

Determination of Key Flavor Components in Methylene Chloride Extracts from Processed Grapefruit Juice

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The relative correlation of 52 aroma and 5 taste components in commercial not-from-concentrate grapefruit juices with flavor panel preference was determined. Methylene chloride extracts of juice were analyzed using GC/MS with a DB-5 column. Nonvolatiles determined included limonin and naringin by HPLC, °Brix, total acids, and °Brix/acid ratio. Juice samples were classified into low, medium, or high categories, based on average taste panel preference scores (nine-point hedonic scale). Principal component analysis demonstrated that highest quality juices were tightly clustered. Discriminant analysis indicated that 82% of the samples could be identified in the correct preference category using only myrcene, β -caryophyllene, linalool, nootkatone, and °Brix. Nootkatone alone was not strongly associated with preference scores. The most preferred juices were strongly associated with low myrcene, low linalool, and intermediate levels of β -caryophyllene.

Keywords: *Flavor; grapefruit juice; principal component analysis; canonical discriminant analysis; sensory analysis*

INTRODUCTION

Citrus juices are popular due to their desirable flavor and perceived health benefits. The color, taste, and aroma quality of citrus juices have a pronounced influence on consumer preferences and purchase decisions. Considerable effort has been expended toward the isolation, identification, and quantitation of compounds that were thought to contribute to the taste of grapefruit juice (Attaway, 1977; Rouseff et al., 1980; Fellers et al., 1986). These studies found that an inverse relationship exists between bitterness and preference.

Extensive research has been conducted to identify and quantify volatile components in grapefruit products (Moshonas et al., 1971; Núñez et al., 1985; Cadwallader, 1994), but few workers have evaluated the relative sensory significance of these compounds. A total of 126 volatile constituents were identified in grapefruit by Demole et al. (1982). Nootkatone was suggested as the key flavor impact compound of grapefruit as early as 1970 (Stevens et al., 1970). However, Shaw and Wilson (1981) found that nootkatone added to oil and juice had a significant flavor impact in oil but little impact in the juice. They concluded that there must be other components which affect the flavor of grapefruit juice. Subsequently, 1-*p*-menthene-8-thiol was reported as a key aroma impact compound in grapefruit juice. It is one of the most potent flavor compounds found in nature, with an aqueous threshold of 1×10^{-7} ppm (Demole et al., 1982). However, until an analytical procedure is developed to quantify this compound at the

levels at which it exists in juice, it will not be possible to evaluate its relative contribution to grapefruit juice flavor.

Flavor is unquestionably one of the most important attributes of the food we eat. It is perceived as taste by the tongue and mouth and also through the release of the volatile components in the mouth, which are sensed retronasally by the olfactory epithelium in the nose (Ohloff, 1990). Previous workers have developed flavor models based on the correlations between quantified volatiles and sensory evaluators (Jennings et al., 1977; Pino et al., 1986a,b). Other workers, using primarily nonvolatile measurements, have evaluated the relationships between these measurements and sensory scores using multiple regression (Attaway, 1972) or principal component analysis (PCA) (Rouseff and Nagy, 1982). Multivariate statistical procedures such as PCA can reveal underlying relationships that exist between variables (Chien and Peppard, 1992). Using multiple linear regression, Pino (1982) concluded that volatiles such as limonene, α -terpineol, linalool, and myrcene were most relevant in explaining grapefruit juice sensory differences. Velez et al. (1993) used GC data with PCA to classify orange juices stored at different times and temperatures. Butanol, α -terpineol, and furfural correlated strongly with increasing storage temperatures, while linalool and terpinen-4-ol correlated best with increasing storage time.

To our knowledge, no systematic study to determine the relative flavor impact between both aroma and taste compounds in grapefruit juice has been reported. Pino et al. (1986b) used canonical and cluster analysis on grapefruit juice volatile measurements to classify 24 commercial single-strength grapefruit juices from different production days and storage conditions. They observed that nootkatone and an unknown component correlated positively with flavor preference, while an-

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other unidentified component correlated negatively. In another paper, Pino et al. (1986a) correlated sensory and GC measurements from grapefruit juice extracts using multiple linear regression. Methyl butyrate, ethyl butyrate, limonene, decanal, and nootkatone correlated with pleasant flavor, while *trans*- and *cis*-epoxydihydrolinalool and α -terpineol correlated with unpleasantness of grapefruit juice.

