Chemical composition and antioxidant activity of the volatile fraction extracted from air-dried fruits of Tunisian *Eryngium maritimum* L. ecotypes

Running title: Volatile components of *E. maritimum* from Tunisia

Houda Ben Lajnef,[a] Federico Ferioli,[b] Federica Pasini,[c] Joanna Politowicz,[d] Abdelhamid Khaldi,[e] L. Filippo D'Antuono,[b] Maria Fiorenza Caboni,[b,c] and Nizar Nasri,[a*]

[a] Université de Tunis El-Manar, Faculté des Sciences de Tunis, Tunis 2092, Tunisia.

[b] Department of Agri-Food Science and Technology, Food Science University Campus, Alma Mater Studiorum–University of Bologna, Piazza Goidanich, 60, 47521 Cesena (FC), Italy.

[c] Inter-Departmental Centre for Agri-Food Industrial Research (CIRI Agrifood), University of Bologna, Piazza Goidanich 60, 47521 Cesena (FC), Italy.

[d] Wrocław University of Environmental and Life Sciences, The Faculty of Food Science, Department of Chemistry, Norwida 25, 50-375 Wrocław, Poland.

[e] Université de Carthage, INREGREF, BP 10, Ariana 2080, Tunisia.

* Corresponding author: nizar.nasri@fst.rnu.tn (NN).

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Abstract

BACKGROUND: *Eryngium maritimum* L., also known as "sea holly", is a typical dune plant species belonging to the *Apiaceae* family and commonly used in Tunisia for therapeutic purposes in folk medicine. In the present study, the chemical composition and antioxidant activity of the volatile fraction extracted from air-dried fruits of five Tunisian *E. maritimum* ecotypes were determined.

RESULTS: The total volatile amount ranged from 0.31 to 0.93% (w d.w.$^{-1}$). Sixty-six volatile components were identified by means of GC-MS and accounted for 77.05-86.65% of the total extracted volatile oil. The majority of the identified metabolites were hydrocarbon sesquiterpenes and oxygenated sesquiterpenes, amounting on average to 46.69 and 30.01% of total volatiles, respectively. The principal individual components were germacrene D (13.62 to 31.71%), 15-hydroxy-α-muurolene (12.04 to 18.58%), and germacrene B (6.77 to 15.04%). Significant differences were noticed among *E. maritimum* populations. The volatile profile of *E. maritimum* fruits was consistently different from those of the aerial parts and roots of plants of the same species reported in previous investigations. Average radical scavenging capacity of the volatile fraction, as determined by DPPH and ABTS tests, was twice higher than that of the Trolox control.

CONCLUSION: This study characterised for the first time the fruits of *E. maritimum* for the composition and radical-scavenging capacity of their volatile fraction. The growth location confirmed as a pivotal factor in influencing the volatile profile of the fruits.

Keywords: *Eryngium maritimum* L.; volatile compounds; antioxidant activity; sesquiterpenes

INTRODUCTION

Essential oils (EOs) are volatile biochemical components, namely terpenes, terpenoids, phenylpropenes and phenolics, that show a lipophilic nature and are synthesised and stored in complex secretary structures (glandular trichomes, secretory cavities and resin ducts) by more than 17,000 aromatic plants, usually belonging to angiospermic families *Apiaceae*, *Lamiaceae*, *Rutaceae*, *Myrtaceae*, *Zingiberaceae*, and *Asteraceae*.[1] They are commonly used as flavorings in the preparation of food products, perfumes (fragrances and aftershaves), cosmetics and pharmaceuticals for their functional properties.[2]

The interest in plant EOs is still increasing since these volatile mixtures of bioactive compounds have shown strong antimicrobial and antioxidant properties. EOs could play an essential role in the development of environmentally friendly and natural plant-based food preservatives and represent a safer and more effective alternative to synthetic chemicals.[3,4]

Several factors including genetic variation, plant ecotype or variety, plant nutrition, application of fertilisers, geographic location of the plants, surrounding climate, seasonal variations, stress factors during growth or maturity and also the post-harvest drying and storage, may affect the chemical profile of plant EOs from a qualitative and quantitative standpoint.[5] In addition to these, the part of plant analysed is another key factor in determining both the yield and composition of EO fraction.[5]

Tunisia, located on the shores of the Mediterranean Sea, is a rich repository of medicinal plants and represents one of the biggest biodiversity centers having approximately 12,000 species of vascular plants.[6] *Eryngium maritimum* L., also known as “sea holly” in England and “panicaut de mer” in France, is one of the medicinal plants commonly used in Tunisia. It belongs to *Eryngium* genus of the *Apiaceae* family. This species is a perennial plant, growing usually in sandy seashores to 0.5 m, with metallic bluish flowers and intensely whitish-glaucous leaves. In many parts of the

world *E. maritimum* is a protected species, but in Tunisia it can be found along coasts and used by locals.

This plant has been reported to exhibit therapeutic uses in folk medicine as diuretic or hypoglycemic.[7] In particular, infusions of the aerial parts and roots of this species are used as antitussive, diuretic, appetiser, stimulant, and aphrodisiac.[8] The root part also causes menstruation, promotes flatulence, cystotonic, urethritis remedy, stone inhibitor and removes obstructions in liver, kidney and gall-bladder.[8]

Recent researches have dealt with the determination of the profile and antioxidant activity of EOs recovered from the aerial parts of *E. maritimum* populations located in the Mediterranean isles of Corsica and Sardinia.[7,9,10] The present investigation was carried out with the aim to characterise for the first time the chemical composition and radical-scavenging capacity of the volatile fraction extracted from *E. maritimum* fruits collected from five different Tunisian costal locations.

EXPERIMENTAL

Sample description

E. maritimum fruits were collected in September 2014 from five littoral locations sited in a wide area located over than 300 km along the eastern side of Tunisian shoreline (**Table 1)**. Samples were air-dried and stored at room temperature in a dark and dry place until analyses. To express data on a dry basis, the residual dry matter content was determined gravimetrically as the mass loss of 5 g of air-dried fruits, until a constant weight was reached. Each analytical assay was performed in duplicate on each sample.

Reagents and chemicals

Chemicals were of analytical grade and purchased from Sigma-Aldrich (Saint Louis, MO, USA). Deionised water was obtained by an Elix 10 water purification system from Millipore (Bedford, MA, USA).

