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Differentiation of *Achillea millefolium*, *A. crithmifolia*, and *A. nobilis* through Analysis of Volatile Constituents using HS-SPME-GC/MS and Chemometric Techniques

Bledar Pulaj, Behxhet Mustafa and Avni Hajdari*

Department of Biology, Faculty of Mathematics and Natural Science, University of Prishtina, Mother Theresa, St. 10000 Prishtinë, Kosovo

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Abstract: *Achillea millefolium, A. crithmifolia* and *A. nobilis* are distinct species that share morphological similarities, making their differentiation challenging. This study utilized gas chromatography-mass spectrometry (GC/MS) combined with headspace-solid phase microextraction (HS-SPME) to analyze the volatile components of these species and employed chemometric methods for species differentiation. A total of 109 volatile compounds from the three studied species were identified. Of these compounds, 71 were identified in *A. millefolium*, 75 in *A. crithmifolia*, and 58 in *A. nobilis*. The primary volatile compounds of *A. millefolium* were germacrene D, 1,8-cineole, sabinene, and β-pinene; in *A. crithmifolia*, the main compounds were caryophyllene, 1,8-cineole, camphor, ascaridole, and *o*-cymene, while in the essential oil of *A. nobilis*, camphor, lavandulyl acetate, camphene, and isobornyl acetate were determined as the main volatile compounds. The study demonstrated that the HS-SPME-GC/MS techniques, combined with chemometric methods such as discriminant analysis (DA) and principal component analysis (PCA), effectively distinguished the samples of *A. millefolium*, *A. crithmifolia*, and *A. nobilis* based on the differences in the chemical composition of their essential oils.

Keywords: *Achillea millefolium*; *A. crithmifolia; A.nobilis;* HS-SPME-GC/MS; discriminant analysis (DA); principal component analysis (PCA). © 2024 ACG Publications. All rights reserved.

1. Introduction

The genus *Achillea* L. (Asteraceae) comprises about 130 species worldwide, distributed mainly in Europe and the temperate regions of Asia and North America [1]. Based on existing records (Krasniqi, 1972, 1987; Rexhepi, 1986, 1994, 2000), in Kosovo are present 18 *Achillea* species, including *Achillea millefolium* L., *Achillea crithmifolia* Waldst. et Kit, and *Achillea nobilis* L. [2-6]

According to Flora Europaea [7], *A. millefolium* and *A. crithmifolia* belong to the *A. millefolium* group, while *A. nobilis* belongs to the *A. nobilis* group. Although they belong to different groups, they share many similarities. On the other hand, *A. millefolium* and *A. nobilis* especially exhibit high polymorphism [7], making their identification challenging.

Comparing the botanical characteristics of these species, they all have erect stems in common. *A. millefolium* can grow up to 8-90 cm, *A. crithmifolia* 20-60 cm, while *A. nobilis* up to 10-60 cm [7]. The middle cauline leaves of *A. millefolium* measure 3-5 x 0.5-1.2 cm and are lanceolate in outline, 2

* Corresponding author: E-Mail: avni.hajdari@uni-pr.edu; Phone: +383 38 249 872; Fax. +383 38 226 104

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(-3) pinnatisect, and more or less pubescent; the cauline leaves of the *A. crithmifolia* are 4-6 x 0.8-2 cm, ovate to lanceolate in outline, plane, pinnatisect, and more or less pubescent, while *A. nobilis* has cauline leaves that measure 1.5 x 3 to 1-1.5 cm, which are ovate in outline, pinnatifid to pinnatisect, and more or less pubescent [7]. Additionally, the ligules of *A. millefolium* are usually white; the ligules of *A. crithmifolia* are white to pale yellow, while the ligules of *A. nobilis* are white [7]. These shared main characteristics of stem length, leaf morphology, and ligule colour make challenging the accurate identification of these species, especially for local communities who traditionally use these species and local collectors who trade them.

Despite morphological similarities, the chemical composition of the essential oil exhibits significant variability within and among these species. In this regard, the variability in the chemical composition of the essential oil of *A. millefolium* originated from different countries was reported by various authors [8-12]. Similarly, the variability reported for the chemical composition of the essential oil of *A. crithmifolia* [8,13-15] and *A. nobilis* [15-17]. So, one approach to differentiate the morphologically similar species such as *A. millefolium, A. crithmifolia* and *A. nobilis* is to analyze the chemical composition of the essential oils.

The diverse chemical composition of the essential oils obtained from the abovementioned species may contribute to the diverse biological activities and traditional uses associated with these species. In Kosovan traditional medicine, *A. millefolium* has been used to enhance general health and treat a variety of conditions, including respiratory inflammations, urinary system infections, gastrointestinal disorders, skin diseases, cardiovascular diseases, neuromuscular disorders, and ophthalmological infections [18-24]. Furthermore, since ancient times, it has been used to make recreational tea and prepare beverages for enjoyment [22].

