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RESEARCH ARTICLE

TLC-GC/MS Method for identifying and selecting valuable essential Oil Chemotypes from Wild Populations of *Mentha longifolia* L.

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Abstract

Wild populations of aromatic plants can be considered resources of natural biodiversity for selecting valuable genotypes in order to obtain well-characterized cultivars with defined essential oil (EO) profiles suitable for different purposes due to their biological activity. Often, their high intrapopulational variability requires preliminary screenings based on a significant number of individuals and, therefore, simple and fast methods to identify EO profiles are required. For this purpose, a TLC-GC/MS method has been proposed in order to identify chemotypes occurring in a selected wild population. Stolons of 50 individuals of Mentha longifolia L were planted and bred in greenhouse conditions before growing in experimental plots. Individual sampling and extraction of leaves were performed to determinate TLC individual profiles. They were validated by preparative TLC and GC/MS analysis of discriminant spots. Fresh material belonging to each defined TLC profile was collected in full flowering stage and subjected to SDE extraction and GC/MS analysis. Five chemotypes were characterized: A (piperitone and piperitenone oxides); B (piperitone oxide + pulegone); C (α-terpineol acetate + carvone acetate); D ((*E*) – dihydrocarvone) and *E* (pulegone + isomenthone + menthol). This TLC method was applied to individuals coming from another population. All of them could be clearly identified as belonging to chemotype E, which was afterwards confirmed by GC/MS analysis.

Keywords: TLC, *Mentha longifolia*, essential oil, chemotype

Introduction

Over the last decades, there has been a tendency to use essential oils (EO) and other natural products as healthier and ecological alternatives in food preservation, medicine, pest management, etc., based on their chemical composition (Newman & Cragg, 2007; Dias et al., 2012; Harvey et al., 2015). Both the occurrence of valuable compounds and the absence of those showing adverse effects demand to fulfil several stability requirements in order to be suitably standardized. This way, well-characterized cultivars with specific EO profiles are required.

These cultivars can be obtained by means of conventional selection methods or new ones based on more recent advances in Biotechnology, such as micropropagation techniques or genetic engineering. With this aim, selecting and breeding valuable genotypes from wild populations as resources of natural biodiversity can be considered the first step. In relation to EOs, to study the chemical variability of wild germplasm can lead to identify promising chemotypes with regard to their biological activity or yield improvement. Many researches have been conducted in this way concerning different aromatic plant species: *Thymus vulgaris* L. (Delpit et al., 2009), *Rosmarinus officinalis* L. (Mulas et al., 2001), *Agonis fragrans* (Robinson, 2006), *Achillea*

species (Rahimmalek et al., 2009), *Lippia alba* Mill. (Rufino et al., 2012), *Lavandula multifida* L. (Zuzarte et al., 2015), *Origanum* species (Goliaris et al., 2003; Turgut et al., 2016).

Owing to the frequent high intrapopulational variability in EO chemical composition related to genetic factors, preliminary screenings of wild populations should be based on a significant number of individuals. Furthermore, studies on how each genotype is affected by environmental and ontogenetic factors are also required (Figueiredo et al., 2008). Consequently, simple and fast methods to process a great number of samples are demanded in order to obtain significant data allowing for well-defined chemotypes. Several methods have been reported for identifying EO chemical profiles such as vibrational spectroscopic methods (Schulz et al., 2003, Gudi et al., 2015) or the electronic nose (Vouillamor, 2009). Because of its simplicity, speed, reliability and economy, thin-layer chromatography (TLC) methods can be also useful (Pothier et al., 2001), mainly, in field research, away from well-equipped laboratories (Franz, 2010).

In this work, a method combining TLC and GC/MS has been proposed to identify EO chemical profiles from individuals belonging to wild populations in order to be propagated for obtaining chemical homogeneous cultivars. It has been applied to *Mentha longifolia* L. As reported in the literature, EO from this species shows a well-known chemodiversity (Lawrence, 2006; Sharopov et al., 2012; Abedi et al., 2015). Two principal chemical profiles can be distinguished according to the molecular skeleton of their main components:

Menthane skeleton oxygenated in C2. Samples containing carvone and dihydrocarvone (*cis* and *trans* isomers) as major compounds, as reported by Kokkini & Lanaras, (1995); Mastelic et al., (2002); Dzamic et al., (2010); Bertoli et al., (2011).

