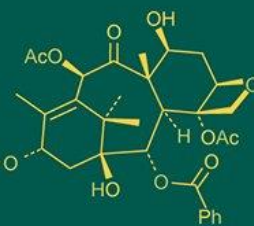
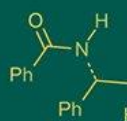


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Authentication of multifloral honey using headspace gas-chromatography mass spectrometry combined with Fourier transform infrared spectroscopy

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Abstract

Honey authentication is a necessary step in building consumer confidence for increasing adulteration posing a significant challenge to its authenticity. The rapid and reliable analytical methods capable of distinguishing authentic honey from adulterated products are need of the hour for quality assurance and regulatory enforcement. This study evaluated the combined application of Headspace Gas Chromatography Spectrometry (HS-GCMS) and Fourier Transform Infrared Spectroscopy (FTIR) for authentication of multifloral honey collected from local beekeepers. HS-GCMS is employed to characterize volatile compounds, while FTIR spectroscopy generated molecular fingerprints of the samples. FTIR analysis revealed characteristic differences within the carbohydrate fingerprint region, whereas HS-GCMS identified volatile markers associated with authentic multifloral honey. The results demonstrate that the combined HS-GCMS and FTIR approach provides a rapid, reliable and non-destructive analytical platform for routine authentication of multifloral honey produced.

Keywords: Multifloral honey, honey authentication, HS-GCMS, FTIR

1. Introduction

Honey is a natural product synthesized by honey bees from floral nectar and is valued for its nutritional, medicinal, and antioxidant properties (Otero & Bernolo, 2020) ^[18]. Growing consumer demand and increasing market value have resulted in widespread adulteration using inexpensive sugar syrups, making authentication an important aspect of food quality assurance (Puranik *et al.*, 2023) ^[19]. The multifloral honey is produced when bees collect nectar from numerous flowering plant species within a particular geographical area (Wang *et al.*, 2022) ^[24]. Although all samples may belong to the same botanical category differences in local vegetation, climatic conditions, soil characteristics, and bee keeping practices influence their volatile composition and physicochemical properties (Mahmoud & Zhang, 2024a) ^[15]. Consequently, geographical origin can contribute to subtle variations in the chemical fingerprint of multifloral honey (Valverde *et al.*, 2022; Wang *et al.*, 2022) ^[23, 24]. Headspace Gas Chromatography Mass Spectrometry (HS-GCMS) enables characterization of volatile organic compounds that contribute to honey aroma and serve as authenticity markers (Mahmoud & Zhang, 2024a; Starowicz, 2021) ^[15, 22]. Fourier Transform Infrared spectroscopy (FTIR) rapidly characterizes molecular functional groups and carbohydrate composition without extensive sample preparation (Halagarda *et al.*, 2024; Kasprzyk *et al.*, 2018) ^[5, 10]. The integration of these complementary analytical techniques offers improved discrimination capability compared with either technique alone. This study characterize multifloral honey samples collected from different regions of Southern Karnataka using HS-GCMS while identifying volatile compounds associated and FTIR spectral fingerprints for authenticating the multifloral honey and to assess the suitability of the combined HS-GCMS and FTIR approach for routine honey authentication.

2. Materials and Methods

2.1 Honey Samples

Five authentic multifloral honey samples were collected directly from beekeepers located in different regions in Southern Karnataka, India. The sample locations included: Nettigere (HM1), Manankalgi (HM2), Rangasamudra (HM3), Hanur (HM4), and Antarahalli (HM5).

Each honey sample was collected immediately after harvesting, stored in sterile amber glass bottles, transported under ambient conditions, and maintained at 25 °C until analysis.

2.2 Chemicals

Analytical-grade glucose, fructose, sucrose, sodium chloride, methanol, and certified volatile reference standards were obtained from Sigma-Aldrich. Ultrapure water was used throughout the study.

