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The Effect of Seasonal Variation on Sweet Orange (Citrus Sinensis) Leaf Components

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ABSTRACT: Studies have shown that oxygenated compounds are important in food products. It seems that seasonal variation has a profound influence on this factor. The goal of the present study is to investigate on seasonal variation of sweet orange leaf components. About 500 g of leaves were collected from many parts of the same trees during the vegetation season (in March, June, September and December 2012). Leaf components were extracted using water distillation method and then analyzed using GC and GC-MS. Data were analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range tests. The amount of oxygenated compounds ranged from 26.04% to 37.60%. Among four seasons examined, September showed the highest content of oxygenated compounds. As a result of our study, we can conclude that the seasonal variation can influence the quantity of oxygenated compounds present in the oil.
Key words: Flavor Components, Leaf Oil, Seasonal Variation, Water-Distillation.

INTRODUCTION

Citrus is one of the most economically important crops in Iran. In the period 2009- 2010, the total Citrus production of Iran was estimated at around 87000 tonnes [1]. Shahsavari orange is a local cultivar of sweet orange that cultivated extensively in the Mazandaran province located in the north region of Iran [2, 3]. It has been regarded as a Citrus fruit with potential commercial value because of its attractive and pleasant aroma. Also; it is one of the most important orange cultivars used in Iran. Although it is as important cultivar, the leaf components of Shahsavari orange have been investigated very little previously [4].

Citrus oils occur naturally in special oil glands in flowers, leaves, peel and juice. These valuable essential oils are composed of many compounds including: terpenes, sesquiterpenes, aldehydes, alcohols, esters and sterols. They may also be described as mixtures of hydrocarbons, oxygenated compounds and nonvolatile residues. Citrus oils are commercially used for flavoring foods, beverages, perfumes, cosmetics, medicines and etc [5]. In addition, recent studies have identified insecticidal, antimicrobial, antioxidative and antitumor properties for Citrus oils [6].

The quality of an essential oil can be calculated from the quantity of oxygenated compounds present in the oil. The quantity of oxygenated compounds present in the oil, is variable and depends upon a number of factors including: seasonal variation [7-10], rootstock [11], varieties [12], organ [13], extraction method [14] and etc.

Branched aldehydes and alcohols are important flavor compounds extensively used in food products [5]. Several studies have shown that oxygenated terpenoids such as linalool, neral, geranial [15], nonanal, decanal and linalool [16] are important in sweet orange flavor. The quality of a honey can be calculated from the amount of oxygenated components present in the honey (17, 18). In addition, type of flowers may influence the quality of volatile flavor components present in the honey. The effect of oxygenated compounds in the attraction of the pollinators has been proven. Therefore, the presence of oxygenated compounds can encourage the agricultural yield [19, 20].

In this paper, we compared the leaf compounds isolated from sweet orange with the aim of determining whether the quantity of oxygenated compounds influenced by the season.

MATERIAL AND METHODS

Sweet orange scions

In 1989, sweet orange scions that grafted on sour orange rootstock, were planted at 8×4 m with three replication at Ramsar research station [Latitude 36° 54' N, longitude 50° 40' E; Caspian Sea climate, average rainfall and temperature were 970 mm and 16.25°C per year respectively; soil was classified as loam-clay, pH ranged from 6.9 to 7)]. Sweet orange was used as plant material in this experiment (Table 1).

Preparation of leaf sample

About 500 g of leaves were collected from many parts of the same trees during the vegetation season (in March, June, September and December 2012), early in the morning (6 to 8 am) and only during dry weather

Leaf Extraction Technique

In order to obtain the volatile compounds from the leaf, 500 g of fresh leaves were subjected to hydro distillation for 3 h using a Clevenger-type apparatus. N-hexane was used to isolate the oil layer from the aqueous phase. The hexane layer was dried over anhydrous sodium sulphate and stored at -4°C until used [11].

GC and GC-MS

An Agilent 6890N gas chromatograph (USA) equipped with a DB-5 (30 m 0.25 mm i.d; film thickness = 0.25 m) fused silica capillary column (J and W Scientific) and a flame ionization detector (FID) was used. The column temperature was programmed from 60 to C (3min) to 250 o C (20 min) at a rate of 3 o C/ min. The injector and detector temperatures were 260 o C and helium was used as the carrier gas at a flow rate of 1.00 ml/min and a linear velocity of 22 cm/s. The linear retention indices (LRIs) were calculated for all volatile components using a homologous series of n-alkanes (C9-C22) under the same GC conditions. The weight percent of each peak was calculated according to the response factor to the FID. Gas chromatography- mass spectrometry was used to identify the volatile components. The analysis was carried out with a Varian Saturn 2000R. 3800 GC linked with a Varian Saturn 2000R MS .