Extraction of volatile compounds

Volatiles were recovered according to the method employed by Ferioli *et al.* on fennel leaves, dried florets, and fruits.[11] Fifty grams of *E. maritimum* dried fruits, previously added with 500 mL of water, underwent a simultaneous water distillation-solvent extraction (SDE) procedure in a Likens-Nickerson apparatus, using 60 mL of diethyl ether as organic solvent. SDE was carried out for two hours, starting from volatile oil distillation. Ethereal extracts were dried for two hours over anhydrous sodium sulfate at 4°C, and the organic solvent was then evaporated under reduced pressure at 20°C. Volatile fraction was gravimetrically quantified and stored in an amber glass vial at -18°C until gas chromatographic analyses.

Identification and quantification of individual volatile components by gas chromatography-mass spectrometry (GC-MS)

A gas chromatograph (mod. 7820A) from Agilent Technologies (Santa Clara, CA, USA), equipped with an autosampler (mod. G4567A) and coupled with a mass spectrometer (mod. 5977E), was used. Compound separation was carried out on a capillary column ZB-WAX (30 m × 0.25 mm; film thickness: 0.25 μm; stationary phase: 100% polyethylene glycol) from Phenomenex (Torrance, CA, USA). Before analyses ten μL of each extract were diluted in 1 mL of *n*-hexane. Operating GC conditions were as follows: injection volume: 1 μL; injection mode: split; split ratio: 1:40; carrier gas (He) flow and linear velocity: 1.0 mL min^{-1} and 36.3 cm sec^{-1}, respectively; injector temperature: 250°C; oven temperature: 40°C for 2 min, from 40 to

250°C at 3°C min^{-1}, 250°C for 10 min. MS operative parameters were the following: solvent delay time: 2.5 min; transfer line temperature: 250°C; ion source and quadrupole temperature: 230 and 150°C, respectively; ionisation energy: 70 eV; scan range: 40-400 *m/z*; scan frequency: 4 scan sec^{-1}.

Data were filed and processed by MassHunter Workstation Software-Qualitative Analysis (ver. B.06.00) from Agilent Technologies. The percentage of each compound was determined from its peak area and the sum of the areas of all peaks detected in the total ion current (TIC) trace. The identification of volatile compounds was performed by computer matching of peak spectra to those present in NIST 2014 Mass Library by means of MassHunter Workstation software and NIST Mass Spectral Search Program (ver. 2.2) software. A further confirmation to peak identity was carried out by comparison of peak retention indices (RIs) relative to a C_8-C_{20} alkane mixture with indices on a polar column given by NIST Mass Spectral Search Program on the basis of literature data. For each identified component, RI was calculated on the basis of its retention time according to the formula described by Adams.[12]

Determination of the antioxidant activity of volatile fraction

The antioxidant capacity of volatile extracts was evaluated spectrophotometrically, testing their scavenging activity on the radical chromogens $ABTS^{\bullet+}$ (radical cation of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) and $DPPH^{\bullet}$ (free radical of2,2-diphenyl-l-picrylhydrazyl). A double beam spectrophotometer (mod. UV-1800) from Shimadzu (Kyoto, Japan) was used.

DPPH assay The procedure described by Parejo *et al.*[13] was followed with some modifications, as briefly reported. One hundred μL of a methanolic solution containing different amounts (10, 20, 30, and 40 μg) of each volatile extract were added to 2.9 mL of 100 μM DPPH in methanol. After briefly shaking, the mixture was incubated at 23°C

for 30 min in the dark and its absorbance was then read against methanol at 515 nm when the reaction reached a plateau. Methanol was used to zero the spectrophotometer. Inhibition of DPPH free radical was calculated as percentage (I%) using the following equation: $I\% = 100\times(A_b-A_s)/A_b$, where A_b and A_s stand for the absorbance of the control reaction and the absorbance of the sample, respectively. The sample concentration providing a 50% inhibition (IC_{50}) was calculated from the graph of I% against the sample concentration. IC_{50} is defined as the concentration of the potential antioxidant needed to decrease by 50% the initial absorbance of the colored solution and then to scavenge 50% of free radicals present in the test solution. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as positive control.

ABTS assay The ABTS radical scavenging activity was determined according to the methods described by Re *et al.*[14] and Eberhardt *et al.*[15], with some modifications. Briefly, ABTS was dissolved in water to a 7 mM concentration. The corresponding radical cation ($ABTS^{\bullet+}$) was obtained by reacting ABTS water solution with 2.45 mM potassium persulfate (final concentration) and allowing the solution in the dark overnight. Before use, $ABTS^{\bullet+}$ solution was diluted in ethanol to an absorbance of 0.70 ± 0.02 at 734 nm at 30°C. One mL of ethanolic $ABTS^{\bullet+}$ solution was added with 10 µL of a solution prepared dissolving different amounts of volatile extract (2.5, 5, 10, and 20 µg) in 100 µL of methanol. Ethanol was used to zero the spectrophotometer and as reference during spectrophotometric readings. Inhibition of $ABTS^{\bullet+}$ was calculated using the same equation previously reported for DPPH assay. The sample scavenging activity was expressed as IC_{50}. Trolox was used as positive control.

Statistical analysis

Data underwent analysis of variance (ANOVA). Multiple comparisons to assesses differences among ecotypes were carried out for all analytical traits by means of

protected least significance difference (LSD) test. A principal component analysis (PCA) was performed to better discriminate *E. maritimum* populations and to detect analytical variables that gave significant contribution to this discrimination. The antioxidant capacity determined both by DPPH and ABTS test, the total content of volatile oil expressed on a dry basis and the percentage of each individual component were employed in PCA. All statistical analyses were carried out employing the SYSTAT 10.0 package from Systat Software (Chicago, IL, USA).