On the other hand, there is no ethnobotanical evidence indicating that *A. crithmifolia* and *A. nobilis* have been traditionally used as medicine or food in Kosovo. However, despite the lack of data on their traditional use as medicine or food, these species may have been used instead of *A. millefolium* due to their morphological similarities. Therefore, these species can be considered "ethnospecies," meaning that multiple scientifically distinct species are used as a single species and used for similar purposes by the local community [25].

In Kosovo, *A. millefolium* is an economically significant medicinal and aromatic plant. Local people collect it from the wild population and export it. The exported amount of *Achillea* sp. (exact species not specified) from Kosovo in 2021 was 14 tonnes [26], while in 2022, the export was 10 tonnes [27]. However, the reports use the English name "yarrow," which is the common name for *A. millefolium.* Therefore, it is very likely that these species were included in the *A. millefolium* data because local collectors could not distinguish these taxa.

The possibility of adulteration of *A. millefolium* raw material with *A. nobilis* due to similar morphological characteristics has been previously reported [29] and references therein. The misidentification of the species may lead to replacing one species with another, which may decrease the quality of the final product [28] and might result in unintended side effects.

Furthermore, chemo-polymorphism within the species, hybridization and polyploidization, genetic polymorphism and ecological plasticity were also reported for *A. millefolium* group [30-31], which further complicates the accurate identification of this species.

Essential oils volatile constituents from *Achillea* sp. are usually extracted using the hydro distillation method. However, this method requires a lot of plant material, is labour-intensive, and is time-consuming. In this context, headspace solid-phase microextraction (HS-SPME) seems to be an alternative extraction method for analysing the volatile constituents of *Achillea* sp. This method is simple, doesn't require solvents, takes less time, and can be automated [32]. Moreover, plant species with similar morphological characteristics can be discriminated by applying chemometrics methods, including principal component analysis (PCA) and discriminant analyses (DA), by using the variation of the main volatile components of the essential oils. This approach was previously used to identify variations and commonalities among complex sample clusters [32].

There is limited data available on the chemical composition of the essential oil of *A. millefolium* from Kosovo, whereas, to the best of our knowledge, there are no previous reports on the chemical composition of the essential oil of *A. crithmifolia* and *A. nobilis*. This study aims to assess the differences in the chemical composition of essential oils volatile constituents among *A.*

millefolium, A. crithmifolia, and *A. nobilis* in Kosovo and to test analytical HS-SPME-GC/MS techniques and statistical multivariate methods for discriminating these plants.

2. Materials and Methods

2.1. Plant Materials

The inflorescences of *A. millefolium, A. crithmifolia* and *A. nobilis* were gathered from the natural population in Kosovo, the Novobërdë municipality (Latitude: 42°37'10"; Longitude: 21°27'80"; Altitude: 1248m), during the blooming season of 2023. The inflorescences gathered in a small area within approximately half a square kilometre, where all three species coexisted. This sampling approach was selected to observe changes that can be attributed more to the species' genetic background and minimize changes in the chemical composition of the species' essential oils that arise due to environmental impact. The plant materials were collected in eight plots in total, and due to slight morphological variability within the species, *A. millefolium* and *A. crithmifolia* were collected in more than one plot. Thus, the *A. crithmifolia* was collected in four plots, *A. millefolium* in three, and *A. nobilis* in one (Table 1). Five samples per plot were collected, each obtained from an individual plant. Plant species were identified by Dr. A. Hajdari using the local Floras and Flora Europaea [34-37]. Herbarium samples were prepared from the identified plant samples and deposited in the Herbarium of the University of Prishtina (Table 1).

The collected plant materials were dried for four days at 35°C in a dry and light-proof cabinet. The dried samples were packaged, labelled, and stored in plastic bags under a vacuum in a dry, light-proof environment.

Table 1. Plant species, sample code and herbarium specimens accession information

Plant species	Sample codes	Specimens accession no.
Achillea nobilis	*A. nobilis* (AN)	00002021
Achillea crithmifolia	1 *A. crithmifolia* (1AC)	00002022
	2 *A. crithmifolia* (2AC)	00002024
	3 *A. crithmifolia* (3AC)	00002025
	4 *A. crithmifolia* (4AC)	00002023
Achillea millefolium	1 *A. millefolium* (1AM)	00002028
	2 *A. millefolium* (2AM)	00002026
	3 *A. millefolium* (3AM)	00002027

2.2. SPME Extraction of the Essential Oils

The volatile components of the species were extracted using the headspace solid phase microextraction (HS-SPME) technique with an autosampler (HTA, model: HT2800T, Brescia, Italy). 0.5 g of plant material (inflorescences) was placed in a 20 mL vial, incubated for 20 minutes, and then extracted for 30 minutes at 70°C with shaking intervals of 0.1 minutes within 3 minutes. The adsorption was performed using DVB/CAR/PDMS fibre size 23 ga, followed by desorption of the absorbed compounds at 250°C for 3 minutes.

