

CHARACTERIZATION OF VOLATILE COMPOUNDS IN THE ESSENTIAL OIL OF SWEET LIME (*Citrus limetta* Risso)

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The essential oil of citrus fruit contains components pleasant sensory characteristics that are appreciated in food, pharmaceutical, and cosmetics industries. In the case of sweet lime (*Citrus limetta* Risso), is necessary to characterize the essential oil components, to identify potential uses of this fruit. The essential oil of sweet lime was obtained from lime flavedo in four different maturation stages. Steam distillation was employed and then compared with hexane extraction. The identification of the components in the essential oil was carried out by gas chromatography and mass spectrometry. A total of 46 components were found in the essence of lime, among which the highest concentration of compounds present were aldehydes such as limonene. Linalool, sabinene, and bergamol were more abundant than in other varieties. The best extraction method was steam distillation, and the concentrations in stage III from the main terpenic portion were d-limonene with 74.4%, bergamol with 8.23%, and β -pinene with 7.62%.

Key words: Sweet lime, *Citrus limetta*, essential oil, steam distillation, maturation stages.

The citrus species are a potential source of valuable oil which might be utilized for edible and other industrial applications (Anwar *et al.*, 2008), and essential oils are broadly used as pharmaceutical components, in nutritious supplements, and for cosmetic industry and aromatherapy (Kondo *et al.*, 2000; Misharina and Samusenki, 2008). Essential oils are a product obtained from vegetable raw materials (Berger, 2007). The essences are complex mixtures whose composition may include volatile terpenic compounds, which have the formula (C₅H₈)_n, where the compounds are monoterpenes if n = 2, sesquiterpenes when n = 3, diterpenes when n = 4, etc. The terpenoids are oxygenated derivatives of terpenes, which may contain hydroxyl or carbonyl groups (Smith *et al.*, 2001). These are secondary metabolites in plants (Mazen, 2002) and are responsible for the characteristic aroma on the fruit. The terpenoids are synthesized in the flavedo, an outer layer that forms part of the exocarp or peel. This section of peel contains the essential oil in circular cavities (oil glands) (Berger, 2007). The production is approximately 1 mL essential oil per 100 cm² exocarp (Mazen, 2002).

The composition of the citric fruits is generally composed of 90% terpenes, 5% oxygenated compounds, and less than 1% non-volatile compounds such as waxes and pigments (Kondo *et al.*, 2000). D-Limonene, the most abundant terpene has antimicrobial properties, primarily the exhibition of antibacterial activity against Gram-positive bacteria, and also increases the effectiveness of sodium benzoate as a preservative (Murdock and Allen, 1960; Roger *et al.*, 1970; Berger, 2007). Among the monoterpenes are citral which has antifungal properties, linalool which has fungistatic properties, and linalool, limonene and β -pinene, all of which have a repellent effect on *Drosophila melanogaster* (Yamasaki *et al.*, 2007). The essential oils may be extracted through different methods whose use depends on the desired yield and quality and the location of the oil glands (Mazen, 2002). Some of these methods are: steam distillation, cold pressing, peel compression, hydrodistillation, and supercritical fluid extraction (Mazen, 2002; Frizzo *et al.*, 2004; Gil and Sáez, 2005; Atti-Santos *et al.*, 2005). After the extraction, the identification of the compounds in the oil is important; chromatography is the most-used alternative (Mondello *et al.*, 2003).

In the analysis and comparison of the essential oils of lemon (*Citrus aurantifolia* Swingle), bergamot (*Citrus bergamia* Risso), mandarin (*Citrus deliciosa* Tenore), sweet orange (*Citrus sinensis* L. Osbeck) and bitter orange (*Citrus aurantium* L), it was found that most of the compounds in these citric essential oils are terpenes, such as α -thujene, α -pinene, camphene, sabinene,

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β -pinene, myrcene, α -terpinene, *p*-cymene, linalool, and d-limonene; this last terpene was the most abundant compound in the four citric fruits, with a concentration of 90% in sweet orange and bitter orange. Likewise, each citrus fruit has particular components present in minor quantities; these components differ between fruits and can be used in identifying the various oils and controlling their quality and authenticity (Mondello *et al.*, 2003). The sweet lime, according to W.T. Swingle classification (Nicolosi, 2007), belongs to *Citrus* genus, *limetta* species, Risso variety, Aurantiaceas subfamily and Rutaceae family. In Mexico, the sweet lime does not have as much importance as the amount attached to the diverse lemon varieties, and it still lacks commercial value, although it is produced in 14 states of the Mexican Republic. Additionally, this crop has begun to disappear from the state of Guanajuato, due to a lack of marketing and incorporation into products that can be made easily available on the market.