Menthane skeleton oxygenated in C3. Two typical profiles related to alternative metabolic pathways involving the double bond C4-C8 reduction can be distinguished: (a) piperitenone, piperitone and their epoxides (Maffei, 1988; Saeidi et al., 2012; Segev et al., 2012; Jamzad et al., 2013; Moradalizadeh et al., 2014) and (b) pulegone, menthone or isomenthone, menthofuran and other menthol derivatives as well (Mkaddem et al., 2009; Hajlaoui et al., 2009; Segev et al., 2012).

Furthermore, samples whose major components show other chemical structures have been also reported such as the menthane skeleton oxygenated in C8. For example, 1,8-cineol is usually found together with the above mentioned two molecular structures as relative major component (Koliopoulos et al., 2010; Moradalizadeh et al.,2014). It is even reported as main compound by Fleisher & Fleisher (1998). It is closely related to α-terpineol as it derives from it by means of an ether bond between C1 and C8. On the other hand, α-terpinyl acetate and terpinen-4-ol (menthane skeleton oxygenated in C4) are reported by Baser et al. (1999) as major components in samples from Turkey. Molecular structures based on the sabinene skeleton have been also reported. For example, (*E*)-sabinenhydrate has been found the principal compound in some samples from Crete (Kokkini & Papageorgiou, 1988) and Turkey, belonging to different subspecies (Baser et al., 1999). Samples rich in borneol (bornane skeleton) from Pakistan have been cited by Hussain (2010) and linalool (acyclic structure) as major compound has been also reported by Baser et al. (1999) in Turkish samples, as well.

Taking as starting point the results of a previous research (Llorens-Molina et al., 2015), in which a noticeable intrapopulational variability was found in a population of *Mentha longifolia* L. located in Teruel (Spain). The aims of this work are the following; a) To develop and validate a TLC – GC/MS method for screening wild populations and to identify EO chemical profiles from individuals in order to be propagated for obtaining chemical homogeneous cultivars, b) To use this method as a quick way to identify individuals coming from any other accessions.

Material and methods

Material

50 small pieces of stolon from a wild population located in the confluence of Jiloca and Pancrudo rivers in Teruel (Spain) (40º 58' 11.89'' N, 1º 8' 49.40'' W) were cut during the winter period (Figure 1). They were planted in pots and bred in greenhouse conditions (Figure 2). Once these individuals were classified at the end of identification process, vouchers belonging to each one of chemotypes were kept at the herbarium of the Universitat Politècnica de València (VALA 9576-9580) and the individuals belonging to same EO profile were transplanted and grown together in field plots as seen in Figure 3.

Figure 1. Stolon pieces from wild population

Methods

Sampling and extraction of mint leaves for Thin Layer Chromatography (TLC) analysis

A non-destructive sampling was employed for isolating volatile and semivolatile components according to Wagner and Bladt, (2002). A small amount of material coming from each individual at flowering stage was subjected to solvent extraction: 2 mL of dichloromethane were added to 0.2 g of fresh material previously cut in small pieces in 5 mL vials. Then, they were placed on an orbital stirrer for 30 min. Afterwards, each extract was dried with a little amount of Na_2SO_4 and filtered with a syringe filter. 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 into each vial. They were sealed and kept in refrigerator at 4°C until TLC analysis.

TLC analysis

The extracts were applied on the TLC plates and developed according to Wagner and Bladt (2002) method for essential oils. Volumes of 10 µL were spotted with capillary tubes (Blaubrand intraMark, 10 µL) on the silica gel plates (DC-Fertigfolien Alugram Sil G/UV 254). In order to classify individuals with similar profiles, they were developed in duplicate using toluene:ethyl acetate (93:7) as mobile phase and further sprayed with sulfuric vanillin and sulphuric anisaldehyde (UV 265 nm), respectively, as visualization reagents, as described by Wagner and Bladt (2002). Five initial profiles were distinguished according the

presence/absence of discriminant spots. With the aim of checking this first classification, a new TLC analysis was performed with three different individuals belonging to each one of the defined profiles. Retention factor (R_f) values were calculated for these discriminant spots.

