Role of Hydroxycinnamic Acids and Vinylphenols in the Flavor Alteration of Blood Orange Juices

Biagio Fallico,[†] Maria C. Lanza,[†] Emanuele Maccarone,*[,†] Carlo Nicolosi Asmundo,[†] and Paolo Rapisarda[‡]

Istituto di Industrie Agrarie, Università di Catania, Via Valdisavoia 5, 95123 Catania, Italy, and Istituto Sperimentale per l'Agrumicoltura, Corso Savoia 190, 95024 Acireale, Italy

Free and total ferulic and *p*-coumaric acids were determined in two processed blood orange juices produced in Italy: one freshly squeezed and the other from concentrate. Hydroxycinnamic acids and their corresponding decarboxylation products, *p*-vinylguaiacol and *p*-vinylphenol, were also determined in the juices for up to 4 months of storage at 4 and 25 °C. The flavor threshold of authentic vinylphenols in aqueous solution and in the blood orange juice was evaluated by sensory analysis. Concentration of these off-flavors in the stored juices abundantly exceeded the threshold values, especially in the juice from concentrate. The content of vinylphenols might provide a reliable index of blood orange juice quality.

Keywords: *Hydroxycinnamic acids; vinylphenols; blood orange juice; sensory analysis*

INTRODUCTION

trans-4-Hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, and sinapic) are very common in fruits and vegetables in the form of esters and glycosides. They carry out important functions in the maturation processes and in plant defense, and enhance fruit flavor quality. Chlorogenic acid (3-caffeoylquinic) is the main cinnamic compound found in apples and pears (Spanos and Wrolstad, 1992), caftaric acid (caffeoyltartaric) is typical of grapes (Lee and Jaworsky, 1987), and ferulic acid esters are characteristic of citrus fruits (Risch and Herrmann, 1988; Peleg *et al*., 1991). The cinnamic derivatives, together with other phenolic compounds, may be considered as markers which characterize and differentiate commercial juices (Fernandez de Simon *et al*., 1992).

The orange juice producing industry has been interested to these studies because ferulic acid esters may hydrolyze and the free acids may decarboxylate during thermal treatments and storage, with formation of *p*-vinylguaiacol (PVG), an unpleasant compound with a very low perception threshold (Tatum *et al*., 1975; Lee and Nagy, 1990). Panel tests have revealed that PVG is the most detrimental compound among other off-flavors, since it gives the characteristic "old fruit" flavor to juice. Other studies conducted by Peleg *et al*. (1992) on ferulic acid containing model orange juice revealed that PVG formation was reduced by storage under nitrogen and by the presence of Cu(II) ions. PVG accumulation is also limited by adding L-cysteine, which favors direct transformation of ferulic acid into vanillin (Naim *et al*., 1993). Naim *et al*. (1992, 1994) provided detailed reviews of progress in the field.

No studies have been conducted on the hydroxycinnamic acids of the blood oranges produced in Italy (Moro, Tarocco, and Sanguinello cultivars), even if the presence of these acids bound to anthocyanidin glucosides has been pointed out (Maccarone *et al*., 1985a).

* Author to whom correspondence should be addressed.

[†] Università di Catania.

[‡] Istituto Sperimentale per l'Agrumicoltura.

Table 1. Analytical Data of the Blood Orange Juices[a]

juice	freshly squeezed	reconstituted from concentrate
pH	3.18	3.25
Brix	11.3	11.3
acidity[b] (g/100)	1.43	1.38
reducing sugars (g/100)	4.28	4.16
total sugars (g/100)	7.95	6.80
ascorbic acid (mg/100 mL)	63.2	53.9
anthocyanins[c] (mg/L)	55.5	52.5

[a] Average values of two analytical determinations. [b] As anhydrous citric acid. [c] As cyanidin 3-glucoside (Rapisarda *et al*., 1994).

Following previous studies on the stabilization of blood orange juice (Maccarone *et al*., 1985b, 1987, 1988), the present study reports an investigation on total and free *p*-coumaric and ferulic acids, as precursors of vinylphenols, in two processed juices: a frozen freshly squeezed juice and a long-life pure juice reconstituted from frozen concentrate. The content of hydroxycinnamic acids was determined by HPLC in samples stored for up to 4 months at 4 and 25 °C. The quantities of *p*-vinylphenol (PVP) and PVG in the juices were also determined by gas chromatography (GC). The sensory detection threshold of the latter compounds in the juice was performed to evaluate their contribution to flavor alteration.

