# Honey of Authenticity: An Analytical Approach

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**Abstract**

The safety and quality of any food product is a primary concern. Adulteration of honey increases day by day in the market. Authentication is very important to confirm purity. Honey is a natural food product that is ready to eat with a high nutritional value which provides several health advantages. Adulterations of honey with sugar or syrups are common practice. Chemical tests and different analytical techniques are used to detect the adulterant in honey. Diverse ranges of the analytical techniques are employed for the analysis of honey-like chromatography, electro-analytical methods, and spectrophotometer technique. Estimation of adulterants even in low quantity can be detected by sophisticated instrument. Analysis in every step is required—part of preliminary screening, processing, and product standards. Most of the analytical methods provide information of pollen distribution, physicochemical parameters, and profile analysis of phenolic, flavonoid, carbohydrate, amino acids, aroma, and individual marker components.

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Honey · Adulterants · Authentication · Analytical technique · Chromatography

## 6.1 Introduction

Honey has been used by human beings since ancient times. Sumerian tablet was a written evidence found in 2100–2000 BC (Crane 1975) "A natural sweet substance formed by bees (Apis mellifera) from a different source of plants like nectar, living parts, and excretion, collect it and transform by combining with specific substances of their own" defined by the European Union (European Commission 2002; Zielinski et al. 2014). Classified honey based on origin, harvest, and process. Further, based on origin, it is categorized into blossom, honeydew, monofloral, and multifloral kinds of honey. Honey obtained through the nectar of flowers was named blossom honey, and honeydew is formed by bees from plant saps. Monofloral honey has more than 45% of the total pollen content of single species of plant. Honey is also classified on the basis of sources such as plant-like citrus, manuka, and acacia honey (Alvarez-Suarez et al. 2010b). The botanical source of multifloral or polyfloral honey is meadow and forest. Based on the secretion of plants, honey can be categorized in to blossom honey produced from nectar of flower and honeydew made from secretions of all livings parts other than flowers. The botanical origin of nectars and secretion of plants are the chief concern of honey's composition and its properties, and carbohydrate is the main constituents (Bertelli et al. 2010).

### 6.1.1 Chemical Composition

The main ingredient of honey is carbohydrate; dextrose and laevulose are two important sugars, and several other sugars are there in small fraction including disaccharides and trisaccharides. Examples of disaccharides are maltose, sucrose, maltulose, turanose, isomaltose, laminaribiose, nigerose, kojibiose, gentiobiose, and trehalose, and examples of trisaccharides are maltotriose, erlose, melezitose, 1-kestose, isopanose, isomaltotriose, panose, and endorse. All different kinds of plants have these sugars in small quantities (Bogdanov et al. 2004). Apart from sugars, many kinds of organic acids exist, for example, gluconic, lactic, formic, butyric, tartaric, pyruvic, acetic, citric, oxalic, succinic, malic, maleic, α-ketoglutaric, glucose-6-phosphate, pyroglutamic, and glycolic acid. The most common acid is gluconic acid that is formed from oxidation of glucose's first carbon by the enzyme glucose oxidase. The presence of an enzyme in honey makes it unique in a contest of a medicinal point of view. Yeasts, nectar, pollen, bee, and microorganisms are sources of enzyme. Key enzymes are glucose oxidase, catalase, acid phosphatase, invertase, and diastase. Enzyme activities are destroyed on heating (Hebbar et al. 2003).

The physical and biological properties depend upon the quality and quantity of organic acids; role of enzymes are cardinal. Mineral contents are the influencing factor for storage; rich contents are less suitable at low temperature. Honey of floral origin has mineral content varying from 0.02 to 0.1% (Olga et al. 2012). Quality of honey concerning nutritional, granulation, flavor, texture, and its medicinal value are controlled by the presence of ingredient such as moisture content, reducing sugars, electrical conductivity, free acids, sucrose content, and hydroxymethylfurfural (HMF). For the production of honey in industry, physicochemical properties are very important. The International Honey Commission (IHC) imposed fixed composition to maintain the quality of honey. The chief ingredient is sugar, 95% weight. It is a complex mixture of concentrated sugar solution and has main ingredients fructose and glucose (Aljadi and Kamaruddin 2004). Ratio of glucose to fructose in any kind of honey depends upon the nectar's source. Other bioactive substances like organic acids, proteins, amino acids, minerals, polyphenols, vitamins, and aroma also have impact on the quality of honey (Ferreira et al. 2009; Ramanauskiene et al. 2012). Taste and color qualities of honey come up with the presence of sugars, amino acids, minerals, and phenolic compounds, but aroma is because of volatile substance presence. The percentage of protein reported in different kinds of honey with a small portion of enzymes is less than 0.5% (Yao et al. 2005).

To maintain the quality, a directive requirement is necessary for the standard composition by the regulatory bodies. At the international level, the Codex Alimentary Standard commission (FAO 1981) imposed the compositional criteria, i.e., fixed the acidity, apparent reducing sugar, 5-hydroxymethylfurfural (HMF), mineral content, moisture, and water-insoluble solids (Belay et al. 2013). In an acidic environment, a chemical reaction takes place reducing sugars and giving HMF. The level of HMF deciding the age and overheating of the honey, its concentration fixed by regulatory bodies with a highest limit of 40 mg/kg exception, 80 mg/kg for tropical honey.