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 kept and sealed in 350 µL insert vials until GC/MS analysis. These extracts were analyzed by GC/MS in order to identify their composition.

Chemotypes characterization

Leaves coming from the plants in full flowering stage belonging to each profile were put together and homogenised. EO was extracted by Simultaneous Distillation Extraction (SDE) (Chaintreau, 2001) and analyzed by GC/MS.

GC/MS analysis

The analysis of samples was carried out by GC-MS using a Clarus 500 GC (Perkin-Elmer Inc., Wellesley, PA, USA) chromatograph equipped with a fused-silica capillary column ZB-5 (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex Inc, Torrance, CA, USA). The injection volume was 1 µL. 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 mode (mass range m/z 45-500 uma). The GC oven temperature was programmed in the following way: 60°C for 5 min, raised at 3°C/min up to 180°C and then at 20°C/min up to 280°C, hold for 10 min. Helium was the carrier gas (1.2 mL min^{-1}). For analysis of selected TLC spots, a shorter oven temperature program was used (60°C for 3 min, raised at 10°C/min up to 180°C and then at 20°C/min up to 280°C, hold for 3 min.) The total ion chromatograms (TIC) 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 (Adams, 2007). 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.

Application to individuals coming from other populations:

Another way of using this method is to perform a first screening of nearby populations of the same species. An individual sample collected in Tuejar (Valencia) (39º 45’ 44’’ N; 1º 01’ 09’’ W) was processed with five samples (all of them were taken in vegetative stage) corresponding to identified profiles. All of them were processed according the method above described. Taking into account the presence/absence of discriminant spots, this new sample was classified according the five defined profiles. In order to check the applied method, this sample was also analyzed by GC/MS and its TIC chromatogram was compared with those taken as reference.

Results and Discussion

Preliminary TLC profiles

According the first visual examination of the 50 individuals' TLC profiles (one example is shown in Fig. 2) five patterns of composition were defined according the presence/absence of discriminant spots. Results of spraying with sulphuric vanillin and sulphuric anisaldehyde (UV 365nm) are displayed in Figure 3. The new TLC analysis based on three individuals of each defined profiles gave similar results (Figure 4a, 4b), so the R_f values were measured in order to apply them to find out by GC/MS the chemical identity of spots (Table 1).

Figure 2. Example of some individual profiles obtained by TLC, using sulphuric vanillin as spray reagent.

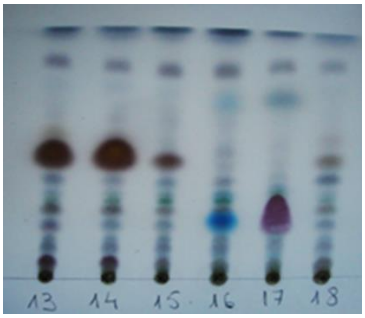


Figure 3. Five EO profiles were defined from the 50 individuals being tested.

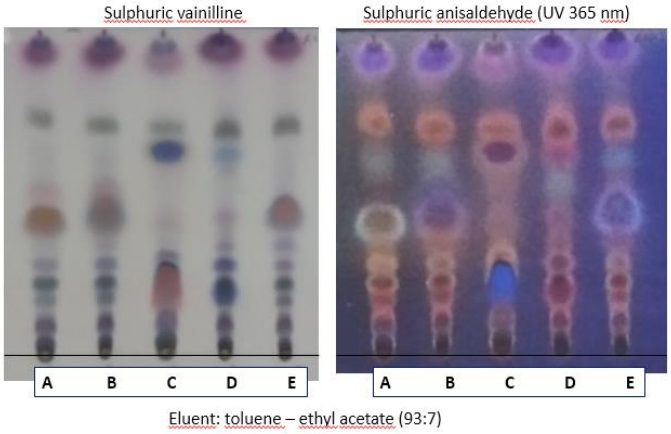
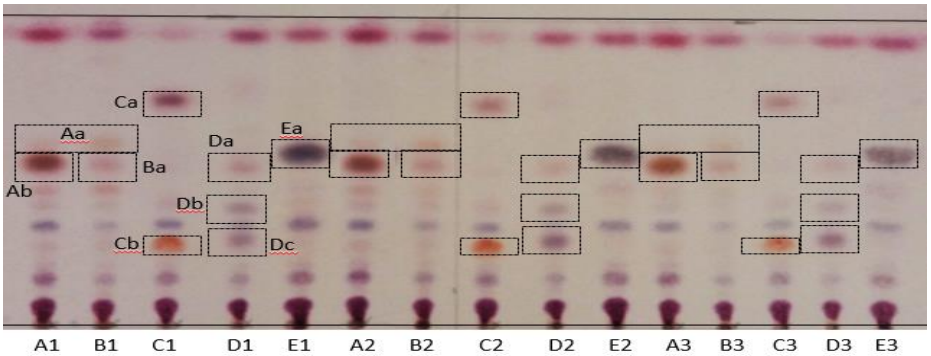