An important step in the study of sweet lime is the characterization of the components of its essential oil for the purpose of identifying potential uses of the fruit. In a recent research, Perez *et al.* (2010) report that sweet lime (*Citrus limetta* Risso) leaves extract antagonizes the hypertensive effect of angiotensin II, suggesting an important bioactive capability of sweet lime. Few studies have been focused on the extraction and analysis of the essential oil of the sweet lime. In the present work, the essential oil of sweet lime was obtained by steam distillation and extraction with hexane in different stages of maturity; to identify main compounds by gas chromatography and mass spectrometry.

MATERIALS AND METHODS

Plant material

Nine sweet lime (*Citrus limetta*) samples (three fruits per sample) were collected from the region of San Juan de la Vega, Celaya, Guanajuato, Mexico (20°38'00" N, 100°46'00" W). The sweet lime juice was extracted and characterized physicochemically for their amount of soluble solids (°Brix) (JAOAC, 1983) and titration acidity (expressed as percentage of citric acid) (AOAC, 1990). The limes were weighed, peeled, squeezed, and classified according to their maturity stage using as a base the relationship between °Brix and the acidity expressed in grams of citric acid per 100 mL of juice (Louche *et al.*, 2000). The fruit's maturity stages were defined as I, II, III, and IV conform to the soluble solids content was increased and the peel color variation with advancement of fruit maturation. In this work, we proposed Stage I: Intense green, Stage II: Green, Stage III: Green-Yellow, Stage IV: Intense yellow. This labeling was proposed based in Mexican lemon (*Citrus aurantifolia* Swingle) norm (NMX-FF-087-SCFI-2001, 2001). Lab essays were performed by triplicate.

Extraction of essential oil

The outline of the equipment used for extraction consisted of a two-neck angled round flask, in which a lime peel previously weighted were deposited; the flask was then placed in a heating mantle. Saturated steam enters through one opening and passes through the oil mixture. The steam-oil mixture then enters a condenser, where the condensate is recovered in a separating funnel, with the oil retained and the water drained. A total of 100 g of lime peel in its four stages of maturity was used with an extraction time of 30-35 min. The extraction using hexane was performed using a hexane-peel at a 1:2 w/v ratio and continuing maceration for 24 h; the essence was extracted by distillation. The essays were performed by triplicate.

Analysis of essential oil

The extracted essences were placed in vials to quantify and analyze according the Mexican Norm NMX-F-062-1974 (1974) Mexican lemon essential oil (*Citrus aurantiifolia* (Christm.) Swingle distillate. The essence was injected into a GC Claurus 500 gas chromatograph coupled to a Claurus 500 MS mass spectrophotometer (Perkin-Elmer Inc., Wellesley, Massachusetts, USA). The column used was a capillary column (30 m \times 0.25 mm i.d.), coated with INNOWAX (0.5 μ m phase thickness) (Agilent Technologies, Palo Alto, California, USA). Helium (99.999% high purity) was used as the carrier gas (inlet pressure 82 737 Pa (12 PSI)) at a flow rate of 1.6667 \times 10⁻⁸ m³ s⁻¹ (1 mL min⁻¹) (splitting ratio 10:1). The oven temperature was programmed from 60 (8 min-hold) to 250 °C (30 min-hold) at 5 °C min⁻¹ (Colecio-Juárez, 2007). The volume injected was 1 μ L. The injector temperature was 220 °C. In the mass spectrophotometer, the temperatures of the ionization chamber and the transfer line were maintained at 180 and 200 °C, respectively. The electron energy was 70 eV, and the mass range used was 30 to 450 m/z (mass/charge number).

