Journal of Agricultural Sciences
(Tarim Bilimleri Dergisi)

J Agr Sci-Tarim Bili
e-ISSN: 2148-9297
jas.ankara.edu.tr

JAS

Characterization of the Volatile Profile of Bee Venom from Different Regions in Türkiye Using Gas Chromatography-Mass Spectrometry

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ARTICLE INFO

Research Article

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Received: 01 February 2024 / Revised: 30 June 2024 / Accepted: 29 July 2024 / Online: 14 January 2025

Cite this article

Aydeniz-Guneser B, Guneser O, Kekeçoğlu M, Kolayli S (2025). Characterization of the Volatile Profile of Bee Venom from Different Regions in Türkiye Using Gas Chromatography-Mass Spectrometry. *Journal of Agricultural Sciences (Tarim Bilimleri Dergisi),* 31(1):22-32. DOI: 10.15832/ankutbd.1430185

ABSTRACT

The volatile organic compounds of bee venoms from four different populations of *Apis mellifera anatoliaca*, came from different regions in Türkiye, were analyzed using solid phase microextraction technique combined with gas chromatography-mass spectrometry. A total of 144 volatile compounds were identified in the bee venom samples. The identified volatile compounds included esters, terpenoids, alcohols, acid esters, aldehydes, ketones, and hydrocarbons. It was determined that ester-type volatile compounds characterized the bee venom obtained from the Central Anatolia Region, while bee venom from the Western Black Sea Region had a higher amount of volatile terpenes with spicy and woody aromas. Further studies are required to understand the volatile profile of bee venom, which consists of plant and animal secondary metabolites.

Keywords: Bee Venom, *Apis Mellifera Anatoliaca*, Volatile Compounds, Gas Chromatography-Mass Spectrometry

1. Introduction

Bee venom (Apitoxin) is a complex mixture of natural compounds, characterized by a yellowish-brownish colour, a sharp odour, and a crystalline form. It is secreted by worker bees of *Apis mellifera anatoliaca* (Bogdanov 2016; Flanjak et al. 2021; Uzuner et al. 2021). Honeybees have two separate glands in their sting as the acid venom gland and the alkaline gland, which collectively form Dufour's gland. The venom gland, which plays a crucial role in the production and storage of bee venom, is the most important defence mechanism for the bee. The secretion of bee venom begins in the venom gland with the emergence of a new adult bee (Ali 2014; Özkök 2018). Although immature young bees have some venom, they cannot produce it effectively because their stings have not yet hardened. Bees reach their full venom production potential within 16-19 days. The capacity for venom production varies according to several factors, including season, race, and nutritional status. Depending on the honeybee race and the collection period, a honeybee produces an average of 0.05 mL to 0.3 mL of venom per day (Özkök 2018).

Bee venom consists of many biologically active molecules. Among these, peptides like melittin and apamin, as well as histamine, epinephrine, phospholipase A2, and hyaluronidase have been identified as major components in bee venom (Melda et al. 2021). The quantity and quality of bee venom are influenced by various environmental and genetic factors, including the bees' nutrition sources, the season and period of production, the collecting techniques used, and the genetic origin of bee races (Ramos et al. 2018; Somwongin et al. 2018; Kekeçoğlu et al. 2022). Different characteristics of bee venom from various honeybee races have been determined by numerous studies. For instance, Africanized bee venom has been found to be less active than European bee venom in different species of mice (Kumar et al. 2014; Zidan et al. 2018; Hussein et al. 2019).

The popularity of bee venom has increased for therapeutic use in apitherapy, showing promise in the treatment of various autoimmune diseases and conditions affecting the nervous system, as well as rheumatoid arthritis, osteoarthritis, scleroderma, psoriasis, and skin cancer (Chung et al. 2012; Hwang et al. 2015; Tanugur-Samanci & Kekeçoğlu 2021; Uzuner et al. 2021). Bee venom has traditionally been used for treating skin diseases, and inflammation or pain in muscles, joints, or fibrous tissues. It has also been used in traditional medicine as a preventive agent for various acute and chronic diseases. Moreover, that recent

studies have shown bee venom has anti-carcinogenic activity against some types of cancer, such as breast, liver, and prostate cancer (Park et al. 2011; El Sharkawi et al. 2015; Jung et al. 2018; Ağan & Kekeçoğlu 2020; El-Didamony et al. 2022).