Figure 4a. TLC analysis by triplicate for each one of defined profiles. Characterization of discriminant spots (sulphuric vanillin as spray reagent)



For each profile (A1-E1), the discriminant spots are specified as: capital letter, profile; small letter: spot in decreasing order of R_f. A2-E2 and A3-E3 are repetitions.

Figure 4b. TLC analysis by triplicate for each one of defined profiles. Characterization of discriminant spots (sulphuric anisaldehyde –UV 365 nm- as spray reagent)

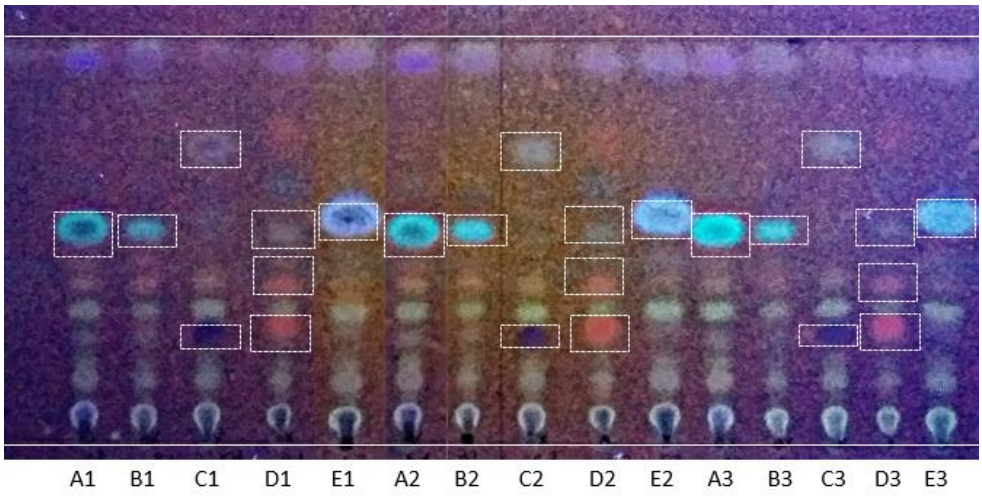


Table 1. Validation by GC/MS of TLC selection method. R_f, colour and composition for discriminant spots.

	Discriminant spots features			**Main GC/MS peaks**		**Other secondary GC/MS peaks**	
Profile	**(R_f range)**	**Reagent**		**RI**	**compound**	**RI**	**Compounds**
		Vanillin	Anisaldehyde (UV 365 nm)				
A	Aa[a]: 0.56-0.64	Reddish-brown (weak)	Blue green (strong)	1365	Piperitenone oxide	1031	1,8-cineole
	Ab: 0.49-0.56	Reddish-brown (strong)	Blue green (strong)	1263	Piperitone oxide (Fig.6a)		
B	Ba: 0.39-0.56	Reddish-brown (weak)	Blue green	1193 1237	(*Z*)-dihydrocarvone pulegone	1031 1174 1365	1,8-cineole Menthol Piperitenone oxide
C	Ca: 0.68-0.76	Purple	Dirty-white	1348	α-terpineol acetate (Fig. 6b)		
	Cb: 0.23-0.29	Orange	Dark	1573	Carvone acetate		
D	Da: 0.62-0.73	Reddish (weak)	Dirty-white	1193	(*Z*)-dihydrocarvone	1031	1,8-cineole
	Db: 0.31-0.40	Grey-violet (weak)	Reddish	1201	Dihydrocarveol neo	1238 1337	Dihydrocarveol neoiso Dihydrocarveol acetate iso
	Dc: 0.15-0.25	Grey-violet (weak)	Reddish	1205	Dihydrocarveol (Fig. 6c)	1366	Dihydrocarveol acetate neoiso
E	Ea: 0.27-0.58	Dark	Blue green (strong)	1237	Pulegone (Fig. 6d)	1031 1174	1,8-cineole menthol

