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Identification and Aroma Impact of Norisoprenoids in Orange Juice

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Four norisoprenoids, α-ionone, β-ionone, β-cyclocitral, and β-damascenone, along with their putative carotenoid precursors, were identified in Valencia orange juice using time-intensity GC-O, GC-MS, and photodiode array HPLC. α-Ionone and β-cyclocitral are reported in orange juice for the first time. GC-O aroma peaks were categorized into seven groups with similar sensory qualities: citrus/minty, metallic/mushroom/geranium, roasted/cooked/meaty/spice, fatty/soapy/green, sulfury/solventy/medicine, floral, and sweet fruity. The four norisoprenoids contributed approximately 8% of the total aroma intensity and 78% of the total floral aroma category. The putative carotenoid norisoprenoid precursors, α- and β-carotene, α- and β-cryptoxanthin, and neoxanthin, were identified in the same orange juice using photodiode array HPLC retention times and spectral characteristics.

KEYWORDS: ***SPME; GC-O; HPLC; MS; carotenoid degradation; citrus; aroma activity***

INTRODUCTION

Orange juice lacks a specific character impact compound. Its aroma is the result of a complex mixture of volatiles blended in specific proportions. Numerous studies (*1*−*3*) have identified and quantified the major volatiles in orange juice in an effort to duplicate this aroma. However, when combined, the volatiles present at the highest concentrations do not duplicate orange juice aroma. Early orange juice GC-O studies (*4*−*6*) have shown that many aroma-active compounds in orange juice are potent low-level volatiles that are difficult to detect using typical FID or MS detectors. Conversely, the major volatiles in orange juice have little to no aroma activity.

Norisoprenoids are volatile C_9−C_{13} fragments from the degradation of C_{40} carotenoids, which have extremely low aroma thresholds. They can be formed as a result of in vivo enzymatic degradation or postharvest thermal degradation of foods containing carotenoids. Free norisoprenoids have also been observed from the release of glycosidically bound norisoprenoids in wine (*7*). β-Ionone has been reported in a few studies (*5*, *8*), and β-damascenone has been recently reported in orange juice using SNIF GC-O (*9*). Norisoprenoids have significant aroma impact in other fruits such as grapes (*10*), apples (*11*), lychee (*12*) starfruit (*13*), and mango (*14*).

Carotenoids are widely distributed in the plant kingdom, and orange juice is a particular rich and complex source of these compounds (*15*). Known carotenoid norisoprenoid precursors such as β-carotene, α-carotene, neoxanthin, β-cryptoxanthin, lutein, violaxanthin, and canthaxanthine (*16*−*20*) have been identified in orange juice (*21*) using HPLC. Since orange juice contains many known carotenoid precursors for a wide range of norisopernoids, the purpose of this study was to determine if aroma-active norisoprenoids other than β-ionone were present in fresh orange juice and determine relative aroma impact of these compounds.

MATERIALS AND METHODS

Orange Juice Sample. Approximately 41 kg of late season Valencia oranges were obtained from Haines City Citrus Growers Association, Haines City, FL. The oranges were juiced using a commercial FMC juice extractor model 291 with standard juice settings. As in commercial practice, juices went through an FMC model 035 juice finisher (0.02 in. screen with typical screw press pressure). The freshly squeezed juice was immediately chilled to 4 °C, and NaCl (36 g/100 mL of juice) was added to inhibit enzymatic reactions. The juice had a Brix value of 11.7°, an acid content of 0.67% citric acid, a Brix/acid ratio of 17.5, and an oil level of 0.0196%.

Chemicals. Standard aroma compounds were obtained from the sources previously reported (*22*). Their identities were confirmed by mass spectra, retention indices, and odor qualities. β-Damascenone and

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p-1-menthene-8-thiol were obtained from Givaudan (Lakeland, FL). β-Ionone and β-cyclocitral were purchased from Aldrich (Milwaukee, WI). α-Ionone was obtained as a gift from Danisco (Lakeland, FL). 4-Mercapto-4-methyl-2-pentanone and 4-mercapto-4-methyl-2-pentanol were synthesized in our laboratory. 3-Mercaptohexan-1-ol was bought from Interchim (Montlucon, France).

