Mentha suaveolens Ehrh. Chemotypes in Eastern Iberian Peninsula. Essential Oil Variation and Relation with Ecological Factors.

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Introduction

The genus *Mentha (*Labiatae) is a paleotemplate taxon including aromatic perennial herbs long cultivated as flavouring, mint sauce and jelly, spearmint oil, antiparasitic and insecticide [1], [2], etc. *Mentha suaveolens* Ehrh. ("apple or woolly menthe", "mentastro" in vernacular name), is one of the five basic Eurosiberian and Mediterranean *mentha* species, common in grasslands on periodically flooded soils (Cl. Phragmitetea Tx. Et Presing, 1942), or moist soils with high phreatic level (Cl. Molinio Arrhenatheretea Tx., 1957). It is included, as a characteristic, in the association Mentho suaveolentis-Juncetum inflexi, [3].

Three subspecies are recognized in their natural dispersion area. The subsp. *insularis* is distributed along west Mediterrannean isles [4]; the subsp. *timija* is extended by plains and valleys of the macaronesic region whereas subsp. *suaveolens* (L.) Hudson is the most geographically widespread and largely employed in the traditional medicine. It is a common wild plant growing in wet places of Europe and northern Africa, as well as introduced in temperate countries of the Southern hemisphere [5]. It grows in Eastern Iberian Peninsula beside watercourses and nitrophile wet grasslands, such as reedy places, margins of channels, etc., both in coastal plains with thermomediterranean bioclimate the same way that mesomediterranean and supramediterranean bioclimates, [6], [7], [8].

As for essential oil (EO) chemical composition, differences among the above mentioned subspecies are based on the presence and different concentration levels of major compounds such as piperitenone oxide, piperitone oxide, pulegone and menthol derivatives [9-13]. The piperitenone oxide is the most common and representative compound as it is sometimes reported

as the only main compound [3], [13], [14]. Nevertheless, the presence of other compounds in leaves and inflorescences, such as limonene and carvone, has been reported as characteristics of the chemotypes in Northern Africa [11], [12].

M. suaveolens subsp. *suaveolens*, the taxon with major dispersion area (S and C. Europe, SW Asia), shows a high presence of oxygenated compounds in C3 as piperitone, piperitenone and their epoxides as well as menthol and pulegone. Other compositions have been also reported as *cis*-piperitol and 1,8-cineol chemotypes [5]. This subspecies has the major economic interest because of its pharmacological and insecticidal properties [3], [13], [15]. Besides, the piperitenone oxide is useful as starting point for heterocycle synthesis [16].

Concerning *insularis* and *timija* subsp., their EO composition has also widely reported, showing a great chemical variability. Menthone, pulegone and isomenthone are characteristic compounds of *timija* subspecie [9], [17]. For *M. suaveolens* subsp. *insularis,* endemic of central and occidental Mediterranean islands, pulegone and *cis-cis-p*-menthenolide constitute the main and differential compounds [4], [18].

The influence of general environmental factors affecting EO chemical profiles has been referred for the populations in Northern Morocco [9], describing three typical profiles: piperitenone and piperitone oxides, piperitone oxide and pulegone. Nevertheless, no evident relationships between chemical composition and ecological factors (bioclimatic specific indices) have been reported.

On the other hand, despite of its ethnobotanical relevance, there are not chemical references from populations in Iberian Peninsula except for methanolic and dichlormethanolic extracts [19]. Likewise, comparative data relating organ (flowers and leaves) and diurnal variations have not been reported so far.

Based on the above mentioned evidences, the aim of this work has been to study EO profiles of *M. suaveolens* subsp. *suaveolens* leaves and flowers, as well as their diurnal variation, of samples coming from two populations in Eastern Iberian Peninsula with marked differences in environmental conditions

Results and discussion

Soil and climate characteristics

Soil characteristic correspond to loamy-sand and sand-loam with differences in carbonates (% CO_3^{2-}), organic matter (% OM) and electric conductivity (EC) between the two localities Tavernes (TAV) and Calamocha (CAL) (table 1).

Table 1. Soil characteristics of the sampling zones (Mean values ± standard error)

Location	Horizon (*)	pH	EC ($\mu S/cm^2$)	% OM	% CO_3^{2-}	Sand %	Texture
TAV (n=3)	A	8.4±0.1	171.3±8.0	3.8±0.4	64.1±11.4	83.2±2.5	Loamy sand
	B	8.4±0.0	151.9±2.0	2.4±0.5	50.3±4.0	82.4±2.2	Loamy sand
CAL (n=3)	A	8.0±0.0	220.3±6.1	3.1±0.5	16.9±2.7	76.3±2.2	Sandy loam
	B	8.2±0.0	172.9±18.6	1.8±0.2	16.3±1.8	77.2±1.4	Sandy loam

(*) A (15-20 cm); B (20-40 cm).