The adaptation of *A. mellifera* races is affected by floral morphologies and climatic conditions. The morphological and physiological structures of honeybees, especially their gland size and secretion, can be differentiated according to environmental adaptation. As a result of this differentiation of morphological and physiological structures of honeybees, the physico-chemical characteristics and bioactivities of bee-collected and brewed products such as honey and propolis, as well as bee secretion products such as bee venom, beeswax were varied (Castro-Vázquez et al. 2008; Miguel & Antunes 2011; Kekecoglu et al. 2021; Kaziur-Cegla et al. 2022). For instance, Ali et al. (2019) observed that the accumulation of lipofuscin and the size of acini in the hypopharyngeal glands of honeybees, responsible for the synthesis of the main royal jelly proteins, were different in foragers and nurses' bees compared to exotic bee races of *A. mellifera carnica* Pollmann and *A. mellifera ligustica* Spinola during the summer and winter seasons. Al-Ghamdi et al. (2011) investigated the differences in hypopharyngeal glands of *Apis mellifera jemenitica* and Carniolan hybrid bees. The researchers observed that the number of secretory cells was higher in the Carniolan hybrid than in *Apis mellifera jemenitica*, while the cytoplasm of both strains was found to have similar characteristics based on the results of haematoxylin and eosin staining analysis.

Studies on the composition of bee venom has predominantly focused on identifying its protein components, while studies on the volatile compound profile of bee venom is scarce, indicating a need for further researches on this subject (Abd El-Wahed et al. 2021; Çaprazlı & Kekeçoğlu 2021; Kaziur-Cegla et al. 2022). The identification and characterization of the volatile compounds of bee venom are essential for revealing its bioactivity and therapeutic properties in apitherapy. In this context, different classes of volatile compounds in bee venom were identified in a most recent study by Isidorov et al. (2023). One hundred forty-nine volatile compounds were identified in bee venom samples from Poland. The researchers found that fresh bee venom samples had higher amounts of lactones and terpenoids than dried bee venom samples. 2-nonanone, 2-nonanol, acetic acid, isoamyl acetate, toluene, α-pinene, limonene, dihydromyrcenol, and γ-heptalactone were found to be major volatiles in fresh bee venom, while dried bee venom samples contained 2-heptanone, isopentanol, acetic acid, octanoic acid, isoamyl-3-methy-2-butenoate, ethyloctanoate, and methylsalicylate at high levels.

The present study aims to reveal the volatile profiles of the bee venom samples from the Anatolian race in various regions of Türkiye using the solid phase microextraction (SPME) technique with gas chromatography-mass spectrometry (GC-MS), which is a powerful and efficient tool for volatile metabolite studies on certain agricultural products.

2. Material and Methods

2.1. Materials

Bee venom (BV) samples were collected using an electroshock-based device (Almışlar Bee Venom Collecting Machine, Product No. 1568, İzmir, Türkiye), following the method described by Samancı & Kekeçoğlu (2019) with minor modifications. The specifications of the bee venom collector are as follows: input voltage: 12 VDC, timer on: 3 sec, timer off: 7 sec, collector frames: 40 cm x 50 cm, and maximum operating time: 15-20 min. Sampling was conducted from inside the hives between 02:00 pm and 05:00 pm, once every 15 days throughout 2022 (May-August). All bee venom samples were stored at -18 °C prior to analysis.

2.2. Analysis of the volatile profiles of bee venom samples

The volatile compounds of bee venom samples were extracted using the solid phase microextraction (SPME) technique (Pawliszyn 1999). SPME is solvent-free, simple, and fast sample preparation technique that easily couples with chromatographic instruments. The SPME technique effectively reduces the sample preparation, extraction and concentration steps to a single step in the analysis of a broad range of volatile compounds from various biological matrices, such as foodstuffs. Despite the advantages of SPME, it has limitations, including fiber lifetime, and limited capacity in high analyte concentrations. The SPME technique was chosen in this study for the extraction and concentration of the volatile compounds in bee venom due to low sample amount and low volatile concentrations (Pawliszyn 1999; Souza-Silva et al. 2015).

Approximately 0.5 g of bee venom was weighed into 40 mL SPME vials, to which 2.5 µL of internal standard was added. Then, the vials were placed in a 40 °C water bath for 20 minutes. Afterwards, an SPME needle (2 cm-50/30 µm divinylbenzene/carboxene/polydimethylsiloxane stable flex, Bellefonte, USA) equipped with a septum cap (tan PTFE/silicone septa, Supelco, USA) was inserted into the vial headspace to extract volatile compounds for 20 minutes at a depth of 2 cm. Following extraction, the SPME needle was immediately injected into the GC-MS system (HP 6890, 7895C MS, Agilent, USA) operating in splitless mode (Güneşer & Yüceer 2017).

Chromatographic separation of volatile compounds was achieved using a polar HP-INNOWax column (Polyethylene Glycol column, 60 m × 0.25 mm ID, 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA). The GC-MS conditions were programmed as follows: helium carrier gas at a constant flow rate of 1.0 mL/min, initial oven temperature of 40 °C held for 10 minutes, ramped to 250 °C at 5 °C/min, final temperature of 250 °C held for 10 minutes. Electron impact ionization at 70 eV was

used for mass spectra acquisition in the range of 35–350 amu. The capillary direct interface temperature was set at 280 °C with a scan rate of 4.45 scans/s.

