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From Farm to Table: High-Tech Solutions for Honey Purity and Therapeutic Integrity to authenticate Medicinal Values

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1. Abstract

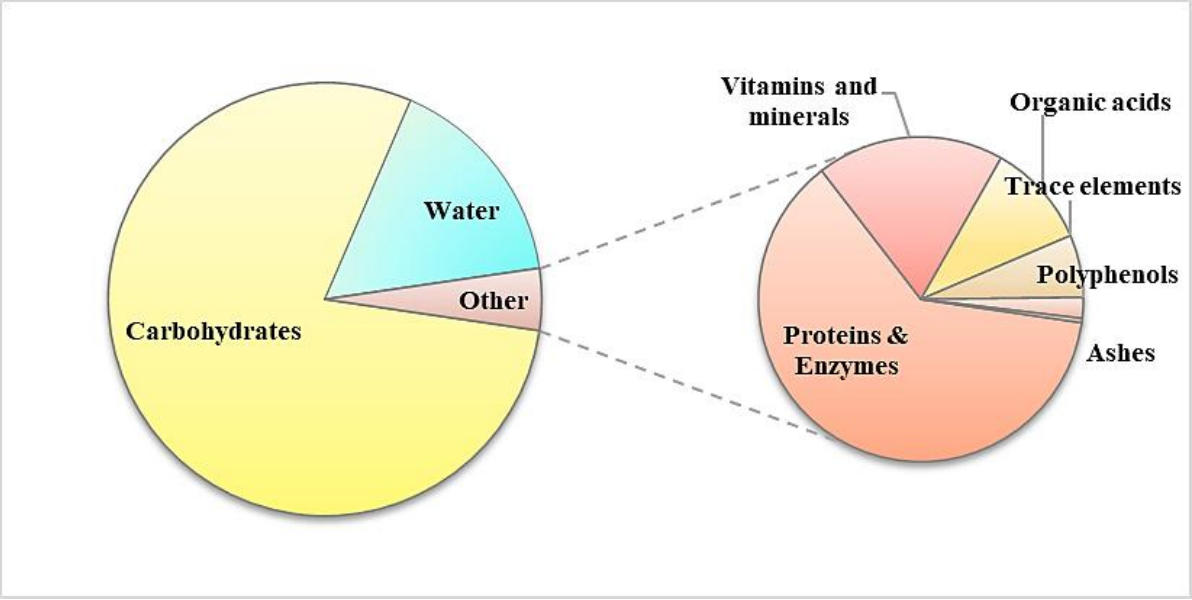
The nectar of various flowers combined with a variety of enzymatic processes occurring within their honey sacs allows honeybees (Apis mellifera Linnaeus 1761) to produce honey, a key biogenic substance. In the domains of agriculture, microbiology, immunology, and pharmaceuticals, the physicochemical characterization and identification of bioactive chemicals with nutritional qualities found in honey are crucial. Honey has a number of well-established health benefits, such as anti-inflammatory, anti-microbial, and antioxidant qualities, as well as cardioprotective, hepatoprotective, and neuroprotective qualities. Studies of honey's melissopalynology yield a wealth of knowledge about its botanical and geographic origins. In order to obtain the analytical foundation for a thorough evaluation of the chemical composition and quality assessment of honey, traditional and high-throughput technologies, such as spectroscopic and chromatographic analysis, offer crucial information encompassing a number of parameters, such as the identification of sugars, amino acids, organic acids, enzymes, vitamins, and phenolic complexes; the detection and quantification of bioactive compounds; and the presence of pollen, pesticides, heavy metals, and environmental pollutants. From identifying plant taxa to detecting the floral origin, DNA metabarcoding and Next Generation Sequencing (NGS) examination of honey yield important botanical information. Additionally, entomological aspects should be taken into account, as they can be an effective way to evaluate the marketable value of honey in relation to pricing and public health advantages. To sum up, these analytical and biochemical methods clarify the practical benefits and use of thorough honey analysis.

2. Keywords
Honey, Pollen biomarkers, Spectroscopy, Chromatographic analysis, DNA Metabarcoding, Next Generation Sequencing (NGS)

3. Introduction
Due to its many nutritional and therapeutic benefits as well as its extensive use in herbal therapies, honey is a pleasant natural substance that has enormous relevance in enhancing health and is regarded as a nutraceutical [1]. Nectar, which contains water with different percentages of dissolved sugars ranging from 25 to 70%, is collected by honey bees from either a single plant species (monofloral) or from several plant species (polyfloral). Eventually, honey with more than 80% sugar and ideally fewer than 20% water is generated after the nectar's excess water evaporates during the next few days in the honeycomb [2]. Honey can be categorized according to its entomological origin (A. mellifera, A. cerana, A. dorsata, and A. florea), botanical origin (unifloral, multifloral, and honeydew), and processing method (comb, strained, chunk, and extracted) [3,4]. Due to its antibacterial and anti-inflammatory properties, honey has therapeutic value in the areas of hepatoprotection, cardioprotection, neuroprotection, ophthalmology, dermatology, and wound healing. The nutritional value and biotherapeutic efficacy of honey are greatly influenced by its physicochemical characteristics. To precisely measure the bioactive components of honey, a variety of traditional techniques can be used in conjunction with cutting-edge bioanalytical technology to evaluate its quality [5]. Two important methods for analyzing and assessing the quality of honey are chromatography and mass spectroscopy. Along with chromatography in conjunction with mass spectrometry, which offers thorough analysis of honey and is essential in identifying any pollutants and adulterates already present, GS, LC, HPTLC, HPLC, and UHPLC are frequently used in chromatography analysis for honey. With its multivariate analysis, mass spectrometry offers information about the botanical, entomological, and geographic origins of honey. It is also useful for detecting environmental pollutants and quantifying the chemical components of honey [6]. Advanced methods for analyzing honey include honey pollen DNA-metabarcoding, honey protein ELISA, and the discovery of genomic, transcriptomic, and proteomic information through the investigation of gut-associated microorganisms in honey bees [7]. This review article highlights the use of high-throughput screening approaches in honey nutritional and value assessment by thoroughly discussing several modern analytical techniques in honey authentication and quality evaluation. Additionally, the antibacterial, anti-inflammatory, and antioxidant properties of honey, along with its many other health-promoting qualities, have been emphasized as part of its medicinal effects.

4. Characteristic Components of Honey with Biotherapeutic Significance
Honey has different levels of chemical components depending on its biochemical and geographic origin. Honey is mostly made up of sugar and water, but it also contains a number of bioactive ingredients, including vitamins, organic acids, amino acids, enzymes, and phenolic compounds. According to Cianciosi, et al. [8], honey bee species, floral origin, geographic location, climate, and honey processing and storage conditions are the primary significant aspects that lead to changes in honey compositions. The general composition of honey and its potential health-protective properties are shown in Figure 1.

Figure 1: Average compositions of honey.

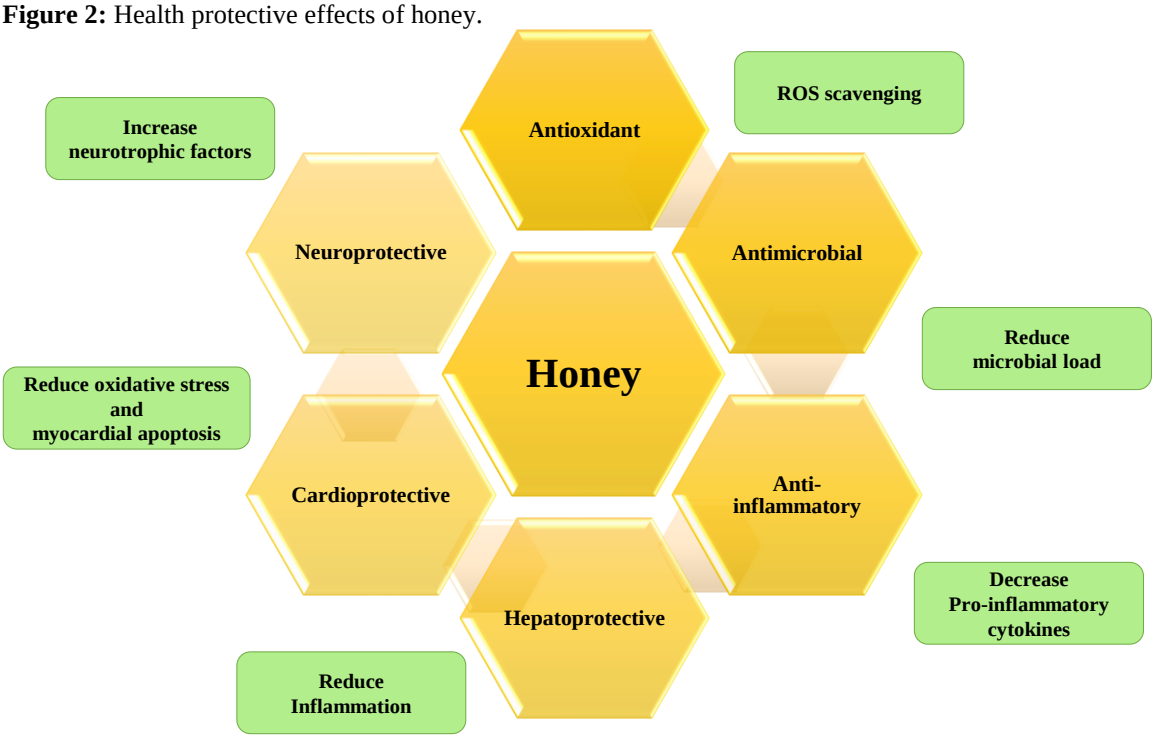


5. Honey as a Modulator of Biogenic Therapeutics
In addition to its hepatoprotective, neuroprotective, and cardioprotective qualities, honey has been identified as a natural therapeutic compound with a number of antimicrobial and antioxidant properties, making it a fundamental ingredient with numerous health advantages [9]. Figure 2 illustrates honey's many health-protective properties.

5.1. Antioxidant properties of pure honey
The main sources of honey's antioxidant qualities are its diverse range of phenolic chemicals, which include flavonoids and phenolic acids. Phenolic component signature

analysis serves as a reliable biochemical diagnostic of honey's botanical and geographic origin. The key factors influencing antioxidant efficacy include floral origin and environmental variations, such as temperature and moisture content [10]. It has been demonstrated that the main antioxidant properties range from metal ion chelation to free radical scavenging to activating antioxidant enzymes. The presence of pigments in honey contributes to its antioxidant efficacy; darker honey (such as manuka and chestnut) typically has higher antioxidant qualities, while lighter honey (such as orange and clover) also has higher phenolic acid concentrations [9]. The antioxidant capacity can be estimated using the Total Phenolic Contents (TPC) in mg GAE/g, where GAE stands for gallic acid equivalents, and the Total Flavonoid Contents (TFC) in mg QE/g or mg CAE/g, where QE is for quercetin equivalents and CAE for catechin equivalents. The Folin–Ciocalteu assay (absorbance measurement at 750 nm) and colorimetric assay (absorbance measurement at 510 nm) are used to determine TPC and TFC, while the ferric reducing antioxidant power (FRAP) assay (absorbance measurement at 593 nm) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (absorbance measurement at 517 nm) are used to estimate antioxidant activity [11]. The main flavonoids in honey are quercetin, apigenin, naringenin, kaempferol, and chrysin, whereas the main phenolic acids are p-hydroxybenzoic, caffeic, vanillic, syringic, p-coumaric, ferulic, benzoic, and cinnamic. The honey is more susceptible to processing and storage conditions due to its greater polyphenol concentrations. Honey's antioxidant activity may be diminished by higher processing temperatures [10].