2.3. GC/FID and GC/MS Analyses

The volatile constituents were quantified using gas chromatography coupled with a flame ionization detector (GC/FID) (Agilent 7890A, Agilent Technologies). The separation was performed on an HP-5MS column (30m × 0.25mm, 0.25μm film thickness). Helium was used as the carrier gas with an initial flow rate of 0.6 mL/min. The front inlet was set at 250°C, while the split ratio was set at 50:1. The GC oven temperature program started at 60°C and increased to 280°C at a rate of 5°C/min. The FID detector operated at 250°C with an airflow of 350 mL/min and a hydrogen flow of 35 mL/min.

The qualitative analyses of the essential oils were carried out using gas chromatography coupled with mass spectrometry (GC/MS) (GC: Agilent 7890A; MS: 5975C, Agilent Technologies). The GC

operation conditions were as described for the GC/FID analyses. The MS ionization energy was set to 70 eV, and the mass range was set at 40-400 *m/z*. The injection volume for each sample was 1.0 µL.

The essential oil volatile constituents were identified by comparing their Arithmetic Retention Indices (ARI) with those reported in the literature [33], by comparing the mass spectra of each constituent with those stored in NIST 08.L and WILEY MS 9th databases and with mass spectra from the literature [33]. The percentage of oil was calculated using a normalization method without correction factors based on the GC peak areas.

2.4. Statistical Analysis

The primary volatile constituents, higher than 2% and highlighted in bold in Table 2, were considered for the statistical analysis (PCA and DA). The statistical analyses were performed by XLSTAT software, Version 2023.1.2 (New York, NY, USA).

3. Results and Discussion

3.1. Chemical Analyses of the Essential Oils

The essential oils from 40 samples were investigated. These samples were categorized into eight groups based on morphological characteristics (Table 1), each representing the average of five plants. *A. crithmifolia* comprised four groups (20 samples), *A. millefolium* comprised three groups (15 samples), and *A. nobilis* comprised the remaining group (five samples) as indicated in Table 1 and 2.

109 volatile compounds were separated from the essential oils of three analysed plant species, 71 from *A. millefolium*, 75 from *A. crithmifolia*, and 58 from *A. nobilis*. Out of the 109 volatile compounds, 106 were successfully identified.

The major components of the *A. millefolium* in the studied samples were germacrene D (11.5-25.0%), followed by 1,8-cineole (7.7-22.5%), sabinene (3.4-20.4%), β-pinene (12.8-18.7%) and β-caryophyllene (8.4-15.0%). El-Kalamouni [9] also reported that germacrene D is one of the primary volatile constituents in *A. millefolium* sample collected from France. In addition, other researchers (from France, Serbia, Romania, and Bosnia and Hercegovina) [8-12] reported similar data for 1,8-cineole in the essential oil of *A. millefolium*. However, contrary to the data in these reports, in our study, the camphor and borneol percentages were found to be very low, 0.2-1.5% and 0.2-0.3%, respectively.

1,8-cineole (27.8-40.2%), camphor (5.0-28.8%), ascaridole (7.4-15.6%) and *o*-cymene (6.6-10.7%) were the most abundant volatile compounds in the essential oil of *A. crithmifolia*. Other authors have also identified 1,8-cineole as the main compound in the essential oil of *A. crithmifolia* [8], [13-15]. Furthermore, similar to our findings, camphor was one of the main volatile constituents of the essential oils of *A. crithmifolia* [13-15]. Ascaridole, as one of the primary constituents in our samples, was reported by Stanković *et al*. [13] to have a low concentration. However, artemisia alcohol, *trans*-chrysanthemum, and artemisia ketone, which were identified as major volatile constituents in the research reports mentioned above, were not detected in our samples.

The primary volatile constituents of *A. nobilis* in our samples were camphor (45.2%), lavandulyl acetate (11.0%), camphene (5.4%) and isobornyl acetate (4.8%). However, other studies have reported different chemical profiles for the essential oil, i.e. 1,8-cineole (eucalyptol) (22.9%), chrysanthenone (14.3%), and endo-borneol (9.9%) [16]; 1,8-cineole (eucalyptol) (21.0%), α-thujone (11.0%), and camphor (8.5%) [17]; α-thujone (25.7%), artemisia ketone (14.8%) and borneol (9.9%) (8.2%) was reported in the literature on the species [15].

