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EXPERIMENTAL PAPER

The effect of different development stages on the quantity and quality of the essential oil of *Citrus aurantifolia* (Christm.) Swingle in Iran

RAZIEH EBADATI ESFAHANI[1]*, PEJMAN MORADI[2]

[1]Department of Horticultural Science
Horticultural Science Student
Islamic Azad University of Saveh, Iran

[2]Department of Horticultural Science
Islamic Azad University of Saveh, Iran

*corresponding author: e-mail: rz.ebadati@gmail.com

Summary

Introduction: The Mexican lime tree with the scientific name of *Citrus aurantifolia* (Christm.) Swingle have great economic value because of its essential oil with a unique flavour. **Objective:** The essential oils from the peel of *C. aurantifolia* were collected during three development periods. **Methods:** The essential oil was analyzed by capillary GC and GC-MS. **Results:** The essential oil yields (v/w%) were 1.54%, 0.88% and 1.23%, respectively. The highest oil yield was obtained at stage I (1.54% v/w). The analysis of the essential oil indicated that limonene, *β*-pinene, geranial, neral and *γ*-terpinene were the main compounds of all samples. At the first stage, the highest percentages belonged to limonene (39.38%), geranial (14.32%) and neral (11.01%). On the other hand, the highest percentages of *β*-pinene and *γ*-terpinene (24.25% and 8.92%, respectively) were found at the final stage. **Conclusion:** Therefore, it is concluded that the harvest time has a considerable effect on the content and amount of lime fruit essential oil.

Key words: *Citrus aurantifolia, development stages, essential oil, GC-MS, limonene, geranial, neral*

INTRODUCTION

The Mexican lime tree with the scientific name of *C. aurantifolia* (Christm.) Swingle (*Rutaceae*) belongs to the citrus genus [1]. This tree has long and spherical fruits, thin and leathery skin and greenish yellow colour [1, 2]. Unique aroma makes the lime essential oil one of the most important citrus oils in business. Much research has focused on the chemical composition of the essential oil from the Mexican lime peel. The major aromatic compounds obtained from the essential oil include limonene, *α*-terpineol, *β*-terpineol, cineol-1,4, cineol-8,1, *p*-cymene, *β*-pinene, *β*-bisabolene, citral, geranial and neral [3, 4]. This essential oil is one of the crucial commercial citrus product utilised in numerous industries, including the beverage, sweet, chocolate, medicine, perfume, and cosmetic industries. New researches have shown that the essential oil of this plant is highly effective in lowering cholesterol, reducing cardiovascular diseases, boosting the immune system, preventing cancer, infectious diseases, and colorectal cancer, improving the quality of bones, preventing osteoporosis, and curing [5-7]. Research reveals that the quality of essential oil in medicinal and aromatic plants depends on different factors. Among them are the climatic conditions as well as the chemotype and biotype of the plant [8]. Unfortunately, there have been few research projects focusing on volatiles during the *C. aurantifolia* fruit ripening. Changes in the peel essential oil compounds of *C. aurantifolia* during growth indicated that limonene, *β*-pinene, neral, geranial and terpinen-4-ol were the main compounds at different growth stages. Additionally, the percentages of limonene and *β*-pinene increased in mature fruits. On the other hand, the highest percentages of neral, geranial and terpinen-4-ol were found in immature fruits [9]. Droby *et al.* [10] reported that the percentage of limonene in (*C. paradise*, *C. sinensis* and *C. reticulata*) decreased from March to August and the percentage of limonene in immature fruits were considerably greater than that in the mature fruits. Bourgou *et al.* [4] reported that the yield of essential oil in growth stages in (*Citrus aurantium, C. limon, C. sinensis, C. reticulata*) were very different such that, the highest percentage of essential oil's yield in *C. aurantium* was in stage 3 (mature), in *C. limon* was in stage 1 (immature) and in *C. sinensis*, *C. reticulata* was in stage 2 (semi-mature). Given that the essential oil of lime in different industries like pharmaceutical industry is crucial and that the components of the essential oil change over different phases of plant growth and that these compounds and their amounts at different stages of this plant growth in Iran have never been studied, this research studies the effect of the three phases of growth in lime fruit on the quantity and quality of the essential oil in order to specify the best harvest time of lime fruit with the high quantity and quality of the essential oil.