The oven condition, injector and detector temperatures, and column (DB-5) were the same as those given above for the Agilent 6890 N GC. Helium was the carrier gas at a flow rate of 1.1 mL/min and a linear velocity of 38.7 cm/s. Injection volume was 1 μL.

Identification of components

Components were identified by comparison of their Kovats retention indices (RI), retention times (RT) and mass spectra with those of reference compounds [21, 22].

Data analysis

SPSS 18 was used for analysis of the data obtained from the experiments. Analysis of variations was based on the measurements of 9 leaf components. Comparisons were made using one-way analysis of variance (ANOVA) and Duncan's multiple range tests. Differences were considered to be significant at $P < 0.01$. The correlation between pairs of components was evaluated using Pearson's correlation coefficient.

RESULTS

Leaf compounds of the sweet orange during the deferent seasons

GC-MS analysis of the compounds extracted from sweet orange leaf using water distillation allowed identification of 47 volatile components (Table1, 2, Fig1): 24 oxygenated terpenes [6 aldehydes, 13 alcohols, 5 esters] and 23 non oxygenated terpenes [17 monoterpens, 6 sesqiterpens].

Aldehydes

Six aldehyde components that identified in this analysis were citronellal, decanal, neral, geranial, β-sinensal and α-sinensal (Table 3). In addition they were quantified from 5.07% to 8.04%. The concentrations of citronellal and geranial were higher in our samples. Citronellal has Citrus-like aroma [23] and is considered as one of the major contributors to sweet orange flavor. Among four seasons examined, September showed the highest content of aldehydes (Table 3). Since the aldehyde content of citrus oil is considered as one of the most important indicators of high quality, season apparently has a profound influence on this factor. (Table 3).

Alcohols

Thirteen alcohol components identified in this analysis were linalool, Isopulegol, terpinen-4-ol, -terpineol, Myrtenol, (z)-piperitol, trans-carveol, β-citronellol, cis-carveol, nerol, geraniol, elemol and (E)-nerolidol (Table 3). The total amount of alcohols ranged from 19.06% to 27.24%. Linalool was identified as the major component in this study and was the most abundant. Linalool has been recognized as one of the most important components for Citrus flavor [23]. Linalool has a flowery aroma [23] and its level is important to the characteristic favor of Citrus. Among four seasons examined, September showed the highest content of alcohols (Table 3).

Esters

Five ester components that identified in this analysis were linalyl acetate, terpinyl acetate, citronellyl acetate, neryl acetate and geranyl acetate. The total amount of esters ranged from 1.73% to 2.32%. Among four seasons examined, September showed the highest content of esters (Table 3).

Monoterpene hydrocarbons

The total amount of monoterpene hydrocarbons ranged from 54.26% to 68.77%. Sabinene was identified as the major component in this study and was the most abundant. Sabinene has a woody aroma [24] and is considered as one of the major contributors to Citrus flavor. Among four seasons examined, December showed the highest content of monoterpenes (Table 3).

Sesquiterpene hydrocarbons

The total amount of sesquiterpene hydrocarbons ranged from 3.43% to 5.02%. (Z)-β-caryophyllene and (Z)-β-farnesene were identified as the major component in this study and were the most abundant. Among four seasons examined, March showed the highest content of sesquiterpenes (Table 3).

Results of statistical analyses

Differences were considered to be significant at $P < 0.01$. These differences on the 1% level occurred in citronellal, geranial, linalool, β-citronellol, sabinene, δ-3-carene, limonene and (E)-β-ocimene. The non-affected oil component was terpinen-4-ol (Table 3).

Results of correlation

Simple intercorrellations between 9 components are presented in a correlation matrix (Table 4). The highest positive values or r (correlation coefficient) were observed between β -citronellol and geranial (99%); linalool and geranial (98%); β -citronellol and linalool (97%). The highest significant negative correlations were observed between (E)-β-ocimene and geranial (82%); (E)-β-ocimene and β -citronellol (79%). (Table 4).

