Solid-Phase Microextraction (SPME) Analysis of Six Italian Populations of *Ephedra nebrodensis* Tineo ex Guss. subsp. *nebrodensis*

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Headspace solid-phase microextraction (HS-SPME) coupled with GC/FID and GC/MS was applied for the first time in the analysis of the volatile fraction of an *Ephedra* species. Notably, six Italian populations (Marche, Abruzzo, and Sardinia) of *Ephedra nebrodensis* subsp. *nebrodensis*, covering almost the entire Italian area, were investigated to examine the chemical variability and to support the taxonomy of the species. A fiber screening with polymethylsiloxane (PDMS), Carboxen™/polymethylsiloxane (CAR/PDMS), and polymethylsiloxane/divinylbenzene (PDMS/DVB) coatings, together with an optimization of the extraction conditions were carried out before analysis of the six populations. A total of 119 volatiles were identified in the headspace of different samples, accounting for 63.35–100.00% of the total volatiles. A great variability was found in the qualitative composition of different samples, since only 18 components were in common among all populations. The headspace composition was dominated by sesquiterpene hydrocarbons (52.30–88.32%), with β-maaliene (traces–7.49%), β-patchoulene (traces–1.29%), β-panasinsene (traces–6.85%), α-isocomene (traces–31.25%), α-*trans*-bergamotene (traces–6.95%), alloaromadendrene (traces–33.20%), α-acoradiene (traces–9.41%), and γ-muurolene (0.61–16.33%) being the most abundant constituents. Noteworthy is the occurrence in a sample of two major unknown sesquiterpenes, one hydrocarbon (24.49%, *RI:* 1396) and one oxygenated compound (10.37%, *RI:* 1591), whose mass spectra were reported for the first time. Multivariate chemometric techniques, such as cluster analysis (CA) and principal component analysis (PCA), were used to characterize the samples according to the geographical origin.

Introduction. – The genus *Ephedra* L. is the only one belonging to the taxonomically isolated Ephedraceae family, within the Gnetales order, which is the closest living relative of the Angiosperm [1]; it comprises 40 species scattered in semi-arid regions of the Mediterranean area, Asia, and America [2]. These species are evergreen, perennial, and dioecious shrubby plants that can reach 1 m in height, with slender and joined stems. Less often, they include also lianas, creepers, and, rarely, small trees. Their leaves are reduced to sheaths and grow in opposite pairs of triplex whorls. Many species of this genus have been used as medicinal remedies. In particular, *E. sinica* Stapf. is used in China since 5000 years as stimulant and antiasthmatic, due to its ephedrine-alkaloids content [3].

In Italy, the genus *Ephedra* is represented by six species: *E. distachya* L. subsp. *distachya*, *E. fragilis* Desf., *E. major*, *E. helvetica* C. A. Mey, *E. negrii* J. Nouviant, and *E. nebrodensis* Guss. subsp. *nebrodensis* [4].

In particular, the last one occurs in Sardinia, Sicily, and Southern and Central Italy on rocky places, up to 1400 m above the sea level (*Fig. 1*). Owing to the human activities, its habitat is reducing; therefore, large populations are currently rare.

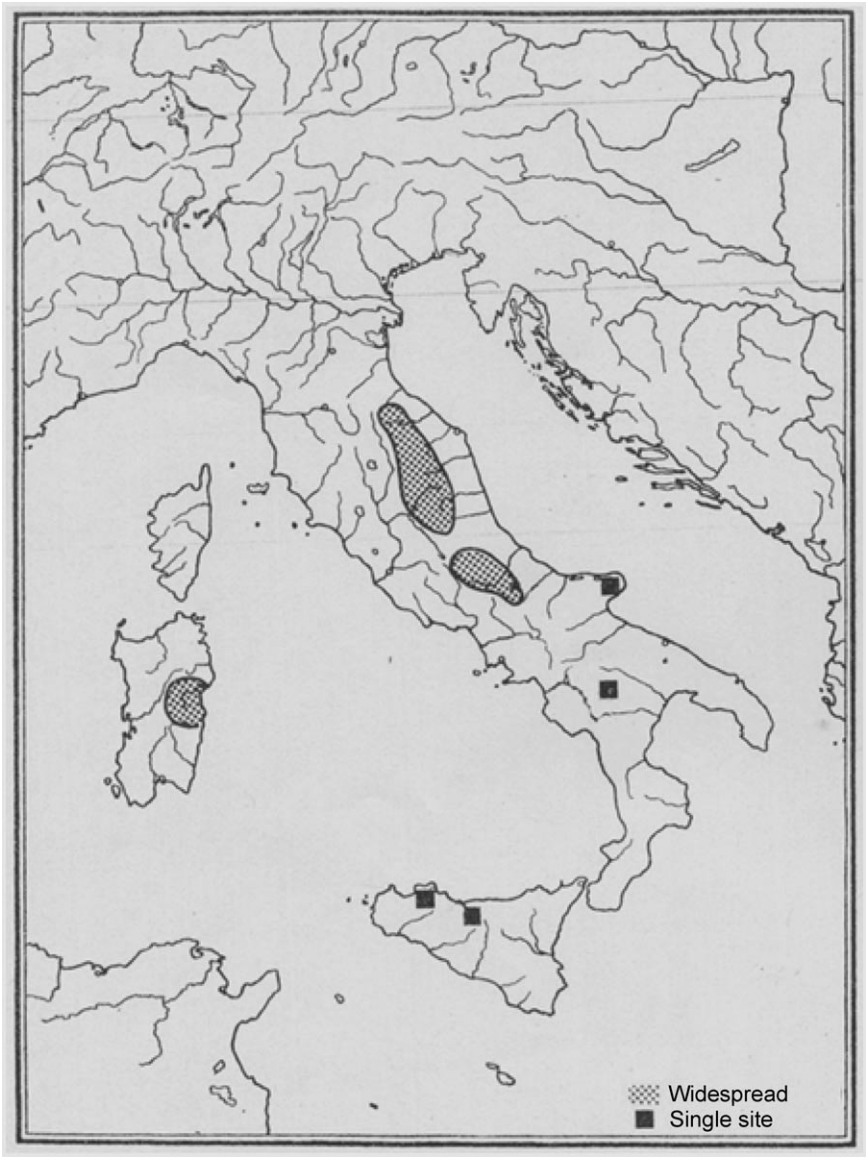


Fig. 1. *Distribution of* Ephedra nebrodensis *in Italy* (taken from [5])

E. nebrodensis was confused in the past with *E. major* HOST (syn. *E. foeminea* FORSSK.) [5], but nowadays it is considered to be taxonomically different [4]: it differs morphologically with the reddish or blackish-brown pith of older twigs, for the male

inflorescences with 2–4 pairs of flowers, and for the female inflorescences with one flower [2].

Besides ephedrine alkaloids, *Ephedra* species contain also volatile compounds [6–8] that may be useful as chemotaxonomic markers. To the best of our knowledge, volatile fractions of the Italian *Ephedra* species have not been exhaustively investigated.

There has been only one study related to the components of the essential oils of *E. distachya* L., *E. fragilis* Desf., and *E. major* Host [9]. In particular, the main components of the oil of *E. major* were eugenol (4.3%), α-terpineol (3.7%), and methyl linoleate (3.5%), while the most abundant group was represented by oxygenated monoterpenes (28%). *E. nebrodensis* subsp. *nebrodensis* was recently investigated by our group for essential-oil composition, and the oxygenated monoterpene citronellol (29.7%) was the most abundant component [10].

Solid-phase microextraction (SPME) has never been applied in the analysis of volatile components of *Ephedra* species. SPME is a non-destructive and non-invasive method [11], enabling the use of considerably smaller amounts of plant material than in other extraction techniques; the sampling time is much shorter, minimizing the possibility of sample contamination; the absence of solvents prevents the loss of volatiles during the concentration of the extractive solutions; the low temperatures normally used during sampling prevent chemical changes in the natural flavor pattern and the formation of artefacts; and the higher concentration capability of this technique permits the identification of many compounds [12].

The aim of this work was to apply for the first time a SPME analysis to *E. nebrodensis* subsp. *nebrodensis* Italian populations (*Table 1*) that may be used to further support the botanical classification of the species, to gain knowledge of the chemical differences between populations, and to find out possible sources of pharmaceutical and cosmetic products.