The volatile organic compounds in the bee venom samples was tentatively identified by comparing obtained mass spectra with those in the National Institute of Standards and Technology (NIST) and Wiley Registry of Mass Spectral database (7th Edition, Wiley) with a mass spectral match threshold of >50. The quantification of volatile organic compounds was expressed as relative abundance (µg/kg) using semi-quantitative techniques (Equation 1) (Güneşer & Yüceer 2017; Avsar et al. 2004). An internal standard comprising 2-methyl-3-heptanone (1 µL/L, for neutral/basic volatiles) and 2-methylpentanoic acid (6 µL/L, for acidic volatiles) in diethyl ether was prepared.

$$\text{Concentration of volatile compounds } \left(\frac{\mu g}{kg}\right) = \frac{\text{Amount of of internal standart x peak area of the compound}}{\text{peak area of internal standart}} \quad \text{(Eq. 1)}$$

3. Results and Discussion

A total of 144 volatile compounds were identified in the bee venom samples obtained from different regions of Türkiye within this study (**BV1**: Central Anatolia Region, **BV2**: Aegean Region; **BV3**: South Marmara Region; **BV4**: Western Black Sea Region of Türkiye. The volatile profile of the bee venom samples is shown in Table 1. The volatile compounds consists of acid esters, alcohols, aldehydes, esters, hydrocarbons, ketones, and terpenoids, The most abundant volatile compounds in the bee venoms were alcohol, esters and terpenes, which are associated with fruity, floral, and plant nuances (Jeleń & Gracka 2016).

As can be seen in Table 1, BV1, BV2, BV3, and BV4 consisted of 67, 67, 68, and 59 volatile compounds, respectively. Isoamyl acetate, octyl acetate, isoamyl alcohol, phenyl ethyl alcohol, (E) 2-decen-1-ol, 2-ethyl-1 hexanol, 1 octanol, 2-nonanol, cyclooctyl alcohol, d-limonene, α-pinene, and phenol were identified in all bee venom samples. The variability in the amounts of alcohol and terpene volatiles in the bee venom samples can be attributed to the diversity of floral resources in each region and regional differences. Thus, it was reported by Ouradi et al. (2021) that different botanical origins, unifloral or multifloral localities have strong influences in the formation of volatiles such as alcohols, aldehydes, esters, ketones and terpenes in honey samples.

Bee venom samples had a similar number of compounds in terms of acidic and alcohol-derived volatile compounds, while aldehydes, ketones, and especially esters volatiles were observed to be quite different. The volatiles including ethyl pentanoate, ethyl senecioate, 2-heptanol, ethyl heptanoate, 2,5-dimethyl pyrazine, dimethyl trisulfide, 2-nonanone, 2-ethyl-5-methyl-pyrazine, trimethyl pyrazine, camphor, bornyl acetate, isobornyl acetate, methyl decanoate, menthol, ethyl benzoate, 1-decanol, 2-acetylpyrrole, and 2-formyl pyrrole were found only in sample BV1. Moreover, it was determined that BV1 had the highest amount of esters volatile compounds compared to other venom samples (Table 1), Ester volatiles, including ethyl acetate, ethyl propionate, ethyl butanoate, are associated with floral and fruity nuances (Jeleń & Gracka 2016). Therefore, BV1 sample was considered to have a fresher and floral flavour than the other samples in terms of sensory characteristics. It is thought that the higher esters content in BV1 sample could be used chromatographic fingerprint to monitor authentication for bee venom obtained from Central Anatolia Region. Hence, it is well known that certain ester type volatiles are the sting alarm pheromones for honeybees, contributing to defensive behaviours. All sting alarm pheromones components are still being investigated for their roles (recruitment, flight, orientation etc.) in bees (Wager & Breed 2000). In this context, the findings obtained in BV1 could be used to reveal defensive behaviour in honeybees from Anatolian Region in Türkiye.

A further point to consider in the volatile profile of BV1 is that the only sulphur-based volatile present in this sample. Dimethyl trisulfide has been reported to be capable of converting highly toxic cyanide into less toxic thiocyanate in rats. Therefore, it exhibits impressive activity in preventing cyanide poisoning, and this pharmacokinetic behavior of the compound has attracted much attention as an encouraging cyanide antidote (Rockwood et al. 2016; Bhadra et al. 2019).

In addition to sulphur-based volatiles, pyrazine-derived volatiles (trimethyl pyrazine, 2,5-dimethyl pyrazine, 2-ethyl-5-methyl-pyrazine, and 2-formylpyrrole) with their pleasant nutty, roasted, and coffee-like aroma (Maga & Sizer 1973; Mortzfeld et al. 2020) were detected only in BV1. Pyrazines and pyrroles can be used as heat markers in food processing and occur in pyrolysis reactions during the destruction of nitrogen sources. Pyrazine-derived volatiles in honeys have been reported to be stored at 10 °C, 20 °C and at 40 °C (Castro-Vázquez et al. 2008). Electrical stimulation can be used to induce bees to sting during the venom extraction. The duration of electrical stimulation can vary from 15 minutes to 8 hours, the temperature of the device from -5 °C to 40 °C (El-Saeady et al. 2016). These electrical impulses may produce pyrazines. Similar to pyrazines, thiazoles are also secondary metabolites formed from amino acids (Kumar & Aggarwal 2019). The occurrence of 2-mercapto-4-phenyl thiazole may be related to the heat treatment or electrical impulse applied to BV1. Pyrazines and thiazoles were also detected only in BV1. A possible explanation for these findings might be that BV1 is exposed to higher thermal conditions compared to other bee venoms.