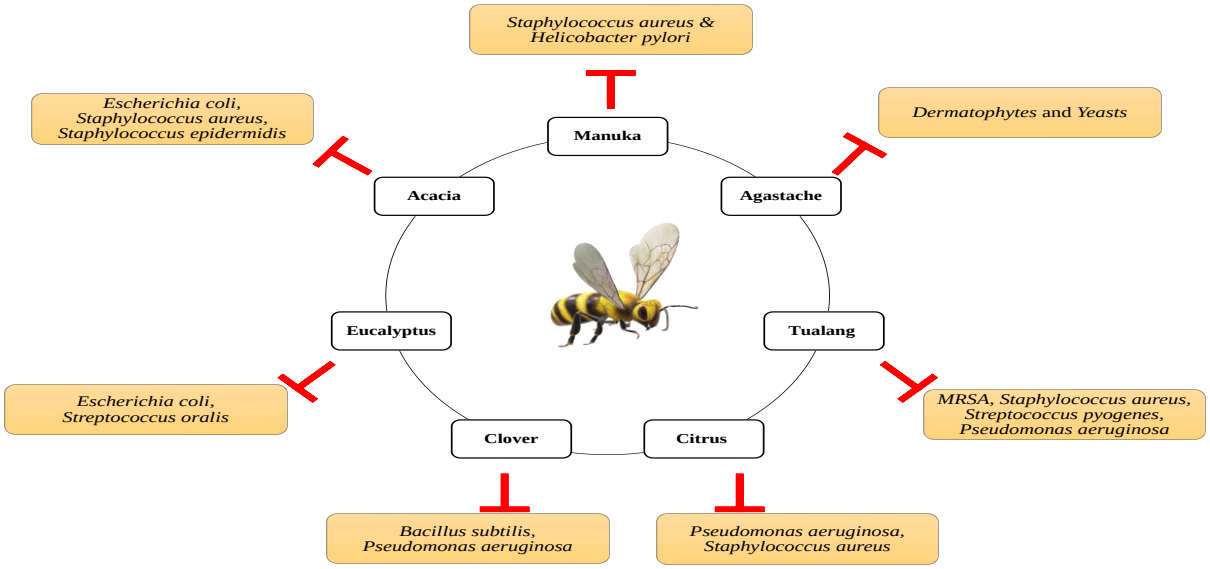
Figure 2: Health protective effects of honey.



5.2. Antimicrobial activities exhibited by pure honey

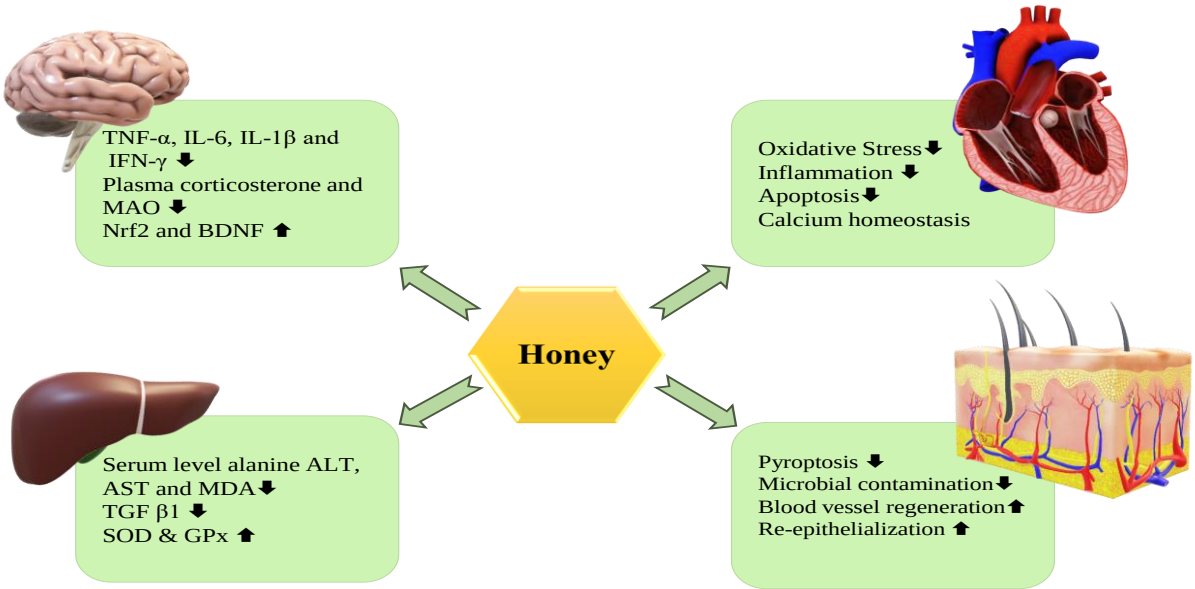
Because of its acidic pH (3.5-4.5), which is caused by the presence of organic acids, increased carbohydrate content (80–85%), low moisture percentage (less than 20%), flavonoids and phenolic compounds, methylglyoxal, and defensin-1, honey has strong antibacterial activity [8]. Because honey contains polyphenols, ascorbic acid, hydrogen peroxide (H2O2), and enzymes, it effectively reduces microbial load by depolarizing the microbial membrane potential, which lowers the expression of genes related to quorum sensing and stress response, and promoting drug intake-associated membrane cell lysis [12]. Honey has antibacterial properties against a wide range of microorganisms; honey from a variety of floral sources, such as manuka, acacia, eucalyptus, clover, and astaxanthe, has the strongest antibacterial activity [13,14]. The varied antibacterial potencies of different monofloral honeys are shown in Figure 3.

Figure 3: Antimicrobial influence of monofloral honey.



The various processes of honey's neuroprotective, hepatoprotective, cardioprotective, anti-inflammatory, and wound-healing properties are depicted in Figure 4. The next sections provide a thorough discussion of the biochemical changes that promote disease prevention and quicker recovery.

Figure 4: Schematic illustration of therapeutic aspects of honey.



5.3. Anti-inflammatory properties and wound-healing properties of pure honey

Several cellular and inflammatory modulations, blood vessel regeneration, re-epithelialization, and tissue resolution are among the sequential, highly integrated, and extremely time-sensitive bio-physiological stages of wound healing [15]. Inhibiting pyroptosis and modifying pro-inflammatory effector molecules can be useful in conjunction with antimicrobial treatment strategies because pyroptosis, dysregulation of pro-inflammatory cytokines, and changes in the inflammasome signaling pathway all contribute to a persistent inflammatory response [16,17]. By lowering inflammatory response, reducing ROS-mediated oxidative damage, inhibiting pyroptosis, lowering microbial contamination, and generally accelerating the healing process, honey showed encouraging results in relation to wound healing [18]. By boosting Keap1-Nrf2 signaling, manuka honey has been demonstrated to shield macrophages from LPS-induced oxidative damage. In 2018, Gasparrini, et al. [19] Hydrogel exhibits a wide range of uses in wound healing and possesses numerous biochemical properties relevant to regenerative therapies. Honey-loaded hydrogel dressings have immunomodulatory, antibacterial, and wound-healing properties in the treatment of skin ulcers, wounds, and burns. This limits microbial infestation and lowers the risk of infection [20]. Because of its high carbohydrate content and antioxidant and antibacterial properties, honey-integrated scaffolds function as a shielding barrier, effectively lowering microbial contaminations [21,22].

5.4. Hepatoprotective properties of honey

Honey reduces liver damage through the decrease of inflammation mediated by polyphenols. The healing qualities of honey were clarified by a number of animal experiments. Apis cerana honey decreased liver damage caused by bromobenzene in a mouse model, Vitex honey helped with liver damage caused by paracetamol, and Manuka honey made cancer cells in hepatocellular carcinoma more susceptible to doxorubicin [23-25]. Overall, cellular and animal research clarified honey's hepatoprotective qualities, showing that the molecular mechanisms depend on its antioxidant and anti-inflammatory capabilities, as demonstrated by decreased levels of the inflammatory biomarker TGFβ1, aspartate aminotransferase (AST), ALT, and malondialdehyde (MDA), as well as elevated levels of

glutathione peroxidase (GPx) and superoxide dismutase (SOD). Cu(2+)-mediated oxidation of lipoprotein was decreased by vitex honey, which also increased the serum's ability to absorb oxygen radicals, increased SOD and 8-hydroxy-2'-deoxyguanosine, and decreased ALT and AST [23]. Higher percentages of phenolic chemicals are found in citrus honey, trigona, and dorsata. Growing phenolic concentrations directly support the honey's antioxidant and free radical-scavenging properties, which may help reduce metabolic disorders brought on by oxidative damage [26]. A number of honey's polyphenolic components that support hepatoprotective properties are shown in Figure 5.

5.5. Cardioprotective properties of honey

Honey's cardioprotective properties include lowering blood pressure of low-density lipoproteins (LDLs), improving the mitochondrial function of heart cells, and reducing oxidative stress, inflammation, apoptosis, and lipid peroxidation in general [27]. Polyphenols primarily prevent heart attacks and strokes by inhibiting platelet aggregation in the circulation, improving coronary vasodilation, and suppressing the oxidation of LDL cholesterol [28]. Caffeic acid increases the bioavailability of nitric oxide (NO), kaempferol maintains calcium homeostasis and mitochondrial function, galangin lowers serum troponin and creatine kinase-MB, isorhamnetin protects against cisplatin (CP)-induced toxicity, and luteolin reduces the size of myocardial infarct [29]. Honey actively maintains heart health because of its exceptional cardioprotective properties. The various polyphenolic components of honey that benefit heart health are shown in Figure 6.

Figure 5: Hepatoprotective aspects of polyphenolic components of honey.