The essence chromatogram obtained was analyzed, and each peak was checked by determining the percent area on the chromatogram, the retention time, the spectrum and the base peak and then referring to the characteristic mass spectra of compounds listed on the National Institute of Standards and Technologies using the software Windows Search Program Version 2.0 Perkin Elmer.

RESULTS AND DISCUSSION

According to the physicochemical analysis (Table 1), the amount of juice, soluble solids and total sugars mainly increases with the stage of maturation, which is probably due to the biosynthetic processing or undergoing hydrolysis of polysaccharides occurring as the lime reaches its greatest size and weight in the maturity stages III and IV. As the concentration of soluble solids increased, the concentration of organic acids decreased and therefore the ratio °Bx/% acidity increases (Louche *et al.*, 2000).

Table 1. Physicochemical characteristics of sweet lime (*Citrus limetta*) in different maturity stages.

Maturity stage	Fruit weight	Juice volume	Soluble solids	Acidity	°Bx/% acidity ratio
I	67.45a±6.56	20.78a±3.940	7.11a±0.485	0.016a±0.0033	444:1
II	72.55b±8.72	30.44b±6.064	7.44ab±0.391	0.012a±0.0022	620:1
III	103.34c±12.05	35.88c±6.822	7.94b±0.726	0.009a±0.0014	882:1
IV	94.70d±20.20	36.71c±9.320	10.38c±0.485	0.0067a±0.0010	1549:1

Values are average ± standard deviation. Values with different letters indicate significant differences between treatments according to Tukey test ($p < 0.005$).

Extraction of essential oil

The characteristics of the essential oil obtained by steam-carrying distillation and hexane at different stages of maturity are compared in Table 2. The most essential oil is extracted when the lime is in the maturity stages I and II. The greatest quantity is found in stage I, exceeding 50% of the remaining steam distillation extractions and 20% of the extractions with hexane. This result is probably obtained because when the fruit is completely green, there are a high number of oil glands used by the plant as a defense against predators, with this number decreasing in the last stages of maturity (Smith *et al.*, 2001). When hexane was used, a green-yellow coloration was observed in the extract due to the co-extraction of chlorophyll and other compounds that are of interest in the study; the sample undergoing steam distillation was colorless and practically free of undesirable compounds. An extraction time of 45 min using steam-carrying distillation was enough to recover most of the essential oil; the amount of time used for this procedure is lower than that reported by other authors, who recommend an extraction time of 2 h (Gil and Sáez, 2005). The refractive index reported is similar in both essences and this presents no significant variation through fruit ripening. However, the percentage evaporation residue makes a significant difference in both extractions, showing that values of the essential oil extracted by steam distillation in all stages of maturity were of 1.534% on average. Mexican norm establishes the average values of 0.2-2.2%, so that lie within the allowable range, unlike the essential oil extracted with hexane whose average values of percentage evaporation residue were of 7.683% on average.

Table 2. Comparison between steam distillation and hexane extraction.

Maturity stage		Volume	Density	Refraction index	Evaporation residue
I	S	1.460a±0.461	0.8656a±0.020	1.476a±0.003	1.534a±0.139
	H	0.830b±0.034	0.7425b±0.096	1.477b±0.003	7.683b±0.068
II	S	0.650a±0.050	0.8868a±0.036	1.477a±0.004	1.534a±0.139
	H	0.770b±0.052	0.6762b±0.036	1.477b±0.000	7.683b±0.068
III	S	0.590a±0.017	0.8470a±0.022	1.471a±0.004	1.534a±0.139
	H	0.400b±0.104	0.7770b±0.082	1.477b±0.000	7.683b±0.068
IV	S	0.260c±0.029	0.8631a±0.003	1.472a±0.004	1.534a±0.139
	H	0.282c±0.042	0.7297b±0.052	1.477b±0.000	7.683b±0.068

S: steam distillation; H: hexane extraction.

The established parameters in the Mexican norm: Density = [0.855 - 0.863]

Refraction index = [1.4745 - 1.477] and % evaporation residue = [0.2 - 2.2].

Values correspond to average value ± standard deviation.

