Effect of origin, harvesting time and leaf age on the yield and content of thymol in essential oils from *Lippia origanoides* H.B.K.

Efecto del origen, la época de recolección y la edad de las hojas en el rendimiento y el contenido de timol de aceites esenciales de Lippia origanoides H.B.K.

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Abstract

The purpose of this research was to evaluate the effect of the origin (4 localities), the plants leaves age (young and mature) and the season (rainy and dry season) on the essential oils (EOs) yield and thymol content of *Lippia origanoides* growing wild in the Alto Patía region (south-west of Colombia). The extractions were performed through microwave-assisted hydrodistillation technique (MWHD) and the EOs were analyzed by gas chromatography. Yields of EOs varied between 2.5 and 3.3% and were obtained where the origin factor showed statistical differences ($P \leq 0.05$) over the yielding. Taking into account the thymol content, differences related to the season were observed. The highest thymol content in the EOs was obtained in the dry season. The major compounds identified in the EOs were thymol (50.8 - 81.6%), ρ-cymene (7.5 - 19.5%) and γ-terpinene (2.3 - 7.4%).

Key words: Essential oil, *Lippia origanoides*, MWHD, thymol, wild oregano.

Resumen

En este estudio se evaluó el efecto del origen (cuatro zonas), la época de recolección (lluviosa y seca) y la edad de las hojas (jóvenes y maduras) sobre el rendimiento y el contenido de timol del aceite esencial (AE) de plantas de *Lippia origanoides*, que crecen en estado silvestre en la región del Alto Patía al suroccidente de Colombia. Las extracciones de AE se realizaron mediante la técnica de hidro-destilación asistida por radiación de microondas (MWHD) y fueron analizadas por cromatografía de gases. Los rendimientos de AE variaron entre 2.53 y 3.28% y sólo se encontraron diferencias significativas ($P \leq 0.05$) para la zona de origen de las plantas. Para el contenido de timol se observaron diferencias relacionadas con la época de recolección, siendo mayor el contenido en las muestras recolectadas en época seca. Los compuestos principales identificados en los AE fueron timol (50.8 - 81.6%), ρ-cimeno (7.5 - 19.5%) y γ-terpineno (2.3 - 7.4%).

Palabra clave: Aceite esencial, *Lippia origanoides*, MWHD, orégano de monte, timol.

Introduction

Lippia origanoides H.B.K. is a plant known as wild oregano, it grown till 3 m tall, has very aromatic green oval shaped leaves and white, axillary and cluster inflorescences. In Colombia it is frequently found in the departments of Guajira, Magdalena, Cauca, Cundinamarca, Norte de Santander and Santander (García-Barriga, 1992).

This plant is used in traditional medicine to treat stomachaches, nausea, flatulence and as an antiseptic for mouth and throat (Pascual *et al.*, 2001). Due to the high essential oil (EO) extraction yield and its antimicrobial and antioxidant properties demonstrated in several studies (Dos Santos *et al.*, 2004; Olivera *et al.*, 2007; Muñoz *et al.*, 2007) this is a promising species to obtain compounds for pharmaceutical, cosmetic and food industry use.

Essential oil composition of *L. origanoides* is affected by the edaphic and environmental conditions during plant growth, such as soil characteristics, solar light duration, temperature, water stress and plant age (Dos Santos *et al.*, 2004).

Due to the factors that affect *L. origanoides* EO composition and yield, it is convenient to study its biological activity which varies according to genetics and local environmental parameters, therefore, it is necessary to characterize the production niche and to define the potentiality to develop plants with a excelled profile related to specific ingredients that are biologically active, and to ensure product quality and efficiency. In this sense, among the most important factors that affect wild oregano´s EO composition are, plant origin zone, plant organ used, developmental stage, weather and growth conditions)temperature, soil, fertilization), as well as extraction method and storage conditions (Cosentino *et al.*, 1999; McGimpsey *et al.*, 1994).

The objective of this research work was determining the effect of origin, leaf age and harvesting season (rainy or dry) on yield and content of thymol on wild oregano (*L. origanoides*) essential oil from the Alto Patía region in Colombia, in order to get information for a better use with commercial purposes.

Materials and methods

Lippia origanoides leaves were manually collected in the Alto Patía area in Taminango town, between the Departments of Nariño and Cauca at the southwestern of Colombia. This micro-region has a tropical dry weather, 24 °C mean temperature, semiarid soils, low precipitation (700 900 mm/year) and with typical subxerophytic vegetation. Leaf material harvesting was done in June and July (dry season) and February and March (wet season). In each season two types of leaves were collected from the same plant, young and mature leaves. Young leaves were those small, turgid and green ones; whereas the mature leaves were larger, less turgid and yellow in color.