Table 1. Common and botanical names for citrus taxa used as scions and rootstock [30].

Common name	botanical name	Parents	category
Sweet orange (scion)	*Citrus sinensis cv. shahsavari*	*Unknown*	Sweet orange
Sour orange (Rootstock)	C. aurantium (L.)	*Mandarin ×Pomelo*	Sour orange

Table 2. Seasonal variations on sweet orange leaf components. (*There is in oil)

	Component	March	June	September	December	KI		Component	March	June	September	December	KI
1	α-thujene	*	*	*	*	**925**	25	(Z)-piperitol	*	*	*	*	**1211**
2	α-Pinene	*	*	*	*	**935**	26	Trans-carveol	*	*	*	*	**1219**
3	Sabinene	*	*	*	*	**974**	27	β -citronellol	*	*	*	*	**1229**
4	β-pinene	*	*	*	*	**978**	28	Cis-carveol	*	*	*	*	**1231**
5	β- myrcene	*	*	*	*	**989**	29	Nerol	*	*	*	*	**1233**
6	δ - 3-carene	*	*	*	*	**1023**	30	Neral	*	*	*	*	**1240**
7	α- terpinene	*	*	*	*	**1012**	31	Geraniol	*	*	*	*	**1257**
8	p-cymene	*	*	*	*	**1027**	32	Linalyl acetate	*	*	*	*	**1259**
9	Limonene	*	*	*	*	**1031**	33	Geranial	*	*	*	*	**1269**
10	β - phellandrene	*	*	*	*	**1032**	34	α-Terpinyl acetate	*	*	*	*	**1353**
11	(Z)- β- ocimene	*	*	*	*	**1042**	35	Citronellyl acetate	*	*	*	*	**1355**
12	(E)- β- ocimene	*	*	*	*	**1052**	36	Neryl acetate	*	*	*	*	**1365**
13	γ- terpinene	*	*	*	*	**1061**	37	α -copaene	*	*	*	*	**1380**
14	Cis-sabinene hydrate	*	*	*	*	**1068**	38	Geranyl acetate	*	*	*	*	**1384**
15	(Z)- Linalool oxide	*	*	*	*	**1071**	39	β -elemene	*	*	*	*	**1395**
16	(E)- Linalool oxide	*	*	*	*	**1085**	40	(Z)- β- caryophyllene	*	*	*	*	**1416**
17	α- terpinolene	*	*	*	*	**1091**	41	(Z)- β - farnesene	*	*	*	*	**1451**
18	Linalool	*	*	*	*	**1100**	42	α -humulene	*	*	*	*	**1461**
19	Citronellal	*	*	*	*	**1154**	43	Elemol	*	*	*	*	**1556**
20	Isopulegol	*	*	*	*	**1162**	44	(E)-Nerolidol	*	*	*	*	**1567**
21	Terpinene-4-ol	*	*	*	*	**1182**	45	Caryophyllene oxide	*	*	*	*	**1581**
22	α - terpineol	*	*	*	*	**1194**	46	β -sinensal	*	*	*	*	**1699**
23	Myrtenol	*	*	*	*	**1198**	47	α - sinensal	*	*	*	*	**1755**
24	Decanal	*	*	*	*	**1205**							

Table 3. Statistical analysis of variation in leaf flavor Components during different seasons. Mean is average composition in % over the different seasons used with three replicates. St. err = standard error. F value is accompanied by its significance, indicated by: NS = not significant, * = significant at P = 0.05, ** = significant at P = 0.01.

Compounds	March		June		September		December		
	Mean	**St.err**	**Mean**	**St.err**	**Mean**	**St.err**	**Mean**	**St.err**	**F**
Oxygenated compounds									
a) Aldehyds									
1) Citronellal	1.69	0.15	1.81	0.16	2.31	0.23	1.73	0.09	**F****
2) Decanal	0.08	0.01	0.10	0.03	0.12	0.02	0.09	0.01	
3) Neral	0.84	0.07	1.02	0.12	1.57	0.12	0.86	0.09	
4) Geranial	1.48	0.15	1.79	0.16	2.81	0.10	1.42	0.11	**F****
5) β-sinensal	0.64	0.04	0.71	0.06	0.79	0.06	0.67	0.05	
6) α- sinensal	0.34	0.03	0.40	0.05	0.44	0.04	0.36	0.03	
total	5.07	0.45	5.83	0.58	8.04	0.55	5.13	0.37	