Table 1. *Geographic and Botanical Informations about the* Ephedra nebrodensis *subsp.* nebrodensis *Populations Investigated*

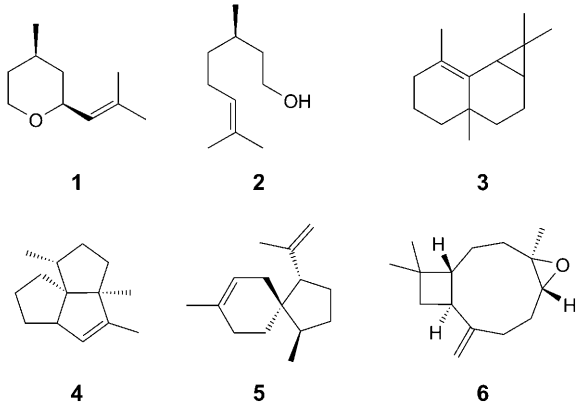
Sample	Locality	Region of collection	Date of collection	GPS Coordinates	Altitude [m]	Plant material [g]	Voucher codes[a])
1	Visso, Val Nerina	Marche	09/09/2007	42°56′06″ N 13°05′43″ E	721	5.163	CAME 23633
2	Camerino, Madonna di Val Povera	Marche	05/06/2007	43°06′33″ N 13°00′06″ E	851	0.933	CAME 9586
3	Forca di Penne, Monte Scarafano	Abruzzo	16/05/2008	42°17′34″ N 13°49′59″ E	1100	5.083	APP 37431
4	Ofena, Monte la Serra	Abruzzo	16/05/2008	42°19′42″ N 13°45′48″ E	620	0.763	APP 25033
5	Orgosolo	Sardinia	02/05/2007	40°11′54″ N 9°20′47″ E	692	0.493	CAGL
6	Gola Gorropu	Sardinia	02/05/2007	40°10′59″ N 9°29′59″ E	621	0.783	CAGL

[a]) Accession number in: CAME, Herbarium Camerinensis, School of Environmental Sciences, University of Camerino, Camerino, Italy; APP, Herbarium of Centro Ricerche Floristiche dell'Appennino, Barisciano, Italy; CAGL, Herbarium of University of Cagliari, accession numbers are unknown.

Results and Discussion. – To the best of our knowledge, this is the first investigation in which headspace (HS)-SPME coupled with GC/FID (flame ionization detector) and GC/MS was applied in the analysis of the volatile fraction of an *Ephedra* species. The previously published data all referred to the chemical composition of essential oils obtained from Asian and European *Ephedra* samples by hydrodistillation [6][7][9][10].

1. *Fiber Screening.* Three fibers, *i.e.*, polydimethylsiloxane (PDMS; 100 μm), *Carboxen*™/polydimethylsiloxane (CAR/PDMS; 75 μm), and polydimethylsiloxane/divinylbenzene (PDMS/DVB; 65 μm), were evaluated for the analysis of the volatiles of *E. nebrodensis* subsp. *nebrodensis.* The fiber screening was conducted on *Sample 1* (Visso) under the following experimental conditions: extraction temperature, 60°; extraction time, 30 min; particle size, 1 mm; amount of plant material, 30 mg; desorption time, 3 min. It was based on sensitivity and reproducibility for six selected marker compounds (representing various chemical classes with different chromatographic behaviors) and total volatiles.

The CAR/PDMS fiber achieved an almost three and ten times higher extraction efficiency than the PDMS and DVB/PDMS fibers, respectively (*Fig. 2*). The reproducibility of the extraction as a function of the fiber coating was evaluated by performing the analyses in triplicate and calculation of the relative standard deviation (RSD). RSD Values of the individual peak areas of the marker compounds **1**–**6** and the total peak areas are compiled in *Table 2*.



Analyses with PDMS and DVB/PDMS coatings provided a good reproducibility with RSD values lower than with CAR/PDMS coating that gave rise to RSD values up to 33.4%. Running blank samples between analyses revealed residual compounds on the CAR/PDMS fiber. This probably accounted for the observed higher RSD values. Although the CAR/PDMS fiber has a porous, bipolar coating that gives rise to higher relative signal responses of low-molecular-mass volatiles (C_2–C_{12}), larger molecules ($>C_{12}$) were retained on the surface of the CAR particle and imbedded in the coating, therefore difficult to desorb [13]. Considering the retention capability, reproducibility, and avoiding time-consuming extra cleaning steps that are time-consuming, before sampling, the PDMS fiber was judged suitable for the aim of the study. Although

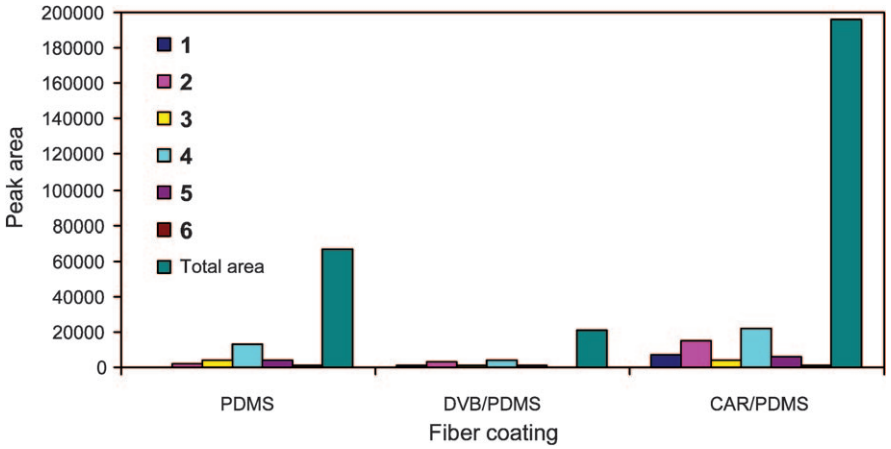


Fig. 2. *Uptake of marker compounds and total volatiles of* E. nebrodensis *subsp.* nebrodensis *by three types of SPME fiber coating under the following analytical conditions: extraction temperature, 60°; extraction time, 30 min; particle size, 1 mm; sample amount, 30 mg; desorption time, 3 min.* Data obtained by GC/FID analysis. Peak identification: **1**, *cis*-rose oxide; **2**, citronellol; **3**, β-maaliene; **4**, α-isocomene; **5**, α-acoradiene; **6**, caryophyllene oxide.

PDMS is an apolar coating, it can be applied successfully also to more polar compounds, particularly after optimizing the extraction conditions [12].

2. *Optimization of SPME Parameters.* Both the peak area of selected marker compounds and the total area of all volatiles were considered to evaluate the influence of the SPME parameters such as temperature, extraction time, sample amount, and H_2O addition on the extraction efficiency of PDMS fiber. The extraction temperature significantly affects the extraction efficiency, because the vapor pressure influences the distribution coefficients of the volatiles between both the plant matrix and the headspace, and between the headspace and the fiber.

Fig. 3,*a*, shows the effect of different temperatures (20, 40, 60, and 80°) on the extraction of marker compounds and total volatiles, keeping the other parameters as follows: extraction time, 30 min; amount of plant material, 30 mg; particle size, 1 mm; desorption time, 3 min. Markers **2** and **6** could not be detected at 20°. It was found that, except for marker **1**, all peak areas increased steadily from 20° to 60°. The peak areas of markers **2** and **6** increased from 60° to 80°, whereas the peak area of markers **1**, **5**, **3**, and **4** decreased. The total peak area was observed to remain approximately constant comparing extraction temperatures of 60° and 80°. From *Table 2*, it can be clearly seen that extraction at 60° gave very good RSD values. For this reason, this temperature was set up for the evaluation of the other extraction parameters. The results of the extraction of marker compounds and total volatiles varying with the extraction time (10, 20, 30, and 60 min) are shown in *Fig. 3*,*b*. The other parameters were set as: temperature, 60°; amount of plant material, 30 mg; particle size, 1 mm; desorption time, 3 min. A steadily increasing tendency can be observed from 10 to 30 min. Extension of the extraction time to 60 min can still slightly improve the extraction efficiency, but the increase in peak areas is too small to push for doubling the extraction

Table 2. *Relative Standard Deviation* (RSD%) *Values* ($n=3$) *Obtained for Marker Compounds* **1**–**6**, *and Total Volatiles of* E. nebrodensis *subsp.* nebrodensis *During the Fiber Screening and Optimization of the Extraction Parameters*[a])

Fiber coating	Marker compounds[b])						Total peak area
	1	**2**	**3**	**4**	**5**	**6**	
PDMS	20.1	4.9	2.7	1.9	1.2	7.5	5.9
DVB/PDMS	3.0	3.9	0.4	7.5	1.3	10.1	6.2
CAR/PDMS	14.9	27.0	18.1	15.2	13.3	33.4	18.5
Optimization of extraction parameters							
Temperature [°]							
20	37.4	–[c])	9.9	9.4	15.2	–[c])	13.0
40	51.4	87.5	17.0	16.1	7.8	105.9	16.0
60	20.1	4.9	2.7	1.9	1.2	7.5	5.9
80	173.2	40.0	22.4	23.6	23.7	31.6	28.4
Extraction time [min]							
10	13.4	58.4	1.9	2.2	1.0	54.2	8.0
20	12.4	11.7	13.4	13.7	13.5	23.1	15.4
30	20.1	4.9	2.7	1.9	1.2	3.1	5.9
60	20.3	10.6	6.1	6.0	5.6	9.3	4.7
Sample amount [mg]							
10	26.8	19.2	4.3	5.6	3.9	17.4	5.8
30	20.1	4.9	2.7	1.9	1.2	7.5	5.9
60	41.2	28.2	11.8	12.0	10.2	11.0	5.6
Added H_2O [µl]							
0	20.1	4.9	2.7	1.9	1.2	7.5	5.9
20	32.7	39.1	20.0	18.0	17.8	29.1	23.7
40	26.1	15.3	23.2	25.5	14.3	32.0	19.5
60	51.3	47.0	33.5	20.3	18.8	40.5	34.3