[a] Capital letter: Profile; small letter: spot in decreasing order of R_f

Validation of TLC analysis by GC/MS

Examples of TIC chromatograms for some discriminant spots (marked in table 1) are displayed in Figure 5a-5e. These results confirm the discriminant character of selected spots.

Figure 5a. TIC chromatogram of extract from the spot containing piperitone oxide.

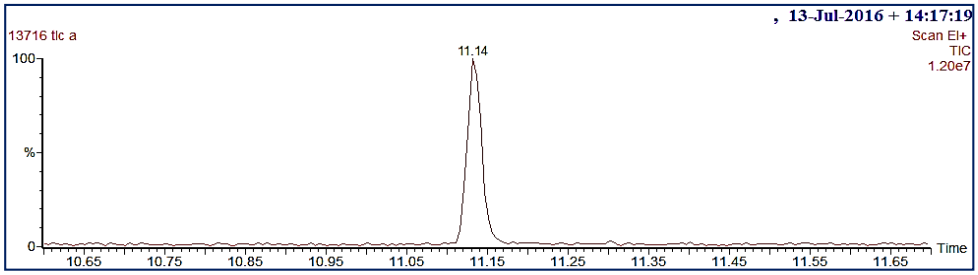


Figure 5b. TIC chromatogram of extract from spot containing α-terpineol acetate.

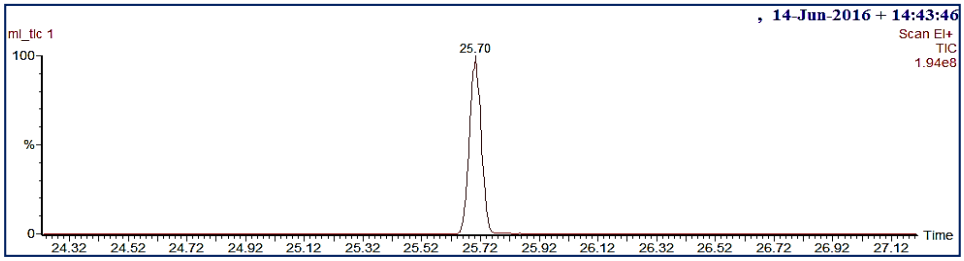


Figure 5c. TIC chromatogram of extract from spot containing dihydrocarveol.

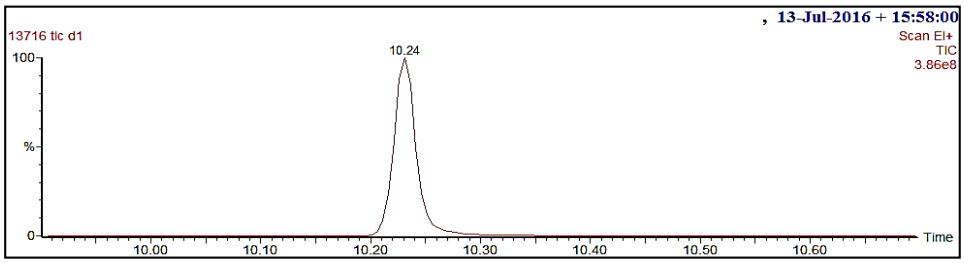
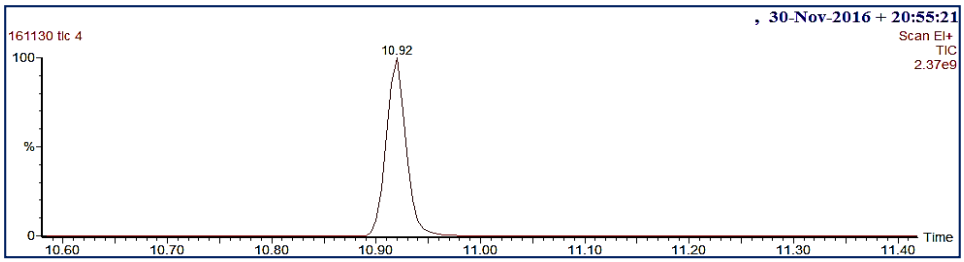