From bioclimatic viewpoint, both locations can be classified as thermomediterranean (Tavernes, Valencia) and supramediterranean (Calamocha, Teruel) according their bioclimatic indices (table 2) [8], [20]. The major differences were found in index of thermicity (It), mean of minimum temperature (Tmin), ombrothermic index (Io) and continentality index (Ic).

Table 2. Bioclimatic indices of Calamocha (Teruel) and Tavernes (Valencia)

Bioclimatic indices (*)	T	m	M	Tmax	Tmin	Ppv	PE	Tp	P	It	Ic	Iar	Io
Calamocha	11,7	-1,8	9,1	29,8	3,7	10,5	57,2	14,1	360,0	189,9	26,2	5,4	0,7
Tavernes	18,5	7,2	16,4	26,4	18,4	1,3	46,8	22,2	385,0	420,7	8,0	2,9	0,1

(*) T: yearly average temperature, m: average temperature of the minima of the coldest month of the year, M: average temperature of the maxima of the coldest month of the year, T max: average temperature of the warmest month, T min: average temperature of the coldest month, Ppv: precipitation of the three warmest months of the year, PE: annual mean of evapotranspiration (Thornthwaite), Tp: mean of positive temperatures, P: total yearly precipitation, Ic: Continentality index, T max - T min., Iar: Dryness index, PE/P. Io: Ombrothermic index, (P/12)*10/∑Tp.

Essential oil profiles

A total of 50 compounds were identified accounting for 88.1 -99.6 % of the volatile constituents (Table 3). Despite the great intrapopulation variability which was noted in some compounds, two major oxygenated monoterpenes could be distinguished: piperitenone oxide (0.5-91.3 %), and

piperitone oxide (0.0-74.3 %). Other compounds accounting for noticeable amounts were limonene (0.7-10.1 %) and germacrene-D (0.4-4.8 %).

Table 3. EO chemical composition (% peak areas) of _Mentha suaveolens_ subsp. _suaveolens_ from Easthern of Iberian Peninsula populations.