RESULTS AND DISCUSSION

Volatile total content and chemical composition

The volatile total amount is reported in **Table 2**. Only TAZ sample showed a significantly different and higher value in comparison to other ecotypes, whereas no significant differences were assessed among HAM, MEN, SOL and MAH. Sixty-six volatile compounds, most of them mono- and sesquiterpenes and amounting on average to 81.62% of volatile fraction, were identified by means of GC-MS (**Table 3**). For some identified volatile compounds, NIST library did not give the corresponding RI on a polar column. For other compounds RI was different from RI given by the library. Nevertheless, RI was not used as primary identification tool since the comparison of RIs obtained from a specific polar coating column against the mean database values can be misleading. Indeed, the chemical properties of the coatings of similar polar capillary columns may vary from one column to another one depending on the column brand and be different from the average DB WAX coating.[16]

Hydrocarbon sesquiterpenes and oxygenated sesquiterpenes, amounting on average to 46.69 and 30.01% of volatiles (**Table 4**), respectively, were the dominating chemical classes and represented more than 70% of individual components in all populations. Hydrocarbon monoterpenes and oxygenated monoterpenes were detected at lower

amounts and accounted on average for 0.89 and 1.24% of volatiles, respectively. Other non-terpenic compounds (aldehydes, ketones, oxygenated heterocycles, cycloalkanes, and aromatic hydrocarbons) represented on average 2.79% of volatiles. Remarkable differences were noticed among ecotypes as regards the relative amount of individual volatiles (**Table 4**). The main component was germacrene D that ranged from 13.62 (HAM) to 31.71% (TAZ) of volatiles and accounted on average for 21.86% of volatile fraction. A significant variability was also noticed as regards the two other main volatile compounds: 15-hydroxy-α-muurolene, ranging from 12.04 (MAH) to 18.58% (TAZ) and representing on average 15.49% of volatiles, and germacrene B, ranging from 6.77 (MAH) to 15.04 % (HAM) and amounting on average to 10.58% of volatiles. Other compounds to be mentioned and representing on average more than 2% of volatiles were epicubenol (3.68-4.75%), δ-cadinene (2.21-3.21%), bicyclogermacrene (1.48-2.86%), β-caryophyllene (1.97-2.23%) and spathulenol (1.47-3.10%).

Significant differences in comparison to our results were noticed in recent investigations dealing with the essential oil (EO) composition in the aerial parts and roots of *E. maritimum* samples and briefly illustrated as follows. Even in these studies EOs were extracted by means of hydrodistillation.

Aslan *et al.*[17] focused on EO extracted from the aerial parts of a Turkish sample of *E. maritimum* which was found to be consistently rich in spathulenol and caryophyllene oxide, respectively accounting for 19.0 and 8.2% of EO. EO from roots of the same sample consistently differed and showed high contents of 2,4,5-trimethylbenzaldehyde (39.8%), 2,3,6-trimethylbenzaldehyde (29.0%) and γ- muurolene (23.5%).

In a Corsican *E. maritimum* population, oxygenated sesquiterpenes were determined in the fresh aerial parts at a higher percentage in comparison to hydrocarbon sesquiterpenes, with the two classes amounting to 57.9 and 38.0% of EO, respectively.[7] 4βH-Cadin-9-en-15-al, germacrene D, 4βH-cadin-9-en-15-ol, and 4βH-muurol-9-en-15-

al were found as the major individual components, accounting for 36.5, 31.6, 8.3 and 6.5% of EO, respectively. EO yield was 0.089%.

The same authors compared the fresh aerial parts collected from Corsican and Sardinian *E. maritimum* samples for their EO profile.[10] EO yield ranged from 0.06 to 0.13%. Geographical origin significantly affected the chemical composition but differences were also verified within samples of the same origin. Hydrocarbon sesquiterpenes ranged from 37.8 to 55.7% and from 20.4 to 37.4% of EO, whereas oxygenated sesquiterpenes were in the range 37.4-49.8% and 46.9-62.3% in Corsican and Sardinian populations, respectively. Germacrene D (13.7-45.9%), 4βH-cadin-9-en-15-al (18.5-26.1%), 4βH-cadin-9-en-15-ol (5.2-14.3%) and 4βH-muurol-9-en-15-al (5.2-9.2%), were the major individual components. Noticeable differences were also assessed among separated organs of the aerial parts of the same Corsican sample whereas isomers of trimethylbenzaldehyde in its isomer forms accounted for almost 70% of EO in roots.

15-hydroxy-α-muurolene and germacrene B, the second and the third most abundant of individual components, respectively, of our samples, were not detected both in *E. maritimum* aerial parts and roots surveyed in these researches. On the contrary, no sesquiterpene aldehydes, detected in large amounts in *E. maritimum* aerial parts were noticed in the volatile fraction of fruits herein analysed.

The chemical composition of EOs extracted from other *Eryngium* species was remarkably different in comparison to our results. Main results from these studies are briefly summarised as follows. In two species, EOs were dominated by oxygenated compounds such as phenylpropanoids or terpene alcohols. Cobos *et al.* determined in the air-dried inflorescence of *E. paniculatum* an EO yield amounting to 0.45%, whereas *trans*-anethole (52.6%) and α-pinene (19.1%) were the two major individual components.[18] Morteza-Semnani focused on EO extracted from the aerial parts *E.*

bungei and yielding to 0.98%. In this *Eryngium* species, the major EO constituents were cumin alcohol (55.3%), terpinolene (14.6%), carvacrol (8.9%) and limonene (7.5%).[19]

In other species hydrocarbon terpenes were the dominating constituents of EO. Palá-Paúl *et al.* identified β-caryophyllene (20.3%), germacrene D (19.2%) and α-humulene (8.8%) as the principal components of EO extracted from the winter leaves of *E. vesiculosum*, while the summer leaves contained bicyclogermacrene (22.2%), β-caryophyllene (15.6%), germacrene D (15.8%) and α-humulene (8.1%) as major components.[20] Sefidkon *et al.* recovered EO, yielding to 0.6%, from the air-dried aerial parts of *E. billardieri*.[21] The main compounds were α-muurolene (42.0%), β-gurjunene (17.0%), δ-cadinene (6.2%) and valencene (5.7%). Flamini *et al.* analysed EOs obtained from the leafy parts, flowers, and fruits of Italian *E. amethystinum*.[22] EO yields were lower in comparison to our results and amounted to 0.18, 0.29, and 0.20% in flowers, leafy parts, and fruits, respectively. α-Pinene was the major compound in the flowers (26.6%) whereas it accounted from 11.8 and 17.0% in the leafy parts and fruits, respectively. Germacrene D was the major components in the leafy parts (31.3%), whereas lower percentages were determined in the flowers (14.5%) and fruits (7.6%).

Antioxidant activity of volatile fraction

Volatile extracts showed an antioxidant activity significantly higher than Trolox since IC_{50} values of DPPH and ABTS radical-scavenging capacity were at least twice lower than those observed for the reference compound (**Table 2**).