Our primary objective in this study was to develop a flavor model based on both volatile and nonvolatile components in processed grapefruit juice that could be used to predict sensory preference. A secondary objective was to determine the relative contribution of volatile and nonvolatile compounds to the flavor (preference) model.

MATERIALS AND METHODS

Materials. Twenty-nine not-from-concentrate (NFC) grapefruit juice samples were obtained from processors with processing dates ranging from November 1995 to June 1996 and stored at -8°C until analyzed. Both red/pink and white samples were used in this study. Authentic standards and solvents were purchased from Fisher Scientific (Pittsburgh, PA).

Extraction of Volatiles. Extraction of volatiles was accomplished with methylene chloride using the method described by Parliament (1986) and modified by Klim and Nagy (1992). Eight milliliters of juice was added to 4 mL of methylene chloride and mixed using a Mixxor-like apparatus. The emulsion was broken by centrifuging for 10 min (15000*g*). The lower solvent layer of ≈ 3 mL was collected for analysis. An internal standard, 6 μL of 100 ppm propyl benzene, was added, and the extract was concentrated to ~ 30 μL . The concentrated extract was immediately injected into the GC. Each juice sample was extracted twice and each extract analyzed in duplicate.

Gas Chromatographic Analysis. The individual volatile constituents were separated using a HP-5890 GC (Palo Alto, CA) with a flame ionization detector and a 30 m \times 0.25 mm i.d. \times 0.5 μm film thickness low-bleed DB-5 column (J&W Scientific, Folsom, CA). The oven temperature was programmed from 35 to 275 $^{\circ}\text{C}$ at 6 $^{\circ}\text{C}/\text{min}$ with helium at a flow rate of 1.55 mL/min. Injector temperature was maintained at 250 $^{\circ}\text{C}$ and detector temperature at 320 $^{\circ}\text{C}$. Nitrogen gas was maintained at 19 mL/min, while air and hydrogen were maintained at 296 and 35 mL/min, respectively. Extract injection volume was 1 μL . Injection was splitless. Chromatograms were recorded and integrated using an APEX Chromatography Workstation (Autochrom Inc., Milford, MA) with a four-channel data system. The data acquisition rate was 0.4 s/point.

GC/MS Conditions. All GC/MS data were collected using a Finnigan GCQ Plus system (Finnigan Corp., San Jose, CA) using helium (99.999%) for the GC carrier gas and the collision/bath gas in the ion trap. Injector temperature was 250 $^{\circ}\text{C}$. Samples (0.2–1.0 μL) were injected using the splitless mode with a purge time of 1.5 min. The initial column temperature was held at 35 $^{\circ}\text{C}$ for 3 min followed by a 4 $^{\circ}\text{C}/\text{min}$ temperature ramp to 221 $^{\circ}\text{C}$, which was followed by a 10 $^{\circ}\text{C}/\text{min}$ ramp to 275 $^{\circ}\text{C}$, which was held for 1.1 min to elute high-boiling components in extracts. Flow rate was 31.9 cm/s through a 30 m \times 0.25 mm i.d., 0.25 μm RTX5-MS column (Restek Corp., Bellefonte, PA). Transfer line and ion source temperatures were 275 and 170 $^{\circ}\text{C}$, respectively. The mass spectrometer had a delay of 4 min to avoid the solvent peak and then scanned from *m/z* 40 to *m/z* 300 to achieve 7 spectra/s. Ionization energy was set at 70 eV.

Peak Identification. Peaks from the chromatograms were identified using mass spectra and Kovats retention indices (Kovats, 1965). Calculation of the retention indices for individual peaks was done using retention time data from a series of alkane standards run under the same conditions. Peak

areas that were not reproducible or well resolved were not included in the data set.