Leaves were separated from sample contaminants, weighted and dried at room temperature under the shadow during eight days until they reached 12% humidity, then they were packed on polyethylene bags and stored at a clean and dry environment until processing.

A multi-factor categorical design was used, with two replicates to study the effect of the origin (four production zones), leaves age (young and mature) and harvesting time (rainy and dry) on thymol yield and content on *L. origanoides* essential oil. The selected production zones were El Cardo and Las Juntas, localized between 740 and 990 MASL and San Juanito and Alto de Mayo between 1100 and 1200 MASL.

Extraction of volatile secondary metabolites was done in the Natural Products Lab at the University of Nariño by means of microwave-assisted hydrodistillation technique (MWHD), using a microwave oven (Electrolux, EME281D28S, 120 v, 60 Hz, 850 W) with a hole in the top to connect a Clevenger distillation equipment according to the procedure described by Stashenko *et al.* (2004). 50 g of leaves chopped in 1.18 mm size or larger, were used for extraction of every sample, they were submerged in 300 ml of water. Essential oil was separated from the water by decantation and dried out with anhydro Na_2SO_4. For chromatographical analyses an oil aliquot (20 µl) was diluted in 1 ml dichloromethane.

Analyses to determine essential oil composition was performed in the Specialized Labs at the Universidad de Nariño using a gas chromatographer Shimadzu GC 17A version 3 equipped with a split/splitless injector at 250 °C, split ratio 1:100 and a fire ionization detector (FID) (280 °C). Chromatographical data were obtained and processed with Shimadzu Class VP 4.3 software. It was used for mix separation an apolar column DB-5 (J&W) 30 m x 0.25 mm D.I. and 0.25 µm with a 5% phenyl-polyethylsiloxane stationary phase. Oven temperature was programmed from 40 °C (5 min) till 250 °C at 5 °C/min. Carrier gas and auxiliary gas was helium (99.9995%, Aga-Fano S.A) flux 1 ml/min, FID flux velocity for combustion gases was 300 ml/ml for hydrogen, and the extraction injected volume was 1.0 µL. Mass spectra were obtained in the SCAN mode on a mass interval of 38 to 450 m/z.

Compound identification was done using Kovats retention indexes with α n-alcane (C_6-C_{32}) series and by comparison of the obtained mass spectra with the Wiley spectra library. Quantification was performed by calculating the relative area percentage of each compound considering those compounds with concentration higher than 0.1%.

Results and discussion

Essential oil composition.

On table 1 is presented the identification of the volatile secondary metabolites obtained by MWHD and their relative percentages in EO coming from mature leaves harvested in the dry season in Alto de Mayo. Main identified components in the EO were thymol with a mean concentration in all the analyzed samples of 72.6%, ρ-cymene (10.7%) and γ-terpinene (4.8%).

Variations in chemical composition of secondary metabolites in plants of the same species allowed the designation of chemotypes. Castañeda *et al.* (2007) studied the chemical composition of leaves essential oils from ten Colombian aromatical plants, among them *L.*

Table 1. Relative quantity (%) and identification of volatile secondary metabolites from *Lippia origanoides* extracted by MWHD.

Pick (No.)	Rt (min)	Area (%)	Experimental IK	Compound*
1	11.449	0.39	924	Tricyclen
2	11.758	0.22	931	α-Pinene
3	14.416	2.76	991	Myrcene
4	15.082	0.29	1006	α-Felandreno
5	15.600	1.21	1018	α-Terpinene
6	16.100	10.23	1029	ρ-Cymene
7	16.190	0.68	1031	Limonene
8	17.500	3.85	1061	γ-terpinene
9	17.990	0.31	1073	Cis-Hydrate Sabinene
10	19.290	1.20	1102	Linalool
11	23.473	0.57	1205	Terpinene-4-ol
12	24.482	0.79	1231	cis-Carveol
13	24.460	0.47	1230	Thymol methyl eter
14	27.562	69.19	1311	Thymol
15	27.590	0.41	1313	Carvacrol
16	28.490	0.64	1350	Timilo acetate
17	30.448	0.22	1392	Timpol isomer / ainilla
18	31.606	1.54	1423	Trans-Caryophylene
19	32.448	0.23	1446	ß-Humulene
20	32.940	0.61	1459	α-Humulene
21	33.773	0.39	1481	γ-Muurolene
22	34.831	0.17	1510	ß-Bisabolene
23	37.598	1.00	1589	Caryophylene oxide

