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RESEARCH ARTICLE

Individual Monitoring to Study Organ and Diurnal Variations in *Mentha longifolia* L.

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Abstract

Growing aromatic plants as a source of bioactive compounds and extracts requires the production of raw materials with a standardized chemical composition. For this purpose, besides the selection of most suitable chemotypes, organ variations and phenotypic plasticity should be taken into account. The aim of this work is to carry out a first approach to diurnal variation for leaves and inflorescences in *Mentha longifolia* L. by means of individual monitoring as a way to bear in mind the intrapopulational variability. A total amount of 36 samples were processed from 3 individuals that were monitored during two days with different meteorological conditions. The extraction was performed by means of an ultrasound assisted method at low temperature in order to avoid the formation of artifacts. Two individuals showed α-terpineol acetate and carvone acetate as major compounds. No organ and diurnal variations were observed in neither of both individuals. Pulegone was found the major compound in the third individual, although considerable amounts of 1,8-cineole, isomenthone, trans-isopulegone, carvone acetate (only in inflorescences) and sesquiterpenes were also observed. Some great changes affecting major compounds were registered in this individual, both regarding organ differences and diurnal variations. Nevertheless, the most noteworthy result was the differences found related to meteorological conditions. This way, hot and dry periods could have a different and relevant influence on volatile chemical profile according to the *M. longifolia* chemotype.

Keywords: Seasonal variation, organ variation, diurnal variation, *Mentha longifolia*, ultrasound assisted extraction

Introduction

Genotypic variability and phenotypic plasticity are the two sources of infraspecific variability in essential oils composition. In that respect, the genus *Mentha* L. is characterized by a high level of genetic polymorphism, with a pronounced tendency to hybridization (Lawrence, 2006). Regarding *Mentha longifolia* L., its chemodiversity related to some ways of biological activity, has been extensively described in the literature (Baser, Kürkçüoglu, Tarimcilar & Kaynak, 1999; Lawrence, 2006; Viljoen et al., 2006; Pal Singh et al., 2008) with several noteworthy recent contributions (Hussain, Anwar, Nigam, Ashraf & Gilani, 2010; Dzamic et al., 2010; Bertoli, Leonardi, Krzyzanowska, Oleszek & Pistelli, 2011; Sharopov, Sulaimonova & Setzar, 2012; Iqbal, Hussain, Chatha, Naqvi & Bokhari, 2013; Stanisavljevic et al., 2014) coming from a wide range of geographical origins. Many oxygenated monoterpenic compounds can be considered as the most representative ones to describe the chemotypes, mainly: dihydrocarvone, piperitone, piperitenone and their oxides, 1,8-cineole, carvone, dihydrocarvone, pulegone, menthone, isomenthone, menthofurane, etc. It is worth to bear in mind that these components are recognized because of their allelopathic potentiality, so *M. longifolia* may be also

a promising source of useful bioactive compounds (Duke, 2003; Kordali, Cakir & Sutay, 2007; Young & Bush, 2009).

On the other hand, this plant has asexual reproduction via rhizome and, as reported by Segev, Nitzan, Chaimovitsh, Eshel & Dudai (2012), the propagated plants keep the essential oil composition as well as the chemotype, in such a way that the wild *M. longifolia* sampled can be horticulturally conserved. In addition, if the influence of environmental factors on each chemotype is known, a high control of essential oil composition can be achieved.

This study has been conducted to carry out a pilot study about diurnal and organ variations in *M. longifolia*. Results from an early study in the same population, sampled by means of mixing material from randomly selected individuals (Llorens-Molina et al., 2012), make evident high intrapopulational variability. For this reason, individual monitoring was selected as a suitable methodology to study phenotypic plasticity related to diurnal variations and environmental influence. Given that flowers play a specific ecologic role as a source of attractive compounds for pollination by insects, leaves and inflorescences composition were studied separately.

Material and methods

Plant material

Three plants of *M. longifolia* were selected in a watercourse belonging to fluvial network of Jiloca river, in Teruel (Spain) keeping a distance of 20 m among them, approximately. These individuals had reached enough amount of biomass to not suffer significant damages because of the sampling process. This population is located in the following coordinates: 40º 56' 11.59'' N; 1º 18' 06.37'' W, at 890 m.a.s.l. A Voucher specimen is kept at the herbarium of Agroforestal Mediterranean Institute (Universitat Politècnica de València, Spain).

Sampling and extraction processes

The sampling process was performed in the following way: three leaves and three inflorescences were cut at 8 AM, 2 PM and 8 PM. After taking an approximate representative mass of 1 g for each sample, each one of them were kept at once in glass vials containig 5 mL of dichloromethane for gas chromatography (Merck™). Once sealed, they were frozen (-18ºC) until laboratory processing.