Nonvolatile Analysis Using High-Pressure Liquid Chromatography (HPLC). *Sample Preparation.* Limonin and naringin were analyzed according to the method developed by Widmer et al. (1994). In a 10 mL flask, 5 mL of juice was measured and equilibrated for 5 min at 90 $^{\circ}\text{C}$. The sample was diluted to 10 mL with 40% acetonitrile and filtered through a Whatman GDX 0.45 μm filter. About 2 mL of filtered sample was placed into a glass vial, which was sealed, and used for further analysis.

HPLC Instrumentation. A Thermo Separations (San Jose, CA) LC system (Spectra Focus Optical Scanning detector and P4000 gradient pump) with a Spectra Physics AS 3000 (San Jose, CA) autosampler was used for the analysis of limonin. A Waters 6000A pump (Milford, MA) with a Waters 440 two-channel UV absorbance detector equipped with a 280 nm filter was used to determine naringin. Chromatograms were recorded and integrated with a Thermo Separations 4290 integrator and Winner on Windows 4290. Separations were achieved using a 4.6 mm \times 150 mm, 5 μm , CN analytical column (MacMod Analytical Inc., Chadds Ford, PA) for limonin and a 4.6 mm \times 150 mm, 5 μm , C₁₈ analytical column (Kromasil C₁₈, Higgins Analytical, Mountain View, CA) for naringin. The mobile phase consisted of water/acetonitrile (80.5:19.5) for naringin analysis and water/acetonitrile (63:37) for limonin analysis. Injection volume was 40 μL , and flow rates of 1.0 mL/min were used.

Sensory Analysis. Taste threshold characteristics of individual taste panelists were determined using 5–50 ppm of limonin and 150–950 ppm of naringin solutions. Twenty-four untrained panelists were used. A nine-point hedonic scale was used with 0 indicating dislike extremely, 9 indicating like extremely, and 5 indicating neither like nor dislike. Panelists were presented with three samples under illumination with red light and asked to rate their preference. Samples were coded with random three-digit numbers randomly arranged on serving trays and then presented to panelists. For comparison purposes all juices were ranked on the basis of average hedonic preference score and divided into three approximately equal categories. There were 10 juices in the "low" category. Average hedonic scores were ≤ 4.75 . There were 9 juices in the "medium" category. They had preference scores between 4.75 and 5.75. The 10 juices in the highly preferred category were rated > 5.75 .

Statistical Analysis. PCA in SAS (version 6.11, SAS Institute, Cary, NC) was used to evaluate the data set. Univariate statistics and stepwise multiple regression (forward) with Wilk's lambda were also employed to identify those components that would be most differentiating between sensory preference classifications. Statistica (version 5, Stat Soft, Tulsa, OK) was used to identify the components responsible for the differences between the preference classes. The cross-validation component in this section was employed to determine the classification significance for each sample. The data set included analytical values from all volatile and nonvolatile components measured.

RESULTS AND DISCUSSION

Figure 1 represents a typical chromatogram from a grapefruit juice methylene chloride extract. It is important to note the relative absence of early-eluting (low-boiling) components. All components identified in Figure 1 have been reported by Núñez et al. (1985) and others. Identification of each peak was based on Kovats retention index values and mass spectral data.

Table 1 shows the univariate correlations between preference scores of the panelists and individual component peak areas. Myrcene, decanal, linalool, linalool oxides, and several unidentified peaks were found to correlate negatively with sensory preference. β -Caryophyllene, α -humulene, and several unidentified peaks

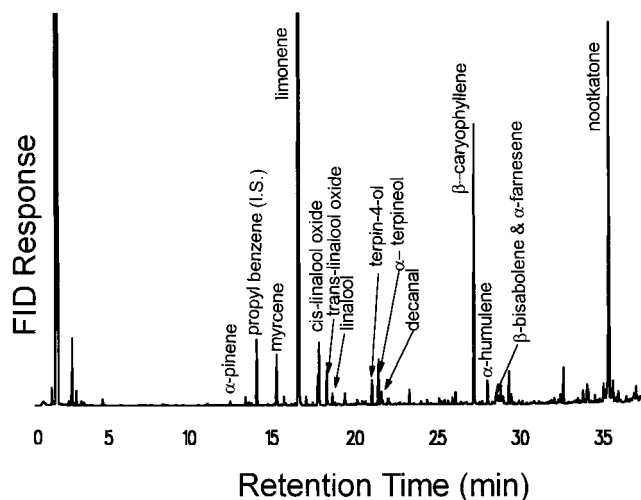


Figure 1. Chromatogram of methylene chloride extract of pasteurized (NFC) grapefruit juice on a DB-5 column. Chromatographic conditions are given in the text.