COMPOUNDS[a,b]	Id. Method[c]	RI[d]	TAV LEAVES MORNING[e]			TAV LEAVES EVENING[f]			TAV INFLORESCENCES MORNING			TAV INFLORESCENCES EVENING			CAL LEAVES MORNING			CAL LEAVES EVENING			CAL INFLORESCENCES MORNING			CAL INFLORESCENCES EVENING		
α-Pinene	MS, RI, PS	935	0.5	0.6	0.5	0.6	0.6	0.5	1.5	2.2	0.9	0.6	0.7	0.7	0.2	0.3	0.3	0.2	0.2	0.1	0.6	0.4	0.9	0.5	0.2	0.4
Sabinene	MS, RI	973	0.4	0.3	0.4	0.8	0.5	0.5	0.8	1.2	0.5	0.7	0.3	0.4	0.2	0.4	0.2	0.2	0.5	0.1	0.5	0.5	0.5	0.3	0.2	0.3
β-Pinene	MS, RI, PS	978	0.6	0.8	0.8	0.8	0.6	0.5	1.6	2.4	0.9	0.8	0.5	0.7	0.3	0.5	0.4	0.3	0.3	0.2	0.9	0.9	1.0	0.6	0.3	0.5
Myrcene	MS, RI, PS	988	0.6	0.4	0.6	1.1	0.6	0.8	1.6	2.2	0.9	0.8	0.6	0.7	0.5	0.6	0.5	0.5	0.5	0.3	1.3	0.9	1.0	0.7	0.5	0.6
Mentha-1(7),8-diene	MS, RI	1001	tr[g]	tr	0.1	0.5	0.1	0.1	tr	-[h]	tr	0.1	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-
Limonene	MS, RI, PS	1031	1.9	1.8	1.9	2.4	1.9	0.9	9.5	8.9	4.8	4.2	2.4	2.5	2.7	1.6	1.1	1.6	1.2	0.7	10.6	7.0	7.5	2.7	2.5	2.6
(E)-β-Ocimene	MS, RI, PS	1045	0.7	0.7	0.8	1.1	0.6	0.7	0.3	tr	-	-	tr	-	0.3	0.2	0.3	0.3	0.2	0.3	0.2	0.1	0.2	0.1	-	0.1
γ-Terpinene	MS, RI, PS	1060	0.1	-	0.1	1.0	0.2	0.3	-	-	0.1	0.1	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-
Terpinolene	MS, RI, PS	1083	2.1	-	3.3	13.0	6.8	7.1	0.1	tr	1.0	2.0	1.7	1.2	-	-	-	-	-	-	-	-	-	-	-	-
HYDROCARBON MONOTERPENES			**6.9**	**4.7**	**8.6**	**21.2**	**12.0**	**11.6**	**15.5**	**17.2**	**9.0**	**9.6**	**6.2**	**6.4**	**4.2**	**3.4**	**2.9**	**3.0**	**2.9**	**1.7**	**14.2**	**9.8**	**11.0**	**4.8**	**3.7**	**4.5**
Terpinen-4-ol	MS, RI, PS	1171	0.4	0.1	0.3	0.2	0.2	0.6	0.3	0.2	0.2	0.1	0.1	0.2	0.1	tr	0.1	0.1	0.1	0.1	0.1	0.1	0.1	-	-	-
Borneol	MS, RI, PS	1178	0.1	tr	0.1	-	tr	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	tr	0.1	tr	0.1	0.1	0.2	0.1	0.2	0.2
α-Terpineol	MS, RI	1181	0.2	tr	0.2	1.4	0.4	0.5	0.1	-	0.2	0.3	0.2	0.2	-	-	-	-	-	-	-	-	-	-	-	-
Pulegone	MS, RI	1237	1.2	0.4	0.6	0.1	0.3	0.5	0.1	-	0.1	-	0.1	0.2	0.1	-	0.1	0.1	0.1	0.1	0.1	-	-	-	0.1	-
Carvone	MS, RI	1241	0.9	0.7	0.6	-	0.6	0.6	0.1	-	0.5	0.5	0.4	0.4	-	-	-	-	-	-	-	-	-	-	-	-
Piperitone oxide	MS, RI	1251	69.1	74.3	64.1	59.5	60.5	65.8	53.3	55.5	39.3	35.2	37.6	60.2	0.3	0.3	0.5	0.3	0.3	0.3	0.2	0.5	0.6	0.5	0.7	0.6
Piperitenone	MS, RI	1267	0.3	-	-	-	1.0	0.5	0.1	0.1	0.4	0.3	0.3	0.4	-	-	-	-	-	-	-	-	0.1	-	-	-
Isopiperitenone	MS, RI	1271	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.2	tr	0.1	tr	0.1	0.5	0.5	0.5	0.5	0.5
Bornyl acetate	MS, RI	1289	tr	-	-	-	tr	tr	0.2	-	0.9	0.9	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-
Piperitenone oxide	MS, RI	1379	2.3	1.3	1.1	1.5	1.5	0.5	22.5	22.5	42.8	48.6	48.8	26.8	88.7	88.4	86.7	88.7	87.3	88.5	80.7	86.0	83.9	90.6	91.3	90.3
OXIGENATED MONOTERPENES			**74.5**	**76.9**	**67.0**	**62.7**	**64.5**	**69.2**	**76.8**	**78.5**	**84.6**	**86.0**	**88.3**	**88.5**	**89.3**	**88.9**	**87.6**	**89.2**	**87.8**	**89.0**	**81.2**	**87.1**	**85.3**	**91.8**	**92.7**	**91.6**
β-Bourbonene	MS, RI	1390	0.1	tr	0.1	0.1	0.1	0.1	tr	-	-	-	-	-	0.1	0.1	0.1	0.1	0.1	0.1	-	tr	-	-	-	-
β-Elemene	MS, RI	1394	0.2	tr	0.3	0.2	0.2	0.3	0.1	-	0.1	-	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1	-	tr	-	-	-	-
(E)-β-Caryophyllene	MS, RI	1422	1.7	2.2	1.8	2.3	1.8	1.6	1.3	1.1	0.7	0.9	0.6	0.4	0.4	1.4	0.9	0.7	1.2	1.0	0.2	0.1	0.1	0.4	0.4	0.3
β-Copaene	MS, RI	1433	0.6	0.4	0.8	0.1	0.6	1.5	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-β-Farnesene	MS, RI	1450	0.1	tr	0.2	0.5	0.3	0.4	0.2	-	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.1	-	0.1	0.1	0.1	0.1
α-Humulene	MS, RI	1455	0.5	0.4	0.4	0.3	0.5	0.9	0.3	0.2	0.3	0.1	0.2	0.3	0.5	0.2	0.3	0.5	0.3	0.6	0.2	0.2	0.2	0.1	0.2	0.1
Aromadendrene (allo)	MS, RI	1459	1.1	2.1	1.8	0.4	0.7	1.2	0.4	0.2	0.3	0.3	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-
Germacrene-D	MS, RI	1484	3.2	2.7	4.8	4.8	4.3	4.1	1.5	0.6	0.9	0.9	0.8	0.6	2.4	3.2	3.1	3.2	3.7	3.9	0.8	0.4	0.6	0.7	0.8	0.7
Bicyclogermacrene	MS, RI	1494	0.2	0.3	-	0.2	0.1	0.1	tr	0.1	-	0.1	-	-	-	0.1	-	-	0.1	-	-	-	-	-	-	-
HYDROCARBON SESQUITERPENES			**7.6**	**8.3**	**10.3**	**8.7**	**8.5**	**10.1**	**4.4**	**2.1**	**2.4**	**2.3**	**1.8**	**1.6**	**3.5**	**5.2**	**4.7**	**4.8**	**5.7**	**5.9**	**1.2**	**0.6**	**0.9**	**1.4**	**1.5**	**1.3**
Caryophyllene oxide	MS, RI	1579	0.1	-	0.1	0.1	0.1	0.1	0.1	-	-	-	-	-	0.6	0.4	0.4	0.6	0.6	0.6	0.1	0.1	0.1	0.1	0.1	0.1
Cubenol <1,10-di-epi>	MS, RI	1619	0.1	-	0.1	-	0.1	0.2	0.1	-	tr	-	-	0.1	0.1	0.1	0.2	0.1	0.1	0.1	-	-	-	-	-	tr
Eudesm-7(11)-en-4-ol	MS, RI	1700	0.2	-	0.1	-	tr	0.2	tr	-	-	-	-	-	0.1	0.1	0.2	0.1	0.1	0.1	-	-	-	-	-	-
OXIGENATED SESQUITERPENES			**0.3**	**-**	**0.3**	**0.1**	**0.3**	**0.5**	**0.2**	**-**	**-**	**-**	**-**	**0.1**	**0.7**	**0.5**	**0.8**	**0.8**	**0.8**	**0.9**	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**
1-Octen-3-ol	MS, RI	980	0.9	0.2	0.8	1.9	0.8	0.6	1.4	0.7	0.4	0.8	0.5	0.3	0.9	0.7	0.8	0.5	0.7	0.5	0.8	0.8	0.9	0.6	0.7	0.5
3-Octanol	MS, RI	991	0.2	0.1	0.3	0.3	0.2	0.1	-	tr	tr	-	-	-	tr	tr	-	-	-	-	0.1	0.1	0.1	0.1	0.1	0.1
1-Octenil acetate	MS, RI	1008	0.4	0.3	0.3	0.6	0.7	0.1	-	0.1	-	-	-	-	-	-	-	tr	tr	tr	-	0.1	-	0.2	0.1	0.1
1-Octen-3-yl acetate	MS, RI	1113	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.1	0.3	0.3	0.2	0.3	-	-	-	0.1	0.1	0.1
(Z)-Jasmone	MS, RI	1393	1.0	0.4	0.7	0.1	0.8	0.9	0.1	0.3	0.6	0.1	0.8	0.9	0.4	0.6	0.8	0.7	0.8	0.8	0.2	0.4	0.6	0.5	0.6	0.6
OTHERS			**2.5**	**1.0**	**2.0**	**2.7**	**1.6**	**0.8**	**1.5**	**1.1**	**1.0**	**0.8**	**0.5**	**1.2**	**1.5**	**1.4**	**2.0**	**1.5**	**1.7**	**1.6**	**1.0**	**1.3**	**1.5**	**1.3**	**1.6**	**1.4**
TOTAL ID.			**91.9**	**90.8**	**88.1**	**95.4**	**86.9**	**92.2**	**98.4**	**98.9**	**97.0**	**99.6**	**97.3**	**97.8**	**99.2**	**99.4**	**97.9**	**99.3**	**98.9**	**99.1**	**97.8**	**98.8**	**98.9**	**99.4**	**99.4**	**98.9**