Table 1- Volatile profile of bee venom samples

No	*Volatile compounds*	*Class*	*Odor description*	*Concentration of Volatile Compounds [mg/kg bee venom]*[a]			
				BV1	***BV2***	***BV3***	***BV4***
1.	Acetic acid	Acid	sharp, pungent, sour vinegar	-	2.54±1.10 [a]	14.94±0.14	6.52±1.57
2.	Propionic acid	Acid	pungent, acidic, cheesy	0.87±.24	-[b]	-	-
3.	Butanoic acid	Acid	sharp, acetic, cheesy	-	0.76±0.10	1.36±0.14	1.64±0.39
4.	Pentanoic acid	Acid	acidic, sharp, cheesy	1.23±0.44	0.14±0.01	1.31±0.12	-
5.	Heptanoic acid	Acid	rancid, sour, cheesy	1.29±0.58	0.21±0.04	1.29±0.06	-
6.	Octanoic acid	Acid	goaty, lamb, mutton fatty	105.08±13.52	45.85±26.06	-	-
7.	Nonanoic acid	Acid	waxy, dirty, cheesy dairy	0.67±0.30	1.60±0.22	0.87±0.01	-
8.	3-methyl butanoic acid	Acid	sour sweaty, cheesy, tropical	7.24±0.40	0.64±0.17	2.73±0.37	2.92±0.85
9.	3-methyl-2-butenoic acid	Acid	green, phenolic, dairy	187.67±13.37	27.67±3.27	73.66±0.46	67.76±18.07
10.	Hexanoic acid	Acid	sour, fatty, sweaty cheesy	2.35±0.60	0.41±0.04	-	0.69±0.16
11.	2-ethyl hexanoic acid	Acid	herbal, earthy	-	-	0.22±0.01	0.18±0.04
12.	Ethanol	Alcohol	solvent	6836.56±158.22	147.36±19.73	2737.85±479.30	1040.30±70.83
13.	Isoamyl alcohol	Alcohol	fusel, alcoholic, whiskey	1644.40±113.15	669.96±47.57	6999.36±1821.54	18208.12±1949.98
14.	3-methyl-1-pentanol	Alcohol	fusel, cognac, winey	-	-	574.27±27.34	-
15.	2-heptanol	Alcohol	fresh, lemongrass, herbal	132.99±17.19	-	-	-
16.	1-hexanol	Alcohol	pungent, ethereal, fusel oily	-	113.30±6.02	944.57±192.32	1202.53±105.85
17.	2-octanol	Alcohol	fresh, spicy, green	-	-	-	584.79±75.11
18.	1-heptanol	Alcohol	musty, leafy, violet	-	90.09±3.54	-	-
19.	2-ethyl-1 hexanol	Alcohol	citrus, fresh, floral,	1094.19±120.47	119.18±1.00	8426.57±1652.59	541.33±52.93
20.	2-nonanol	Alcohol	waxy, green, creamy	1798.54±278.59	207.04±3.70	1429.23±290.29	1642.60±163.76
21.	2-formyl-1-methyl pyrrole	Alcohol	musty, beefy, coffee	347.24±41.39	-	422.92±180.64	-
22.	1-nonanol	Alcohol	fresh, fatty, floral	-	161.78±27.39	-	-
23.	Endo borneol	Alcohol	earthy, minty, camphoreous	42.97±4.82	24.59±2.35	133.16±48.46	-
24.	2-tetra decanol	Alcohol	fruity, waxy, coconut	-	3.93±0.51	-	-
25.	1-butanol	Alcohol	oily, sweet, balsamic	-	-	167.97±84.79	226.51±3.79
26.	1-decanol	Alcohol	fatty, waxy, floral, orange	144.97±53.40	-	-	-
27.	(E) 2-decen-1-ol	Alcohol	waxy, ozone, citrus rose	379.27±100.21	171.54±8.40	864.88±156.53	1874.01±146.98
28.	*p*-propenylanisole	Alcohol	sweet, anise, licorice	-	-	-	258.15±1.56
29.	Benzyl alcohol	Alcohol	floral, rose, phenolic	-	241.61±1.43	1385.79±254.65	7166.39±765.81
30.	Phenyl ethyl alcohol	Alcohol	floral, rose, rose	202.23±21.17	147.44±6.35	768.40±153.13	1320.78±132.16
31.	2-acetylpyrrole	Alcohol	musty, nutty, coumarinic	55.03±7.58	-	-	-
32.	2-phenoxy ethanol	Alcohol	floral, rose, dried rose	67.55±15.63	98.65±0.12	-	-
33.	4-methyl decane	Hydrocarbon	-	-	-	-	143.75±13.24
34.	3,6-dimethyl decane	Hydrocarbon	-	344.03±12.26	150.29±43.12	-	175.24±18.51
35.	5- ethyl-2-methyl octane	Hydrocarbon	-	-	106.07±28.09	-	-
36.	3,7 dimethyl undecane	Hydrocarbon	-	-	104.53±14.33	-	-