HAM sample showed in ABTS test the highest IC_{50} value and then the lowest antioxidant capacity of all populations herein analysed. No significant differences were assessed among other populations. This pattern was not confirmed by DPPH test that did not allow a discrimination among populations as regards their antioxidant capacity since no significant difference was noticed in the radical scavenging properties of the

corresponding volatiles. A previous research comparing ABTS and DPPH test in evaluating the antioxidant capacity of plant materials highlighted as interfering substances extracted along with antioxidant compounds may significantly affect the response of the two chromogen-based methods in different ways.[23] In particular at wavelengths nearer to the visible region such as those employed in DPPH test, the antioxidant activity measures was underestimated in comparison to ABTS assay due to sample interferences.

On the basis of a recent and aforementioned study,[10] the lower antioxidant capacity of HAM ecotype appeared related to its fraction of oxygenated sesquiterpenes which was the lowest of all Tunisian ecotypes. Indeed, oxygenated volatiles were proposed in that investigation as the compounds bearing most of the scavenging properties of the essential oil recovered from the aerial parts of *E. maritimum*. Nevertheless, the same authors suggested that overall antioxidant capacity was depending not only on the total amount of oxygenated compounds but also on a more complex synergistic interaction of major and minor oxygenated components and their relative content.

Principal component analysis (PCA)

PCA yielded four rotated factors, explaining 98.2% of total variance, that were retained for discussion. The four principal components (PCs) led to a discrimination of *E. maritimum* ecotypes, and contributed to better identify differences on the basis of the composition of their volatile fraction and antioxidant capacity. In **Table 5** the loadings of analytical traits employed for PCA are shown whereas **Figure 1** presents the layout of Tunisian populations in the planes of PCs.

PC1, accounting for 39.8% among variance (**Figure 1A**), showed remarkable and positive correlations to less volatile components, in particular oxygenated mono- and sesquiterpenes. MAH, sited on the positive side of PC1 axis, was effectively

discriminated from other samples because of its higher percentages in comparison to other accessions with respect to all oxygenated compounds significantly correlated with PC1.

PC2, explaining 25.8% of variance (**Figure 1A**), appeared to be mainly related to more volatile compounds, in particular to minor constituents whose percentage was never higher than 1%. Even if detected in low amounts, these substances can give an important contribution in characterising the sensory profile of the fruits. PC2 better discriminated samples TAZ, HAM, SOL and MEN that were less differentiated by PC1. PC2 is also negatively and significantly related to the total amount of volatile oil.

PC3 explained 25.0% of variance (**Figure 1B**) and separated HAM sample, sited on the positive side of PC3 axis, from others. PC3 showed a high and positive relation to the antioxidant capacity assayed by ABTS test and to some minor hydrocarbon sesquiterpenes. PC3 was also significantly connected to the relative amount of the major components of volatile fraction: germacrene D (negative correlation), germacrene B (positive correlation), and 15-hydroxy-α-muurolene (negative). These correlations well accounted for the discrimination of HAM along PC3 axis from other Tunisian populations. PC3 was also highly related to two oxygenated sesquiterpenes that on average amounted together to less than 2% of volatiles.

PC4, explaining only 7.5% of total variance (**Figure 1C**), contributed to discriminate SOL from other samples, however with a poor and not clear relation with analytical traits.

CONCLUSIONS

A fist survey characterizing the fruits of five Tunisian ecotypes of *E. maritimum* L. for the composition and antioxidant activity of their volatile fraction was carried out. Even

the number of samples was not high, ecotypes here analysed came from a wide area spread over the eastern shoreline of Tunisia. Sixty-six individual components, accounting for more than 80% of total volatiles, were identified by means of GC-MS. Hydrocarbon sesquiterpenes were the dominating chemical class in all five ecotypes, followed by oxygenated sesquiterpenes, whereas Germacrene D, 15-hydroxy-α-muurolene and germacrene B were the most abundant individual components. Growth area confirmed as an important factor in influencing the chemical composition of volatile fraction since remarkable differences were assesses among Tunisian populations. Fruits here analysed also showed a volatile profile completely different with respect to the aerial parts and roots of samples belonging to the same species and studied in previous researches. Volatile oils showed a significantly higher radical scavenging capacity in comparison to a standard antioxidant compound, highlighting the possibility to expand the use of this plant from the food to the pharmaceutical field.

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FIGURE LEGENDS

Figure 1. Layout of *E. maritimum* ecotypes in the planes of the rotated principal components (PCs). For details about the name and origin of each ecotype see **Table 1**.

Table 1. Collection site, tag, and geographical coordinates of *E. maritimum* ecotypes grown in Tunisia.

Collection site	Population working tag	Geographical coordinates

		Longitude	Latitude
Tazarka	TAZ	10.48 E	36.33 N
Hammamet	HAM	10.62 E	36.40 N
Menzel Horr	MEN	10.96 E	36.73 N
Soliman	SOL	10.52 E	36.72 N
Mahdia	MAH	10.95 E	35.50 N

Table 2. Total amount, DPPH- and ABTS-radical scavenging activity of the volatile fraction extracted from Tunisian *E. maritimum* fruits.

E. maritimum ecotypes[1]	Total volatile amount (%, w d.w.$^{-1}$)	Antioxidant activity – IC_{50} (µg mL^{-1})	
		DPPH assay	ABTS assay

TAZ	0.93	141	39
HAM	0.45	135	71
MEN	0.41	104	39
SOL	0.32	122	40
MAH	0.31	136	50
Trolox[2]	-	310	138
Significance[3]	**	**	**
LSD[4]	0.24	42	17

[1] For details about the name and origin of each ecotype see **Table 1**.

[2]Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; compound chosen as reference antioxidant in radical-scavenging activity tests.

[3] * $p \leq 0.05$; ** $p \leq 0.01$.

[4] LSD: least significant difference ($p = 0.05$).

Table 3. Retention time, name, chemical class, retention index, and amount range of the compounds identified in the volatile fraction of *E. maritimum* fruits.

