

Determination of Vanillin in Orange, Grapefruit, Tangerine, Lemon, and Lime Juices Using GC–Olfactometry and GC–MS/MS

Kevin L. Goodner,* Prashanthi Jella,[†] and Russell L. Rouseff

Citrus Research and Education Center, University of Florida, 700 Experiment Station Road,
Lake Alfred, Florida 33850

The presence of vanillin in orange, tangerine, lemon, lime, and grapefruit juices has been identified and confirmed using high-resolution GC retention index values, mass spectra, and aroma quality. The impact of vanillin on the flavor score for grapefruit juice is discussed and reported to be minimal. Vanillin concentrations are determined to be in the low parts-per-million range for the various citrus juices. The calculated concentrations in the orange, tangerine, lemon, lime, and grapefruit juices are 0.20, 0.35, 0.41, 0.35, and 0.60 ppm, respectively. Pasteurization produced an average 15% increase in the concentration of vanillin in grapefruit juices. Vanillin did not correlate well with the overall flavor score despite a rather intense signal using OSME gas chromatography–olfactometry software.

Keywords: *Vanilla*; GC-O; gas chromatography; mass spectrometry; flavor; olfactometry; orange; tangerine; lemon; lime

INTRODUCTION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a unique and highly prized flavor compound used in the flavoring of many foods such as ice cream and bakery products. The natural source of vanillin is from the seeds of the vanilla plant, a member of the orchid family. In the vanilla bean, vanillin, the characteristic aroma compound of vanilla, is released from its nonvolatile glucoside during the fermentation process by the action of vanilla β -glucosidase on vanillin glucoside (Arana, 1943). Over 100 species have been reported, but only 3 species, *Vanilla planifolia*, *V. tahitensis*, and *V. pompona*, have any commercial relevance.

Vanillin has been reported in many fruits and fruit products. In mango, it has been found as “free” vanillin as well as vanillyl glucoside (Sakho et al., 1997). It has also been found in elderberry juice (Poll and Lewis, 1986), blueberries (Hirvi and Honkanen, 1983), orange juice (Marin et al., 1992), strawberries (Pyysalo et al., 1979), passion fruit juice (Huet, 1973), lychee (Ong and Acree, 1998), and wines (Spillman et al., 1997). Vanillin has also been reported in such diverse food products as popcorn (Buttery et al., 1997), commercial liquid smoke flavorings (Guillén and Ibargoitia, 1998), extruded oat flour (Guth and Grosch, 1993), apple cider brandy (Mangas et al., 1997), mushrooms (Lizárraga-Guerra et al., 1997), and chocolate. Vanillin is typically added to milk chocolate in trace amounts as a flavor modifier, yet it is one of the most intense aroma compounds in the commercial product (Schnermann and Schieberle, 1997).

A major biosynthetic source of vanillin from plant sources is ferulic acid. Ferulic acid is a hydroxycinnamic acid and is often found as a bound component of plant cell wall material. Free ferulic acid can be degraded into either vanillin or 4-vinylguaiacol (Peleg et al., 1992). Both vanillin and 4-vinylguaiacol have very low aroma thresholds. Therefore, one would expect either or both of these compounds to contribute to the aroma of plant materials through thermal or enzymatic action by releasing ferulic acid from its cell wall matrix which, in turn, is thermally or enzymatically converted into either or both vanillin or 4-vinylguaiacol. It has been known since antiquity that the aroma profiles of wines and spirits (brandy, cognac, whisky, rum, etc.) can be altered by storing them in contact with wood. The lignin of the inner surface of the cask leaches out of the wood into the alcoholic medium, where it forms ethanol–lignin complexes that are later broken down (i.e., producing ferulic acid), finally giving rise to aromatic aldehydes such as vanillin. The wood is often charred to increase the concentration of these aromatic aldehydes in the stored product (Clyne et al., 1993).