37.	1-octene	Hydrocarbon	-	-	-	-	622.30±23.72
38.	2-pentene	Hydrocarbon	-	-	-	-	503.63±121.12
39.	Dichloromethane	Hydrocarbon	-	938.04±288.95	-	-	-
40.	Hexanal	Hydrocarbon	green, fatty, leafy	-	-	-	197.37±29.87
41.	Benzaldehyde	Aldehyde	almond, fruity, powdery	-	224.01±33.02	13469.91±2218.85	24434.86±2228.58
42.	4- methoxy benzaldehyde	Aldehyde	sweet, powdery, anise	-	15.01±0.32	-	-
43.	Decanal	Aldehyde	sweet, aldehydic, orange	-	657.07±67.93	386.28±282.72	1189.32±177.00
44.	2-methyl-3-phenyl propanal	Aldehyde	fresh, marine, ocean		59.93±11.51	-	-
45.	3-methyl butanal	Aldehyde	ethereal, aldehydic, chocolate	-	-	-	364.92±24.73
46.	Octanal	Aldehyde	aldehydic, waxy, citrus	-	-	3267.49±1269.27	5544.78±692.17
47.	Nonanal	Aldehyde	waxy, aldehydic, rose	-	2364.41±124.79	7389.97±692.31	7421.09±1025.25
48.	E-2- octenal	Aldehyde	fresh, cucumber, fatty	-	-	-	3873.49±356.43
49.	Acetone	Ketone	ethereal, apple, pear	-	-	2129.01±410.47	1499.98±75.28
50.	2-pentanone	Ketone	fruity, ethereal, winey	-	-	134.75±18.02	-
51.	6-methyl-5-hepten-2-one	Ketone	citrus, green, musty	-	-	662.26±137.33	536.02±68.29
52.	2-nonanone	Ketone	sweet, waxy, soapy	285.76±33.75	-	-	-
53.	2-decanone	Ketone	orange, floral, fatty	129.42±48.53	11.80±0.30	-	-
54.	Butyrolactone	Ketone	creamy, oily, caramellic	229.95±29.44	60.80±4.74	-	-
55.	Geranyl acetone	Ketone	rose, leafy, floral,		-	123.90±26.05	-
56.	α-isomethyl ionone	Ketone	orris, woody, floral	76.86±47.52	294.18±34.11	-	-
57.	Camphor	Ketone	camphoreous	152.41±16.54	-	-	-
58.	α-amorphene	Terpene	-	-	-	-	381.81±25.02
59.	Epizonarene	Terpene	-	-	-	155.21±45.77	-
60.	L-verbenone	Terpene	verbenone	-	45.90±2.86	95.17±2.79	-
61.	α-muurolene	Terpene	woody	-	-	269.75±97.77	426.58±44.47
62.	Piperitone	Terpene	herbal, minty, camphoreous	-	382.00±9.21	-	-
63.	L-calamenene	Terpene	herbal, spice	-	-	462.87±137.46	405.34±4.10
64.	α-calacorene	Terpene	woody	-	-	42.10±18.39	-
65.	Δ-selinene	Terpene	woody	-	-	84.95±21.86	
66.	α-terpineol	Terpene	lilac, floral, terpenic	100.51±10.97	53.41±7.15	-	-
67.	Geraniol	Terpene	sweet, floral, fruity, rose	-	97.26±13.47	-	-
68.	Cedrol	Terpene	cedarwood, woody, dry sweet	-	-	186.43±66.52	-
69.	Eugenol	Terpene	sweet spicy, clove, woody	-	170.46±21.48	-	-
70.	Thymol	Terpene	herbal, thyme, phenolic	36.98±10.00	21.20±2.87	-	597.75±102.60
71.	Bulnesol	Terpene	spicy	-	-	43.08±12.88	-
72.	α-bisabolol	Terpene	floral, peppery, balsamic	-	-	28.48±7.86	-
73.	Beta eudesmol	Terpene	woody, woody, green	-	-	197.01±59.93	222.31±34.31
74.	β-linalool	Terpene	citrus, orange, floral	121.10±6.56	1751.04±78.83	-	819.13±60.24
75.	Guaiol	Terpene	guaiacwood, rose tea, woody	51.22±5.70	-	126.59±28.74	-
76.	1 octanol	Terpene	waxy, green, orange, rose	2558.04±203.41	1007.09±50.70	5629.25±1207.17	4727.97±456.01
77.	Z-3-octen-1-ol	Terpene	fresh, fatty, greasy, melon	-	-	-	268.49±31.07