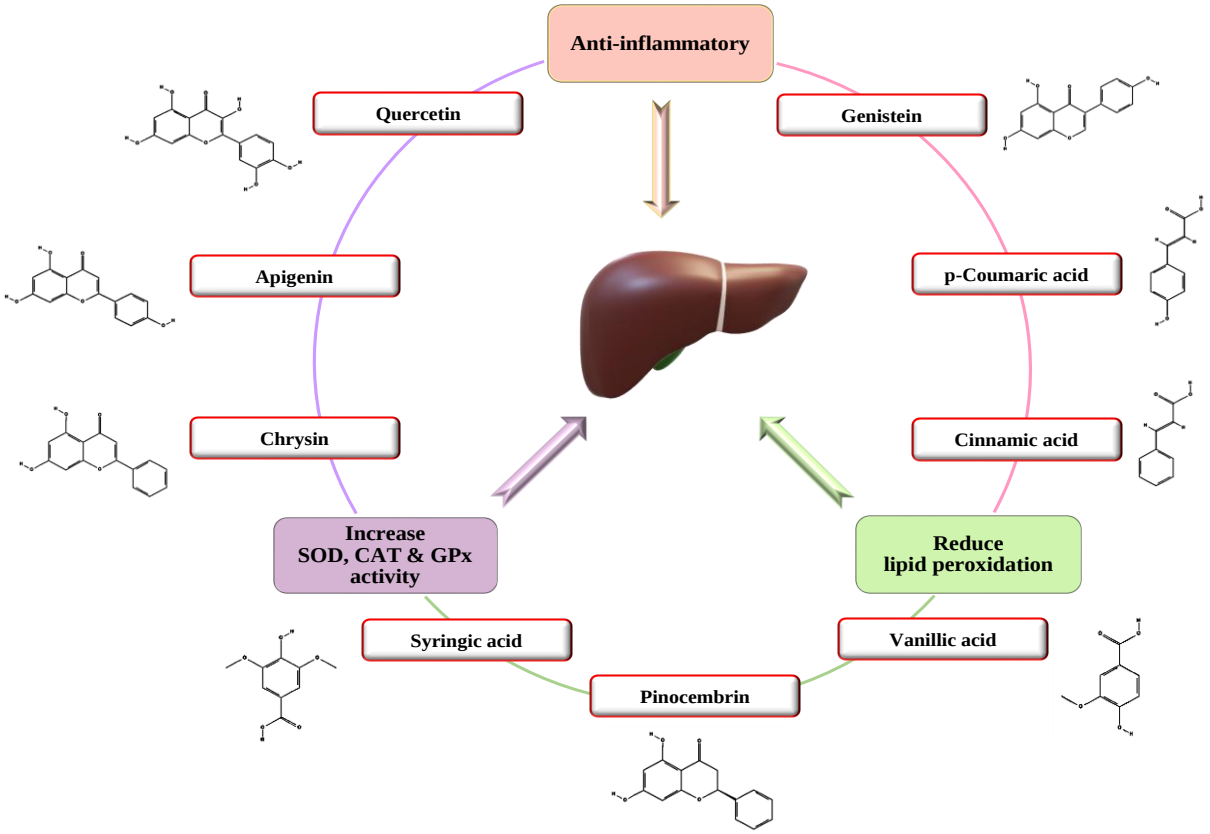
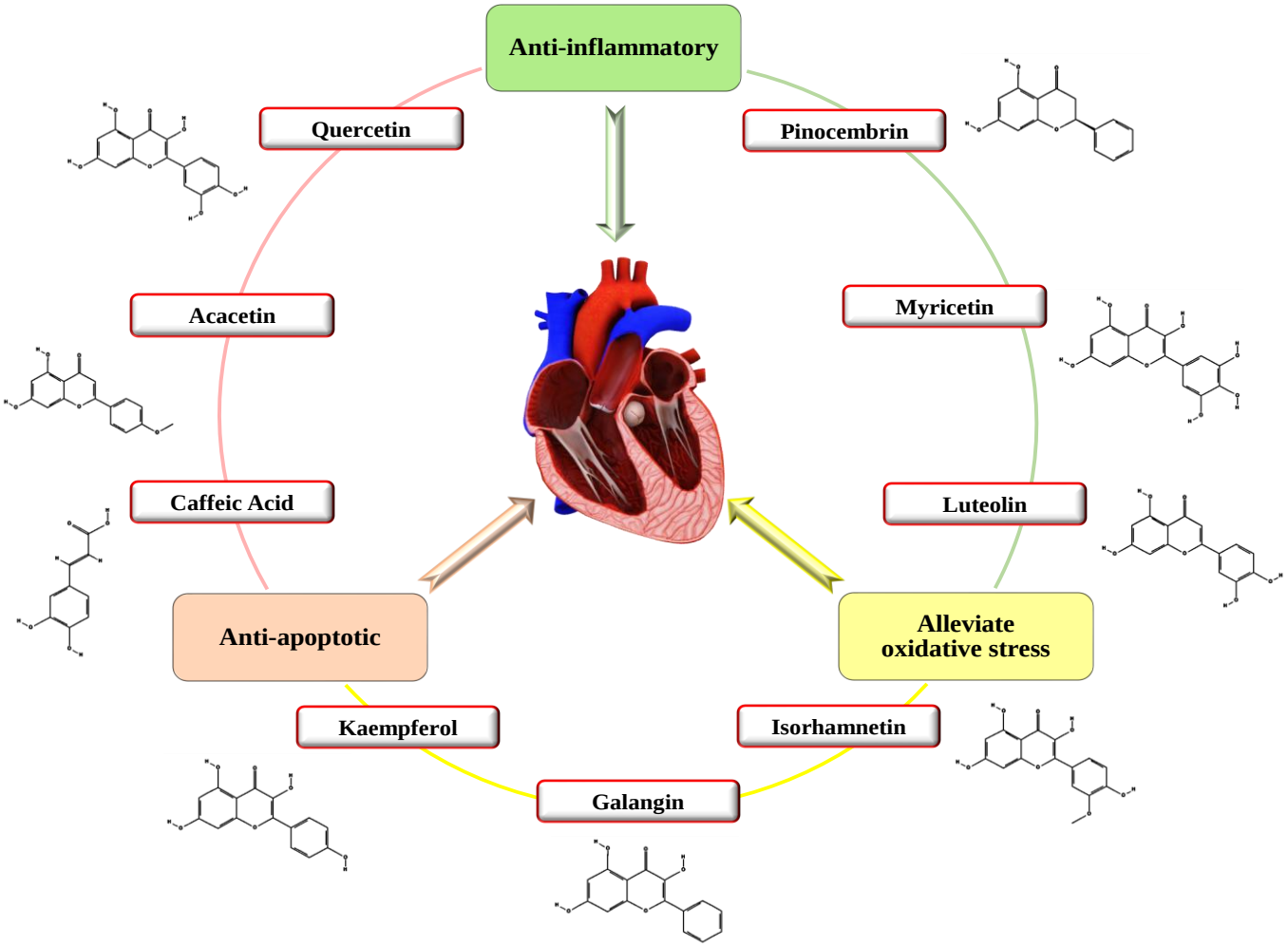


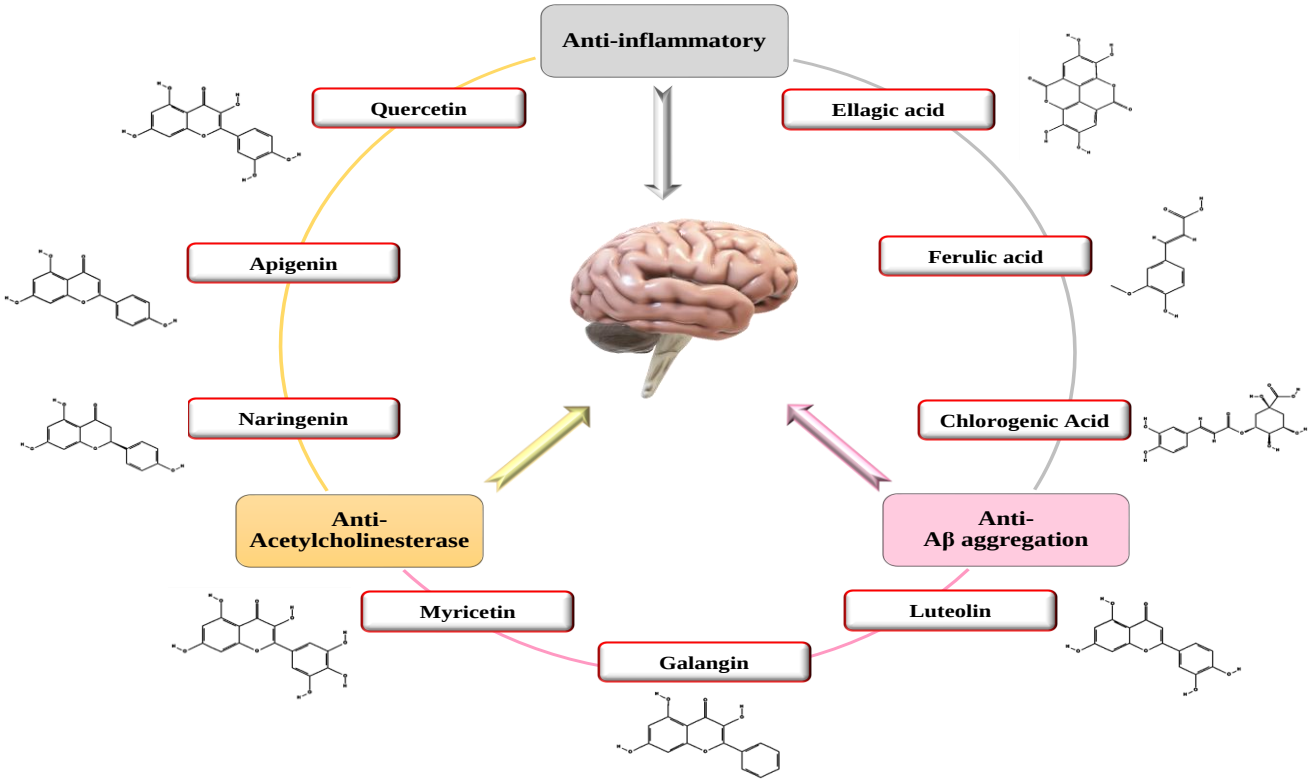
Figure 6: Cardioprotective aspects of polyphenolic components of honey.



5.6. Neuroprotective properties of honey

Due to its antioxidant and anti-inflammatory components, honey has a wide range of neuroprotective properties, including improving memory, reducing stress-induced brain damage, and easing neurodegenerative illnesses. It has been demonstrated that honey consumption helps with a variety of neurodegenerative illnesses, such as Parkinson's, Alzheimer's, and dementia. Due to the anti-inflammatory and anticholinesterase properties of its polyphenolic components, a number of mono- and polyfloral honeys continue to have neuroprotective properties. Of these, honey from Kelulut, Zantaz, Thyme, and Tualang exhibits remarkable neuroprotective qualities because of its higher TPC [30,31]. According to Hossen, et al. [32] and Iftikhar, et al. [33], the main contributing elements that exhibit neuroprotection include myricetin, naringenin, quercetin, luteolin, isorhamnetin, kaempferol, apigenin, and galangin. Among the functional mechanisms are the reduction of plasma corticosterone, acetylcholinesterase (AChE), and monoamine oxidase (MAO); inhibition of inflammatory cytokines TNF-α, IL-6, IL-1β, and IFN-γ; inhibition of the aggregation of amyloid beta (Aβ); and the restoration of brain-derived neurotrophic factor (BDNF) by increasing Nrf2 [30-35]. Figure 7 shows how honey's different polyphenolic components offer protection against neurodegeneration.

Figure 7: Neuroprotective aspects of polyphenolic components of honey.



6. Factors Influencing the Purity and Quality of Honey as Food and Drug

In addition to several ecotoxicology criteria like bioaccumulation, acute and chronic toxicity, and mutagenicity, honey is a crucial environmental marker for detecting changes in important environmental variables such as pollution, climate, and heavy metals [36]. According to Cunningham, et al. [37], honey bees and their byproducts, such as honey, pollen, and wax, as well as the gut microbiota of bees, can play a significant role in biomonitoring and biosurveillance.

6.1. Pollution and climate change

Increased greenhouse gas emissions that cause the ozone layer to thin, global warming, and rising water levels are some of the factors affecting environmental determinants. These factors also affect plant species distribution, which changes the chemical makeup and quality of honey. The quality of honey is indirectly impacted by rising air pollution because it causes more suspended particles in the air, which harms honey bee health and makes it harder for pollinators to identify certain plant species [38]. Because different plant species flower at different times, climate change also affects how bees forage. The native habitat of bees has been altered, and their numbers have generally decreased as a result of unfavorable environmental factors such as forest fires, higher emissions of greenhouse gases (COx, NOx, and SOx), and an overall rise in global temperatures [39]. Monitoring the essential amounts of important contaminants, primarily suspended particulate matter (SPM), such as metal oxide fumes and polycyclic aromatic hydrocarbons (PAHs), in honey is necessary to preserve its quality and nutritional value.

6.2. Increasing application of pesticides in agriculture

The annihilation and eradication of dangerous pests and honey insects are among the negative consequences of pesticides. One example of this is colony collapse disorder (CCD), in which insecticides cause a significant number of honey bee colonies to disappear. The sensitivity of the detection method is indicated by the limit of quantification (LOQ, the lowest quantifiable analyte concentration) and limit of detection (LOD, the lowest detectable analyte concentration) values [40,41]. According to Hisamoto, et al. [42], LC/MSMS analysis of Japanese honey bee (Apis cerana japonica) colonies revealed a link between land usage and pesticide presence, with LOQ (ng/g) as low as 0.01. The main insecticides found were clothianidin, acetamiprid, and dinotefuran. Herbicides, pesticides, and the breakdown components of these substances were detected in Apis mellifera honey from Queensland, Australia, according to LC-MS/MS and GC-MS/MS analyses [43].