To perform the extraction process, an ultrasound assisted method was adapted (Alissandrakis, Daferera, Tarantilis, Polissiou & Harizanis, 2003; Esclapez, García-Pérez, Mulet & Cárcel, 2011; Picó, 2013). The vials were introduced in an ultrasound bath Selecta™ for 15 min. The bath temperature was kept at 0-5 ºC to avoid vials heating up. Afterwards, a little amount of anhydrous sodium sulphate was added to remove water from extract. This was filtered with a syringe filter and kept into chromatographic vials up to chromatographic analysis.

Relative humidity and temperature were recorded *in situ* using a HOBO U12 data logger (Fig. 1).

GC and GC/MS analysis

The analysis of samples was carried out by GC-FID and GC-MS. A Clarus 500 GC (Perkin-Elmer Inc., Wellesley, PA) chromatograph equipped with an FID detector and capillary column ZB-5 (30 m x 0.25 mm i.d. x 0.25 μm film thickness; Phenomenex Inc., Torrance, CA) was used for quantitative analysis. The injection volume was 1 μL. The GC oven temperature was programmed from 50ºC 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 250ºC. The percentage composition

of the essential oil was computed from GC peak areas without correction factors by means of the software Total Chrom 6.2 (Perkin-Elmer Inc.)
Analysis by GC-MS was performed using a Clarus 500 GC-MS with the same capillary column, carrier and operating conditions above described 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 software Turbomass 5.4 (Perkin-Elmer Inc.).
Retention indices were determined by injection of C8–C25 n-alkanes standard (Supelco/ Sigma-Aldrich, Química SL, Madrid, Spain) under the same conditions. The essential oil components were identified by comparison of their mass spectra with those of computer library NIST MS Search 2.0 and available data in the literature (Adams, 2007). Identification of the following compounds were confirmed by comparison of their experimental RI with those of authentic reference standards (Sigma-Aldrich, Química SL): α-pinene, β-pinene, camphene, myrcene, limonene, (Z)-β-ocimene, camphor, terpinolene, β-thujone, borneol, terpinen-4-ol, bornyl acetate, geraniol and linalool.

Results and Discussion

A total amount of 36 samples were processed and a 77.3-87.5 % of total composition was identified. The chromatographic profiles of these extracts are noteworthy similar to the ones of essential oils from hydrodistillation, except for the occurrence of a fraction of unidentified low-volatile compounds accounting for a 10-15 % of total peaks area.

The chemical composition referred to major compounds for extracts from leaves and flowers during the sampling days is shown in Table 1. Genotypic and phenotypic variation sources affecting chemical composition can be observed. Individuals 1 and 2 show very similar and consistent results when both sampling days are compared. Only carvone acetate shows slight differences in their diurnal change. As this individual monitoring does not involve any statistical analysis, only noteworthy differences can be taken into account. This way, no diurnal changes can be considered regarding the composition of individuals rich in α-terpineol acetate and carvone acetate. Likewise, organ variations cannot be considered either.

However, composition of individual 3 is completely different, the same way that its organ and diurnal variations for each one of sampling days (Fig. 2, 3). Pulegone was found the major compound, accounting for a 46,3-78,7 %. In addition, significant amounts of 1,8-cineole (2-3.7 %), isomenthone (0.4-9.1 %), (*Z*)-isopulegone (0.2-10.1 %), carvone acetate (only in inflorescences, 0.2-27.9 %) and sesquiterpenes (1.8-5.5 %) were also observed.

On the first day, a great increase in isomenthone and trans-isopulegone rates was observed at 2 PM, at the same time that an important decrease in pulegone. These variations are very similar both in leaves and inflorescences (Fig. 2, 3). This consistence can be interpreted as a sign of data reliability. On the other hand, the rest of main compounds show stable rates, except for sesquiterpenes. This fraction show different behavior in leaves (no diurnal changes are observed) and flowers (high increase at midday).

Nevertheless, the most interesting changes concern to differences between 1st and 2nd day. As shown in Fig. 1, noticeable differences in temperature and relative humidity variations were found over the day. Indeed, from these data, the first day was a typical clear summer one, with a high temperature and dryness period from midday on. The 2nd day was characterized by a more stable temperature and higher level of relative humidity (it was raining the previous night). This day, a stable and very low amount of isomenthone

and trans-isopulegone was observed, whereas pulegone shows the higher rate in the evening. The most noticeable difference between these two days affects to the presence of carvone acetate in flowers. This compound is hardly found the first day but achieves a relative great amount (21.6-27.9%) in the 2nd day up to afternoon, to be almost not found in the evening (Fig. 3).