Table 2. Composition (%) of the volatile constituents of *Achillea millefolium, A. crithmifolia* and *A. nobilis**

Compound[a]	**ARI**[b]	**ARI**[c]	**1AM**	**2AM**	**3AM**	**1AC**	**2AC**	**3AC**	**4AC**	**AN**
Tricyclene	923	925	-	-	t	t	t	t	t	0.47
α-Thujene	924	923	0.16	0.32	0.12	t	t	0.18	0.12	-
α-Pinene	932	932	1.59	1.49	4.27	4.85	0.88	1.23	0.75	1.62
Camphene	**946**	**945**	**0.10**	**t**	**0.22**	**0.20**	**0.83**	**1.62**	**0.89**	**5.36**
Benzaldehyde	952	948	0.38	0.46	0.15	0.40	0.14	0.25	0.13	0.13
Sabinene	969	966	**7.21**	**20.39**	**3.37**	**0.20**	**0.77**	**1.22**	**0.95**	**t**
β-Pinene	**974**	**972**	**16.57**	**12.84**	**18.74**	**0.30**	**1.81**	**0.77**	**0.74**	**1.08**
Dehydro-1,8-Cineole	989	989	1.51	1.48	0.66	0.16	t	0.54	t	-
α-Phellandrene	1002	999	-	-	-	1.03	-	-	-	-
α-Terpinene	1014	1012	0.15	0.26	t	0.10	0.62	1.31	1.10	0.72
***o*-Cymene**	**1022**	**1019**	**0.51**	**0.35**	**t**	**7.24**	**9.60**	**6.58**	**10.70**	**0.12**
Limonene	1024	1020	1.09	0.82	0.86	0.17	-	0.19	t	0.30
1,8-Cineole	**1026**	**1021**	**22.45**	**17.26**	**7.73**	**27.84**	**40.24**	**30.85**	**34.95**	**0.13**
(E)-β-Ocimene	1044	1043	0.73	0.81	0.91	t	t	-	t	-
γ-Terpinen	1054	1051	0.67	0.78	0.20	0.26	1.13	1.78	0.82	0.10
***cis*-Sabinene hydrate**	**1065**	**1064**	**0.40**	**0.45**	**0.16**	**t**	**0.50**	**2.26**	**0.46**	**0.31**
ρ-Mentha-2,4(8)-diene	1085	1082	0.18	0.17	0.11	0.22	0.43	0.50	0.36	0.36
Butyl pentanoate	1092	1091	0.34	0.34	0.15	0.19	0.50	1.11	0.48	-
Linalool	1095	1091	-	-	t	0.75	t	-	-	-
cis-*ρ*-Menth-2-en-1-ol	1115	1110	t	t	-	0.55	-	0.28	0.21	-
trans-*ρ*-Menth-2-en-1-ol	1119	1114	-	-	-	1.45	1.49	1.20	1.43	-
Camphor	**1141**	**1135**	**0.32**	**0.19**	**1.45**	**28.76**	**4.96**	**16.83**	**13.33**	**45.20**
iso-Isopulegol	1155	1153	t	t	-	0.31	0.39	0.18	0.39	-
Sabina ketone	1154	1149	t	t	t	0.28	t	0.15	0.16	0.26
trans-Pinocamphone	1158	1153	t	t	0.28	0.93	0.43	0.17	0.25	-
cis-Chrysanthenol	1161	1156	-	-	-	-	-	-	-	0.75
Borneol	1165	1163	0.31	0.20	0.27	0.78	1.17	0.99	0.88	-
Umbellulone	**1167**	**1163**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**4.40**

Volatile constituents of *Achillea sp.*

cis-Linalool oxide	1170	1163	-	-	-	-	-	-	-	0.10
Terpinen-4-ol	**1174**	**1170**	**0.43**	**0.66**	**0.24**	**0.42**	**2.46**	**3.12**	**1.86**	**0.42**
ρ-Cymen-8-ol	1179	1172	-	-	-	0.29	0.36	0.34	0.40	-
α-Terpineol	**1186**	**1180**	**4.54**	**2.87**	**0.79**	**0.73**	**1.29**	**2.03**	**1.92**	**-**
Myrtenol	1194	1191	0.11	t	0.24	0.29	0.70	0.15	0.26	0.12
trans-Piperitol	1207	1206	-	t	-	t	0.24	0.14	0.21	-
Verbenone	1204	1198	t	-	0.12	0.57	t	t	0.16	-
trans-Carveol	1215	1210	-	t	t	0.33	t	0.20	0.16	-
neoiso-Dihydro carveol	1226	1225	-	-	-	t	0.11	-	0.23	-
cis-Carveol	1226	1219	-	-	-	-	-	-	-	0.11
Ascaridole	**1234**	**1227**	**t**	**t**	**-**	**7.41**	**15.57**	**7.47**	**12.05**	**-**
Carvone	1239	1234	0.21	t	t	0.18	t	0.34	0.22	-
Piperitone	1249	1243	-	-	-	0.37	0.32	0.12	0.24	-
Linalool acetate	1254	1251	-	-	-	-	-	-	-	t
trans-Piperitone epoxide	1252	1246	-	-	-	0.11	0.27	0.18	0.19	-
cis-Chrysanthenyl acetate	1261	1261	-	-	0.17	0.42	-	0.18	0.26	1.82
o-Guaiacol acetate	1261	1256	-	-	-	-	-	-	-	0.33
Perilla aldehyde	1269	1264	-	t	0.48	t	0.22	1.02	0.42	-
Isobornyl acetate	**1283**	**1280**	**-**	**-**	**-**	**0.35**	**0.56**	**0.17**	**0.64**	**4.81**
Lavandulyl acetate	**1288**	**1284**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**10.95**
Thymol	1289	1284	0.19	0.15	-	0.38	0.40	0.20	0.44	0.41
Perilla alcohol	1294	1288	-	-	-	0.14	0.23	t	0.13	-
Carvacrol	1298	1295	-	-	-	-	-	-	-	0.66
trans-Carvyl acetate	1339	1339	-	-	-	-	-	-	-	0.28
Unknown 1	**1311**	**1311**	**-**	**-**	**0.19**	**3.12**	**3.64**	**2.80**	**3.56**	**-**
Linalool propanoate	1334	1330	-	-	t	t	-	0.64	0.61	-
trans-Carvyl acetate	1339	1337	-	t	t	0.40	0.17	0.16	0.27	-
α-Cubebene	1348	1346	-	-	-	-	-	0.19	0.24	-
α-Longipinene	1350	1345	-	-	-	-	-	-	-	0.24
Eugenol	1356	1354	0.55	0.34	0.15	0.13	0.34	0.17	0.17	0.38