Compounds present at level < 0,1%: of total samples: (*Z*)-β-ocimene, γ-cadinene. Compounds in Tavernes samples at level < 0,1%: α-thujene, camphene, 1,8-cineole, *p*-cymen-8-ol, germacrene-A, germacrene D-4-ol, 2-hexenal. 2-hexenol, 1-nonen-3-ol, 3-octanol acetate, Compounds in Calamocha samples at level < 0.1%: edulan I dihydro.

Identificación method: MS (mass spectra), RI (Kovats retention index), PS (pure standards)

[a]Compounds listed in order of elution

[b]Kovats retention indices measured to *n*-alkanes (C8-C22) on the DB-5 column

[c] Morning sampling time (8.00 AM)

[d] Evening sampling time (8.00 PM)

[e]traces (tr)

[f]no detected (-)

According to these data, two typical profiles could be clearly characterized when the results were considered as a whole, without any distinction between leaves and flowers or sampling time in any location. These are shown in table 4 and they match up with some of the most frequently reported in the literature [3], [9], [13], [14], [21].

This way, a first approach to chemotype identification allows assigning the piperitone oxide chemotype to Tavernes (TAV) population and the pure piperitenone oxide chemotype to Calamocha (CAL) (Table 4).

Table 4. Characteristic differential compounds of the *M. suaveolens* subsp. s*uaveolens* chemotypes from Tavernes and Calamocha based on leaves and inflorescences EO composition (Results expressed as mean ± standard error, n = 12).