Aroma threshold data (Boidron et al., 1988) suggest that vanillin can have a strong influence on wine aroma. However, gas chromatography–olfactometry (GC-O) studies on Scheurebe and Gewürztraminer white wines found vanillin at below aroma threshold values (Guth, 1997), but other workers reported high Charm (aroma strength) values in Chardonnay wines (Moio et al., 1994). Because vanillin or vanillic acid has been reported in so many fruits and because the relative aroma strength of vanillin in many of these products is uncertain, we wanted to determine how widespread the occurrence of vanillin might be in different citrus juices. We had preliminary GC-O data for grapefruit juice extracts suggesting the presence of vanillin, but no confirmatory evidence. Therefore, the object of this study was to confirm the presence and determine the concentration of vanillin in citrus juices.

* Address correspondence to this author at USDA, ARS, SAA, U.S. Citrus and Subtropical Products Laboratory, 600 Avenue S, N.W., Winter Haven, FL 33881 [telephone (863) 293-4133, ext. 127; fax (863) 299-8678; e-mail goodner@citrus.usda.gov].

[†] Present address: Coca-Cola Foods, One Coca-Cola Plaza, Atlanta, GA 30313.

MATERIALS AND METHODS

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. Authentic standards and solvents were purchased from Fisher Scientific (Pittsburgh, PA). Orange juice was obtained directly from a commercial processing plant (Sun-Pure, Lakeland, FL). Retail tangerine juice was obtained from a local market. Lemons and limes were hand reamed in the laboratory, and the juices were chilled and analyzed immediately.

Extraction of Volatiles. Extraction of volatiles was accomplished with a 50:50 mixture of pentane and diethyl ether. Four milliliters of pentane/ether solution and an internal standard, 6 μL of 100 ppm propylbenzene, were added to 4 mL of juice and mixed using a Mixxor-like apparatus. The emulsion was broken using a refrigerated centrifuge for 10 min (15000g). Approximately 3 mL of the pentane/ether layer was collected and concentrated to $\sim 30\ \mu\text{L}$ for analysis. The concentrated extract was immediately injected into the GC.

Gas Chromatographic (GC) Analysis. The individual volatile constituents were separated with an HP-5890 GC (Palo Alto, CA) utilizing a flame ionization detector (FID) using a $30\ \text{m} \times 0.25\ \text{mm i.d.} \times 0.5\ \mu\text{m}$ film thickness low-bleed DB-5 column (J&W Scientific, Folsom, CA). The oven temperature was programmed from 35 to 275°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°C and detector temperature at 320°C . Nitrogen gas was maintained at 19 mL/min, while air and hydrogen were maintained at 296 and 35 mL/min, respectively. The splitless injection volume was 1 μL . Chromatograms were recorded and integrated using Chromperfect (Justice Innovations, Inc., Mountain View, CA) with a data acquisition rate of 0.1 s/point.

GC-Olfactometry (GC-O). GC-Olfactometry data were collected using a modified Hewlett-Packard HP-5890 GC with a $30\ \text{m} \times 0.25\ \text{mm i.d.} \times 0.5\ \mu\text{m}$ film thickness low-bleed DB-5 column (J&W Scientific). After passing through activated charcoal, drierite, and a molecular sieve 5A (Alltech, Deerfield, IL), the purified air was directed through a constant-temperature, water-filled, round-bottom flask to provide humidified air. The purified, humidified air was adjusted to a flow rate of 11 L/min and combined with the GC column effluent. Trained assessors rated the intensity of the aromas on a 16-point sliding scale (linear taper variable resistor) with a score of 0 given if no aroma is perceived and 15 for extremely intense aroma. The sliding scale was interfaced with a personal computer using the OSME software (Lopez et al., 1992) that collects time-intensity data. Aroma descriptors along with the respective retention times were recorded manually for later concatenation with the time-intensity data collected with the OSME system.