Table 1. Univariate Correlations of Selected Volatile and Nonvolatile Data with Preference Category

variable	<i>r</i>	variable	<i>r</i>
<i>allo</i> -ocimene	0.42	naringin	-0.47
β -caryophyllene	0.27	linalool	-0.49
α -humulene	0.22	acid	-0.51
RI-954 ^a	0.21	decanal	-0.53
^o Brix/acid	0.18	RI-1796 ^a	-0.57
limonin	-0.02	RI-935 ^a	-0.61
nootkatone	-0.14	myrcene	-0.61
<i>trans</i> -linalool oxide	-0.39	RI-1047 ^a	-0.62
γ -terpinene	-0.42	BRIX	-0.67

^a RI, Kovats retention indices.

were found to correlate positively. Pino et al. (1986a) also reported that the linalool oxides contributed negatively toward grapefruit flavor. The positive flavors reported by Pino and co-workers consisted primarily of top notes such as methyl butyrate and ethyl butyrate, in addition to decanal and nootkatone. The extraction procedure used in this study did not favorably extract methyl and ethyl butyrate. Therefore, the relative importance of these highly volatile compounds was not assessed in this study. In terms of nonvolatiles, the bitter naringin and sour total acid correlated negatively with preference. There was no significant correlation of limonin ($r = -0.02$) with preference. Similar findings were reported by Pino and Cabrera (1988). However, earlier studies (Rouseff et al., 1980; Barros et al., 1983) found significant negative correlation between limonin and preference.

Sensory judgments of the panel were limited to a simple hedonic score based on degree of like or dislike (preference). It should be kept in mind that the score for each juice represents preference rather than defined flavor. This could cause some scatter in sensory scores as some panelists might respond differently to various flavor aspects from others; nevertheless, the majority of the panel typically responds in a similar fashion. Some of the scatter is reduced as the highest and lowest scores are typically discarded before the remaining scores are averaged. This sensory approach was chosen as it more closely reflects marketplace consumer attitudes.

Multivariate Analysis. *PCA.* PCA can be used to determine the inherent structure of the data and identifies the most differentiating variables within the

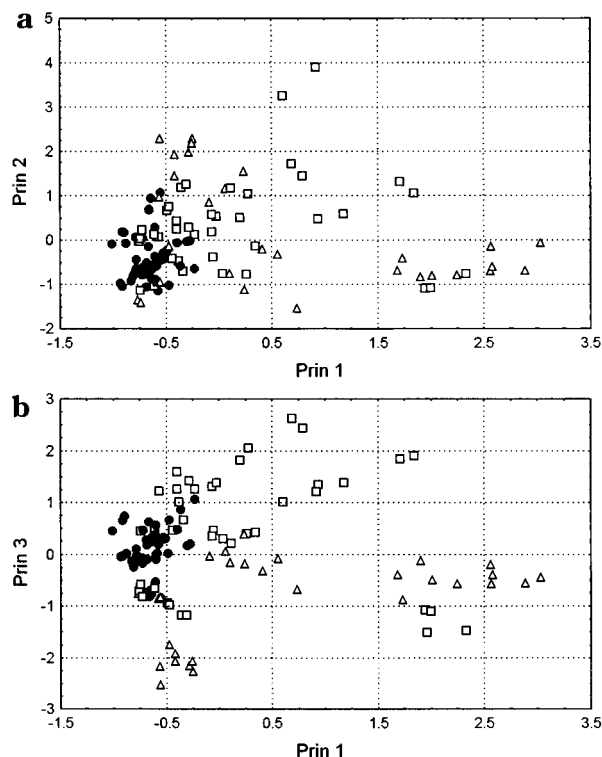


Figure 2. Eigenvector values of PC 1 vs PC 2 (a) and PC 1 vs PC 3 (b) from PCA of all 57 volatile and nonvolatile components: (●) high-preference category; (□) medium-preference category; (△) low-preference category.