	Leaves			Inflorescences		
	CAL	TAV	P value	CAL	TAV	P value
Compounds						
Limonene	1.47 ± 0.28	1.80 ± 0.21	**0.0157**	3.05 ± 0.80	5.38 ±1.79	0.9852
Piperitone oxide	0.30 ± 0.04	65.56 ± 2.26	**0.0000**	0.52 ± 0.10	46.85 ± 6.18	**0.0000**
Piperitenone oxide	88.03 ± 0.35	1.37 ± 0.22	**0.0000**	87.13 ± 2.48	35.33 ± 7.37	**0.0000**
Germacrene D	3.24 ± 0.22	3.99 ± 0.34	0.0994	0.67 ± 0.09	0.88 ± 0.19	0.1707
Monoterpenes	3.02 ± 0.34	10.96 ± 2.40	**0.0083**	8.00 ± 2.47	10.65 ± 2.68	0.3041
Ox. Monoterpenes	88.61 ± 0.31	69.17 ± 2.28	**0.0000**	88.28 ± 2.62	83.78 ± 2.89	0.1221
Sesquiterpenes	4.99 ± 0.35	8.99 ± 0.42	**0.0000**	1.15 ± 0.20	2.43 ± 0.58	**0.0066**
Ox. Sesquiterpenes	0.77 ± 0.05	0.25 ± 0.08	**0.0003**	0.10 ± 0.00	0.05 ± 0.05	**0.0444**

Fig. 1. Diferential compounds of the *M. suaveolens* subsp. *suaveolens* chemotypes: piperitone oxide and piperitenone oxide (Mean values ± standard error, in % peak areas, I from inflorescenses; L, from leaves).
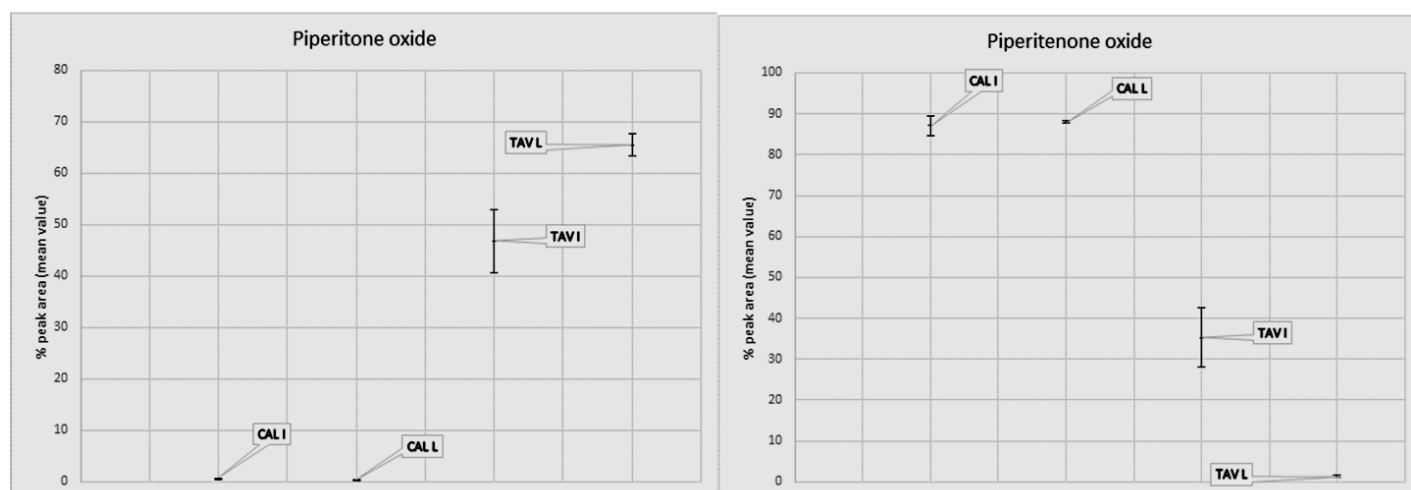

Environmental influences

In order to explain the origin of these differences, EO chemical composition data were related to bioclimatic indices and soil parameters, taking into account the organ (leaves or inflorescences) from which the oil was extracted (Fig.2).

The chemotype TAV, in thermomediterranean bioclimate, showed high concentration of piperitone oxide (59.5 – 74.3 %) in leaves and very low presence (0.5-2.3 %) of piperitenone oxide. Inversely, populations of CAL had the major concentration of piperitenone oxide in leaves (86.7 – 88.7 %) and minimum of piperitone oxide (0.3-0.5 %).

As for inflorescences, TAV samples showed a more balanced proportion for piperitone and piperitenone oxides (35.2-60.2 % and 22.5-48.8 % respectively). However, the proportion of these compounds found in the inflorescences from CAL samples was similar to that registered in CAL leaves (0.2-0.7 % and 80.7-91.3 % for piperitone and piperitenone oxides, respectively) (Fig. 1).

From data soil analysis and bioclimatic characteristics of the two studied areas, the canonical correspondence analysis permit us to consider the influence of the ombrothermic bioclimatic index (I_O), and some edaphic factors, such as salinity (EC) and water field capacity (WFC), on piperitenone oxide, and monoterpene and sesquiterpene oxides (chemotype CAL). On the other hand, carbonates, sand proportion, organic matter and major indices of mean of minimum temperatures (values correlated to positive axis F1, Fig. 2), were related to piperitone oxide, hydrocarbon monoterpenes and sesquiterpenes (chemotype TAV), showing a greater relative dispersion (Fig. 2).