origanoides, finding two chemotypes for the EO of this species. Chemotype A (typical) with main components: carvacol (36.6%), ρ-cymene (14%), γ-terpinene (13.3%) and thymol (9.1%). For chemotype B (atypical) had as main components: ρ-cymene (15.7%), trans-β-caryophylene (9.4%), α-phellandrene + δ-3-carene (8.7%) and limonene (6.9%).

Generally essential oils are complex mixes of 100 or more compound which chemical composition can correspond to aliphatic compound of low molecular mass (alcanes, alcohols, aldehydes, ketones, esters and acids), monoterpens, sesquiterpenes and phenylpropanes (Lee *et al.*, 2003). Chemical composition of wild oregano EO from Alto Patía region, corresponds in its major part to aromatic monoterpenes thymol (72.6%), ρ-cymene (10.7%) and γ-terpinene (4.8%), which is very similar to the composition of *Thymus vulgaris*, which has the same main components (Solomakos *et al.*, 2008). Oregano is the commercial name given to those species rich in phenolic monoterpenes, mainly carvacrol and thymol. In the international markets is generally accepted that the greek oregano (*Origanum vulgare hirtum*) has the best essential oils qualities and its main components are carvacrol and/or thymol, varying its basic composition according to the geographic areas of the crop. In contrast with

Table 2. Thymol yield and content on essential oils from *Lippia origanoides* according to the production zone.

Experiment	Zone	Leaves age	Harvesting time	Yield	Thymol
1	San Juanito	Young	Rainy	2.7	69.2
2	San Juanito	Young	Rainy	3.1	59.9
3	San Juanito	Mature	Rainy	3.0	70.2
4	San Juanito	Mature	Rainy	3.0	50.8
5	Alto de Mayo	Young	Rainy	3.5	58.0
6	Alto de Mayo	Young	Rainy	3.2	52.3
7	Alto de Mayo	Mature	Rainy	3.2	64.2
8	Alto de Mayo	Mature	Rainy	3.2	67.8
9	El Cardo	Young	Rainy	3.1	70.3
10	El Cardo	Young	Rainy	3.2	56.6
11	El Cardo	Mature	Rainy	2.7	67.8
12	El Cardo	Mature	Rainy	2.9	68.1
13	Las Juntas	Young	Rainy	2.6	81.6
14	Las Juntas	Young	Rainy	2.5	64.3
15	Las Juntas	Mature	Rainy	2.5	68.2
16	Las Juntas	Mature	Rainy	2.5	69.7
17	San Juanito	Young	Dry	3.7	79.8
18	San Juanito	Young	Dry	3.4	79.4
19	San Juanito	Mature	Dry	4.0	83.4
20	San Juanito	Mature	Dry	3.4	83.0
21	Alto de Mayo	Young	Dry	2.7	78.1
22	Alto de Mayo	Young	Dry	2.7	77.1
23	Alto de Mayo	Mature	Dry	2.4	86.7
24	Alto de Mayo	Mature	Dry	2.7	87.3
25	El Cardo	Young	Dry	2.6	74.2
26	El Cardo	Young	Dry	3.5	74.2
27	El Cardo	Mature	Dry	2.9	82.1
28	El Cardo	Mature	Dry	3.0	82.5
29	Las Juntas	Young	Dry	2.0	77.2
30	Las Juntas	Young	Dry	1.9	77.5
31	Las Juntas	Mature	Dry	2.9	82.1
32	Las Juntas	Mature	Dry	3.6	80.9

the composition found in the *L. origanoides* oil, the one from *O. vulgare hirtum* presents a high level of carvacrol (90.3%) and low thymol (3.5%) (Ariza *et al.*, 2011).

Results obtained in the most abundant compounds in the essential oil of oregano from the Alto Patía region (thymol 72.6%, ρ-cymene 10.7% and γ-terpinene 4.8%) differed from the chemotypes found by Castaeda *et al.* (2007), however, they agree with the chemotype II cited in the work of Ruiz *et al.* (2007), which more frequent compounds are thymol (56.3%), ρ-cymene (11.8%) and γ-terpinene (7.3%), and with the chemotype C described by Stashenko *et al.* (2010) with thymol as the most abundant compound (56%), followed by ρ-cymene (9%) and γ-terpinene (5%), however the Alto Patía oregano essential oil differs in its high thymol content.

