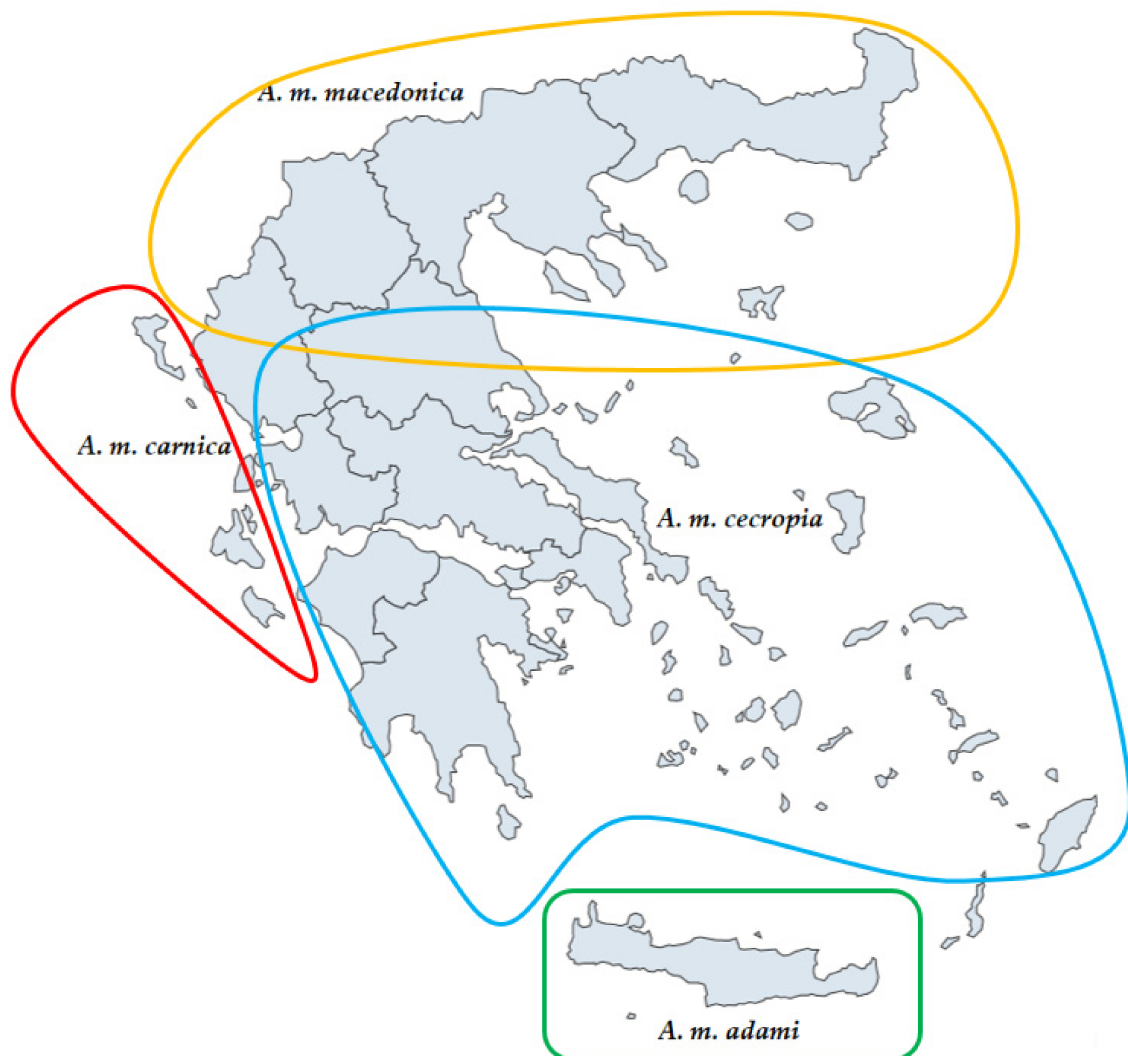


Figure 1. Four common *Apis mellifera* L. subspecies in the Greek region.

Beekeeping plants provide nectar, honeydew, and/or pollen to honeybees. “Blossom honey” is produced from flower nectar, while “honeydew honey” is from honeydew secretions from insects parasitizing the plants; various mixtures are also produced. The period when a plant provides food is called the “flowering period”. Greece has a wide variety of indigenous and nonindigenous melliferous plants. The most common botanical species producing monofloral honeys in Greece are included in Table 1. Greek legislation has set more strict criteria (Table 2) compared to the European legislation regarding the eight most common monofloral honeys [4].