[a]) Extraction conditions under which each parameter was optimized are reported in *Figs. 2* and *3*. [b]) Marker compounds: **1**, *cis*-rose oxide; **2**, citronellol; **3**, β-maaliene; **4**, α-isocomene; **5**, α-acoradiene; **6**, caryophyllene oxide. [c]) No data available due to absence of the marker compound.

time. Therefore, 30 min were considered suitable for reaching the equilibrium between the stationary phase (fiber), the headspace of the vial, and the extracted analytes. The RSD values for compounds extracted during 20, 30, and 60 min revealed sufficient reproducibility, with RSD values generally not exceeding 20% (*Fig. 2*). Taking into consideration the reproducibility of the method, the extraction efficiency, and the total time of analysis, an extraction time of 30 min was chosen for further analyses. *Fig. 3*, *c*, visualizes the influence of the sample amount on the extraction efficiency, keeping the other parameters as follows: temperature, 60°; extraction time, 30 min; particle size, 1 mm; desorption time, 3 min. Generally, a maximal GC/FID response can be observed after extraction of 30 mg of plant material. Peak areas representing compounds **1** and **6**, the most and least volatile compound selected as marker, respectively, displayed a slightly aberrant trend, both reaching maximal observed peak area after extraction of 60 mg of plant material. The RSD values in *Table 2* show that reproducibility of the method is not highly affected by varying the extracted sample amount for compounds **3**,

4, **5**, and **6**, and for the total area of all peaks. Still considerably worse RSD values were obtained with compounds **1** and **2** with a sample amount of 30 mg. Taking into account the above data and the small amount of sample material available, 30 mg of plant material were chosen to perform the extractions on. Finally, we considered to add H_2O to the dry plant material, as H_2O has previously proven to facilitate release of analytes from the matrix [14]. The influence of the addition of H_2O to the dry sample appeared to be unpredictable, as no similar pattern (*Fig. 3*, *d*) could be revealed for all marker compounds. On the one hand, a clear negative effect on the peak areas was observed for marker compounds **3** and **4**. On the other hand, the peak areas of markers **1**, **2**, and **5** were fluctuating as a function of the amount of added H_2O, while marker **6** seems to be independent of it. In accordance with the RSD values of the experiments (*Table 2*), the total peak area was chosen as the most important parameter, thus, further experiments were conducted without adding H_2O, as this gave rise to a maximal GC/FID response for the total peak area. In the case of *E. nebrodensis*, the addition of H_2O to the plant matrix generally introduced a lower amount of extracted volatiles and a lower repeatability.

3. *Headspace Volatiles Profile*. The headspace volatiles of six Italian populations of *E. nebrodensis* subsp. *nebrodensis* are compiled in *Table 3*. A total of 119 volatiles were identified in the different samples, accounting for 63.35–100.00% of the total volatiles. *Sample 1* (Visso) was the richest, with 80 identified components, whilst *Sample 3* (Forca di Penne) the poorest, with 46 identified components. A great variability was found in the qualitative composition of the headspace of different samples, since only 18 components (five aldehydes, one ketone, three alkanes, one aromatic and two oxygenated monoterpenes, five sesquiterpene hydrocarbons, and one norisoprenoid) were in common among all populations. However, in all cases, volatile fraction was dominated by sesquiterpene hydrocarbons (52.30–88.32%; *Fig. 4*), with β-maaliene (**3**; traces–7.49%), β-patchoulene (**7**; traces–11.29%), β-panasinsene (**8**; traces–6.85%), α-isocomene (**4**; traces–31.25%), α-*trans*-bergamotene (**9**; traces–6.95%), alloaromadendrene (**10**; traces–33.20%), α-acoradiene (**5**; traces–9.41%), γ-muurolene (**11**; 0.61–16.33%) being the most abundant components. Their chemical structures are given in *Fig. 5*.

Minor contributions were provided by aromatics (2.53–6.18%) and oxygenated monoterpenes (3.06–7.51%) in *Samples 1* (Visso) and *2* (Camerino), alkanes (15.40%) and norisoprenoids (10.34%) in *Sample 3* (Forca di Penne), and oxygenated sesquiterpenes (12.36%) in *Sample 4* (Monte la Serra). The most abundant representatives of these classes were citronellol (**2**; 2.71–4.22%), thymol methyl ether (1.68–2.82%), pentadecane (5.84%), and heptadecane (6.37%), and (*E*)-β-ionone (8.14%), respectively.

Extracted from *Leontopodium alpinum* Cass. (*Asteraceae*), α-isocomene (**4**) previously proved to be able to increase significantly the extracellular level of acetylcholine and amplify cholinergic transmission in the brain of rats. Consequently, this compound may have potential as antidementia agent in brain diseases caused by cholinergic deficiency [17]. Isolated from spikenard (*Nardostachys grandiflora* DC., Valerianaceae), β-maaliene (**3**) was reported, in the context of aromatherapy, to act as sedative agent after inhalation [18]. The norisoprenoid (*E*)-β-ionone was stated to dispose of chemopreventive and antitumor activities [19]. Furthermore, (*E*)-β-ionone

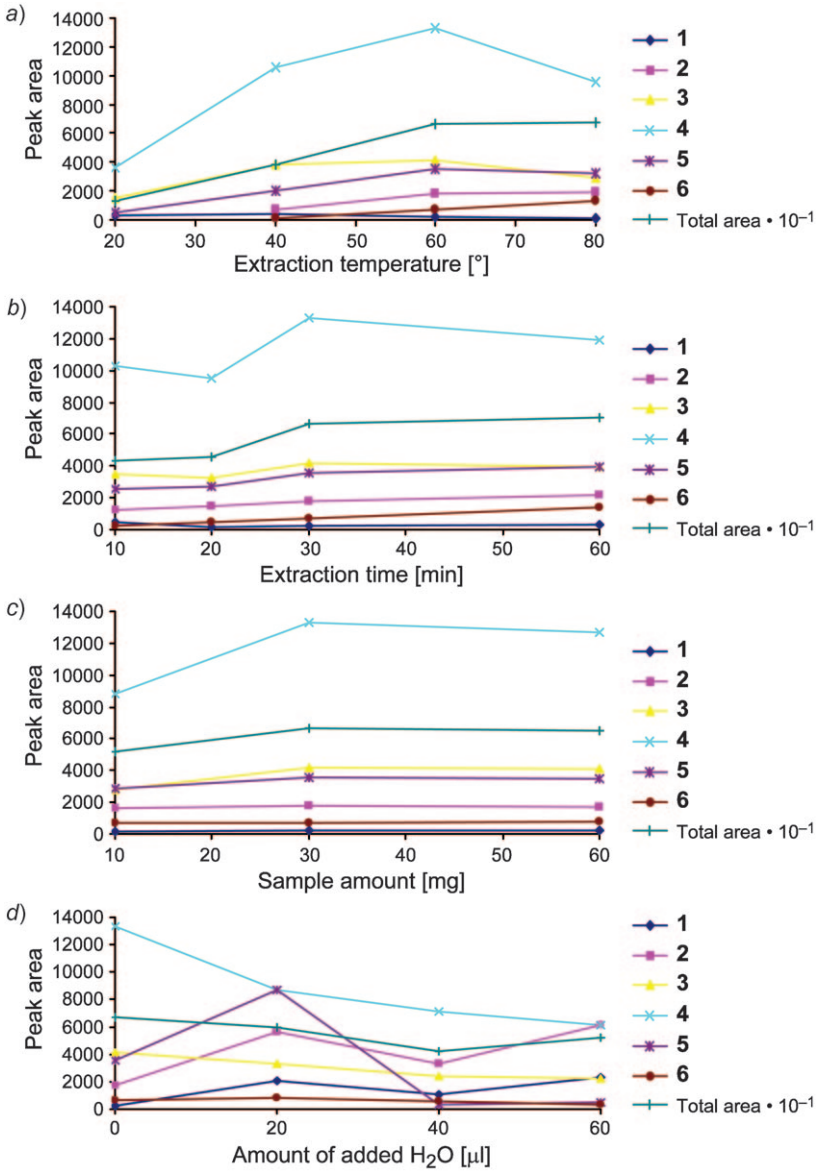


Fig. 3. *Effect of temperature* (*a*), *extraction time* (*b*), *sample amount* (*c*), *and* H_2O *addition* (*d*) *on the peak area of the marker compounds and total volatiles of* E. nebrodensis *subsp.* nebrodensis (*Sample 1*) *captured by the PDMS fibre. a*) Extraction time, 30 min; no added H_2O; particle size, 1 mm; sample amount, 30 mg; desorption time, 3 min; *b*) temperature, 60°; no added H_2O; particle size, 1 mm; sample amount, 30 mg; desorption time, 3 min; *c*) temperature, 60°; extraction time, 30 min; no added H_2O; particle size, 1 mm; desorption time, 3 min; *d*) temperature, 60°; extraction time, 30 min; sample amount, 30 mg; desorption time, 3 min. All the experiments were performed in triplicate. Peak identification: **1**, *cis*-rose oxide; **2**, citronellol; **3**, *β*-maaliene; **4**, *α*-isocomene; **5**, *α*-acoradiene; **6**, caryophyllene oxide. The total peak areas are represented on the graphs divided by ten to improve readability.