Volatile constituents of *Achillea sp.*

cis-Carvyl acetate	1365	1364	-	-	-	-	-	-	-	1.74
Cyclosativene	1369	1370	-	-	t	t	-	0.15	0.19	-
Linalool isobutanoate	1373	1372	-	-	-	-	-	-	-	0.34
α-Ylangene	1373	1370	t	t	-	0.12	t	0.17	t	-
trans-p-Menth-6-en-2,8-diol	1371	1366	0.15	0.13	0.28	0.11	0.11	0.34	0.16	-
α-Copaene	1376	1374	0.61	0.56	1.90	-	-	-	-	-
Geranyl acetate	**1379**	**1375**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**2.72**
β-Bourbonene	1387	1388	0.22	0.19	0.32	0.69	t	t	-	-
7-Epi-Sesquithujene	1391	1390	-	-	-	-	-	-	-	1.08
(Z)-Jasmone	1392	1387	0.26	0.14	0.19	1.71	1.18	0.52	1.42	0.25
Methyl eugenol	**1403**	**1401**	**2.24**	**1.32**	**t**	**-**	**-**	**t**	**-**	**-**
Unknown 2	1416	1416	0.16	0.14	0.34	0.12	t	-	t	0.27
Caryophyllene	**1419**	**1418**	**8.43**	**10.33**	**15.04**	**0.43**	**0.73**	**1.75**	**0.47**	**-**
Lavandulyl isobutanoate	1421	1420	-	-	-	-	-	-	-	0.12
Linalool butanoate	1423	1422	-	-	-	-	-	-	-	0.40
β-Copaene	1430	1431	0.10	0.12	t	t	-	-	-	0.36
(*Z*)-β-Farnesene	1440	1435	-	-	-	-	-	-	-	0.18
Aromadendrene	1439	1433	0.36	0.39	0.40	0.15	0.26	0.27	0.10	-
α-Humulene	1454	1453	1.29	1.43	1.89	t	t	0.13	t	-
(*E*)-β-Farnesene	1454	1451	t	0.10	0.37	t	t	0.10	t	-
γ-Gurjunene	1475	1472	-	-	-	-	-	-	-	0.59
γ-Himachalene	**1481**	**1480**	**1.30**	**0.91**	**2.54**	**t**	**0.27**	**t**	**0.17**	**0.71**
Germacrene D	**1480**	**1476**	**12.16**	**11.51**	**25.00**	**0.76**	**0.87**	**1.22**	**0.63**	**3.01**
γ-Curcumene	1481	1477	-	-	-	0.18	-	0.27	-	-
α-Zingiberene	**1493**	**1489**	**6.50**	**4.85**	**1.79**	**-**	**-**	**-**	**-**	**-**
α-Muurolene	1500	1498	0.37	0.36	1.18	t	t	0.12	t	0.35
β-Himachalene	1500	1495	t	t	-	-	0.17	-	0.37	-
Lavandulyl isovalerate	1509	1507	-	-	-	-	-	-	-	1.23
γ-Cadinene	1513	1510	0.13	0.11	0.15	-	-	-	-	0.21
β-Curcumene	1515	1511	0.21	0.15	0.26	t	t	t	t	-