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 in the electron impact (EI) mode. Injector temperature was 250°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°C for 3 min followed by a $4^{\circ}\text{C}/\text{min}$ temperature ramp to 221°C , which was followed by a $10^{\circ}\text{C}/\text{min}$ ramp to 275°C , which was held for 1.1 min to elute high boiling temperature components in extracts. Carrier gas velocity was 31.9 cm/s through a $30\ \text{m} \times 0.25\ \text{mm id.}, 0.25\ \text{m RTX5-MS}$, column (Restek Corp., Bellefonte, PA). Transfer line and ion source temperatures were held at 275 and 170°C , respectively. The mass spectrometer detector had a delay of 4 min to avoid the solvent peak, and scans were made from m/z 40 to 300 to achieve seven spectra per second. 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 Kovats retention indices for individual peaks was done using retention time data from a series of alkane standards. The Kovats indices were calculated

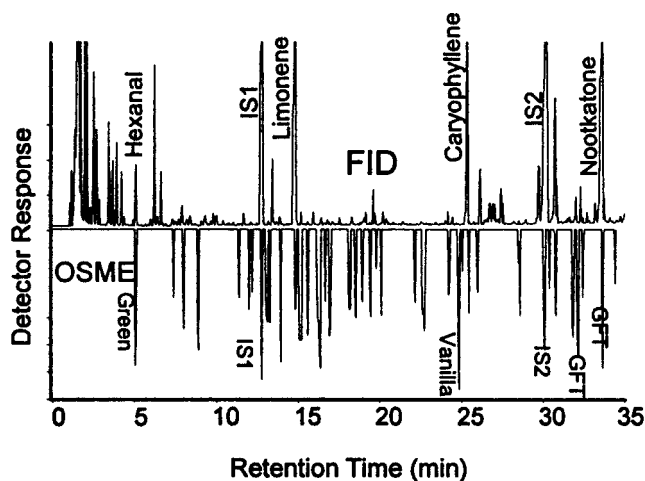


Figure 1. GC-FID (top) and GC-O (bottom) chromatograms illustrating the different responses. Internal standards are propylbenzene (GC-FID), benzaldehyde (GC-O), and methyl jasmonate (GC-O).

for both the unknown and known samples as well as compared to literature values.

Quantitation. A standard curve was generated using three concentration levels of 1, 5, and 10 ppm of vanillin along with 5 ppm of an internal standard (2-heptadecanone). Each concentration level was done in triplicate. The internal standard was added to the juice prior to extraction. The juice samples were extracted twice, with each extract being analyzed in duplicate.

Grapefruit Sensory Evaluation. The attributes rated by the 12 trained panelists were grapefruit aroma intensity, grapefruit aroma quality, bitterness, balance of sweetness/tartness, and overall flavor quality. Panelists rated four samples and a reference juice using a 16-point scale with 0 indicating least intensity and 15 indicating highest intensity. The panel consisted of approximately equal numbers of men and women ranging in age from 25 to 65 that were food scientists by trade and have had many years of experience prior to this session. A more complete description has been reported in a study that was more focused on the sensory evaluation of grapefruit juice (Jella, 1998).

The pasteurized NFC reference juice was obtained from a local juice processor and stored at -8°C in amber glass bottles. The panelists were given the reference juice six times with the attributes averaged. These averages were marked on the ballot as reference points for the samples. Consistency was maintained by presenting the reference juice after every 10 grapefruit juice samples.

Samples were coded with random three-digit numbers, which were randomly arranged on serving trays and then presented to panelists.

RESULTS AND DISCUSSION

The identification of an unknown compound in a complex mixture requires more than a single form of identification. In the case of vanillin in grapefruit juice, the authors are presenting three independent forms of identification.