### 6.1.2 Biological Activities

Honey has several biological properties such as antimicrobial, antiviral, anti-inflammatory, wound and sunburn healing, antioxidant, antiparasitic, antidiabetic, antimutagenic, and anticancer activities (Gomes et al. 2010). Pharmacological study research by a team of scientist reported that natural honey has the potential to cure gastric and cardiovascular disease without an increase in body weight, apart from these observed advantageous effects on fertility by enhancing the effects of hormones related to fertility (Alvarez-Suarez et al. 2010a; Mosavat et al. 2014). Patients of type I and II diabetes are advised to take honey because of its lower glycemic index value. Uses of honey by diabetic patients find pharmacological change that helps to cure the disease, raise the hemoglobin level, restore secretion of insulin, reduce blood glucose level, and refine lipid profile. The phenolic content of honey establishes antioxidant properties and intensity of color reported by the researcher (Piljac-Žegarac et al. 2009). Other therapeutic effects exhibited by honey are

anticarcinogenic, anti-inflammatory, antiatherogenic, antithrombotic, immune-modulating, and analgesic activities because of phenolic contents (Yaghoobi et al. 2008). Athletes generally take honey as a source of energy. Infection by bacteria either gram-positive or gram-negative including aerobes and anaerobes is studied in around 60 types. Treatment of such bacterial infection by honey has been reported (Molan 2006). In Egypt, back to 1553–1550 BC, medical practitioners uses honey for the treatment of wound, urination, and obesity. Similarly, Galen, a renowned physician prescribed honey to cure the disease of poisoning and intestinal disturbance. Honey was prescribed by the greatest medical authority of medieval times Ibn-Sina (Ave-Sina) to cure the diseases like runny nose, digestion of food, improve appetite, boost up the memory power, increase the blood circulation, and enhance the intelligence. Advanced research published that honey can cure several diseases and boost up the immune system of the body. A balanced composition of various content improves the resistance against the pathogenic organism. Intestinal infection caused by nematodes, including ascariasis and hookworm is also cured by honey (Sajid and Azim 2012). The presence of glycoproteins and peptides are responsible for immunomodulatory properties which exhibit as these molecules are interfering with the innate immune system in humans (Mesaik et al. 2014). The nutritive and medicinal values of natural honey with unique flavor make it very expensive and demanding. To fulfil the requirements of the consumer with low cost leads to adulteration. Used adulterants are very difficult to detect; they replace the natural properties and finally lead to decreasing both nutritive and medicinal values. Thus, global authenticity is a very important concern for the consumer as well as the manufacturer.

## 6.2 Adulteration

Adulteration makes the quality poorer, as well as the safety of the product is questionable. The adulterants are chemical substances, which lower the medicinal and nutritive values which harm human beings; almost 128 chemicals are reported as adulterants. In the market, adulterants available are generally starch syrup, inverted syrup, starch or inverted syrup fed to bees, and in some places low-grade honey is mixed with standard quality of honey. The process of adulteration may be a direct or indirect method. If a substance is directly added to honey, it is called direct method, and when honey bee is fed with chemicals and industrial sugars, it is called indirect method. So the detection of adulterants added through indirect method is very difficult as compared to adulterants added through direct method (Fig. 6.1). Examples of industrial sugar as adulterants that are frequently used are high fructose corn syrups (HFCS), high fructose insulin syrups (HFIS), invert syrups (IS), and corn syrups (CS). Syrup and invert sugar adulterants have been used which are chemically similar to a pure substance in which the concentration of honey is increased and is very difficult to detect. In quality control by the analyst, it is very difficult to determine the differences between pure and adulterated honey by analytical methods (Mehryar and Esmaiili 2011). Rice syrup as adulterants is also available in the market.