Today, most consumers are looking for authentic foods [5]. This growing demand is directly connected with market globalization, e-commerce, food chains, and national and international trade. In addition, due to strong economic motivations, more types of fraud are observed, including mislabeling and false declaration regarding origin (Figure 2). Food authentication according to the CEN Workshop Agreement 17,369:2019 is “a food product where there is a match between the actual food product characteristics and the corresponding food product claims; when the food product actually is that the claim says that is” [6]. Moreover, Codex Alimentarius described fraud as “any deliberate action of businesses or individuals to deceive others in regards to the integrity of food to gain undue advantage” [7].

Table 1. Melliferous species and honeys in Greek region.

Scientific Name	Flowering Period	Nectar	Pollen	Honeydew	Honey Name	Commercially Widespread
Blossom Honeys						
<i>Arbutus unedo</i> L.	November–December	3 *	2	-	Strawberry tree	+ **
<i>Castanea sativa</i> Miller	June	2–3	3	1–2	Chestnut	++
<i>Ceratonia siliqua</i> L.	September–October	3	3	2	Carob	+
<i>Citrus</i> spp.	March–April	3	2	-	Citrus, orange etc.	++
<i>Erica arborea</i> L.	October–November	2–3	2–3	-	Spring Heather	++
<i>Erica manipuliflora</i> Salisb.	March	3	2–3	-	Autumn Heather	++
<i>Eucalyptus</i> spp.	May–July	2–3	2–3	-	Eucalyptus	+
<i>Gossypium hirsutum</i> L.	July–September	-	-	-	Cotton	++
<i>Helianthus annuus</i> L.	June–August	2–3	2–3	-	Sunflower	+
<i>Paliurus spina-christi</i> Miller	May–June	2–3	2	-	Jerusalem thorn	+
<i>Phlomis</i> spp.		2–3	-	-	Jerusalem sage	+
<i>Pimpinella anisum</i> L.		1–2	1–2	-	Anise	+
<i>Polygonum aviculare</i> L.	July–August	2	2	-	Common knotweed	+
<i>Salvia officinalis</i> L.		2–3	2	-	Sage	+
<i>Thymbra capitata</i> L.	June–July	2–3	2	-	Thyme	+++
Honeydew Honeys						
<i>Abies cephalonica</i> Link.	May–July	-	-	3	Fir	++
<i>Pinus</i> spp.	March–April, June–August, September–October	-	-	3	Pine	+++
<i>Quercus</i> spp.		-	3	3	Oak	+

* Number 1: low contribution; number 2: medium contribution; number 3: high contribution; dash (-): no contribution. ** high (+++), medium (++), and low (+) commercially widespread.

Table 2. Greek legislation criteria of eight common monofloral honeys.

	Pine	Fir	Chestnut	Heather	Thyme	Citrus	Cotton	Sunflower
Moisture (%)	-	≤18.5	-	-	-	-	-	-
Electrical conductivity (Ms cm ⁻¹)	≥0.9	≥1.0	≥1.1	-	≤0.6	≤0.45	-	-
Main pollen (%) of pollen of nectar plants	-	-	≥87	≥45	≥18 *	≥3	≥3	≥20
HDE/P **	varies	varies	-	-	-	-	-	-
TPG/10g ***	varies	varies	≥100,000	-	<90,000	<70,000	<90,000	<55,000
	major presence of characteristic honeydew elements	minor presence of characteristic honeydew elements	-	-	-	-	-	-

* The percentage of accompanying pollen grains of a plant species should not exceed 45%. ** Honeydew elements/pollen. *** Total number of pollen grains.