No.[1]	RT (min)[2]	Compound name	Chemical class	RI[3]	LRI[4]	Identification[5]	Amount range (mg kg^{-1}d.m.)[6]
1	2.64	*cis*-2,5-Dimethyltetrahydrofuran	Oxygenated heterocycle	-	-	MS	TR-0.1
2	2.83	*trans*-2,5-Dimethyltetrahydrofuran	Oxygenated heterocycle	-	-	MS	TR-0.4
3	3.82	1-(1-methyl-2-cyclopenten-1-yl)-Ethanone	Ketone	958	-	MS	0.9-2.7
4	4.88	α-Pinene	Hydrocarbon monoterpene	1018	1013±3	MS, RI	20.8-57.4
5	6.51	Hexanal	Aldehyde	1083	1083±8	MS, RI	0.9-2.5
6	7.50	Sabinene	Hydrocarbon monoterpene	1117	1124±8	MS, RI	0.3-1.3
7	9.11	β-Myrcene	Hydrocarbon monoterpene	1166	1161±7	MS, RI	1.7-8.7
8	9.82	Heptanal[7]	Aldehyde	1184	1184±9	MS, RI	1.1-2.0
9	10.11	Limonene	Hydrocarbon monoterpene	1192	1166±0	MS	2.0-4.0
10	11.60	2-Amylfuran	Oxygenated heterocycle	1233	1231±9	MS, RI	TR-1.4
11	11.76	6-Methyl-2-heptanone	Ketone	1237	1237±10	MS, RI	1.0-2.4
12	13.31	1,2,4-Trimethylbenzene	Aromatic hydrocarbon	1276	1282±7	MS, RI	4.4-8.2
13	13.81	Octanal	Aldehyde	1287	1289±9	MS, RI	5.7-11.1
14	20.42	α-Cubebene	Hydrocarbon sesquiterpene	1450	1463±6	MS, RI	3.6-12.5
15	20.75	Aciphyllene	Hydrocarbon sesquiterpene	1458	-	MS	5.2-13.1
16	20.98	δ-Eiemene	Hydrocarbon sesquiterpene	1463	1470±9	MS, RI	2.9-12.4
17	21.30	α-Ylangene	Hydrocarbon sesquiterpene	1471	1491±3	MS	1.3-4.8
18	21.64	α-Copaene	Hydrocarbon sesquiterpene	1479	1492±7	MS, RI	29.7-86.9
19	21.72	Daucene	Hydrocarbon sesquiterpene	1481	1495±9	MS, RI	5.3-21.6
20	22.69	β-Bourbonene	Hydrocarbon sesquiterpene	1503	1526±9	MS	14.9-41.0
21	23.59	β-Cubebene	Hydrocarbon sesquiterpene	1528	1545±5	MS	2.8-34.7
22	23.87	γ-Cadinene[7]	Hydrocarbon sesquiterpene	1535	-	MS	3.9-14.6
23	24.83	β-Ylangene	Hydrocarbon sesquiterpene	1560	1562±0	MS, RI	8.5-26.4
24	25.00	*cis*-Chrysanthenol acetate	Oxygenated monoterpene	1564	1562±20	MS, RI	1.5-8.3
25	25.26	8-Isopropenyl-1,5-dimethyl-1,5-cyclodecadiene	Hydrocarbon sesquiterpene	1570	-	MS	1.1-5.3
26	25.48	β-Copaene	Hydrocarbon sesquiterpene	1576	1586±11	MS, RI	7.1-36.1
27	25.68	β-Caryophyllene	Hydrocarbon sesquiterpene	1581	1595±16	MS, RI	60.5-198.1
28	26.01	1,4-Dimethyladamantane[7]	Cycloalkane	1588	-	MS	28.1-75.6
29	26.55	*cis*-Muurola-4(14),5-diene	Hydrocarbon sesquiterpene	1601	-	MS	0.7-2.8
30	27.55	γ-Elemene	Hydrocarbon sesquiterpene	1629	1642±9	MS, RI	34.1-138.2
31	27.83	*cis*-2-Decenal[7]	Aldehyde	1637	1622±19	MS, RI	4.8-10.8

32	28.43	Humulene	Hydrocarbon sesquiterpene	1653	1667±14	MS, RI	21.2-43.8
33	28.54	(+)-epi-Bicyclosesquiphellandrene	Hydrocarbon sesquiterpene	1656	-	MS	1.6-4.9
34	28.95	*trans*-β-Famesene	Hydrocarbon sesquiterpene	1666	1664±6	MS, RI	11.6-50.5
35	29.15	Verbenol	Oxygenated monoterpene	1672	1674±6	MS, RI	6.3-15.2
36	29.30	γ-Muurolene	Hydrocarbon sesquiterpene	1676	1692±12	MS, RI	18.7-64.3
37	30.06	Germacrene D	Hydrocarbon sesquiterpene	1695	1710±14	MS, RI	553.9-2954.0
38	30.84	Bicyclogermacrene	Hydrocarbon sesquiterpene	1716	1735±14	MS, RI	76.4-137.3
39	31.07	*p*-Mentha-1,5-dien-8-ol[7]	Oxygenated monoterpene	1723	1689±19	MS	1.2-2.7
40	31.86	δ-Cadinene	Hydrocarbon sesquiterpene	1745	1758±13	MS, RI	88.6-205.6
41	32.42	Longipinene[7]	Hydrocarbon sesquiterpene	1761	-	MS	5.9-16.0
42	32.64	Cubenene	Hydrocarbon sesquiterpene	1767	1786±13	MS, RI	2.2-4.6
43	33.03	α-Cadinene	Hydrocarbon sesquiterpene	1777	1815±0	MS	3.2-6.1
44	34.20	Germacrene B	Hydrocarbon sesquiterpene	1809	1819±19	MS, RI	208.6-948.1
45	34.41	Calamenene (isomer not identified)	Hydrocarbon sesquiterpene	1816	-	MS	0.8-2.2
46	34.49	Calamenene (isomer not identified)	Hydrocarbon sesquiterpene	1818	-	MS	1.2-3.3
47	36.48	Epicubebol	Oxygenated sesquiterpene	1876	1900±0	MS	4.2-9.9
48	36.63	2,4,5-Trimethylbenzaldehyde	Aldehyde	1880	1896±0	MS	2.7-9.5
49	37.18	1,5-Epoxysalvial-4(14)-ene	Oxygenated sesquiterpene	1896	1945±0	MS	12.4-27.7
50	37.30	α-Calacorene	Hydrocarbon sesquiterpene	1899	1919±21	MS, RI	3.1-8.4
51	38.22	Cubebol	Oxygenated sesquiterpene	1927	1957±0	MS	5.4-12.5
52	39.24	Caryophylleneoxide	Oxygenated sesquiterpene	1958	1989±19	MS	19.5-50.2
53	39.47	2,4,6-trimethylbenzaldehyde	Aldehyde	1965	1929±0	MS	30.8-55.6
54	40.12	Salvial-4(14)-en-1-one	Oxygenated sesquiterpene	1984	2037±0	MS	29.2-69.4
55	41.09	Humulene-1,2-epoxide	Oxygenated sesquiterpene	-	2071±0	MS	13.6-33.4
56	41.42	Tricyclo[4.4.0.0(2,7)]dec-3-ene-3-methanol, 1-methyl-8-(1-methylethyl)	Oxygenated sesquiterpene	-	2578±0	MS	22.8-79.9
57	41.79	Germacrene D-4-ol	Oxygenated sesquiterpene	-	2069±0	MS	39.4-97.7
58	42.89	Elemol	Oxygenated sesquiterpene	-	2080±10	MS	17.5-47.4
59	43.95	(1R,7S)-Germacra-4(15),5,10(14)-trien-1β-ol	Oxygenated sesquiterpene	-	-	MS	7.9-22.7
60	44.09	Spathulenol	Oxygenated sesquiterpene	-	2136±8	MS	57.6-134.6
61	45.58	Cadinol T	Oxygenated sesquiterpene	-	2169±16	MS	9.7-17.7
62	46.07	15-Hydroxy-α-muurolene	Oxygenated sesquiterpene	-	2599±0	MS	373.7-1722.3
63	46.30	4-Camphenylbutan-2-one[7]	Oxygenated monoterpene	-	-	MS	29.1-71.7
64	47.34	α-Cadinol	Oxygenated sesquiterpene	-	2226±9	MS	28.7-59.7
65	49.26	Ylangenal[7]	Oxygenated sesquiterpene	-	-	MS	8.1-25.6
66	54.61	Epicubenol	Oxygenated sesquiterpene	-	2067±21	MS	119.6-366.7
-	-	*Total identified compounds*	-	-	-	-	2397.1-8036.1