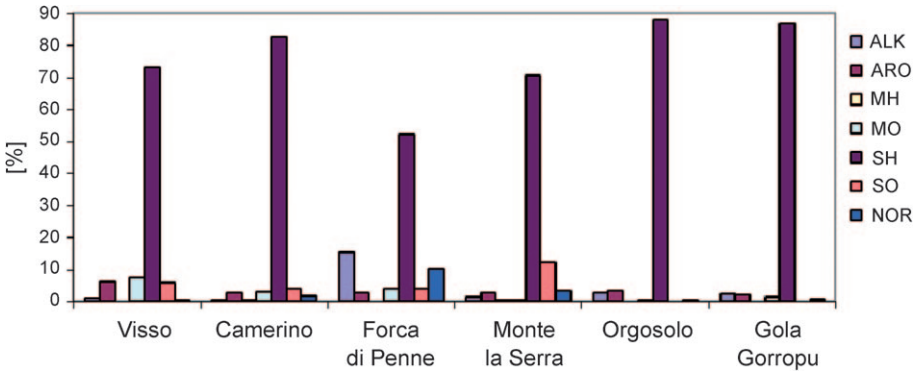


Fig. 4. *Percentages of the main classes of volatiles in the headspace of* E. nebrodensis *subsp.* nebrodensis. MH, monoterpene hydrocarbons; ALK, alkanes; ARO, aromatics; MO, oxygenated monoterpenes; SH, sesquiterpene hydrocarbons; SO, oxygenated sesquiterpenes; NOR, norisoprenoids.

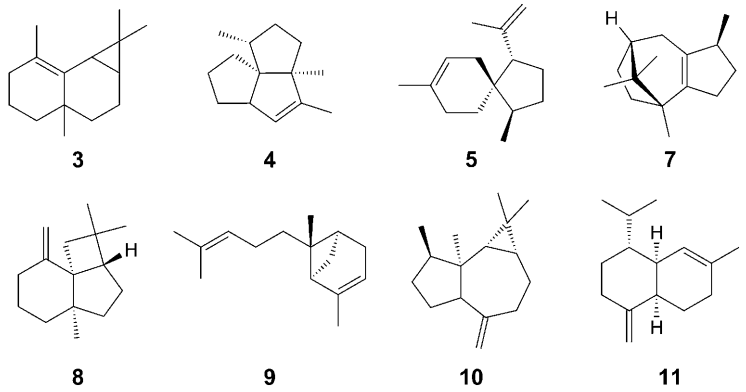


Fig. 5. *Structures of major compounds identified in the headspace of* E. nebrodensis *subsp.* nebrodensis.

is one of the main contributors to the aroma of roses. Also noteworthy is the presence of several other volatiles useful as perfuming agents in cosmetics and pharmaceutical preparations. These include *cis*-rose-oxide (**1**), citronellol (**2**), β-patchoulene (**7**), heptadecane, and pentadecane.

Identification of at least 96% of the total detected area was accomplished for five of the six samples. In spite of our attempts, the *Sample 4* originating from Monte la Serra was only identified for 63.4%. The remaining area to be identified is almost exclusively constituted by only two unknown compounds that were also found in traces in the *Sample 2* from Camerino: a hydrocarbon sesquiterpene (24.49%, *RI:* 1396) and an oxygenated sesquiterpene (10.37%, *RI:* 1591), whose mass spectra, lacking in the MS commercial libraries used, are reported in *Fig. 6*.

To interpret the obtained SPME/GC/FID data of six populations of *E. nebrodensis* subsp. *nebrodensis* on a statistical basis, they were subjected to hierarchical cluster cluster analysis (CA) and principal-component analysis (PCA). The UPGMA method

Table 3. *Volatile Compounds Identified in* E. nebrodensis *subsp.* nebrodensis *after HS-SPME Using the PDMS Fiber*

Entry	Component[a])	*RI*[b])	*RI* from literature		*Samples*[c])						ID[d])
			Adams[e])	NIST 08[f])	*1*	*2*	*3*	*4*	*5*	*6*	
1	Hexanal	797	802	799	tr.[g])	0.1±0.0	tr.	0.2±0.0	tr.	tr.	Std
2	(*E*)-Hex-2-enal	850	855	847	–	tr.	–	–	–	–	MS, *RI*
3	Heptanal	903	902	903	–	0.1±0.0	tr.	tr.	tr.	tr.	MS, *RI*
4	6-Methylheptan-2-one	955	955	955	0.6±0.3	0.3±0.1	tr.	tr.	0.3±0.1	0.1±0.0	MS, *RI*
5	Benzaldehyde	962	960	959	tr.	0.1±0.0	1.2±0.7	0.2±0.1	–	tr.	Std
6	6-Methylhept-5-en-2-one	989	985		tr.	tr.	–	–	–	–	MS, *RI*
7	Octanal	1005	998	1006	–	tr.	–	–	–	–	Std
8	*p*-Cymene	1024	1024	1025	tr.	0.1±0.0	–	0.2±0.0	–	–	Std
9	(*E*)-Oct-2-enal	1059	1054		tr.	tr.	–	–	–	–	MS, *RI*
10	Undecane	1099	1100	1100	tr.	–	–	–	–	–	Std
11	Nonanal	1104	1100	1105	tr.	0.3±0.1	2.8±0.3	0.3±0.0	0.4±0.1	0.2±0.0	MS, *RI*
12	2,6-Dimethylcyclohexanol	1107	1100	1110	tr.	–	–	–	–	–	MS, *RI*
13	*cis*-Rose oxide (**1**)	1110	1106	1111	0.5±0.1	0.3±0.0	tr.	0.1±0.0	tr.	0.1±0.0	MS, *RI*
14	*trans*-Rose oxide	1126	1127	1127	0.3±0.0	tr.	–	–	–	–	MS, *RI*
15	4-Ketoisophorone	1144		1152	tr.	0.2±0.0	2.3±0.3	0.4±0.1	–	0.8±0.0	MS, *RI*
16	Citronellal	1153	1153	1153	tr.	–	–	–	–	–	MS, *RI*
17	Ethyl benzoate	1170	1173	1170	–	tr.	tr.	tr.	tr.	tr.	MS, *RI*
18	α-Terpineol	1189	1188	1189	–	tr.	–	–	–	–	Std
19	Methyl salicylate	1192	1191	1191	0.1±0.0	–	–	–	–	–	MS, *RI*
20	Safranal	1196	1196	1197	tr.	–	–	–	–	–	MS, *RI*
21	Dodecane	1198	1200	1200	0.1±0.0	–	tr.	tr.	–	tr.	Std
22	Decanal	1203	1201	1203	tr.	tr.	tr.	0.2±0.0	0.2±0.1	0.3±0.1	MS, *RI*
23	α-Citronellol	1216		1214	tr.	–	–	–	–	–	MS, *RI*
24	β-Cyclocitral	1218	1219		0.1±0.0	0.1±0.0	tr.	0.1±0.0	–	–	MS, *RI*
25	Citronellol (**2**)	1226	1225	1227	4.2±0.2	2.7±0.2	–	0.3±0.0	0.4±0.0	1.4±0.2	Std
26	Thymol methyl ether	1233	1235	1233	2.8±0.3	1.7±0.1	tr.	1.1±0.1	1.5±0.2	0.5±0.0	MS, *RI*
27	Citronellyl formate	1273	1271	1275	tr.	tr.	–	–	–	–	MS, *RI*
28	Safrole	1285	1287	1287	–	–	–	–	tr.	0.1±0.0	MS, *RI*
29	Tridecane	1298	1300	1300	tr.	tr.	–	tr.	tr.	tr.	Std
30	Undecanal	1304	1306	1305	tr.	tr.	tr.	tr.	tr.	0.2±0.0	MS, *RI*
31	(*Z*)-Hex-3-enyl tiglate	1322	1321		tr.	0.6±0.0	–	0.6±0.1	–	–	MS, *RI*

Table 3 (cont.)

