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Research Article

Comparison of leaf components of sweet orange and sour orange (*Citrus sp.*)

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A B S T R A C T

Studies had shown that oxygenated compounds were important in food products. It seems that Citrus species had a profound influence on this factor. The goal of the present study was to investigate on flavor components of two Citrus species. In the early week of June 2012, about 500 g of leaves were collected from many parts of the same trees. 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 and Duncan's multiple range tests. The amount of oxygenated compounds ranged from 29.14% to 85.64%. Between two species examined, sour orange showed the highest content of oxygenated compounds. As a result of our study, can be concluded that the species used can influence the quantity of oxygenated compounds present in the oil.

Introduction

The genus *Citrus*, belonging to the Rutaceae family, comprises of about 140 genera and 1,300 species. *Citrus sinensis* (sweet orange) and *Citrus aurantium* (sour orange) are two of the most important species of the genus *Citrus* (Kamal et al., 2011).

Citrus is one of the most economically important crops in Iran. The total Citrus production of Iran was estimated at around 87000 tonnes in the period 2009- 2010 (FAO, 2012). Shahsavari orange is a local cultivar of sweet orange that cultivated extensively in the Mazandaran province located in the north region of Iran (Zaare-Nahandi et al., 2008, Ebrahimzadeh et al., 2004). It has been regarded as a Citrus fruit with potential commercial value because of its attractive and pleasant aroma (Babazadeh, 2013).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 not been investigated previously.

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,

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perfumes, cosmetics, medicines and etc (Salem, 2003). In addition, recent studies have identified insecticidal, antimicrobial, antioxidative and antitumor properties for Citrus oils (Shahidi and Zhong , 2012).

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: rootstock (Babazadeh, 2011a), Citrus species (Minh-Tu et al., 2002; Kostadinovic et al., 2005), seasonal variation (Babazadeh et al., 2011b), organ (Babazadeh, 2011c), extraction method (Babazadeh, 2011d).

Branched aldehydes and alcohols are important flavor compounds extensively used in food products (Salem, 2003). Several studies have shown that oxygenated terpenoids such as linalool, neral, geranial (Baldwin, 2002), nonanal, decanal and linalool (Kostadinovic et al., 2005) are important in sweet orange flavor. The quality of a honey may be calculated from the amount of oxygenated components present in the honey (Alissandrakis et al., 2003; Alistair et al., 1993) and various 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 (Kite et al., 1991; Andrews et al., 2007).

In this paper, we compared the leaf components isolated from two different Citrus with the aim of determining whether the quantity of oxygenated compounds influenced by the species.

Materials and methods

Citrus 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 970 mm per year and average temperature16.25°C; soil was classified as loam-clay, pH range (6.9 to 7)]. Shahsavari orange and sour orange were used as plant materials in this experiment (Table 1).

Table 1. Common and botanical names for Citrus taxa used as scion and rootstock (Fotouhi and Fattahi, 2007).

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

Preparation of leaf sample

In the early week of June 2012, about 500 g of leaves were collected from many parts of the same trees, located in Ramsar Research Station, 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 (Lota et al., 2001; Babazadeh, 2011a).

GC and GC-MS

An Agilent 6890N gas chromatograph (USA) equipped with a DB-5 (30 m $\times$0.25 mm i.d ; film thickness = 0.25 μm) fused silica capillary column (J&W Scientific) and a flame ionization detector (FID) was used. The column temperature was programmed from 60 ^{o} 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 (Babazadeh, 2011d).

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 (Adams, 2001; McLafferty & Stauffer, 1991).

Data analysis

SPSS 18 was used for analysis of the data obtained from the experiments. Analysis of variations was based on the measurements of 6 leaf components. Variations between two Citrus species were analyzed using one-way analysis of variance. The correlation between pairs of components was evaluated using Pearson's correlation coefficient.

Results

Leaf compounds of the sweet orange and sour orange

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

Also, GC-MS analysis of the compounds extracted from sour orange leaf using water distillation allowed identification of 31 volatile components (Table 2): 12 oxygenated terpenes [2 aldehydes , 6 alcohols, 4 esters] and 19 non oxygenated terpenes [16 monoterpens, 3 sesqiterpens].