2.4 Fourier-transform infrared spectroscopy (FT-IR) analysis

FTIR spectra of all honey samples were obtained using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) with a spectrometer (Spectrum Two, 99,065 NIOS2 software) equipped with a MIRacle ATR accessory. Spectra were collected over the wavenumber range of 4000-500 cm^{-1} , with each spectrum recorded as an average of 16 scans to enhance the signal-to-noise ratio (Ioannou *et al.*, 2023; Kędzierska-Matysek *et al.*, 2023) [6, 11]. All measurements were conducted at ambient temperature (25 ± 3 °C). The acquired spectra were subjected to baseline correction and basic spectral smoothing using the instrument software to minimize background noise.

2.3 Headspace Gas Chromatography Mass Spectrometry Analysis

Gas Chromatography-Mass Spectrometry (GC-MS) system (TSQ 9000 Thermo Scientific, Triple Quadrupole) was used to analyze the flavor compounds in honey. The GC-MS system having Column Rtx-5MS (5% Diphenyl/95% Dimethyl poly siloxane), 30m x 0.25mm ID x 0.25 μm df, 5 μl sample injected, 1 ml per min carrier gas, Split 20:1, X caliber software with NIST Library, Oven temperature programme being: 110 °C hold for 4 min; Upto 200 °C at the rate of 10 °C/min with no hold; upto 280 °C at the rate of 5 °C min^{-1} for 12 min hold; Injector temperature being 280 °C, Inlet line temperature for MS programme being 290 °C, with source temperature of 250 °C, and electron energy at 70 eV was employed for the study. The samples was methylated and extracted with

petroleum ether and analyzed through GC-MS for identification of different compounds (FSSAI, 2016) [4].

3. Results and Discussion

3.2 FTIR Spectral Characteristics

The FTIR spectra revealed the molecular fingerprinting of honey samples revealing their authenticity (Figure.1). All the samples exhibited typical absorption peaks corresponding to hydroxyl stretching at 3200-3600 cm^{-1} , C-H stretching at 2930 cm^{-1} , carbohydrate vibrations at 1200-900 cm^{-1} , and water bending at 1640 cm^{-1} (Ioannou *et al.*, 2023; Kasprzyk *et al.*, 2018; Kędzierska-Matysek *et al.*, 2023; Matwijczuk *et al.*, 2022) [6, 10, 11, 17]. Even though the samples originated from different regions, only minor variations were observed in band intensity (Kasprzyk *et al.*, 2018) [10]. This reflects regional differences in nectar composition, local vegetation, climatic conditions and environmental factors (Halagarda *et al.*, 2024) [5].

The broad absorption band (3600-3000 cm^{-1}) was notable in all samples and corresponds to the hydroxyl (O-H) stretching vibrations. This region is primarily associated with extensive hydrogen bonding network formed by water molecules and monosaccharides, mainly fructose and glucose present in honey. HM1 and HM2 samples exhibited slightly broader absorption profiles in this region compared to HM4 and HM5, which may indicate small differences in moisture content or hydrogen-bonding interactions. Similar variations have been reported in natural honey (da Silva *et al.*, 2016) [3]. A comparatively weaker absorption band observed near 2930 cm^{-1} is attributed to asymmetric C-H stretching vibrations of aliphatic- CH_2 and CH_3 groups from carbohydrate molecules. The absorption around 1640 cm^{-1} corresponds to the bending vibration of molecular water with a possible contribution from carbonyl-containing compounds and organic acids present in honey (Kelly *et al.*, 2006) [12]. The region from 1200 to 900 cm^{-1} exhibited the greatest spectral variability among the five honey samples. Strong absorption bands centered near 1145, 1080, 1045, and 995 cm^{-1} correspond to C-O stretching, C-C stretching, and glycosidic bond vibrations of fructose and glucose molecules, which together constitute the principal carbohydrate matrix of honey (Irudayaraj *et al.*, 2003; Islam *et al.*, 2024) [7, 8].