78.	Cyclooctyl alcohol	Terpene	-	402.80±51.78	508.55±16.17	1016.44±676.45	3700.31±395.74
79.	Ethyl-2-formyl pyrrole	Terpene	burnt, roasted, smoky	551.24±67.20	-	-	-
80.	Menthol	Terpene	cooling, mentholic, minty	598.94±383.98	-	-	-
81.	Trans-pinocarveol	Terpene	woody, balsamic, fennel	-	60.38±8.17	320.43±93.34	-
82.	β-gurjunene	Terpene	woody, balsamic	-	-	46.13±12.05	-
83.	β-myrecene	Terpene	peppery, terpenic, spicy	48.23±4.42	40.42±3.89	105.54±26.14	-
84.	D-limonene	Terpene	citrus, orange fresh, sweet	2679.12±33.88	15.34±5.11	1101.11±139.08	393.31±29.39
85.	β-pinene	Terpene	woody-green, pine-like	311.09±50.23	50.31±2.61	553.31±132.49	-
86.	β-cymene	Terpene	fresh, citrus, terpenic	152.13±8.05	53.22±22.59	397.12±128.79	-
87.	Styrene	Terpene	sweet, balsamic, floral	114.14±18.32	-	-	318.36±7.19
88.	Copaene	Terpene	woody, spicy, honey	-	-	-	282.28±16.83
89.	Caryophyllene	Terpene	sweet, woody, spicy,	-	317.98±19.65	896.63±180.11	-
90.	Alloaromadedrene	Terpene	woody	-	-	-	337.69±10.90
91.	Δ-cadinene	Terpene	thyme, herbal, woody	-	-	-	91.27±2.96
92.	α-pinene	Terpene	woody, pine, terpenic	578.06±177.93	32.39±2.08	2068.96±756.30	2167.01±285.01
93.	α-terpinen	Terpene	woody, terpenic, lemon	-	-	299.97±55.67	-
94.	Camphene	Terpene	camphoreous, pine, woody	-	-	-	74.61±6.06
95.	β-cyclocitral	Terpene	tropical saffron, herbal	-	-	-	277.22±4.99
96.	*p*-xylene	Terpene	sweet	-	-	127.62±20.75	257.28±35.55
97.	α-N-Methyl ionone	Terpene	orris, violet, powdery	-	34.89±4.38	-	-
98.	β-ionone	Terpene	floral, woody, fruity	45.51±8.56	33.83±4.47	-	-
99.	Lilial	Terpene	muguet	-	145.99±36.87	-	-
100.	Piperonal	Terpene	cherry, vanilla, sweet cherry	-	12.03±	-	-
101.	E,E 2,5 heptadiene	Terpene	-	-	83.92±8.75	-	-
102.	Methyl acetate	Ester	ethereal, sweet, fruity	1265.64±107.75	-	-	-
103.	Ethyl acetate	Ester	ethereal, fruity, sweet	4466.35±900.32	161.24±17.23	665.52±242.00	
104.	3-octen-1-ol acetate (Z)	Ester	fresh, fatty, greasy	-	115.74±8.29	-	-
105.	Isoamyl acetate	Ester	sweet, banana, ripe estery	596.67±108.86	268.85±13.23	3261.12±640.52	2274.55±237.14
106.	Ethyl pentanoate	Ester	fruity, apple, pineapple	244.24±8.35	-	-	-
107.	Hexyl acetate	Ester	fruity, green, apple	-	-	971.76±372.48	188.70±21.69
108.	Octyl acetate	Ester	green, earthy, mushroom,	423.96.84±113.63	252.22±15.08	3725.35±599.55	1651.74±265.54
109.	Bornyl acetate	Ester	woody, camphoreous, menthol	70.38±11.37	-	-	-
110.	Isobornyl acetate	Ester	balsamic, camphoreous, herbal	117.20±29.13	-	-	-
111.	Pentyl propionate	Ester	-	-	-	-	558.53±47.56
112.	Ethyl butanoate	Ester	fruity, juicy, pineapple	529.31±91.12	-	294.12±70.42	94.01±8.16
113.	Ethyl senecioate	Ester	-	19.01±4.89	-	-	-
114.	Ethyl hexanoate	Ester	-	940.19±97.11	-	-	249.68±35.34
115.	Geraniol acetate	Ester	floral, rose, lavender	-	214.09±27.11	-	-
116.	Ethyl propionate	Ester	sweet, fruity, rummy	1056.48±260.51	-	-	-
117.	Ethyl heptanoate	Ester	fruity, pineapple, cognac	620.85±156.73	-	-	-