Volatile constituents of *Achillea sp.*

δ-Cadinene	**1522**	**1520**	**2.03**	**1.58**	**0.95**	**t**	**t**	**t**	**t**	**-**
β-Sesquiphellandrene	1521	1516	-	-	-	-	-	-	-	0.42
cis-Sesquisabinene hydrate	1544	1542	-	-	-	-	-	-	-	0.17
Elemol	1548	1542	0.13	t	t	-	-	-	-	-
Unknown 3	1561	1561	-	-	-	-	-	-	-	0.15
(*E*)-Nerolidol	1561	1558	0.20	0.13	0.13	-	0.23	0.40	-	-
epi-Longipinanol	1562	1559	-	-	-	-	-	-	-	0.22
Spathulenol	1577	1574	t	0.10	0.14	0.17	-	-	0.14	0.79
Caryophyllene oxide	**1582**	**1579**	**1.06**	**1.42**	**2.43**	**0.69**	**1.32**	**2.83**	**0.81**	**0.27**
Allo-cedrol	1589	1584	t	-	t	0.25	0.15	0.26	0.11	-
Cubeban-11-ol	1595	1589	0.15	0.13	0.22	t	t	t	t	-
cis-Dihydro-Mayurone	1595	1590	-	-	-	-	-	-	-	0.18
Ledol	1602	1598	0.13	0.13	t	0.26	0.14	0.29	t	-
Humulene epoxide II	1608	1603	t	t	0.19	t	0.13	0.13	t	-
Caryophylla-4(12),8(13)-dien-5α-ol	1639	1639	t	t	0.91	-	0.10	0.15	-	-
1,7-Diepi-α-Cedrenal	1639	1638	-	-	-	-	-	-	-	0.26
β-Eudesmol	1649	1645	-	-	-	0.78	0.42	0.30	0.80	-
Selin-11-en-4-α-o	1659	1658	0.23	0.16	0.24	-	-	-	-	1.34
neo-Intermedeol	1658	1653	-	-	-	-	-	-	-	0.25
Valeranone	1674	1672	0.28	0.17	t	-	-	-	-	-
Germacra-4(15),5,10(14)-trien-1-α-ol	1685	1681	-	-	-	-	-	-	-	0.32
		Monoterpenes	14.73	16.28	15.47	16.62	28.95	38.29	28.99	10.22
		Oxygenated monoterpenes	73.22	72.06	69.27	71.16	31.35	24.14	12.98	53.00
		Sesquiterpenes	2.78	2.75	4.61	2.45	33.92	32.65	51.96	7.14
		Oxygenated sesquiterpenes	2.23	2.59	4.44	2.07	2.38	2.46	4.56	4.86
		Others	7.05	6.21	6.07	7.55	3.40	2.47	1.29	24.79

[a]Compounds listed in order of elution from a HP-5MS column; [b]ARI - Arithmetic Retention Indices from the literature [33]; [c]ARI - Arithmetic Retention Indices calculated against a mixture of C9- C28n alkanes; Compounds marked in boldface (with relative concentrations higher than 2%) chosen for HCA and PCA statistical analyses; - = compound not detected; t = trace < 0.1%.; *Species names are abbreviated according to the format used in Table 1.

Considering the main classes of volatile constituents of *A. millefolium*, the predominant constituents were found to be oxygenated monoterpenes (6.3-73.2%), followed by monoterpenes (14.7-16.3%), other volatile constituents (6.1-7.1%), oxygenated sesquiterpenes (2.2-4.4%), and sesquiterpenes (2.8-4.6%). In the essential oil of *A. crithmifolia*, the main classes of volatile constituents are oxygenated monoterpenes (13.0-71.2%), followed by sesquiterpenes (2.5-52.0%), monoterpenes (2.5-33.9%), other constituents (1.3-7.6%), and oxygenated sesquiterpenes (2.1-4.6%). Additionally, the primary main classes of volatile constituents of *A. nobilis* include oxygenated monoterpenes (53.0%), followed by other constituents (24.8%), monoterpenes (10.2%), sesquiterpenes (7.1%), and oxygenated sesquiterpenes (4.9%) [15].

The variations in the chemical composition of essential oils within the species may be due to environmental factors (climate conditions, soil characteristics, etc.), altitude, geographic isolation, habitat composition, plant growth period, harvest timing, and genetic factors like hybridization [39-43].

3.2. Principal Component Analysis and Discriminant Analysis

The DA and PCA were utilized to evaluate the variation in the volatile composition of *A. millefolium*, *A. crithmifolia*, and *A. nobilis*. In both cases, the localities were used as grouping variables to classify or discriminate between different species based on the main chemical constituents of the essential oils as independent variables.