Table2. Leaf components of Citrus species

	Component	Sweet orange	Sour orange	KI	Component		Sweet orange	Sour orange	KI
1	α -thujene	*	*	925	**26**	(Z)-piperitol	*		1211
2	α - Pinene	*	*	933	**27**	Trans-carveol	*		1219
3	Sabinene	*	*	974	**28**	β -citronellol	*		1229
4	β - Pinene	*	*	978	**29**	Cis-carveol	*		1231
5	β -myrcene	*	*	989	**30**	Nerol	*	*	1233
6	δ - 3-carene	*		1023	**31**	Neral	*	*	1240
7	α -terpinene	*	*	1017	**32**	Geraniol	*	*	1255
8	p-cymene	*		1027	**33**	Linalyl acetate	*	*	1259
9	Limonene	*	*	1030	**34**	Geranial	*		1269
10	β -phellandrene	*	*	1032	**35**	α-Terpinyl acetate	*	*	1353
11	(Z)-β-ocimene	*	*	1035	**36**	Citronellyl acetate	*		1355
12	(E)-β-ocimene	*	*	1052	**37**	Neryl acetate	*	*	1365
13	γ- terpinene	*	*	1057	**38**	α -copaene	*		1380
14	Cis-sabinene hydrate	*	*	1068	**39**	Geranyl acetate	*	*	1384
15	(Z)- Linalool oxide	*	*	1071	**40**	β -elemene	*		1395
16	(E)- Linalool oxide	*	*	1085	**41**	(Z)-β-caryophyllene	*	*	1417

17	α- terpinolene	*	*	1088	**42**	(Z)- β -farnesene	*	*	1451
18	Linalool	*	*	1100	**43**	α -humulene	*		1461
19	Allo ocimene		*	1126	**44**	(Z)-a-bisabolene		*	1513
20	Citronellal	*	*	1155	**45**	Elemol	*		1556
21	Isopulegol	*		1162	**46**	(E)-nerolidol	*	*	1562
22	Terpinen-4-ol	*	*	1179	**47**	Caryophyllene oxide	*		1581
23	α - terpineol	*	*	1192	**48**	β -sinensal	*		1699
24	Myrtenol	*		1198	**49**	α - sinensal	*		1755
25	Decanal	*		1209			**47**	**31**	