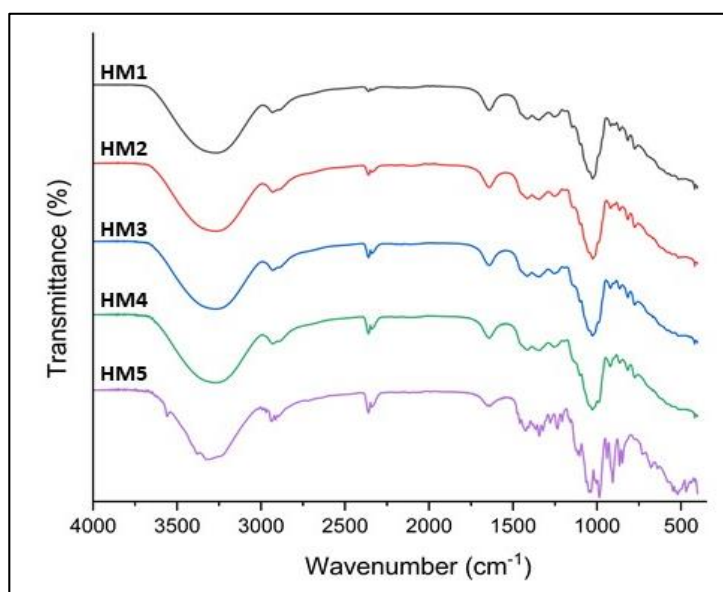


Fig 1: FTIR spectra of honey samples

Overall, the ATR-FTIR spectra demonstrate that all five honey samples possess the characteristic spectral profile expected for authentic multifloral honey. The similarity observed in the major absorption bands confirms that the samples have common chemical composition consisting of carbohydrate and water (Anjos *et al.*, 2015) ^[1]. Nevertheless, subtle differences in spectral intensity within the carbohydrate region indicate measurable compositional variability among samples collected from different regions (Islam *et al.*, 2024) ^[8]. This variability is attributed to the multifloral nature of honey reflecting the collective nectar contribution of locally available flowering plants, whose composition changes with geography, climate and seasonal floral availability (Kaškonienė & Venskutonis, 2010) ^[9].

3.1 Headspace Gas Chromatography Mass Spectrometry Volatile Profile

The Headspace Gas Chromatography Mass Spectrometry (HS-GCMS) analysis identified numerous volatile organic compounds associated with multifloral honey volatile profile. The principal compounds detected include Benzaldehyde, Furfural, Methyl syringate, Nonanal, Linalool oxide, and Phenylacetaldehyde. In spite of all samples being multifloral honey, characteristic differences in composition and relative abundance of volatile constituents were observed (Table 1). The relative abundance of these compounds varied moderately among five honey samples, reflecting differences in surrounding floral resources (Figure 2). A measurable difference in volatile composition was observed among the samples revealing the multifloral composition of honey attributed to local floral biodiversity and environmental conditions (Figure 3). Among the identified compounds, 2-furanmethanol, 5-ethenyltetrahydro- α,α ,5-trimethyl-, cis- was consistently predominant volatile compound in all five samples, constituting about 39.82%, 36.12%, 29.35%, 29.11% and 49.96% of the total volatile area in samples HM1, HM2, HM3, HM4, and HM5 respectively. The widespread occurrence of this oxygenated furan derivative suggests that it represents an important component of the aroma profile of multifloral honey (Song & Liu, 2018) ^[14]. Furan derived compounds are commonly formed through nectar metabolism and mild sugar degradation reactions and have been reported as characteristic constituents of natural honey (da Silva *et al.*, 2016; Starowicz, 2021) ^[3, 22]. Sample HM5 exhibited the highest abundance of this compound (46.96%), whereas samples HM3 and HM4 contained comparatively lower proportions (29.35% and 29.11%, respectively). Such variation may reflect differences in nectar sources, climatic conditions, and enzymatic transformations occurring during honey maturation. Furan derivatives represented one of the dominant chemical groups across all five samples. Furfural and its closely related derivatives were detected in every sample, however their relative abundance varied considerably (Brattoli *et al.*, 2013) ^[2]. Sample HM4 exhibited the highest concentration (7.02%), followed by sample HM3 (5.00%), sample HM5 (2.48%), sample HM1 (2.27%), and sample HM2 (1.09%). Oxygenated monoterpenes constituted another major class of compounds detected in the analysed honey samples. Sample A contained a relatively high proportion of trans-linalool oxide (25.065), while sample HM2 contained