-	-	Hydrocarbon monoterpenes	-	-	-	-	26.0-70.8
-	-	Oxygenated monoterpenes	-	-	-	-	38.3-97.9
-	-	Hydrocarbon sesquiterpenes	-	-	-	-	1223.0-5045.4
-	-	Oxygenated sesquiterpenes	-	-	-	-	986.2-2660.2
-	-	Other compounds	-	-	-	-	95.0-161.7

[1] Order of elution.

[2] RT: retention time.

[3] RI: retention index herein calculated on the basis of compound RT and RTs of an alkane (C_8-C_{20}) standard mixture.

[4] LRI: retention index and corresponding uncertainty given on a polar column by identification Software NIST Mass Spectral Search Program on the basis of literature data

[5] Identification method: MS: comparison of mass spectra, RI: comparison of retention indices on a polar column.

[6] TR: traces.

[7] Compound tentatively identified.

Table 4. Percentage of individual components and main chemical classes of the volatile fraction of *E. maritimum* fruits.

Individual volatile compounds (%)	*E. maritimum* ecotypes[1]						
	TAZ	HAM	MEN	SOL	MAH	Significance[2]	LSD[3]
cis-2,5-Dimethyltetrahydrofuran	TR	TR	TR	<0.01	TR	ns	-
trans-2,5-Dimethyltetrahydrofuran	TR	<0.01	0.01	0.01	<0.01	**	<0.01
1-(1-methyl-2-cyclopenten-1-yl)-Ethanone	0.03	0.03	0.05	0.03	0.03	**	0.01
α-Pinene	0.64	0.78	0.88	0.64	0.71	**	0.13
Hexanal	0.01	0.03	0.06	0.03	0.03	**	0.01
Sabinene	0.01	0.02	0.03	0.02	0.01	**	0.01
β-Myrcene	0.10	0.08	0.12	0.07	0.05	**	0.01
Heptanal	0.01	0.04	0.05	0.03	0.04	**	0.01
Limonene	0.04	0.06	0.07	0.07	0.06	**	<0.01
2-Amylfuran	TR	0.02	0.03	0.02	0.01	**	0.01
6-Methyl-2-heptanone	0.03	0.03	0.04	0.03	0.04	**	<0.01
1,2,4-Trimethylbenzene	0.06	0.18	0.14	0.16	0.14	**	0.01
Octanal	0.10	0.22	0.27	0.21	0.19	**	0.02
α-Cubebene	0.13	0.12	0.11	0.11	0.16	**	0.02
Aciphyllene	0.14	0.14	0.13	0.16	0.17	ns	-
δ-Eiemene	0.13	0.18	0.14	0.20	0.10	**	0.03
α-Ylangene	0.05	0.04	0.03	0.04	0.06	**	0.01
α-Copaene	0.93	0.74	0.91	0.96	0.97	**	0.09
Daucene	0.12	0.48	0.24	0.27	0.17	**	0.03
β-Bourbonene	0.45	0.48	0.49	0.48	0.58	ns	-
β-Cubebene	0.37	0.25	0.14	0.16	0.09	**	0.02
γ-Cadinene	0.06	0.32	0.16	0.18	0.13	**	0.02
β-Ylangene	0.28	0.22	0.25	0.26	0.30	**	0.03
cis-Chrysanthenol acetate	0.09	0.11	0.06	0.04	0.11	**	0.01
8-Isopropenyl-1,5-dimethyl-1,5-cyclodecadiene	0.06	0.07	0.05	0.06	0.04	**	0.01
β-Copaene	0.39	0.18	0.24	0.22	0.32	**	0.04
β-Caryophyllene	2.13	2.09	1.99	2.23	1.97	**	0.13
1,4-Dimethyladamantane	0.82	0.93	1.09	1.30	0.92	**	0.10
cis-Muurola-4(14),5-diene	0.03	0.02	0.02	0.02	0.03	**	0.01
γ-Elemene	1.49	2.23	1.38	1.80	1.11	**	0.09
cis-2-Decenal	0.08	0.24	0.16	0.19	0.16	**	0.01
Humulene	0.47	0.58	0.62	0.65	0.74	**	0.04
(+)-epi-Bicyclosesquiphellandrene	0.05	0.04	0.06	0.05	0.06	**	0.01
trans-β-Famesene	0.22	1.13	0.62	0.79	0.37	**	0.10
Verbenol	0.17	0.23	0.19	0.19	0.29	**	0.02
γ-Muurolene	0.69	0.61	0.56	0.57	0.87	**	0.06
Germacrene D	31.71	13.62	24.37	21.66	17.94	**	1.39
Bicyclogermacrene	1.48	1.72	2.62	2.65	2.86	**	0.22
p-Mentha-1,5-dien-8-ol	0.03	0.04	0.04	0.04	0.04	*	0.01
δ-Cadinene	2.21	2.27	2.70	2.73	3.21	**	0.17
Longipinene	0.14	0.36	0.26	0.29	0.19	**	0.02
Cubenene	0.05	0.09	0.07	0.07	0.08	**	0.01
α-Cadinene	0.07	0.09	0.10	0.10	0.13	**	0.02
Germacrene B	10.22	15.04	8.87	12.00	6.77	**	0.46
Calamenene (isomer not identified)	0.02	0.03	0.02	0.03	0.05	**	<0.01
Calamenene (isomer not identified)	0.04	0.05	0.04	0.04	0.07	**	0.01
Epicubebol	0.11	0.17	0.14	0.13	0.28	**	0.01
2,4,5-Trimethylbenzaldehyde	0.10	0.12	0.07	0.12	0.14	**	0.01
1,5-Epoxysalvial-4(14)-ene	0.30	0.35	0.39	0.38	0.67	**	0.02