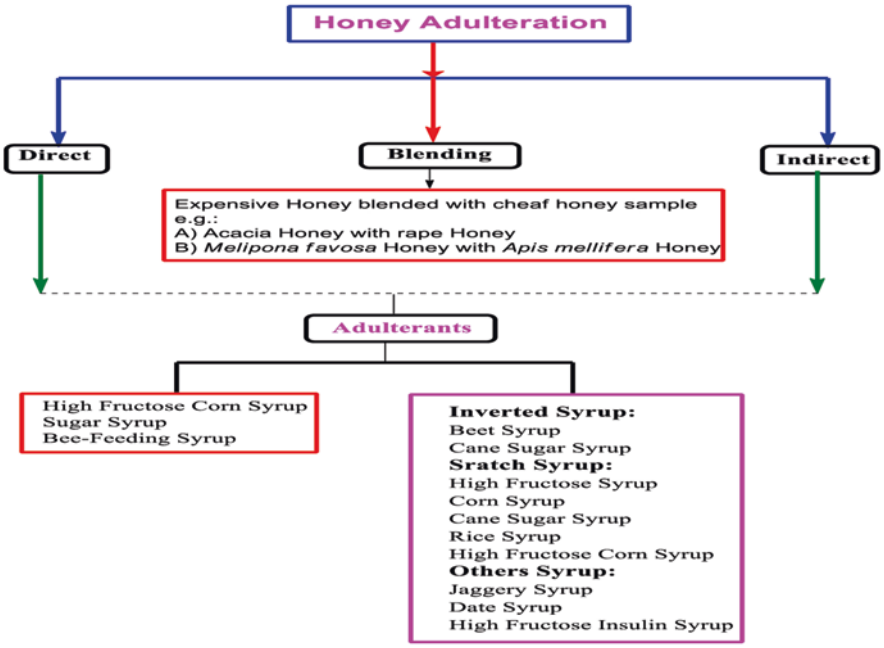


**Fig. 6.1** Different types of honey adulterations

## 6.3 Authenticity

Presently, few authorities like Codex Alimentarius standard, the EU Honey Directive, and other legislations work at the national level to regulate the food authenticity. But, growing business globally and its medicinal uses require making an international standard regulation for authenticity. For the perfect authenticity of honey, we need to focus on two important points, first is its origin and second is the mode of production. Origin of honey may be either geographical or botanical while its production is defined by the way of harvesting and processing. The regulatory bodies International Honey Commission lately examined the Codex Alimentarius (CA) standards and European Community standards. In several countries, few adulterants are very easily available for adulteration in the market such as cheaper sweeteners from beet or canes like corn syrups (glucose), high fructose corn syrup (HFCS), saccharose syrups, and invert sugar syrups (Tosun 2013). Artificial honey is also manufactured in several places in the world adulterated with bee feeding of sugars or syrups (Bogdanov and Gallmann 2008). Monofloral honey has high market value as it is recognized by the consumer, but it is adulterated with cheaper multifloral honey (Soares et al. 2015). Authenticity of honey, and to find out its purity, is a very important task. The purity of honey must be checked by the processors, retailers, consumers, and regulatory authorities in every step by analytical methods followed by the regulation of regulatory bodies at national and international levels.

## 6.4 Detection Methods and Techniques

Authentication of honey of botanical origin by classical approach is common practice and monofloral origin by sensory and physicochemical analyses; melissopalynological analysis examines floral pollen grains in honey by microscopic origin (Bogdanov et al. 2004). Many factors are responsible for fraction of pollen content in honey such as species of plant, collection time, and nectar yield from a male or female flower. Pollen is sometimes collected from the bee's honey sac and illegally added to honey (Donarski et al. 2008). This method is not sufficient for the identification, but it has to be analyzed by the sensory method and physicochemical characteristics as pollen contents have some natural variation. Authentication with this method is a very tedious job, and numbers of physicochemical parameters are obligatory for the characterization. Classical authentication techniques have a limitation; modern analytical techniques are used to find out the origins of honey which is more reliable. Modern instruments like liquid chromatography and mass spectrometer (LCMS), infrared spectrometer, Raman spectrometer, nuclear magnetic resonance (NMR), and flame ionization detectors (FID) or sensor arrays are used. Research has been reported that adulterants like exogenous sugars or the addition of sugar syrups have been detected by different analytical techniques (Baroni et al. 2006). Sugar composition of honey is studied by high-performance liquid chromatography (HPLC) and chemometric method. Volatile composition and floral origin are determined by solid-phase microextraction (SPME) and gas chromatography coupled with mass spectrometry (GC–MS) (Baroni et al. 2006). By chemometric analysis, principal component analysis (PCA) and linear discriminate analysis (LDA) have been employed to estimate the controlling variables and, a likeness of honey samples. The molecular genetics approach is used to find out the composition, and geographical and entomological origins of honey (Chin and Sowndhararajan 2020) (Fig. 6.2).

### 6.4.1 Qualitative Physicochemical Analysis for Honey Identification

Honey quality is based on physicochemical parameters; it could be useful for the assessment of its origin. Routine determination of physicochemical parameters, water content, electrical conductivity, sugar content, fructose/glucose ratio, enzyme activity, color, ash value, optical rotation, pH value, acidity, and hydroxymethylfurfural (HMF) content is commonly used for both QC and processing control of honey. Several factors influence the final values of these parameters in honey. For water content, the most important factor is air humidity. The self-life of honey degrades when the content of water is high, low density, and high electrical conductivity. The density of honey is influenced by water content, lesser density in high water content and vice versa, less than 5% sucrose in honey indicates good quality of honey, and if the percentage is more than 5%, it indicates sucrose may be unripe and it is not completely converted by enzyme invertase into glucose and fructose (Ouchemoukh et al. 2007).