MATERIAL AND METHODS

Plant material

This study was done in a randomized complete blocks design with 3 replications in order to evaluate the effect of different stages of fruit growth on the quantity and quality of the essential oil of lime fruit [*Citrus aurantifolia* (Christm.) Swingle]. The harvest was carried out in three stages, that is, the phase I (the green peel of the immature fruit in July), the phase II (the greenish yellow peel of the semi-mature fruit in September) and the phase III (the yellow peel of the mature fruit in November). The Mexican lime tree fruits were collected from the commercial orchard in Fasa City in Iran (28°58′N 53°46′E; 1554 MAMSL) from Spring to Autumn and the *C. aurantifolia* authentication was done by professors of botany in the Centre for Agriculture and Natural Resources in Fars Province, Iran. The sampled trees were of the same age, height and crown size and their fruits were selected randomly from different crown directions. This experiment consists of three replications. In all three stages, the fruits were peeled carefully with the help of a sharp knife to avoid damage of oil glands; after that, the peels were kept in darkness for about 24 hours, then dried and chopped in small pieces for better hydrodistillation. In order to reduce the percentage of error in the essential oil yield, the percentage moisture was assessed by Moisture meter apparatus Sartorius Model MA 100. Due to the same conditions, the percentage of moisture was almost equally in all stages.

Essential oil isolation

To isolate essential oil, the samples were transported to the Centre for Agriculture and Natural Resources in Fars Province for the measurement and analysis of the essential oils. 80 g of the dried samples was hydrodistillated for 3 hours Clevenger apparatus and the related percentage was evaluated. Oil recovered was dried over anhydrous sodium sulphate and then kept at 4°C until dissolution.

In order to analyse the essential oil components, the Gas Chromatograph (GC) systems and Gas Chromatograph Mass Spectrometry (GC-MS) with the following specifications were used.

Gas chromatography analysis

The analysis was conducted using the Model 7890A gas chromatography system of the Agilent technologies, HP-5 column with 30 m long and 0.32 mm in diameter, stationary phase thickness of 0.25 μm, and column temperature between 60 and 210°C with the incremental temperature of 3 degrees per minute. For the temperatures of 210 till 240°C, it was set at the incremental temperature of 20°C per minute and the final temperature was kept for about 8.5 minutes. The type of detector is FID, the temperature is 290°C, the carrier gas is nitrogen at a rate of 1 ml per minute, and the injection temperature is 280°C using the Chemstation software.

Gas Chromatography-Mass Spectrometry analysis

The GC-MS analysis of the essential oil volatile components was carried out with use of the model 5975C, system of Agilent technologies, HP-5MS column 30 m long and 0.25 mm in diameter, stationary phase thickness of 0.25 μm, column temperature programming as before, MS with the temperature 280°C, injector temperature 280°C, the ionization energy 70 eV, carrier gas: helium.

Compound identification

Identification of essential oil volatile compounds was based on the calculation of their retention indices (RI) relative to (C8–C25) n-alkanes. Further identification was made by matching their recorded mass spectra with those stored in the Wiley/NBS mass spectral library of the GC-MS data systems and other published mass spectra and by comparing with MS literature data [4, 11].

Statistical analyses

The statistics were performed using the SPSS software where Duncan's multiple range test compared the mean values at 1% and 5% levels.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS

The results showed that the average percentages of the essential oil yield obtained from the lime peel in three growth phases (immature, semi-mature and mature) were 1.54%, 0.88% and 1.23%, respectively (v/w) (tab. 1). According to the results obtained from the analysis of variance, we found that the effect of the different fruit growth stages in Fasa City is significant at 5% level of the Duncan test (tab. 2). The comparison between mean values of treatments showed that the maximum yield of the essential oil (1.54%) has been achieved in the first phase – from immature fruit skins. As compared with other phases, it was a remarkable difference. The least essential oil (0.88%) was yielded in the second growth phase – from the semi-mature fruit peels. In figure 1, the chromatogram of Mexican lime peel oil is presented. Table 3 presents the identified compounds of the essential oil at various lime fruit growth stages, as well as the percentages of each compound with the retention indices. Overall, in all stages of growth 30, 27 and 29 compounds were identified, making up to 98.71%, 98.49% and 98.45% of total available compounds, respectively. The analysis of the volatile compounds obtained from three stages of fruit growth showed that major constituents of essential oil (over 4%) were limonene, β-pinene, geranial, neral and γ-terpinene (tab. 4). Limonene was found to be the most prevalent component of the essential oil at the three stages, with values of 39.38%, 36.23% and 32.81%, respectively. The greatest amount of limonene was achieved in the first growth phase from immature fruits, showing a significant difference, as compared to the amounts yielded in the other phases. The amounts of *β*-pinene, the second major compound of the essential oil, were 17.81%, 16.75% and 24.25%, respectively. The maximum occurred in the fruit mature phase and as compared to the immature and semi-mature stages, it showed a remarkable difference. The results indicated that the third major component of the lime essential oil in all three growth stages is geranial, with the values of 14.32%, 11.53% and 9.31% for the consecutive phases, respectively. Like limonene, the maximum amount of this compound was achieved from the first phase that, as compared to the other phases, showed a significant difference. The maximum amount of neral (11.01%) was achieved from the first growth phase in the immature fruits (fig. 2). The amounts of neral obtained from the first phase of harvest have shown a significant difference, as compared with other phases.