GC-O. GC-O is a powerful tool in the detection and identification of unknown flavor compounds. It offers a highly selective detector (the human nose) that can detect aroma active compounds for which more routine detectors may show little to no signal. A good example is the comparison of a GC-O chromatogram to a GC-FID chromatogram as shown in Figure 1. The top chromatogram was generated using the FID detector and the bottom using the OSME detector. As can be

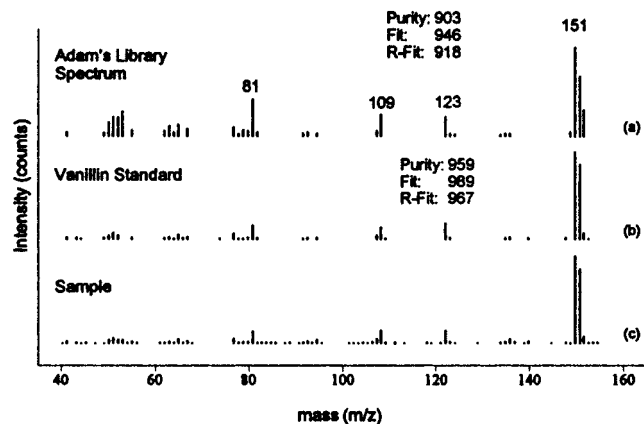


Figure 2. Mass spectral matching of a grapefruit juice extract (c) with the library spectrum (a) and a vanillin standard (b).

seen, many peaks in the FID chromatogram have a corresponding peak in the OSME chromatogram such as hexanal (green note) at ~ 5 min. However, there are many peaks in the FID chromatogram that do not have a corresponding OSME response such as the large peak following hexanal. Correspondingly, there are peaks in the OSMEgram that have small or nonexistent responses in the FID chromatogram. An example of this is at ~ 25 min, where a peak in the OSMEgram is labeled "vanilla", which has a very small response in the FID chromatogram. The aroma descriptor "vanilla" is fairly specific and associated with a relatively small number of compounds, the most obvious of which is vanillin. This was one of the first clues that led to the investigation of vanillin in citrus juices.

The aroma descriptor "vanillin" was attributed to the same compound that produced the mass spectra shown in Figure 2.

Mass Spectral Identification. The mass spectra shown in Figure 2 can be used for positive identification of vanillin. Sample c is from an NFC grapefruit juice sample that was extracted according to the method described. As can be seen, the library spectrum a (Adams, 1995) is a fairly good match with sample c with purity, fit, and reverse fit (r-fit) scores of 903, 946, and 918, respectively. These scores have a range of 0–1000. Purity measures the similarity between the sample mass spectrum and the library reference mass spectrum. Fit measures the degree to which the library spectrum is contained within the sample mass spectrum. This is useful for coeluting compounds. R-fit is the opposite of fit in that it measures the degree to which a sample mass spectrum is contained in the library mass spectrum. These scores are high enough to be considered a match. However, to be more certain, a sample of vanillin was diluted with ethanol and injected as a standard. The resulting mass spectrum (b) is also shown in Figure 2. The purity, fit, and r-fit scores are 959, 989, and 967, respectively. The better fit is due to the standard being analyzed by the same instrument and operating conditions without which minor differences in mass spectra can occur, as seen by a comparison of the mass spectra from the standard (b) and the sample (c). The high degree of similarity of the sample mass spectrum to both the Adams' library and standard show the likelihood of correct identification.

GC-MS/MS Identification. The Finnigan GCQ utilizes an ion-trap mass spectrometer that is capable of performing MS/MS. This is accomplished by introducing

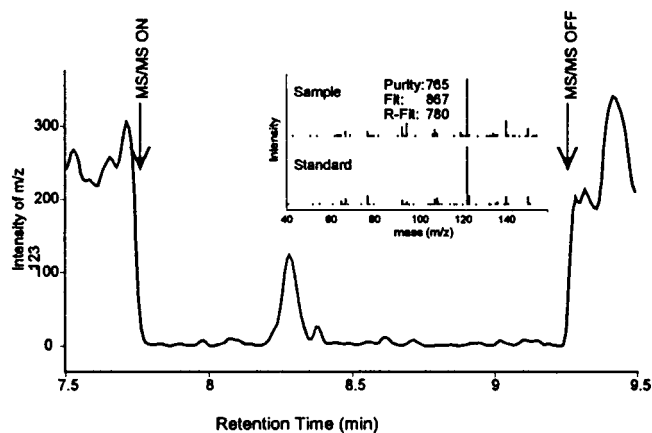


Figure 3. MS/MS chromatogram based on m/z 123, which is the major ion product of vanillin. Insert contains ion trap mass spectra of vanillin in a grapefruit juice compared with that obtained from standard vanillin.