MATERIALS AND METHODS

Preparation of Samples and Standards. The reagents and solvents used were analytical or HPLC grade. *p*-Coumaric and ferulic acids and ethylguaiacol used as standards were available on the market (Sigma-Aldrich, Milan, Italy). The freshly squeezed juice (11.3° Brix, from FMC in line process) and concentrated juice (55° Brix, from taste multiple effect evaporator) came from the same lot of orange fruits and were supplied by Ortogel (Caltagirone, Catania, Italy) in March 1994. The 55° Brix concentrated juice was reconstituted at 11.3° Brix by diluting it with distilled water. The results of standard analysis of the juices are reported in Table 1. Sodium benzoate (400 mg/L) was added to the juices to prevent fermentation during storage. The samples were placed in 100 mL glass bottles with crown caps and stored for 4 months at 4 and 25 °C. Two bottles of each juice were randomly removed every month to determine hydroxycinnamic acids and vin-

S0021-8561(95)00331-1 CCC: $12.00

Table 2. ^{1}H NMR and Mass Spectra of *p*-Vinylphenol and *p*-Vinylguaiacol

	R = H: *p*-vinylphenol	R = OCH_3: *p*-vinylguaiacol
	^{1}H NMR: Chemical Shifts in $CDCl_3$	
H_a	6.57, 6.63, 6.66, 6.72, quartet (1H)	6.55, 6.61, 6.64, 6.69, quartet (1H)
H_b	5.09, 5.15, doublet (1H), J = 12 Hz	5.08, 5.14, doublet (1H), J = 12 Hz
H_c	5.55, 5.64, doublet (1H), J = 18 Hz	5.53, 5.62, doublet (1H), J = 18 Hz
H_1	7.28, 7.32, doublet (2H), J = 8.4 Hz	6.88, multiplet (3H)
H_2	6.77, 6.81, doublet (2H), J = 8.4 Hz	6.88, multiplet (3H)
OH	5.34, broad singlet (1H)	5.73, broad singlet (1H)
OCH_3		3.86, singlet (3H)
	Mass Spectra: *m*/*z* (Abundance %)	
	120 (95%) M^+	150 (90%) M^+
	91 (100%) $C_7H_7^+$	135% (100%) $M^+ - CH_3$
	65 (35%) $C_5H_5^+$	107 (65%) $C_7H_7O^+$
	39 (45%) $C_3H_3^+$	77 (75%) $C_6H_5^+$

ylphenols. PVG and PVP used as standards for GC and for sensory analysis were prepared by decarboxylation of ferulic and *p*-coumaric acids with copper in quinoline (Klaren-De Wit *et al*., 1971; Naim *et al*., 1988), and twice purified by column chromatography on silica gel. The structures were assessed by ^{1}H NMR and mass spectra (Table 2). The purity was >99.9%, checked by GC.

Hydroxycinnamic Acids. Extraction and HPLC of hydroxycinnamic acids were performed using procedures similar to those described by Naim *et al*. (1988) and Rouseff *et al*. (1992).

Free Acids. The juice was centrifuged at 5000 rpm for 20 min, and then 10 mL of the clear juice was acidified with 2 N HCl to pH 2 and extracted with three 20 mL portions of ethyl acetate. The three fractions were pooled and evaporated under reduced pressure. The residue was recovered using 5 mL of water−acetic acid−methanol solution (65−15−20).

Total Acids. Ten milliliters of 2 N NaOH was added to 10 mL of centrifuged juice and the solution was stored at room temperature in the dark for 4 h. The solution was then acidified with 2 N HCl to pH 2 and extracted using ethyl acetate, according to the same procedure described for free acids. Twenty microliters of solution was injected into a liquid chromatograph (Waters Model 600E) equipped with a W-484 spectrophotometer and a W-746 Data Module integrator. A Hypersil ODS 5 μm column (25 cm × 4.6 mm i.d.; Policonsult Scientifica, Milan, Italy) was used. Chromatograms were recorded using a 280 nm UV lamp, flow 1 mL/min. Elution was performed using a two-solvent mixture of A, tetrahydrofuran−water (80−20), and B, water−acetic acid (98−2), with the following program: isocratic A 2% and B 98% for 5 min, gradient up to A 7% and B 93% up to 10 min (curve 3), isocratic A 7% and B 93% up to 25 min, linear gradient up to A 20% and B 80% up to 40 min. Concentrations of *p*-coumaric and ferulic acids were calculated from the experimental peak area by analytical interpolation in standard calibration lines. The results of the linear regressions were the following: for *p*-coumaric acid, slope 249 777 ± 3512, R = 0.9993 (P < 0.001); for ferulic acid, slope 147 430 ± 1672, R = 0.9996 (P < 0.001). Extraction and HPLC analysis of hydroxycinnamic acids were carried out in duplicate.