Headspace Sampling. A 10 mL aliquot of orange juice was added to a 40 mL screw cap glass vial with Teflon-coated septa containing a micro stirring bar. The bottle and contents were placed in a combination water bath and stirring plate set at 40 °C. A SPME fiber (50/30 μm DVB/Carboxen/PDMS on a 2 cm StableFlex fiber, Supelco, Bellefonte, PA) was inserted into the headspace of the sample bottle and exposed for 45 min. Subsequently, the fiber was thermally desorbed in the GC injector port for 5 min (220 °C).

Gas Chromatography. FID/Olfactometer. Separation was accomplished with a HP-5890 GC (Palo Alto, CA) using either a DB-wax column (30 m × 0.32 mm. i.d. × 0.5 μm, J&W Scientific; Folsom, CA) or Zebron ZB-5 column (30 m × 0.32 mm. i.d. × 0.5 μm, Phenomenex, Torrance, CA). Column oven temperature (for DB-wax) was programmed from 40 to 240 °C (but 40 to 265 °C for ZB-5) at 7 °C/min with a 5 min hold. Helium was used as carrier gas at flow rate of 1.55 mL/min. Injector and detector temperature were 220 °C and 290 °C, respectively. A small diameter injection port liner of 0.75 mm was employed to improve peak shape and chromatographic efficiency. The separation was conducted in the splitless mode. The GC effluent was split between a FID and olfactometer as previously described (*4*). A time-intensity approach was used to evaluate odor quality and intensity at the sniffing port under GC conditions. Assessors rated aroma intensity continuously throughout the chromatographic separation process using a linear potentiometer whose output was recorded and quantified using chromatography software. Retention times and verbal descriptors were recorded to permit aroma descriptors to be coupled with computerized aroma time-intensity plots. Two trained assessors evaluated the sample in duplicate, thus producing four individual time-intensity aromagrams. Intensities of aroma-active compounds of each GC-O run were normalized so the highest intensity was given a score of 10. The normalized intensities of all the runs were then averaged, providing a similar aroma activity detected at least half the time at that retention time. If the compound was not detected in one run its value was treated as missing, not zero. Average intensity from the four runs was calculated for each odorant detected. An averaged time-intensity aromagram was constructed by plotting average intensity versus retention time. Chromatograms and aromagrams were recorded and integrated using Chromperfect version 5.0, Justice Laboratory Software (Palo Alto, CA). Identification of the aroma-active components was based on the combination of sensory descriptors, standardized retention indices, and identification confirmed by comparison with standards and GC-MS.

Mass Spectrometry. GC-MS was employed to confirm the identities of the aroma-active volatiles identified in the GC-O experiments. SPME volatiles were separated and analyzed using a Finnigan GCQ ion trap mass spectrometer (Finnigan, Palo Alto, CA) equipped with a DB-5, 60 m × 0.25 mm I.D., capillary column (J&W Scientific, Folsom, CA). Injector and transfer line temperatures were 200 and 250 °C, respectively. Helium was used as the carrier gas at 1 mL/min. The oven temperature program consisted of a single thermal gradient from 40 to 275 °C at 7 °C/min. The MS was set to scan from mass 40 to 300 at 2.0 scans/s in the positive ion, electron impact mode. The ionization energy was 70 eV. Chromatographic peaks were identified using NIST 98 and Wiley, sixth edition, databases. Only those compounds with spectral fit values equal to or greater than 800 were considered positive identifications. Final identification was based on the combination of spectral matches and standardized alkane retention index values (Kovat's Index) and aroma characteristics. Standards were used to confirmation identification, by comparing the resulting fragmentation pattern, retention index value, and aroma descriptor (*23*).

Carotenoids Extraction and HPLC. Carotenoids in orange juice were extracted according to Lee et al. (*24*). HPLC was employed to determine carotenoids in Valencia orange juice which could serve as norisoprenoid precursors. Carotenoid pigments were analyzed according to Rouseff et al. (*21*) by reverse phase HPLC using ternary gradient of water, methanol, and methyl *tert*-butyl ether (MTBE) with photodiode array detection (PDA).

