DETERMINATION OF BIOACTIVE VOLATILE ORGANIC COMPONENTS OF *LIPPIA CITRIODORA* USING ULTRASONIC ASSISTED WITH HEADSPACE SOLID PHASE MICROEXTRACTION COUPLED WITH GC-MS

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In this study the chemical composition of the essential oil extracted from fresh leaves of *Lippia citriodora* (*Verbenaceae*) using ultrasonic assist with headspace solid phase microextraction (UA-HS-SPME) combined with GC and GC–MS determined in full bloom. The highest extraction efficiency was achieved with a 100 μm polydimethylsiloxane (PDMS) fiber. Different experimental parameters such as fiber's coating type, sonication time, extraction time and temperature, and desorption time were investigated. As a result, 15 constituents, representing 93.07% of the oil, were identified. Comparison of the UA-HS-SPME and the commonly used HD method showed that the UA-HS-SPME method is simpler and require smaller samples and shorter extraction times in addition to the greater ease of trapping and extracting the volatile and thermo-sensitive compounds. Also, GC-MS analysis of essential oils revealed that 1, 8-Cineole l (23.66%), α-curcumene (14.83%), Geranial (13.74%), limonene (13.40%) and caryophyllene oxide (6.60%) were the main components of essential oils of *L. citriodora*, respectively.

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1. Introduction

The genus *Lippia* (*Verbenaceae*) includes approximately 200 species of herbs, shrubs and small trees. The genus *Lippia* shows a rich genetic diversity, enabling it to synthesize a variety of essential oil constituents in plants grown in different parts of the world. [1-2]. Lemon verbena, *Lippia citriodora* H.B.K., [syn. *Lippia triphylla* (L'Her.) Kuntze; *Aloysia triphylla* (L'Her.) Britton], is indigenous to South America and was introduced into Europe at the end of the 17th century. It is cultivated mainly due to the lemon-like aroma emitted from its leaves that are utilized for the preparation of herbal tea, which is reputed to have antispasmodic, antipyretic, sedative and digestive properties. Lemon verbena has a long history of folk uses in treating asthma, spasms, cold, fever, flatulence, colic, diarrhea, indigestion, insomnia and anxiety [3-6].

Distillation (water and/or steam) is a conventional method for the extraction of essential oils from plant materials, in which the plant materials are mixed (or not) with water followed by heating or by the introduction of water system. The resulting vapors are cooled by condenser and collected in a separator and essential oil separates from water. However, distillation have several disadvantages including labor intensive/time consuming, loss of target compounds due to thermal

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degradation, etc. [7-8], have led to search for find new benefice and efficient alternatives for isolation methods. Among several recently introduced alternative techniques, the coupling of headspace solid-phase microextrction (HS-SPME) sampling with gas chromatography mass spectrometry (GC/MS) has been shown to be very fast, handy, reliable and inexpensive extraction tool for organic volatiles. Theoretical bases and various applications of SPME are presented by Pawlisyzn [9] as well as in numerous publications. The UA-HS-SPME technique is based on the employment of the energy derived from ultrasound to enhance the extraction of analytes from the solid sample by the organic solvents [10-12].

The chemical composition of the essential oil from the leaves of *L. citriodora* has been previously reported [1, 3, 5, 13-20].

The purpose of this paper is to analyze the composition of the essential oil from leaves of *L. citriodora* cultivated in Iran, for the first time using Ultrasonic assist with headspace solid phase microextraction (UA-HS-SPME) analyzed by GC and GC–MS.

2. Material and Methods

Fresh leaves of *L. citriodora* H.B.K. were collected from plants growing in the gardens of the Agricultural College of Razi University in May 2009, at flowering stage. About100 g of fresh leaves dried in room temperature by spreading them on a clean aluminum foil in laboratory. 10 g portions of air-dried sample were subjected to a household coffee grinder in order to make them to a coarse powder. The ground samples were stored in nylon bags and placed in refrigerator until use and further analysis.

The SPME fiber, 100 µm polydimethylsiloxane (PDMS) was purchased from Supelco (Bellefonte, PA, USA) and conditioned prior to use according to suppliers prescriptions. Helium, 99.999%, used as carrier gas, was purchased from Roham Gas Company (Tehran, Iran). The alkane mixture consisting of the C_8-C_{26} alkanes (concentration of 40 mg/mL in hexane) was purchased from Fluka. All other chemicals were of the highest purity available from Merck or Fluka. Doubly distilled deionized water was used.