Table 1.

Mean amounts and standard deviations of essential oil yield in *Citrus aurantifolia* peel during three growth stages

Growth stages	Mean and SD of essential oil (v/w)
Stage I	1.54 ± 0.001^{a}
Stage II	0.88 ± 0.105^{b}
Stage III	1.23 ± 0.180^{ab}

Means of three replicates. (Values with different superscripts are significantly different at $p<0.05$. Small superscripts (comparison between stages).

Table 2.

Variance analysis of essential oils of three growth stages of *Citrus aurantifolia*

	Sum of squared	Degree of freedom	Mean square	Frequency	Significance level
Trimming effect	0.66	2	0.33	5.174	0.049*
Error	0.383	6	0.064		
Total	1.043	8			

*The difference is significant at the level of 5%.

Table 3.

Chemical components of peel essential oil in three growth stages of *Citrus aurantifolia* fruit

				Stage I	stage II	stage III
No	Compounds	RI	RI^{L}	[%]	[%]	[%]
1	*α*-Thujene	924	925	0.16	0.20	0.33
2	*α*-Pinene	932	934	1.15	1.31	1.67

				Stage I	stage II	stage III
No	Compounds	RI	RI[L]	[%]	[%]	[%]
3	Camphene	947	950	0.09	0.09	0.11
4	Sabinene	974	976	-	2.48	-
5	*β*-Pinene	982	982	**17.81**	**16.75**	**24.25**
6	Myrcene	989	991	1.06	1.02	0.94
7	*α*-Phellandrene	1003	1006	0.23	0.18	0.10
8	*α*-Terpinene	1017	1018	0.27	0.32	0.50
9	(Z)-*β*-Cimene	1036	1036	-	0.26	0.20
10	Limonene	1037	1037	**39.38**	**36.23**	**32.81**
11	(E)-*β*-Ocimene	1047	1046	0.53	0.45	0.37
12	*γ*-Terpinene	1060	1060	3.81	4.96	**8.92**
13	Terpinolene	1088	1090	0.30	0.38	0.58
14	Linalool	1103	1104	1.39	1.23	1.31
15	n-Nonanal	1105	1106	0.10	-	0.19
16	cis-Limonene oxide	1142	1138	0.05	-	0.28
17	Citronellal	1145	1147	0.06	-	-
18	trans-*p*-Menth-2-en-1-ol	1153	1155	0.20	0.30	0.33
19	Borneol	1169	1171	0.09	-	-
20	Terpinen-4-ol	1181	1183	1.26	1.39	2.31
21	*α*-Terpineol	1196	1198	2.06	2.17	3.02
22	*γ*-Terpineol	1205	1207	0.21	0.29	0.32
23	Citronellol	1236	1236	0.93	2.74	1.11
24	Neral	1252	1252	**11.01**	**8.82**	**6.99**
25	Geraniol	1263	1262	1.004	2.94	1.04
26	Geranial	1284	1283	**14.32**	**11.53**	**9.31**
27	Neryl acetate	1366	1367	0.09	-	0.12
28	Geranyl acetate	1385	1387	0.35	0.35	0.24
29	(*β*)-Caryophyllene	1420	1422	0.29	0.61	0.24
30	trans-*α*-Bergamotene	1434	1433	0.13	0.28	0.16
31	*β*-bisabolene	1507	1508	0.29	0.99	0.62
32	Germacrene B	1557	1559	0.09	0.22	0.08
Total identified (%)				98.71	98.49	98.45
Monoterpene hydrocarbons				64.79	64.54	72.09
Oxygenated monoterpenes				32.58	31.41	24.71
Sesquiterpene hydrocarbons				0.8	2.1	1.1
Non-terpene				0.54	0.35	0.55

RI: The retention indices, RI[L]: The retention indices literature, Constituents in bold (> 5.0), (-): didn't identify.

Table 4.