Vinylphenols. Vinylphenols were extracted from the juices according to the Peleg *et al*. (1992) procedure, modified as follows: 10 mL of juice centrifuged at 5000 rpm for 20 min was passed onto a C18 Sepak (Waters) cartridge which had been pretreated with 5 mL of methanol, 5 mL of water, and 5 mL of 0.01 N HCl. The cartridge was then washed with 5 mL of water and the vinylphenols were eluted with 6 mL of methanol. The methanol solution was evaporated under reduced pressure, and the residue was recovered with 1 mL of methanol solution containing ethylguaiacol as internal standard. Of this 1 μL was injected into a gas chromatograph (Hewlett Packard, Model 5890) equipped with flame ionization detector and recorder−integrator (HP Model 3397A). Analyses

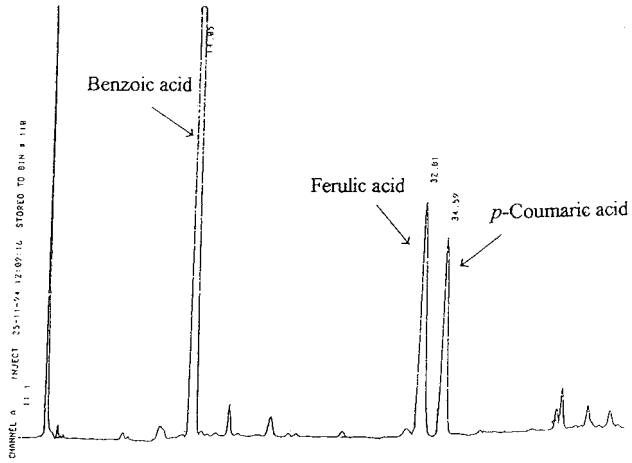

Figure 1. HPLC of the total hydroxycinnamic acids extracted from freshly squeezed blood orange juice.

conditions were as follows: capillary column SE54 (30 m, 0.25 mm), helium gas carrier, injector and detector at 230 and 260 °C respectively, oven temperature programmed between 150 and 230 °C with 2 °C/min gradient, split 1/50 and 26 psi column pressure (linear velocity 27.8 cm/s).

Sensory Detection Threshold of Vinylphenols. *Panel*. Tests were carried out with the collaboration of students, aged between 20 and 25, of the Agricultural Faculty in the University of Catania. Assessors were selected among 36 subjects (25 females and 11 males), and trained during different sessions using the difference from control test method (Amerine *et al*., 1965). A preliminary session was devoted to explain the aim of the work and the behavior recommended both before and during the test. After this selection−training phase the number of trained assessors amounted to 20 (14 females and 6 males).

Difference from Control Test. The difference from control test was carried out with an aqueous solution of PVG (0.041 ppm) and PVP (0.064 ppm) and pure water. The scores were rated on a scale indicating the degree of difference from control (PVG, 0.041 ppm), ranging from "not different" (corresponding to 1) to "extremely different" (corresponding to 9). Assessors were requested to compare, for smell and taste, samples presented in random order with the control sample. Assessors were also asked to indicate in what respect they considered the sample different.

Threshold Test. To define the threshold value of PVG and PVP in water, samples of aqueous solution containing increasing concentration of PVG and PVP were employed (0.01−0.040 ppm). Assessors were requested to recognize the sample difference, for odor and taste, with respect to reference sample (water). The same test was repeated using freshly squeezed blood orange juice with PVG and PVP added (0.02−0.07 ppm).

Statistical Analysis. Statistical analysis of sensory data was carried out using the SPSS for Windows, Version 6.1.2 (Norusis, 1995). One-way analysis of variance was used to evaluate data scattering around means; Duncan's multiple range test was used to evaluate the significance of difference. Significance at various probability levels of threshold values was established by the number of correct judgments (Stone and Sidel, 1993).

