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RESEARCH ARTICLE

Chemodiversity of wild populations of aromatic plants as source of valuable essential oil profiles. A study on *Thymus vulgaris* L. from Valencia (Spain)

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Abstract

Chemodiversity of wild populations of aromatic plants is a valuable source of essential oils, whose composition may be suitable for specific purposes according their biological activity. Furthermore, knowing the intrapopulational variability based on individual analysis has allowed characterizing atypical profiles, which can reach high levels of active compounds. Obviously, it requires the treatment of a high number of individual samples. In this work, a methodology to characterize *T. vulgaris* profiles in an area of recognized biodiversity was proposed and applied. After Thin Layer Chromatography (TLC) screening data of 85 individual samples, 7 groups, and 13 individuals were classified. Then, 20 samples were subjected to GC/MS and GC/FID analysis, respectively. These data were subjected to Hierarchical Agglomerative, Discriminant Analysis and ANOVA, which finally highlighted five profiles: (1) based on the camphane skeleton (camphene, camphor and borneol), (2) rich in the oxygenated sesquiterpenic fraction, (3) rich in 1,8-cineole, with appreciable amounts of camphor and borneol (typical chemotype from Eastern Iberian Peninsula), (4) camphor and terpinen-4-ol as major compounds, and (5) linalool chemotype. It should be noted that the percentages of the main compounds in these groups were higher than some of those described in the literature for similar chemotypes. In summary, the preliminary screening by TLC, grouping individuals with similar profiles, allowed establishing a quick first approximation to the chemodiversity of *T. vulgaris* in the studied area. Furthermore, the analysis of unclassified and potentially atypical individuals has also provided valuable information to establish the final profiles.

Keywords: *T. vulgaris*, thin layer chromatography, chemodiversity, atypical individuals, wild populations

Introduction

Over millions of years of evolution, the nature has provided us with an enormous amount of secondary plant metabolites whose biological activity makes them potentially useful natural resources (Verpoorte, 1998; Cordell, 2000; Bakkali et al., 2008). Among them, essential oils (EOs) are particularly promising from the perspective of sustainability and environmental protection. Indeed, during the last two decades an extensive review literature is available on specific fields such as food processing and conservation (Sanchez-González et al., 2011; Calo et al., 2015, Anupama et al., 2019), pest and weed control in agriculture (Tworkoski, 2002; Tripathi et al., 2009), or aromatherapy and health care (Edris, 2007; Koo, 2017).

Comparing the cultivation of aromatic plants for EOs production with their collection from wild populations, the first allows to fulfil quality requirements due to the standardization of their chemical profiles (Kindlovits

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& Németh, 2012). On the other hand, it also contributes to improve the biodiversity conservation, especially as a consequence of the protection of certain species, sometimes endemic. In this way, the selection and domestication of plants from wild populations to create chemically uniform cultivars has been widely applied in numerous aromatic plants (Bernáth, 2002; Dudai, 2011). With respect to *Thymus* species, some experiences have been reported in a great diversity of environmental conditions (Salas, 2014). Concerning *Thymus vulgaris* L. (thyme), the work reported by Delpit (2000) on the clonal selection of sabinene hydrate rich individuals, and the improvement of its micropropagation *in vitro* (El-Banna, 2017), can be cited as representative examples.

The high intrapopulational chemodiversity of EOs coming from wild populations of many species of aromatic and medicinal plants can be considered as an opportunity for finding particularly useful genotypes (Németh, 2016). Furthermore, to study in which way each genotype is affected by environmental and ontogenetic factors is also required to ensure the chemical identity of new developed cultivars (Figueiredo et al., 2008).

This methodological approach demands, beyond the study of EO chemical profiles from bulk population samples, a research focused on the analysis of individual plants. It has revealed the existence of atypical profiles, apart from the known chemotypes, which may be of particular interest for their propagation and cultivation (Judzentiene, 2016). Thus, the key point is how to identify these valuable profiles in such a way that a significant number of samples could be processed. It requires simple and fast methods to achieve a first path to explore the chemodiversity of wild populations. In that respect, as reported by Pothier et al., (2001); Taylor, (2001); Njenga, (2005); Chapman, (2009) and Franz, (2010); thin layer chromatography (TLC) can be considered as a useful methodology because of its simplicity, speed, reliability, economy and possibility of using in field conditions.

Many recent reviews enhanced the increasing interest of thyme cultivation due to its wide range of applications derived from its biological activity and sensory characteristics: it is used in medical and pharmacological applications (Sáez, F. & Stahl-Biskup, 2002; Reddy et al., 2014; Hosseinzadeh, 2015; Miraj & Kiani, 2016; Kuete, 2017), and in food preservation (Embuscado, 2015), for example. On the other hand, *T. vulgaris* shows a noticeable chemical polymorphism which has been extensively studied and several main chemotypes based on the oxygenated monoterpenic compounds have been reported. They can be grouped in two main types specifically related to their potential applications: (a) phenolic, with thymol and carvacrol as more representative compounds, typical of milder and drier Mediterranean environment; and (b) non-phenolic chemotypes, which are characterized by the predominance of cyclic and acyclic aliphatic oxygenated monoterpenes, adapted to a wider range of habitats, even to extreme climates (Kulevanova et al., 1996)

As reported by Thomson et al. (2003), four of these non-phenolic chemotypes were found in Southern France and European countries (Satyal et al., 2016), defined by the predominance of geraniol, α-terpineol, thujan-4-ol and linalool. In addition, another nonphenolic chemotype rich in 1, 8-cineole was reported as endemic of Iberian Peninsula (Guillem & Manzanos, 1998; Jordan et al., 2006, Torras et al., 2008), although its occurrence in Southern France has also been cited (Keefover-Ring, 2009). Other chemotypes based on the bornane skeleton (camphene, borneol and camphor) have also been reported from several countries (Giordani et al (2004), such as Morocco (Imelouane et al., 2009); Iran (Kazemi, 2015); Brazil (Kohiyama et al., 2015) or Italy (Mancini et al. 2015).

The aim of this work was to study the chemodiversity of *T. vulgaris* in a relatively small area located in La Safor, a coastal region in the Valencian Community (Spain), which is characterized by its high biodiversity due to the wide range of edaphoclimatic conditions. A methodological approach is proposed in order to carry out this type of studies. It can be summarized according the following process:

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(1) A first stage of field work, in which a high number of individuals is selected trying to obtain a representative set of the different environmental conditions that can influence the composition of the EO, mainly altitude and orientation, due to its relationship with the possible microclimatic differences.
(2) Screening by TLC of individual extracts. Afterwards, TLC profiles are visually grouped according the presence of discriminant spots, whose chemical composition is identified both by comparing with pure standards and preparative TLC followed by the GC/MS. Unclassified or doubtful individuals are considered as potentially atypical, to be subsequently independently analysed.
(3) Once the classification has been performed, the plant material coming from the grouped and potential atypical individuals is subjected to hydrodistillation or simultaneous distillation extraction (SDE) according the amount of available material. Then, these samples are analysed by GC/MS and GC/FID.

Material and Methods

Area of study

The study area was located in the Mondúver mountain, a calcareous mountainous massif. From the geomorphological point of view, Mondúver is a cretacic massif with Jurassic stratums, in which karstic landforms are widely represented. All these geographical features, together with a complex orography, give rise to a great diversity of microclimates and soil types, which lead to a high plant biodiversity. It covers an area of approximately 25 km^2, where a protected natural area of 650 ha (Parpalló-Borrell) and a micro-reserve of flora (0.95 ha) are placed, as well as a meteorological station (Barx-La Drova, 39° 0' 18.00" N, 00° 17' 24.36" W, 379 m asl), to which the following data correspond: yearly average temperature: 15,9 ºC; yearly average precipitation: 928,6 mm; compensated thermicity index = 360 (Rivas, 2004). According to these data, this area can be classified belonging to upper thermomediterranean bioclimatic floor. As for characterizing its climatic conditions, it is important to indicate the irregularity of rainfall, largely influenced by the NE-SW orientation, with a marked summer drought and relatively frequent episodes of torrential rains (which can exceed 200 mm daily). In addition, frosts are not frequent (eight days a year, on average) due to maritime influence.

Plant material

The sampling of plant material was performed in such a way that different orientations and altitudes were considered, avoiding areas with special protection (Figure 1). Samples formed by small cuts of 85 individuals were collected during full flowering stage (August 2018). Each individual plant was marked in order to carry out possible subsequent analysis or to obtain material for its propagation. A voucher specimen of each individual sample was submitted to identification in the Mediterranean Agroforestry Institute (IAM) of Universitat Politècnica de València (UPV).

After TLC screening, vouchers of material from grouped individuals according the similarity of their TLC profiles and unclassified ones were deposited at the herbarium of IAM, UPV (VALA 9581-9600). The rest of material was kept in sealed bags at -40 ºC for further analysis.

It should be noted that in the studied area had been reported two species of the genus *Thymus*: *Thymus piperella* (phenolic, and clearly distinguishable morphologically) and *T. vulgaris*, of which three subspecies have been identified: *T. vulgaris* ssp. *vulgaris, T. vulgaris* ssp*. aestivus* and *T. vulgaris* ssp. *mansanetianus*, with certain specific morphological characteristics (Gallego et al., 2013; Mateo & Crespo, 2014).