data set as a whole. Variables or measurements that help to separate the data points are given more weight or emphasis. This weighting system is usually expressed as a loading factor. The larger the loading factor, the more differentiating the measurement. The results of the combined data set for the first three principal components (PC) are shown in Figure 2. The first three eigenvectors accounted for 66% of the total variance of the data. As seen in these figures, the highly preferred juice samples were tightly clustered but not completely separated from the low- and medium-preference juices. In general, the most preferred juices had the lowest PC 1 eigenvector values. The second PC axis was not especially effective in separating the three categories of juices. In PC 3, the highest preferred juices had eigenvector values close to zero. The least preferred juices had negative eigenvector values, and the medium preference juices had positive values. The loadings in PC 3 are not easy to interpret. As indicated earlier, the highly preferred juices had eigenvalues very close to zero. Thus, the *balance* between negative and positively loaded measurements will be associated with preference. For example, α -humulene and acid have equal but opposite loadings and could contribute to an eigenvalue of approximately zero.

Component Analysis. PCAs are typically calculated in the *correlation* mode. However, it is also possible to employ PCA in the *covariance* mode. In this mode, those nonredundant measurements that can best account for the maximum variance in the data are given maximum loading. In the covariance mode, PCA 1, the loading is almost exclusively in favor of nootkatone (0.95). This indicates that nootkatone is the single variable that can account for most of the variance in the data regardless of preference category. PCA 2 most heavily loads β -caryophyllene (0.94), whereas the load-

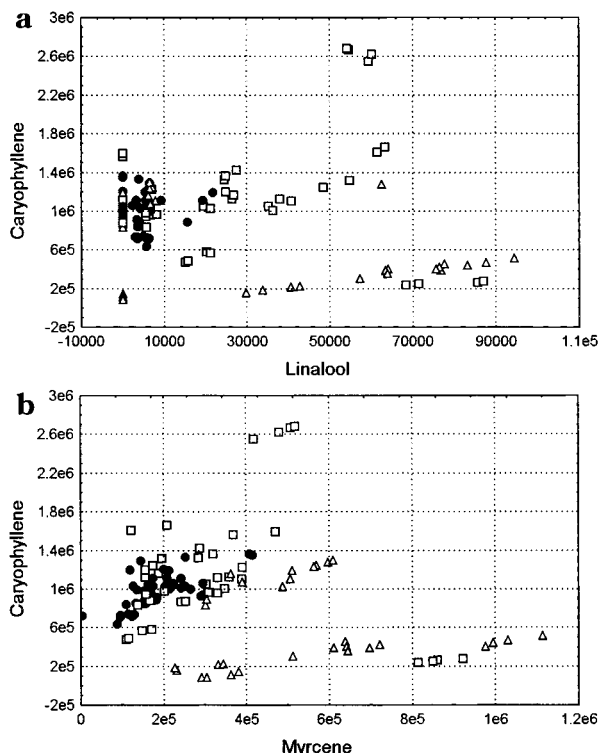


Figure 3. Peak areas of linalool and caryophyllene (a) and myrcene and caryophyllene (b) from 29 grapefruit juice extracts analyzed in triplicate: (●) high-preference category; (□) medium-preference category; (△) low-preference category.

ing in PCA 3 is weighted between myrcene and linalool (0.88 and 0.30, respectively). Essentially 97% of the variance can be explained with these three eigenvectors. These compounds may be highly effective in accounting for the variance in the total data set, but they may or may not be effective in discriminating between samples in the three preference categories.

To determine if these four components might also discriminate with respect to preference category, the univariate correlation coefficients were compared from Table 1. It can be seen that nootkatone, which was effective in accounting for the total variance in all samples, was almost completely ineffective in differentiating between juices of various preference categories. On the other hand, myrcene, which was also effective in accounting for total variance between all samples, was reasonably effective ($r = -0.61$) in differentiating between juices of various preferences. Of the four measurements that accounted for most of the variance in the total data set, three, myrcene, β -caryophyllene, and linalool, were also effective in differentiating between juices of various preference. In Figure 3, and various combinations of the peak areas for these three components are plotted against each other. It can be seen that essentially the same degree of separation between juices of various flavor preference using peak areas from these three compounds was achieved from the eigenvector value plots from all 57 components shown in Figure 2.