*There is in oil

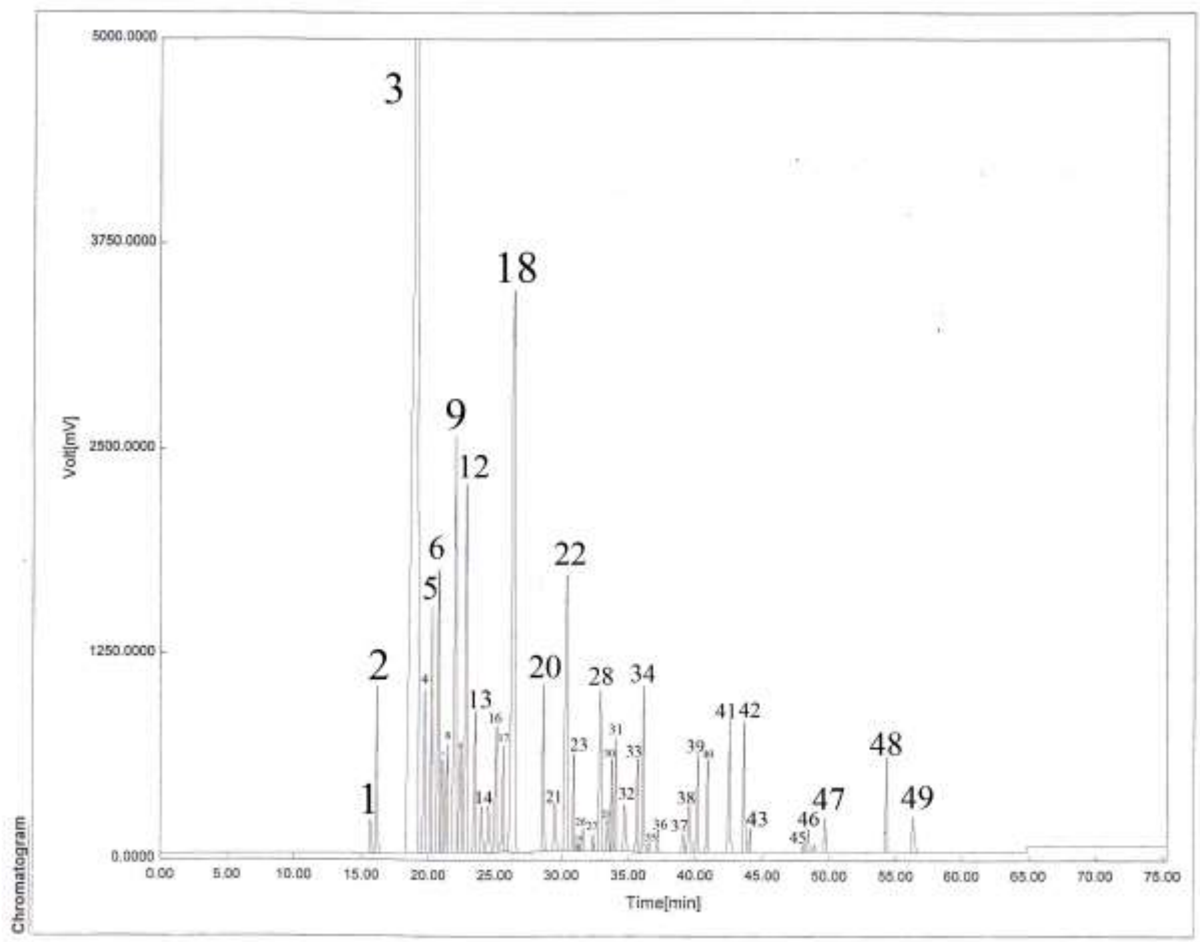

Figure 1. HRGC chromatograms of sweet orange leaf oil

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 0.09% to 5.83%. The concentrations of citronellal and geranial were higher in our samples. Citronellal has Citrus-like aroma (Buettner et al., 2003) and is considered as one of the major contributors to sweet orange flavor. Between two species examined, sweet orange showed the highest content of aldehydes (Table 3). Since the aldehyde content of Citrus oil was considered as one of the more important indicators of high quality, species apparently had a profound influence on this factor.

Sweet orange aldehydes were also compared to those of sour orange in this study. Decanal, geranial, β-sinensal and α-sinensal were identified in sweet orange, while they were not detected in sour orange. Amount of aldehydes in sweet orange was 64.77 times higher than sour orange (Table 3).

Alcohols

Thirteen alcohol components identified in this analysis were linalool, Isopulegol, terpinen-4-ol, α-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 21.30% to 46.81%. 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 (Buettner et al., 2003). Linalool has a flowery aroma (Buettner et al., 2003) and its level is important to the characteristic favor of Citrus. Between two species examined, sour orange showed the highest content of alcohols (Table 3).

Sweet orange alcohols were also compared to those of sour orange in this study. Isopulegol, Myrtenol, (z)-piperitol , trans-carveol, β-citronellol, cis-carveol, elemol was identified in sweet orange, while it was not detected in sour orange. Amount of alcohols in sour orange was 2.19 times higher than Sweet orange (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 2.01% to 38.74%. Between two species examined, sour orange showed the highest content of esters (Table 3).

Monoterpene hydrocarbons

The total amount of monoterpene hydrocarbons ranged from 11.51% to 62.36%. Sabinene was identified as the major component in this study and was the most abundant. Sabinene has a woody aroma (Sawamura et al., 2004) and is considered as one of the major contributors to Citrus flavor. Between two species examined, sweet orange showed the highest content of monoterpenes (Table 3).

Sesquiterpene hydrocarbons

The total amount of sesquiterpene hydrocarbons ranged from 0.45% to 4.10%. (Z)-β-caryophyllene and (Z)-β-farnesene were identified as the major component in this study and were the most abundant. Between two species examined, sweet orange showed the highest content of sesquiterpenes (Table, 3).

Results of statistical analyses

Statistically significant differences on the 1% level occurred in linalool, α-terpineol, linalyl acetate, geranyl acetate, sabinene and limonene. (Table 3).

Table 3. Statistical analysis of variation in leaf Components of Citrus species

Compounds	Sweet orange		Sour orange		
	Mean (%)	SE	Mean (%)	SE	F value
Oxygenated compounds					
a) Aldehyds					
1) Citronellal	1.81	0.16	0.04	0.006	
2) Decanal	0.10	0.03			
3) Neral	1.02	0.12	0.04	0.006	
4) Geranial	1.79	0.16			
5) β-sinensal	0.71	0.06			
6) α- sinensal	0.40	0.05			
total	5.83	0.58	0.09	0.01	
b) Alcohols					
1) Linalool	12.44	0.92	29.34	1.29	F**