Fig. 2. Characteristics oil components of *M. suaveolens* chemotypes in Eastern Iberian peninsula (TAV and CAL) and ecological factors related (EC, electric conductivity; I_O, ombrothermic index; WFC, water field capacity; OM, organic matter; $CaCO_3$, carbonates; TMIN, mean of minimum temperatures) . CCA, vector scaling: 1.16.
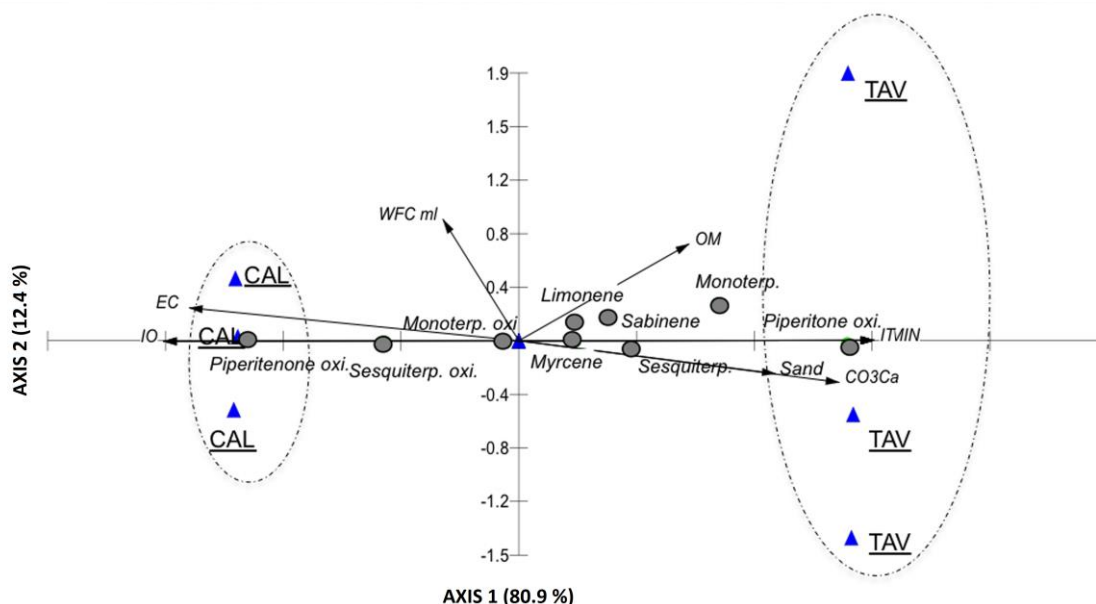
Diurnal variation of volatile scent compounds

Diurnal variation of principal groups of EO compounds for leaves and inflorescences are displayed in table 5.

Table. 5 Diurnal changes (morning (M) –evening (E)) of principal groups of EO compounds in leaves (L) and inflorescences (I) of *M. suaveolens* subsp. *suaveolens* (Values in % peak areas, average value ± standard error, P value, n = 3).

LOC.	ORGAN	PERIODE	MONOT.	MONOT. OXI.	SESQUIT.	SESQUIT. OXI.
CAL	L	M	3.50 ± 0.37	88.59 ± 0.54	4.49 ± 0.48	0.68 ± 0.07
		E	2.53 ± 0.44	88.63 ± 0.43	5.50 ± 0.35	0.86 ± 0.02
		P value	0.1695	0.9284	0.1824	0.1619
	I	M	11.69 ± 1.31	84.54 ± 1.75	0.89 ± 0.19	0.10 ± 0.02
		E	4.36 ± 0.36	92.00 ± 0.34	1.38 ± 0.04	0.11 ± 0.02
		Pvalue	**0.0032**	**0.0091**	0.0641	0.7087
TAV	L	M	6.80 ± 1.16	72.83 ± 3.01	8.78 ± 0.80	0.22 ± 0.11
		E	15.13 ± 3.17	65.52 ± 1.92	9.20 ± 0.44	0.27 ± 0.12
		Pvalue	0.0696	0.1082	0.7199	0.5484
	I	M	13.91 ± 2.51	80.22 ± 2.26	2.96 ± 0.70	0.09 ± 0.07
		E	7.49 ± 1.11	87.69 ± 0.78	1.94 ± 0.21	0.04 ± 0.02
		Pvalue	0.0710	**0.0340**	0.2064	0.8227

Hydrocarbon monoterpene fraction significantly decreases at evening in CAL inflorescences, whereas the oxigenated monoterpene one significantly increases from morning to evening (Tab. 5). The same evolution was observed in TAV inflorescences, hydrocarbon monoterpenes decrease although only marginally significant, meanwhile oxygenated monoterpenes significantly increase in the evening samples. No significant change was noticed for these fractions according both CAL and TAV leaves.