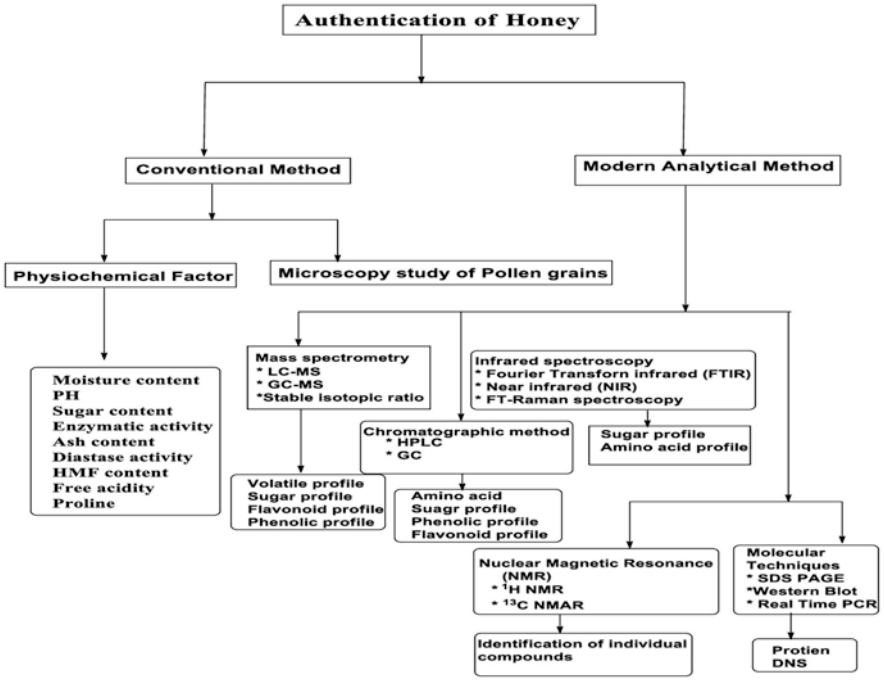


**Fig. 6.2** Classical and modern analytical methods used for honey authentication

Quality indicators of honey, i.e., invertase activity, diastase, and HMF indicate freshness and overheating of honey. The presence of low quantity diastase indicates low natural amylase in honey (Pasias et al. 2017). The purity of honey is checked by the presence of HMF, higher content indicates overheated, aged, and the duration of storage is long and in poor condition. Recommended standard is (80 mg/kg). The self-life of honey is 1 year and should be consumed (Khalil et al. 2010). Mineral content determined the electrical conductivity (EC), its value varies from region to region, for example, the values of electrical conductivity are 4.18 and 1.98 ms/cm for geographical regions Yemen and Egypt, respectively, and 0.53 and 0.67 ms/cm for Saudi and Kashmir, respectively (El Sohaimy et al. 2015). Acidity is because of the presence of gluconic acid, esters, lactones, and inorganic ions of chloride and phosphate, and the pH value of honey varies as the season of extraction varies. The pH values below 3.5 spoilage the honey. The pH value of fresh honey is 4.1–4.6, and the standard range is 3.4–6.1 (Codex Alimentarius 2001). Conversion of sugar into organic acid is fermentation; in such condition, the acidic value of honey is high, and acidity of honey influences the microbial spoilage and sustains honey flavor.

### 6.4.2 Microscopic Analysis of Pollen Grains

The subject, learning for pollen grains and spores is called palynology (early branch is called Melissopalynology). Resin, nectar, water, and pollen are a source of energy

for the honeybee. In pollens, the amounts of protein, minerals, vitamins, and fats are abundant. To know about the origin of honey, either it is geographical or botanical, the fingerprint of pollen and honeydew elements are very helpful. With microscope pollens, it can be identified, and traditionally it is used for quality analysis (Hermosín et al. 2003). The procedure adopted for microscopic analysis is weigh accurately 10 g of honey and dissolve in a volume of 20 mL warm water (40 °C) after that at 2000 rpm centrifuge for two times for a duration of 10 min. Dregs are taken out, dried, put on a slide having chemical glycerin, gelatin, and stained with fuschin alcohol solution. Observe this slide under a microscope for the examination of pollen. This analysis indicates only morphological characteristics, not genus or species. Results of the study are analyzed and categorized into four types: very frequent, frequent, rare, and sporadic percentage of pollen in each category is 45%, 16–45% 3–15%, and less than 3%, respectively. Microscopic analysis along with physicochemical analysis is very helpful to recognize the standard of honey for the business purpose (Louveaux et al. 1970).

### 6.4.3 Detection of Adulterants by Microscope

A routine practice to detect the adulterants by a microscope is a classical method. Adulterants like cane sugar and acid hydrolyzed cane sugar syrup,in cane sugar, epidermis cells and single rings of ring vessel particles are present along with sugar cane starch and sclereids. With microscopic analysis, other instrumental techniques like HPLC are used for the estimation of glucose, sucrose, fructose, and HMF along with the determined the value of pH, water content, and electrical conductivity that is very helpful to find out the adulterants (Kerkvliet et al. 1995). Chemical analysis data reveal whether the honey is adulterated or heated. Qualitative physiochemical analysis for honey identification is presented in Table 6.1 (Sadasivam and Manickam 1996; Patil 2016).

### 6.4.4 Analytical Technique

Spectroscopic techniques are quick and noninvasive and do not require tedious sample preparation, which often involves laborious work and consumption of large amounts of organic solvents, reagents, and time. Therefore, they can be considered a green analytical alternative. The noninvasive nature of spectroscopic techniques allows investigation of intact food samples, which is particularly appropriate for high-throughput screening, especially in commercial production plants quality control.