6.3. Presence of heavy metals

Arsenic (As), lead (Pb), cadmium (Cd), and mercury (Hg) are the main heavy metals. They cause cytotoxicity by blocking important enzymes that are essential for performing basic functions. Target enzymes include δ-aminolevulinic acid dehydrogenase (ALAD), coproporphyrinogen III oxidase, ferrochelatase, and pyruvate dehydrogenase (PDH) complex for arsenic, adenosine-5'-di-phosphate and phosphocreatine for cadmium, and amylase, lipase, lactase, maltese, and glucose-6-phosphatase for mercury. Inductively coupled plasma mass spectrometry (ICP-MS), flame atomic absorption spectroscopy (FAAS), and inductively coupled plasma optical emission spectroscopy (ICP-OES) can all be used to identify heavy metal pollutants [44].

7. Physicochemical Properties of Honey for Quality Evaluation

Numerous important details about the composition and

authenticity of honey can be obtained by investigating and evaluating it in light of its physicochemical characteristics, which include its color, acidity or basicity, viscosity and rheology, moisture content, electric conductivity (EC), carbohydrate, amino acids, proline, phenol, organic acids, vitamin, mineral compositions, pollen content, enzymes, sensory analysis, ash content, hydroxy methyl furfur, total solids soluble (°Brix), and microbial content. Additionally, the presence of organic acids, honey proteins, and the gut microbiota of honey bees can be evaluated. Table 1 shows the important physicochemical characteristics of honey and their importance in evaluating its quality.

Table 1: Physicochemical properties and their significance in quality assessment.

Physicochemical properties	Importance in Quality Evaluation	References
Color	Varies from medium light to dark amber, owing to plant pigments (anthocyanins, carotene, xanthophylls), polyphenols, flavonoids, amino acids and minerals, USDA considered optical density (OD) 0.0945 to 3.008.	Pascual-Mate, et al. [45] Smetanska, et al. [46]
pH	Acidic (3.5 - 4.5), existence of organic acids (including gluconic, galacturonic, pyruvic, citric and malonic acids etc.); pH meter	El Sohaimy, et al. [47]
Viscosity & Rheology	Function of moisture content (14 - 24%), Pa. s range 14.73 to 4.17 (25 °C), Varies due to handling and storing temperature also protein content, measured by Rheometer	Garcia, et al. [48] Bogdanov, et al. [49]
Moisture content	Usually less than 20%; honey collected in humid season has high moisture content, causes fermentation; Measured by refractometric (Abbe & Electronic) method, also IR, FT-NIR, FT-MIR-ATR & FTIR	Singh and Singh [50] Ananias, et al. [51] Pataca, et al. [52]
Electric conductivity (EC)	Mineral salts, organic acids and amino acids are determining factor of EC, more than 0.8 in honeydew honey and < 0.8 ms.cm^{-1} inblossom honey; Measured by dissolved honey in Electrical Conductivity Cell	Kaškonienė, et al. [53]
Carbohydrate	80-85% of honey, Mainly Glucose & Fructose, also signature carbohydrates including melezitose, turanose and erlose; Percentage depends on the honey type, HPAEC-MS, HPLC & GC	De La Fuente, et al. [54] Tedesco, et al. [55]
Amino acids	Major source of amino acid is pollen, amino acids in flower honey (0.1%-1.5%) and honeydew honey (3.0%) ^{1}H NMR, LC-ECD	Cotte, et al. [56] Iglesias, et al. [57]
Proline content	proline amount to almost 50–85% of the amino acid (Total), 873.00 mg/kg; LC–MS/MS	Iglesias, et al. [57] Kowalski, et al. [58]
Phenol content	p-Coumaric acid, Caffeic acid, Gallic acid, Vallinic acid, Syringic acid, Chlorogenic acid; Evaluated by CE, GC, TLC, HPLC, and colorimetric assays	Alvarez-Suarez, et al. [59] Trautvetter, et al. [60]
Organic acids	Gluconic, malic, and citric acids, approximately 0.57% of honey; measured by stable isotope labeling assisted LCMS	An, et al. [61]
Vitamin	Vitamin A, B complex, C, E & K; around 0.05–0.11 g/100g, analyzed by HPLC-RP	Bogdanov, et al. [62]
Mineral compositions	K, Ca, Mg, Na, S, and P; Trace elements: Fe, Cu, Zn, and Mn, almost 0.5–0.9 g/100g; evaluated by acid digestion followed by FAAS, GF-AAS, ET-AAS, ICP-OES, and ICP-MS	Solayman, et al. [63] Pohl, et al. [64]
Pollen content	Taxonomical origin, Biomonitoring, Source of amino acid; Microscopy & DNA sequencing	De-Melo, et al. [65]
Enzymes	Diastase activity More than 8 DN Invertase activity range 0.8 to 25.9 IN. Glucose oxidase range 25.58 to 402.47 μg $H_2O_2/h^{-1}g^{-1}$	Sakač and Sak-Bosnar [66] Serra Bonvehí J, et al. [67] Sahin, et al. [68]
Sensory analysis	Electrical nose & Electrical tongue	Veloso, et al. [69] Gonçalves, et al. [70]
Ash content	about 0.1–0.4g/100g, depend on the mineral content & determined by thermogravimetry	Felsner, et al. [71]
Hydroxy methyl furfur	Less than 40 mg/kg, although fresh honey has a minimal HMF (0–0.2 mg/kg). Heat treatment increases HMF content, due to dehydration of fructose; evaluated by reverse phase HPLC coupled with ultraviolet detector, ^{1}H NMR	Anese, et al. [72] Shapla, et al. [73] Chernetsova, et al. [74] del Campo, et al. [75]
Total solids soluble (°Brix)	Calculated from the refractive indextable (20 °C) and °Brix	Albu, et al. [76]
Microbial content	yeasts & bacteria; least colony forming units per gram (cfug)	Adenekan, et al. [77]

ET-AAS electro thermal atomic absorption, *FAAS* flame atomic absorption, *FT-NIR* Fourier transform near-infrared spectrometry, *FT-MIR-ATR* Fourier transform mid-infrared spectrometry with attenuated total reflectance, *GF-AAS* graphite furnace atomic absorption, *HPAEC-MS* High Pressure Anion Exchange Chromatography coupled with Mass Spectroscopy, *ICP-OES* inductively coupled plasma optical emission, *ICP-MS* inductively coupled plasma mass spectrometry, *IR* infrared spectrometer, *LC-ECD* Liquid chromatography-electrochemical detection.

8. Chromatography Coupled Spectroscopic

Analysis for Purity Checking of Honey

8.1. Mass spectrometry

The main component of honey's composition is carbohydrates. The abundance of carbohydrates gives honey its sweet flavor and high calorie content. GC-MS/MS, LC-MS/MS, and UHPLC-MS are more commonly used chromatography coupled mass spectroscopy techniques that are essential for the identification and quantitative analysis of existing carbohydrates, particularly higher order oligosaccharides. These techniques include HPAEC-MS, UHPLC/ESI Q-Orbitrap MS, Nano-ESI-MS, and UHPLC-Q-ToF-HRMS.

8.1.1. HPAEC-MS: It is possible to profile honey's carbohydrates with a high degree of precision using high-performance anion-exchange chromatography coupled with mass spectrometry (HPAEC-MS). When monofloral, polyfloral, and honeydew honey are analyzed using HPAEC-MS, it is shown that fructose and glucose make up the majority of the carbohydrates (73% to 89%). Variability in the carbohydrate concentration and composition has been noted, depending on the honey's genesis. Multifloral honey had higher levels of melezitose (3%), lactose (3%), turanose (2%) and erlose (1%) than monofloral honey with Acacia, where the most common carbohydrates are lactose (2%), turanose (2%) and erlose (2%); dandelions (4%), lactose (3%), turanose (3%), and palatinose (2%); rhododendron (6%), erlose (3%), lactose (2%) and turanose (2%); and honeydew (12%), erlose (3%) and raffinose (2%) [78]. These observations could effectually certify the pharmacological superiority among different mono and polyfloral honey samples around different agroclimatic and geographical realms.

8.1.2. LC-MS/MS: Biochemical investigations (enzymatic) and coupled chromatography and mass spectrometric analysis can be used to quantify the organic acids in honey. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) is a crucial tool for identifying and measuring the organic acids present in honey. Gluconic acid (2995.6 ± 1602.3 mg/kg), malic acid (148.6 ± 346.4 mg/kg), citric acid (73.4 ± 84.2 mg/kg), succinic acid (28.6 ± 25.2 mg/kg), and tartaric acid (8.0 ± 4.6 mg/kg) were detected in honey samples from China, Japan, Canada, Argentina, Romania, the United States, Spain, and New Zealand, among other geographical locations [79].

8.1.3. UHPLC/ESI Q-Orbitrap MS: Honey metabolite profiling offers important information on the composition, geographic origin, entomological origin, and feeding habits of various species which could serve as essential dossier for the pharmacologists to explore these samples for biotherapeutic explorations. The presence of 3-amino-2-naphthoic acid and methyl indole-3-acetate from A. cerana honey and kynurenic acid from A. mellifera honey was successfully detected using ultrahigh-performance liquid chromatography electrospray ionization quadrupole Orbitrap high-resolution mass spectrometry (UHPLC/ESI Q-Orbitrap MS). Finding molecular authenticity markers for confirming entomological origin is much easier with the help of this extremely sensitive honey metabolite profiling [80].

8.1.4. Nano-ESI-MS: UPLC-Q/TOF-MS multivariate study of honey using nanoliter electrospray ionization mass spectrometry (Nano-ESI-MS) reveals the presence of a differential metabolic biomarker, which contains taxonomical, geographical region-specific, and entomological biomarkers. The honeys of Castanopsis (CH), Triadica cochinchinensis (TH), Eurya (EH), and D. dentiger (DH) were all subjected to metabolite profiling. Phenethylamine for CH, tricoumaroyl spermidinex for EH, and (+/-)-abscisic acid for DH are potential biomarkers of particular honey types [81]. Bees that feed on the manuka tree (*Leptospermum scoparium*), which is primarily found in Pacific regions (Australia and New Zealand), produce manuka honey. Manuka honey has enormous nutritional and commercial value due to its antibacterial and antioxidant qualities. Honey metabolic indicators are obtained by combining liquid chromatography with high-resolution mass spectrometry and quadrupole-time-of-flight. The economic significance of honey greatly depends on the analytical identification of biomarkers unique to honey. Methylglyoxal, Dihydroxyacetone (a precursor to Methylglyoxal), and 19 other manuka markers have been found in manuka honey; the signature markers are 2′-methoxyacetophenone, 2-methoxybenzoic acid, 3-phenyllactic acid, and 4-hydroxyphenyllactic acid [82].

8.1.5. GC-MS/MS studies to authenticate volatile compounds in honey: Gas chromatography coupled with mass spectrometry (GC-MS) can be used to do both qualitative and quantitative examination of volatile and gaseous complexes. While GC helps separate the components of a sample, MS makes it easier to detect substances. In order to ensure nutritional validation and proper value assessment, GC-MS's evaluation of honey adulteration and authentication aids in the identification and quantification of the volatile compounds present in honey by identifying floral markers and unifloral signature aromatic profiles [61,83]. Thyme honey has the most volatile components, according to a thorough GC–MS investigation of cotton, Erica, fir, pinus, and thyme honey, which showed a distinct volatile profile chromatogram [84]. Characteristic volatile organic compounds (VOCs) were found in 51 honey samples from 12 monofloral honey samples; these VOCs can serve as useful botanical biomarkers to identify regional variations in monofloral honey [85]. VOCs, such as cis- and trans-linalool oxide and hotrienol, serve as a crucial marker of the changed fragrance profile of honey after extended storage [86]. Table 2 contains a list of signature monofloral volatile chemicals.