the ions into the trap and isolating the masses of interest (in this case m/z 151 and 152, which are the major ions of vanillin as can be seen in Figure 2). This is the first "MS" of MS/MS. Once the target ions are isolated, they are excited so that they will fragment. This is called collision-induced dissociation (CID). The resulting fragments are collected to produce a mass spectrum, is the second "MS" of MS/MS. In complex matrices, such as orange juice extracts, the first MS is used as a filter to reduce chemical noise, with the second MS providing a means of identification. For a compound to be detected using MS/MS, it must first elute from the GC at the correct time, have the correct mass ions for isolation, and fragment in the same manner as a standard that can be compared in user-generated libraries. Figure 3 is an example of how this technique was employed to produce further evidence and quantitation. The chromatogram shown is a single ion chromatogram (SIC) illustrating how the mass of 123 changes with retention time for an extract from an early-season unpasteurized NFC grapefruit juice. One can see the sharp decrease in the background at 7.75 min due to the start of the MS/MS, the vanillin peak at 8.3 min, and the sharp increase of the background due to the end of the MS/MS. The insert shows how the grapefruit sample compares to a vanillin standard that was run under identical MS/MS conditions. Comparison of the sample extract with the standard contained in the user library gave purity, fit, and r-fit scores of 765, 867, and 780, respectively. The purity, fit, and r-fit scores are lower in the MS/MS mode due to signal-to-noise levels. One can see that the sample extract and standard are the same, which provides an additional form of identification along with reduced chemical noise resulting from coeluting compounds for improved quantitation.

Retention Time Identification. The retention time of the GC-MS and GC-O peaks were converted to Kovats index for cross-instrument comparisons. The Kovats indices for the GC-MS peak and GC-O peak were 1404 and 1401, respectively. The Kovats index reported in the Adams mass spectral library is 1391. Likewise, the Kovats index for the vanillin shown in Figure 2b was calculated as 1405. The four Kovats indices have an average of 1400 with a relative standard deviation of 0.46%. This level of agreement is a strong indication that the identification is correct.

Scheme 1. Chemical Breakdown Pathway of Ferulic Acid into Vanillin

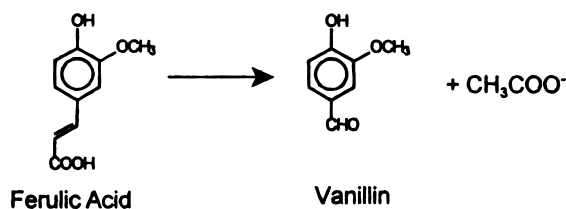


Table 1. Vanillin Concentrations in Various Citrus Juices

juice type	vanillin concn (ppm)	%RSD
orange	0.20	23.5
tangerine	0.35	11.7
lemon	0.41	18.6
lime	0.35	15.5
early-season unpasteurized grapefruit	0.52	20.8
early-season pasteurized grapefruit	0.62	17.0
midseason unpasteurized grapefruit	0.59	16.2
midseason pasteurized grapefruit	0.66	13.8

Ferulic Acid Degradation. Scheme 1 is an illustration of the ferulic acid degradation that can lead to the production of vanillin. There was some concern that ferulic acid could be degraded in the hot (250 °C) GC injector and produce vanillin as an artifact. To test this possibility, a test solution of 10 ppm ferulic acid was injected under the same conditions. As a more strenuous test, a 620 ppm solution was also injected under the same conditions. No vanillin was observed in either chromatogram.