18.12% of the same compound. Linalool derivatives are among the most frequently reported volatile compounds in floral honey and originate primarily from nectar terpenoids produced by flowering plants and considered as an indicator of floral origin because these compounds contribute pleasant floral and sweet aroma notes. The samples HM3, HM4, and HM5 contained several alternative terpenoid compounds such as 2-(5-Methyl-5-vinyltetrahydro-2-furanyl)-2-propanol, 1,7-Octadiene-3,6-diol, 2,6-dimethyl-, Myroxide, 3-Carene-10-al, and cis-Verbenol (Mahmoud & Zhang, 2024b) ^[15]. The presence of these compounds suggests variation in surrounding vegetation attributing to the multifloral nature of the honey. Sample HM5 displayed the greatest diversity of terpenoid compounds, including Myroxide (5.09%), cis-p-mentha⁻¹7,8-dien-2-ol (0.82%), Spirobicycloheptane-oxirane (0.77%), 3-Carene-10-al (2.73%), and cis-Verbenol (0.81%). The presence of multiple terpene derivatives may contribute to a more complex aroma profile and reflects greater diversity of nectar sources in corresponding geographical location (Wang *et al.*, 2023) ^[25].

Several ester compounds were identified particularly in samples HM1, HM2, and HM3. Sample HM2 contained higher proportion of Benzoic acid, ethyl ester (33.19%), while samples HM3 and HM1 contained 14.41% and 1.17%, respectively. Benzoic acid esters contribute sweet fruity, and floral aroma characteristics and may originate from both plant secondary metabolites and enzymatic transformations during honey ripening (Wang *et al.*, 2023) ^[25]. Additional esters like Acetic acid, phenyl-, ethyl ester, Butanedioic acid, diethyl ester, and Dodecanoic acid, and ethyl ester were detected at lower concentrations. The occurrence of these esters contributes to the overall complexity of honey aroma and supports the multifloral nature of the analysed samples (Li *et al.*, 2024) ^[14].

The observed variability confirms that HS-GCMS can capture subtle geographical influences. These observations support previous findings that multifloral honey does not possess a single uniform volatile profile. Instead, local floral biodiversity, seasonal flowering patterns, climatic conditions, and bee foraging behavior collectively influence the final volatile composition (Kozłowicz *et al.*, 2020) ^[13]. The present results demonstrate that HS-GCMS successfully differentiated multifloral honey samples based on their volatile composition. While dominant oxygenated furan compounds represent a common characteristic of all samples, differences in terpene derivatives, aldehydes, esters, and aromatic alcohols provide additional discriminatory information (Kozłowicz *et al.*, 2020; Xagoraris *et al.*, 2021) ^[13, 26]. These volatile fingerprints may therefore serve as complementary authenticity markers when integrated with FTIR spectral data.

Unlike FTIR, which primarily reflects the carbohydrate matrix and major functional groups, HS-GCMS captures volatile metabolites directly associated with floral origin and aroma characteristics. The integration of these complementary analytical techniques is expected to enhance the robustness of honey authentication by simultaneously evaluating both volatile and non-volatile chemical signatures (Sotiropoulou *et al.*, 2021; Xagoraris *et al.*, 2021) ^[21, 26].