Compounds	Sweet orange		Sour orange		
	Mean (%)	SE	Mean (%)	SE	F value
2) Isopulegol	0.43	0.05			
3) Terpinen-4-ol	3.55	0.40	0.14	0.02	
4) α-terpineol	0.69	0.06	9.55	0.30	F**
5) Myrtenol	0.08	0.006			
6) (Z)-piperitol	0.23	0.04			
7) (Z)-carveol	0.16	0.02			
8) β -citronellol	1.75	0.19			
9) (E)-carveol	0.37	0.06			
10) Nerol	0.71	0.08	1.95	0.21	
11) Geraniol	0.54	0.06	5.74	0.33	
12) Elemol	0.08	0.006			
13) (E)-nerolidol	0.26	0.04	0.09	0.01	
total	21.30	1.912	46.81	2.16	
d) Esteres					
1) Linalyl acetate	0.71	0.06	31.24	1.19	F**
2) α-terpinyl acetate	0.09	0.006	0.10	0.02	
3) Citronellyl acetate	0.23	0.03			
4) Neryl acetate	0.15	0.02	2.58	0.20	
5) Geranyl acetate	0.84	0.06	4.83	0.39	F**
total	2.01	0.17	38.74	1.79	
Monoterpenes					
1) α-thujene	0.42	0.05	0.02	0.00	
2) α-pinene	1.80	0.10	0.23	0.02	
3) Sabinene	33.00	2.00	0.47	0.03	F**
4) β-Pinene	1.75	0.11	3.10	0.20	
5) β-myrcene	2.66	0.19	2.29	0.18	
6) δ-3-carene	3.59	0.29			
7) α-terpinene	0.64	0.06	0.07	0.006	
8) p-cymene	0.92	0.08			
9) Limonene	6.50	0.50	0.82	0.07	F**
10) β-phellandrene	0.01	0.00	0.08	0.01	
11) (Z)-β-ocimene	0.58	0.50	0.99	0.08	
12) (E)-β-ocimene	3.91	3.42	2.70	0.18	
13) γ-terpinene	0.87	0.76	0.09	0.006	
14) Cis-sabinene hydrate	0.30	0.26	0.02	0.00	
15) (Z)-Linalool oxide	0.29	0.25	0.05	0.006	
16) (E)-Linalool oxide	0.77	0.67	0.03	0.00	
17) α-terpinolene	0.60	0.53	0.51	0.05	
18) Allo ocimene			0.03	0.006	
total	62.36	4.53	11.51	0.83	
Sesquiterpenes					
1) α-copaene	0.42	0.04			
2) β-elemene	0.68	0.05			

Compounds	Sweet orange		Sour orange		
	Mean (%)	SE	Mean (%)	SE	F value
3) (Z)-β-caryophyllene	1.25	0.13	0.23	0.02	
4) (Z)-β-farnesene	1.24	0.15	0.04	0.006	
5) α-humulene	0.19	0.02			
6) (Z)-a-bisabolene			0.17	0.02	
7) Caryophyllene oxide	0.32	0.04			
total	4.10	0.42	0.45	0.05	
Total oxygenated compounds	29.14	2.662	85.64	3.96	
Total	95.6	7.612	97.6	4.84	

Results of correlation

Correlations coefficients between 6 components were presented in a correlation matrix (Table 4). Not only linalool and α-terpineol showed a high positive correlation with each other but also they showed a high positive correlation with linalyl acetate and geranyl acetate.

Sabinene showed a high negative correlation with linalool, α-terpineol, linalyl acetate and geranyl acetate. Limonene also showed a high negative correlation with α-terpineol and linalyl acetate.

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

	Linalool	α-terpineol	Linalyl acetate	Geranyl acetate	Sabinene
α-terpineol	0.99**				
Linalyl acetate	0.99**	0.99**			
Geranyl acetate	0.99**	0.99**	0.98**		
Sabinene	-0.99**	-0.99**	-0.99**	-0.99**	
Limonene	-0.98**	-0.99**	-0.99**	-0.98**	0.98**

***=significantat 0.05**

****=significant at 0.01**

Discussion

Our observation that different species had an effect on some of the components of Citrus oil was in accordance with previous findings (Minh-Tu et al., 2002; Kostadinovic et al., 2005). The compositions of the leaf oils obtained from different species of Citrus were very similar. However, the relative concentration of compounds was differed according to the type of species.

Comparison of our data with those in the literatures revealed some inconsistencies with previous studies (Lota et al., 2001; Baalioumer et al., 1988). It may be related to cultivar, rootstock and environmental factors that can influence 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 (Kesterson et al., 1974). 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 (Hay and Waterman, 1995). The pronounced enhancement in the amount of oxygenated compounds, when sour orange was used as the plant material, showed that either the synthesis of geranyl pyrophosphate was enhanced or activities of both enzymes increased.

High positive correlations between pairs of terpenes suggest a genetic control (Scora et al., 1976)] and such correlation between pairs of terpenes was due to derivation of one from another that was not known. Similarly, high negative correlations between pairs of terpenes indicated 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 by sour orange.

Conclusion

In the present study we found that the amounts of leaf compositions were significantly affected by species and there was a great variation in most of the measured characters between two species. The present study demonstrated that volatile compounds in leaf can vary when different species are utilized. Between two species examined, sour orange showed the highest content of oxygenated compounds. Studies like this is very important to determine the amount of chemical compositions existing in the species that we want to use, before their leaves can be utilized in food industries, aromatherapy, pharmacy, cosmetics, hygienic products and other areas. Further research on the relationship between species and essential oil (oxygenated terpenes) is necessary.

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