Regarding hydrocarbon and oxygenated sesquiterpenic fractions neither significant change according organ nor sampling time were observed. (Tab.5)

Nevertheless, having a look on table 3, this description is not so simple since noticeable differences related to specific compounds could be detected. Concretely, it is worth to carefully analyze changes in limonene, as it is the most abundant compound in the hydrocarbon monoterpene fraction. A high content of limonene was also found in flowers of *M. suaveolens* cultivated in Egypt. [11]. For this reason, an ANOVA focused on this compound was performed separately for leaves and flowers in order to clarify its ecological role (Fig.3)

As described in figure 3, the limonene reach the major concentrations from floral extracts at morning (8.4 % in CAL ; 7.7 % in TAV), reaching minimum values at evening (2.59% in CAL; 3.0% in TAV). On the contrary, minor values and no significant changes were observed in extracts from leaves.

According recent studies, scent compounds and flower smell can vary according hour of the day and nocturnal rhythms [22], temperature, relative humidity, sun exposition and pollinator activity. [23], [24, [25]. Likewise, as described by Figueiredo et al. [26], appreciable differences in volatile profiles can be expected according the plant organ because the typically entomophilous nature of *Mentha* species flowers.

Fig. 3 .-Limonene concentration (mean values of % peak areas ± standar error) in CAL and TAV chemotypes of *M. suaveolens*. Diurnal changes in inflorescences and leaves (M: morning; E, evening)
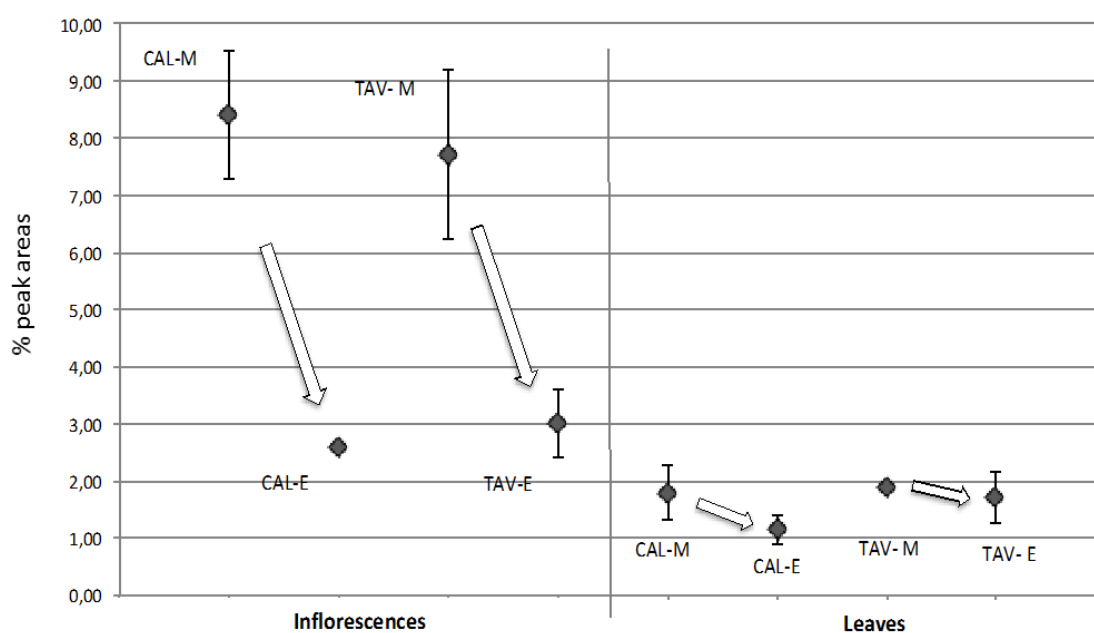
Conclusions

Well defined typical chemotypes of *M. suaveolens* Ehrh. subsp. *suaveolens*, have been identified in the studied populations. Statistical analysis confirmed that the existence of different chemotypes is significantly related to specific environmental factors, mainly the bioclimatic ones, of each location. In addition, some edaphic parameters could also have some influence. Thus, the piperitenone oxide chemotype can be related to harsher climatic conditions and soils showing relative major salinity in A horizon and water field capacity. On the other hand, piperitone oxide chemotype comes to be associated to milder climate conditions and high levels of carbonates, sand and organic matter. These results suggest that plant EO could vary responding to climate stress and soil characteristics.

Combined analysis of diurnal variations gives relevant information contributing to clarify the ecological role of volatile composition in leaves and inflorescences. Diurnal variations do not affect in the same way neither both chemotypes nor leaves and inflorescences. Piperitenone oxide chemotype shows a noticeable stability throughout the day in leaves EO composition whereas significant changes happen in flowers. Piperitone oxide chemotype shows diurnal variations both in leaves and flowers. All these changes affect specifically to monoterpenic (hydrocarbon and oxygenated) fraction, whereas sesquiterpenic compounds show major stability for leaves and flowers of both chemotypes. These differences can be interpreted from the ecological point of view. Diurnal variation affecting the most volatile fraction (hydrocarbon monoterpenes) was mainly due to changes in limonene abundance, which is maximum during the morning for both chemotypes, probably playing an important role for the attraction of pollinators.