Table 2: Characteristic volatile compounds of monofloral honey.

Honey category	Characteristic Volatile Compounds	References
Acacia Honey	cis-linalool oxide, 3-methyl-3-buten-1-ol and heptanal	Machado, et al. [85]
Buckwheat Honey	3-methylbutanal, 2-methylbutanal, (3-methylbutyric acid	Panseri, et al. [87] Machado, et al. [85]
Chestnut	Benzaldehyde, 2-aminoacetophenone, acetophenone, 1-phenylethanol, phenylacetic acid, 3-hexen-1-ol, 2-methyldihydrofuranone	Soria, et al. [88] Machado, et al. [85]
Cotton	1- nonanal, phenylacetaldehyde and phenylethyl alcohol	Machado, et al. [85]
Dandelion	3-methylpentanenitrile and phenylacetonitrile	Jerković, et al. [89] Machado, et al. [85]
Eucalyptus	Phenylacetaldehyde, nonanol and nonanoic acid, terpenes, linalool and linalool	Castro-Vázquez, et al. [90]

	oxides, norisoprenoids	Machado, et al. [85]
Pine	Nonanal, nonanol, decanal and octanal	Tananaki, et al. [91] Machado, et al. [85]
Strawberry	α-Isophorone, β-isophorone, 4-oxoisophorone, 2,5-dimethylfuran, 2,3-butanedione	Seisonen, et al. [92]
Honeydew	Pinene, octane, and nonanal	Tananaki, et al. [84]
Erica	Isophorone and furfural	
Thyme	Benzeneacetaldehyde, benzealdehyde, and benzyl nitrile	
Orange	α-Pinene, cis-myrcenol, methyl anthranilate	Machado, et al. [85]
Strawberry	2,3,5-trimethylphenol, 3,4,5-trimethylphenol	

8.1.6. Application of GC-MS/MS, LC-MS/MS & UHPLC-MS in detection of pesticides in honey: The destruction and eradication of dangerous pests and honey insects are among the detrimental impacts of pesticides on the production of honey. One example of this is colony collapse disorder (CCD), in which insecticides cause a significant number of honey bee colonies to disappear. The sensitivity of the detection technique is shown by the limit of quantification (LOQ, the lowest quantifiable analyte concentration) and limit of detection (LOD, the lowest detectable analyte concentration) values. The European Union (EU) has implemented Maximum Residue Levels (MRLs) to guarantee honey quality and consumer health safety in relation to the presence of pesticide residues in honey.

Pesticides in honey have been detected and quantified at the ppb (parts per billion) level using gas chromatography-tandem mass spectroscopy (GC-MS/MS) and liquid chromatography-tandem mass spectroscopy (LC-MS/MS) analysis. Both consumer health and honey's economic value are negatively impacted when different pesticide residues, such as neonicotinoids, organochlorines, organophosphates, triazoles, carbamates, dicarboximides, and dinitroaniline, are found in honey. The honey obtained from several regions of Kerala has levels of Amitraz, Parathion, Parathion methyl, Deltamethrin, Anthraquinone, and 2 phenyl phenol over the limit of quantification (LOQ) according to LC-MS/MS screening for pesticide residues in honey [93]. Among all the pesticides found, Thiabendazole, Carbendazim, Azoxystrobin, Chlorpyrifos, Imidacloprid, and Thiamethoxam were found to be present in more honey samples, according to LC-MS/MS and GC-MS/MS analyses of commercial honey samples from six Brazilian states **[40]**. In the actual honey and pollen samples taken from the Island of Ireland, a number of contaminants have been identified using GC-MS/MS and UHPLC-MS. These include 2,4-D (herbicide), azoxystrobin (fungicide), boscalid (fungicide), coumaphos (insecticide-acaricide), cyprodinil (fungicide), fludioxonil (fungicide), MCPA (herbicide), propargite (insecticide-acaricide), quizalofop (herbicide), tau-fluvalinate (insecticide-acaricide), trifluralin (herbicide), DDAC (fungicide), and propargite (insecticide-acaricide). Propargite was discovered to be the most prevalent pesticide among these **[94]**. Insecticides were detected in honey samples from Apiário Cambará, Rio Grande do Sul, Brazil, using UHPLC-MS/MS with modified QuEChERS preparation. Although honey has been found to contain pesticide residues such as cypermethrin, permethrin, imidacloprid, clothianidin, chlorpyrifos, and dimethoate, their amounts were below the limit of quantification. For honey quality assurance and appropriate nutritional value maintenance, these pesticide residues must be regularly monitored **[95]**. In Indian context more extensive research to detect the presence of pesticides in honey needs to be warranted for greater welfare of Indians.

8.2. Application of Fluorescence spectroscopy in purity checking

The presence of fluorophores, comprising phenolic chemicals, including phenolic acids, flavonoids, aromatic amino acids, and vitamins is indicated by the fluorescence emission spectra of honey from Unifloral, Acacia, and Sidr plants. The presence of fluorophores in Sidr honey and an increasing right shift towards the wavelength of 507 nm of fluorescence emission spectra with increasing concentration of Sidr honey are demonstrated by a comparative analysis of adulterated honey made with varying concentrations of sugar syrup and Sidr honey [507 nm Sidr honey fluorescence emission maxima] **[96]**. The existence of fluorophore markers as botanical identifiers can be further understood by comparing Sidr honey with commercial samples, polyfloral honey, and unifloral honey from (Elettaria cardamomum, Citrus reticulata, and Grewia asiatica) **[97]**. The existence of numerous phenolic compounds is confirmed by the analysis of unifloral honey samples obtained from the four distinct taxonomical sources (citrus, pine, fir, and thyme) and three-dimensional emission matrices (3D-EEM) Spectra of Phenolic Compounds **[98]**. To clarify the spectral components ratio derived from the phenol and proteins (Ph/Pr) components of honey, fluorescence spectroscopy in conjunction with multivariate curve resolution alternating least squares (MCR-ALS) was utilized **[99]**. Real honey, fake honey, and HFCS-55 (high fructose corn syrup) may all be distinguished using fluorescence spectroscopy and soft independent modeling of class analogy (SIMCA) analysis. Fake honey and HFCS-55 show identical peaks at the wavelength of 378 nm, but the fluorescence spectra of real honey (SBH) showed a prominent peak at 480 nm **[100]**. Moreover, combined liquid chromatography-fluorescence detection (LC-FLD) can be used to identify the presence of sulfonamide and its derivatives **[101]**.

8.3. Application of ultraviolet-visible spectroscopy in purity checking

In order to identify and quantify polyphenols, including flavonoids and polyphenols in honey, as well as to detect artificial sweeteners and colorants, UV-vis spectroscopy is used in conjunction with multivariate statistical analysis **[102]**. In order to identify adulterated honey, it is essential to use UV-vis spectroscopy to determine the percentage of aromatic content present. Suhandy, et al. [103] state that the absorbance range of proteins, phenolic compounds, and amino acids-primarily tryptophan-is 260-300 nm, the absorbance range of sugars, particularly glucose and fructose, and phenolic compounds is 200-260 nm, and the absorbance range of flavonoids is 300-340 nm. While the spectral data of pure honey shows increased absorption at a wavelength window between 250 and 300 nm, a discernible change is seen at 280 nm. This drop in absorbance intensity has been seen in honey containing high fructose corn syrup (HFCS) [104]. The identification of fillers such as corn syrup, agave

syrup, sugarcane molasses, and other sweeteners is essential for honey authenticity and quality control. When combined with one-class pattern recognition techniques, such as OC-PLS and DD-SIMCA, UV-vis spectroscopic analysis yields extremely sensitive and precise results when identifying sugar-based adulterants in honey [105].

8.4. Application of Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy

By displaying the FTIR spectral band attribution of particular groups and chemicals, Fourier Transform Infrared Spectroscopy (FTIR) examination of honey yields botanical information. To identify distinct peaks and distinctive spectrum features, FTIR spectroscopy uses the frequency of molecular vibrations of single bonds (C-H, O-H, N-H), double bonds (C-O, C-N), triple bonds (C-C, C-N), and fingerprint zones. The ratio of oligosaccharides and the group-specific frequency range (cm^{-1}) in FTIR spectra reveal information about the components of honey [106]. For the identification of adulteration in honey, FTIR in conjunction with principal component analysis (PCA) and cluster analysis (CA) has been used. Spectral region analysis has been studied between 4000 and 650 cm^{-1}, while cluster analysis has been considered between 1800 and 650 cm^{-1}. In order to differentiate between pure and contaminated honey made of simple sugar, the spectral range between 1800 and 750 cm^{-1} is thought to be extremely important. Certain functional groups and their changed molecular bond vibration can be identified using the wave number (cm^{-1}) of pure honey and the shifting wave number of contaminated honey [107]. The origin of honey can also be ascertained using FTIR absorption spectra; for example, unifloral and multifloral honey showed notable FTIR spectral alterations, indicating differences in honey content. According to the study's comparison analysis, Honeydew honey had the sample group's greatest absorbance based on FTIR absorption spectra [108]. An Amide II spectral absorption peak was detected at 1547 cm^{-1}, inside the predetermined FTIR spectral area of 1580-1500 cm^{-1}. An increase in absorbance signifies a higher concentration of royal jelly in honey; this discovery shows a result that is synonymous with HPLC (R2 = 0.99) [109].

8.5. Attenuated total reflectance (ATR)-FTIR spectroscopy for purity checking

An FTIR technique called ATR-FTIR spectroscopy is used to identify and measure the inorganic ions and organic functional groups in aerosol samples [110]. Using the spectral variations in the 4000-650 cm^{-1} spectral range, honey samples are analyzed. The five regions (Region 1-5, which consist of sequential wave numbers 3000-2800 cm^{-1}, 1700-1600 cm^{-1}, 1540-1175 cm^{-1}, 1175-940 cm^{-1}, and 940-700 cm^{-1}) in the FTIR spectra have been separated in order to identify monofloral honey from various taxonomical sources. Each region lies in a specific wave number and represents specific bond movements in macromolecules present in honey [111]. Eighty four unifloral honey samples from Sardinia, Italy (strawberry, asphodel, thistle, and eucalyptus) were subjected to ATR-FTIR and chemometric analysis. The results showed a distinctive phenolic proportion in the ATR-FTIR Spectra at region 1540-1175 cm^{-1} [112].

8.6. Isotope ratio mass spectrometry (IRMS) for quality checking

Different photosynthesis cycles (C4 and C3 plants) produce carbon isotope ratios 13C/12C, which can be distinguished using element analyzers (EA) and liquid chromatography (LC) in conjunction with isotope ratio mass spectrometry (IRMS) analysis. It is possible to ascertain the isotope ratio of specific sugars (δ13Cglucose (δ13Cg), δ13Cfructose (δ13Cf), δ13Cdisaccharide (δ13Cds), and δ13Ctrisaccharide (δ13Cts) by conducting a δ13C survey of 33 honey samples. By using the highest absolute differences between all δ13C values (Δδ13Cmax), the authenticity of honey has been established; a higher Δδ13Cmax value indicates more adulteration. EA IRMS results revealed a higher percentage of C4 sugar (HFCS) in adulterated honey, while LC IRMS results of 33 honey samples from various Lebanese locations showed 13 samples of adulterated honey [113]. If the Δδ13Cmax (abs) is less than ±2.1%, it is deemed pure honey. A value of Δδ13Cmax (%) that was outside of the range of ±2.1% was deemed to be contaminated in the EA/LC-IRMS analysis. About 70% of the honey samples failed the δ13C-LC-IRMS test, according to an analysis of a variety of honey samples from Acacia (n = 29), Rape (n = 33), Vitex (n = 11), Cotton (n = 10), Linden (n = 4), Jujube (n = 3), and Sunflower (n = 4) [114]. Honey saccharide profiling is provided by IRMS analysis, which is primarily used to identify instances of C4 sugar adulteration in honey. Additionally, values of δ13C and δ15N of honey and honey proteins provide valuable information to distinguish between unique mono- and polyfloral honey origins [115].