α-Calacorene	0.09	0.14	0.12	0.10	0.15	**	0.01
Cubebol	0.14	0.23	0.18	0.17	0.37	**	0.01
Caryophylleneoxide	0.55	0.72	0.78	0.60	1.22	**	0.04
2,4,6-trimethylbenzaldehyde	0.52	1.23	0.75	1.17	1.37	**	0.07
Salvial-4(14)-en-1-one	0.75	0.90	1.09	0.90	1.61	**	0.04
Humulene-1,2-epoxide	0.29	0.74	0.48	0.42	0.68	**	0.05
Tricyclo[4.4.0.0(2,7)]dec-3-ene-3-methanol, 1-methyl-8-(1-methylethyl)	0.30	1.77	0.80	0.87	0.73	**	0.08
Germacrene D-4-ol	0.43	1.11	1.48	1.48	3.14	**	0.14
Elemol	0.52	0.96	0.44	0.55	0.56	**	0.05
(1R,7S)-Germacra-4(15),5,10(14)-trien-1β-ol	0.25	0.32	0.31	0.24	0.53	**	0.03
Spathulenol	1.47	1.99	2.03	1.77	3.10	**	0.19
Cadinol T	0.19	0.31	0.36	0.30	0.54	**	0.04
15-Hydroxy-α-muurolene	18.58	12.77	16.37	17.70	12.04	**	0.55
4-Camphenylbutan-2-one	0.78	1.04	0.76	0.90	1.05	**	0.10
α-Cadinol	0.65	0.87	1.11	0.88	1.74	**	0.14
Ylangenal	0.28	0.32	0.32	0.25	0.56	**	0.04
Epicubenol	3.98	4.75	4.02	3.68	4.59	**	0.45
Total identified compounds (%)	86.65	77.05	82.53	84.47	77.42	**	1.17
Hydrocarbon monoterpenes	0.79	0.94	1.10	0.80	0.84	**	0.14
Oxygenated monoterpenes	1.07	1.42	1.06	1.18	1.48	**	0.10
Hydrocarbon sesquiterpenes	54.25	43.33	47.32	48.87	39.67	**	2.56
Oxygenated sesquiterpenes	28.78	28.27	30.32	30.32	32.35	**	1.52
Other compounds	1.76	3.07	2.73	3.30	3.08	**	0.13

[1] For details about the name and origin of each ecotype see **Table 1**.

[2] * $p \leq 0.05$; ** $p \leq 0.01$; ns: not significant.

[3] LSD: least significant difference ($p = 0.05$).

Table 5. Loadings of four principal components (PCs) on analytical variables and percentage of total variance explained by each PC.

Variables[1]	Rotated principal components[2]			
	PC1	PC2	PC3	PC4
DPPH	0.038	***-0.810***	0.186	-0.090
ABTS	0.142	-0.177	***0.902***	0.059
TOTVOL	*-0.604*	***-0.611***	-0.258	0.362
trans-2,5-Dimethyltetrahydrofuran	-0.335	***0.858***	0.277	-0.267
1-(1-methyl-2-cyclopenten-1-yl)-Ethanone	0.441	***0.743***	-0.105	0.484
α-Pinene	0.344	***0.691***	0.542	0.325
Hexanal	0.443	***0.870***	0.097	0.188
Sabinene	-0.277	***0.923***	0.244	0.091
β-Myrcene	-0.512	***0.604***	-0.089	*0.601*
Heptanal	0.487	***0.767***	0.416	0.010
Limonene	0.404	***0.903***	0.114	-0.087
2-Amylfuran	-0.286	***0.908***	0.292	0.081
6-Methyl-2-heptanone	***0.742***	*0.581*	-0.047	0.318
1,2,4-Trimethylbenzene	0.317	0.558	***0.670***	-0.371
Octanal	0.241	***0.914***	0.322	0.029
α-Cubebene	0.419	***-0.820***	0.038	0.300
Aciphyllene	0.173	***0.929***	0.027	0.112
δ-Elemene	***-0.668***	0.330	0.499	-0.411
α-Ylangene	0.181	***-0.955***	-0.135	0.104
α-Copaene	-0.322	0.012	***-0.918***	-0.191
Daucene	-0.196	0.272	***0.932***	-0.130
β-Bourbonene	0.429	-0.162	***0.724***	0.497
β-Cubebene	***-0.780***	*-0.587*	0.077	0.193
γ-Cadinene	-0.089	0.294	***0.929***	-0.149
β-Ylangene	0.163	*-0.647*	***-0.699***	0.202
cis-Chrysanthenol acetate	0.234	***-0.627***	*0.632*	0.376
8-Isopropenyl-1,5-dimethyl-1,5-cyclodecadiene	***-0.804***	0.028	*0.580*	-0.088
β-Copaene	-0.126	***-0.689***	*-0.592*	0.394
β-Caryophyllene	***-0.898***	-0.024	0.166	-0.387
1,4-Dimethyladamantane	-0.324	***0.765***	0.040	-0.554
cis-Muurola-4(14),5-diene	0.221	***-0.760***	-0.191	0.533
γ-Elemene	*-0.609*	0.053	***0.747***	-0.256

