## Scientific note
# RELATIONSHIP BETWEEN REFRACTIVE INDEX AND THYMOL CONCENTRATION IN ESSENTIAL OILS OF *Lippia origanoides* Kunth

## Nota científica
# RELACIÓN ENTRE EL ÍNDICE DE REFRACCIÓN Y LA CONCENTRACIÓN DE TIMOL EN ACEITES ESENCIALES DE *Lippia origanoides* Kunth

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**ABSTRACT**

The composition of essential oils extracted from plants can present great variability, even between individuals of the same species due to different conditions, such as extraction methods, climate and soil composition, among others. Most methods available for the determination of essential oil composition, such as gas chromatography-mass spectrometry (GC/MS), are expensive and time consuming, which prevents farmers or producers from determining the true value of their product or performing in-line quality control in production. Therefore, practical tools are required to test the quality of essential oils in a quick and inexpensive way. The aim of this study was to determine the relationship between the concentration of thymol and refractive index of essential oils as a quality control measure in *Lippia origanoides* Kunth, an aromatic and medicinal plant with a large content of thymol. Essential oils were extracted by steam distillation from leaves of 5-month-old plants. A Clevenger type apparatus was used for extraction. The refractive index was measured with an Abbe refractometer, while the concentration of thymol was determined by GC-MS. It was found that thymol concentration and refractive index are adjusted in a linear equation $y = 0.00030x + 1.4893$ with a correlation coefficient of 0.9439, demonstrating that the refractive index of the essential oils of *Lippia origanoides* Kunth can be used to determine the thymol concentration of the oil.

**Key words:** Medicinal plants, secondary metabolites, analysis GC/MS.

**RESUMEN**

La composición de los aceites esenciales extraídos de plantas puede presentar gran variabilidad, aún entre individuos de la misma especie, debido a diferentes condiciones como el método de extracción, el clima, la composición del suelo, entre otros. La mayoría de los análisis disponibles para determinar la composición como la cromatografía de gases acoplado a espectrometría de masas (CG-MS) son costosos y toman tiempo, lo que impide que el productor pueda determinar el verdadero valor

de su producto y hacer un control de calidad oportuno. Por esto es necesario contar con herramientas prácticas que permitan controlar la calidad de los aceites esenciales de manera ágil, rápida y a bajo costo. El objetivo de este estudio fue determinar la relación presente entre la concentración de timol y el índice de refracción de aceites esenciales en plantas de *Lippia origanoides* Kunth, una planta aromática y medicinal, con alto contenido de timol. Los aceites esenciales fueron extraídos por destilación por arrastre con vapor usando un aparato tipo Clevenger, de las hojas de plantas de 5 meses de edad, se midió el índice de refracción con refractómetro tipo Abbe y la concentración de timol se determinó por CG-MS. Se encontró que la concentración de timol y el índice de refracción se ajustan a una ecuación lineal y = 0,00030 + 1,4893 con coeficiente de correlación 0,9439. Demostrando que el índice de refracción de los aceites esenciales de *Lippia origanoides* Kunth puede ser usado para determinar la concentración de timol en el aceite.

**Palabras clave:** Plantas medicinales, metabolitos secundarios, análisis GC-MS.

## INTRODUCTION

Thymol is a natural cyclic monoterpene found in essential oils of some plant species of genus *Thymus, Origanum, Lippia*, and others used for medicinal, aromatic and seasoning purposes. As products of secondary metabolism of plants, their production is minor in relation to the production of biomass.

At present, thymol is used as a biocide with different technologies of application: encapsulated in nanospheres for cosmetic applications (Wattanasatcha et al., 2012), vapors for postharvest treatment of fruits of the genus Prunus (Svircev et al., 2007), powder to control *Varroa destructor* mites in apiculture (Papachristoforou et al., 2011). It is also used as a tool in biochemistry to study enzymatic mechanisms of muscle contraction (Tamura and Iwamoto, 2004) and, in synthesis, as a starting point for other molecules such as L-menthol (Dudas and Hanika, 2009).