8.7. Application of stable isotope ratio mass spectrometry (SIRMS)

A total of 116 commercial honeys from the following countries have undergone thorough EA/IRMS and LC/IRMS analyses: Japan (34), Spain (13), Italy (13), France (10), New Zealand (10), China (9), Canada (4), Hungary (4), EU (4), Argentina (1), Australia (1), Bulgaria (1), Kuba (1), Mexico (1), Romania (1), Taiwan (1), America (1). Table 3 presents the δ13C values of protein, glucose, fructose, disaccharides, trisaccharides, and organic acids in pure honey, together with the δ13C value of pure honey itself [116]. Based on honey adulteration, SIRMS analysis can detect C4 sugars (cane and corn syrup) with excellent accuracy. A C4 sugar adulteration evaluation using the ISCIRA method was conducted on accessible honey samples from the Philippine market. The findings indicated that the percentage of C4 adulteration in honey purchased locally from local stores and online was 75% and 86.5%, respectively. The genuine Philippine honey samples from Apis mellifera (11), Tetragonula biroi (13) and Apis breviligula (14), have been compared with this differential analysis. With the use of a continuous-flow EA-IRMS system, the carbon-13 isotope abundance (δ13C) of all 38 samples was determined to range between -29.57 and -23.79‰, which is characteristic of C3 plants. But in (64 of 74) Philippines honey that was bought online, the bulk honey's δ13C (‰) ranged from -10.71 to -18.73, and the apparent C4 sugar content (%) was determined to be between 7 and 100% [between 7 and 10 (3), between 11 and 50 (1), between 51 and 70 (1), 71 and 80 (1), 81 and 90 (7), and between 91 and 100 (51)]. Lao, et al. [117] Stable isotope detection has been found to be more effective with two-dimensional (2D)-LC. High-precision organic acid identification in honey has been achieved by using the heart-cutting two-dimensional LC/IRMS method for honey sample analysis. Gluconic, malonic, and citric acids were found in chromatograms of 25 purchased honey samples from Japan (10), New Zealand (5), China (3), Spain (2), Canada (2), Romania (1), the United States (1), and Argentina (1), according to 2D-LC/IRMS analysis. However, only gluconic acid was found, indicating the presence of other organic acids in lesser concentrations. According to Suto, et al. [118], the

2D-LC/IRMS data obtained for glutonic acid revealed a δ13C value of -31.7 to -28.5‰ (mean: -30.0 ± 0.7 %) and a concentration of 415 to 6043 mg kg^{-1} (mean: 2674 ± 1635 mg kg^{-1}).

Table 3: The stable isotope ratio of individual sugars and their δ13C values.

Stable Isotope	δ13C values	References
Glucose	from -27.0 to -23.8 ‰	Kawashima, et al. [116]
Fructose	from -28.4 to -23.8 ‰	
Disaccharides	from -30.2 to -24.0 ‰	
Trisaccharides	from -29.2 to -22.8 ‰	
Organic Acids	from -33.6 to -26.5 ‰	
Pure Honey	from -29.1 to -24.3 ‰	

8.8. Application of Inductively coupled plasma mass spectrometry (ICP-MS)

When it comes to assessing food adulteration and screening for authenticity, ICP-MS is an incredibly accurate investigative investigation. ICP-MS is helpful for determining the isotopic fraction, multi-elemental composition, and quantification of various components [119]. The elemental profile of Romanian honey samples made from sunflower, linden, acacia, and rape has been investigated using inductively coupled plasma quadrupole mass spectrometry (ICP-Q-MS) by measuring the concentration of several elements. The mineral fingerprint profile of honey can be a helpful indicator of origin and botanical identification, with the most common elements being K (248.70 mg kg^{-1}), Ca (59.97 mg kg^{-1}), Mg (20.54 mg kg^{-1}), and Na (11.92 mg kg^{-1}) [120]. Significant quantities of main elements (K, Mg, Mn, and Na) and trace elements (Cu) were found in another study that screened 18 elements of diluted honey samples from Poland [121]. In order to identify mineral concentration profiles as a sign of the botanical origins of honey samples, monofloral honey of buckwheat and rape honey and honeydew from Poland have been examined for various element proportions in conjunction with CA and PCA analysis. The key elements identified using principal components analysis (PCA) were Al, Cd, K, Ni (PC 1), Ba, Na, Pb (PC 2), Cu, Mg (PC 3), and Zn (PC 4). Honeydew honey has K, Al, Ni, Cd, and Zn profiles, while rape honey has Ba, Na, and Pb profiles [122]. Using the HPLC-ICP-MS technology, mineral elements were examined in honey samples as a potential indicator of pollution. Arsenic species, such as As(III), As(V), AsB, DMA, and MMA, as well as water-soluble arsenic, have been identified and quantified in 90 honey samples from Poland and Ukraine. The mean concentration of arsenic in Polish multiflowered honey was found to be 6.1 μg kg^{-1}, while the mean concentration in Polish acacia honey was 13 μg kg^{-1}. In contrast, the mean concentrations in Ukrainian multiflowered and acacia honey were found to be 1.0 μg kg^{-1} and 2.5 μg kg^{-1}, respectively [123]. The geographical provenance of honey samples has been ascertained by ionomics analysis based on ICP-MS. To distinguish A. cerana honey from various geographic locations, the HPLC-DAD (Diode Array Detector) approach together with multivariate data analysis of the mineral element content of honey samples has been carried out. The heat map analysis revealed distinct features in the honey samples from Liuba, Yangxian, and Longxian [124].

8.9. NMR spectroscopy to detect purity of honey

One important factor in confirming the quality of honey and ensuring the safety of consumer health is the identification and measurement of potentially hazardous substances in honey samples. Benchtop nuclear magnetic resonance (NMR) spectrometers have been used to identify pollutants in honey, including drug residues and pharmaceutical components. Tadalafil (37), sildenafil (5), and a combination of flibanserin and tadalafil (1) were found in 50 French honey samples that were analyzed [125]. By measuring the different percentage of sugars, amino acids, and carboxylic acid, 1H NMR in conjunction with multivariate data analysis offers insights about the geographical origin of honey [126]. Furthermore, 1H NMR metabolomics analysis may also identify the presence of C3 and C4 adulterants in stingless bee honey (SBH) [127]. HPLC and 1H-NMR in conjunction with chemometrics analysis have been used to identify and measure non-sugar components (NSC) in monofloral honey samples, such as jujube honey (15), chaste honey (16), and locust honey (19). NSC was found to be 780.77 ± 8.98 mg/100 g in jujube honey, 707.94 ± 7.86 mg/100 g in locust honey, and 292.43 ± 5.67 mg/100 g in chaste honey [128]. It is possible to distinguish between seasonal bees using the standard 1D 1H NMR spectra of the body components of honey worker bees. Three of the 28 metabolites-fructose, sucrose, and trehalose-have been found to be significant markers of honey bee seasonal fluctuation. Summer bees contain more free amino acids, while winter bees have more adenosine monophosphate (AMP), fructose, sucrose, trehalose, oxidized nicotinamide adenine dinucleotide (NAD+), and O-phosphocholine. Seasonal changes in physiological requirements are exemplified by the outcome [129]. Analyzing the 1D and 2D 1H and 13C-NMR spectra of honey samples from North Macedonia (16) and Bulgaria (22) can be a useful and practical method for determining their botanical and geographic origin. Together with the distinctive carbohydrate profiles of honey, the 2D score PCA and hierarchical clustering can be effective determinants in identifying and authenticating the honey's origin [130]. The following metabolites were taken into consideration during the 1H NMR-based metabolomic study of honey samples (9) from Vietnam: HMF, Citric acid, Acetic acid, Lactic acid, Ethanol, Alanine, Tyrosine, and Phenylalanine. Given that honey is thought to have both nutritional and therapeutic benefits, quality control (no artificial additives) and freshness monitoring (no HMF) can be crucial in the authenticity screening process [131].

8.10. Application of raman spectroscopy

In order to authenticate honey, phytochemical analysis and the identification of adulterants might be very helpful. To ascertain the physicochemical properties, 189 honey samples from Campeche, Mexico, were subjected to a low-cost examination using Raman spectroscopy. Raman spectra that were taken into consideration for analysis ranged from 201 cm^{-1} to 2000 cm^{-1}, however there were notable spectrum shifts found between 330 cm^{-1} and 1460 cm^{-1}, with 13 distinctive segments linked to certain bond vibrations (bending and stretching) [132]. Evaluation of the honey sugar profile, it is also possible to distinguish between honey from

different sources and identify honey that has been adulterated with HFCS and other sugar syrups using Raman spectroscopy. According to Xu, et al. [114], there are several methods for conducting spectral analysis of honey, including partial least square (PLS), principal component analysis (PCA), principal component regression (PCR), and partial least square discrimination analysis (PLS-DA). In order to identify potential adulteration, 97 pure honey samples from 12 different nations were sourced and subjected to HPLC-RI with Soft Independent Modeling of Class Analogy (SIMCA) data analysis. The 200–2500 cm^{-1} range of Raman spectra taken into consideration for analysis shows that 17% of the samples have adulteration in the 400-1400 cm^{-1} spectra fingerprint region [133]. Silver-coated gold nanoparticles (Au@Ag NPs) and the highly accurate SERS probe 8-thioguanosine (8-TG) were used in the intrinsic Raman signal amplification approach for the rapid detection of methylglyoxal (MGO) in manuka honey. Probed by 8-TG MGO produced a peak at 631 cm^{-1} when 8-TG reacted with it to form N2-(1carboxyethyl)-thioguanosine (CETG), while Au@Ag NPs displayed a characteristic Raman signal at 700 cm^{-1}. Consequently, analysis has been conducted using the Raman intensity ratio (I631/I700), and MGO encourages an increase in this ratio. It was determined that the assay's limit of detection for MGO detection was 0.392 µg mL^{-1} [134]. 900 adulterated honey samples (containing increasing concentrations of various sugar syrups) and 56 pure honey samples (acacia, honeydew, sunflower, tilia, and polyfloral) from Romania were examined using Raman spectroscopy, which ranges from 250 to 2339 cm^{-1} to identify specific spectral changes depending on the concentration of the adulterant. Significant spectral shift must be seen among the spectral profiles of several adulterants when the concentration of malt increases [135]. Reliable and practical green Raman spectroscopy analysis is demonstrated by comparing raw honey samples with heat-treated and water-diluted honey. Acacia, honeydew, raspberry, thyme, sunflower, lime, coriander, and rape honey have all been shown to exhibit discrete spectrum alterations [136].

9. State-of the-Art Advanced Techniques in Honey Analysis

Advanced analytical techniques, such as genetic marker-based identification technologies like PCR, transcriptome sequencing, and high throughput sequencing of DNA fragments present in honey, could supplement traditional palynology with increasingly accurate and sensitive comprehensive information. This information could include molecular markers for floristic genes, geographical region, entomological origin, bee foraging behavior, plant species diversity monitoring, the influence of various climatic changes, including potential effects of global warming, and more. The key methods in honey analysis that guarantee honey authenticity while tracking environmental factors and biodiversity are DNA metabarcoding, Enzyme-Linked Immunosorbent Assay (ELISA), honey zymography, and metagenomic analysis of honey bee gut microbiota studies, as explained in Table 4.

Table 4: Advanced techniques in honey analysis.