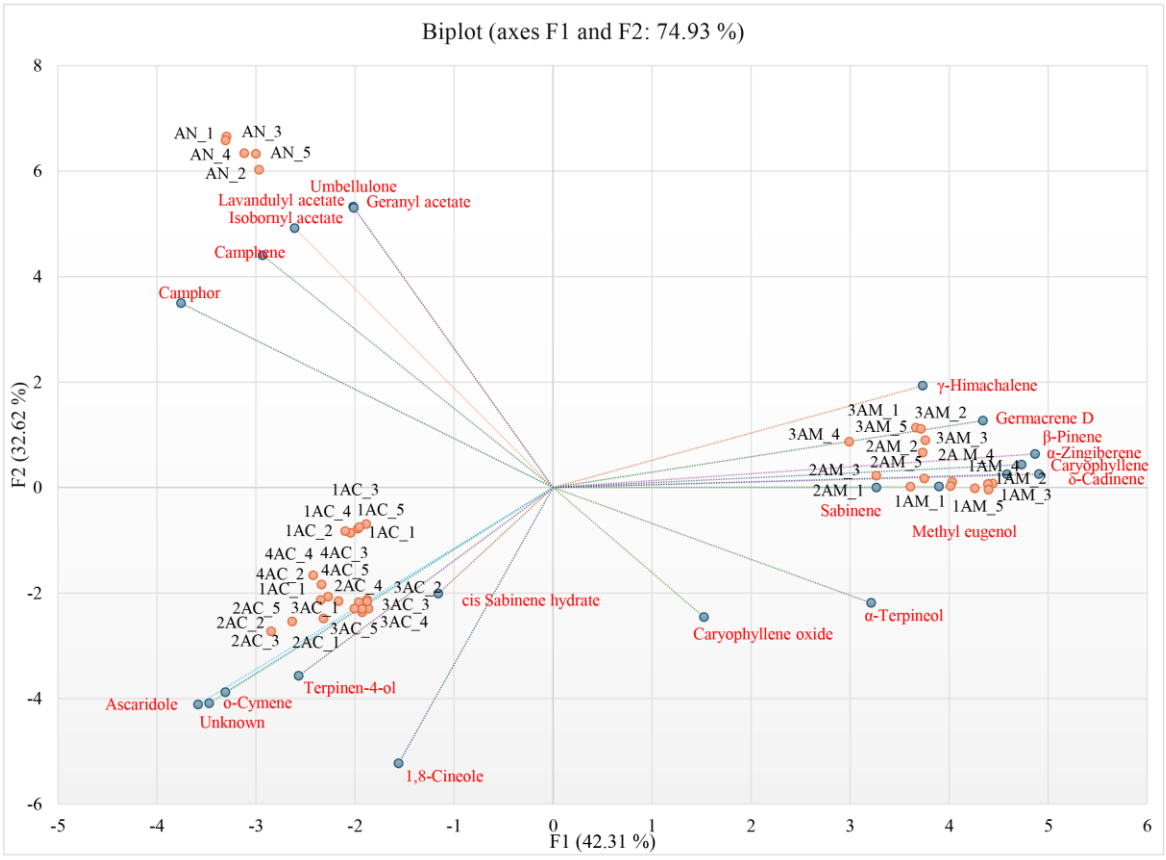

Figure 1. PCA scatter plot of the main essential oil constituents obtained from *A. millefolium* (AM), *A. crithmifolia* (AC), and *A. nobilis* (AN).

For PCA statistical analysis, 22 primary volatile constituents of the essential oils were used; the ones with a concentration of more than 2% are highlighted in bold in Table 2. The PCA revealed that

the first two principal components accounted for 74.9% of the total variance. The first axis, which accounts for 42.3% of the total variance (Eigenvalue variability 9.3%), demonstrates positive contributions from volatile compounds such as caryophyllene oxide, α-terpineol, methyl eugenol, sabinene, δ-cadinene, caryophyllene, α-zingiberene, β-pinene, germacrene D, and γ-himachalene. Conversely, negative contributions are associated with camphene, *o*-cymene, 1,8-cineole, *cis*-sabinene hydrate, camphor, umbellulone, terpinen-4-ol, ascaridole, isobornyl acetate, lavandulyl acetate, unknown 1, and geranyl acetate. The second PCA axis accounted for 32.6% of the total variance (Eigenvalue variability 7.2%) and was primarily influenced by umbellulone, geranyl acetate, isobornyl acetate, lavandulyl acetate, camphene, camphor, γ-himachalene, germacrene D, β-pinene, α-zingiberene, caryophyllene, and δ-cadinene. Conversely, it was negatively affected by sabinene, *o*-cymene, 1,8-cineole, *cis*-sabinene hydrate, terpinen-4-ol, α-terpineol, ascaridole, unknown 1, methyl eugenol, and caryophyllene oxide (Figure 1). The PCA scatter plot (Figure 1) indicates a differentiation among the studied samples based on species.

Similarly, the DA scatter plot (Figure 2) further validates the species-based discrimination of the samples. This suggests that the variation in the chemical composition of essential oils of the analyzed species may be attributed to the species' genetic background, as the plant material for all species was collected in a limited area, which minimized the impact of environmental factors like habitat composition, altitude, and microclimatic conditions.

However, in both the DA and PCA plots, intermediate samples between the species were absent, indicating a lack of hybridization among the analyzed species.