Classical techniques have limitations for authentication; advanced analytical techniques have adopted for the examination of botanical and geographical origins of honey. Also, carbohydrate profile, mineral content, aroma profiles, and phenolic and flavonoid composition were studied (Ouchemoukh et al. 2010; Jerkovic et al. 2009). Chromatographic techniques like thin-layer chromatography (TLC)

**Table 6.1** Qualitative physiochemical analysis for honey identification

| Test | Procedure |
|---|---|
| Molisch's test | Detection of carbohydrate: take accurate volume of 2 mL sample in the test tube then add few drops of Molisch reagent +1 mL of concentrated $H_2SO_4$. If a red-violet color ring develops at the junction of the two liquids, it specifies the presence of carbohydrates in the honey sample |
| Fehling's test | Detection of reducing sugar: transfer a specific volume of sample in a test tube then add equal volume of Fehling's solution A and B in the test tube. After shaking, keep it in a boiling water bath for few minutes. Development of a brownish-red precipitate indicates the presence of reducing sugar |
| Benedict's test | Detection of reducing sugar: 2 mL of Benedict's reagent is mixed with a small volume of samples; heat the mixture for 5 min on a boiling water bath, then the mixture is cooled under tap water. Development of green, yellow, or red color indicates the presence of reducing sugar in honey samples |
| Seliwanoff's test | Detection of ketose sugar like fructose: two drops of each sample solution is heated with 2 mL Seliwanoff's reagent in boiling water bath. Development of a deep red color specifies the presence of ketose sugar. Colored is formed with in 30 s |
| Adulteration confirmation test | The collected honey samples were analyzed for adulterants. Following physical tests are carried out to identify the purity and adulterants added to the sample |
| Flame test | The presence of added water in each honey sample is determined by putting a drop of honey on a laboratory Bunsen burner by using cotton wick. The presence of added water is confirmed by the observation of cracking sound without flame. Pure honey gives smokeless flame |
| Fiehe's test | Detection of added sugar: weigh accurately 2 g of honey sample dissolved in 10 mL of water and mix properly. Extract the sample with solvent diethyl ether ($C_2H_5OC_2H_5$) of volume 30 mL in a separating funnel. Prepare resorcinol solution (1 g of resublimed resorcinol in 100 mL of hydrochloric acid), take fresh 2 mL of this solution and add to extract. Shake the solution properly. Development of cherry red color within a minute indicates the presence of added sugar. No significance of other color |

employed for amino acid determination. Gas chromatography (GC) and high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), and high-performance anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) were used for the estimation of adulterants like high fructose corn syrups (HFCS) and corn syrups (CS) in the sample (Verzera et al. 2014). The analytical methods also used for the determination of adulterant are differential scanning calorimetry (DSC), electrochemical analysis, enzymatic methods, vibrational spectroscopy like mid-infrared (MIR), near-infrared (NIR) spectroscopy, Raman techniques, isotope ratio mass spectrometry coupled with an elemental analyzer, low-field nuclear magnetic resonance, stable isotope analysis, and others such as flame ionization detectors (FID) or sensor arrays (Wang et al. 2010; Kropf et al. 2010). In developing countries, microscopic analysis is a method of choice, and modern analytical techniques are bearable. Apart from the discussed technique, several special methods are used to find out the adulterants like three-dimensional fluorescence spectroscopy (3DFS) coupled with multivariate calibration, electronic honey quality analyzer, fiber-optic displacement sensor (FODS),

and an electronic tongue. Detection and estimation of adulterants in the sample are very easily performed with adulterant kits, development for an enzyme label, which can make a difference in the color of the sample matrix (Table 6.2: important merits and demerits of analytical techniques).

**Table 6.2** Important merits and demerits of the discussed techniques

| Detection technique | Merits | Demerits | References |
|---|---|---|---|
| Melisso palynological analysis and other physicochemical parameters detection | Simple or no sample preparation; best for unifloral honeys of same geographical origin | Wide range of thresholds; could not work for honey from close geographical zones | Castro-Vazquez et al. (2014) |
| Chromatographic analysis | Complex, volatile and nonvolatile, wide variety of analytes are readily analyzed | Honey origin is difficult to be identified | Kamboj et al. (2013) |
| High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) | Did not require derivatization; shorter total analysis time | Need specialized equipment to handle to high-pH mobile phases; no method flexibility to resolve an interfering peak | Xue et al. (2013) |
| Front phase fluorimetric spectroscopy | Botanical origin of polyfloral honeys can be identified easily; highly sensitive in comparison to other spectroscopic technique | Geographical origin estimation could not be done accurately | Ruoff et al. (2006) |
| Fourier transform infrared spectroscopy | Botanical origin of polyfloral honeys can be identified easily; short analysis time | Geographical origin estimation could not be done | Wang et al. (2010) |
| Fourier transform Raman spectroscopy | No water interference and minimal fluorescence interference; detect adulteration from the same plant source | Aqueous, dark colored samples at high temperatures increase interferences | Pierna et al. (2011) |
| Stable isotope ratio mass spectrometry (SIRMS) | Wide applicability and versatility to be coupled with several different interfaces | Lack of availability of SIRMS standards and standardized methods; not suitable for routine analysis | Cengiz et al. (2014) |
| Ultra-performance liquid chromatography-quadrupole/time of flight-mass spectrometry (UPLC-Q/TOF-MS) | It was possible to identify several components which cannot be detected by diode array using combination of detection with retention time for accurate molecular mass to obtain phenolic acids and flavonoids from ethyl acetate extracts of different honeys (sunflower, lime, clover) | Deficiency of this high sensitive's technique and not suitable for analysis | Trautvetter et al. (2009) |

(continued)