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b) Alcohols									
1) Linalool	11.74	0.41	12.44	0.92	16.00	1.00	11.23	0.56	**F****
2) Isopulegol	0.38	0.05	0.43	0.05	0.52	0.05	0.36	0.03	
3) Terpinen-4-ol	2.97	0.29	3.55	0.40	3.82	0.37	3.11	0.38	**NS**
4) α-terpineol	0.62	0.10	0.69	0.06	1.11	0.09	0.61	0.06	
5) Myrtenol	0.07	0.02	0.08	0.006	0.09	0.01	0.06	0.006	
6) (Z)-piperitol	0.21	0.02	0.23	0.04	0.25	0.03	0.20	0.02	
7) (Z)-carveol	0.14	0.02	0.16	0.02	0.18	0.02	0.13	0.02	
8) β -citronellol	1.52	0.12	1.75	0.19	2.97	0.15	1.57	0.10	**F****
9) (E)-carveol	0.33	0.04	0.37	0.06	0.42	0.01	0.31	0.02	
10) Nerol	0.66	0.07	0.71	0.08	0.79	0.06	0.63	0.05	
11) Geraniol	0.62	0.06	0.54	0.06	0.71	0.05	0.59	0.05	
12) Elemol	0.07	0.006	0.08	0.006	0.09	0.01	0.06	0.01	
13) (E)-nerolidol	0.21	0.02	0.26	0.04	0.31	0.02	0.19	0.02	
total	19.55	1.22	21.29	1.93	27.24	1.88	19.06	1.31	
d) Esteres									
1) Linalyl acetate	0.65	0.06	0.71	0.06	0.78	0.05	0.68	0.06	
2) α-terpinyl acetate	0.07	0.006	0.09	0.006	0.10	0.01	0.08	0.01	
3) Citronellyl acetate	0.20	0.03	0.23	0.03	0.25	0.02	0.21	0.02	
4) Neryl acetate	0.12	0.02	0.15	0.02	0.17	0.02	0.13	0.01	
5) Geranyl acetate	0.68	0.07	0.84	0.06	1.02	0.12	0.75	0.05	
total	1.73	0.19	2.02	0.18	2.32	0.22	1.85	0.14	
Monoterpenes									
1) α-thujene	0.47	0.04	0.42	0.05	0.35	0.04	0.53	0.05	
2) α-pinene	2.54	0.11	1.80	0.10	1.51	0.09	2.65	0.11	
3) Sabinene	36.09	1.01	33.00	2.00	32.00	0.92	37.57	0.50	**F****
4) β-Pinene	2.22	0.09	1.75	0.11	1.49	0.08	1.91	0.08	
5) β-myrcene	2.73	0.13	2.66	0.19	2.14	0.13	3.44	0.19	
6) δ-3-carene	3.44	0.22	3.59	0.29	2.97	0.19	3.67	0.16	**F****
7) α-terpinene	1.17	0.09	0.64	0.06	0.59	0.06	1.07	0.09	
8) p-cymene	0.80	0.07	0.92	0.08	0.99	0.07	0.77	0.06	
9) Limonene	8.22	0.38	6.50	0.50	6.04	0.32	8.13	0.42	**F****
10) β-phellandrene	0.02	0.00	0.01	0.00	0.007	0.001	0.03	0.006	
11) (Z)-β-ocimene	0.64	0.08	0.58	0.50	0.48	0.04	0.67	0.06	
12) (E)-β-ocimene	4.50	0.37	3.91	3.42	3.27	0.25	4.80	0.31	**F****
13) γ-terpinene	1.12	0.07	0.87	0.76	0.76	0.04	1.18	0.08	
14) Cis-sabinene hydrate	0.37	0.04	0.30	0.26	0.26	0.02	0.34	0.04	
15) (Z)-Linalool oxide	0.36	0.03	0.29	0.25	0.22	0.02	0.34	0.02	
16) (E)-Linalool oxide	0.86	0.06	0.77	0.67	0.69	0.06	0.83	0.06	
17) α-terpinolene	0.79	0.08	0.60	0.53	0.50	0.04	0.82	0.06	
total	66.34	2.85	58.61	9.77	54.26	2.34	68.77	2.28	
Sesquiterpenes									
1) α-copaene	0.47	0.05	0.42	0.04	0.38	0.04	0.45	0.04	
2) β-elemene	0.74	0.06	0.68	0.05	0.62	0.05	0.73	0.05	
3) (Z)-β-caryophyllene	1.80	0.10	1.25	0.13	0.88	0.05	1.55	0.08	
4) (Z)-β-farnesene	1.37	0.15	1.24	0.15	1.14	0.11	1.33	0.08	
5) α-humulene	0.24	0.02	0.19	0.02	0.14	0.02	0.20	0.02	
6) Caryophyllene oxide	0.39	0.05	0.32	0.04	0.27	0.02	0.37	0.03	
total	5.02	0.42	4.1	0.43	3.43	0.28	4.63	0.28	
Total oxygenated compounds	26.35	1.86	29.14	2.69	37.6	2.65	26.04	1.82	
Total	97.71	5.13	91.85	12.89	95.29	5.27	99.44	4.38	