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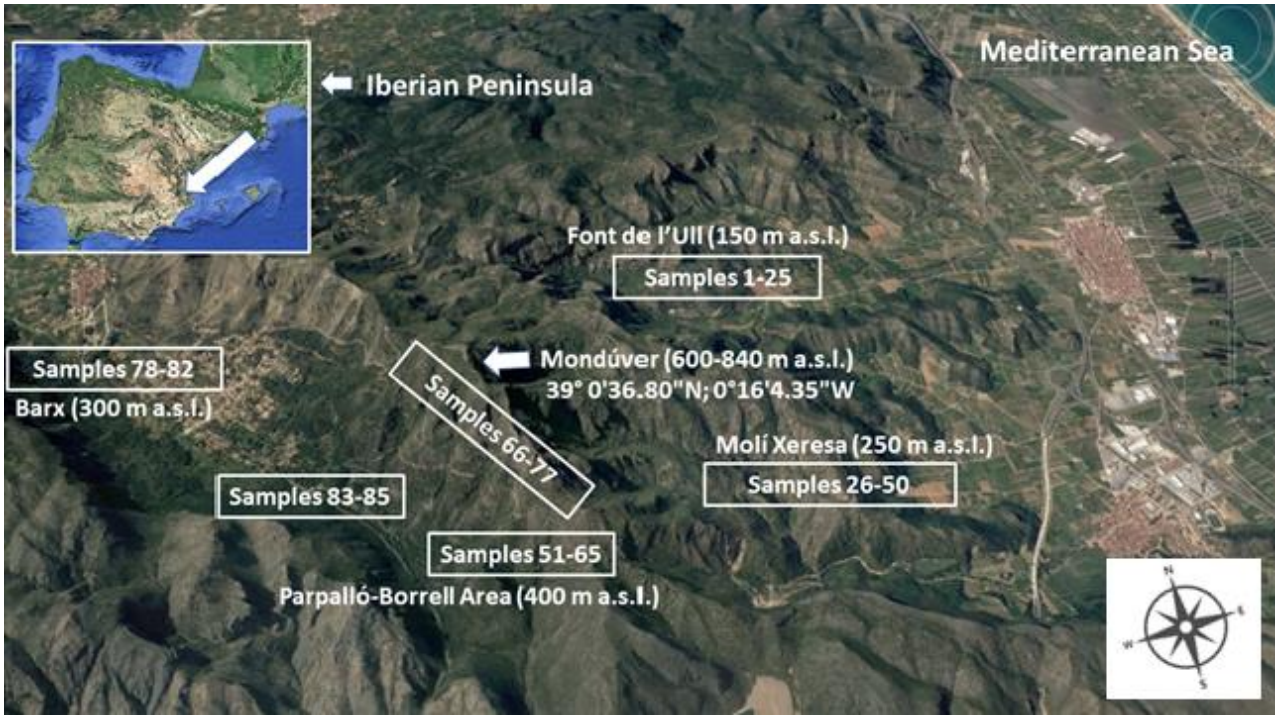
Figure 1. Geographical distribution of the samples



TLC screening

Extraction

The extraction and TLC analysis were performed according to Wagner & Bladt (1996). Small amounts of individual samples (0,2 g) with 2 mL of dichloromethane (Sigma-Aldrich™, for GC analysis) were subjected to stirring in 5 mL glass vials for 30 min. Afterwards, they were dried with anhydrous sodium sulphate and filtered with a syringe filter (Teknokroma™). Dried extracts were transferred to other vials and placed opened in fume hoods up to total solvent evaporation. Finally, 100 μL of toluene were added to each vial. They were sealed and kept in refrigerator at 4º C until TLC analysis.

TLC analysis

From each extract, 10 μL were spotted with capillary tubes (Blaubrand intraMark, 10 μL) on the silica gel plates (DC-Fertigfolien Alugram Sil G/UV 254). They were developed in duplicate using toluene:ethyl acetate (93:7) as a mobile phase, and further stained with sulphuric vanillin and *p*-anisaldehyde (UV 265 nm), respectively, as stain reagents, as described by Wagner & Bladt (1996). From the visual analysis of plates, spots showing different colour and/or retention factor (R_f value) were marked for further validation to ensure their chemical identity.

TLC method validation

TLC plates were spotted with 25 μL of sample extracts, and developed in the same conditions, but they were not sprayed with any visualization reagent. Silica gel layer, matching to measured range for discriminant spots, was scrapped and extracted with 0.5 mL of dichloromethane. The filtered extract was allowed to evaporate, until its volume was reduced to approximately 300 μL, which was kept and sealed in 350 μL insert vials until GC/MS analysis. These extracts were analysed by GC/MS in order to identify their composition. The presence of 1,8-cineole, borneol, camphor and linalool were also confirmed by comparing with pure standards.

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Characterization of chemical profiles

SDE extraction

Plant material coming from grouped individuals was put together and homogenized to obtain representative samples (5-10 g). These samples and those originated from each of the unclassified individuals, were subjected to simultaneous extraction distillation using a Godefroot type apparatus (De Frutos et al., 1988) using dichloromethane (≥99.9%, capillary GC grade, Sigma-Aldrich™) as solvent, for 3 h. The extracts were dried with anhydrous sodium sulphate and concentrated under reduced pressure at room temperature up to 1.5 mL. They were kept in sealed chromatographic vials and stored at -18ºC until GC analysis.

GC and GC/MS analysis

The analysis of samples was carried out by gas chromatography with flame ionization detector (GC-FID) and mass spectrometry (GC-MS). A Clarus 500 GC (Perkin-Elmer Inc. Wellesley. PA. USA) chromatograph equipped with a FID detector 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 the 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 percentage composition of the EO was calculated 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 (Perkin-Elmer Inc.) apparatus equipped with the same capillary column, carrier and operating conditions described above for GC-FID 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 C_8–C_{25} *n*-alkanes standard (Supelco, Bellefonte, PE, USA) under the same conditions.

The EO components were identified by comparison of calculated retention indices and high probability matches according to mass spectra computer library search (NIST MS 2.0) and available data from literature (Adams, 2007). A shorter run was applied to identify discriminant spots. Identification of the following compounds was also confirmed by comparison of their experimental lineal retention index (LRI) with those of authentic reference standards (Sigma-Aldrich™): α-pinene, β-pinene, camphene, myrcene, limonene, (*Z*)-β-ocimene, camphor, terpinolene and terpinen-4-ol.

Statistical analysis

All the data obtained from GC-FID analysis were processed using Statgraphic Centurion XVI. Agglomerative Hierarchical Clustering (Square Euclidean Distance and Wards method as aggregation criterions) was performed on percentages of compounds (over 5 % at least on three samples) in order to group the most characteristic EO profiles. Then, these data were subjected to Discriminant Analysis to validate the classification.

The significance of differences among the final defined profiles was determined by means of analysis of variance (ANOVA) using Statgraphics 5.1. Software. Tukey's HSD multiple-range test at $P < 0.05$ was used to find out significant differences among average values. As the original data were expressed like percentage (%) peak areas, they were subjected to arcsin [square root (%/100)] transformation and previously their homocedasticity was tested.

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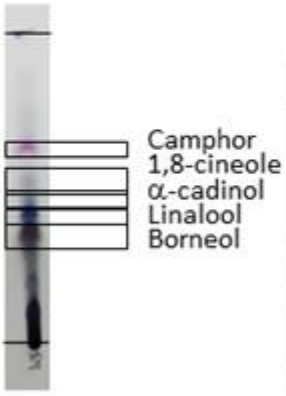
Results and Discussion

Preliminary screening

From the 85 individual profiles, 47 were found clearly similar, showing a pattern of spots like the one displayed in Fig. 2A (majority profile). Among the other ones, some groups could be visually established based on the presence with remarkable intensity or the absence of certain discriminant spots which are described in Fig. 2. Their GC/MS analysis allowed to determine their composition, so that the following groups were defined according the occurrence of the following representative compounds: B. Linalool (5 individuals: 9,49,51,52,53), C. Borneol (4 individuals: 10, 56, 57, 67), D. α-Cadinol (8 individuals: 58, 60, 61, 71, 72, 73,74,75), E. 1,8-Cineole (2 individuals: 17, 18), F. Camphor and borneol, (2 individuals: 12,65) G. 1,8-Cineole and borneol (4 individuals: 15, 50, 62, 66).

Figure 2. TLC screening: (A) representative profiles, (B-G) discriminant spots (Rf range) and mass spectra.