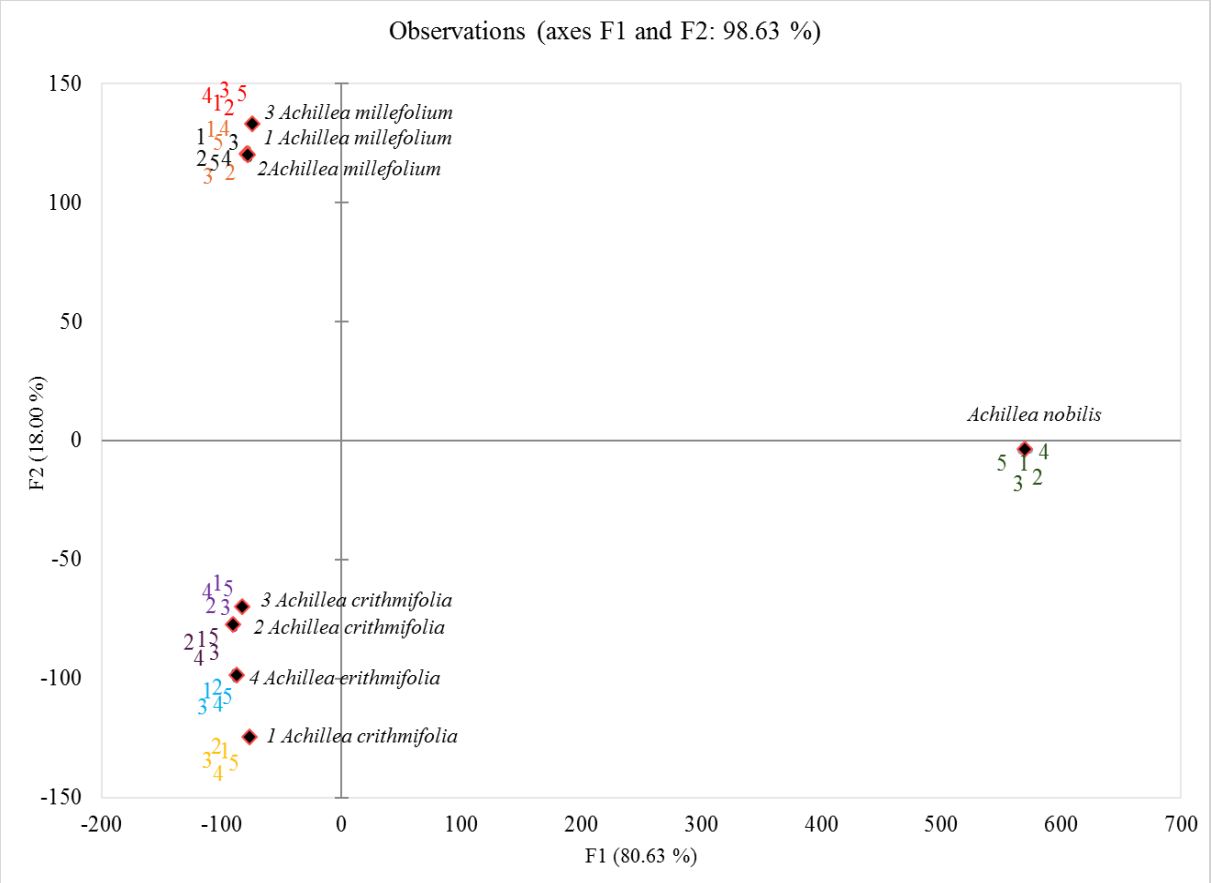

Figure 2. A scatter plot of discriminant analysis obtained by analyzing the essential oils *A. millefolium*, *A. crithmifolia*, and *A. nobilis*

4. Conclusions

In this study, we effectively utilized the HS-SPME-GC/MS method to identify the volatile constituents from *Achillea* species. This solvent-free method, which is simple and rapid, allowed us to separate 109 volatile constituents from the essential oils of three analyzed plant species. The main volatile constituents of the essential oil of *A. millefolium* were germacrene D, followed by 1,8-cineole, sabinene, β-pinene, and caryophyllene. The essential oil of *A. crithmifolia* was dominated by 1,8-cineole, camphor, ascaridole, and o-cymene, while the main volatile constituents of *A. nobilis* were camphor, lavandulyl acetate, camphene, and isobornyl acetate. Chemometric tools, like DA and PCA, were successfully used to differentiate the samples of *A. millefolium*, *A. crithmifolia*, and *A. nobilis* based on their differences in volatile chemical composition. The study revealed that genetic background significantly impacted the chemical composition and variability of essential oils of the analyzed species, as also indicated by the PCA and DA statistical analyses. However, it is important to note that other factors, such as micro-environment, vegetative periods, altitude, geographic isolation, habitat composition, etc. can also influence the volatile chemical composition of the essential oil. Thus, due to the small sampling area, our data does not reflect the environmental impact, which is a limitation of this research. Therefore, further studies using more extensive samples and populations from various geographical locations supported by molecular DNA analyses are needed.

ORCID
Bledar Pulaj: 0000-0003-0543-090X
Behxhet Mustafa: 0000-0002-1052-5252
Avni Hajdari: 0000-0001-5688-9679

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