*L. origanoides* (Creole oregano) is an aromatic and medicinal plant, native to several countries in Central America and the Caribbean (Mexico, Guatemala, Cuba) and northern South America (Venezuela, Brazil and Colombia) (Pascual et al., 2001). It is different from Orégano (*Origanum vulgare*), which it is grown in South American countries like Chile, but this also contains large amounts of thymol in its essential oil. In Colombia, accessions presented contents of thymol between 30% and 68% of total secondary metabolic byproducts, and a total content exceeding 4% in the essential oil (Delgado et al., 2015). A study conducted by Ruiz et al. (2007) reported that the major components of total volatile secondary metabolites were ǫ-cymene and 1,8-cineole for chemotype I and thymol for chemotype II when examining two *L. origanoides* chemotypes native to Santander, Colombia.

Many factors affect the quality, quantity and composition of essential oils, such as the type of extraction method used (hydrodistillation, steam distillation, supercritical fluids, microwave assisted hydrodistillation) (Staschenko et al., 2004), climate, soil composition, plant organ, age, and vegetative cycle stage (Angioni et al., 2006). Since factors are variable and can not be entirely controlled, it is necessary to have practical tools to measure the quality of essential oils quickly and inexpensively.

The refractive index (RI) is used to identify compounds, to determine their purity, and to analyze the ratio of homogeneous binary mixtures of known components. The latter occurs because the RI of a mixed solution is a weighted average of the RIs of the components of the mixture. Thus, it is useful as a rapid measure of purity and quality.

Various techniques are available for the determination of RI in liquids. The relationship between RI and density of a fluid has been subject of many theoretical and experimental studies. The effects of wavelength and temperature on RI have also been studied and it has been determined that temperature presents the greatest sensitivity in the analysis (Khodier, 2002). The Abbe refractometer provides reliable measurements of RI. In fact, accuracy of Abbe devices varies from $10^{-4}$ to $10^{-5}$ refractive index units (Räty and Peiponen, 2015), correcting possible interference in the determination.

In vegetable oils, RI is related to the degree of saturation, as cis/trans double bonds and can be transformed by the incident light used in the assay. In essential oils, RI is influenced by the chemical composition of the oil. In this case, even though composition is varied, a main component can be generally found.

The objective of this research was to determine the relationship between RI and the concentration of thymol in the essential oil of chemotypes of *L. origanoides*. To the best of the authors' knowledge, no studies have reported on this to date.

## MATERIALS AND METHODS

The study was conducted in greenhouses and laboratories of the Universidad Nacional de Colombia, campus Palmira (03°30′45″ N; 76°18′30″ W), located at 930 m.a.s.l. in the town of Palmira, Valle del Cauca State, with average temperature of 26°C and relative humidity of 65%. Plants of three chemotypes of *L. origanoides* (Patía, Cítrica y Típica) of 5 months of age were used. Material was collected from different regions of Colombia and also obtained from the *in vivo* collection of native medicinal, aromatic, and seasoning plants of the Universidad Nacional de Colombia.

### Essential oil extraction

The leaves of *Lippia origanoides* Kunth were dried at ambient conditions for two days after harvest (approximately 12% humidity). Then, an amount of 40.00 g was placed in the Clevenger type extraction apparatus and stripping was performed with steam for 2 hours. The essential oils obtained were then dried with anhydrous sodium sulfate and volume was measured. The yield was expressed as the volume of essential oil obtained from 100 g of dried leaves (% v/w).

### Measurement of the refractive index and density

Essential oils were subjected to tests of index of refraction according to Colombian Technical Standard 289 (2002a) (Normas Técnicas Colombianas, NTC) using an Abbe refractometer (Atago DR-A1,Tokio, Japón), while density was determined according to the NTC 336 (2002b) using density bottle (Blaubrand, Germany) in accordance with Gay-Lussac.

### Chromatographic analysis of the essential oils

The chemical composition of the volatile and semi-volatile fractions of the essential oils was determined by gas chromatography coupled to mass spectrometry (GC-MS). GC-MS analysis was performed by direct injection of essential oil into the gas chromatograph (Agilent Technologies 6890 Plus, Wilmington, Delaware, USA) equipped with a mass selective detector (MSD, Agilent Technologies 5973) operated in full scan mode. ADB-5MS (J & W Scientific, Folsom, California, USA) 60 m X 0.25 mm i.d. capillary column coated with 5%-phenyl poly (methylsiloxane) (0.25 μm film thickness) was used. The injection was performed in split mode (30:1), with an injection volume of 2 μl. The identification of the compounds was established based on their retention index and mass spectra (Electron Ionization, 70eV) using the databases of Adams, Wiley 138 and W8N08. The temperature of the oven was programmed from 45°C to 275°C, while the temperature of the ionization chamber and the transfer line was set at 230 and 285°C, as described by Ruiz et al. (2007). The data obtained were subjected to analysis of variance (ANOVA) and means tests (Duncan) using SAS (2007) version 9.2 software .