A (Majority profile)



Discriminant spots			Main peak CG/MS	
Rf range	Stain reagent		LRI	Compound
	Vanillin	Anisaldehyde		
0,59-0,65	Pink	Purple	1146	Camphor
0,47-0,56	Blue	Purple	1031	1,8-Cineole
0,42-0,48	Blue	Pink	1096	Linalool
0,38-0,43	Deep blue	Pink	1646	α-Cadinol
0,30-0,38	Deep brown	Brown	1096	Borneol

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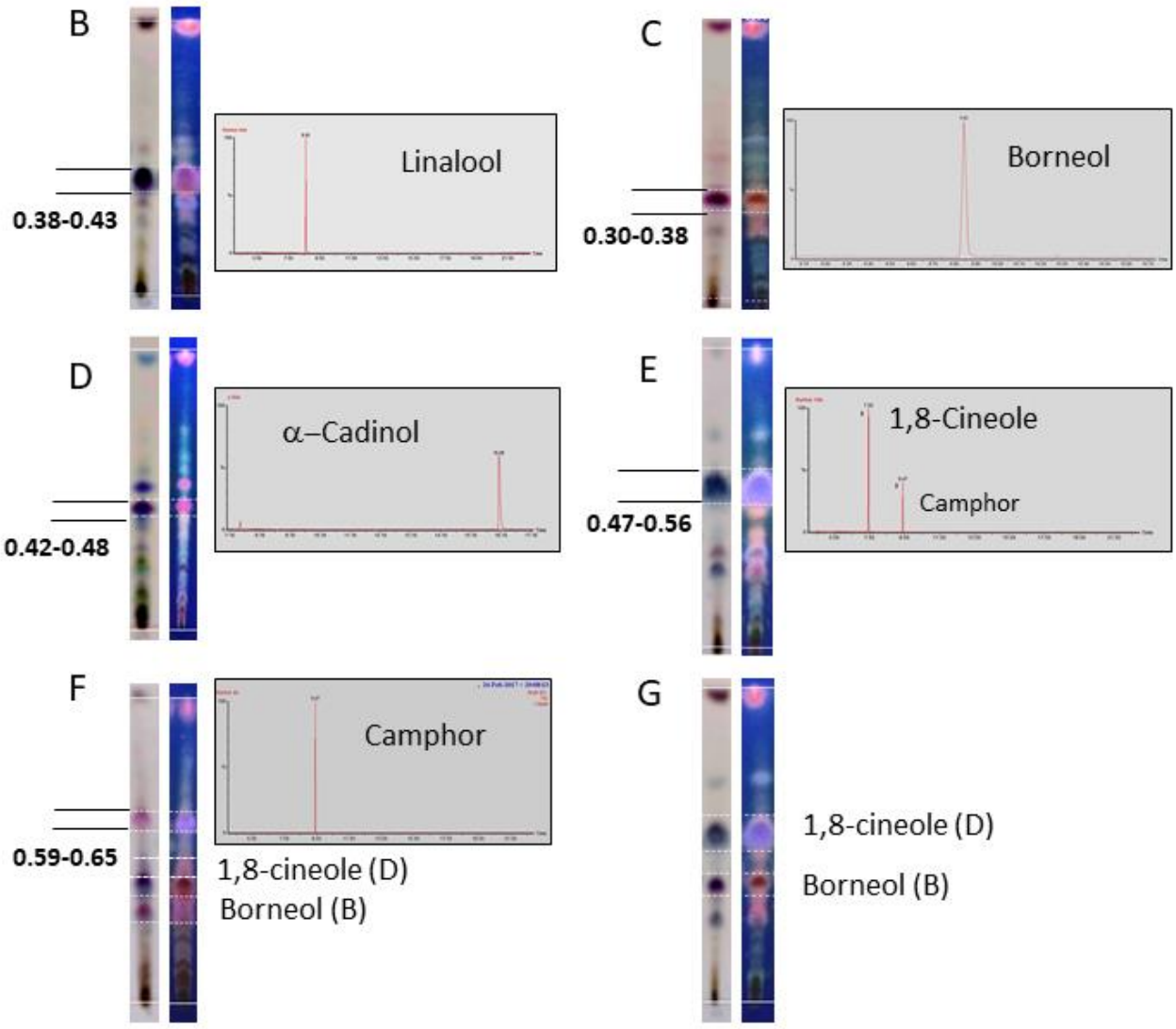
Figure 2. (Cont.)



As an example, individuals grouped according their high level of linalool are shown in Fig.3. Finally, the profiles of 13 individuals could not be clearly classified, so they were directly analysed by GC/MS and/or GC/FID.

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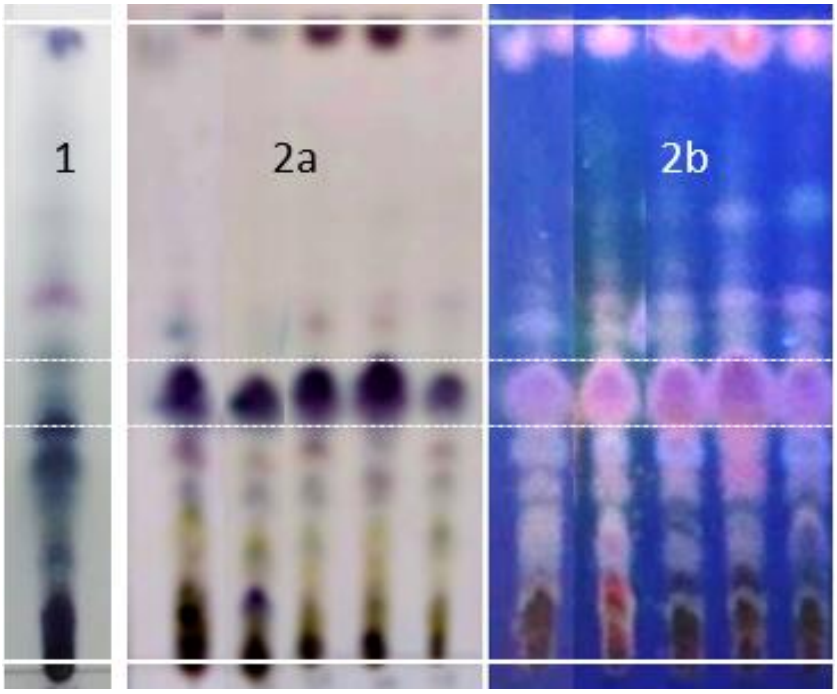
Figure 3. The five individuals grouped according their high amount of linalool



1: Representative TLC plate of predominant profile. 2a: stained with vanillin sulphuric. 2b: stained with anisaldehyde sulphuric.

This screening methodology was applied to select chemotypes of *Mentha longifolia* (L.) Huds. from wild populations (Llorens-Molina et al., 2017) in order to obtain chemical homogeneous plots of this species. Seasonal changes of these selected accessions have been recently studied (Llorens-Molina et al., 2020). To the best of our knowledge, this type of methodology has not been applied so far to study the chemodiversity of wild populations or to identify atypical EO profiles.

GC results

Samples from grouped individuals

In summary, 20 samples (one from each group A-G and the 13 non-classified individuals) were subjected to GC/MS and GC/FID analysis. As example, Total Ion Chromatograms (TIC) of the samples A, B, D, F are displayed in Figures 4-7.

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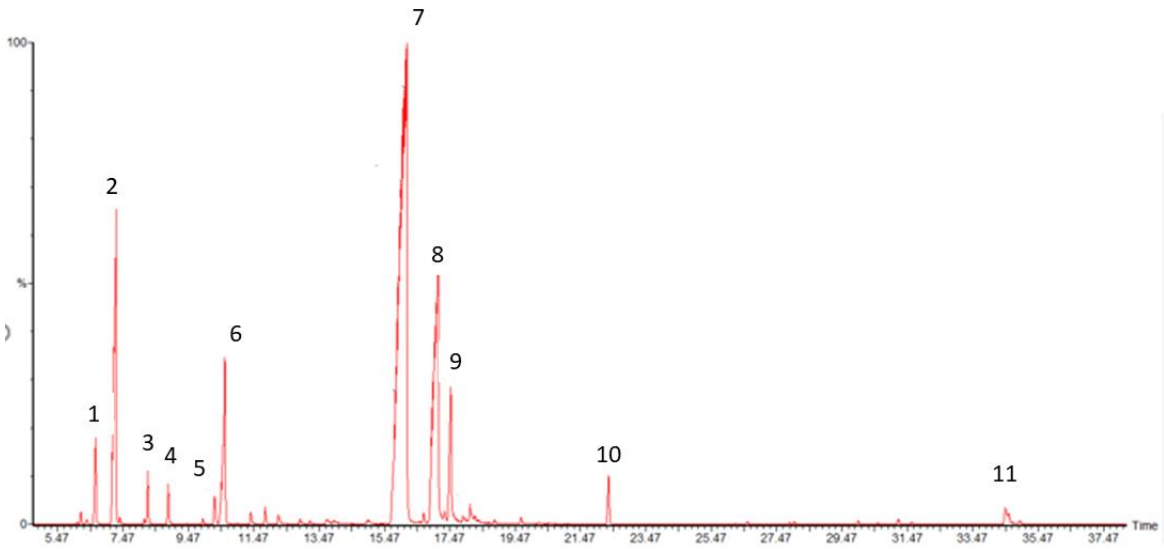
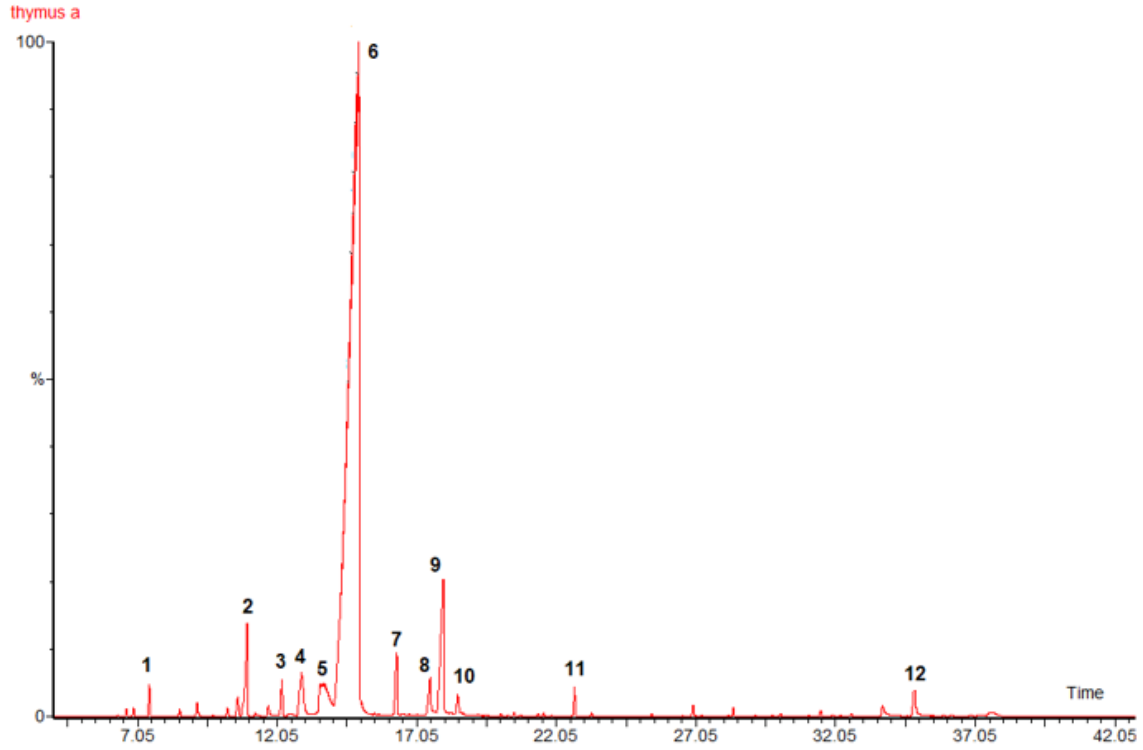
Figure 4. Chromatographic profile (TIC) of group A (predominant composition).