**Table 6.2** (continued)

| Detection technique | Merits | Demerits | References |
|---|---|---|---|
| Nuclear magnetic resonance | Fingerprint technique so easy to identify a specific biomarker for a class of sample; minimal sample processing; non-destructive nature | Extensive chemometric analysis is required which makes it complicated for routine analysis | Consonni and Cagliani (2008) |
| Western blot | Development of a novel method based on honey proteins to determine floral origin of honey samples using SDS-PAGE immune blot or Western blot techniques | To sort the proteins by size, charge, or other differences in individual protein bands | Baroni et al. (2002) |
| Atomic absorption spectrophotometer | Characterized different types of honey produced in the Canary Islands according to their mineral contents using atomic absorption spectrophotometer | Only solutions can be analyzed, relatively large sample quantities are required | de Alda-Garcilope et al. (2012) |

#### 6.4.4.1 Infrared Spectroscopy

Infrared spectrometer is a very useful technique for honey sample analysis. Some research has been reported. Infrared different vibration range was employed for the estimation of honey in botanical origin, eight monofloral and polyfloral honey sample authenticated by near-infrared spectrometer also, performed a quantitative examination of various type of sample. FTIR and chemometrics were used for botanical origin studies (Ruoff et al. 2005; Kelly et al. 2004). Fourier transform infrared spectrometer (FTIR) with attenuated total reflectance (ATR) is used for the determination of various food parameters. Organic compounds present in honey give signals in the range MIR (4000–400 $cm^{-1}$) and NIR (10,000–4000 $cm^{-1}$) originate from the vibrational and rotational modes (stretching, bending, and rotating). The signals that originate due to NIR are complex overtones and high-frequency combinations of fundamental vibrations at shorter wavelengths. The wavelength range of MIR gives sharper, resolved, and informative peaks which indicate the botanical and geographical origins reported by Ruoff et al. FT-MIR gives more efficient information of 11 types of unifloral (acacia, alpine rose, chestnut, dandelion, heather, lime, grape, fir honeydew, metcalfa honeydew, and oak honeydew) and polyfloral kinds of honey ($n = 411$ samples; 15). The characteristic spectral line between 800 and 1500 $cm^{-1}$ is observed. Spectra of various samples of honey correspond to the C–O and C–C stretching regions of the saccharides between 950 and 1050 $cm^{-1}$ (Wu et al. 2017). IR spectroscopy and Raman spectroscopy have a disadvantage: during the analysis of the sample, the duration exposed to heat may lead to sample destruction. Overcome such problem by adopting a procedure to expose less irradiation duration and increasing the number of the scan experimented attenuated total reflection (ATR). Prominent absorption lines in the mid-IR region indicate the presence of water in the samples. The botanical origin of honey is indicated by

FTIR spectroscopy and attenuated total reflection (ATR) sampling technique (de la Mata et al. 2012). Differentiate HFCS-adulterated and unadulterated honey by spectra obtained through fiber optic diffuse reflectance NIR spectrometer acquired within the 10,000–4000 $cm^{-1}$ range. A perfect PLS model used to differentiate pure and adulterated honey samples is within the range of 6000–10,000 $cm^{-1}$ (Chen et al. 2011).

#### 6.4.4.2 Nuclear Magnetic Resonance (NMR)

For structure determination, this instrument is best for different organic compounds present in honey sample and gives a better understanding of the complex structure (Cazor et al. 2006). Compare to the other analytical technique, NMR provides better information about the sample composition and metabolites also, have property non-invasive nature, the relative ease and rapidity of data execution in a single run (Ribeiro et al. 2014). Data produced by NMR extract useful information with multivariate analysis, several published paper indicate methods which are useful to categorize honey samples according to their botanical origin. NMR analysis performed as per the demand several choices are available such as principal component analysis (PCA), hierarchical cluster analysis (HCA), K-nearest neighbor (KNN), soft independent modeling of class analogies (SIMCA), and orthogonal PLS (OPLS)-DA. In addition to the one-dimensional (1D) technique, two-dimensional (2D) NMR experiments were also employed for the analysis (Lolli et al. 2008). Five types of botanical origin (robinia, chestnut, citrus, eucalyptus, and polyfloral) honey have been differentiated by $^{1}$H-NMR and heteronuclear multiple-bond correlation (HMBC) experimented by taking 72 honey samples. Developed general DA models that had cross-validation accuracy rates of 92% in the case of D2O $^{1}$H–$^{13}$C HMBC spectra and 97% in the case of DMSO-d6 $^{1}$H–$^{13}$C HMBC spectra (Simova et al. 2012). $^{1}$H- and $^{13}$C-NMR analyzed, confirmed the protons and methylene group carbon in quercitol on TOCSY spectroscopy for the given sample. Honey sample of oak honeydew is differentiated from the other honey sample by the presence or absence of quercitol. $^{1}$H-NMR is more useful as compare to $^{13}$C-NMR because of sensitivity is high. $^{13}$C-NMR technique is employed for the estimation of saccharides in authentic Greek honey samples, a method which was developed for the estimation has been validated as according to ICH guideline, performed experiment for validation on accuracy, linearity, range, limit of detection, etc. The samples taken have either single sugar molecules or artificial mixtures of isoglucose (glucopyranose and fructose) (Kazalaki et al. 2015). Monofloral honey ingredients like carboxylic acids, amino acids, ethanol, and hydroxymethylfurfural estimated by $^{1}$H-NMR (Beretta et al. 2009). Various samples from different botanical origin were analyzed by HPLC-DAD-ESI-MS and multidimensional diffusion-ordered (DOSY) NMR, and ingredients like quinoline alkaloids and the biosynthetic precursor, i.e. kynurenic acid (KA), have been estimated. Concentration level of quinoline alkaloids distinguishes chestnut honey from others (Cho et al. 2015).