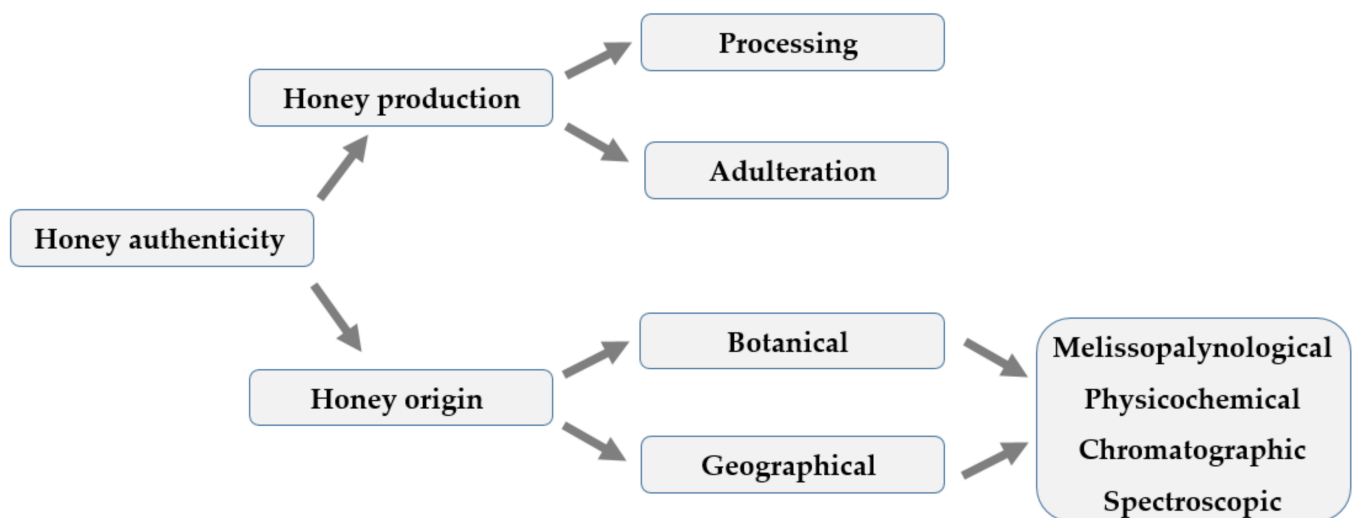


Figure 2. A summary of the honey authenticity fields.

The notion of honey authenticity has received great interest worldwide and increased focus in the last twenty years. However, prior to the commentary of the honey authenticity techniques one must distinguish the concept of “honey quality”, “honey standardization”, and “honey packaging” (Figure 3). Honey quality is a summary of characteristics that are considered important for determining the degree of acceptance by the consumer. Honey standardization is the process by which specifications are established of its production, the composition, and the properties. Finally, the packaging is their placement inside a packaging material to be protected from physical, chemical, and biological hazards and to be transported.



Figure 3. From “honey quality” to “honey packaging”.

According to the Scopus database, the most studied authenticity issue is the honey botanical origin differentiation. From reviewing, the most frequent analytical methods of honey botanical discrimination are classical and instrumental chemistry analyses. However, emphasis was given to specific botanical markers and/or in representative “fingerprint” spectra. Table 3 gives an overview of the most ordinary methods for honey authentication.

Table 3. A summary of the methods for the botanical differentiation of honey.

Analytical Technique	Abbreviation	Main Analytes and Parameters
Melissopalynological and Physicochemical techniques		
Optical microscopy	OM	Pollen analysis
Scanning Electron Microscope	SEM	
Conductimetry		Electrical conductivity
Refractometer		Moisture
Colorimetry-Photometry		Diastase (Heat abuse)
		Hydroxymethylfurfural (HMF) (Heat abuse)
Potentiometry		Acidity
International commission on Illumination	CIE	Lightness, color, hue
Viscometer		Rheological properties
pH-meter		pH
Chromatographic techniques		
High-Performance Liquid Chromatography Diode-Array Detector	HPLC-DAD	Hydroxymethylfurfural (HMF)
		Phenolics
High-Performance Liquid Chromatography Refractive Index Detector	HPLC-RID	Sugars
High-Performance Liquid Chromatography Fluorescence Detector	HPLC-FS	Amino acids
		Phenolics
High-Performance Liquid Chromatography Pulsed Amperometric Detector	HPLC-PAD	Sugars
High-Performance Thin-Layer Chromatography	HPTLC	Phenolics
		Non-volatile components
		Sugars and/or fructose/glucose ratio
Liquid Chromatography Mass Spectrometry	LC-MS	Hydroxymethylfurfural (HMF)
		Phenolics
Gas Chromatography Mass Spectrometry	GC-MS	Volatiles
		Semi-volatiles
Spectroscopic techniques		
Ultraviolet-Visible Spectroscopy	UV-Vis	Spectrum of phenolics
Raman Spectroscopy	Raman	Sugars spectra and minor components
Fourier-Transform Mid-Infrared Spectroscopy	FT-MIR	Sugars spectra and minor components
Fourier-Transform Near-Infrared Spectroscopy	FT-NIR	Sugars spectra and minor components
Fluorescence Spectroscopy	FS	Spectra of amino acids, phenolics, Maillard reaction by-products
Nuclear Magnetic Resonance	NMR	Sugars, untargeted and targeted screening

Table 3. Cont.