Main compounds (>0.5 % TIC peaks área): 1. α-Pinene (2,0 %); 2. Camphene (9,9 %); 3. β-Pinene (1,4 %); 4. Myrcene (0,8 %); 5. *p*-Cymene (0,7 %); 6. 1,8-Cineole (5,6 %); 7. Camphor (51,1 %); 8. Borneol (17,7 %); 9. Terpinen-4-ol (4,1 %); 10. α-terpineol (2,0 %); 11. Spathulenol (1,3 %)

Figure 5. Chromatographic profile (TIC) of group B (rich in linalool).



Main compounds (>0.5 % TIC peaks area): 1. Camphene (0,5 %); 2. 1,8-Cineole (2,3 %); 3. γ-Terpinene (0,7 %); 4. (Z)-linalool oxide furanoid (2,0 %); 5. (*E*)-linalool oxide furanoid (3,5 %); 6. Linalool (78,6 %); 7. Camphor (1,4 %); 8. Borneol (1,0 %); 9. Terpinen-4-ol (4,5 %); 10. α-terpineol (0,5 %); 10. Isobornyl acetate (0,5 %); 11. Spathulenol (0,9

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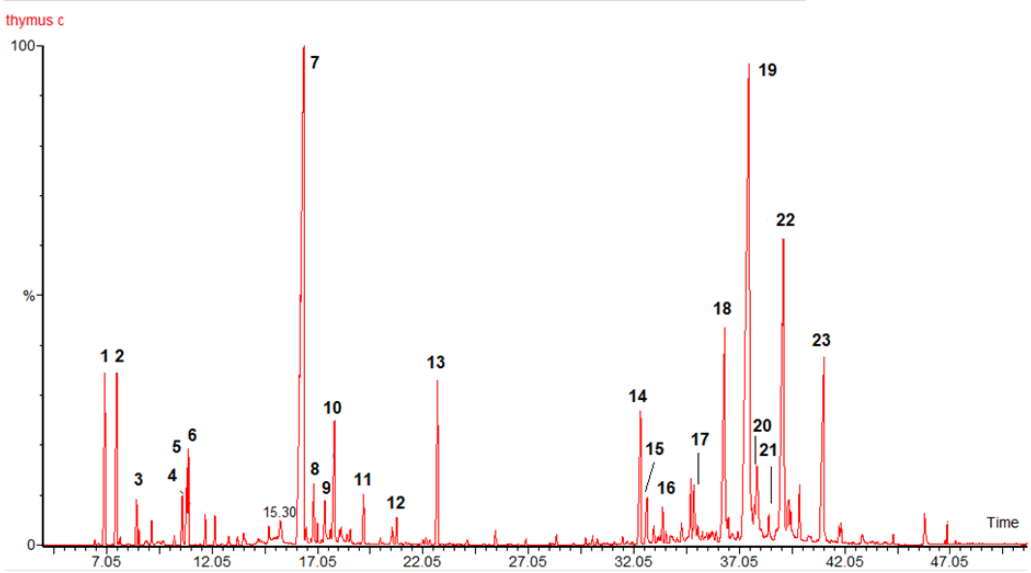
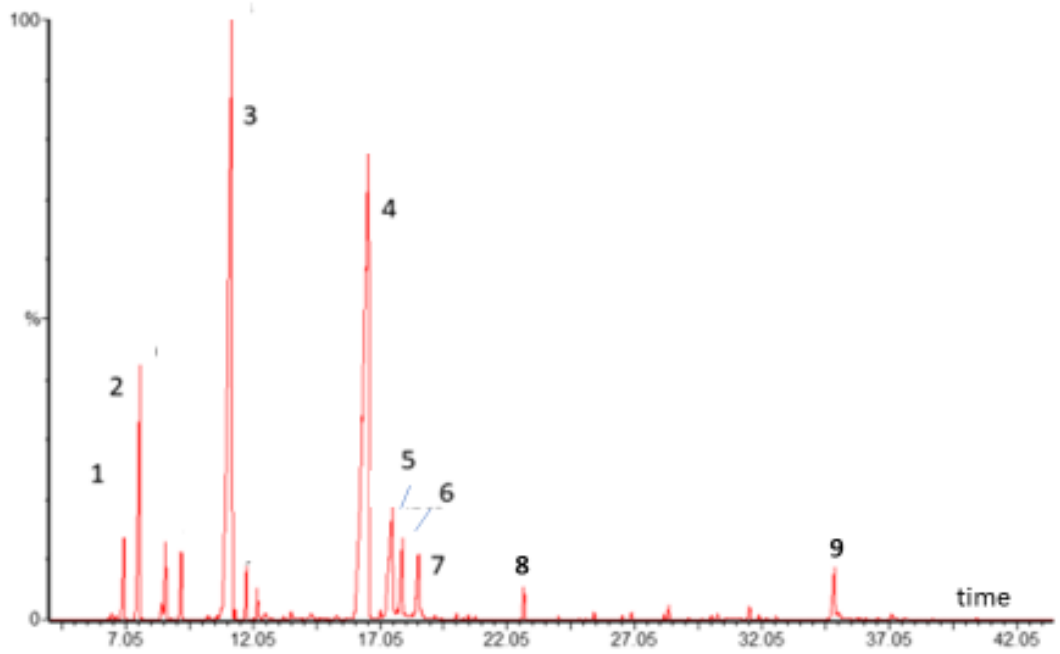
Figure 6. Chromatographic profile (TIC) of group D (rich in oxygenated sesquiterpenes).



Identified main compounds (>0.5 % TIC peaks área): 1. α-pinene (2,8 %); 2. Camphene (2,7 %); 3. Sabinene (0.6 %); 4. *p*-Cymene (0.8 %); 5. Limonene (1.3 %); 6. 1,8-Cineole (1,1 %); 7. Camphor (19.7 %); 8. Sabina ketone (1,0 %); 9. Borneol (0.8 %); 10. Terpinen-4-ol (2,3 %); 11. Verbenone (0.8 %); 12. Carvone (0.5 %). 13. Isobornyl acetate (3,0 %); 14. γ-Cadinene (2,9 %); 15. δ-Cadinene (1,1 %); 16. α-Calacorene (0,5 %); 17. Spathulenol (1,1 %); 18. Cubenol <1,10-di-epi> (5,8 %); 19. Cadinol <epi-α> (20,9 %); 20. α-cadinol (1,7 %); 21. Cadalene (0,5 %); 22. Muurol-5-en-4-one <cis-14-nor> (10,1 %); 23. Cyclocolorenone (5,5 %)

Figure 7. Chromatographic profile (TIC) of group F (rich in camphene, 1,8-cineole, camphor and borneol).