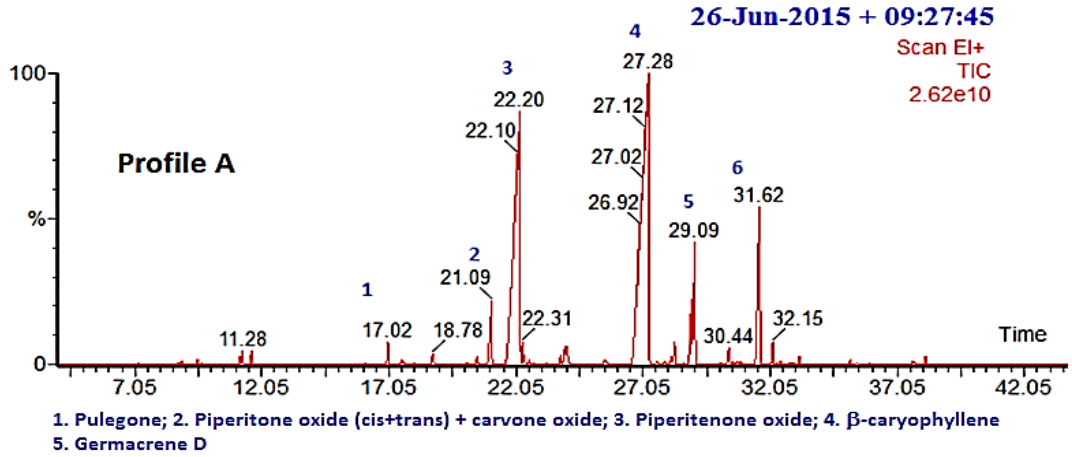
Figure 5d. TIC chromatogram of extract from spot containing pulegone.



Chemotypes characterization

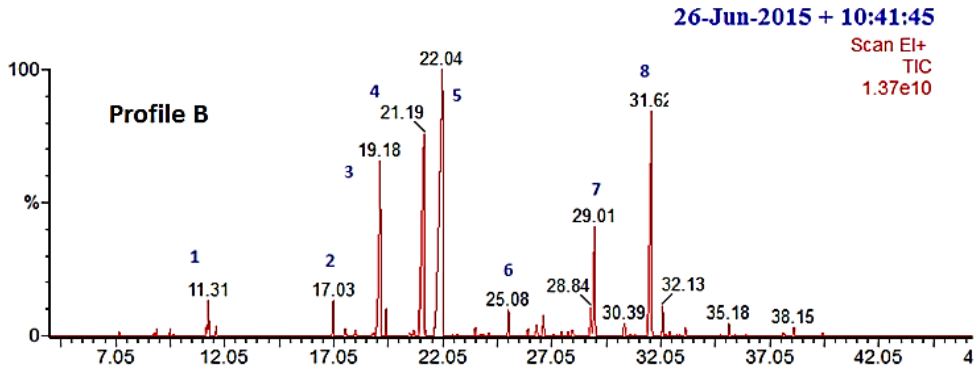
GC/MS TIC chromatograms of defined profiles are shown in Figure 6a-6e (major compounds are marked)