## RESULTS

### Chemical composition of the volatile and semi-volatile fraction of the essential oils

The GC-MS analysis showed that the main component in the three essential oils of *L. origanoides* chemotypes was thymol, reaching levels well above other compounds (Table 1).

In the chemotype Patía, a total of 40 compounds were identified by GC-MS as described in the methodology. The major components were thymol (60.3-60.5%), ǫ-cymene (8.1-8.7%), γ-terpinene (6.6-8.7%), *trans* β-caryophyllene (3.4-4.7%) and β-myrcene (3.1-3.3%). These five components corresponded to 84% of all the compounds present; the others had relative concentrations lower than 3%.

In the chemotype Cítrica, a total of 57 compounds were identified by GC-MS. The major components were thymol (50.9–68.4%), ǫ-cimene (4.6–5.6%), *trans* β-caryophyllene (3.8–5.3%), α-humulene (2.1-3.2%) y γ-terpinene (2.9–4.4%). These five corresponded to 71% of all the compounds present.

In the chemotype Típica, a total of 58 compounds were identified by GC-MS. The major components were thymol (30.1–47.8%), *trans* β-caryophyllene (5.0–8.5%), ǫ-cimene (4.4–6.3%), α-humulene (3.0–5.7%), γ-terpinene (2.5–4.6%), α-felandreno (2.7–4.0%), α-eudesmol (1.1–3.8%) y δ-cadinene (1.8–3.0%). These eight components corresponded to 66% of all the compounds present.

### Refractive index and composition

The RI of the essential oil is a weighted average of the RI of each of its components, which takes into account the molar fractions of the components in the oil. The essentials oils of chemotypes of *L. origanoides* contain thymol as the major component, so that RI of the oil will be close to that of thymol.

The RI of pure thymol is 1.5227 nD 20. The RI of the other major components are: ǫ-cymene 1.4906 nD 20; α-terpinene 1.4784 nD 20; γ-terpinene 1.4754 nD 15.6; *trans* β-caryophyllene 1.5030 nD 17; β-myrcene 1.4680 to 1.4740 nD 20; α-phellandrene 1.4700 to 1.4750 nD 20; 1,8-cineole 1.4550 to 1.4600 nD 20. All of these have an RI lower than that of thymol; thus, when thymol concentration decreases, RI of the essentials oils also decreases.

**Table 1. Chemical composition and relative amount of the *Lippia origanoides* Kunth volatile and semi-volatile fractions in three chemotypes.**

**Tabla 1. Composición química y cantidad relativa de las fracciones volátiles y semi-volátiles de tres quimiotipos de *Lippia origanoides* Kunth.**

| $t_R$ (min) | Compound | PATÍA Relative amount (%) Repetition | | | CÍTRICA Relative amount (%) Repetition | | | TÍPICA Relative amount (%) Repetition | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 20.17 | β-Myrcene | 3.2 | 3.3 | 3.1 | 1.5 | 1.9 | 1.2 | 0.9 | 2.0 | 1.2 |
| 21.04 | α-Felandreno | 0.2 | 0.2 | 0.2 | 1.8 | 0.1 | 1.2 | 3.6 | 2.7 | 4.0 |
| 21.52 | α-Terpinene | 2.5 | 2.5 | 2.0 | 0.9 | 1.1 | 0.7 | 0.8 | 1.0 | 0.6 |
| 21.85 | ϱ-Cymene | 8.5 | 8.7 | 8.1 | 5.6 | 5.5 | 4.6 | 4.4 | 6.3 | 4.7 |
| 22.23 | 1.8-Cineole | <0.1 | <0.1 | <0.1 | 2.2 | <0.1 | 1.6 | 2.6 | 2.6 | 2.3 |
| 23.25 | γ-Terpinene | 8.7 | 8.3 | 6.6 | 3.3 | 4.4 | 2.9 | 4.6 | 3.7 | 2.5 |
| 32.23 | Thymol | 60.5 | 60.3 | 60.4 | 50.9 | 68.4 | 57.9 | 33.6 | 47.8 | 30.1 |
| 37.54 | trans β-Caryophyllene | 3.5 | 3.4 | 4.7 | 5.2 | 3.8 | 5.3 | 8.5 | 5.0 | 7.9 |
| 38.75 | α-Humulene | 2.0 | 2.0 | 2.8 | 3.2 | 2.1 | 3.2 | 5.3 | 3.0 | 5.7 |
| 40.56 | δ-Cadinene | - | - | - | 1.1 | <0.1 | 1.0 | 2.7 | 1.8 | 3.0 |
| 44.64 | α-Eudesmol | - | - | - | 1.7 | <0.1 | 1.4 | 3.8 | 1.1 | 2.1 |