Vanillin's Affect on Flavor Scores. Vanillin did not correlate with flavor score (correlation coefficient of -0.2) in NFC grapefruit juice. This is an unusual result as the GC-O aroma intensity for vanillin in grapefruit juice is fairly high. There are several compounds that have GC-O aroma intensities lower than that obtained from vanillin but have significantly higher ratios of concentration/threshold than vanillin and significantly greater effects on flavor. We suggest a few possibilities for this seeming inconsistency. First, the aroma assessor might be overestimating the aroma intensity in the OSME analysis due to the distinctiveness of the aroma. Second, there could be either a synergistic or an antagonistic effect with another compound in the juice. Because vanillin is not observed in taste testing, we feel that there is some tertiary effect (synergistic or antagonistic) masking the vanillin aroma in the juice. Last, the flavor unit (as measured as a ratio of the concentration to the flavor threshold) may be low.

Vanillin Concentration in Citrus Juices. Table 1 shows the vanillin concentration in various citrus juices. The concentration of vanillin is similar in the different samples, differing by only a factor of 3 from the least concentrated (orange) to the most (pasteurized mid-season grapefruit). Pasteurization of grapefruit juice yields an $\sim 15\%$ (90% confidence level) increase in the concentration of vanillin. One possible explanation for this increase is the degradation of ferulic acid as shown in Scheme 1 due to the heat used during the pasteurization process.

The concentrations reported here are all greater than or equal to the thresholds reported by Cartwright and Kelley (1952). These researchers determined the threshold for vanillin to be 0.2 ppm in water. This explains the lack of correlation between the concentration of

vanillin and overall flavor quality and lends credibility to the idea that the panelists over-rated the intensity of the vanilla aroma.

Conclusions. We have presented three independent forms of identification for the presence of vanillin in grapefruit juice. Mass spectral identification using both a spectral library and a vanillin standard coupled with the characteristic vanilla aroma and excellent Kovats retention index match leads us to assert, with some assurance, that vanillin is indeed present in grapefruit, lemon, lime, and tangerine juices.

LITERATURE CITED

- Adams, R. P. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy*; Allured Publishing: Carol Stream, IL, 1995.
- Arana, F. Action of a β -glucosidase in the curing of vanilla. *Food Res.* **1943**, *8*, 343–351.
- Boidron, J. N.; Chatonnet, P.; Pons, M. Influence du bois sur certaines substances odorantes des vins (Effects of wood on aroma compounds of wine). *Connaiss. Vigne Vin* **1988**, *22*, 275–294.
- Buttery, R. G.; Ling, L. C.; Stern, D. J. Studies on popcorn aroma and flavor volatiles. *J. Agric. Food Chem.* **1997**, *45*, 837–843.
- Cartwright, L. C.; Kelly, P. H. Flavor quality and strength of propenyl guaethol as a vanilla extender or replacement. *Food Technol.* **1952**, *6*, 372–376.
- Clyne, J.; Conner, J. M.; Paterson, A.; Piggott, J. R. The effect of cask charring on Scotch whisky maturation. *Int. J. Food Sci. Technol.* **1993**, *28*, 69–81.
- Guillén, M. D.; Ibargoitia, M. L. New components with potential antioxidant and organoleptic properties, detected for the first time in liquid smoke flavoring preparations. *J. Agric. Food Chem.* **1998**, *46*, 1276–1285.
- Guth, H. Quantitation and sensory studies of character impact odorants of different white wine varieties. *J. Agric. Food Chem.* **1997**, *45*, 3027–3032.
- Guth, H.; Grosch, W. Geruchsstoffe von extrudiertem Hafermehl, Veränderungen bei der Lagerung (Aroma compounds of extrusion products of oat meal—changes during storage). *Lebensm. Unters. Forsch.* **1993**, *196*, 22–28.
- Hirvi, T.; Honkanen, E. The aroma of blueberries. *J. Agric. Food Chem.* **1983**, *34*, 992–996.
- Huet, R. The aroma of passion fruit juice. *Fruits* **1973**, *28*, 397–403.
- Jella, P. Ph.D. Thesis, University of Florida, Gainesville, FL, 1998.
- Kovats, E. *Advances in Chromatography*; Dekker: New York, 1965; Vol. 1.
- Lizárraga-Guerra, R.; Guth, H.; López, M. G. Identification of the most potent odorants in huitlacoche (*Ustilago maydis*) and austern pilzen (*Pleurotus* sp.) by aroma extract dilution analysis and static headspace samples. *J. Agric. Food Chem.* **1997**, *45*, 1329–1332.
- Lopez, M. R.; Libbey, L. M.; Watson, B. T.; McDaniel, M. R. Odor analysis of Pinot noir wines from grapes of different maturities by a gas chromatography-olfactometry technique (Osme). *J. Food Sci.* **1992**, *57*, 985–993.
- Mangas, J. J.; Rodríguez, R.; Moreno, J.; Suárez, B.; Blanco, D. Furanic and phenolic composition of cider brandy. A chemometric study. *J. Agric. Food Chem.* **1997**, *45*, 4076–4079.
- Marin, A. B.; Acree, T. E.; Hotchkiss, J. H.; Nagy, S. Gas chromatography-olfactometry of orange juice to assess the effects of plastic polymers on aroma character. *J. Agric. Food Chem.* **1992**, *40*, 650–654.
- Moio, L.; Schlich, P.; Etievant, P. Acquisition and analysis of aromagrams of Chardonnay Burgundy wines. *Sci. Aliments* **1994**, *14*, 601–608.