Figure 6a. Essential oil profile of chemotype A



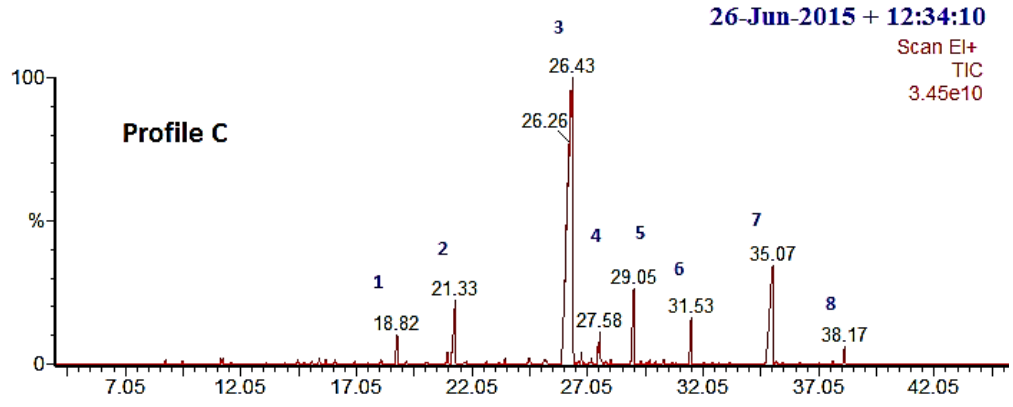
1. Pulegone; 2. Piperitone oxide (cis+trans) + carvone oxide; 3. Piperitenone oxide; 4. β-caryophyllene
5. Germacrene D

Figure 6b. Essential oil profile of chemotype B



1. 1,8-cineole; 2. Menthone; 3. (*E*)-Dihydrocarvone; 4. Pulegone; 5. Piperitone oxide; 6. Piperitenone
7. β-caryophyllene; 8. Germacrene-D

Figure 6c. Essential oil profile of chemotype C



1. α-terpineol; 2. Carvone; 3. α-terpineol acetate; 4. β-bourbonene; 5. β-caryophyllene; 6. Germacrene D
7. Carvone acetate; 8. Hymachalene.

Figure 6d. Essential oil profile of chemotype D

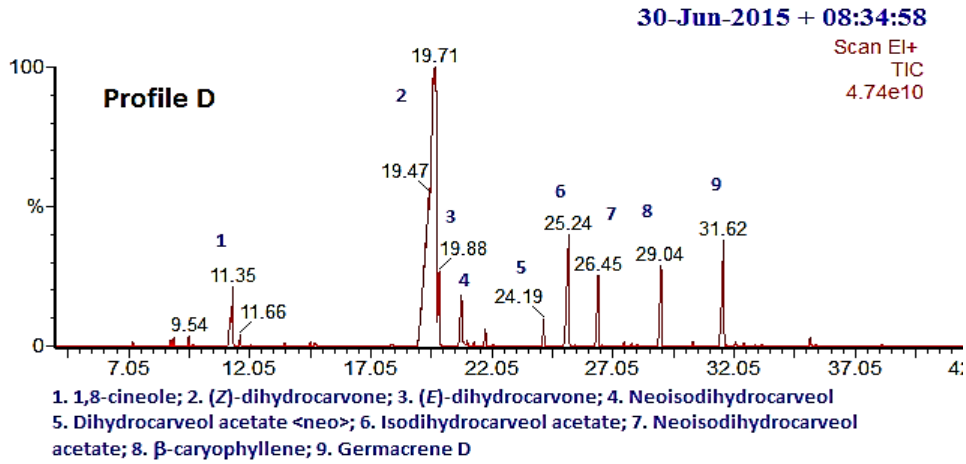
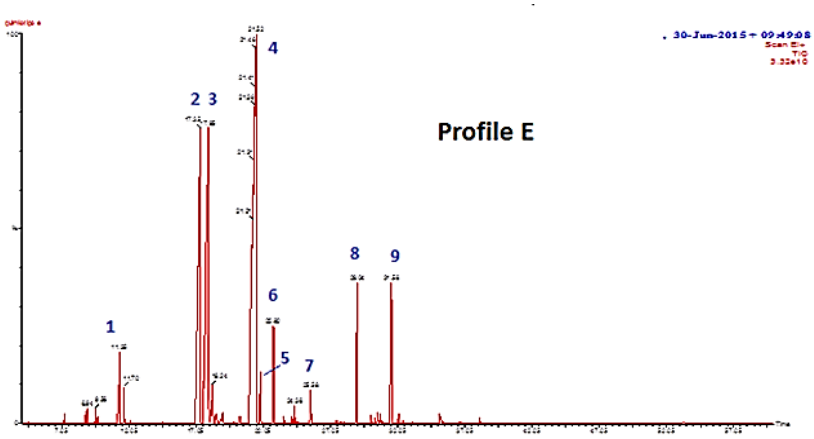