Techniques	Principle	Applications in honey analysis	References
DNA Metabarcoding	Amplification and sequencing of environmental DNA (eDNA)	Identification of Taxonomical source of Honey	Wirta, et al. [137]
ELISA	Assay based on affinity of Ab to quantify Ab or Ag by use of an enzyme-linked antibody by forming a colored reaction product	Detection of antibiotic contaminants and possible allergens in Honey	Poungmalai, et al. [138]
Proteomics & Zymography	Protein purification & SDS PAGE, Enzymatic activity assay	Recognize signature protein and enzymatic profile of honey	Alaerjani, et al. [139]
Metagenomic analysis of honey bee Gut microbiota	Extraction, amplification and sequencing of 16S rRNA gene for detecting microorganism evolution & bioinformatics-based gene ontology study	Identification of Entomological origin & microorganism biodiversity	Ellegaard, et al. [140]

9.1. DNA Metabarcoding

In order to identify plant species and their diversity in a specific geographic area, amplification has been carried out using a marker of the Internal Transcribed Spacer (ITS2) and the large subunit of ribulose bisphosphate carboxylase, RuBisCO (rbcL), after pollen DNA was extracted from 196 honey samples [141]. Primers rbcLaf and rbcLr506 were used in the rbcL Illumina MiSeq to sequence pollen DNA and find taxonomical biomarkers. Honey bees have been shown to exhibit distinct seasonal preferences and shifts in their feeding habits. Bees favor *Brassica* spp., *Prunus* spp., *Ulex* spp., and *Salix* spp. in April, and *Rubus* spp. in June, July, and August. In addition to *Trifolium repens*, *Cirsium/Hypochaeris/Centaurea* spp., *Rosa* spp., and in September, **Hedera helix**, *Impatiens glandulifera*, and *Camellia* spp., the honey bees were found to favor these plants as the main preferable selection. This pollen DNA Barcoding finding has led to the conclusion that honey undergoes season-specific modification, showing seasonal change in honey's nutritional content [142]. Two metabarcoding markers (ITS2 and trnL) have been used in sequencing-based biomonitoring to examine the feeding habits of European honey bees (Apis mellifera) in the Jerrabomberra wetlands, an urban reserve in Canberra, Australia. According to Milla, et al. [143], pollen DNA metabarcoding showed that Eucalyptus (26.7%) was the most common genus, while Plantago (75% of plots), Hypochaeris (62%), and Sonchus (50%) were the three most commonly found genera. The most distinctive taxa were found to be Fabaceae, Asteraceae, Myrtaceae, Brassicaceae, Macadamia integrifolia, and Melaleuca nodosa based on the trnL and ITS2 sequencing data of pollen DNA metabarcoding of honey from several Australian locations [144]. A comparison between honey from *Apis cerana* (ACH) and *A. mellifera* (AMH) revealed that honey bees have species-specific floral preference. The findings showed that, of the 56 taxa that were found, the presence of both unique taxa (ACH 23 & AMH) 15 and shared taxa (Actinidia, Diospyros, Flueggea, and Anacardiaceae) clarified the pattern of interspecies competition [145]. Amplification and sequencing of these

genes revealed the presence of pathogens, as well as the geographical and floral source of honey. Metabarcoding of environmental DNA (eDNA) from various sources derived from honey includes microorganism-specific (16s rRNA), botanical origin-specific (chloroplast trnL), and honey bee-specific (mitochondrial cytochrome oxidase subunit I (COI) genes [146]. DNA evidence of bacteria (16S rRNA gene), fungi (ITS), and plants (ITS2, rbcLa, and trnL) were found in honey samples gathered from northern European nations (Estonia, Finland, and Sweden). The findings showed microbiological and taxonomical variety [137]. Samples of honey and beebread were collected from 43 beehives in Finland between June and August 2021, and the samples were then subjected to pollen DNA metabarcoding analysis. In addition to indicating seasonal change in honey metabolites, the results clarified the floral selectivity of honey bees, as only 32 and 30 of the 73 genera studied were present in honey and beebread, respectively [147]. Table 5 lists significant molecular markers in honey analysis. Environmental DNA metabarcoding yields a wealth of information about environmental materials through the use of integrated sequencing technologies, bioinformatics, and statistical techniques. With great accuracy and specificity, genetic marker-based technologies may discriminate between ecosystem health, monitoring biodiversity, honey's geographical provenance, pollinator species variety, feeding patterns, and seasonal changes (Figure 8).

Figure 8: Steps followed in DNA metabarcoding to authenticate honey samples [148].

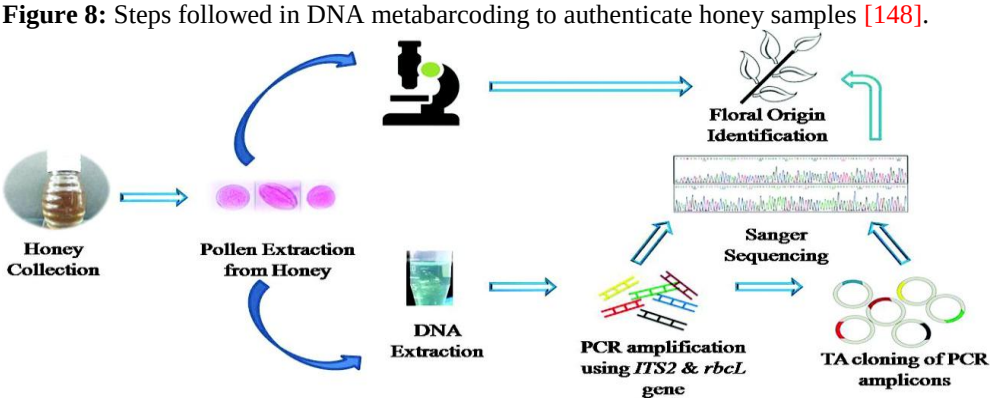


Table 5: Molecular markers in honey analysis.

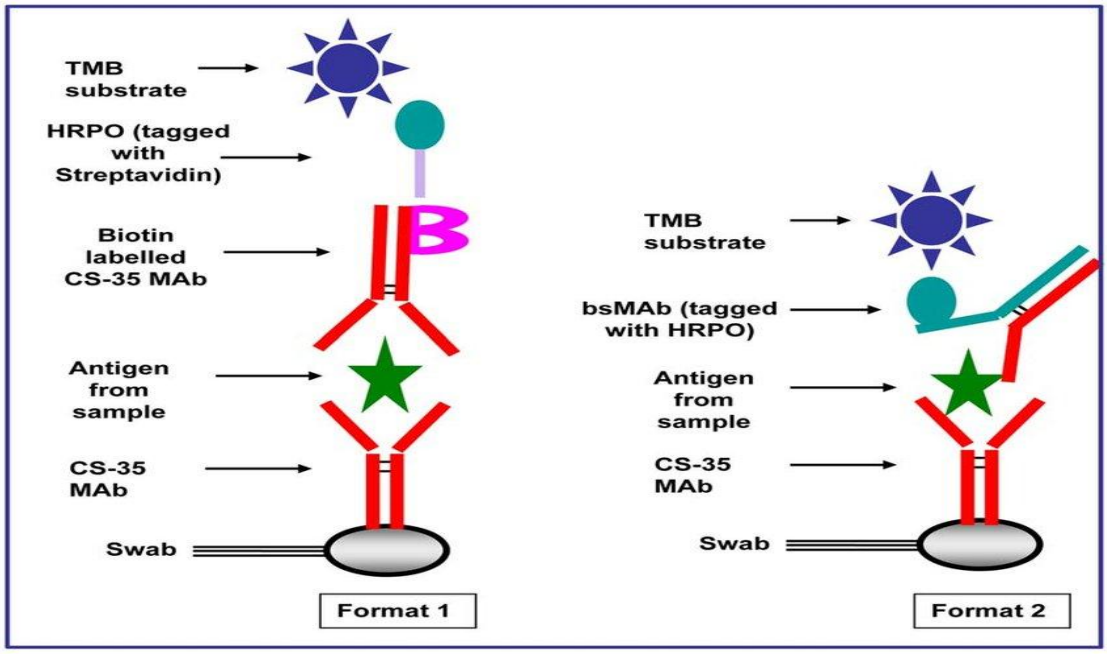
Gene code	Gene name & source	Significance in Honey analysis	References
RuBisCO (*rbcL*)	Large Subunit of Ribulose Bisphosphate Carboxylase; Plant	Taxonomical & diversity biomarkers	Khansaritoreh, et al. [141]
trnL	Chloroplast; Plant		Milla, et al. [144]
ITS2	Internal Transcribed Spacer; Plant		Milla, et al. [143]
COI	Mitochondrial Cytochrome Oxidase Subunit I; Honeybee	Entomological origin	Pathiraja, et al. [146]
ITS	Nuclear ribosomal internal transcribed spacer; Fungi	Microbial diversity biomarkers	Wirta, et al. [137]
16s rRNA	16S ribosomal RNA; Microorganism		Pathiraja, et al. [146]

9.2. ELISA

The existence of antibiotics and antibiotic residues in consumable goods might have detrimental effects and can disrupt current treatment plans, leading to severe illnesses. ELISA technology based on monoclonal antibodies (mAb) can be used to identify antibiotics found in health items. Antibiotic residues can be detected with excellent accuracy and at a much lower detection limit thanks to ELISA's specificity and sensitivity. Using indirect competitive ELISA (icELISA), honey has been found to contain chlortetracycline (CTC), with a detection limit of 0.1 ng/ml [138]. Upon screening 40 honey samples for chloramphenicol (CAP), it was found that all of the samples contained CAP (the reporting limit is 0.1 µg kg^{-1}), with 2.2 µg kg^{-1} being the highest concentration [149]. Sulfonamide antibiotic residue was found in 23.75% (19) of the 80 honey samples from different antibiotic doses ranging from 10 to 120 ng/g, according to a semi-quantitative ELISA technique [150]. In addition to revealing an IC50 of 0.12 ng/mL, the ic-ELISA screening of commercially available honey samples for Sparfloxacin (SPFX) also revealed the presence of cross-reaction (CR) with pertinent antibiotic substances such as Flumequine, Fleroxacin, Enrofloxacin, Ciprofloxacin, and Ractopamine [151]. Along with having the ability to cross-react (CR) with Narasin, Maduramycin, Monensin, Nigericin, and Lasalocid, the development of an ic-ELISA that could detect both Salinomycin (SLM) and Methyl Salinomycin (MLN) in honey samples showed an IC50 of 0.86 ng/mL and a detection limit of 0.28 ng/mL [152]. Tulathromycin (TULA), an antibiotic used to treat respiratory diseases in cattle, can cause hypersensitive allergies and the establishment of antibiotic-resistant strains if it is ingested. According to Liu, et al. [153], the immunochromatographic test strip designed for TULA detection has shown lower detection limits of 4 ng/mL and cut-off values of 10 ng/mL. With detecting limits of 0.4 µg/kg, the new honey antibiotic detection system (HADS), which is based on a lateral-flow immunochromatographic assay (LFIA)-based strip, can simultaneously detect tetracyclines (TCs) and sulfonamides (SAs) [154]. In 40 samples of honey, thiacloprid (insecticide) residues were detected in the detection range of 0.003 to 0.06 mg/kg. The ic-ELISA showed IC50 and IC20 values of 0.38 ng/mL and 0.097 ng/mL, respectively [155]. In comparison to immunoassay-based procedures for honey analysis, these screening techniques exhibit a number of benefits, such as easy experimentation, short procedure, cost effectiveness, extremely sensitive and accurate detection, and rapid data analysis and interpretation. These analytical methods make it

simple to maintain the effectiveness of honey's nutritional benefits, which require strict quality testing and ongoing monitoring.