#### 6.4.4.3 Hyphenated Technique (Mass Spectrometer Coupled with Chromatography Techniques)

The semi-volatile and volatile substances in honey separated and identified by LC–MS and GC–MS techniques. Flavor of honey-based on the concentration of volatile substance and its variation in concentration are related to floral origin. Estimation of these substances by technique headspace (HS) solid-phase micro-extraction (SPME) (Escriche et al. 2011), with this technique followed by GC–MS of various volatile substances, is identified and quantified in a different honey sample. Various types of samples were analyzed: 35 volatile components (Spanish honey), 62 compounds (Greek honey), 31 compounds (16 samples from European countries), and 26 compounds (70 authentic Turkish honey) (Alissandrakis et al. 2007; Senyuva et al. 2009). Italian thistle honey was analyzed by the HS-SPME method, and 40 volatile compounds were characterized and reported by Bianchi et al. (2011). HS-SPME/GC-MS extended with chemometric studies to estimate the organic volatile compounds, and 42 unifloral samples of five floral origins were studied (Spanik et al. 2014). SPME-GC-MS instrument used for the investigation of volatile consituents have chiral carbon. Flavonoid component of honey was studied using TLC–MS diode array detection system and electrospray ionization mass spectrometry (LC–DAD–ESI/MS). Seven types of Slovenian honey samples were extracted by solid-phase method followed by liquid chromatography, and their botanical origin was reported. Different types of honey-like strawberry tree honey, chaste honey, and rape honey studied floral origin by the high-performance liquid chromatography–diode array detection–tandem mass spectrometry (HPLC–DAD–MS/MS) method (Zhou et al. 2014). Floral markers such as kaempferol, morin, and ferulic acid are used to distinguish chaste honey from rape honey (Oelschlaegel et al. 2012). Ultra-performance liquid chromatography–photodiode array detection–mass spectrometer (UPLC–PDA–MS/MS) had been used to examine the volatile composition of Manuka honey sample; solid-phase extraction was first performed. Constituents like kojic acid, unedone, 5-methyl-3-furan carboxylic acid, 3-hydroxy-1-(2-methoxyphenyl) penta-1,4-dione, and lumichrome were identified in Manuka honey sample. Advanced technique ultra-performance liquid chromatography–quadrupole/time of flight mass spectrometry (UPLC–Q/TOF-MS) was used to detect phenolic acids and flavonoids based on the retention time of individual compound of a sample; sunflower, lime, clover, rape, and honeydew were extracted with solvent ethyl acetate (Trautvetter et al. 2009).

#### 6.4.4.4 Inductively Coupled Plasma–Mass Spectrometry (ICP–MS)

Atomic absorption spectrometric technique is one of the key instruments used for food analysis. This technique is very popular in the food industry. This instrument is used for the estimation of multiple elements in the sample, and the advantage is that very high sensitivity detects low-level concentration elements. Brazilian kinds of honey of the geographical origin have been analyzed by ICP-MS with combination data mining approaches (Batista et al. 2012).

#### 6.4.4.5 Stable Isotopic Ratio Mass Spectrometry (SIRMS)

This analytical technique used for the detection of more sophisticated adulterations. The method is based on the differences in the metabolic enrichment of the $^{13}C$ isotope due to the different photosynthetic pathways of the C3 and the C4 plants. The slower-reacting $^{13}CO_2$ is depleted to a larger extent in C3 plants than in C4 plants during $CO_2$ fixation, making it possible to detect the addition of cheap C4 sugar because of its different $\delta^{13}C$ value (i.e., the $^{13}C/^{12}C$ isotope ratio related to Vienna Pee Dee Belemnite as a standard reference material, expressed as a percentage; 100). The method was later improved by using the isolated honey protein as an internal standard, which enhanced sensitivity and lowered the LOD for C4 sugars from around 20 to 7%. However, the main drawbacks of this technique are the impossibility to detect the addition of C3 syrups, which is why their fraudulent use is on the rise and the working hypothesis that assumed a correlation between the floral origins of honey and its proteins (Cotte et al. 2007). The $\delta^{13}C$ value indicates the adulteration, the addition of C4 sugar in pure honey changes the value of $\delta^{13}C$, if it is less negative than 23.5, it indicates to be adulterated (Padovan et al. 2003).

#### 6.4.4.6 Chromatographic Methods

Chromatographic techniques provide reliable separation and quantification of macro and micro components of highly similar chemical structures in complex matrixes such as food products. The chromatographic fingerprint profile is a well-established assay of authenticity concerning botanical and geographical origins of honey. Fingerprint analysis can be defined as a set of characteristic chromatographic signals leading to sample pattern recognition (classification). However, authentication of adulteration is usually done by matching measured compound profiles with predetermined target values. In these studies, LC was applied for the identification of proteins, amino acids, carbohydrates, vitamins, phenolic compounds, triglycerides, chiral compounds, and pigments, whereas GC was used for the analysis of naturally volatile or semi-volatile molecules (Doner et al. 1979). Maltose/isomaltose ratios in different kinds of honey and high fructose corn syrup were determined using gas chromatographic (GC) method.