The aerial parts of the plant were dried at room temperature by spreading them on clean aluminum foil in the laboratory. 10-g portions of air-dried sample were ground to a coarse powder using a household coffee grinder. The ground samples were stored in nylon bags and placed in a refrigerator prior to analysis. Extraction of the volatiles from the plant sample using SPME fibers was achieved by placing 0.5 g of ground sample into a 40-ml vial to which 500 µl double-distilled water was added as a matrix modifier, the vial was then vigorously shaken by hand to ensure homogeneous dispersal of the spiked water. The sample vial was then placed into an ultra-sonicator and incubated for 15 min to allow the volatiles to equilibrate between the headspace and sample matrix, during which time the sample was heated to 70 ºC. The actual SPME extraction of volatile compounds was accomplished by incubation with a polydimethylsiloxane (PDMS) fiber at 70 ºC for 40 min. Ultrasonic irradiation (18 kHz, 450 W) was applied by means of a PFO100 5RS Series ultrasonicator (Italy) equipped with a water bath in which the extraction vials were placed. The samples were sonicated to create stress in the sample matrix to facilitate the release of the analytes, and control the temperature during the extraction process. The alkane mixture (C_8-C_{26}), 40 mg/mL in hexane) was used for the calculation of retention indices (RIs). Loading the alkane mixture onto the fiber was carried out using a 5-min head space extraction from the 10-ml SPME vial including 1 ml double-distilled water spiked with 10 µl of the above-mentioned mixture.

Gas chromatography was performed with a Shimadzu model GC-17A (Kyoto, Japan) instrument equipped with a Shimadzu Quadropole-MS (QMS) model QP5050 detector. Separation was achieved using a 30 m × 0.25 mm I.D capillary fused silica column 6 coated with a 0.25 µm film of DB5-MS (5% Phenyl-95% Polydimethyl Siloxane), and a split/splitless injector with a 1 mm internal diameter glass liner. Ten-, 20- and 40-milliliter sample vials sealed with PTFE coated silicone septa (Supelco) were used for extraction. SPME fibers with PDMS (100 µm, non-bonded) coating, were used as commercial fibers for the extraction of analytes. The fibers were handled using a manual SPME fiber holder provided by Supelco (Bellefonte, PA, USA). Analytes extracted onto the fiber were injected into the injection port of the GC system. The fiber was kept

in the injection port for additional 2 min after injection to ensure the complete desorption of the compounds from the fiber. Every 10 analyses on a GC run were carried out in the presence of the fiber but without sampling to assure complete desorption. The injector was set at 220 ºC. The carrier gas was helium and flowed at a rate of 1 ml/min. The GC was operated in a splitless mode. The column temperature was initially set at 40 ºC and increased to 200 ºC at a rate of 4 ºC/min, and remain at 200 ºC for 1 min, resulting in a total GC run time of 45 min. The temperature of the ion source was kept at 220 ºC, and the transfer line temperature at 250 ºC. The mass fragments were collected in the range from m/z 40 to 450 with an acquisition rate of 1000 to provide a satisfactory number of points per peak for effective spectral resolution. The ionization energy of 70 eV and the detector voltage of 1700 V were applied to the QMS detector.

Most constituents were identified by gas chromatography through comparison of their retention indices (RIs) with those of the literature [21-22] or with those of authentic compounds available in our laboratories. The retention indices (RIs) were determined in relation to a homologous series of n-alkanes (C8–C24) under the same operating conditions. Further identification was made by comparison of their mass spectra on both columns with those stored in NIST 98 and Wiley 5 Libraries or with mass spectra from literature [21-23]. Component relative concentrations were calculated based on GC peak areas without using correction factors.

3. Results and discussion

The chemical composition of the essential oil of *L. citriodora* is summarized in Table 1 (in the order of elution from the DB5-MS column). Fifteen compounds (15) were identified, representing 93.07% of the total oil. 1, 8-Cineole l (23.66%), α-curcumene (14.83%), Geranial (13.74%), limonene (13.40%) and caryophyllene oxide (6.60%) were found to be the main components available in the essential oil of *L. citriodora*, respectively (Table 1). These results are in agreement with previous reports [3, 6, 16, 19-20].