118.	Ethyl octanoate	Ester	-	5964.08±451.27	-	204.12±149.16	-
119.	Methyl octanoate	Ester	waxy, green, sweet orange	-	133.32±15.13	826.16±205.16	-
120.	Ethyl nonanoate	Ester	waxy, cognac, fruity	2429.75±129.84	94.14±15.08	1459.07±353.03	-
121.	Methyl decanoate	Ester	oily, winey, fruity	194.95±71.82	-	-	-
122.	Ethyl decanoate		Sweet, waxy, fruity	950.71±73.22	-	126.51±57.13	-
123.	Ethyl benzoate	Ester	minty, sweet, wintergreen	182.76±57.00	-	-	-
124.	2-ethyl-3-hydroxy hexyl-2-methylpropanoate	Ester	-	41.87±10.62	-	-	-
125.	Isoamyl butanoate	Ester	fruity, green, apricot	-	-	1459.30±232.78	1765.97±88.56
126.	A-phenyl ethyl acetate	Ester	sweet, honey, floral	-	-	252.72±74.48	-
127.	4- tert-butylcyclohexyl acetate	Ester	woody, cedar, floral	82.12±29.53	27.58±2.60	-	-
128.	Benzyl acetate	Ester	sweet, floral, jasmin	-	12.13±4.20	267.83±69.92	1693.93±147.79
129.	Phenylmethyl acetate	Ester	sweet, floral, honey	-	-	-	169.80±30.41
130.	E-2-decenyl acetate	Ester	waxy, fatty	-	-	-	851.71±104.79
131.	Trimethyl pyrazine	Pyrazine	nutty, nut skin, earthy	101.88±40.74	-	-	-
132.	2,5 dimethyl pyrazine	Pyrazine	cocoa, roasted, nutty beefy	984.66±127.46	-	-	-
133.	2-ethyl-5-methyl-pyrazine	Pyrazine	nutty, coffee, hazelnut	393.55±59.12	-	-	-
134.	2-formylpyrrole	Pyrazine	musty, beefy, coffee	43.78±2.41	-	-	-
135.	2 methoxy phenol	Phenol	phenolic, smoky, spicy	92.90±39.73	-	207.69±52.64	-
136.	2 methoxy-4-methyl phenol	Phenol	spicy, clove, vanilla	-	-	178.72±29.39	-
137.	2-methoxy-4-ethyl phenol	Phenol	coffea, coffea arabica,	-	-	66.54±20.85	-
138.	Phenol	Phenol	phenolic, plastic, rubbery	76.96±5.76	13.69±0.72	89.17±18.25	540.79±51.55
139.	2,4,bis (1.1-dimethyl-ethyl) phenol	Phenol	phenolic	-	128.35± 3.14	-	-
140.	Dimethyl trisulfide	Organic trisulfide	sulfurous, garlic, onion	77.87±20.52	-	-	-
141.	2-mercapto-4-phenyl thiazole	Thiazole	-	187.74±7.10	-	-	-
142.	Elemicin	Alkenylbenzene	spicy, floral	-	32.24± 7.05	-	-
143.	Myristicin	Alkenylbenzene	spicy, warm, balsamic	-	13.38±2.91	-	-
144.	Butylated hydroxytoluene (BHT)	Synthetic anti-oxidant	phenolic, camphoreous	189.41±154.32	16.09±3.93	148.16±28.39	-

Abbreviations: BV1: Central Anatolia Region, BV2: Aegean Region; BV3: South Marmara Region; BV4: Western Black Sea Region.
[a]Mean relative abundance = [concentration of internal standard × peak area of compound]/[peak area of the internal standard], [b]:not detected

The pharmacological activities of pyrazines and thiazoles have been confirmed by numerous clinical studies. Reported biological activities range extensively from anti-microbial, anti-helminthic, anti-inflammatory, and anti-convulsant properties to anti-proliferative and anti-tumor effects. The potential therapeutic effects of pyrazines and thiazoles may contribute to the medical use of BV1. In general, the findings obtained from bee venoms encourage further research into the development of drug formulations (Ali & Sayed 2021; Huigens et al. 2022).

In the case of BV2 sample, 5-ethyl-2-methyl octane, 3,7-dimethyl undecane, 3-octen-1-ol acetate (Z), 1-nonanol, 2-tetra decanol, piperitone, 2-methyl-3-pheynyl propanal, 4-methoxy benzaldehyde, α-N-methyl ionone, piperonal, geraniol acetate, geraniol, lilial and eugenol were detected only in this sample. Geraniol, geraniol acetate, lilial, and eugenol have characteristic fruity-floral, lavender-like, lily-like, clove-like, and spicy odour notes, respectively (Caputi & Aprea 2011; Jeleń & Gracka 2016).

Elemicin and myristicin belong to the class of alkenylbenzene and are secondary metabolites synthesized by various plants (nutmeg, sweet basil, sweet bay, tarragon etc.) (Götz et al. 2022). It is noteworthy that elemicin and myristicin were found only in BV2 among the different bee venom samples. Although these alkenyl benzenes are considered as potential toxicants in food products, a recent review on these compounds emphasized that elemicin and myristicin have several pharmacological and therapeutic activities such as anti-inflammatory, anti-proliferative, and antioxidant properties based on *in vitro* and *in vivo* studies. The researchers emphasized that, considering the ethnopharmacology of myristicin, further clinical studies are urgently needed to confirm its use as a therapeutic agent. Bee venom is a natural source of myristicin with promising potential for its future use in medicine (Seneme et al. 2021; Götz et al. 2022).