Comparison of the major compound average content in *Citrus aurantifolia* peel essential oil in three growth stages with Duncan test at the level of 5%

	β-Pinene	Limonen	*γ*-Terpinene	Neral	Geranial
Stage I	17.81[b]±0.29	39.38[a]±0.37	3.81[c]±0.63	11.01[a]±0.35	14.32[a]±0.44
Stage II	16.75[c]±0.39	36.23[b]±0.29	4.96[b]±0.31	8.82[b]±0.14	11.53[b]±0.29
Stage III	24.25[a]±0.35	32.81[c]±0.44	8.92[a]±0.40	6.99[c]±0.31	9.31[c]±0.31

Averages with the same letters were the non-significant difference at the level of 5% in the Duncan test.

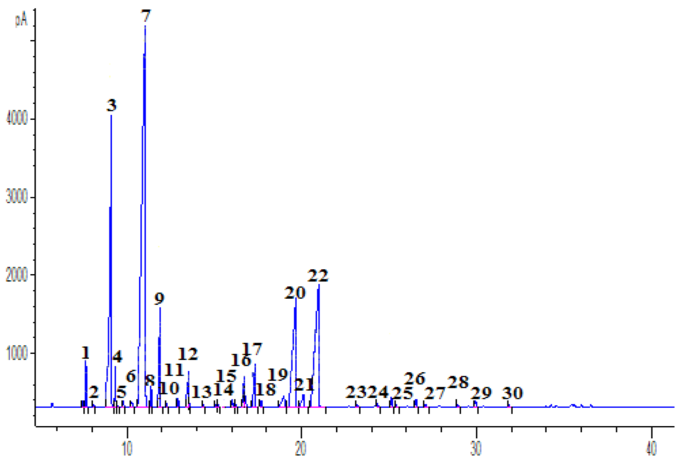

Figure 1.

Chromatogram of *Citrus aurantifolia* peel essential oil at first growth stage

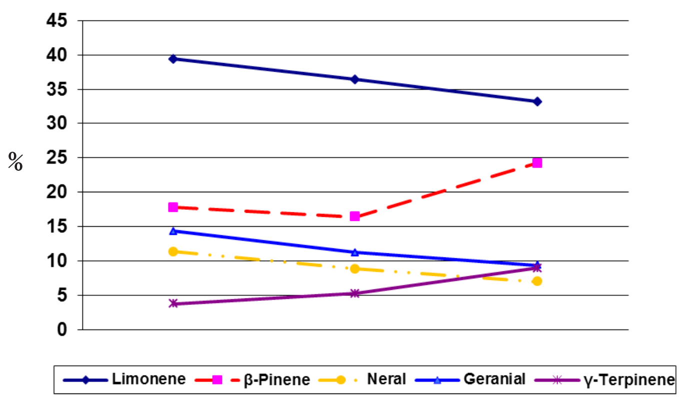

Figure 2.

Changes in major essential oil ingredients of *Citrus aurantifolia* fruit peel during three stages of growth