Entry	Component[a])	*RI*[b])	*RI* from literature		*Samples*[c])						ID[d])
			Adams[e])	NIST 08[f])	*1*	*2*	*3*	*4*	*5*	*6*	
32	(6*S*)-2,3,8,8-Tetramethyltricyclo-[5.2.2.0^{1,6}]undec-2-ene	1327			0.6±0.1	–	–	0.6±0.0	–	–	MS
33	β-Clovene	1345			0.7±0.0	–	–	0.3±0.0	1.1±0.1	1.1±0.0	MS
34	α-Longipinene	1347	1352	1348	–	0.5±0.0	–	–	–	–	Std
35	Citronellyl acetate	1351	1352	1351	0.3±0.0	–	–	–	–	–	MS, *RI*
36	Cyclosativene	1361	1374	1363	–	0.2±0.1					MS, *RI*
37	β-Maaliene (**3**)	1363		1359	7.3±0.2	–	2.0±0.5	2.0±0.0	6.9±0.1	7.5±0.2	MS, *RI*
38	α-Ylangene	1368	1375	1368	–	7.1±0.1	–	–	–	–	MS, *RI*
39	α-Copaene	1372	1376	1372	–	0.5±0.0	0.2±0.0	0.8±0.0	–	1.9±0.1	Std
40	Isoledene	1375	1376	1373	–	1.2±0.2	–	–	–	–	MS, *RI*
41	β-Panasinsene (**8**)	1376	1382		4.5±0.1	–	2.3±0.2	1.9±0.0	6.9±0.1	7.3±0.1	MS, *RI*
42	2-Epi-α-funebrene	1377	1382	1386	–	tr.	–	–	–	–	MS, *RI*
43	β-Patchoulene (**7**)	1378	1379		11.3±0.2	–	4.3±0.3	2.4±0.0	9.4±0.1	7.8±0.1	MS, *RI*
44	Sativene	1386	1391	1396	0.1±0.0	0.3±0.0	–	–	–	–	MS, *RI*
45	α-Isocomene (**4**)	1387	1388		22.8±0.3	–	8.2±0.3	6.9±0.1	31.2±0.2	24.5±0.3	MS, *RI*
46	Longiborn-8-ene	1391			3.5±0.3	–	tr.	–	3.9±0.1	4.1±0.3	MS
47	Unknown sesquiterpene hydrocarbon[h])	1396			–	tr.	–	24.5±0.4	–	–	
48	Tetradecane	1398	1400	1400	–	–	0.8±0.1	–	–	–	Std
49	α-Chamipinene	1397	1396		tr.	0.7±0.0	–	–	–	–	MS, *RI*
50	Longifolene	1400	1407	1400	1.9±0.0	0.4±0.1	–	0.9±0.0	1.7±0.1	1.8±0.2	Std
51	Dodecanal	1403	1408	1405	tr.	–	1.0±0.1	3.2±0.1	tr.	1.6±0.1	MS, *RI*
52	α-Gurjunene	1405	1409	1404	–	1.8±0.1	–	–	–	–	Std
53	α-Cedrene	1407	1414	1408	1.1±0.0	0.4±0.1	2.7±0.1	–	0.1±0.0	0.2±0.0	Std
54	Acora-3,7(14)-diene	1410			–	0.4±0.1	–	–	–	–	MS
55	1,7-Dimethylnaphthalene	1415	1418	1419	2.5±0.0	–	1.4±0.1	–	1.6±0.1	–	MS, *RI*
56	(*E*)-Caryophyllene	1415	1419	1415	1.6±0.0	2.8±0.0	–	5.2±0.3	1.0±0.0	4.7±0.2	Std
57	β-Copaene	1423	1432		–	0.4±0.1	–	tr.	–	–	MS, *RI*
58	α-*trans*-Bergamotene (**9**)	1432	1434	1433	0.9±0.0	0.3±0.0	7.0±0.2	tr.	1.8±0.0	0.2±0.0	MS, *RI*
59	Coumarin	1434	1432	1432	tr.	–	tr.	0.5±0.0	–	0.3±0.0	Std
60	3,3,7,11-Tetramethyltricyclo-[6.3.0.0^{2,4}]undec-8-ene	1437		1440	–	2.1±0.0	–	0.9±0.0	–	–	MS, *RI*

Table 3 (cont.)

Entry	Component[a])	*RI*[b])	*RI* from literature		*Samples*[c])						ID[d])
			Adams[e])	NIST 08[f])	*1*	*2*	*3*	*4*	*5*	*6*	
61	α-Himachalene	1447	1451		0.8±0.0	3.7±0.1	–	–	4.9±0.0	4.6±0.2	MS, *RI*
62	α-Neoclovene	1447	1454	1451	2.3±0.1	–	1.5±0.1	2.1±0.1	–	–	MS, *RI*
63	α-Humulene	1447	1454	1447	–	–	–	–	1.0±0.0	2.0±0.1	Std
64	Geranyl acetone	1447	1455	1446	2.1±0.0	–	4.1±0.4	0.2±0.0	–	–	MS, *RI*
65	Alloaromadendrene (**10**)	1458	1460	1458	0.7±0.0	33.2±1.1	–	15.7±0.3	–	–	Std
66	α-Acoradiene (**5**)	1462	1466	1459	6.2±0.0	0.8±0.0	–	–	9.4±0.1	8.6±0.3	MS, *RI*
67	2,6-Di(*tert*-butyl)quinone	1464	1472	1458	–	–	2.7±0.2	1.8±0.0	–	–	MS, *RI*
68	(*E*)-β-Farnesene	1465	1456		–	–	–	–	1.2±0.0	–	MS, *RI*
69	β-Neoclovene	1468	1475		tr.	–	–	0.4±0.0	0.9±0.1	1.0±0.1	MS, *RI*
70	β-Acoradiene	1471	1470	1483	2.5±0.0	1.3±0.0	–	–	–	–	MS, *RI*
71	γ-Selinene	1474		1473	–	–	–	0.5±0.1	–	1.0±0.1	MS, *RI*
72	γ-Muurolene (**11**)	1474	1479		0.6±0.0	3.9±0.1	16.3±1.0	1.1±0.1	2.2±0.0	1.0±0.1	MS, *RI*
73	α-Amorphene	1478	1484		–	3.3±0.1	–	0.4±0.0	–	–	MS, *RI*
74	(11α*H*)-Himachala-1,4-diene	1479	1486		0.7±0.0	–	–	0.1±0.0	0.9±0.0	0.2±0.2	MS, *RI*
75	(*E*)-β-Ionone	1483	1487		3.2±0.2	1.5±0.2	8.1±0.5	2.0±0.1	0.5±0.0	0.8±0.0	Std
76	β-Selinene	1483	1490	1483	–	–	–	0.7±0.0	–	0.8±0.0	MS, *RI*
77	δ-Selinene	1488	1492		–	–	–	–	–	tr.	MS, *RI*
78	10,11-Epoxycalamenene	1489	1492		0.8±0.2	0.2±0.1	–	–	tr.	–	MS, *RI*
79	Viridiflorene	1492	1496	1493	–	2.4±0.3	–	–	–	–	MS, *RI*
80	α-Selinene	1492	1498		0.4±0.1	–	–	1.6±0.0	–	1.3±0.5	MS, *RI*
81	Pentadecane	1495	1500		tr.	–	5.8±2.0	–	1.5±0.8	0.8±0.0	Std
82	α-Muurolene	1498	1500		–	3.2±0.1	0.6±0.1	0.6±0.0	–	1.8±0.0	MS, *RI*
83	β-Himachalene	1498	1500	1499	tr.	–	–	–	1.7±0.1	–	MS, *RI*
84	Tridecanal	1506	1510	1510	–	0.4±0.1	2.2±0.3	0.5±0.0	0.2±0.0	tr.	MS, *RI*
85	δ-Amorphene	1504	1512		–	0.3±0.1	–	–	tr.	–	MS, *RI*
86	γ-Cadinene	1511	1513		–	0.7±0.0	–	tr.	–	0.2±0.0	MS, *RI*
87	α-Dehydro-*ar*-himachalene	1511	1517		0.2±0.1	0.3±0.0	–	–	tr.	–	MS, *RI*
88	*trans*-Cycloisolongifol-5-ol	1517	1513		–	tr.	–	–	–	–	MS, *RI*
89	*trans*-Calamenene	1520	1522	1520	0.6±0.2	0.1±0.0	2.1±0.6	0.9±0.0	0.4±0.1	2.2±0.1	MS, *RI*
90	Zonarene	1520	1529		–	–	–	–	–	0.2±0.0	MS, *RI*
91	δ-Cadinene	1521	1523		–	2.8±0.1	4.2±1.2	0.1±0.0	–	–	MS, *RI*

Table 3 (cont.)