Analytical Technique	Abbreviation	Main Analytes and Parameters
Other techniques		
Isotope-Ration Mass Spectrometry	IRMS	Isotope ration of H, C, N, S, and/or ¹³ C ratios
Inductively Coupled Plasma Mass Spectrometry	ICP-MS	Chemical elements

2. Harvest, Honey Identity, and Authenticity Issues

2.1. Honey Harvesting

Honey harvesting is the most significant step before any further analysis. Honey is primarily a concentrated solution of sugars with other compounds such as organic acids, enzymes, vitamins, minerals, phenolics, and volatiles [8].

Honey composition is dependent on the plants that honeybees visit. Most beekeepers know the floral sources from which their honeybees collect nectar and pollen. This is because they consciously choose the flowering period and location of the hive. However, some beekeepers move the hives to more than one area in order to collect nectar sources from a wider area. In those cases, multifloral honey is produced. In addition, honey composition can be affected by beekeeper's manipulations, postharvest processing [9], and storage conditions and length [10]. After harvesting, honey is subjected to various postharvest processing steps including extraction and sometimes dehumidification, liquefaction, heating, or pasteurization [11]. Finally, packaged honey must remain under cool and shady conditions before further use.

2.2. Classical Methods for Honey Authentication

Generally, melissopalynology is a microscopic analysis of honey and it is the basic method for determination of their botanical origin. Blossom honeys are considered mainly from one or more sources of pollen grains. According to legislation criteria, when the pollen content is over- or under-represented, honey can be characterized as unifloral or polyfloral. In addition, for honeydew honeys, the ratio of honeydew elements/pollen (HDE/P) is taken into account for botanical determination. Melissopalynological analysis constitutes a classic and widely used method for detecting botanical origin of Greek honey [12–18]. Tsigouri et al. [15] gave some palynological characteristics of 208 different monofloral honeys including fir, pine, chestnut, cotton, citrus, and thyme. Karabournioti et al. [13] carried out melissopalynological analysis in 135 thyme honeys and quantitated 65,000 pollen grains per 10 g of thyme honeys. More recently, Rodopoulou et al. [17] applied microscopic analysis to determine the botanical origin of thyme honeys, while they investigated the effects of over-presented pollen grains in blend honeys. However, they concluded that in some cases pollen analysis did not give trustworthy results and should be combined with other analyses. Recently, Tsiknakis et al. [18] applied machine learning to classify Cretan pollen grains with overall detection accuracy of 92%.

Pollen grains come mainly from the plants foraged by honeybees, while these pollens provide the botanical origin [19]. However, melissopalynological analysis requires specialized staff with experience in pollen grain recognition. Furthermore, this analysis always needs literature on beekeeping plants and optical or a scanning microscope for greater accuracy. Moreover, the possibility of human error is high with subjectivity in the entire process. Fraudulent counterfeiting actions, such as removing existing pollen and replacing it with another, could alter pollen content.

Reviewing the literature, many studies were based on physicochemical analyses to discriminate monofloral Greek honeys [12,16,17,20–22]. In most cases, physicochemical analyses showed a good success rate in classifying honey. Generally, honeydew honey is characterized by higher values of electric conductivity and acidity compared to blossom honey. On the other hand, blossom honey is richer in monosaccharides, and lighter colored.

Further, physicochemical analyses as defined by Greek legislation provide information for quality (moisture must be lower than 20% *w/w*), freshness (diastase not lower than 8 Schade and HMF not higher than 40 mg/kg), stability, and shelf life of honey. Even so, sporadically, the dispersion of the above values, associated with the nature and heterogeneity of honey produces overlapping and reduces their usefulness. Physicochemical analyses overall are time-consuming and non-environmentally friendly techniques. In addition, they require large quantities of honey, a lot of chemical reagents, and trained labor. Even so, physicochemical analyses are a valuable reference and officially recognized methods and are widely used for the evaluation and characterization of blossom and honeydew honey, usually providing accurate and reliable results.

2.3. Analytical Methods for Honey Authentication

2.3.1. Chromatographic Techniques

Chromatographic techniques, including mainly LC and GC, are the most commonly used methodologies for honey authentication. These analytical techniques can be coupled with many detectors for qualitative or quantitative analysis of several compounds. A variety of methods have been developed during the last 20 years to meet with the demand for reliable certification of monofloral honey.