Table 1: Volatile profiling of honey samples

Compound	Sample				
	HM1	HM2	HM3	HM4	HM5
2-Furancarboxaldehyde	✓	✓	✓	✓	✓
5-Methyl furfural	✓	✓			✓
2-Furanmethanol, 5-ethenyltetrahydro- $\alpha,\alpha,5$ -trimethyl-, cis-trans-Linalool oxide (furanoid)	✓	✓		✓	✓
α -Methyl- α -[4-methyl-3-pentenyl]oxiranemethanol	✓				
Benzoic acid, ethyl ester	✓	✓	✓		✓
Butanedioic acid, diethyl ester	✓				
Acetic acid, phenyl-, ethyl ester	✓			✓	
Melezitose	✓				
2-Furancarboxaldehyde,5-methyl-			✓		
2-Furanmethanol,5-ethenyltetrahydro- $\alpha,\alpha,5$ -trimethyl-, cis-			✓		✓
2-(5-Methyl-5-vinyltetrahydro-2-furanyl)-2-propanol			✓		
2,6,6-Trimethyl-2-cyclohexene-1,4-dione			✓		✓
Dodecanoic acid, ethyl ester			✓		
1,7-Octadiene-3,6-diol,2,6-dimethyl-				✓	
1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-					✓
cis-p-mentha ¹ 7,8-dien-2-ol					✓