Entry	Component[a])	*RI*[b])	*RI* from literature		*Samples*[c])						ID[d])
			Adams[e])	NIST 08[f])	*1*	*2*	*3*	*4*	*5*	*6*	
92	γ-Dehydro-ar-himachalene	1527	1532		tr.	0.4±0.1	–	–	0.1±0.0	tr.	MS, *RI*
93	Dihydroactinidiolide	1527		1525	0.3±0.0	0.1±0.0	2.2±0.1	1.3±0.2	–	tr.	MS, *RI*
94	α-Cadinene	1535	1538		0.5±0.1	1.1±0.1	–	tr.	–	–	MS, *RI*
95	α-Calacorene	1540	1545		0.8±0.0	5.0±0.1	tr.	tr.	0.6±0.0	0.3±0.1	MS, *RI*
96	β-Calacorene	1560	1565		tr.	0.4±0.0	–	–	tr.	–	MS, *RI*
97	Palustrol	1566	1568	1567	–	tr.	–	–	–	–	MS, *RI*
98	(*Z*)-Hex-3-enyl benzoate	1566	1566		tr.	0.2±0.0	tr.	0.8±0.2	0.1±0.0	1.0±0.1	MS, *RI*
99	Hexyl benzoate	1572	1580	1576	tr.	0.6±0.1	–	tr.	–	–	MS, *RI*
100	Caryophyllene oxide (**6**)	1580	1583	1580	1.4±0.1	1.3±0.5	–	2.0±0.2	–	–	Std
101	Unknown oxygenated sesquiterpene[i])	1591			–	tr.	–	10.4±0.4	–	–	
102	Hexadec-1-ene	1583		1587	tr.	–	–	–	–	–	MS, *RI*
103	Cubeban-11-ol	1590	1595		–	1.3±0.2	–	–	–	–	MS, *RI*
104	Hexadecane	1593	1600		–	tr.	1.4±0.3	0.7±0.0	tr.	0.1±0.0	MS, *RI*
105	Tetradecanal	1606	1612	1606	0.7±0.2	tr.	0.3±0.1	0.1±0.0	0.1±0.0	0.8±0.0	MS, *RI*
106	β-Himachalene oxide	1609	1616		0.5±0.1	1.0±0.1	–	–	–	–	MS, *RI*
107	α-Corocalene	1618	1623		0.3±0.0	0.5±0.0	–	–	0.5±0.0	–	MS, *RI*
108	α-Muurolol	1643	1646	1643	tr.	–	3.8±0.6	–	–	–	MS, *RI*
109	*cis*-Methyl dihydrojasmonate	1651	1655		tr.	–	–	–	–	–	MS, *RI*
110	Hexadec-1-yne	1665		1664	tr.	–	–	–	–	–	MS, *RI*
111	(*Z*)-Trideca-1,6-diene	1665			–	–	–	–	0.4±0.0	–	MS
112	Cadalene	1674	1676	1674	0.8±0.1	0.6±0.1	0.9±0.4	tr.	0.4±0.0	0.9±0.1	MS, *RI*
113	Heptadecane	1695	1700		0.1±0.0	tr.	6.4±0.8	0.6±0.0	0.8±0.2	1.1±0.2	Std
114	Octadecane	1799	1800		0.3±0.1	0.5±0.2	0.5±0.2	0.1±0.0	0.1±0.0	0.2±0.0	Std
115	Isopropyl myristate	1826		1827	0.4±0.2	–	–	–	–	–	MS, *RI*
116	6,10,14-Trimethylpentadecan-2-one	1845		1845	0.8±0.3	–	–	–	–	–	MS, *RI*
117	Nonadecane	1897	1900		0.2±0.1	tr.	0.4±0.1	tr.	tr.	0.1±0.0	Std
118	Eicosane	1999	2000		0.4±0.1	–	–	–	–	–	Std
119	Hexadecanoic acid	1963	1960	1963	–	–	–	tr.	–	–	Std
	Total identified [%]				97.0±0.1	97.1±1.0	100.0±0.0	63.4±0.5	96.2±0.4	97.6±0.2	
	Identified compounds				80	74	46	61	52	56	

Table 3 (cont.)

Entry	Component[a])	*RI*[b])	*RI* from literature		*Samples*[c])						ID[d])
			Adams[e])	NIST 08[f])	*1*	*2*	*3*	*4*	*5*	*6*	
	Grouped compounds [%]										
	Aliphatic alcohols				tr.	–	–	–	–	–	
	Alkanes				1.1±0.2	0.5±0.2	15.4±2.2	1.3±0.0	2.8±0.9	2.4±0.2	
	Aldehydes and ketons				2.2±0.5	1.7±0.2	6.4±0.4	4.5±0.1	1.2±0.1	3.2±0.1	
	Esters				0.4±0.2	0.6±0.0	–	0.6±0.1	–	–	
	Aromatics				6.2±0.3	2.5±0.2	2.6±0.7	2.6±0.2	3.3±0.2	1.9±0.1	
	Monoterpene hydrocarbons				tr.	0.1±0.0	–	0.2±0.0	tr.	–	
	Oxygenated monoterpenes				7.5±0.3	3.1±0.2	4.1±0.4	0.5±0.1	0.4±0.0	1.4±0.2	
	Sesquiterpene hydrocarbons				73.3±0.7	82.9±1.2	52.3±1.9	70.7±0.6	88.3±0.4	87.1±0.8	
	Oxygenated sesquiterpenes				5.9±0.3	3.8±0.6	3.8±0.6	12.1±0.5	tr.	–	
	Norisoprenoids				0.4±0.0	1.7±0.2	10.3±0.5	3.4±0.2	0.5±0.0	0.8±0.0	
	Others				tr.	0.2±0.0	5.0±0.4	2.2±0.1	–	0.8±0.0	

[a]) Compounds are listed in order of their elution from a *HP-5* column; percentage values are means of three determinations ± standard deviation; they were obtained at FID by peak-area normalization calculating the relative response factor. [b]) Retention index on *HP-5* column, experimentally determined using homologous series of C_8–C_{30} alkanes. [c]) Samples are numbered according to *Table 1*. [d]) Identification methods: MS, by comparison of the mass spectrum with the spectra of the computer mass libraries *Wiley275*, *Adams*, and *NIST 08*; *RI*, by comparison of the *RI* value with the values reported by *Adams* [15] and *NIST 08* [16]; Std, by comparison of the retention time (t_R) and MS of available authentic standard. [e]) Relative *RI* taken from *Adams* [15]. [f]) Relative *RI* taken from NIST 08 [16]. [g]) tr., Traces (mean value below 0.1%). [h]) Unknown compound, *m/z* (ten largest peaks): 189 (999), 175 (908), 81 (897), 107 (878), 79 (856), 123 (853), 93 (844), 91 (808), 105 (747), 121 (603). [i]) Unknown compound, *m/z:* 107 (999), 105 (668), 43 (615), 95 (612), 121 (545), 91 (538), 205 (530), 177 (479), 187 (455), 93 (435).

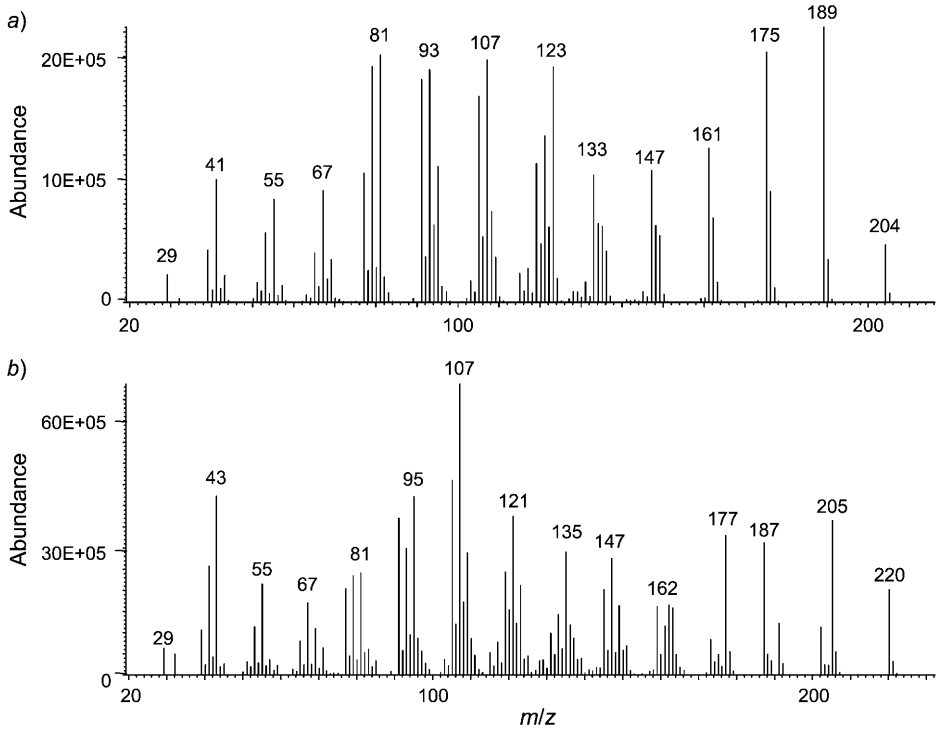


Fig. 6. *MS Fragmentation of the major volatiles occurring in a population of* E. nebrodensis *subsp.* nebrodensis (*Sample 4*)*: a sesquiterpene hydrocarbon* (*a*) *and an oxygenated sesquiterpene* (*b*)