Fig.1. Meteorological conditions during the sampling days

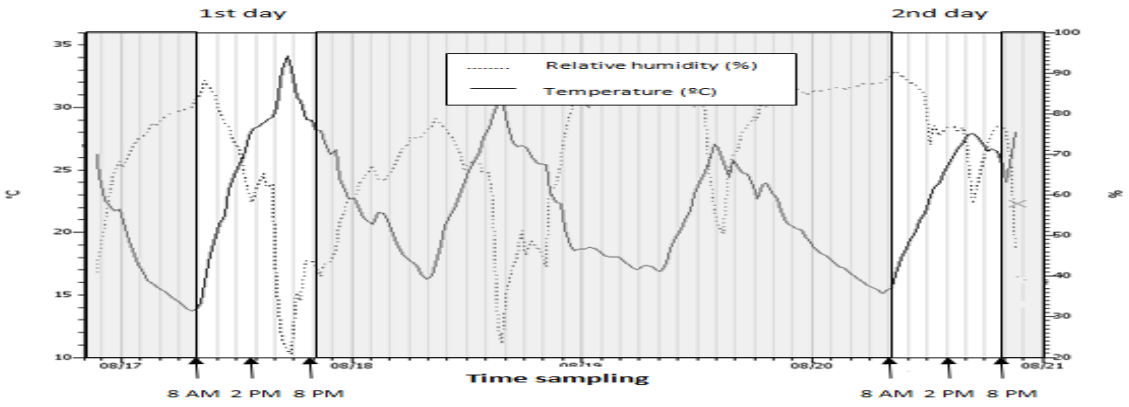

Fig. 2. Diurnal variations in leaves for individual 3 (major compounds)

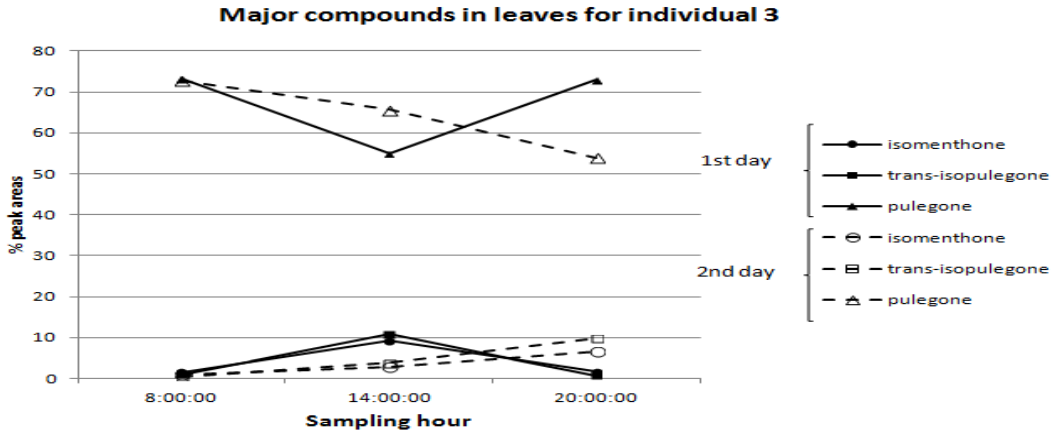

Fig. 3. Diurnal variations in flowers for individual 3 (major compounds)

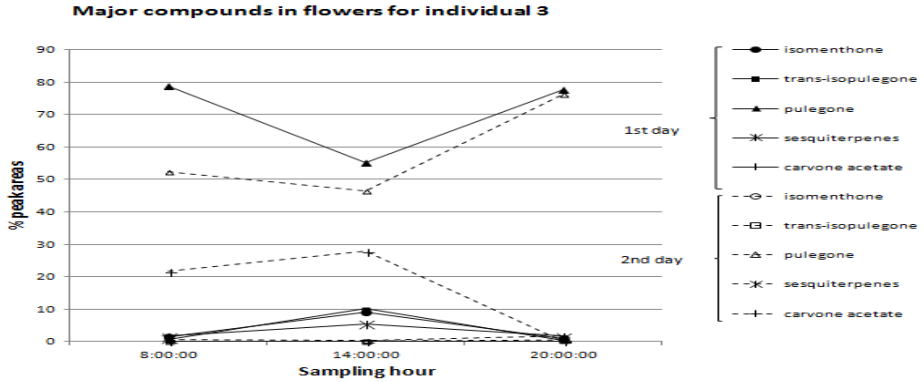

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Table 1. Major compounds in leaves and flowers during the sampling days (major compounds)