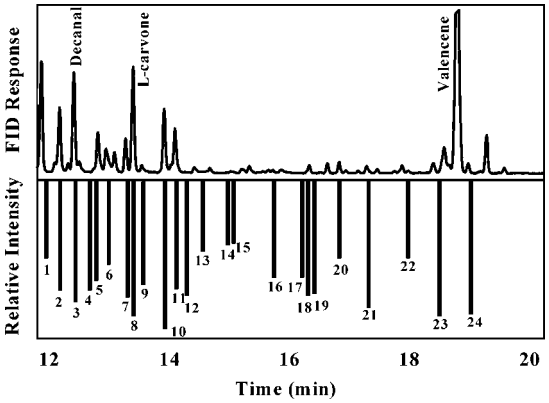

Figure 1. GC–FID (top) and average time-intensity of four GC-O runs by two panelists (inverted, bottom) of fresh orange juice on ZB-5 column. Peaks 5, 19, 21, and 23 correspond to nonisoprenoids, all numbers refers to compounds in **Table 1**.

RESULTS AND DISCUSSION

Extraction of Juice Norisoprenoids Using SPME. Solid-phase microextraction was used to extract and concentrate orange juice volatiles because it was a solventless headspace technique that did not co-extract juice carotenoids. Ethyl acetate and pentane−ether liquid−liquid juice extractions were examined as a potential sample preparation approaches but were abandoned after it became apparent that they coextracted juice carotenoids. Coextracted carotenoids could degrade in the GC's injector (200 °C), possibly introducing thermally generated artifact norisoprenoids. Since carotenoids are essentially nonvolatile and would not be found in the headspace, the possibility of producing artifact norisoprenoids from coextracted carotenoids is eliminated using headspace sampling.

GC-Olfactometry. A total of 59 aroma-active components were detected using SPME headspace sampling of fresh orange juice. Since the primary goal of this study was to determine if additional aroma-active norisoprenoids were present in orange juice, GC-O was concentrated primarily in the region where β-ionone and other norisoprenoid standards elute. Using standards of β-cyclocitral, α-ionone, β-ionone, and β-damascenone, the retention time region was established between 12 and 20 min, and the resulting aromagram and concurrent chromatograms are shown in **Figure 1**.

As noted in **Figure 1**, four aroma peaks corresponding to peaks 5, 19, 21, and 23 were observed at the identical retention times as β-cyclocitral, β-damascenone, α-ionone, and β-ionone, respectively. It is also apparent from the relative intensities shown in **Table 1** that these potential norisoprenoid peaks were among the more intense aromas in this region. β-Ionone was the most intense, and β-cyclocitral was the weakest aroma compound of the four potential norisoprenoids observed. When the samples were rerun on a wax column, the four aroma peaks also were found at retention index values that corresponded with the four potential norisoprenoids. Furthermore, the aroma quality of each juice norisoprenoid corresponded exactly with the aroma description of standards.

MS Norisoprenoid Identifications. Headspace volatiles from orange juice were extracted using SPME and analyzed with capillary GC and an ion trap mass spectrometer. To achieve greater selectivity for the norisoprenoids of interest, selected ion chromatograms were reconstructed in the retention region

Table 1. Identification, Retention Characteristics and Aroma Descriptors of Aroma-Active Compounds in Fresh Orange Juice Observed in the General Region Where Norisoprenoids Elute