(unweighted pair-group method using arithmetic averages) with the Euclidean distance as dissimilarity coefficient was employed on percentages of volatiles, and the obtained dendrogram is shown in *Fig. 7,a*. This statistical method clearly revealed significant differences between the *Ephedra* populations. From CA, three main groups were delineated: group *A*, formed by Sardinian populations (*Samples 5* and *6*) and one Marchigian population (*Sample 1*) characterized by a high content of sesquiterpene hydrocarbons such as α-isocomene (**4**; 31.25, 24.45, and 22.83%, resp.), β-maaliene (**3**; 6.95, 7.49, and 7.32%, resp.), and β-patchoulene (**7**; 9.40, 7.83, and 11.29%, resp.); group *B*, formed by only one Abruzzian population (*Sample 3*), characterized by a lower content of sesquiterpene hydrocarbons (52.30%) dominated by γ-muurolene (**11**; 16.3%), and a higher content of alkanes (15.40%) and norisoprenoids (10.34%); group *C* formed by one Marchigian population (*Sample 2*) and one Abruzzian population (*Sample 4*), characterized by the occurrence of the unknown sesquiterpenes (ranging from traces to 24.29% for the hydrocarbon, resp., and from traces to 10.37% for the oxygenated compound, resp., and by the high content of alloaromadendrene, *i.e.*, 33.20 and 15.65%, resp.). These results showed that the volatile compositions of *E. nebrodensis* are highly variable, and, with the exception of the Sardinian populations, it surprisingly seems to be independent of the geographic origin of the samples. In fact,

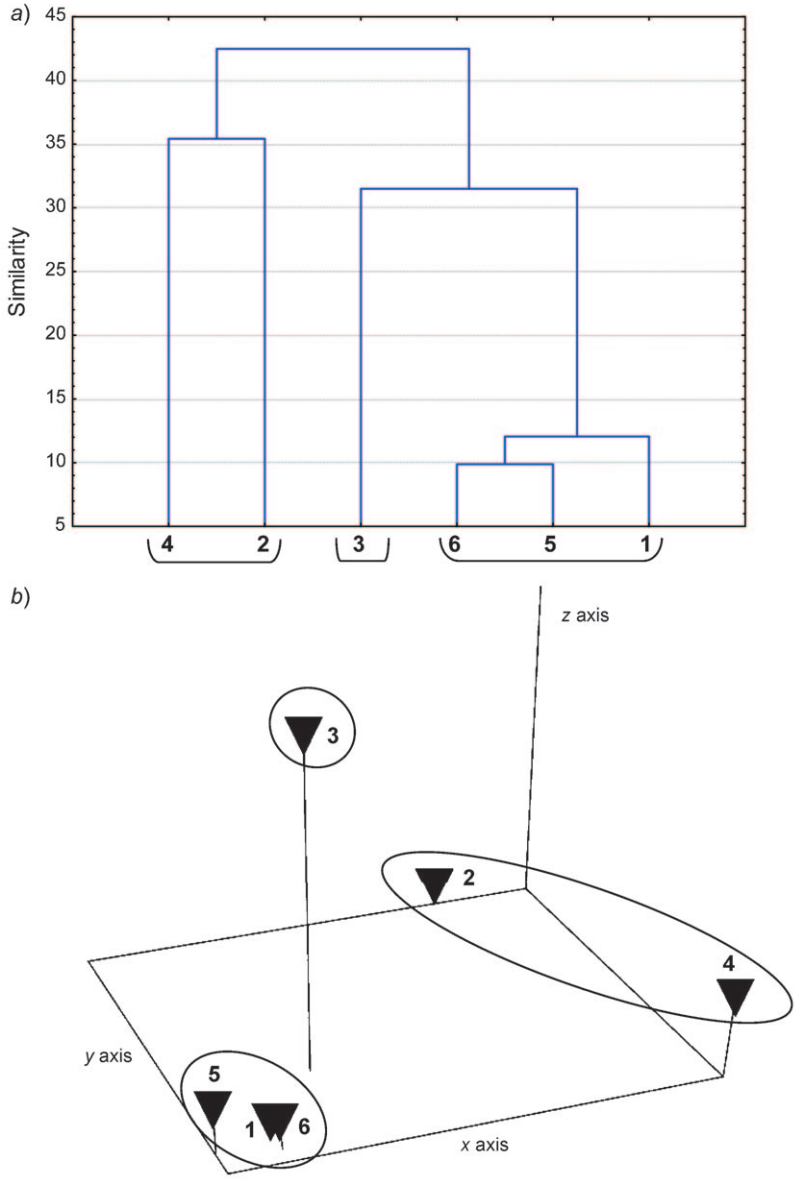


Fig. 7. a) *Dendrogram obtained by hierarchical cluster analysis of headspace volatile compositions of six* E. nebrodensis *subsp.* nebrodensis *Italian populations based on the unweighted pair-group method using arithmetic averages* (UPGMA) *and the Euclidean distance as dissimilarity coefficient.* b) *Principal component analysis based on covariance matrix of the same data.* The *x*, *y*, and *z* axes showed 37.24, 27.80, and 23.30% of variance, respectively, for a total variance of 88.34%. Numbers of populations refer to the *Table 1*.

SPME data did not permit a thorough characterization of peninsular samples with respect to those originating from the island of Sardinia.

Another multivariate statistical method, PCA, was used for definition of the principal components, contributing most to the variability of the studied set and confirmed (*Fig. 7,b*) the grouping obtained with CA. The variability of first two axis (*x* and *y*) was generated mostly by the content of the unidentified sesquiterpene hydrocarbon (values of eigenvectors: 0.49; −0.41), alloaromadendrene (values of eigenvectors: 0.45; 0.28), α-isocomene (values of eigenvectors: −0.30, −0.36), and the unidentified oxygenated sesquiterpene (values of eigenvectors: 0.31, −0.25). The variability of the third axis (*z*) was generated mostly by γ-muurolene, heptadecane, and pentadecane.

The acquired SPME-GC-FID data of the sample collected in Camerino also allowed comparison with the volatile profile of essential oil as previously reported by *Maggi et al.* [10], revealing great differences. The most abundant compound in the essential oil, the oxygenated monoterpene citronellol (**2**; 29.67%), was only found in a little amount in the headspace (2.71%). Oxygenated monoterpenes dominated the essential-oil composition, while sesquiterpene hydrocarbons constituted a minor fraction; the opposite pattern was observed with SPME analysis. Esters constituted the second most abundant group (11.5%) in the essential oil with ethyl hexadecanoate (9.5%) as the main representative, whereas SPME revealed only scant amounts of esters with ethyl hexadecanoate being absent. Globally, more compounds were identified by SPME (74) in comparison with extraction by means of hydrodistillation (59 identified compounds). These observed different profiles revealed a different sensitivity of the extraction techniques, besides sharing only 20 constituents. Still some differences may be accounted for by the invasive/destructive character of hydrodistillation. Unlike SPME, hydrodistillation can entail artefacts caused by high extraction temperatures, oxidations, hydrolysis, and decomposition of the plant matrix or the volatiles themselves [20]. For example, phytol, an oxygenated diterpene, degradation product of chlorophyll, was detected in the essential oil, while it proved to be absent after SPME. The high amount of oxygenated monoterpenes can possibly be caused by oxidation and hydrolysis reactions occurring during hydrodistillation.

The SPME profile from Italian populations was also different from the published data on *E. sinica* [7], obtained with a method combining continuous hydrodistillation of plant material with concurrent SPME. The main constituents of the essential oil were α-terpineol (13.2%), tetramethylpyrazine (7.4%), and 3-methylbut-2-en-1-ol (5.2%), whilst only traces of α-terpineol were found in *E. nebrodensis* originating from Visso with the other compounds being absent.