Identified main compounds (> 0.5 % TIC peaks área): 1. α-pinene (0,5 %); 2. Camphene (6,6 %); 3. 1,8-cineole (34,5 %); 4. Camphor (36,0 %); 5. Borneol (3,1 %); 6. Terpinen-4-ol (2,3 %); 7. α-terpineol (2,4 %); 8. Isobornyl acetate (0,9 %); 9. Spathulenol (1,9 %)

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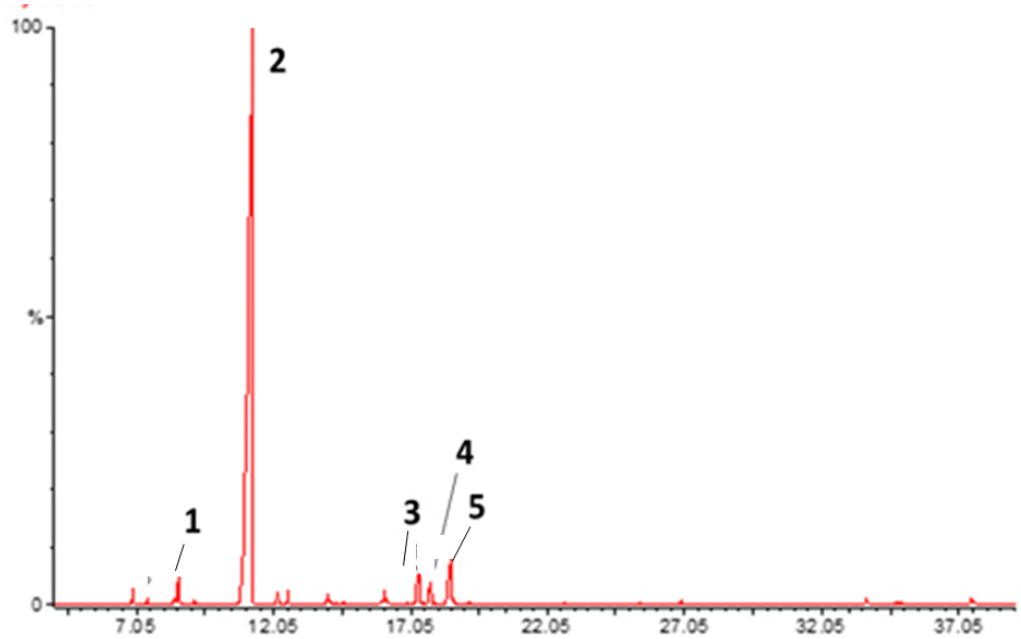
As aforementioned, the individuals not matching any of the established groups were independently subjected to GC-MS and GC-FID analysis. Figure 8 shows an atypical profile highly rich in 1,8-cineole.

Figure 8. Chromatographic profile (TIC) of an atypical individual highly rich in 1,8-cineole



Identified main compounds (> 1 % TIC peaks área): 1. α-Pinene (1,5 %); 2. 1,8-Cineole (83,5 %); 3. δ-Terpineol (2,6 %); 4. Terpinen-4-ol (1,3 %); 5. α-Terpineol (4,5 %)

GC analysis

The composition of the 20 processed samples (one from each of the seven groups and the 13 non-classified individuals) expressed as percentage (%) peak areas in FID chromatograms, is detailed in Tables 1 and 2.

A total of 73 compounds were identified accounting for 88.7-99.6 % of the entire area. The major profile, grouping 47 individuals, contained as main constituents: camphene (17 %), camphor (46.4 %) and borneol (11.5 %). This profile was found very similar to that reported by Imelouane et al. (2009) from Morocco. The group B, as well as the non-classified individuals: 2, 3, 4, 5, 8, exhibited a high amount of linalool (48.3-80.6 %). This composition could be related to chemotype linalool (Thomson et al. ,2003; Giordani et al., 2004). Other well-defined profile was E, which represents the 1,8-cineole chemotype, typical of Eastern Iberian Peninsula. The non-classified individual 1 could be characterized by its high amount of oxygenated sesquiterpenic fraction (α-cadinol, 43.5 % and epi-α-cadinol, 9.8 %). The profile D showed some similarity with this composition (34.4 % oxygenated sesquiterpenes). The rest of groups and the non-classified individuals represented different relative rates of pinene isomers, camphene, camphor, linalool, borneol, terpinen-4-ol and oxygenated sesquiterpenic fraction (considered as a whole, which main constituents were found spathulenol and cadinol isomers).

For a more accurate classification, the aforementioned components accounting for more than 5% in at least three samples, were taken as variables for the Hierarchical Clustering and the Discriminant Analysis. Camphene was not considered because it was highly correlated with camphor (r = 0.96).

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The Cluster analysis (Fig. 9) allowed to propose a first classification to be validated by Discriminant Analysis. The samples were grouped as follows: 1. A, C, F, NC11; 2. D, NC1, NC10; 3. E, G, NC12, NC13; 4. NC6, NC7, NC9; 5. B, NC2, NC3, NC4, NC5, NC8.

Table 1. Chemical composition of grouped samples of *Thymus vulgaris* L coming from Mondúver area (La Safor, València, Spain)

Compounds				**Grouped individual samples (% peak areas)**						
	Id. [b]	**LRI**[c]	**LRI (lit.)**[d]	**A**	**B**	**C**	**D**	**E**	**F**	**G**
Tricyclene	1,2	920	921	0.7	0.2	0.7	0.3	0.3	0.9	0.7
α-Thujene	1,2	924	924	0.3	0.5	0.3	0.1	0.2	0.3	0.3
α-Pinene	1,2,3	931	932	4.9	0.6	5.9	6.6	2.7	6.6	6.1
Camphene	1,2,3	946	946	17.0	1.8	15.1	6.1	0.9	16.9	11.6
Sabinene	1,2	969	969	0.2	0.2	0.1	2.7	2.6	0.2	0.8
β-Pinene	1,2,3	975	974	1.2	0.4	1.2	0.4	5.4	1.3	2.5
1-Octen-3-ol	1,2	979	974	0.1	0.3	0.1	0.2	t	0.1	0.1
3-Octanone	1,2	984	979	-[e]	t[f]	t	-	0.3	t	t
Myrcene	1,2,3	989	988	1.2	0.8	1.0	0.6	3.7	1.2	2.4
3-Octanol	1,2	998	994	-	t	t	-	-	-	-
α-Phellandrene	1,2	1003	1002	t	0.1	-	-	t	-	-
α-Terpinene	1,2,3	1016	1014	0.2	0.5	0.2	0.3	t	0.3	0.2
p-Cymene	1,2,3	1022	1020	1.1	1.3	0.8	-	0.2	1.2	0.6
Limonene	1,2,3	1026	1024	1.9	1.0	2.5	1.4	-	3.2	-
1,8-Cineole	1,2,3	1028	1026	3.4	3.3	0.2	2.1	56.0	0.4	25.8
cis-β-Ocimene	1,2	1035	1032	0.1	0.2	0.1	1.3	-	0.2	0.1
trans-β-Ocimene	1,2	1046	1044	0.5	0.4	1.0	0.6	0.5	2.1	1.0
γ-Terpinene	1,2,3	1055	1054	0.5	1.3	0.5	0.6	0.7	0.8	0.5
cis-Sabinene hydrate	1,2	1069	1065	0.5	3.9	0.5	0.2	0.8	0.7	0.5
Camphenilone	1,2	1079	1078	0.2	-	0.2	0.2	-	0.2	0.1
Terpinolene	1,2,3	1083	1086	0.1	2.9	0.1	0.5	0.2	0.2	0.1
trans-Linalool oxide (furanoid)	1,2	1085	1084	t	-	-	-	-	-	-
Linalool	1,2	1100	1095	0.3	61.3	1.4	0.5	0.8	0.3	0.6
α-Campholenal	1,2	1121	1122	0.1	0.3	0.3	0.3	0.2	0.5	0.3
Camphor	1,2,3	1144	1141	46.4	2.1	43.5	19.9	t	41.3	27.5
Sabina ketone	1,2	1152	1154	0.1	-	0.1	0.7	0.1	0.1	t
Pinocarvone	1,2,3	1159	1160	0.3	-	0.2	0.2	0.4	0.2	0.2
Borneol	1,2,3	1170	1165	11.5	4.9	12.1	3.7	4.0	10.1	6.7
Terpinen-4-ol	1,2,3	1177	1174	2.3	4.1	2.4	2.0	1.8	2.9	2.0
α-Terpineol	1,2	1192	1186	0.5	0.7	0.4	0.5	6.9	0.6	2.1
Dodecane	1,2	1203	1204	0.1	t	t	0.7	0.2	0.1	0.1
Isobornyl methanoate	1,2	1223	1223[g]	0.1	0.1	0.8	-	0.1	0.2	0.1
Carvone	1,2	1237	1239	0.1	-	-	0.4	0.1	0.1	t
Linalool acetate	1,2	1254	1254	-	0.1	-	-	-	-	-
Isobornyl acetate	1,2	1280	1283	1.0	0.4	1.6	2.4	0.1	1.4	0.6
δ-Terpinyl acetate	1,2	1309	1316	-	-	-	-	0.4	-	0.1