no.	compound	aroma descriptor	linear retention ZB-5	linear retention DB-wax	relative [c]intensity
1	terpinen-4-ol[b]	metallic, musty	1175	1619	4.65
2	(*Z*)-4-decenal[a]	green, metallic, soapy	1188	1542	6.75
3	decanal[b]	green, soapy	1198	1508	7.35
4	(*E*,*E*)-2,4-nonadienal[a]	fatty, green	1209	1702	6.66
5	β-cyclocitral[b]	mild floral, sweet, hay-like	1214	1632	6.13
6	nerol[a]	lemongrass	1222	1798	5.11
7	neral[b]	lemongrass	1236	1692	7.06
8	L-carvone[b]	minty	1242	1747	8.19
9	unknown	metallic/ woody	1247		6.27
10	geraniol[a]	citrus, geranium	1265	1853	9.02
11	unknown	soapy, almond	1274		6.57
12	1-*p*-menthene-8-thiol[a]	grapefruit	1281	1619	7.02
13	(E,*Z*)-2,4-decadienal[a]	metallic, geranium	1293	1759	4.32
14	geranial[a]	green, minty	1310	1742	3.90
15	(*E*,*E*)–2,4-decadienal[a]	fatty, green	1314	1819	3.84
16	α-terpinyl-acetate[a]	sweet	1349	1663	5.86
17	4,5-epoxy-(*E*)-2-decenal[a]	metallic, fatty	1375	2010	6.04
18	unknown	sweet nutty	1380		6.98
19	β-damascenone[b]	tobacco, apple, floral	1383	1829	6.86
20	dodecanal[a]	soapy	1403	1722	5.54
21	α-ionone[b]	floral	1426	1863	7.71
22	unknown[a]	fermented, rancid butter	1459		4.72
23	β-ionone[b]	floral, raspberry	1484	1951	8.18
24	unknown	nutty	1510		8.11

[a] Identified by linear retention index on ZB-5 and/or DB-wax, aroma description as compared with standard. [b] Identified by linear retention index on ZB-5 and/or DB-wax, aroma description as compared with standard, and MS. [c] Averages of normalized intensities evaluated by two trained panelists in four replications.

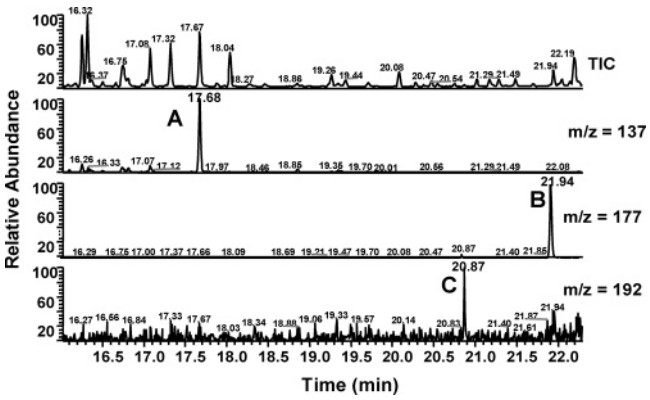

Figure 2. Comparison between total ion chromatograms and selected ion chromatogram (SIC) A: β-cyclocitral (m/z = 137), B: β-ionone (m/z = 177), C: α-ionone (m/z = 192).

where norisoprenoid standards were found to elute. The selectivity achieved is demonstrated in **Figure 2**. Specific m/z values were evaluated to provide the best peak height for each norisoprenoid of interest as well as minimizing interference from nonnorisoprenoid components as well as noise. The following ions were monitored for the specific norisoprenoids: β-cyclocitral, m/z = 137 and 152; β-damascenone, m/z = 175 and 190; α-ionone, m/z = 177 and 192; β-ionone, m/z = 177 and 192. Although only a single ion has been shown for each norisoprenoid, two or more selective ions were employed to detect the presence of specific norisoprenoids. For example, the selected ion chromatogram using m/z 137 was more intense than that from m/z = 152 but not as specific for β-cyclocitral. The selected ions of m/z = 177, 192 were extracted for the determination of α-ionone and β-ionone. Selected ion chromatograms at m/z 177 provided excellent signal strength and selectivity for β-ionone but little signal for α-ionone. This latter norisoprenoid was obviously present at much lower concentrations than β-ionone and needs a more selective ion which was achieved with m/z = 192 (**Figure 2**). Fragments with m/z = 175 and 190 were not detected for β-damascenone. This norisoprenoid also has a major peak at m/z = 121, but this ion is shared with many terpenes which are ubiquitous in citrus samples. β-Damascenone was detected by GC-O but not by FID nor ion-trap MS, demonstrating for β-damascenone that the human nose is more sensitive than the instruments used in this study (odor threshold 0.002 ppb). The ion trap mass spectral data for β-cyclocitral, α-ionone, and β-ionone were as follows: β-cyclocitral; 41 (100), 137 (55), 79 (45), 83 (30), 109 (28), 67 (25), 123 (21), 81 (16), 94 (15), 119 (14), 152 M^+(7), α-ionone; 121 (100), 93 (90), 177 (67), 91 (64), 192 M^+(29), 77 (24), 109 (22), 136 (21), 92 (19), 159 (12), β-ionone; 177 (100), 105 (20), 133 (19), 91(14), 189 (14), 178 (14), 161 (10), 107 (9), 147 (9), 119 (9), 192 M^+ (0.32).