	Leaves									Flowers								
	Individual 1			Individual 2			Individual 3			Individual 1			Individual 2			Individual 3		
1st day (times sampling)	**8:00**	**14:00**	**20:00**	**8:00**	**14:00**	**20:00**	**8:00**	**14:00**	**20:00**	**8:00**	**14:00**	**20:00**	**8:00**	**14:00**	**20:00**	**8:00**	**14:00**	**20:00**
Hydrocarbon monoterpenes	0.8	0.9	1.2	0.5	0.8	0.8	2.3	2.4	2.3	0.9	1.1	1.3	1.1	1.2	1.3	2.0	2.1	2.2
1,8-cineole	0.9	1.1	1.0	0.0	0.0	0.0	3.5	3.4	3.3	0.2	0.2	0.2	0.0	0.0	0.2	2.8	3.4	3.7
Isomenthone	0.0	0.0	0.0	0.1	0.0	0.0	0.0	1.5	9.3	1.7	0.0	0.0	0.0	0.0	0.0	1.5	9.1	1.0
Trans-isopulegone	0.0	0.0	0.0	0.0	0.0	0.0	1.0	10.8	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.1	0.4
Pulegone	0.0	0.0	0.0	0.0	0.0	0.0	73.4	55.0	72.9	0.0	0.0	0.0	0.0	0.0	0.0	78.7	55.2	77.6
Alpha-terpineol acetate	9.8	9.7	10.2	14.0	14.4	13.1	0.1	0.1	0.1	10.6	10.7	11.2	12.1	12.5	12.7	0.1	0.2	0.1
Sesquiterpenes	2.7	2.5	2.6	2.3	2.5	2.6	5.3	5.3	5.8	1.7	2.0	2.2	2.4	2.2	2.1	1.8	5.5	1.8
Carvone acetate	70.6	69.1	70.1	60.8	65.0	55.8	0.0	0.1	0.0	66.5	68.3	68.4	61.0	64.0	61.1	0.0	0.2	0.0
2nd day (times sampling)																		
Hydrocarbon monoterpenes	1.0	1.3	1.2	0.6	0.7	0.9	1.9	2.2	2.2	1.0	1.2	1.4	1.4	1.0	1.4	1.7	1.9	2.2
1,8-cineole	0.9	0.9	1.0	0.0	0.0	0.0	3.6	3.1	3.3	0.2	0.2	0.3	0.0	0.0	0.0	2.1	2.2	3.2
Isomenthone	0.0	0.0	0.0	0.0	0.0	0.0	1.0	2.8	6.5	0.0	0.,0	0.1	0.0	0.0	0.0	0.6	0.4	1.8
Trans-isopulegone	0.0	0.0	0.0	0.0	0.0	0.0	0.5	3.8	9.7	0.0	0.0	0.2	0.0	0.0	0.0	0.2	0.2	0.6
Pulegone	0.0	1.1	0.2	0.0	0.0	0.0	72.7	65.6	53.9	0.4	0.6	1.2	0.0	0.0	0.0	52.3	46.3	76.4
Alpha-terpineol acetate	9.6	10.3	10.1	12.5	13.1	13.0	0.4	0.2	0.7	10.4	10.3	10.8	13.2	12.2	13.8	5.2	6.4	0.2
Sesquiterpenes	3.3	2.6	2.7	2.4	2.6	3.0	4.4	6.4	6.7	2.1	2.9	3.0	2.3	1.9	2.8	2.1	1.9	2.1
Carvone acetate	70.4	67.1	68.6	65.3	59.3	61.7	2.3	0.1	0.8	64.6	64.8	65.1	64.2	60.5	63.1	21.6	27.9	0.2

Individual sampling and the extraction process allowed the detection of two different chemotypes among three closely collected plants. Despite the number of individual plants in which this work is based on, it seems evident that diurnal variations affect each chemotype in a different way. These results also show how diurnal variations are influenced by environmental factors, such as temperature and humidity. This way, for farming purposes, once the appropriate chemotype is selected, a deep knowledge of factors affecting its chemical profile would be useful. This approach requires carrying out studies involving enough number of individuals which should be sampled at once. In order to deal with this problem and to make feasible a statistical treatment, a method for sampling and the ultrasound assisted extraction are proposed in the present work. This method can be considered a suitable, fast and cheap way to analyze volatile composition in plants. Nevertheless, two weak points should be considered for future researches. Firstly, according the research goals (such as the knowledge of ecological significance of volatile content in leaves and inflorescences, for example), the relationships between volatile content and emissions should be established. Thus, to correlate extracts composition and data from volatile emissions analysed by solid-phase microextraction could be necessary. Secondly, the extraction process could be improved in order to remove the low-volatile fraction.

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