The essential oil of chemotype Patía presented similar values for RI and thymol content (Table 2). However, these values were variable in samples of chemotypes Cítrica and Típica, in which the highest percentage of thymol corresponded to the highest RI value. This indicates that RI is not only a qualitative parameter, but also a metric parameter that can rapidly predict thymol content in this type of essential oils.

The relationship between refractive indices and concentration of thymol in essential oils fit to a linear model (Fig. 1). The value of r is 0.9439. The intercept is 1.4893, with upper and lower confidence limits of 1.4870 and 1.4916. The slope of the graph is 0.00030 with a confidence interval of $\pm 1\sigma$ between 0.00026 and 0.00034.

**Density**

Density is also a measure of the quality of an essential oil (Florido et al., 2013). Like the RI, density is directly related to the composition of the essential oil. However, no relationship between density and composition of the oil was found in this study, although significant differences were observed between density of essential oils of Cítrica and Típica chemotypes (Table 3).

**DISCUSSION**

Essential oils of the three chemotypes have similar chemical composition, mainly consisting of terpenes with small variations in some fractions of minor components. This variation may be mainly due to adaptations of the plants to the different ecosystems in which they have developed. However, it is important to note that thymol is the major component in each of the chemotypes. The total refractive index of the essential oil is expressed as the sum of the refractive indices of the individual components multiplied by their molar

**Table 2. Relationship between the refractive index (RI) and concentration of thymol in the essential oils of *Lippia origanoides* Kunth chemotypes.**

**Tabla 2. Relación del índice de refracción (IR) con la concentración de timol en los aceites esenciales de quimiotipos de *Lippia origanoides* Kunth**

| Sample | Patía RI | Patía Thymol (%) | Cítrica RI | Cítrica Thymol (%) | Típica RI | Típica Thymol (%) |
|---|---|---|---|---|---|---|
| 1 | 15.060 | 60.5 | 15.060 | 50.9 | 14.970 | 33.6 |
| 2 | 15.080 | 60.3 | 15.100 | 68.4 | 15.040 | 47.8 |
| 3 | 15.082 | 60.4 | 15.056 | 57.9 | 15.000 | 30.1 |

**Table 3.  Average density of essential oils from *Lippia origanoides* Kunth chemotypes.**
**Table 3.  Densidad promedio de los aceites esenciales de quimiotipos de *Lippia origanoides* Kunth.**

| | Density (g mL$^{-1}$) | | |
|---|---|---|---|
| Sample | Patía | Cítrica | Típica |
| 1 | 0.8500 a | 0.8385 a | 0.8915 b |
| 2 | 0.8585 a | 0.8400 a | 0.8960 b |
| 3 | 0.8480 a | 0.8650 b | 0.8565 a |
| Means | 0.8522 ± 0.0003 α* | 0.8478 ± 0.0005 α | 0.8813 ± 0.0007 β |