δ-Elemene	1,2	1334	1335	-	-	0.2	0.6	0.8	-	0.1
Isobornyl propanoate	1,2	1381	1383	-	-	0.1	-	-	-	t
β-Bourbonene	1,2	1381	1387	0.1	0.3	0.3	-	0.2	0.2	0.2
β-Caryophyllene	1,2,3	1413	1417	-	0.2	0.3	-	0.4	0.4	0.5
β-Copaene	1,2	1422	1430	-	-	t	-	-	-	t
Aromadendrene	1,2	1430	1439	-	t	-	-	-	-	t
α-Humulene	1,2	1449	1452	-	-	-	-	-	-	-
Alloaromadendrene	1,2	1458	1458	-	-	-	-	-	-	-
Isobornyl *n*-butanoate	1,2	1471	1473	0.1	-	0.2	-	-	0.1	0.1
Germacrene D	1,2	1476	1484	0.3	0.1	0.5	-	-	0.3	0.3
Bicyclogermacrene	1,2	1490	1500	-	0.3	0.8	0.2	0.1	0.4	0.8
α-Muurolene	1,2	1494	1500	0.1	-	-	-	-	-	0.1
γ-Cadinene	1,2	1508	1513	-	-	-	4.0	-	0.1	0.1
δ-Cadinene	1,2	1512	1522	-	0.1	-	0.1	-	0.1	-
Isobornyl 3-methylbutanoate	1,2	1520	1521	-	-	0.1	-	-	0.2	t
Isobornyl 2-methylbutanoate	1,2	1524	1524	-	-	-	-	-	-	-
α-Calacorene	1,2	1542	1544	-	0.7	-	0.2	1.3	0.1	-
Elemol	1,2	1545	1548	-	-	-	-	-	-	-
Amorph-4-ene <1α,10α-epoxy->	1,2	1559	1572	-	-	-	0.2	t	-	t
Spathulenol	1,2	1570	1577	1.6	2.8	2.9	2.2	0.7	1.9	3.0
Caryophyllene oxide	1,2	1576	1582	0.2	-	-	0.4	-	t	-
β-Oplopenone	1,2	1600	1607	-	t	t	0.3	-	-	t
Cubenol <1,10-di-epi>	1,2	1610	1618	-	t	-	4.2	-	t	t
α-Muurolol	1,2	1623	1644	t	0.2	0.1	-	0.5	0.1	t
epi-α-Cadinol	1,2	1638	1638	t	-	-	12.9	0.1	0.5	0.1
α-Eudesmol	1,2	1640	1652	-	-	-	-	-	-	-
α-cadinol	1,2	1650	1652	-	0.8	-	1.7	1.9	0.3	0.2
cis-Calamenen-10-ol	1,2	1659	1660	-	0.2	-	0.4	-	-	-
Elemol acetate	1,2	1670	1680	-	-	-	-	-	-	-
Eudesma-4(15),7-dien-1-β-ol	1,2	1676	1687	-	-	0.1	4.2	0.1	t	0.1
Eudesm-7(11)-en-4-ol	1,2	1683	1700	-	0.1	0.1	2.5	-	0.2	t
Muurol-5-en-4-one <cis-14-nor->	1,2	1692	1688	-	-	-	0.1	-	-	t
Isobicyclogermacrenal	1,2	1725	1733	-	-	-	0.5	-	-	-
Cyclocolorenone	1,2	1732	1759	-	-	-	3.0	-	0.1	t
1-Octenyl acetate	1,2	1108	1110	-	-	-	0.1	-	-	-
(2*Z*)-Hexenyl isovalerate	1,2	1234	1241	-	0.2	0.1	0.2	0.2	0.1	0.1
Manool oxide	1,2	1959	1987	-	0.1	0.1	0.1	0.1	t	t
Grouped compounds										
Hydrocarbon monoterpenes				29.8	12.1	29.4	21.5	17.5	35.3	26.8
Oxygenated monoterpenes				66.9	81.2	64	33.1	71.7	59.1	66.7
Hydrocarbon sesquiterpenes				0.4	1.7	2.0	5.0	2.8	1.6	2.0
Oxygenated sesquiterpenes				1.9	1.6	3.3	34.2	4.6	3.2	3.5
Other				0.2	0.6	0.3	1.4	0.7	0.3	0.4
Total identified				**99.0**	**99.0**	**99.1**	**93.4**	**95.8**	**99.4**	**99.2**

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Table 2. Chemical composition of individual samples (no classified) of *Thymus vulgaris* L coming from Mondúver area (La Safor, València, Spain). The listed compounds and therefore the identification methods and the LRI values are the same as in Table 1 Unclassified individual samples (% peak areas)

Compound	NC1	NC2	NC3	NC4	NC5	NC6	NC7	NC8	NC9	NC 10	NC 11	NC 12	NC 13
Tricyclene	-	-	-	-	0.0	0.2	0.1	0.0	0.0	0.4	0.3	0.0	0.2
α-Thujene	-	-	-	-	t	1.0	0.5	-	1.3	0.3	0.2	0.2	0.8
α-Pinene	1.3	0.3	0.1	0.3	0.1	5.7	2.1	0.1	5.0	4.7	3.1	2.3	2.0
Camphene	0.3	-	0.1	0.2	0.0	17.7	11.6	0.3	15.8	8.7	9.0	2.8	0.7
Sabinene	-	-	-	-	-	t	0.1	-	0.2	5.6	0.1	0.7	1.3
β-Pinene	6.0	0.7	0.1	0.6	0.2	0.2	0.1	-	1.1	0.3	0.7	2.0	2.6
1-Octen-3-ol	-	-	-	0.9	-	1.3	0.9	t	0.1	-	0.1	-	t
3-Octanone	-	-	-	-	-	-	-	-	0.1	-	-	-	-
Myrcene	0.8	1.1	1.1	0.1	0.4	0.1	-	t	0.9	0.6	0.5	0.5	1.6
3-Octanol	-	-	-	-	-	-	-	-	t	-	-	-	-
α-Phellandrene	-	-	-	1.6	-	1.1	0.6	0.1	t	-	-	-	-
α-Terpinene	0.1	-	-	-	t	0.5	0.2	0.1	0.5	0.4	-	-	0.2
p-Cymene	0.6	t	t	-	t	-	-	-	1.6	3.2	1.0	1.3	2.0
Limonene	2.0	1.1	0.9	t	0.7	2.3	1.0	t	-	2.3	2.7	3.1	1.6
1,8-Cineole	0.7	17.3	3.6	26.5	4.3	5.5	6.5	1.3	4.5	1.3	1.5	36.9	38.6
cis-β-Ocimene	-	-	1.4	-	-	0.1	0.1	-	0.2	-	-	-	-
trans-β-Ocimene	3.9	1.2	t	-	t	0.5	0.5	t	1.3	0.7	0.7	-	-
γ-Terpinene	0.3	-	0.1	0.1	0.2	0.9	0.4	-	1.0	0.9	0.7	0.8	3.0
cis-Sabinen hydrate	0.2	0.3	0.8	1.0	1.4	0.7	0.3	1.3	0.7	0.8	1.4	1.8	8.5
Camphenilone	-	-	-	-	-	-	-	-	0.5	-	0.2	-	-
Terpinolene	-	0.2	0.5	-	0.4	0.2	0.2	1.0	0.1	-	0.4	-	0.3
trans-Linalool oxide (furanoid)	-	-	-	0.3	-	t	-	-	-	-	-	-	-
Linalool	0.1	63.3	76.7	48.3	79.2	0.9	1.6	80.6	t	0.5	13.8	4.3	13.1
α-Campholenal	-	t	t	-	-	0.1	0.6	-	t	-	0.2	-	0.6
Camphor	1.3	t	1.0	0.4	t	36.3	42.0	1.3	41.1	27.5	35.4	11.5	2.6
Sabina ketone	-	-	-	-	-	-	-	-	-	1.5	-	-	-
Pinocarvone	-	-	-	-	-	0.1	0.1	-	-	0.2	-	-	-
Borneol	0.2	t	0.4	2.9	0.6	0.1	0.1	0.4	0.2	5.4	11.4	4.9	2.2
Terpinen-4-ol	0.6	-	0.6	-	0.6	13.2	17.5	-	17.0	2.7	3.3	2.6	8.6
α-Terpineol	-	-	-	0.4	-	3.0	2.1	0.2	0.6	0.2	0.5	2.2	3.3
Dodecane	-	-	-	-	-	-	-	-	0.2	0.5	0.2	-	-
Isobornyl methanoate	-	-	-	-	-	-	-	-	0.2	-	0.9	0.1	1.0
Carvone	-	-	-	-	-	-	-	-	0.2	-	-	-	-
Linalool acetate	t	-	-	-	0.1	0.1	-	-	-	-	-	-	-
Isobornyl acetate	0.1	-	-	0.3	-	1.3	1.4	-	1.5	1.3	1.5	0.5	-
δ-Terpinyl acetate	-	-	-	-	-	-	-	-	-	-	-	0.8	-
δ-Elemene	-	-	-	-	0.4	-	-	-	-	0.3	-	2.0	0.1
Isobornyl propanoate	-	1.1	0.4	-	0.6	0.1	-	-	t	-	-	-	-
β-Bourbonene	-	-	-	0.6	-	0.2	0.6	0.7	0.1	-	-	-	-