#### 6.4.4.7 High-Performance Thin-Layer Chromatography (HPTLC)

HPTLC gained very high popularity for the authentication of honey and has an advantage over the other chromatography like GC and HPLC because it is easy to handle and low-cost and has the ability to simultaneously analyze multiple samples on the same plate. Automation increases precision and accuracy of the developed method. By this technique, authenticity of honey production is confirmed, but it is not possible to authenticate the botanical or geographical origin (Morlock and Schwack 2008). Puscas et al. reported adulterants in several samples of Romanian kinds of honey by high-performance thin-layer chromatography (HPTLC) combined with image analysis. In several honey, estimated proline, leucine and phenylalanine and their enantiomeric ratios by HPTLC (Rizelio et al. 2012).

#### 6.4.4.8 High-Performance Liquid Chromatography (HPLC)

Estimation of phenolic and amino acid component experimented by this analytical technique which is used for the assessment of its authenticity (floral and geographical origins). Campone et al. (2014) analyzed the honey sample reported 5 phenolic acids and 10 flavonoids used dispersive liquid-liquid microextraction followed by HPLC analysis. This chromatographic technique developed chromatogram providing very complex information which is helpful to differentiate whether the honey is of botanical, geographical, or entomological origin. The performance of this analysis is increased when this instrument is coupled with MS (Zhou et al. 2014). Determination of phenolics compounds in honey needs to be performed with several steps like isolation from a sample matrix, analytical separation, identification, and quantification.

#### 6.4.4.9 High-Performance Anion Exchange Chromatography (HPAEC)

Carbohydrate is one of the main constituents present in honey, and this analytical tool is very powerful because of its ability to separate all classes of aldols, amino sugar, and mono-, oligo-, and polysaccharides based on their structural features such as size, composition, atomicity, and linkage isomerism. Adulterants, structurally based on carbohydrate, are detected by HPAEC–PAD instruments. HPAEC profiles of polysaccharides are also used for the detection of adulteration with CS after pretreatment of the sample with reversed-phase SPE to remove monosaccharides and small oligosaccharides, and to concentrate traces of polysaccharides.

#### 6.4.4.10 Gas Chromatography (GC)

It is a technique used to analyze volatile organic components (VOCs); mono-, di-, and trisaccharides; and pesticide residues in honey. In the majority of studies evaluating the authenticity of honey, GC was combined with MS to identify different substances within a test sample. GC is also a suitable technique for the detection of honey adulteration due to its relatively high resolution and sensitivity for the determination of mono-, di-, and trisaccharides. A literature survey revealed that GC was used mainly for the detection of honey adulterations carried out by the addition of sugar syrups such as HFCS, CS, and IS (Zhou et al. 2014).

#### 6.4.4.11 Electrochemical Methods

These techniques provide a high level of sensitivity and selectivity, although some of them require cumbersome sample preparation steps. Electrochemical techniques are as fast, simple, and cheap as some of the already-mentioned techniques; however, they also provide valuable information about the redox properties of honey constituents. Therefore, they have been extensively applied in the honey analysis.

#### 6.4.4.12 Voltammetry and Electronic Tongue

Several advantages, such as high sensitivity, versatility, simplicity, and robustness, make voltammetry a powerful electroanalytical technique. So far, cyclic, stripping, pulse, and alternating current voltammetry methods have been developed by a

significant number of researchers for the analysis of different organic and inorganic compounds, as well as antioxidative activity. The electronic tongue (e-tongue) is a novel device consisting of arrays of nonselective gas or liquid sensors coupled with pattern recognition software. The e-tongue analyzes the complex natural sample as a whole without the need for separating it into simpler components. So far, there are just a few papers related to the determination of the botanical origin of honey using an e-tongue. Eight different botanical types of honey and five geographically different acacia kinds of honey were classified using an e-tongue in combination with PCA, HCA, and artificial NNs (105). Voltammetric e-tongue (VE-tongue) has been composed of six working electrodes (gold, silver, platinum, palladium, tungsten, and titanium) in a standard three-electrode configuration and is used for the classification of various kinds of monofloral kinds of honey-based on multifrequency large amplitude pulse.

#### 6.4.4.13 Electrophoresis

Proteins are found as minor components in honey and originate from honey bees, plants, pollen, and nectar. Electrophoresis, although rarely applied as an electrochemical technique in honey analysis, provides the simultaneous analysis of a large number of samples under the same conditions. In 1987, the first silver-staining sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) method was described by Marshall and Williams (109) for the detection of 19 protein bands in Australian kinds of honey of different plant origins. Baroni et al. (2002) reported a novel analytical method for the assessment of floral origin in kinds of honey-based on the study of proteins using SDS-PAGE. The authors used honey proteins as chemical markers for the floral origin of eucalyptus honey. They also found that pollen from different plants could be significantly differentiated through SDS-PAGE coupled with DA (110).

#### 6.4.4.14 Capillary Electrophoresis (CE)

This is becoming a popular electroanalytical technique for the separation and identification of phenolic compounds, carbohydrates, amino acids, organic acids, and cations in honey. High speed, resolution, simplicity, low operating costs, and short analysis times make CE an alternative technique to HPLC for the analysis of different target compounds in honey. Also, MS in combination with CE provides a high level of sensitivity and selectivity.

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