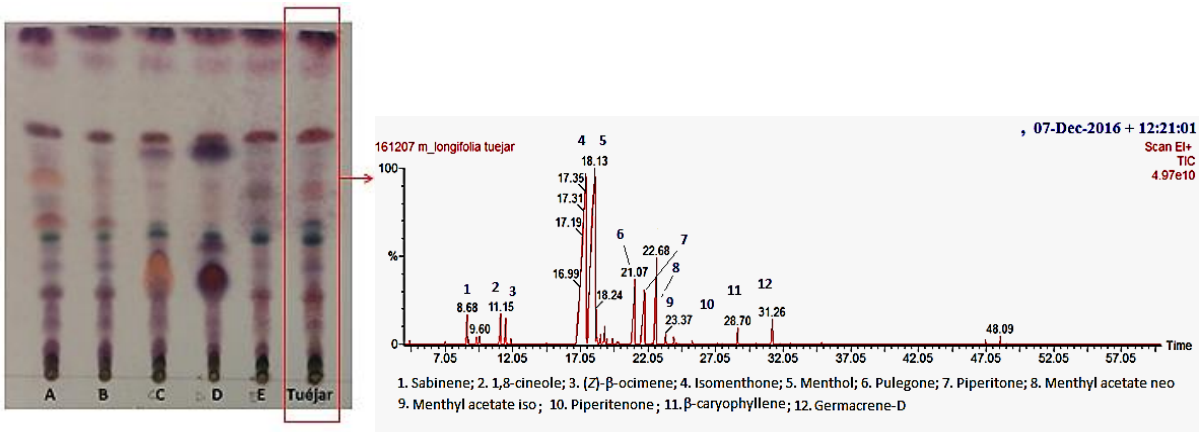
Figure 6e. Essential oil profile of chemotype E



Piperitone oxide and piperitenone oxide are the major compounds in profile A. This composition is similar to that reported by Baser et al. (1999) in Turkey and Singh et al (2008) in India. Profile B is characterized by (*E*) dihydrocarvone, pulegone and piperitone oxide as main compounds. Chemotypes rich in (*E*)-dihydrocarvone have been reported in samples from Serbia (Mimica-Dukic et al, 1991; Matovic & Lavadinovic, 1999; Dzamic et al, 2010). Chemotypes similar to profile C (α-terpineol acetate + carvone acetate) have not been reported so far except for samples coming from the same and nearby populations (Llorens-Molina et al.,2015). Profile D has been found mainly rich in dihydrocarvone isomers and minor amounts of carveol isomers and derivatives. It may be also considered a new specific chemotype given that no similar composition has been reported. Lastly, profile E has been found rich in menthone, menthol and pulegone. A very similar composition has been reported by Hajlaoui et al., (2009) in Tunisia. Other chemotypes rich in menthone and pulegone have been also reported from South Africa (Asekun et al., 2007; Oyedeji & Afaloayan, 2006).

Concerning the usefulness of the proposed TLC-GC/MS method, the results related with the identification of samples coming from Tuejar are promising. TLC profiles of this new sample matches with E chemotype. GC/MS TIC chromatogram confirms its identity (Figure 7).

Figure 7. New sample coming from Tuéjar compared with the five TLC profiles defined. GC/MS analysis confirm the identification of this new sample as belonging to chemotype E.



Conclusions

The proposed methodology contributes with an easy and reliable procedure allowing the screening of wild plant populations in several concerns: a) a first overview of intrapopulational variability, b) identification of chemical profiles potentially useful for their possible biological activities, c) the possibility of propagating these specific individuals in order to obtain chemical homogeneous cultivars. To the extent that this method can be applied to more populations, a greater number of profiles could be defined, thus increasing its usefulness.

On the other hand, the improvement of the photographic techniques used when the plates are developed with UV light, would allow to dispense with the validation by GC/MS, whose necessity is justified especially when the discriminant spots have very close R_f or very similar fluorescence colors.

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