Determination of Furaneol and *p*-Vinylguaiacol in Orange Juice Employing Differential UV Wavelength and Fluorescence Detection with a Unified Solid Phase Extraction

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A unified sample preparation and modified chromatographic procedure were developed to determine two major off-flavors in orange juice, *p*-vinylguaiacol (PVG) and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF or Furaneol). Furaneol was quantified using the absorbance difference between 335 and 292 nm. This procedure helped identify Furaneol by suppressing an interfering peak that developed in juice during storage. The C_{18} solid phase extraction procedure isolated both PVG and Furaneol from orange juice into a single extract. A 30 min reversed-phase HPLC gradient method employing UV and fluorescence detectors in series was also developed for the determination of both off-flavors from a single injection. The ternary solvent system consisted of water, methanol, and acetonitrile. Recovery studies yielded mean recoveries of 99.9% ± 2.3% for Furaneol (0.3, 1.0, and 10.0 ppm) and 100.0% ± 1.2% for PVG (0.05, 2.0, and 5.0 ppm), thus demonstrating that the unified solid phase procedure quantitatively isolated both compounds.

Keywords: *Storage off-flavors; peak suppression; PVG; citrus*

INTRODUCTION

In recent years, off-flavors have received considerable attention as consumers disapprove of their presence and manufacturers strive to improve product quality. The development of undesirable volatile compounds in citrus products during storage is well documented, and the sensory impact of these compounds on quality is profoundly negative (Naim *et al.*, 1994). *p*-Vinylguaiacol (PVG) and 2,5-dimethyl-3(2*H*)-furanone (DMHF or Furaneol) are putatively two of the most detrimental off-flavors that occur during storage of orange juice (Tatum *et al.*, 1975). PVG and Furaneol are produced from the thermal decomposition of ferulic acid and Maillard reactions, respectively. With a flavor threshold of just 50 ppb, PVG contributes an “old fruit” or “rotten” flavor to the juice. Furaneol is reported to produce a pineapple-like aroma typical of aged orange juice and to mask fresh orange juice aroma when it exceeds its flavor threshold of 50 ppb (Tatum *et al.*, 1975).

Due to the thermal instability of Furaneol and the low volatility and strong fluorescence of PVG, analytical methodology favors high-performance liquid chromatography (HPLC) rather than gas chromatography (GC). Reversed-phase HPLC (RP-HPLC) has become the method of choice for the analysis of these compounds because it requires minimal sample preparation and is sensitive and reproducible. Although chromatographic methods have been developed to isolate and quantify PVG and Furaneol individually in orange juice, no method exists for the quantitative determination of both off-flavors in a single assay.

Lee and Nagy (1990) developed one of the earliest SPE procedures for PVG, which was later improved by Rouseff *et al.* (1992a,b). Several investigators have developed solid phase extraction procedures for Furaneol in various matrices (Lee, 1987; Sanz, 1994). However, no method has been reported for isolating both compounds in a single extraction.

The purpose of this research was to develop a single, rapid method for the extraction of the two most detrimental off-flavors in orange juice. A second objective was to generate a chromatographic system to separate and quantify PVG and Furaneol from a single injection.

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MATERIALS AND METHODS

Reagents and Standards. HPLC grade organic solvents from Fisher Scientific (Pittsburgh, PA) were used both for sample preparation and as chromatographic solvents. PVG (2-methoxy-4-vinylphenol, 98% pure) and Furaneol (registered by Firmenich) [2,5-dimethyl-4-hydroxy-3(2*H*)-furanone, 95% pure] were obtained from Aldrich Chemical Co. (Milwaukee, WI). All water was deionized and then distilled. Commercial single-strength orange juice (SSOJ) was obtained from a processor in Florida.

Solid Phase Extraction. SSOJ samples were centrifuged (20 min, 15000*g*, 4 °C). Extraction of PVG and Furaneol from the supernatant was accomplished using C_{18} ODS SPE cartridges (Whatman, Clifton, NJ). The bonded stationary phase of the SPE cartridges consisted of an octadecylsilane, 5% carbon load on a non-end-capped silica surface (40 μm particle size, 85 Å mean diameter, 400 m^2/g surface area). Supernatant juice aliquots (2 mL) were applied to SPE cartridges which had been preconditioned with methanol (2.5 mL) and water (6 mL). Using a luer-lok 10 mL glass syringe, cartridges were then washed with water (1.5 mL) and eluted dropwise with methanol (1.5 mL). Juice samples were stored in 2 mL amber vials with Teflon/silicone septa screw tops until analyzed.