Prior to LC analysis, extraction of target compounds is carried out including sugars, phenolic compounds, amino acids, and other molecules. Phenolic compounds are present in all honeys and largely dependent on the botanical origin, while some of them come from bee propolis. Amino acids originate mainly from pollen resulting in high variability between different floral kinds of honey. As a result, depending on the extraction method and the polarity of solvents, a different fraction of compounds is isolated each time. However, this selective extraction is an important piece of research that can give different results. LC has been used to differentiate Greek monofloral honeys [23–25]. Initially, interest was focused on the separation and identification of phenolic compounds of commercial Greek pine, thyme, and fir honey [26,27]. Then, classification efforts were carried out with an overall rate of 99.2% [24]. Recently, a study characterized the phenolic profile on ten different honey botanical origins by a targeted and untargeted analysis [25]. The main advantage of the above technique is that it allows simultaneous measurement of several compounds in one analysis. Nevertheless, sample isolation procedures, which are often regarded as time-consuming, are needed. In addition, an LC system is expensive and complex.

Numerous studies have been carried out in the last years to evaluate GC analysis in the separation and quantification of volatiles and semi-volatiles. These volatile organic compounds (VOCs) originate from source plants, while some of them are transformed by honeybees or processes like heating and storage. Furthermore, VOCs analytes are dependent on isolation techniques and solvents. Compounds with high volatility isolated by solid-phase micro extraction (SPME) and semi-volatiles in most cases by liquid-liquid extraction (LLE), ultrasound extraction (USE), and solid phase extraction (SPE) using non-polar solvents. Almost exclusively, GC was combined with MS detectors in order to identify the isolated compounds. Alissandrakis et al. [28] used ultrasound-assisted extraction for isolation and relation volatiles and semi-volatiles from citrus honeys and citrus flowers. Thus, GC-MS analysis indicated associations that are mainly due to linalool derivatives. Two years later, Alissandrakis et al. [29] determined a total of 15 volatile compounds that could serve as potent markers for cotton honey. Later on, the determination of volatile compounds based on SPME coupled with GC-MS was adopted to investigate the dominant volatile fraction of Greek citrus and thyme honey [30,31]. In the same period, Tananaki et al. [32] analyzed 22 samples of pine honey from the Greek region and determined their characteristic volatiles. Then, GC-MS was applied for the botanical discrimination of 77 monofloral honeys (chestnut, cotton, fir, heather, pine, thyme, and citrus) [33] with correct classification of samples higher than 98%, while Karabagias et al. [21] tried to classify fir, thyme, pine, and orange honeys with an overall rate of 84.0%. In a more recent study, the volatile fraction of some common and rare honeys by a non-targeted

metabolomics methodology using GC-MS was studied [34] using SPME-GC-MS, while later, 151 honeys were classified into seven groups (clover, citrus, chestnut, eucalyptus, fir, pine, thyme) based on 56 volatile compounds with classification rate of 95.4% using the SPME technique [35]. Xagoraris et al. [36–38] identified key volatiles compounds which were directly associated with the botanical origin of Greek honey (thyme, pine, fir, citrus, heather) and will enable the development of analytical methods based on GC-MS for application in industrial setting for botanical honey authentication. Furthermore, the interest has been focused on rare honey varieties such as strawberry tree honey [39]. GC constitutes a suitable technique with high resolution and reproducibility. However, as in the case of LC, it needs skilled operators and the whole system is expensive.

Following the above chromatographic techniques, multivariate statistical analysis in combination with LC and GC analytical data could be a powerful tool for botanical authentication of honey. These statistical tools can be more powerful when combined with internal and external validation sets to enhance the robustness of the proposed chemometric models and could be used by industry.

2.3.2. Spectroscopic Techniques

UV-Vis, FTIR, Raman, FS, and NMR have been proposed as rapid methods for honey authentication. They provide information of honey fingerprint and can be used for routine analysis. However, only a few studies have evaluated their potential to determinate the botanical origin of Greek monofloral honey.

UV-Vis (200–900 nm) is a traditional spectroscopic technique and this region of absorbance has been related to various compounds such as phenolics and sugars (mainly glucose and fructose). Orfanakis et al. [40] based on UV absorption (200–400 nm) classified blossom and honeydew honeys with a successful estimation of 92.65% and 91.30%, respectively. This result showed that UV spectroscopy can provide an alternative approach to determine the botanical origin of honey.