- Ong, P. K. C.; Acree, T. E. Gas chromatography/olfactory analysis of lychee (*Litchi chinesis* Sonn.). *J. Agric. Food Chem.* **1998**, *46*, 2282–2286.
- Peleg, H.; Naim, M.; Zehavi, U.; Rouseff, R. L.; Nagy, S. Pathways of 4-vinylguaiacol formation from ferulic acid in model solutions of orange juice. *J. Agric. Food Chem.* **1992**, *40*, 764–767.
- Poll, L.; Lewis, M. J. Volatile components of elderberry juice. *Lebensm. Wiss. Technol.* **1986**, *19*, 258–262.
- Pyysalo, T.; Honkanen, E.; Hirvi, T. Volatiles of wild strawberries, *Fragaria vesca* L., compared to those of cultivated berries, *Fragaria* × *ananassa* cv. Senga Sengana. *J. Agric. Food Chem.* **1979**, *27*, 19–22.
- Sakho, M.; Chassagne, D.; Crouzet, J. African mango glycosidically bound volatile compounds. *J. Agric. Food Chem.* **1997**, *45*, 883–888.
- Schnermann, P.; Schieberle, P. Evaluation of key odorants in milk chocolate and cocoa mass by aroma extract dilution analyses. *J. Agric. Food Chem.* **1997**, *45*, 867–872.
- Spillman, P. J.; Pollnitz, A. P.; Liacopoulos, D.; Skouroumounis, G. K.; Sefton, M. A. accumulation of vanillin during barrel-aging of white, red, and model wines. *J. Agric. Food Chem.* **1997**, *45*, 2584–2589.

Received for review May 25, 1999. Revised manuscript received April 11, 2000. Accepted April 26, 2000. We acknowledge the Florida Department of Citrus, Scientific Research Department, for financial support along with the Hunt Brothers foundation and the Hurlong fellowship fund. Mention of a trademark or proprietary product is for identification only and does not imply a guarantee or warranty of the product by the U.S. Department of Agriculture. The U.S. Department of Agriculture prohibits discrimination in all its programs and activities on the basis of race, color, national origin, gender, religion, age, disability, political beliefs, sexual orientation, and marital or family status.

JF990561D