Table 1. The chemical composition of essential oils obtained from L. citriodora

No.	Component	RT	RI	*L. citriodora*
1	α-Pinene	11.97	947	1.50
2	Sabinene	13.55	987	0.74
3	Limonene	15.97	1047	13.46
4	1,8-Cineole	16.21	1053	23.66
5	Trans-Sabinene hydrate	17.71	1090	1.25
6	Trans-Limonene oxide	20.45	1159	0.62
7	α-Terpineol	22.88	1221	1.76
8	Geranial	25.59	1293	13.74
9	Neryl acetate	29.29	1396	2.81
10	α-Copaene	29.42	1399	2.87
11	β-Bourbonene	29.76	1409	2.56
12	β-Caryophyllene	31.1	1449	4.52
13	γ-Gurjunene	32.5	1491	2.15
14	α-Curcumene	33.05	1507	14.83
15	Caryophyllene oxide	36.75	1624	6.60
Total content fraction of determinated compounds				93.07
Yield % (w/w)				1.22
Monoterepen				15.70
Oxygenated monoterepen				43.84
Sesquiterepen				26.93
Oxygenated Sesquiterepen				6.60

The essential oil of *L. citriodora* was characterized by high contents of oxygenated monoterpenes (43.84%), sesquiterpenes (26.93%), monoterpenes (15.70%) and oxygenated sesquiterpenes (6.6%), respectively. The fraction of monoterpene was enriched, mainly due to an increase in limonene's percentage.

According to the literature, limonene is the component found to occur in higher quantities in essential oils of the genus *Lippia*, followed by: *p*-cymene, α-pinene, camphor, β-caryophyllene, linalool and thymol in a decreasing order [5, 17]. In our study, α-pinene, α-Terpineol, Sabinene, γ-Gurjunene and β-caryophyllene were also identified at low percentages, in addition to limonene and 1,8-Cineole and α-Curcumene which were of the major compounds. However, our results did not show the presence of *p*-cymene, camphor and thymol, which have been mentioned in other studies concerning *L. citriodora* [15, 19].

In other investigations the composition, at full bloom, differs significantly from ours. Santos-Gomes et al. [6] and Sartoratto et al. [20] reported the percentage of citral exceeding that of limonene, while Zygadlo et al. [15] detected myrcenone, α-thujone and lippifoli-1(6)-en-5-one as the main components, limonene in low percentage and no citral.

The literature emphasizes that a variety of geographical and ecological factors can lead to qualitative and quantitative differences in the essential oil produced. At the same time, a number of other factors can influence its composition, such as the developmental stage of the plant, its physiology, the age of leaves and the growing conditions. In addition, chemical composition of the essential oils affected by the isolation procedure and analysis conditions [2, 14, 15, 18-20].

In order to provide a complete peak separation of extracted compounds, some preliminary SPME-GC/MS experiments were performed using ground *L. citriodora* sample utilizing a PDMS fiber. From different recorded chromatograms, it becomes clear that the best GC program was as which mentioned previously (the column initial temperature: 40 ºC, with a rate of 4 ºC/min increased to 200 ºC, and remain at 200 ºC for 1 min). The affecting experimental parameters such as fiber's coating type, sonication time, extraction time and temperature, desorption time, and water content of sample were optimized. The optimization of different affecting parameters was accomplished by using simplex method. The optimal conditions were as fiber's coating type: PDMS, sonication time: 15 min, extraction time: 40 min, extraction temperature: 70 ºC, desorption time: 2 min, and water content: 500 µl per 0.5 g of ground sample. The use of a simplex optimization method was of paramount importance in order to select the best working conditions of interrelated variables.

A number of SPME fibers of different polarity and coating thickness are commercially available and have been used for extraction of the volatile compounds in medicinal plants [22]. Among the fibers, PDMS or PDMS-based mixed fibers are most commonly used. Using ultrasonic assist with headspace solid phase microextraction, the highest extraction efficiency was achieved with a 100 µm polydimethylsiloxane (PDMS) fiber and it was found that the oxygenated sesquiterpene and oxygenated monoterpene fractions decreased but the amount of sesquiterpenes and monoterpenes increased.

In order to get access to the absolute mass percentage of the identified compounds, the essential oil of *L. citriodora* was analyzed after extraction by SPME. Our experience showed that SPME could not give the exact mass percentage of the constituents of volatile compounds in comparison with reported hydrodistillation method [2, 3, 16, 19-20, 24] to limited load capacity of micro-scale fibers especially for main components [25-26].

4. Conclusions

In conclusion, the study of the essential oil from fresh leaves of *L. citriodora* of Iran cultivated showed the presence of geranial, 1,8- Cineole and limonene after extraction by SPME. Thus, the experimental parameters such as extraction time, irradiation power and ultra sound effects can be optimized for the particular aim of the SPME, either to obtain a high yield of essential oil, or to obtain essential oils of differing composition [9]. However, SPME is capable to analyze the volatiles with the least extraction time, sample amount, and sample preparation steps. In addition, significant ability of trapping and extracting of compounds which are more volatile.

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