Notes: Values with different letters in each column are statistically different (Duncan, P ≤ 0.05). * Means with different letters are statistically different (Duncan, P ≤ 0.05)
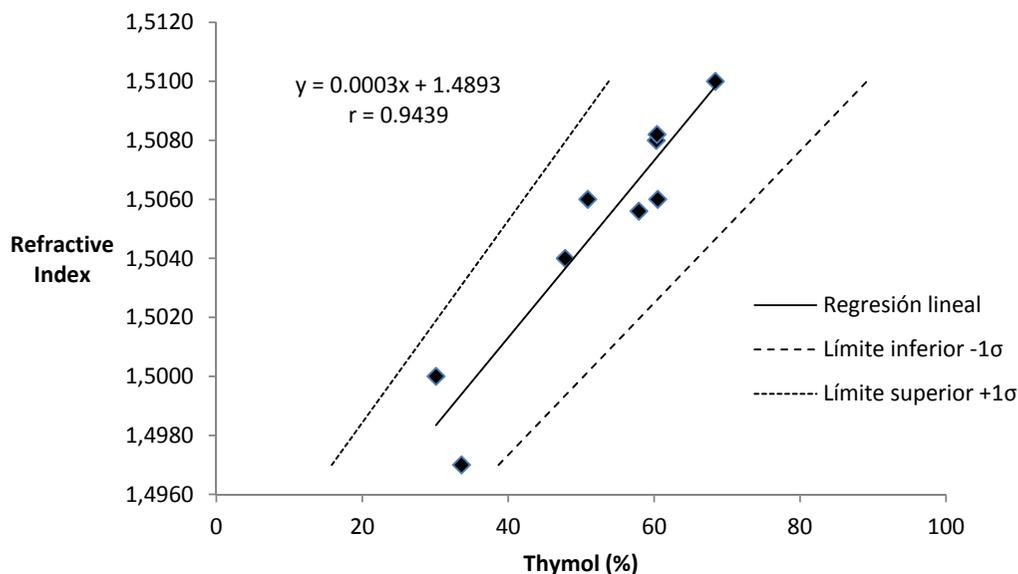


**Fig. 1.  Relationship between refractive indices and concentration of thymol in samples of essential oils from *Lippia origanoides* Kunth chemotypes. Confidence limits at ± 1σ.**
**Fig. 1.  Relación entre el índice de refracción y la concentración de timol en muestras de aceites esenciales de quimiotipos de *Lippia origanoides* Kunth. Límites de confianza a ± 1σ.**

fraction in the mixture (Equation 1); thus, the RI for each essential oil in these experiments was dominated by the contribution from thymol.

$$n_{total} = \sum_{a=1}^{\infty} n_a x_a \qquad (1)$$

where $n_{total}$ is refractive index of the essential oil, $n_a$ is refractive indices of the individual components and $x_a$ is their molar fraction in the mixture.

The linear equation in Fig. 1 shows that the refractive index of essential oil is directly related to the concentration of thymol. In particular, values above 50% are strikingly close to the trend line. Although the correlation coefficient is low and

data statistically better fit to a confidence limit of ± 1σ (Miller et al, 2002), it is possible, within the range of this study and under the conditions of extraction and purification applied, to determine the percentage of thymol in the essential oil of *L. origanoides* simply by measuring its RI. Since this eliminates most of the need for GC-MS analysis in the quality control process, which substantially reduces direct costs and analysis time, it is a cost-saving tool to determine the concentration of thymol in the essential oil.

This same determination can be made for other *L. origanoides* chemotypes and other species (thyme, oregano, among others) that contain thymol as the major component of their essential

oils, and other compounds with lower RIs.

Although a direct relationship between density and thymol concentration was not observed, density may also be a qualitative indicator of impurities contained in an essential oil, such as solvents, or mixtures of mineral, vegetable or animal oils, which are used for adulteration and decrease the quality of essential oils.

## CONCLUSIONS

RI of the essential oils from *Lippia origanoides* Kunth chemotypes can be used to determine the concentration of thymol in the essential oil (within confidence limits of ± 1σ) with no need to repeat the GC-MS analysis, which is expensive and time-consuming. The linear relationship between thymol concentration in the essential oil and composite RI shown in Fig. 1 may be used to accurately infer thymol concentration from RI measurements.

This method of determining the concentration of thymol in an essential oil can be used in other species containing thymol as major component of their essential oils provided the RIs of each one of the other compounds is lower than that of thymol. Therefore, essential oil composition should be determined before this technique is applied to other species.

## ACKNOWLEDGEMENTS

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