Values with different letter indicate significant differences between treatments according to Tukey test ($p < 0.005$).

Chromatographic analysis

A total of 46 compounds were identified by mass spectrometry (Table 3). Most of these are terpenes, which are found in greater amounts than sesquiterpenes, aldehydes, ketones, phenols, and free acids. Alcohols and some terpenes show higher percentage areas in the maturity stages I and II. Maturity stage I showed the presence of α -terpineol, a monoterpene that slows the peroxidation of linoleic acid (Foti and Ingold, 2003). The main component of the citrus peel oils is limonene, which appears in concentrations of 45% in lime up to 96% in orange and grapefruit oil (Steuer *et al.*, 2001); in this work, d-limonene showed a concentration level higher than 70%, followed by bergamot (8%),

Table 3. Area percent in compounds found in different maturity stages of *Citrus limetta* Risso.

Compound	Maturity stages			
	I	II	III	IV
α -Pinene	0.77±0.06	0.89±0.03	0.82±0.05	0.89±0.01
Camphene	0.03±0.01	0.04±0.00	0.04±0.00	0.03±0.00
β -Pinene	8.63±0.00	8.96±0.03	7.62±0.01	6.48±0.02
Sabinene	1.85±0.00	2.12±0.00	1.86±0.02	0.93±0.00
β -Myrcene	1.15±0.05	1.31±0.01	1.43±0.01	1.68±0.01
d-Limonene	66.8±0.04	71.7±0.00	74.4±0.00	77.7±0.00
Nonanal	-	-	0.03±0.00	0.04±0.00
(Z) Sabinene hydrate	0.02±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Nonane	0.09±0.00	0.10±0.01	0.10±0.00	0.12±0.00
Undecanal	0.02±0.00	0.03±0.01	-	-
Linalool	5.92±0.01	3.61±0.01	2.89±0.00	1.98±0.00
Camphor	-	Tr	Tr	-
Bergamot	12.3±0.05	8.76±0.05	8.23±0.05	7.19±0.05
Trans- α -bergamotene	0.36±0.08	0.37±0.00	0.36±0.00	0.35±0.00
Aromadendrene	0.10±0.06	0.10±0.00	0.10±0.00	0.10±0.03
Terpinen-4-ol	-	0.07±0.00	0.05±0.01	0.06±0.03
Epi- β -santalene	-	0.01±0.00	0.01±0.01	0.01±0.02
Trans-sabinene hydrate	0.07±0.00	-	-	-
Farnesol	0.06±0.00	0.05±0.06	0.06±0.02	0.06±0.00
Isopinocarveol	0.13±0.01	-	-	-
Terpineol acetate	0.09±0.07	0.09±0.02	0.08±0.01	0.09±0.00
α -Terpineol	0.33±0.05	-	-	-
Neryl acetate	0.28±0.08	0.28±0.02	0.26±0.03	0.34±0.03
Neral	0.2±0.02	0.2±0.01	-	-
Geranyl acetate	-	0.26±0.00	0.10±0.05	0.20±0.02
Geraniol	-	0.18±0.00	0.18±0.00	0.22±0.00
Cis-geraniol	0.16±0.00	0.18±0.00	0.16±0.03	0.16±0.01
1-Cyclohexen-1-methanol	-	-	0.01±0.02	-
4-1 methylenil acetate	-	-	-	-
Octal cyclopropene	-	-----	0.01±0.01	0.01±0.00
Cis-myrtanol	-	0.01±0.00	0.01±0.01	0.02±0.00
Perillal	-	0.01±0.00	-	0.01±0.00
2-Tridecen-1-ol	-	-	Tr	Tr
3-Cyclohexen-1-ol	-	-	Tr	Tr
2-Cyclohexyl-dodecane	-	Tr	-	-
Bicyclo(2,2,1) Heptane	0.09±0.00	0.08±0.00	-	-
2,2-diethyl-3-methyl	-	-	-	-
P-menth-1-en-8-ol	0.34±0.02	0.31±0.01	0.21±0.03	0.21±0.01
β -Bisabolol	0.60±0.05	0.60±0.01	0.60±0.05	0.059±0.00
Carveol	Tr	0.13±0.01	-	-
α -Farnesene	Tr	-	-	-
α -Bisabolol	0.02±0.00	0.03±0.00	0.02±0.00	0.03±0.00
β -Farnesene	Tr	Tr	Tr	0.01±0.00
Trans- β -santalol	0.01±0.00	-	-	-
α -Santalol	-	0.01±0.00	0.01±0.00	0.02±0.00
Isopropyl palmitate	-	0.01±0.00	-	-
β -Santalene	Tr	0.01±0.00	0.01±0.00	0.01±0.00
Cyclopropamethanol	Tr	0.01±0.00	0.01±0.00	0.02±0.00
α ,2-dimethyl-2,4-methyl-3-pentanyl	-	-	-	-