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β-Caryophyllene	-	1.1	2.1	-	1.3	0.2	0.2	0.0	0.2	-	-	0.4	-
β-Copaene	-	-	-	0.7	-	0.3	0.4	1.3	-	-	-	-	-
Aromadendrene	-	-	-	-	-	-	-	0.1	-	-	-	-	-
α-Humulene	-	-	-	-	-	-	-	0.2	-	-	-	-	-
Alloaromadendrene	1.0	-	-	0.3	-	0.1	0.3	0.2	-	-	-	-	-
Isobornyl *n*-butanoate	-	-	-	-	-	0.3	t	-	0.2	-	0.2	-	-
Germacrene D	1.0	4.3	1.3	2.2	1.9	0.1	0.2	0.4	0.1	-	-	-	-
Bicyclogermacrene	0.2	0.7	1.3	-	2.2	-	-	-	0.2	-	0.4	-	-
α-Muurolene	-	-	-	1.0	-	0.5	0.4	0.8	0.1	-	-	-	-
γ-Cadinene	10.4	0.8	-	-	-	-	-	0.2	-	0.7	-	-	-
δ-Cadinene	-	-	-	-	-	-	-	-	-	-	-	-	-
Isobornyl 3-methylbutanoate	-	-	-	-	-	-	-	-	0.2	1.9	0.2	0.4	-
Isobornyl 2-methylbutanoate	0.8	-	-	0.2	-	-	0.1	0.2	-	-	-	-	-
α-Calacorene	-	-	-	-	-	-	-	-	-	-	-	-	-
Elemol	-	-	4.4	-	1.3	-	-	-	-	-	-	-	-
Amorph-4-ene <1α,10α-epoxy->	-	1.3	-	1.4	-	-	-	0.6	-	0.6	-	-	-
Spathulenol	0.3	0.2	0.4	0.4	0.9	2.8	4.1	5.1	2.2	3.6	3.8	3.2	1.5
Caryophyllene oxide	-	0.4	0.8	0.2	0.4	0.6	1.2	1.2	-	0.8	-	-	-
β-Oplopenone	-	-	-	-	-	-	-	-	-	3.3	1.3	6.3	0.4
Cubenol <1,10-di-epi>	-	-	-	-	-	-	0.1	0.1	-	-	-	-	-
α-Muurolol	-	-	-	-	-	-	-	-	0.1	-	-	-	-
epi-α-Cadinol	9.8	0.1	-	0.8	-	-	-	0.2	-	8.7	-	-	-
α-Eudesmol	-	-	0.2	-	-	0.1	0.1	t	-	-	-	-	-
α-cadinol	43.5	3.2	-	3.6	-	-	0.1	0.1	-	0.7	-	0.1	-
cis-Calamenen-10-ol	2.9	0.1	0.3	0.7	0.1	-	0.3	1.1	-	-	-	-	-
Elemol acetate	0.3	-	0.8	-	0.2	-	-	-	-	2.0	-	-	-
Eudesma-4(15),7-dien-1-β-ol	-	-	-	0.3	-	-	-	-	-	2.1	-	-	-
Eudesm-7(11)-en-4-ol	-	-	0.2	0.5	-	-	-	-	-	-	0.2	-	-
Muurol-5-en-4-one <cis-14-nor->	-	-	-	-	-	-	-	-	-	-	-	-	-
Isobicyclogermacrenal	-	-	-	-	-	-	-	-	-	2.3	-	-	-
Cyclocolorenone	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Octenyl acetate	-	-	-	-	-	t	-	-	t	-	-	t	-
(2*Z*)-Hexenyl isovalerate	-	-	-	-	1.3	0.4	0.4	-	t	0.9	0.2	-	1.0
Manool oxide	-	-	-	-	-	-	-	-	t	-	0.8	2.1	0.9
Grouped compounds													
Hydrocarbon monoterpenes	15.3	4.6	4.4	3.0	2.1	30.3	17.4	1.6	28.9	28.1	19.3	13.6	16.2
Oxygenated monoterpenes	3.2	82	83.5	80.1	86.8	61.7	72.2	85.1	66.7	41.4	70.3	65.6	78.5
Hydrocarbon sesquiterpenes	12.6	6.9	4.7	4.6	5.8	1.5	2.0	3.7	0.6	1.0	0.4	2.4	0.1

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Oxygenated sesquiterpenes	57.5	5.4	7.1	8.0	2.9	3.5	5.9	8.4	2.3	24.3	5.3	10.3	1.8
Other	-	-	-	0.9	1.3	1.7	1.3	t	0.5	1.4	1.4	2.2	1.9
Total identified	**88.7**	**98.9**	**99.6**	**96.6**	**98.9**	**98.3**	**98.7**	**98.9**	**99.1**	**97.6**	**96.8**	**93,6**	**98.6**

[a]Compounds listed in order of elution on DB5 column; [b]Method of identification: 1. MS spectra, 2. LRI, 3. Co-injection with pure standards, [c]Experimental linear retention index; [d]Linear retention index according Adams (2007); [e]No detected; [f]Traces (peak area < 0.05 %); [g]Cao et al. (2011)

Figure 9. Dendogram of Hierarchical Clustering of selected variables

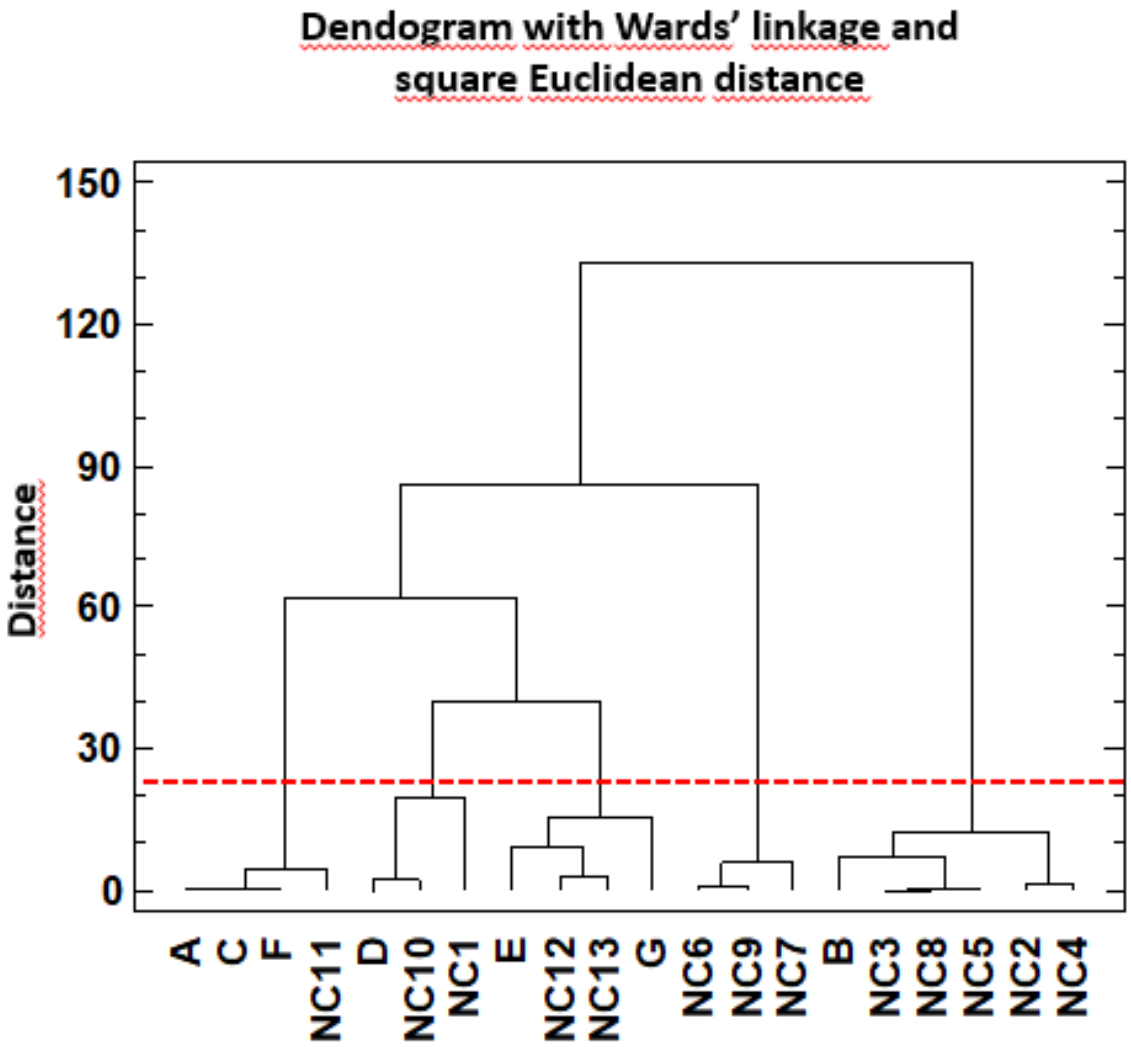

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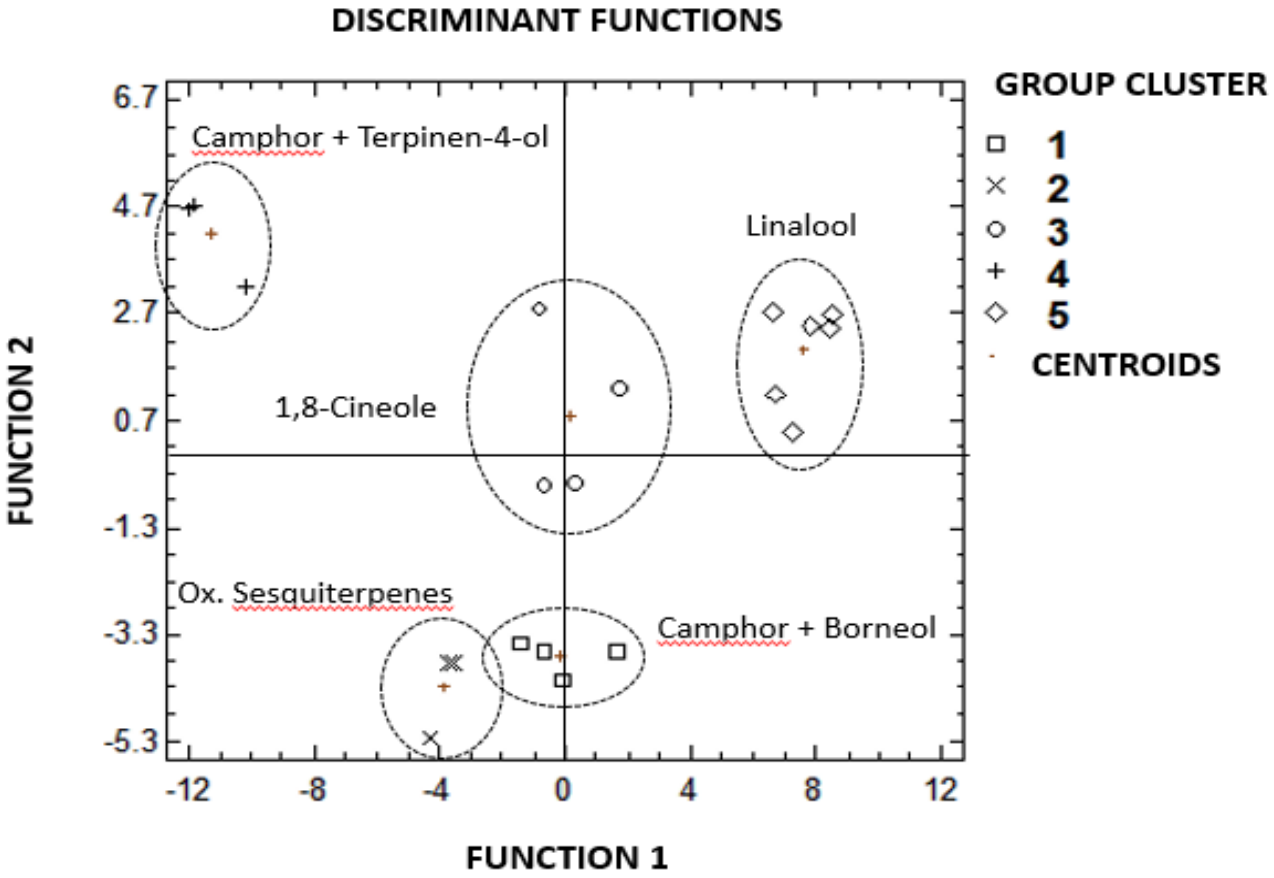
Figure 10. Grouped samples according the two first discriminant functions. Relative eigenvalue percentage: F1: 70,40 %; F2: 17,36 % (P = 0.0000).