Mid FTIR (4000–400 cm^{-1}), especially combined with Attenuated Total Reflection (ATR), is a well-established technique recording the vibration bonds of water, sugars, phenolics, carboxylic acids, and amino acids. However, Mid FTIR have the inability to measure compounds with very low concentrations and difficulty in samples with high presence in water, due to the strong O-H absorption. Honey FTIR spectra consist of a region between 4000 and 1500 cm^{-1} attributed to functional groups and a region between 1500 and 750 cm^{-1} attributed to sugars and is known as a honey “fingerprint”. Orfanakis et al. [40] developed a chemometric model with correct classification rate of 95.56% and 100% for multifloral and thyme honeys, respectively, based on FTIR region between 4000 and 2400 cm^{-1} . In addition, in a previous study, a chemometric model was developed based on 847–803 and 1390–945 cm^{-1} spectral regions with a classified rate of 93% [36]. These monofloral honey samples from four botanical origins (thyme, pine, fir, citrus) were cross-validated with a successful percentage of 82.3%, while external validation identified correctly 84.6% of test set samples [36].

Raman spectroscopy is a simple technique used to study vibrational, rotational, and other low frequency models in a sample such as honey. Moreover, Raman spectra are not affected by the presence of water, constituting an advantage compared to FTIR. However, this analytical technique has barely been studied in evaluation in Greek honey authentication. A recent study proposed a chemometric model for the discrimination of botanical origin of three (thyme, pine, fir) Greek common honeys [16]. The developed model estimates a recognition of standards of 95.3%, whilst cross-validation and external validation were 90.6% and 84.3%, respectively [16].

FS (electronic transition $S_1 \rightarrow S_0$ with timescale of 10^{-9} to 10^{-6}) is rapid and 100–1000 times more sensitive than other spectroscopic techniques. FS spectra are often complicated due to Raman and/or Rayleigh scattering. To avoid scattering, Front-Face Fluorescence (FFS) or Synchronous Fluorescence Spectroscopy (SFS) can be applied. However, it is not necessary that the above scattering affect the spectral area which was investigated. Thus,

right-angle fluorescence spectroscopy can be used. Honey contains intrinsic fluorochrome compounds, including phenolics, flavins, Maillard reaction products, and amino acids. An alternative novel method, based on hydroxycinnamic and other phenyl carboxylic acids, was developed to evaluate the potential of right-angle fluorescence to distinguish and determine the botanical origin of four Greek honey samples (thyme, pine, fir, citrus) [41]. All chemometric models were considered successful and they can be used for routine analysis.

NMR has achieved general acceptance because of its noninvasive characteristics in honey analysis. However, the above technique produces a very complex spectra, with much information mainly for sugar profile. The NMR profile of Greek honey was investigated previously by some studies [42,43]. Nevertheless, to the best of our knowledge, no study using NMR spectroscopy has been performed for Greek honey authentication.

During the last years, spectroscopic methods have become increasingly practicable as a novel application for determining the botanical origin of honey. They have many advantages including high simplicity, speed, repeatability, and accuracy, while they are environmentally friendly, nondestructive, and noninvasive for the samples. In this context, more research is required on their application in botanical differentiation of Greek honey.

2.3.3. Other Analytical Techniques

Mineral content can be identified by ICP analysis combined with MS or Optical Emission Spectrometry (OES). Nevertheless, the profile of mineral content can give information mainly for geographical origin [44–47] and secondarily for botanical origin [22,48] of Greek honey samples.

Other alternative techniques such as Laser Induced Breakdown Spectroscopy (LIBS) [49] and Stable Isotope Ratio MS (IRMS) [50] have been also investigated for their potential as analytical tools for honey authentication. However, all the above techniques must be combined with chemometric tools to extract their information from the data sets.

3. Conclusions

To summarize, the authenticity of Greek honey has acquired increasing interest from consumers, producers, suppliers, and therefore scientists. These increasing demands push the adoption of legislation criteria that will be imposed on honey suppliers worldwide. However, the current melissopalynological and physicochemical criteria are complex, allowing the mislabeling of botanical origin. In recent years, chemometric models based on synchronous chromatographic and spectroscopic techniques have been efficiently used. These techniques include among others, LC, GC, UV-Vis, FTIR, Raman, FS, NMR, ICP, LIBS, and IRMS. Chromatography could be used as a screening tool, while many studies dealt with phenolics and VOCs as potential botanical markers of monofloral Greek honey. Spectroscopy as an application tool is rapid, relatively low-cost, environmentally friendly, and can be applied in both industries and/or fundamental research monitoring the botanical determination of Greek honey.

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