Table 4. Correlation matrix (numbers in this table correspond with main components mentioned in Table 3).

	Citronellal	Geranial	Linalool	Terpinen-4-ol	β -citronellol	Sabinene	δ-3-carene	Limonene
Geranial	0.94**							
Linalool	0.96**	0.98**						
Terpinen-4-ol	0.86**	0.78**	0.82**					
β -citronellol	0.95**	0.99**	0.97**	0.74**				
Sabinene	-0.53	-0.74**	-0.67*	-0.51	-0.67*			
δ-3-carene	-0.46	-0.70*	-0.62*	-0.27	-0.67*	0.91**		
Limonene	-0.67*	-0.78**	-0.71**	-0.61*	-0.74**	0.90**	0.79**	
(E)-β-ocimene	-0.65*	-0.82**	-0.75**	-0.57	-0.79**	0.89**	0.83**	**0.81****

*=significant at 0.05
**=significant at 0.01

DISCUSSION

Our observation that seasonal variations had an effect on some of the components of sweet orange oil was in accordance with previous findings [7-10]. The compositions of the leaf oils obtained by water distillation during different seasons were very similar. However, the relative concentration of compounds was different according to the type of season.

Comparison of our data with those in the literatures revealed some inconsistencies with previous studies [12, 25]. It may be related to varieties, rootstock and environmental factors that can influence the compositions.

However, it should be kept in mind that the extraction methods also may influence the results. Fertilizer and irrigation affects the content of oil present in Citrus [26]. Fertilization, irrigation and other operations were carried out uniform in this study so we did not believe that this variability was a result of these factors.

The discovery of geranyl pyrophosphate (GPP), as an intermediate between mevalonic acid and oxygenated compounds (Alcohols and aldehyds), led to a rapid description of the biosynthetic pathway of oxygenated compounds. The biosynthetic pathway of oxygenated compounds in higher plants is as below:

Mevalonic acid → Isopentenyl Pyrophosphate → 3.3-dimethylallylpyrophosphate→ geranyl pyrophosphate→ Alcohols and Aldehyds

This reaction pathway catalyzed by isopentenyl pyrophosphate isomerase and geranyl pyrophosphate synthase, respectively [27]. The pronounced enhancement in the amount of oxygenated compounds, when September used as the season, showed that either the synthesis of geranyl pyrophosphate was enhanced or activities of both enzymes increased.

Also, the higher proportion of the detected oxygenated compounds in leaf was probably due to seasonal temperature [28], which is the most important environmental factor in the control of endogenous enzymes.

High positive correlations between pairs of terpenes suggest a genetic control [29] and such dependence between pairs of terpenes was due to derivation of one from another that was not known. Similarly, high negative correlations between pairs of terpenes suggest that one of the two compounds had been synthesized at the expense of the other or of its precursor. Non-significant negative and positive correlations can imply genetic and/or biosynthetic independence. However, without an extended insight into the biosynthetic pathway of each terpenoid compound, the true significance of these observed correlations is not clear.

Considering that acetate is necessary for the synthesis of terpenes, it can be assumed that there is a specialized function for this molecule and it may be better served in September.

In the present study we found that the amount of leaf compositions was significantly affected by seasons and there was a great variation in most of the measured characters among four seasons. The present study demonstrated that volatile compounds in leaf can vary when different seasons are utilized. Among four seasons examined, September showed the highest content of oxygenated compounds. The lowest of oxygenated compounds content were produced by December. Studies like this are very important to determine the amount of chemical compositions existing during different seasons. Further research on the relationship between seasonal variations and oxygenated compounds is necessary.

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