Nootkatone was not a particularly discriminating variable in this study. Our observed lack of nootkatone correlation agrees with the reports of Shaw and Wilson (1981) and Pino et al. (1986a,b). The indication that a high °Brix (sweetness) value was strongly associated with the least preferred juices was unexpected. This suggests, however, that highly sweet juices were not

Table 2. Stepwise Discriminant Analysis (Forward)

variable name	partial R^2	Wilk's lambda
°Brix	0.46	0.54
RI-1677	0.29	0.38
α -terpineol	0.23	0.29
β -gurjunene	0.17	0.24
ratio	0.15	0.21
limonin	0.13	0.18
<i>cis</i> -linalool oxide	0.14	0.15
naringin	0.24	0.12
nonanal	0.23	0.09
acid	0.14	0.08
<i>allo</i> -ocimene	0.14	0.07
α -copaene	0.15	0.06

preferred. Finally, in identifying the components that correlate with highly preferred grapefruit juice, it is important to acknowledge that these components only correlate with preference, but may or may not be causative.

Discriminant Analysis. To identify the variables that are most differentiating with respect to preference, discriminant analysis was used (Tables 2 and 3). Discriminant analysis will load heavily those measurements that most effectively distinguish between juices of different preference category. Figure 4a illustrates the results of discriminant analysis using just five components. All three preference category juices are clustered, but several highly preferred samples have overlapped with the medium-preference juices and four medium-preference juices are found in the region of the low-preference juices. However, increased category separation can be achieved if additional terms are used. Figure 4b illustrates the separation that can be achieved with 13 components. One of these components was the peak *allo*-ocimene; the others are noted in the legend. This is the minimum number of components required to achieve 100% separation between juices of different flavor preferences.

Identification of the Peak at RI-1126. The peak with a Kovats index value of 1126 was the single highest positively correlated component among the entire 57 components evaluated. GC/MS was employed to identify this peak. It was noted that the mass spectrum at the front of the peak differed from that at the back. Furthermore, upon examining scan by scan, we found there was a major ion mass of 117 which was evident only during the first portion of the peak and a second major ion mass of 121 which could be seen only during the last half of the peak. This strongly suggested the single peak at the retention index 1126 consisted of two coeluting compounds. When this peak was replotted as two single ion chromatograms, one generated using only the mass of 117 and the second using only the mass of 121, two distinct peaks were observed. By judiciously choosing the mass spectral scans spanning the elution time of the second compound for averaging with the background chosen as the mass spectral scans spanning the elution time of the first compound, it is possible to achieve a mass spectrum that is essentially free from ions due to the coeluting compound. The same procedure can be repeated to produce library-searchable spectra for both compounds. For the second peak the following spectrum was observed: m/z 121, 100%; 105, 53.32%; 136, 49.03%; 91, 35.55%; 79, 27.92%; 93, 20.65%; 77, 15.36%; 19, 11.91%; 22, 9.73%; 103, 8.88%. A library search (Adams, 1995) produced a match for the second peak that had a purity, fit, and rfit of 919, 944, and 954, respectively, with *allo*-ocimene (2,6-

Table 3. Discriminant Analysis Classification Results

group	compound	no. of components	% correct			
			total	low	medium	high
B	linalool + myrcene	2	68	50	50	95
C	B + °Brix	3	78	83	53	98
D	C + β-caryophyllene	4	78	90	58	89
E	D + nootkatone	5	82	90	75	82
B	linalool + myrcene	2	68	50	50	95
C	B + °Brix	3	78	83	53	98
D	C + RI-1677	4	86	77	83	95
E	D + <i>allo</i> -ocimene	5	90	97	80	95
stepwise (backward)	16 components	16	100	100	100	100
stepwise (forward)	19 components	19	100	100	100	100