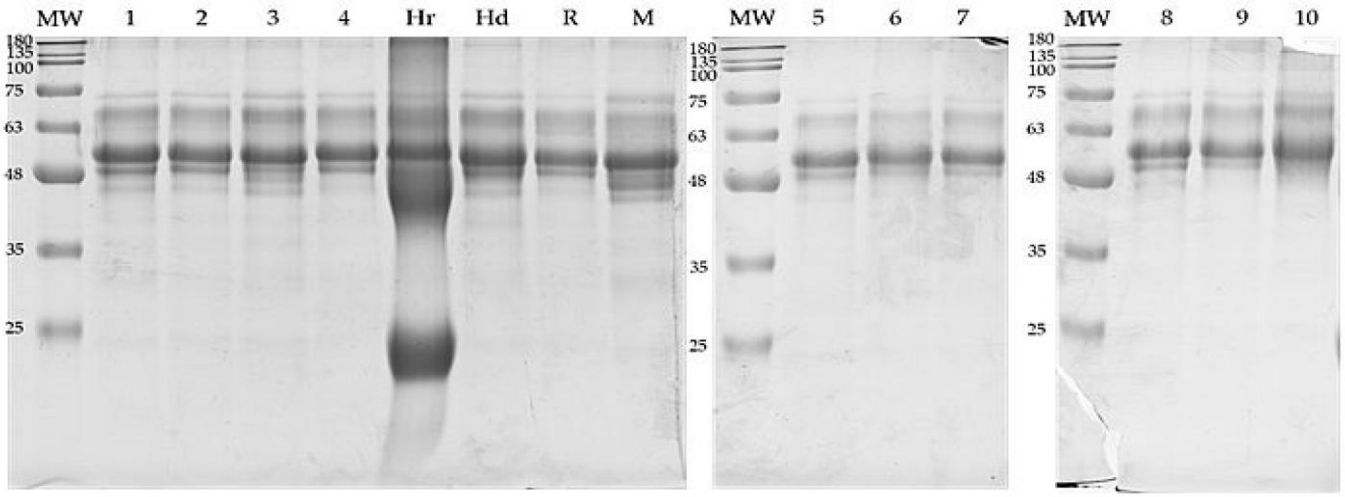
Figure 9: Two formats of sandwich ELISA. Format 1: Monoclonal antibody (MAb) based, where both the capture and detection antibody are MAb's. Format 2: Bispecific Monoclonal antibody (bsMAb) based, where the capture antibody is a MAb but the detection antibody is a bsMAb [156].



9.3. Proteomics & zymography to detect honey purity

Proteases (antimicrobial & antioxidant), glucose oxidase (antibacterial), acid phosphatase (floral origin biomarker & fermentation indicator), diastese (honey quality factor), and invertase (honey storage & processing factor) are the main enzymatic components of honey [139]. For honey to be processed and stored for optimal quality, it is crucial to optimize the factors (temperature, duration, and pH) and their combined impact on honey enzymatic activity [157]. According to Huang, et al. [158], an analysis of 110 honey samples revealed that diastaseactivity is adversely affected by rising temperatures and heating times. The hypopharyngeal glands of honeybees generate the enzymes invertase and glucose oxidase, which serve as a quality biomarker for honey's nutritional value and guarantee of quality. Invertase activity decreased and glucose oxidase activity varied with temperature and time in Turkish honey samples (Anzer flower, Pine, and Oak), which are crucial for determining the optimal extraction temperature [68]. 24 honey samples from Castilla y León, Spain, showed that honey's antibacterial activity against Staphylococcus aureus was mediated by the enzymes catalase (CAT) and glucose oxidase (GOx) [159]. Another helpful way to determine the honey's geographic origin is through protein profiling. 45 honey samples from Belgium, France, Italy, Romania, and Spain were subjected to SDS-PAGE analysis, which revealed distinctive protein patterns as a geographical and palynological biomarker [160]. Two chitinase types found in the examined honey are xylosidase and thaumatin. Floral nectar chitinase can be used as an authenticity biomarker in monofloral honey [161].

Figure 10: Protein profiles of analyzed goldenrod honey samples in comparison to other selected honeys obtained using SDS-PAGE. Abbreviations: 1-10-goldenrod honeys, Hr-heather honey, Hd-honeydew honey, R-rapeseed honey, M-multifloral honey, MW-BlueEasy Prestained Protein Marker [162].

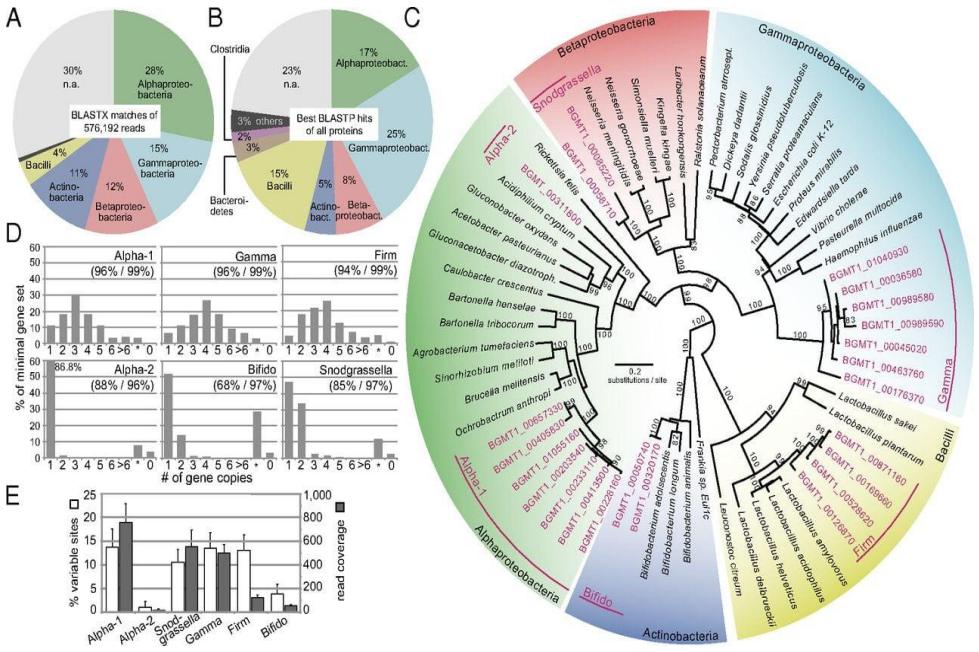


9.4. Metagenomic analysis of honey bee gut microbiota

Diversity screening and profiling of honey bee gut-associated

microorganisms has shown itself to be a highly effective indication of important factors in guaranteeing the nutritional and therapeutic qualities of honey. The entomological origin and health status of honey bees, as well as environmental changes like pollution, climate change, and global warming, can be usefully determined by the diversity of host-specific bacterial communities. Metagenomic study of the gut microbiota of A. cerana and A. mellifera revealed the presence of both species-specific and shared bacterial communities. The study used qPCR assay-based 16S rRNA gene analysis to examine the hindguts of 40 bees (20 each species Bartonella apis, Commensalibacter sp., and Frischella perrara are strains of A. mellifera, while Apibacter sp. is the strain of A. cerana. A. mellifera has been found to have a greater diversity of bacterial strains. But there is a close relationship between these two species. According to Ellegaard, et al. [140], A. mellifera have a larger hindgut, a varied pollen diet, larger colonies, and a global distribution. Bifidobacterium, Bartonella, Frischella, Gilliamella, Lactobacillus, and Snodgrassella were identified as common bacterial communities in the guts of A. mellifera and A. cerana [163]. Variability in the gut microbiota of honey bees can serve as a marker of microbial evolution and environmental shifts. A study of the KEGG gene ontology revealed a potential impact of dietary characteristics on the transport and metabolism of carbohydrates [164]. Gut metabolomics, taking into account four seasonal points (summer, early-, mid-, and late-winter), showed a discernible decrease in pollen-specific metabolites such as tricoumaroyl spermidine (exosporium), spermidine, and 9,10-dihydroxystearic acid (sporopollenin), as well as flavonoids (kaempferol, keracyanin, and quercitrin). showing a decrease in nutrients in the gut of honey bees, which may have an impact on the gut microbiota. Seasonal fluctuation in the stomach microbiota of honey bees was shown by a comparative research between Apis mellifera sub-species, A. m. carnica, A. m. ligustica, and A. m. mellifera. Additionally, TCA cycle, glyoxylate, decarboxylate, porphyrin, and chlorophyll metabolism were found to be predominant during the winter months, according to gene ontology and pathway analysis [165]. By extracting and amplifying the bacterial 16S rRNA (V3-V4 region), it is also possible to identify variations in the honey gut microbiota caused by the nectar of jujube (*Ziziphus jujuba* Mill) flowers. In comparison to healthy bees, honey bees infected with jujube blossom illness exhibited a relative increase in Firmicutes and Actinobacteria and a relative decrease in Proteobacteria [166]. Amplification of the V4 region of the 16S rRNA gene from the stomach microbiome of Swiss honey bees revealed that whereas Gilliamella and Snodgrassella were more common in forager bees, Lactobacillus Firm-4 and Bartonella were more abundant in winter bees [167]. A functional metagenomic approach of gut microbiome deep mining approach is illustrated in Figure 11.

Figure 11: Identification of bacterial species or species groups in the honey bee microbiome and analysis of their genetic diversity. Phylogenetic profile based on (A) classification of 576,192 reads mapping against 31 marker proteins with MetaPhyler (48) and (B) best BLASTP hit distribution of all 112,128 CDSs. n.a., reads or CDSs not assigned. (C) Maximum-likelihood protein phylogeny of UvrC. All eight phylogenies revealed that most sequences from the honey bee microbiome (shown in pink) fall into the same six distinct clusters. These phylogenetic clusters are referred to as Alpha-1, Alpha-2, Snodgrassella, Gamma, Bifido, and Firm. We considered all closely related taxa with available genomes for this analysis. Bootstrap values >80 are shown. (D) Percentages of the minimal gene set present in each bin are depicted in parentheses (only full-length copies/including fragmented genes). Graphs show distribution of genes of the minimal gene set based on identified full-length copies per bin. Asterisks indicate fragmented genes. (E) Average percentage of variable sites and average read coverage for 27 ribosomal protein-encoding genes of each bin [168].



10. Conclusion

Honey has been prized for its medicinal qualities, purity, nutritional worth, capacity to support life at any stage of life, and use as a human elixir since ancient times. It is regarded as a long-standing gem of the living world. Ayurvedic science, apiculture, plant science, horticulture, wildflower gardening, entomology, forestry, and silviculture are just a few of the diverse sectors of research and development that honey has linked together in a variety of intricately intertwined networks. Many modern chromatographic separation techniques have been employed to identify and verify pure honey as well as to detect adulterants in honey samples. Nectar-related genetics research indicates that the spring and summer flowering seasons are when the relevant

genes are mainly activated. Investigating honey quality, precise nutrition, and economic valuation are some of the primary reasons for combining proteomics technologies with data analysis based on honeybee zymography and metabolomics. Honey's many health-protective qualities, including its antibacterial, antiviral, antimicrobial, anti-inflammatory, and antioxidant qualities, have drawn attention from pharmaceutical companies and clinical research. A viable alternative to conventional fixed monitoring stations for ongoing pollution monitoring is the use of honey bees as biomonitors. This method provides a more thorough and nuanced view of environmental health, which is consistent with the One Health idea, which acknowledges the connection between human, animal, and environmental health. We can learn a great deal about the state of the ecosystem and, consequently, the welfare of the communities that live there by keeping an eye on honey bees. However, the amount of research that is currently available in this area is little and frequently concentrates on contrasting landscapes and evaluates similar pollutants without providing enough context. This emphasizes how honey bee biomonitoring studies require more research and standardization. In addition to improving our knowledge of environmental health, these initiatives will help create broadly used and accessible monitoring procedures. Standardized investigations are required from the standpoint of environmental health in order to potentially develop an approachable monitoring strategy and to facilitate a more uniform assessment of the levels present in the beehive matrices.

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