Instrumentation. A Perkin-Elmer (Norwalk, CT) Series 410 LC pump with an SEC-4 solvent environmental control chamber was used for solvent delivery. Sample volumes were injected by a Hewlett-Packard (Waldbronn, Germany) Series 1050 autosampler. A Waters Associates (Milford, MA) 490E multiwavelength detector and a Perkin-Elmer LS40 fluorescence (dual monochrometer) detector were used in series.

S0021-8561(96)00435-9 CCC: $14.00 © 1997 American Chemical Society

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 1.2 points/s. Spectral data was obtained with a Waters Model 990+ UV−visible photodiode array detector and an NEC APCIV computer employing software from the Waters 990+ unit.

Chromatography. Separations were achieved using a 5 μm Bondesil C_{18}-RP, 4.6 mm i.d. × 25 cm (Varian, Palo Alto, CA) column coupled with a Supelguard LC-18 guard column, 4.6 mm i.d. × 2.0 cm (Supelco, Bellefonte, PA). The mobile phase consisted of methanol (A), acetonitrile (B), and aqueous 0.05 M sodium acetate/acetic acid, pH 4.0 (C). Flow rate was 0.8 mL/min, and sample injection volume was 20 μL. The following linear chromatographic gradients were used: 0 min, 10% A, 5% B, 85% C; 16 min, 20% A, 33% B, 47% C; 28 min, 0% A, 95% B, 5% C. The column was washed with 100% acetonitrile followed by a 2 min linear gradient to re-establish initial conditions. The column was equilibrated at initial conditions for 18 min prior to each injection.

Detection and Integration. PVG was measured using fluorescence detection with excitation and emission set at 290 and 335 nm, respectively. For Furaneol, a multiwavelength UV detector set at 292 and 335 nm was used for quantification. By measuring absorbance differences between the two wavelengths, the Furaneol could be quantified in the presence of other possible coeluting components. A difference chromatogram was calculated by subtracting the absorbance value of the chromatogram at 335 nm from the absorbance value of the chromatogram at 292 nm, a point at a time, to provide a final difference chromatogram, which was then integrated.

Recovery Studies. Centrifuged orange juice samples were spiked with three levels of Furaneol (0.3, 1, and 10 ppm) and three levels of PVG (0.05, 2, and 5 ppm) and prepared using the new unified SPE procedure. Percent recoveries were determined by comparing chromatographic peak areas from spiked OJ samples against peak areas generated by direct injection of standard solutions of Furaneol and PVG. Analyses were performed in duplicate using the chromatographic conditions described above.

RESULTS AND DISCUSSION

Solid Phase Extraction. Due to the dissimilar polarities of PVG and Furaneol, it was difficult to extract both off-flavors using a single SPE procedure. A solid phase material had to be found that would retain both PVG and Furaneol. PVG has a strong affinity for C_{18} material. Polar compounds, such as Furaneol, have little affinity for this material and could be lost in solvent washes designed to remove sugars. To remedy this problem, a 5% carbon load, non-end-capped solid phase material was employed. This material was the most polar of the nonpolar phases since more than 60% of the original silica surface hydroxyls were unreacted (Popovich and Southern, 1979). Both the low carbon load and the unreacted stationary phase helped to impart a more polar character to the surface of the solid support, thus improving Furaneol retention.

A protocol was then established to concentrate the juice sample with optimum recovery efficiencies. Since a nonpolar stationary phase was used, elution of moderately polar Furaneol in the washing of the cartridge was a major concern. With this in mind, solvent flow rates in the extraction column were optimized at 0.3−0.5 mL/min. Polar solvent washes used to remove sugars and other polar compounds were analyzed for the compounds of interest. No Furaneol was detected, suggesting that all of the Furaneol was retained by the cartridge.

Lee and Nagy (1990) eluted the components of interest with 6 mL of hexane. Entrapped water was removed with sulfate drying. Excess solvent was reduced via evaporation under nitrogen. Rouseff *et al.* (1992a) eliminated the drying and evaporation steps by eluting with a small volume of tetrahydrofuran (THF), a water-miscible and powerful eluting solvent. Since the sample could be eluted in a small volume, the time-consuming solvent evaporation step was eliminated and overall analysis time reduced.

Table 1. Recovery of Furaneol and PVG Off-Flavors Added to Orange Juice

compd	trial no.	concn in OJ (ppm)	% recovery
Furaneol	1	10	102.3 ± 0.01
	2	1	97.62 ± 0.03
	3	0.3	99.96 ± 0.02
PVG	1	5	101.3 ± 0.02
	2	2	99.45 ± 0.05
	3	0.05	99.28 ± 0.03

[a] Mean and standard deviation from duplicate samples.