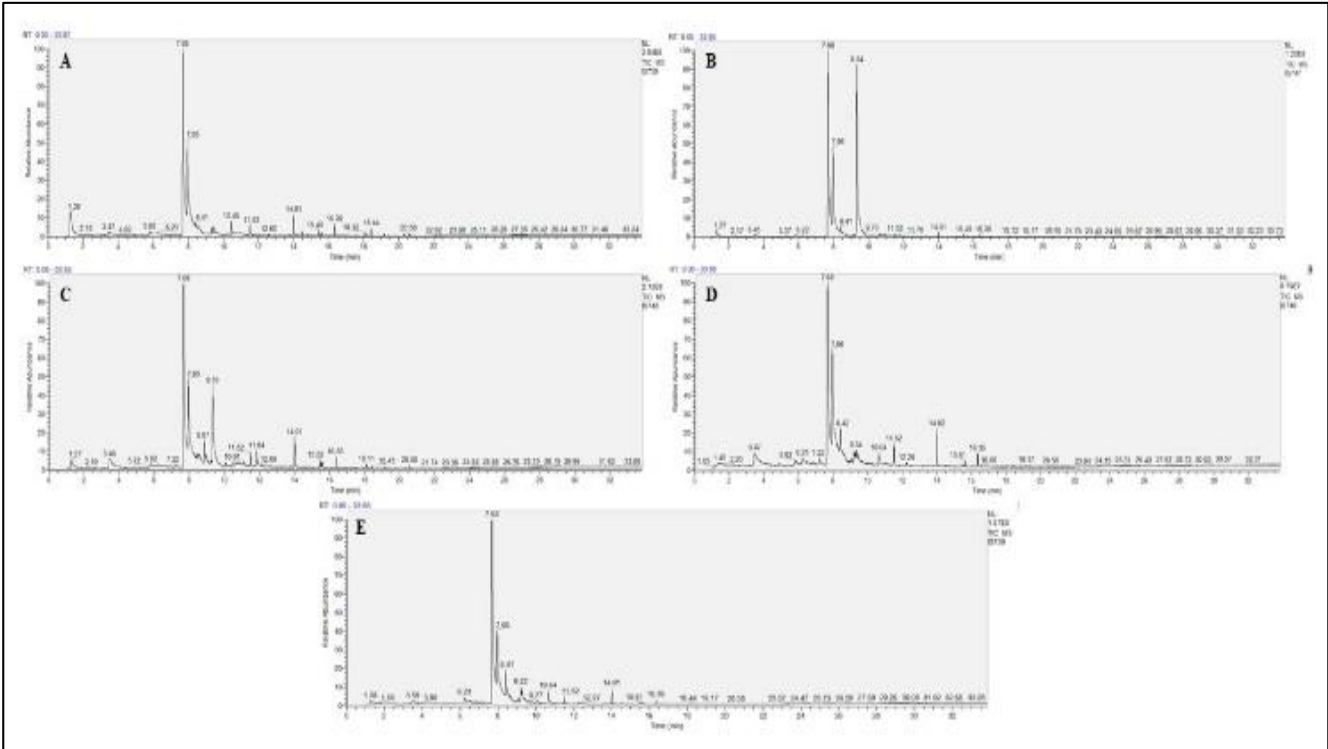
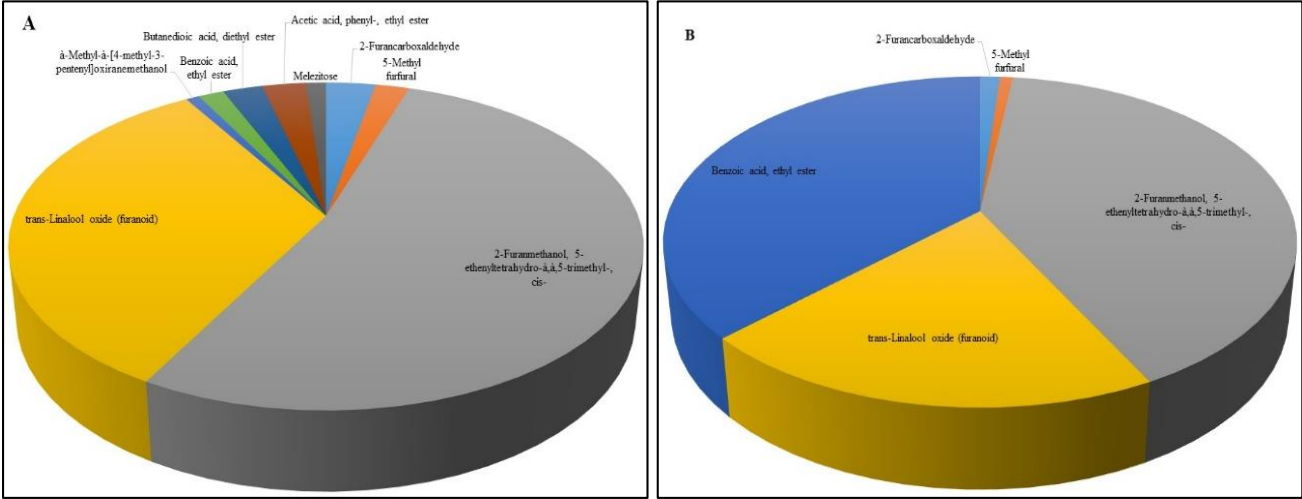


Fig 2: Headspace GCMS Chromatogram of honey showing the volatile profile of samples A:HM1; B:HM2; C:HM3; D:HM4; and E:HM5