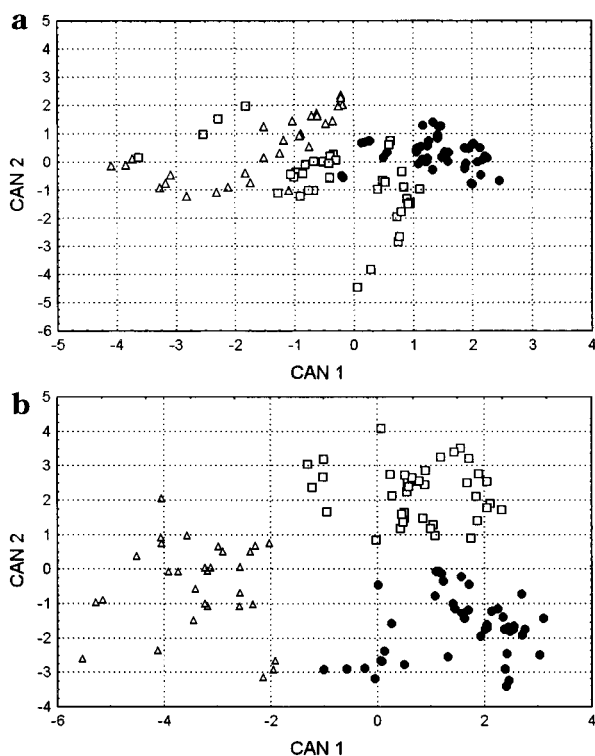


Figure 4. (a) Canonical discriminant analysis using myrcene, linalool, °Brix, and the peaks at RI-1677 and RI-1126; (b) canonical discriminant analysis using 13 variables (Brix/acid ratio, RI-935, *cis*-linalool oxide, nonanal, *allo*-ocimene, α-terpineol, decanal, RI-1299, α-copaene, β-gurjunene, RI-1762, RI-1796): (●) high-preference category; (□) medium-preference category; (△) low-preference category.

dimethyl-2,4,6-octatriene). Not only is the mass spectrum a good match to the library spectrum, but the library spectrum has included with it a Kovats retention index (RI) for each compound. The library RI for *allo*-ocimene was 1129, which very close to the observed 1126. Therefore, the designation is based on two independent means of identification.

The identification of the first eluting peak was more difficult. Its mass spectrum consisted of m/z 43, 100.00%; 117, 96.22%; 71, 67.73%; 89, 44.04%; 55, 41.18%; 69, 28.59%; 41, 22.63%; 42, 21.47%; 97, 21.00%; 75, 18.76%. The two best mass spectral matches were hexyl *n*-hexanoate and butyl *n*-hexanoate. However, these two compounds have RI values of 1383 and 1188, respectively, which were too high to be considered a match. The mass spectra for these esters along with the unknown peak all have a m/z 117 ion as a base peak, which is from the common hexanoic acid part of the ester. The unknown spectrum contains a m/z peak of

43, which is indicative of a propyl fragment. The unknown also contains a m/z 159 ion, which could be from a protonated propyl hexanoate ester. Also, the RI of 1126 would fit the pattern of decreasing RIs for decreasing size of the alcohol portion of the ester. For these reasons, we have suggested the first eluting compound might be propyl hexanoate.

Conclusions. Multivariate statistics helped identify which analytical measurements best correlated with sensory preference measurements. Nootkatone was effective in accounting for the variance in the total data set but relatively ineffective in differentiating between juices of various flavor preference. Myrcene, β-caryophyllene, and linalool could be used to differentiate juices of various flavor preference. Using discriminant analysis they could correctly predict preference category for 74% of the samples. At least 19 components were required to correctly predict the preference category for 100% of the samples using forward stepwise discriminant analysis. However, using backward stepwise discriminant analysis, it was possible to construct a predictive model using only 16 components. It should be emphasized that the models developed to predict flavor preference are based on statistical correlation only and may or may not be causative. These models may not include all of the flavor impact compounds, as it is not necessary to have a statistical model that includes all (or any) causative components as long as other components consistently correlate.

Nonvolatile components were included in almost all predictive models despite the fact that there were 10 times as many volatile components measured. This strongly suggests that nonvolatiles are an important component in any grapefruit juice flavor preference model. However, there were no successful models that contained only nonvolatile components. Therefore, the most successful models will contain both nonvolatile and volatile components.

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