Table 2. Relative Intensities of Aroma Groups Using Time-Intensity GC-O

1. citrus/minty	25
2. metallic/mushroom/geranium	17
3. roasted/cooked/meaty/spice	13
4. fatty/soapy/green	17
5. sulfury/solventy/medicine	10
6. floral	10
7. sweet fruity	8
total	100

Relative Aroma Impact of Norisoprenoids. Many of the 59 aroma-active compounds detected in fresh orange juice had similar aroma qualities. Therefore, they were categorized according to the similarity of their aroma descriptors into seven groups: (1) citrus/minty (1,8 cineole, nonanal, 3-mercaptohexan-1-ol, citronellal, nerol, neral, L-carvone, geraniol, 1-*p*-menthene-8-thiol, geranial, nootkatone, and unknown at LRI 963 of ZB-5 column), (2) metallic/mushroom/geranium (1-octen-3-one, β-myrcene, octanal, terpinolene, (*Z*)-2-nonenal, terpinen-4-ol, (*E*,*Z*)-2,4-decadienal, 4,5-epoxy-(*E*)-2-decenal, β-sinensal, and unknown at LRI 1247 and 1589 of ZB-5 column), (3) roasted/cooked/meaty/spice (methional, 2-acetyl-2-thiazoline, unknown at LRI 1380, 1459, 1510, and 1718 of ZB-5 column), (4) fatty/soapy/green (hexanal, 1-octanol, (*E*)-2-nonenal, (*Z*)-4-decenal, decanal, (E,E)-2,4-nonadienal, (*E*,*E*)-2,4-decadienal, dodecanal, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, (E,Z)-2,6-nonadienal, and unknown at LRI 1274 on a ZB-5 column), (5) sulfury/solventy/medicine (acetaldehyde, carbon disulfide, dimethyl sulfide, dimethyl disulfide, 2-methyl-3-furanthiol, 4-mercapto-4-methyl-2-pentanone, dimethyl trisulfide, 4-mercapto-4-methyl-2-pentanol, and unknown at LRI 818 on a ZB-5 column), (6) floral (linalool, β-cyclocitral, β-damascenone, α-ionone, and β-ionone), (7) sweet fruity (ethyl-2-methylpropanoate, ethyl butyrate, ethyl-2-methylbutyrate, ethyl hexanoate, and α-terpinyl acetate).

All norisoprenoids in this study are in group 6 (floral) which possess a floral note at their juice concentrations. Linalool was also in this category. Linalool and β-ionone are generally described as floral and violet-like and have been reported as among the more odor-active volatiles in freshly squeezed Valencia orange juice (*5*). The additional three aroma-active norisoprenoids are slightly weaker contributors to floral note of orange juice. The sums of the total olfactometry intensities was determined and expressed as 100%, and the relative intensities of the seven aroma groups with similar GC-O descriptors are summarized in **Table 2**. On the basis of peak intensities of the GC-O analysis, the total floral group contributes approximately 10% of the total orange juice aroma. Linalool