In the case of BV3 sample, 3-methyl-1-pentanol, α-phenyl ethyl acetate, α-terpinene, epizonarene, geranyl acetone, 2-methoxy-4-methyl phenol, 2-methoxy-4-ethyl phenol, cedrol, α-calacorene, delta selinene, beta gurjunene, bulnesol, and alpha bisabolol are unique volatiles of this bee venom. 3-methyl-1-pentanol and α-terpinene were the major volatile compounds among these identified aromatic compounds. While 3-methyl-1-pentanol is associated with green, apple with an alcoholic nuance, α-terpinene gives woody and terpenic aromas (Anonymous 2024). BV3 was noteworthy among bee venoms in this study due to its higher terpene and phenol content. In fact, this finding confirms that bee venom can be used for medical purpose. Paulino et al. (2002) highlighted the potential applications of monoterpenes for medical purpose extensively. The researchers pointed out that terpenes exhibit remarkable biological activities including antidiabetic, anti-inflammatory, anti-tumor, cardioprotective, hepatoprotective, and neuroprotective.

Although sample BV4 had the lowest volatile compound profile, the major volatile compounds detected in it, such as, 2-pentene, 2-octanol, copaene, β-cyclocitral, alloaromadedrene, δ-cadinene, α-amorphene, and p-propenyl anisole were not found in other samples. 2-octanol, phenyl methyl acetate, E-2-decenyl acetate, copaene, alloaromadedrene, δ-cadinene and α-amorphene contribute significantly to the unique spicy and woody aroma of BV4 (Anonymous 2024; Jeleń & Gracka 2016). Considering its overall chemical composition, BV4 appears to have lower levels of acidic volatiles, ketones and phenolic contents compared to other bee venoms.

Butylated hydroxytoluene (BHT) is a synthetic antioxidant widely used in many formulations from the food industry to the pharmaceutical, petroleum, and rubber industries (Yehye et al. 2015). The detection of BHT in the bee venom samples except BV4 suggests that synthetic antioxidants contained in oils and bee cakes used to feed honeybees may be transferred to bee products. In an interview with the producers of BV4 (M, Almışlar, September 2022), the manufacturers stated that bee cake is not used to feed bees. The fact that BHT was not detected in BV4 also supports this finding.

α-pinene (Salehi et al. 2019), d-limonene (Anandakumar et al. 2021), isoamyl acetate, and isoamyl alcohol (Ando et al. 2015) are known to exhibit different bioactive effects and are closely related to bee venom's anti-tumor, anti-inflammatory, anti-allergic, analgesic, and anti-microbial activities in the treatment of both acute and chronic conditions. In a recent study by Abd El-Wahed et al. (2021), volatile compounds were identified in beehive air containing a mixture of bee bread, beeswax, honey, royal jelly, propolis, larvae and bee venom. The authors determined a total of 56 volatile compounds, mainly short-chain fatty acids. Similar to our findings, benzyl_alcohol, octanal, (E)-2-octenal, isoamyl_acetate, *n*-octyl acetate, 2-nonanol, benzyl_acetate were detected in the beehive air. Another recently study reported the detection acids, esters, alcohols, and terpenoids in volatile compound analyses carried out on bee products such as propolis. It is noteworthy that terpenes represented a higher proportion (40%) than other volatile compounds (Ding et al. 2021).

4. Conclusions

Bee venom produced by honeybees for self-defence, is also used as a complementary and alternative medicine, which has attracted particular attention due to its various pharmaceutical applications. While the pharmaceutical activity of bee venom is thought to be derived from the venom proteins, such as melittin and apamin, the volatile compounds of bee venom have been relatively neglected. However, bee venom is a holistic entity, and its efficacy should be considered together with its matrix. The findings of the present study regarding bee venoms produced by *Apis mellifera anatoliaca* populations from four different regions in Türkiye revealed that bee venom consists mostly of terpenes and esters. The result of the GC-MS analysis showed that bee

venom samples contain a variety of volatiles, including esters, terpenes, alcohols, organic acids, aldehydes, ketones, and hydrocarbons. It is thought that the differences detected are due to diet, production method and different harvesting times. The volatile compounds of bee venom should be considered when establishing the quality parameters of bee venom. In conclusion, further research is now needed to determine exactly what the essential compounds of bee venom are and what affects them.

Acknowledgements

The authors are greatly indebted to Prof. Dr. Yonca Karagül YÜCEER for her support and permitting our access to her laboratory infrastructure. The data used are confidential. This research does not require ethical approval.

Data availability: Data are available on request due to privacy or other restrictions.

***Authorship Contributions*:** Conceptualization, Data Curation, Formal Analysis, Writing – Review & Editing- Original Draft: B.A.G., O.G.; Conceptualization, Investigation, Review & Editing- Original Draft: M.K., S.K.
All authors reviewed and approved the final version of the manuscript.

Financial Disclosure: The author declared that this study received no financial support.

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