-: Compound not reported; Tr ≤ 0.001 . Values correspond to average ± standard deviation.

β -pinene (7.62%), linalool (2.89%), α -pinene (0.82%) and the remaining compounds, which number over 40 (2.73%). The main flavor components of the fresh lime are limonene, α -terpineol, 4-terpineol, neryl acetate, β -pinene, β -bisabolene, neral, citral, geranial 1,4 cineole, 1,8 cineole, *p*-cymene, α -bergamotene, valencene, and d-germacrene (Yadav *et al.*, 2004); the first seven compounds were found in this work.

The compounds found in *C. limetta* are compared with the compounds reported in other varieties (Table 4) (Shaw *et al.*, 2000; Steuer *et al.*, 2001; Yadav *et al.*, 2004; Mahmud *et al.*, 2009; Bousbia *et al.*, 2009). The d-limonene, β -pinene, β -myrcene, α -pinene, β -bisabolol and α -terpineol levels are in the range reported by the majority of authors. The linalool, sabinene, and bergamol show similar concentration levels with those found for other reported varieties. Sabinene has antimicrobial and antioxidant properties; its concentration in lemon is 1-2%, similar to the amount found in this work.

Previous research described, a practical and convenient synthesis starting from linalool via bergamol or linalyl acetate (Berger, 2007). Geraniol, a compound related to the fruit's aroma, with a citrus-like and menthol-like odor, possesses anticancer activity and can reduce the growth of colon cancer cells by up to 70% (Berger, 2007). Rammanee and Hongpattarakere (2011) report that essential oils from tropical citrus epicarps have inhibitory activities against *Aspergillus* fungi. In sweet lime, geraniol increased with maturity but was not found in stage I; however, it is found in greater quantity than in other varieties. The alcohol β -bisabolol was found in lime for the first time in the year 2004 (Yadav *et al.*, 2004); in this study, it was found with a concentration that was 50% higher. There were no reported values for the content of nonanal and undecanal; these aliphatic aldehydes are related to the quality of citrus (Stuart *et al.*, 2001) and have aromatic properties, first described as having citrus-like and soapy notes (Berger,

Table 4. Comparison between *Citrus limetta* in this work and other authors (% area percent).