Table 3. HPLC Retention Times from C-30 Carotenoid Column with a Ternary Gradient Solvent System and Photodiode Array Spectral Characteristics of Orange Juice Carotenoids. The Three Peaks Correspond to the Three Absorbance Maxima from Each Carotenoid

			observed (nm)			literature (nm)			
peak no.	carotenoid	t_R[a] (min)	peak 1	peak 2	peak 3	peak 1	peak 2	peak 3	ref[b]
1	valenciaxanthin	5.52	351	369	390	351	369	390	E
2		6.00	371	391	414				
3		6.92	420	435	465				
4	neoxanthin	7.35	416	438	468	415	439	467	A
5		11.60	410	431	454				
6		12.95	416	438	467				
7	neochrome	14.68	400	422	448	399.5	421.5	447.5	B
8		15.55	408	429	415				
9		15.90	383	402	425				
10		16.83	411	430	462				
11	*cis*-violaxanthin	17.60	415	437	464	414	437	464	C
12	leutoxanthin	19.07	399	418	443	399.5	419.5	441.5	B
13	mutatoxanthin	20.08	405	429	451	404	427	452	D
14	leutein	20.92	420	445	471	424.5	445.5	471.5	B
15	zeaxanthin	23.80	425	450	476	425	450	478	A
16	isolutein	24.70	418	441	468	418	439.5	467.5	B
17		26.40	429	445	469				
18	α-cryptoxanthin	28.15	420	445	472	420	444	472	D
19	phytofluene	28.83	331	348	367	331	348	367	A
20	β-cryptoxanthin	31.57	425	451	477	425	449	476	A
21	β-carotene, 5,8:5′,8′-diepoxy	34.30	380	400	424	380	400	425	
22	α-carotene	36.33	420	446	472	420	445	472	D
23	ζ-carotene	39.28	379	401	425	378	400	425	A
24	β-carotene	39.77	425	451	477	425	450	478	A

[a] t_R = retention time. [b] A = (*33*); B = (*21*); C = (*34*); D = (*35*); E = (*36*).

contributes 2.2%, α-ionone contributes 2.1%, β-ionone contributes 2.2%, β-cyclocitral contributes 1.7%, and β-damescenone contributes 1.9%. Therefore, the four norisoprenoids contribute about 78% of the total floral group intensity and are the primary contributors to the floral note in orange juice.

Carotenoid Norisoprenoid Precursors. Although over 30 carotenoids have been identified in orange juice, only a few possess the structural requirements to produce the four norisoprenoids observed in this study. Carotenoids with the correct structural features to act as norisoprenoid precursors include: α- and β-carotene, α- and β-cryptoxanthin, and neoxanthin. The same norisoprenoids observed in this study have also been reported in other food systems containing these carotenoids (*25*−*31*). β-Cyclocitral can be formed from oxidative cleavage of the double bond between carbons 7 and 8 of α- and β-carotene and carbons 7′ and 8′ of β-cryptoxanthin. In similar fashion, β-ionone can be formed from the same carotenoids by oxidative cleavage of the double bond between carbons 9 and 10 and between carbons 9′ and 10′ for β-carotene and β-cryptoxanthin. Although there are several possible precursors for β-ionone, α-ionone can only be directly formed from the oxidative cleavage of the double bond between carbons 9′ and 10′ of α-carotene and α-cryptoxanthin. The final norisoprenoid of interest, β-damascenone, cannot be directly formed from any carotenoid found in citrus. Rather it is produced from neoxanthin, in a well documented three step-process (*32*).

HPLC Carotenoid Identification. The potential precursors of the four norisoprenoids observed in this study were detected in the identical Valencia orange juice used in this norisoprenoid study. Their presence is indicated by the data in the shaded rows shown in **Table 3**.

As shown in **Table 3**, the absorbance maxima observed exactly matched those published in the literature or differed at most by 2 nm as in the case of the central peak for β-cryptoxanthin. Since the wavelength accuracy of most photodiode array detectors is only ±1 nm, the agreement is excellent. Since the carotenoids of interest have the same elution and spectral characteristics as α-, β-carotene, α-, β-cryptoxanthin, and neoxanthin, it is reconfirmed that they are present in orange juice and can serve as precursors to the norisoprenoids observed in this study.

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