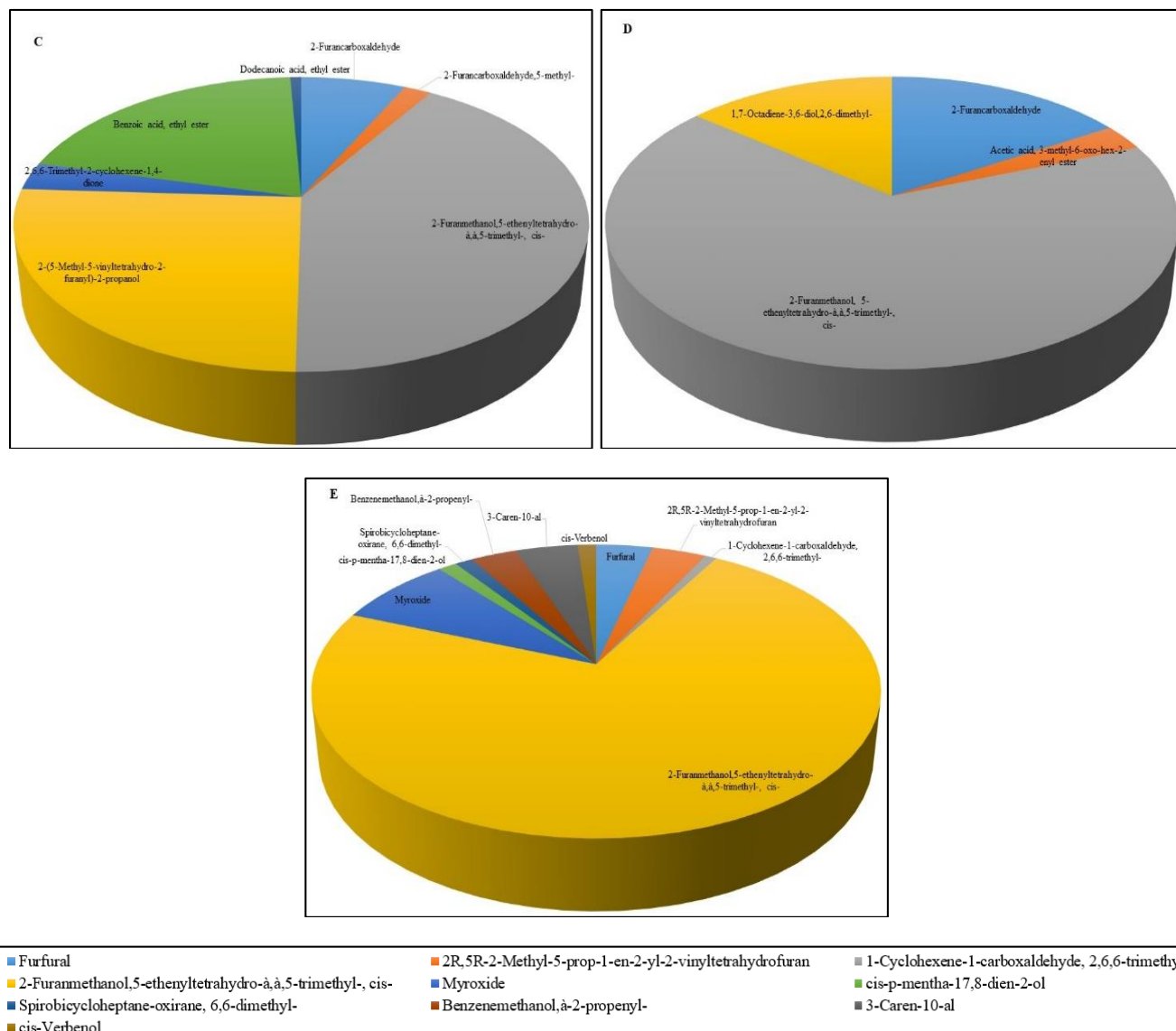


Fig 3: Volatile composition in honey samples A:HM1; B:HM2; C:HM3; D:HM4; and E:HM5

Conclusion

The present investigation demonstrates that the combination of Headspace Gas Chromatography and Fourier Transform Infrared Spectroscopy provides a robust and efficient method for authenticating multifloral honey collected from different regions of Southern Karnataka. FTIR rapidly identified compositional changes associated with sugar syrup adulteration, whereas HS-GCMS characterized volatile compounds that reflected both geographical variation and product authenticity. Integration of chromatographic and spectroscopic information with chemometric analysis significantly improved classification accuracy, making the proposed methodology suitable for routine quality control and food adulteration detection. Future studies involving larger sample numbers collected across multiple harvesting seasons and additional geographical regions would further strengthen the development of regional authentication models.

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