Variables	Rotated principal components[2]			
	PC1	PC2	PC3	PC4
(+)-epi-Bicyclosesquiphellandrene	0.258	-0.112	-0.561	***0.751***
trans-β-Famesene	-0.200	0.354	***0.862***	-0.301
Verbenol	***0.912***	0.039	0.401	-0.036
γ-Muurolene	*0.624*	***-0.757***	-0.058	0.159
Germacrene D	-0.547	-0.254	***-0.759***	0.242
Bicyclogermacrene	***0.664***	*0.643*	-0.198	-0.293
p-Mentha-1,5-dien-8-ol	0.341	***0.757***	0.400	0.168
δ-Cadinene	***0.861***	0.386	-0.248	-0.216
Longipinene	-0.124	*0.579*	***0.789***	-0.156
Cubenene	0.214	0.351	***0.888***	0.198
α-Cadinene	***0.833***	0.475	0.254	0.100
Germacrene B	*-0.650*	-0.006	***0.706***	-0.276
Calamenene (isomer not identified)	***0.908***	-0.291	0.279	0.100
Calamenene (isomer not identified)	***0.908***	-0.305	0.269	0.081
Epicubebol	***0.972***	-0.107	0.195	0.034
2,4,5-Trimethylbenzaldehyde	**0.550**	-0.466	0.400	-0.540
1,5-Epoxysalvial-4(14)-ene	***0.990***	-0.030	-0.113	-0.082
α-Calacorene	***0.699***	0.076	*0.653*	0.258
Cubebol	***0.963***	-0.117	0.240	-0.004
Caryophylleneoxide	***0.991***	0.004	0.028	0.131
2,4,6-trimethylbenzaldehyde	***0.694***	0.118	0.458	-0.543
Salvial-4(14)-en-1-one	***0.990***	0.063	-0.112	0.055
Humulene-1,2-epoxide	*0.685*	0.048	***0.724***	0.028
Tricyclo[4.4.0.0(2,7)]dec-3-ene-3-methanol, 1-methyl-8-(1-methylethyl)	0.026	0.218	***0.967***	-0.123
Germacrene D-4-ol	***0.980***	0.132	-0.066	-0.125
Elemol	0.010	-0.172	***0.976***	-0.128
(1R,7S)-Germacra-4(15),5,10(14)-trien-1β-ol	***0.982***	-0.088	0.056	0.147
Spathulenol	***0.999***	0.028	0.021	0.024
Cadinol T	***0.983***	0.171	0.021	0.005
15-Hydroxy-α-muurolene	*-0.684*	0.084	***-0.710***	-0.134
4-Camphenylbutan-2-one	***0.825***	-0.164	0.428	-0.329
α-Cadinol	***0.991***	0.083	-0.095	0.024

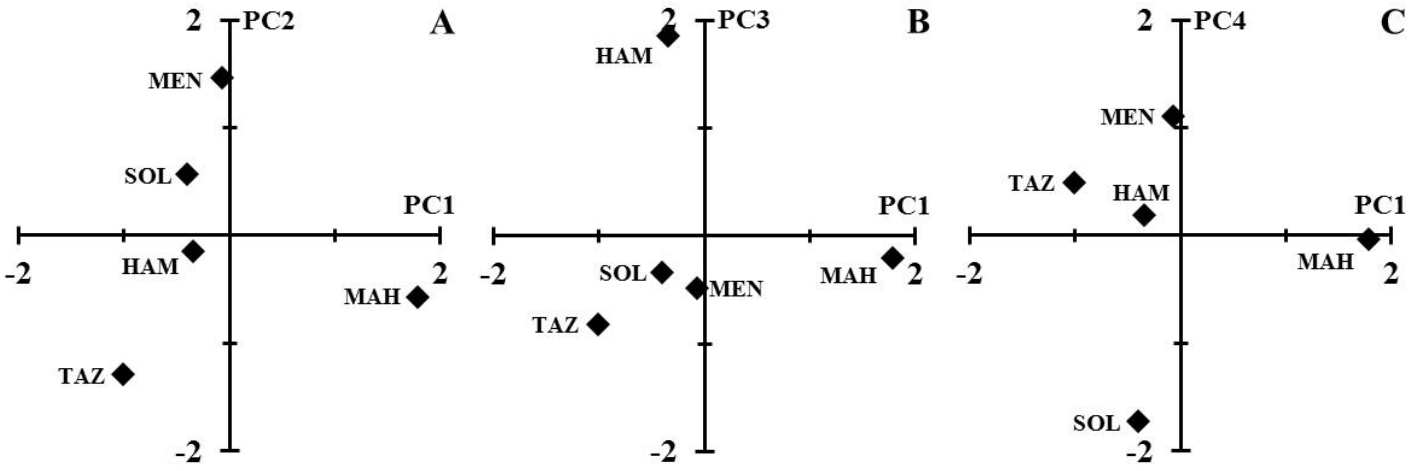
cis-2-Decenal	0.089	0.431	***0.867***	-0.223	Ylangenal	***0.978***	-0.174	-0.009	0.097
Humulene	***0.784***	0.504	0.131	-0.336	Epicubenol	***0.889***	-0.251	0.314	0.201
%EV[3]	39.8	25.8	25.0	7.5	*%EV*[3]	39.8	25.8	25.0	7.5

[1] DPPH and ABTS: radical-scavenging activity determined by DPPH and ABTS test; TOTVOL: total volatile amount expressed on a dry basis.

[2] Italic characters: significant correlation ($p \leq 0.05$); bold characters: highest correlation within each row.

[3] Percentage of total variance explained by each PC.

Figure 1


A
2
PC2
MEN
SOL
PC1
-2
HAM
2
MAH
TAZ
-2
B
2
PC3
HAM
PC1
-2
SOL
MEN
MAH
2
TAZ
-2
C
2
PC4
MEN
TAZ
HAM
PC1
MAH
2
SOL
-2