In the preliminary stages of this research, THF was a candidate for the final eluting solvent due to its ability to elute both PVG and Furaneol. Unfortunately, many compounds normally retained by the SPE cartridge were also eluted. These compounds were chromatographically very late eluting, which lengthened the chromatographic analysis time and reduced retention time reproducibility. Because of these problems, methanol was chosen as the eluting solvent. The solvent strength of methanol was sufficient to elute both compounds without the other late eluting, nonpolar compounds. Both compounds could be eluted with 1.5 mL of methanol, thus eliminating the need to reduce the sample volume through evaporation.

Recovery Study. To accurately assess the effectiveness of the extraction procedure, recovery studies were performed on orange juices containing known added amounts of off-flavor components. Shown in Table 1 are the recovery values for PVG and Furaneol obtained from fresh orange juice spiked with three levels of each standard and then analyzed using the chromatographic procedure developed in this study. The mean recoveries for PVG and Furaneol were 100.0% ± 1.1% and 99.9 ± 2.3%, respectively. This is similar to what has been reported in the literature. In two separate published procedures, Lee and Nagy reported mean recoveries for PVG of 91.2% ± 4.0% (1990) and 92.6% ± 2.3% for Furaneol (1987). They employed a separate C_{18} Sep-Pak cartridge (12% carbon load, end-capped) with different eluting solvents for each analysis.

The analytical precision (reproducibility) of the current method was determined by analyzing the identical sample of orange juice in triplicate. The coefficients of variance (CV) were 1.12% for PVG and 2.34% for Furaneol.

Solvent Selection. The chromatographic separation was optimized by varying experimental conditions (solvent selection, gradient rate, and run time) until the components of the orange juice sample were optimally separated within a minimum time. Since Furaneol was expected to elute quickly, only small amounts of methanol (10%) and acetonitrile (5%) were necessary at initial conditions. A much stronger reversed-phase (more nonpolar) solvent was needed to elute PVG. Thus, a rapid change in solvent strength using a linear gradient was appropriate. Isocratic systems using methanol and water that were strong enough to elute PVG in a reasonable time compressed the front end of the chromatogram. Unfortunately, the Furaneol peak was often merged with other orange juice constituents. Initial

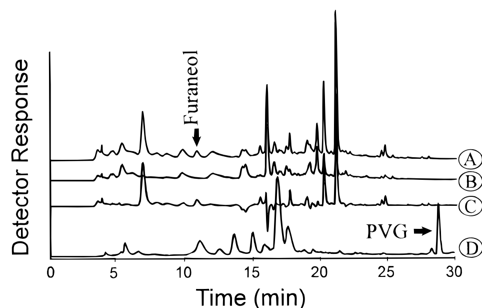

Figure 1. HPLC chromatogram of orange juice stored at 30 °C for 4 months: (A) UV detection at 292 nm: (B) UV detection at 335 nm; (C) "difference" of 292 − 335 nm; (D) fluorescence detection (ex = 290 nm, em = 335 nm).

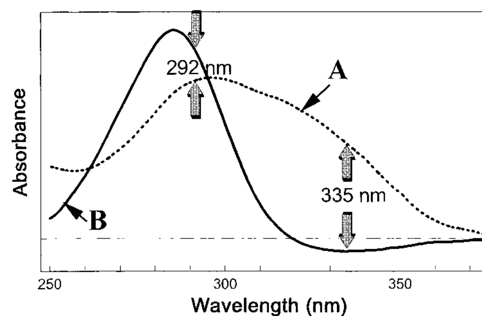

Figure 2. Diode array spectra of (A) interfering peak at RT = 9.4 min and (B) authentic Furaneol. Note the relative absorbances at 292 and 335 nm.

studies were conducted using THF, since it had demonstrated unique selectivity compared to methanol and acetonitrile (Rouseff *et al.*, 1992a,b). However, as THF concentration was rapidly increased to elute PVG, the chromatographic baseline also increased. THF's UV cutoff is 220 nm, but it still has appreciable absorbance at the monitoring wavelength of 292 nm. Therefore, other reversed-phase solvents, which would not produce pronounced baseline shifts with increasing concentration such as methanol and acetonitrile, were examined. Methanol was an excellent solvent for separating Furaneol from other polar components in the juice. However, acetonitrile was found to be the superior choice to elute and resolve the more nonpolar PVG and still separate it from other juice components with a minimum baseline shift.