Compounds	Shaw <i>et al.</i> , 2000	Steuer <i>et al.</i> , 2001	Yadav <i>et al.</i> , 2004	Mahmud <i>et al.</i> , 2009	Bousbia <i>et al.</i> , 2009	This work
	Wild lime <i>Microcitrus inodora</i>	Lime commercial oil (MCI)	<i>Citrus aurantifolia</i> (Chrism.) Swingle	<i>Citrus acida</i> var. sour lime	<i>Citrus aurantifolia</i> (Chrism.) Swingle	
d-Limonene	68.50	49.9	75.5		63.44	71.70 ± 0.00
β -Pinene		4.2	32.1		13.09	8.96 ± 0.03
Bergamol				2.37		12.30 ± 0.05
Linalool	0.13		1.3		0.36	3.61 ± 0.01
Sabinene	0.11	0.5				2.12 ± 0.00
β -Myrcene	1.44	1.3			1.46	1.31 ± 0.01
α -Pinene	0.31	1.4	6.8		1.94	0.89 ± 0.03
β -Bisabolol			0.4			0.60 ± 0.05
β -Bisabolene				5.07	0.81	0.60 ± 0.01
Trans- α -bergamotene			4.7		0.54	0.37 ± 0.00
α -Terpineol	0.02	6.2	13.3		0.37	0.33 ± 0.05
Neryl acetate			0.5		0.60	0.28 ± 0.02
Geranyl acetate			0.6	2.83	0.60	0.26 ± 0.00
Neral	Tr		1.8		1.55	0.20 ± 0.02
Geranial	Tr		4.1		2.05	0.18 ± 0.00
Cis-geraniol					0.07	0.18 ± 0.00
Isopinocarveol						0.13 ± 0.01
Citronellal					0.05	0.13 ± 0.00
nonane						0.10 ± 0.01
Aromadendrene						0.10 ± 0.00
Epi- β -santalene						0.10 ± 0.00
α -Terpineol acetate						0.09 ± 0.02
Terpinen-4-ol			6.8		0.19	0.07 ± 0.00
Trans-sabinene hydrate						0.07 ± 0.00
Farnesol				1.25		0.05 ± 0.06
Camphene						0.04 ± 0.00
Undecanal						0.03 ± 0.01
Nonanal					-	0.03 ± 0.00
α -Bisabolol			0.2		0.02	0.03 ± 0.00
Myrcenil acetate						0.01 ± 0.03
(Z) Sabinene hydrate						0.01 ± 0.00
Octil ester						0.01 ± 0.00
1-Cyclohexen-1-methanol, 4-1 methylenil acetate						0.01 ± 0.00
Trans-nerolidol			14.3			0.01 ± 0.00
Octal cyclopropane						0.01 ± 0.00
Cis-myrtanol						0.01 ± 0.00
Aldehyde peril	Tr					0.01 ± 0.00
β -Farnesene			0.6		0.06	0.01 ± 0.00
Trans- β -santalol						0.01 ± 0.00
Isopropyl palmitate						0.01 ± 0.00
β -Santalene			0.2			0.01 ± 0.00
Camphor						0.001
α -Farnesene						0.001

Compound not reported; Tr = < 0.001.

2007). Other compounds, such as aromadendrene and camphene, are not reported; camphene has a role in the digestion of fat through increasing bile secretion (Berger, 2007). Compounds with values of under 0.03% cannot be compared; camphor and α -farnesene show area percents of 0.001%, and this result indicates that the sensitivity of the column and the chromatographic method are important in the separation and identification of compounds.

CONCLUSIONS

During the characterization of the essential oil of sweet lime (*Citrus limetta*), a higher amount of essential oil was found in maturity stages I and II, which is probably due the greater number of essential oil-producing glands present at these stages. Extraction with hexane produced a higher yield of essential oil, but, in addition to extracting the essence, this approach also extracted compounds such as waxes, pigments and polysaccharides. Therefore, the steam distillation technique provides an essence of higher purity. Using chromatography, compounds with values of 0.001, such as camphene, were identified and isolated.

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Caracterización de compuestos volátiles en aceite esencial de lima dulce (*Citrus limetta* Risso). El aceite esencial de frutos cítricos contiene componentes de características sensoriales agradables que son apreciadas en las industrias alimentaria, farmacéutica y de cosméticos. En el caso de la lima dulce (*Citrus limetta* Risso), es necesaria la caracterización de los componentes de su aceite esencial para identificar usos potenciales de este fruto. El aceite esencial de lima dulce se obtuvo a partir del flavedo de lima en cuatro etapas de maduración diferentes. Se utilizó destilación por arrastre de vapor y se comparó con la extracción con hexano. La identificación de los componentes en el aceite esencial se realizó por cromatografía de gases y espectrometría de masas. Se encontró un total de 46 componentes en el aceite esencial de lima, entre los cuales la mayor concentración de compuestos presentes son aldehídos como el limoneno. Linalol, sabineno y bergamol fueron más abundantes que en otras variedades. El mejor método de extracción fue la destilación al vapor, y las concentraciones en la etapa III de la parte terpénica principal fueron d-limoneno 74.4%, bergamol 8.23%, y β -pineno 7.62%.

Palabras clave: lima dulce, *Citrus limetta*, aceite esencial, destilación por arrastre de vapor, estados de maduración.

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