Conclusions. – The non-destructive and non-invasive SPME technique coupled with GC/FID and GC/MS has been successfully developed to evaluate for the first time the volatile composition of six Italian populations of *E. nebrodensis* subsp. *nebrodensis*. The technique permitted analysis of very small amounts of plant material, since the *Ephedra* populations are threatened by reduction in Italy owing to the human activities. By using the PDMS fiber, the method provided sufficient reproducibility, as generally the obtained RSD values for total volatiles and major compounds did not exceed 20%, which is acceptable considering the biological origin of the samples. Results revealed a

high intraspecies variability, and, with the exception of Sardinian samples, no significant correlation was found between the volatile profile and the geographical distribution of the six populations. In all cases, sesquiterpene hydrocarbons were major constitutents in the headspace profile of the plant, accounting for 52.30–88.32% of all volatiles. This profile revealed to be quite different from those of *Ephedra* essential oils investigated so far. Multivariate analysis applied to SPME data suggested the presence of at least three different chemotypes among the six samples. Several interesting phytochemicals were abundant in the headspace of *E. nebrodensis* subsp. *nebrodensis*. Beside substances used as fragrances, there have been detected metabolites with pharmaceutical potential such as α-isocomene, β-maaliene, and (*E*)-β-ionone having antidementia, sedative, and antitumor activity, respectively. Finally, we revealed two major sesquiterpenes whose structures have never been characterized before. Thus, their identification is challenging and could in the future be achieved by NMR analysis after appropriate preparative chromatographic separation. In spite of the observed highly variable volatile profile within *E. nebrodensis*, future SPME analysis of other *Ephedra* species may prove if the method can be successfully used to support the botanical classification of the genus.

Experimental Part

Plant Material. Aerial parts of *E. nebrodensis* subsp. *nebrodensis*, including young green stems with leaves reduced up to sheaths, were collected in May, June, and September 2007, as well as in May 2008 in six different localities (in all cases constituted by cliffy limestones and rocky places) belonging to Marche (Appennino Umbro-Marchigiano), Abruzzo (Gran Sasso and Monti della Laga National Park), and Sardinia (Gennargentu Montains) regions, and covering the main Italian areal of the species [5] (*Fig. 1*), from 620 to 1100 m above the sea level (*Table 1*). Voucher specimens of Central Italy were identified by *F. M.* and Dr. *Conti* using available literature and deposited with the *Herbarium Camerinensis* (CAME) and with the Herbarium of Centro Ricerche Floristiche dell'Appennino (APP) (both included in the online edition of *Index Herbariorum:* http://sweetgum.nybg.org/ih/) [21] of School of Environmental Sciences, University of Camerino (Italy). Sardinian specimens were authenticated and deposited with the Herbarium of Botanical Sciences of the University of Cagliari, Italy. Because *E. nebrodensis* lives only in impervious places constituted by rocks and cliffy limestones, and therefore threatened of reduction in number and density of population, only 0.493–5.163 g of plant material were collected in the different collection sites, taking into consideration the higher capacity of SPME to analyze considerably smaller amounts of plant material than other extraction techniques.

Sample Preparation. Plant material was stored in the dark at r.t. (*ca.* 22°) until completely dry, then ground in powder by using a blender *MFC* model *DCFH 48 IKA-WERK* (D-Staufen) equipped with sieves of 1-mm size in diameter.

SPME Fiber Screening. A preliminary screening of three types of coating fibers of various polarity and retention capability was carried out in order to select the best type in terms of extraction efficiency and reproducibility for *Ephedra* volatiles. The screening was conducted on *Sample 1* (Visso) using the following anal. conditions: extraction temp., 60°; extraction time, 30 min; particle size, 1 mm; amount of plant material, 30 mg; desorption time, 3 min; it was based on reproducibility and extraction efficiency for six selected marker compounds (representing different chemical classes and having different chromatographic behavior) and total volatiles of *E. nebrodensis* subsp. *nebrodensis*. The following fibers were tested and compared: polydimethylsiloxane (PDMS, 100 μm), *Carboxen*™/polydimethylsiloxane (CAR/PDMS; 75 μm) and polydimethylsiloxane/divinylbenzene (PDMS/DVB; 65 μm). The silica fibers and the manual SPME holder were purchased from *Supelco* (Bellefonte, PA, USA). The coating of all fibers was 1-cm-long. Before GC/FID and GC/MS analyses, each fiber was conditioned in the injector of the GC system, according to the instructions provided by the manufacturer. All analyses were performed

in triplicate, and average values were calculated. A blank was run at the beginning of each day to assure that each fiber was free of impurities and residues, as well as after each sample run to check for carryover.

SPME Procedure. Once the fiber was chosen, ground aerial parts of *E. nebrodensis* were placed into a 4-ml headspace glass vial, and, after being capped with a polypropylene hole cap with PTFE/silicone septa (*Supelco*, Bellefonte, PA, USA), the vial was immersed in a thermostated water bath. Once the desired temp. was reached, the PDMS fiber (*Supelco*, Bellefonte, PA, USA), 100-μm-thick, 1-cm-long, was introduced into the vial by manually penetrating the septum and exposed to the headspace of the sample. The SPME fiber was maintained 10 mm above the solid samples; after absorption, the fiber was retracted and inserted into the injection port with a SPME inlet liner (0.75 mm i.d.; *Supelco*) of gas chromatograph in splitless mode. The extracted compounds were thermally desorbed at 250° for 3 min, that was sufficient to desorb most of analytes from the fiber. Prior to use, the PDMS fiber was conditioned in the GC injector at 250° in order to remove contaminants. To enhance the release of volatiles from plant material, the following exper. parameters were optimized by a single-factor SPME analysis on *Sample 1* (Visso): extraction temp. (20–80°), extraction time (10–60 min), amount of plant material (10–60 mg), H_2O addition (0–60 μl). All experiments and sample measurements were carried out in triplicate, and the average and RSD [%] (Relative Standard Deviation) values were calculated. To obtain the highest reproducibility, all measurements were performed with the same fiber.

GC/FID and GC/MS Analysis. For GC separations, an *Agilent 4890D* instrument coupled with a flame ionization detector (FID) was used. Volatile components were separated on a *HP-5* cap. column (5% (phenylmethyl)polysiloxane, 25 m, 0.32 mm i.d.; 0.17 μm film thickness; *J & W Scientific*, Folsom, CA, USA). The oven temp. was initially maintained at 60° for 5 min and then programmed to 220° at a rate of 10°/min, then 20°/min up to 280°, held for 20 min, for a total run of 42 min. Temps. of the injector and detector were set to 250°. He was used as carrier gas at 1.4 ml/min under splitless mode. A mixture of aliphatic hydrocarbons (C_8–C_{30}; *Sigma*, I-Milan), diluted in hexane, was loaded onto the SPME fiber and injected under the above temp. program to calculate the retention indices (*RI*s; as *Kovats* indices) of each extracted compound. The rel. amounts of volatile components, expressed as percentages, were obtained by FID peak-area internal normalization, by calculating the response factor of the FID for different classes of volatiles occurring in the headspace of plant matrix [22]. Data were collected by using *HP3398A* GC Chemstation software (*Hewlett Packard*, Rev. A.01.01).

GC/MS Analysis was performed using an *Agilent 6890N* gas chromatograph coupled with a *5973N* mass spectrometer equipped with a *HP-5MS* cap. column (5% (phenylmethyl)polysiloxane, 30 m, 0.25 mm i.d., 0.1 μm film thickness; *J & W Scientific*, Folsom), using the same temp. program reported above. Temps. of the injector, transfer line, and quadrupole were set to 250°. He was used as carrier gas at 1.0 ml/min under splitless mode. The mass spectrometer was run in the electron impact (EI) mode with electron energy at 70 eV, scanning the 29–400 amu. Whenever possible, aroma components were identified by comparing the t_R, RI, and MS of the chromatographic peak with that of standard analyzed (obtained from *Sigma–Aldrich*) under the same conditions. Otherwise, the peak assignment was based on computer matching with the *WILEY 275*, *NIST 08*, *ADAMS*, and a home-made (based on the analyses of reference oils and commercially available standards) MS databases, taking into account the coherence of the *RI*s of the analyzed compounds with those reported by *Adams* [15] and *NIST 08* library [16]. Data were analyzed by using MSD ChemStation software (*Agilent*, Version G1701DA D.01.00).

Statistical Analysis. The multivariate chemometric techniques, cluster analysis (CA) and principal component analysis (PCA), were applied to the obtained SPME data in order to interpret the volatile profiles statistically and discriminate between the six *E. nebrodensis* populations using STATISTICA 7.1 (*Stat Soft Italia srl*, www.statsoft.it). The percentage composition of the identified compounds of the six samples was included in the data set of the software program as handling data. The unknown oxygenated sesquiterpene and sesquiterpene hydrocarbon, found in the *Samples 2* and *4* were also included, as they accounted for 10.37 and 24.49% of the volatiles detected in the *Sample 4*, resp. Data with values under 0.1% or missing data were substituted for the purpose of statistic analyses by 0.01%. CA is an unsupervized chemometric technique that enables to disclose the natural groupings existing between samples that are characterized by the dataset. Arcsin transformation was performed on the primary data set to ensure normality. Hierarchical CA with the unweighted pair-group method using arithmetic averages (CA-UPGMA) and the Euclidean distance as dissimilarity coefficient was applied to the

transformed matrix. A further multivariate method, PCA based on covariance matrix, was used for definition of principal components, which contribute the most to the variability of the studied set. PCA enabled also three-dimensional visualization of the position of the samples rel. to each other.

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