Both F1 and F2 allows to consider well established differences among the defined groups according λ (Wilks) values: F1 (λ = 0,00005, p=0,0000), F2 (λ = 0,003, p = 0,0000), (100 % samples well classified). Standardized coefficients for both factors are the following:

F1= 0,487*1,8-cineole - 0,642*Camphor + 1,101*Borneol - 0,581*Terpinen-4-ol - 0,062*Oxygenated sesquiterpenes - 0,078*α+β pinene - 1,278*Linalool

F2= 1,055*1,8-cineole + 0,831*Camphor – 0,624*Borneol + 0,630*Terpinen-4-ol -0,032*Oxygenated sesquiterpenes +0,335*α+β pinene +0,950*Linalool

Based on the selected variables for cluster classification, a discriminant analysis (AD) was performed in order to evaluate it. The first two factors explained 87,8 % of the variability.

According F1 and F2 coefficients, borneol, 1,8-cineole and linalool were the most powerful discriminant compounds in order to define the EO profiles. The relationships camphor-borneol and camphor-terpinen-4-ol in each one of the factors expressed clearly the presence of two well defined profiles in which camphor and each one of these two compounds were the dominant ones.

The best defined profile can be related with the chemotype linalool as expressed when the coefficients values in both factors are compared. The same, but in a less pronounced way, can be said of 1,8-cineole. Oxygenated sesquiterpenes showed negative and low coefficients in both factors, constituting also a well classified group. On the other hand, α+β pinene did not seem to be relevant as discriminatory compounds. The composition

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of the final defined profiles and the significance of the contributions of the main compounds is displayed in Table 3.

Table 3. Average amounts of considered variables of groups defined from Cluster Analysis. Different letters on the same row mean significant differences according the Tukey's HSD test, (P <0.05)

	GROUP CLUSTER				
Compounds	**1**	**2**	**3**	**4**	**5**
α+β Pinene	6,2±1,8 a	6,4±1,2 a	6,4±2,2 a	4,7± 2,2 a	0,6±0,4 b
1,8-Cineole	1,4 ± 1,5 b	1,4 ± 0,7 b	39,3 +12,5 a	5,5±1,0 b	9,4±10,2 b
Linalool	3,9±6,6 b	0,4±0,3 b	4,7±5,9 b	0,8±0,8 b	68,2±12,8 a
Camphor	41,7± 4,6 a	16,2±13,5 b	10,4±12,4 bc	39,8±3,1 a	0,8±0,8 c
Borneol	11,3 ± 0,8 a	3,1±2,6 bc	4,4±1,8 b	0,1±0,1 d	1,6±1,9 cd
Terpinen-4-ol	2,7 ± 0,5 b	1,8±1,0 b	3,7±3,3 a	15,9±2,4 b	0,9±1,6 a
Oxygenated sesquiterpenes	3,4±1,4 b	38,8±17,0 a	5,0±3,7 b	3,9 ± 1,9 b	5,6±2,8 b

The profiles A, C, F and NC 11 (group 1) could be considered as major profiles. In this case, the preliminary screening did not allow for significant distinctions, probably due to differences in the concentration of the plant extracts subjected to the TLC analysis. As displayed in Table 1, these samples also exhibited important amounts of camphene (9,0 %-16,9 %). This way, group 1 could be considered as the representative profile based on the camphane skeleton (camphene, camphor and borneol as the most important components). If compared profile A (predominant in terms of number of individuals) with that described by Imelouane et al. (2009), a higher content of the major compounds was in general observed (17.0%; 46.4% and 11.5% versus 17.19%, 38.54% and 4.92% of camphene, camphor and borneol, respectively).

The group 2 exhibited a relative high amount of sesquiterpenic fraction including NC1, which could be considered as an atypical individual because of its great amount of cadinol isomers. To the best of our knowledge, a similar profile has not been referred to so far in thyme. Only samples accounting up to 14,1 % of τ-cadinol were reported by Mancini et al. (2015) in phenolic (thymol) *T. vulgaris*. It is also worth mentioning its reported pharmacological applications (Zygmunt et al., 1993).

The group 3 was characterized by its high rate of 1,8-cineole, although profile C showed a noticeable amount of camphene and camphor. This profile was mostly observed in a previous study with samples from the East of the Iberian Peninsula (Teruel and Valencia) (Llorens et al., 2017). On the other hand, the content of 1,8-cineole raised up to 56,0 % in sample E, whereas it accounted for a 36,42 % as reported by Jordán et al. (2006) on Spanish *T. vulgaris*, chemotype 1,8-cineole.

The composition of the group 4 exhibited a noticeable presence of terpinen-4-ol, which was not considered in the preliminary screening. It may explain why these individuals were not classified. Its composition could be considered as a specific chemotype: camphor, terpinen-4-ol. It is important referring to the composition described by Quesada et al. (2016) in an oil marketed by a company located close to the area studied. Its composition (camphene (4,78 %); 1,8-cineole (12,3 %), camphor (11,23 %), terpinen-4-ol (5,50 %), borneol (8,87 %)) seems to be balanced between those described for groups 3 and 4.

The group 5 was the best-defined and it can be clearly related to the chemotype linalool, already cited above (Thomson et al., 2002), the same way that the reported samples from Catalonia (Spain) (Torras et al., 2007),

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in which the variation range of linalool was 32.92 - 74.55% versus 48.3 - 80.6% found in this group. This increase could be attributed to the prior selection of individuals.

As mentioned when describing the plant material, it should be noted that in the studied area three *T. vulgaris* subspecies have been reported: *T. vulgaris* ssp *vulgaris*, T. *vulgaris* ssp. *aestivus* and *T. vulgaris* ssp. *mansanetianus*. Nevertheless, the composition reported of *T. vulgaris* ssp. *aestivus* collected in a close geographical area (1,8-cineol (22.18%), geraniol (17.43%) and geranyl acetate (20.01%) as major compounds) is quite different to profiles identified in this work (Blazquez et al., 1990). No information has been described when regards to the chemical composition of *T. vulgaris* ssp. *mansanetianus* EO.

Conclusion

From the final classification obtained through the statistical treatment of the results, the validity of the preliminary TLC screening has been proved as a useful tool to carry out a first classification of the individuals studied. In spite of the difficulties derived from the similarity between the colours and the R_f values of the spots corresponding to the main compounds, the most notable differences among the EO profiles could be identified.

On the other hand, it should be noted that the aforementioned difficulties led to validate the identification of the spots by preparative TLC and GC/MS, instead of the simple comparison with the development of the plates with reference pure standards. In this way, it was possible to group the 85 individuals so that those with a similar profile were part of a single sample. Thus, taking into account that individuals which could not be clearly classified were analysed independently, the number of samples to be processed could be reduced from 85 to 20. Moreover, this prior classification gives a first orientation about the relative abundance of each profile in the population.

Finally, after the statistical treatment of the data, five profiles were defined. As it has been demonstrated by comparing the range of variation of the main compounds in the defined groups and those reported from studies on non-phenolic samples of *T. vulgaris*, a more defined composition can be observed in terms of the proportion of the major components. Insofar as these compounds are related to potentially applicable biological activities, this fact leads to consider the interest of individual screening of populations in order to identify valuable genotypes to create more profitable cultivars.

Another interesting aspect of this methodology is that certain compounds of interest can be obtained with similar percentages in more advantageous species from the agronomic point of view, as can happen, for example, with linalool-rich oils.

The results of this work may be the starting point for further investigations, for example, to establish the possible taxonomic implications of the identified profiles in relation to the different subspecies of *T. vulgaris* present in the area. Moreover, given that the established groups of individuals as well as those considered atypical are localized, those with the greatest potential interest could be propagated.

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