Effects of leaf origin, age and harvesting time in the EO yield

Experimental matrix and obtained results to determine the effect of the harvesting zone, leaves age (young and mature) and the collection time (rainy and dry) on thymol yield and content on *L. origanoides* essential oils are displayed on Table 2.

Significant statistical differences between mean of EO extraction yield according to the harvesting zones were observed (Table 3). With the FISHER minimal significant difference at 95% confidence level, it was determined that the samples derived from San Juanito had the highest yield of extracted oils with an average of 3.3%; the opposite happened with the yield of samples from Alto de Mayo and El Cardo (2.96%) that did not have differences. Meanwhile Las Juntas' samples had the lowest yield (2.6%).

Zone x leaves age, zone x harvesting time and leaves age x harvesting time interactions, showed a significant effect on EO yields ($P < 0.05$). Mature leaves samples from San Juanito zone had the highest yield (3.4%), while the lowest were obtained in young leaves samples from Las Juntas (2.2%). Leaves age x harvesting time interaction was also significant ($P < 0.05$) since in the dry season it was observed an increase in the yield while leaves were maturing, changing from 2.8% in young leaves to 3.1% in mature leaves.

EO extraction yield in dry season agree with the ones find by Stashenko *et al.* (2010) to samples collected in the same zone (3.1 ± 0.2%). It is possible to declare that the harvesting season is not a factor that influences oregano EO yield since the results of this study agree with the ones of Rojas *et al.* (2006), they studied the EO composition from *L. origanoides* collected in dry and rainy seasons in Merida, Venezuela, with the EO extraction yield of 1.1% for both seasons.

Effects of origin, leaves age and harvesting time on thymol content

Analysis of variance (Table 4) showed significant differences between the mean of thymol percentages of *L. origanoides* oil according to the season when the samples were collected. In the dry season, the average content was higher (80.3%) than in the rainy season (64.9%). These results agree with the ones of Rojas *et al* (2006) who found that the contents of this compound were higher in the samples collected during the dry season.

Table 3. Analysis of variance for yield according to the origin, harvesting time and leaves age of *Lippia origanoides*.

Source of variation	Sum of squares	d.o.f	Mean square	F- ratio	P <
A: Zone	2.12344	3	0.707813	10.44	0.0005
B: Leaves age	0.0703125	1	0.0703125	1.04	0.3237
C: Harvesting time	0.0078125	1	0.0078125	0.12	0.7387
AB	0.888437	3	0.296146	4.37	0.0199
AC	1.76094	3	0.586979	8.66	0.0012
BC	0.340312	1	0.340312	5.02	0.0396
ABC	0.603437	3	0.201146	2.97	0.0634
Residue	1.085	16	0.0678125		
Corrected total	6.87969	31			

Table 4. Analysis of variance for thymol content in essential oil of oregano.

Source of variation	Sum of squares	d.o.f.	Mean squares	F-ratio	P <
A: Zone	70.6959	3	23.565313	0.75	0.5364
B: Leaves age	132.438	1	132.438	4.23	0.0563
C: Harvesting time	1898.82	1	1898.82	60.7	0.0000
AB	152.086	3	50.5963	1.62	0.2240
AC	216.318	3	72.1061	2.31	0.1157
BC	40.2753	1	40.2753	1.29	0.2732
ABC	29.7284	3	9.90948	0.32	0.8131
Residue	500.495	16	31.2809		
Corrected total	3040.86	31			

Conclusions

- Wild oregano (*L. origanoides*) from the Alto Patia region (southwestern Colombia) is a highly promising species because it is a chemotype rich in thymol (50.8 - 81.6%), compound with a demonstrated antimicrobial and antioxidant activity. Additionally, the extraction yield average of its essential oil is high (2.76%) compared with other aromatic species.
- It was observed that the origin of the plants had a significant effect on EO extraction yield. Higher yields (3.28%) were obtained on the samples coming from the zones located between 1100 and 1200 MASL (San Juanito) than in the zones between 740 and 990 MASL (2.53%) (Las Juntas). However it is required to enlarge this study considering other aspects such as soil characteristics.
- Harvesting time of oregano leaves (rainy and dry) affected significantly the thymol content on EO, the samples collected during the dry season had higher thymol content (80.3%) than the ones from the rainy season (64.9%).

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