Chromatography. Calibration plots were prepared for PVG and Furaneol using standard solutions with concentrations spanning the range of those likely to be observed in citrus juices. Linear responses were obtained between peak areas and amounts injected. The peak area vs concentration relationship observed for Furaneol at 292 nm and the peak area from the 292 − 335 nm "difference" chromatogram showed there was a slight loss in sensitivity in using the "difference" peak area compared to that from just the 292 nm chromatogram for the measurement of Furaneol. Triplicate injections of each Furaneol standard from 0.745 to 9.29 ppm yielded $r^2 = 0.9999$ and slope = 1.013×10^5. The peak area vs concentration relationship for PVG from 0.6 to 7.47 ppm ($n = 5$) was $r^2 = 0.9996$ and slope = 1.670×10^5.

Four chromatograms from the identical sample of a canned orange juice are shown in Figure 1. All were obtained from a single injection. The first two chromatograms were obtained from UV wavelengths, and the fourth was obtained using fluorescence detection. The third chromatogram was constructed using data from the first two. This chromatogram was produced by subtracting the absorbance value of each point in the second chromatogram (335 nm) from the corresponding point in the first chromatogram (292 nm). Furaneol eluted at 10.26 ± 0.78 min (7.6% RSD) and was identified by comparing the absorbance spectrum obtained from the sample (photodiode array) with the spectrum of authentic Furaneol. Retention time comparisons with authentic Furaneol and resulting peak size changes from fortifying the sample with authentic Furaneol also indicated that the peak in question was due to Furaneol. PVG eluted at 28.37 ± 0.60 min (2.1% RSD) and was identified by comparison of fluorescence excitation and emission spectral data from the peak in question with that from authentic PVG and similar retention time data. Both spectral information and retention time behavior indicated that the peak in question was PVG.

Dual-Wavelength Detection of Furaneol. Prior investigators monitored chromatographic effluent at 280 nm (the λ_{max} for Furaneol). This was adequate for relatively simple matrices. However, the chemical complexity of orange juice made it difficult to completely resolve all of the juice components from Furaneol. The optimum wavelength for Furaneol (280 nm) would also detect other compounds in citrus with similar absorbance spectra (e.g., flavanone glycosides), which are abundant and found in much higher concentrations. Even with solid phase sample cleanup, there were components that eluted in the same region as Furaneol. To further complicate the separation requirements, additional peaks were observed in the same region as the Furaneol peak in juices stored under more severe conditions. Therefore, a second wavelength was chosen to differentiate these coeluting compounds from Furaneol.

The use of a difference chromatogram to obtain improved selectivity and analytical accuracy is a relatively recent development. It is only since the availability of photodiode array detectors capable of monitoring several wavelengths and other detectors capable of monitoring two wavelengths simultaneously that this interference suppression procedure has become practical. The basic procedure has been outlined by Huber (1993) in his section on optimizing photodiode array detectors for selectivity. As shown in Figure 2, both Furaneol and its interfering peak had significant absorbance at 280 and 292 nm. It can be seen that 335 nm was a minimum absorbance wavelength for Furaneol but still a significant absorbance point for the coeluting compound. By subtracting the absorbance value at 335 nm from the corresponding absorbance value at 292 nm, the interfering compound was suppressed and the Furaneol peak easily quantified. The "difference" chromatogram was composed of the point-by-point calculations, and the resulting chromatogram was integrated to determine Furaneol concentrations.

Shown in Figure 3 is a magnified portion of the chromatogram obtained in the region of the Furaneol peak using this peak suppression technique. The top chromatogram was obtained at 292 nm and shows Furaneol on the tailing edge of a larger juice component. This was a difficult peak to accurately integrate, especially at low Furaneol levels. Fortunately, the absorbance readings at 335 nm provided an absorbance pattern that was due almost entirely to the interfering compound. Thus, when the absorbance at 335 at one point in the chromatogram was subtracted from the absorbance at 292 at the same point in the chromato-

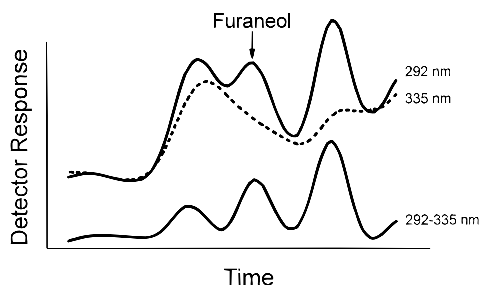

Figure 3. Dual-wavelength detection from 8 to 12 min, showing the peaks from detection at 292 and 335 nm as well as the resulting difference trace for the same components.