DISCUSSION

The variance and mean analyses indicated that the growth stages affected the quantity and quality of *C. aurantifolia* essential oil in Fasa city. The result of the quantity of *C. aurantifolia* essential oil (the ratio of the actual yield on the dried weight) showed that in order to obtain the highest degrees of essential oil, it is better to harvest fruits at the immature stage. It must be pointed out that the results obtained in this research were different from the performed on the *C. reticulata* and *C. aurantifoila*. The authors reported that the highest essential oil yields in *C. reticulata* was in the second growth phase [12] and the highest essential oil yield of *C. aurantifolia* was obtained from full ripe stage [9]. The primary increase in the essential oil content of the fruit peel might be exactly correlated with a surface area of fruit [12]. Such differences between *Citrus* species could be ascribed both to interplay between genetic and environmental factors [4]. Water supply during ripening could increase considerably essential oil content with an improvement under moderate water shortage condition [12]. Furthermore, by comparing the *C. aurantifolia* essential oil during its fruition phase with the research reports from [9] (0.9%) and [13] (0.47%), it was found that the yield percentage in Fasa city is considerably higher than all the reported amounts, probably due to specific climate. The studies on *C. reticulata*, *C. volkameriana* and Shamouti oranges found that the maximum amount of limonene was at the first growth stage or immature species, corresponding to the present research [12]. Interestingly, when limonene showed the lowest level at the mature stage (32.81%), several minor compounds including α-pinene, γ-terpinene, α-terpineol, terpinen-4-ol reached their highest content. Then, at the mature stage, the biosynthesis of limonene is lowered in favour to other cyclic monoterpenes. Moreover, by comparing the limonene percentage during the three growth phases in Fasa with the research reports showed that the limonene percentage were lower than the other reported amounts. Saleem *et al.* [13], Yadav *et al.* [3], Venkateshwarlu and Selvaraj [9] as well as Chamblee and Clark [14] reported the limonene percentage in *C. aurantifolia* were 82.84%, 75.5%, 47.0% and 46.86%. The studies on *C. aurantifolia* and *C. reticulata* found that the maximum amount of β-pinene was at the third growth stage or mature fruits, corresponding to the present research [9,13]. Venkateshwarlu and Selvaraj [9] as well as Chamblee and Clark [14] claimed that the third phase presents more β-pinene, as compared to the other growth phases. This trend is thoroughly opposite to that regarding limonene: the closer to the final phase, the smaller the limonene percentage, and the greater the β-pinene percentage. The percentage of β-pinene found in Fasa showed that this amount is either equal to or greater than other reported during the fruition phase. The percentages of β-pinene in *C. aurantifolia* have been reported as follows: 32.1%, 23.8%, 10.7%, 1.34% and 0.86% [3, 8, 9, 13, 14]. Venkateshwarlu and Selvaraj [9] as well as Vekiari *et al.* [8] reported that the highest value of geranial and neral have been obtained from the first stage of growth or the beginning of the growing season like limonene, corresponding to our research. Those components content decreased from the first phase to the third phase. The percentages of geranial and neral in this study were considerable higher than other research reported on *C. aurantifolia*, 3.6% and 4.2% [9], no and 0.02% [14] as well as 4.1% and 1.8% [3]. On the other hand, the geranial and neral components in *C. aurantifolia* essential oil were not reported by Saleem *et al.* [13]. In this study, monoterpene hydrocarbons increased with the advancement of fruit ripening. As identified before, the value of the major component of the *C. aurantifolia* essential oil changes during the growth period. The evaluation of changes in the value of these components revealed that the percentage of limonene, geranial and neral declines from the immature stage to the mature stage. The result specified that in order to obtain the maximum amounts of limonene, geranial and neral, the fruits must be harvested at the immature stage. On the other hand, the amount of β-pinene and γ-terpinene increases gradually from the immature stage to the mature stage of the fruit growth. Furthermore, to obtain the maximum amount of β-pinene and γ-terpinene from the skin of the Mexican lime fruit, they need to be harvested at the full maturity.

CONCLUSIONS

Therefore, it is concluded that the harvest time has a considerable effect on the content and amount of *Citrus aurantifolia* essential oil. This great effect on the amounts of essential oil and the components can be caused by changes in the metabolic trends and in the secondary metabolism, which is associated with the plant's growth. The maturation processes and plant growth can be used as an indicator. However, further studies are required to investigate the molecular aspects of these changes at different stages of fruit growth.

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Conflict of interest: Authors declare no conflict of interest.

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WPŁYW STADIUM ROZWOJU OWOCU NA ZAWARTOŚĆ I SKŁAD OLEJKU ETERYCZNEGO Z NAOWOCNI LIMONKI *CITRUS AURANTIFOLIA* (CHRISTM.) SWINGLE W IRANIE

RAZIEH EBADATI ESFAHANI[1]*, PEJMAN MORADI[2]

[1]Department of Horticultural Science
Horticultural Science Student
Islamic Azad University of Saveh, Iran

[2]Department of Horticultural Science
Islamic Azad University of Saveh, Iran.

*autor, do którego należy kierować korespondencję: e-mail: rz.ebadati@gmail.com

Streszczenie

Wstęp: Limonka *Citrus aurantifolia* (Christm.) Swingle ma duże znaczenie ekonomiczne ze względu na niepowtarzalny aromat olejku eterycznego. **Cel:** Porównanie wydajności i składu olejku eterycznego z naowocni limonki w trzech stadiach rozwoju owocu. **Metodyka:** Olejek eteryczny analizowano metodą GC i GC/MS. **Wyniki:** Wydajność olejku (v/w%) wynosiła w kolejnych stadiach odpowiednio 1,54%, 0,88% i 1,23%. Najwyższa zawartość olejku była w stadium I - 1,54% v/w. Analiza i identyfikacja składników olejku wykazała, że głównymi związkami we wszystkich próbkach są limonen, *β*-pinen, geranial, neral i *γ*-terpinen. W pierwszym stadium w największej ilości występowały: limonen (39,38%), geranial (14,32%) i neral (11,01%). Z drugiej strony najwyższą zawartość *β*-pinenu (24,25%) i *γ*-terpinenu (8,92%) obserwowano w finalnym trzecim stadium. **Wnioski:** Stadium rozwoju owocu ma istotny wpływ na zawartość i skład olejku eterycznego z naowocni limonki *Citrus aurantifolia*.

Słowa kluczowe: *Citrus aurantifolia, etapy rozwoju, olejek eteryczny, GC/MS, limonen, geraniol, nerol*