Given that the high intrapopulational variability probably hides other significant relationships, more extensive studies based on diurnal monitoring of individuals should be performed [27]. Moreover, this monitoring should be carried at different times throughout the day, according the foreseeable pollinator activity. On the other hand, in order to associate changes in volatile composition with pollinating insects' activity, a more realistic analysis of volatile emissions by means of collection methods such as solid phase microextraction (SPME) could be required.

Author Contribution Statement

Llorens-Molina, J. A.: General coordination of work**, s**ampling process, chemical identification, data processing, paper writing.
Rivero, C: Extraction of samples and soil analysis.
Vacas, S: Chromatographic analysis, English supervision.
H. Boira: General advice, statistical treatments, botanical foundations and identification of vouchers.

Experimental Section

Environmental factors. Soil analysis and bioclimatic indices.

Principal soil characteristics were determined from three soil samples by locality. They were obtained at the same time the plant samples were collected. Soil texture studies were carried out by mechanical sieving and classification according to Boyoucos method [28]; inorganic carbonates were determined by means of a pressure calcimeter (Bernard calcimeter) [29] method, whereas Walkey & Black [30] procedure was followed to determine total organic matter. Measures of conductivity were determined by conductivity meter from aqueous saturation extracts of soil samples.

The study areas were characterized by bioclimatic indices [8] such as positive temperature (T_p), thermicity (I_t), IC (continentality), I_{ar} (aridity) and I_o (ombrothermic index). They were calculated from 30 years climatic data of Calamocha (Teruel, Spain) and Valencia (Spain).

Plant material

In order to study EO composition, three samples for leaves and inflorescences, at morning and evening periods (8 AM and 8 PM) were collected at the flowering stage (July-August) in Tavernes (TAV) (Valencia), (2 m.a.s.l.; 39º 03' 39.03'' N, 0º 13' 13.30'' W; total 24 samples) and Calamocha (CAL) (Teruel), (917 m.a.s.l.; 40º 56' 00.62'' N, 1º 19' 29.26'' W) localities. Each sample was composed by 10 individuals randomly selected in each one of the sampling zones. After removing the lignified stems, inflorescences and leaves were kept separately for each one of the samples. This way, a total amount of 24 samples (2 locations x 2 times, in triplicate) were processed both for leaves and inflorescences. The fresh material was frozen at -40 ºC until extraction. Voucher specimens are deposited in the Herbarium of Mediterranean Agroforestal Institute (VALA, Nos. 9574, 9575), at the Polytechnic University of Valencia (Spain).

Analysis of the essential oils

The volatile compounds were isolated from deep-frozen fresh material by simultaneous distillation-extraction for 3 h, using a Likens-Nickerson type apparatus [31]. The extracts were dried with anhydrous sodium sulphate and evaporated under reduced pressure at room temperature. After adding 1.5 mL of dichloromethane, the extracts were kept in sealed chromatographic vials until analysis.

The analysis of samples was carried out by GC-FID and GC-MS. A Clarus 500 GC (Perkin-Elmer Inc., Wellesley, PA, USA) chromatograph equipped with a flame ionization detector (FID) and capillary column ZB-5 (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Phenomenex Inc, Torrance, CA, USA) was used for quantitative analysis. The injection volume was 1 μL. The GC oven temperature was programmed from 50°C to 250°C at a rate of 3°C min−1. Helium was the carrier gas (1.2 mL min−1). Injector and detector temperatures were set at 250°C. The % peak areas was computed from GC peak areas without correction factors by means of the software Total Chrom 6.2 (Perkin-Elmer Inc., Wellesley, PA, USA).

Analysis by GC-MS was performed using a Clarus 500 GC-MS with the same capillary column, carrier and operating conditions as described above for GC analysis. Ionization source temperature was set at 200°C and 70 eV electron impact mode was employed. MS spectra were obtained by means of total ion scan (TIC) mode (mass range m/z 45-500 uma). The total ion chromatograms and mass spectra were processed with the Turbomass 5.4 software (Perkin-Elmer Inc.). Retention indices were determined by injection of C8–C25 *n*-alkanes standard (Supelco) under the same conditions. The EO components were identified by comparison of retention indices and mass spectra by computer library search (NIST MS 2.0) and available data from literature [32]. Identification of the following compounds was confirmed by comparison of their experimental retention index (RI) with those of authentic reference standards (Sigma-Aldrich): α-pinene, β-pinene, camphene, myrcene, limonene, (Z)-β-ocimene, camphor, terpinolene, terpinen-4-ol, borneol and bornyl acetate.

Data processing

The percentage compositions of the oil samples were used as matrix elements to determine the relationship between the 24 samples from the two studied populations.

Firstly, analysis of variance (ANOVA) and principal component analysis (PCA) were applied to data matrix in order to determine data variability and relation between EO components, respectively (Statgraphics Centurion XVI. 2 statistical package). Canonical correspondence analysis (CCA) was applied to refer the relationship between principal compounds and ecological factors, (Multi-Variate Statistical Package (MVSP) V 3.01).

Original data were subjected to arcsin[square root (%/100)] transformation in order to homogenize the variance.

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