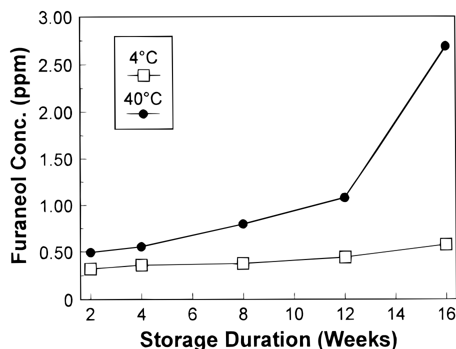

Figure 4. Furaneol content of commercial orange juice stored in cans at 4 and 40 °C during 16 weeks of storage.

gram, the difference was due primarily to Furaneol. The selection of wavelengths for the difference chromatogram could have been different if it was necessary to completely eliminate the interfering peak. For example, 282 − 322 would have completely eliminated the interfering peak but produced a substantially lower Furaneol peak. The wavelengths chosen represent the best compromise between maximizing suppression of the interfering peak and minimizing loss in the resulting Furaneol peak height. The collection of points from the current difference (292 − 335) was then connected to form the "difference" chromatogram shown in the lower plot. It can be seen that this choice of wavelengths did not completely eliminate the interfering peak but greatly reduced its size to the point that it no longer interfered with Furaneol.

Peak Purity. The results of this wavelength difference procedure can also be used as an indicator of peak purity. The symmetry of the "difference" Furaneol peak strongly suggests that the peak is due to a single component. If there were a second unresolved impurity (third component), the resulting "difference" peak would not be symmetrical unless the second impurity exactly coeluted with Furaneol. This approach is similar in principle to wavelength ratio methods which have been employed to determine peak purity in the past. Thus, dual-wavelength detection greatly simplified the Furaneol peak for integration purposes (with the concomitant improvement in analytical accuracy and precision) and produced a peak that appears pure.

Furaneol Accumulation. Furaneol levels increased with increasing storage time and temperature (Figure 4). At 4 °C the increase in Furaneol concentration with time was very gradual and almost linear. However, at 40 °C the rate of Furaneol accumulation is considerably greater and the pattern is almost exponential. After 16 weeks of storage, Furaneol levels in the juices were 0.58 ppm (4 °C), 0.62 ppm (20 °C), 0.93 ppm (30 °C), and 2.69 ppm (40 °C). Juice stored at 40 °C for 16 weeks

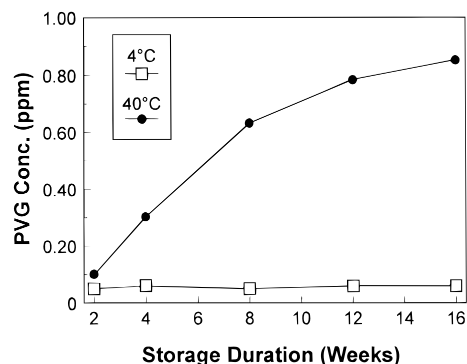

Figure 5. PVG content of commercial orange juice stored in cans at 4 and 40 °C during 16 weeks of storage.

contained about 5 times more Furaneol than the juice stored at 4 °C. Thus, storage temperature is a critical factor in Furaneol accumulation in canned orange juices. Storage time was of secondary importance, at least during the first 12 weeks of storage.

PVG Accumulation. The extent of PVG accumulation in canned orange juice stored for different periods and at different temperatures is shown in Figure 5. Both time and storage temperature are important factors in governing ultimate PVG concentration in canned orange juice. At a storage temperature of 4 °C, the PVG concentration was essentially unchanged during the entire 16 weeks of storage. Juices stored at 40 °C for 16 weeks contained considerably more PVG than the 4 °C sample at any given storage time. The 0.80 ppm PVG observed at 12 weeks (40 °C) is very similar to values reported by Tatum *et al.* (1975), who found concentrations between 0.6 and 1.6 ppm in three different samples of canned orange juice stored for 12 weeks at 35 °C.

Conclusion. Previously, extraction methods and chromatographic separations of PVG and Furaneol had focused on each compound individually. This study isolated both off-flavors in a unified procedure and quantified them from a single injection. The dual-wavelength absorbance difference approach improved chromatographic selectivity and integration accuracy. Recovery studies indicated that the unified SPE procedure quantitatively extracted the compounds of interest from orange juice. After Furaneol and PVG were quantified in orange juices stored for different times and at different temperatures using this new procedure, it was concluded that reducing storage temperature and minimizing the time at elevated storage temperatures are the most important factors in inhibiting the accumulation of these off-flavors.

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Received for review June 19, 1996. Revised manuscript received December 11, 1996. Accepted December 23, 1996.[⊗] This research was supported by Grant I-1967-91 from BARD, The United States−Israel Binational Agricultural Research and Development Fund. Florida Agriculture Experiment Station Journal Series No. R04732.

JF960435Z

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1997.