RESULTS AND DISCUSSION

Figure 1 shows a typical HPLC analysis of the total hydroxycinnamic acids extracted from the orange juice after alkaline hydrolysis. Peak 1 is due to the benzoic acid added to inhibit fermentation, while peaks 2 and 3 have been attributed to ferulic and *p*-coumaric acids, respectively, according to the retention times of standard acids and the chromatograms in the mixture. The content of the free and total acids in the two juices is shown in Table 3. Free acids were initially absent in both juices, apart from small quantities of ferulic acid

Table 3. Free and Total *p*-Coumaric and Ferulic Acids (mg/L)[a] in Processed Blood Orange Juices

months of storage	*p*-coumaric				ferulic			
	free		total		free		total	
	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C
Freshly Squeezed								
0	0.0	0.0	34.1	34.1	1.0	1.0	42.5	42.5
3	0.0	1.9	34.1	28.1	0.6	1.1	41.8	35.1
4	0.0	5.2	21.0	24.8	0.0	3.2	22.5	28.6
Reconstituted from Concentrate								
0	0.0	0.0	35.9	35.9	1.2	1.2	41.2	41.2
3	2.8	3.6	35.7	24.7	2.0	3.7	43.1	28.2
4	2.1	3.8	26.7	23.8	0.2	2.3	27.6	25.7

[a] Average values of two analytical determinations.

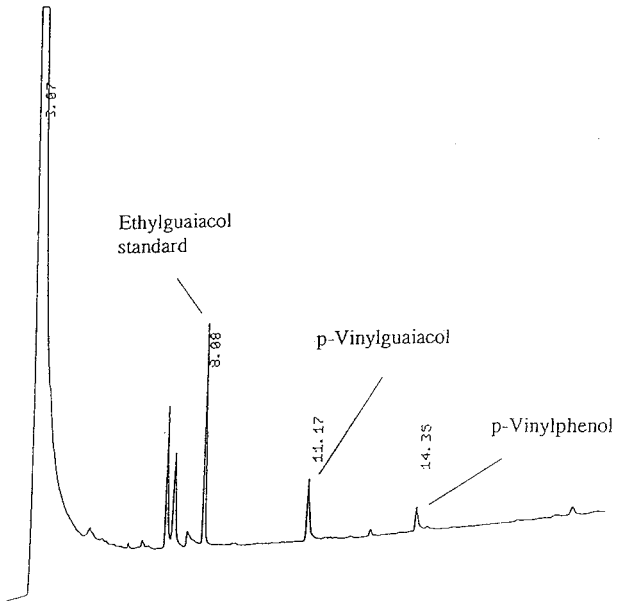

Figure 2. Gas chromatogram of vinylphenols extracted from freshly squeezed blood orange juice after 4 months of storage at 25 °C.

(about 1 mg/L). The total acids showed quantities of ferulic of about 40 mg/L and *p*-coumaric of about 35 mg/L. The overlapping values in the two juices indicate that the thermal evaporation treatment the reconstituted juice underwent did not determine cinnamic ester hydrolysis.

Free and total hydroxycinnamic acids were also determined monthly for a 4 month period. The content remained almost unchanged over the first 2 months, while a significant increase of free acid concentration, and a marked decrease in total acid concentration, was observed after 3−4 months of storage (Table 3). The reduction of the total acids was not compensated by a rise in the free acids, as the latter underwent decarboxylation to vinylphenols. Although free *p*-coumaric acid was absent initially, it was detected in the samples of both juices after 3−4 months of storage, with the concentration being generally higher in the juices stored at 25 °C. Free ferulic acid was practically absent in samples stored at 4 °C and only slightly above initial values in ones stored at 25 °C. There was a 35% reduction in concentrations of total *p*-coumaric and ferulic acids after 4 months, with degradation kinetics being more marked in juices stored at 25 °C.

Figure 2 shows a typical GC analysis of the vinylphenols in a juice sample after 4 months of storage. Initially, neither of the juices contained vinylphenols, nor were they detected within the first 2 months. They were present in traces at 3 months of storage, and in

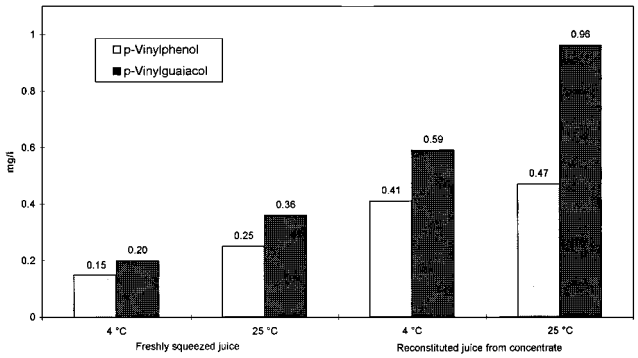

Figure 3. Content of *p*-vinylphenols in processed blood orange juice stored at 4 and 25 °C after 4 months of storage.

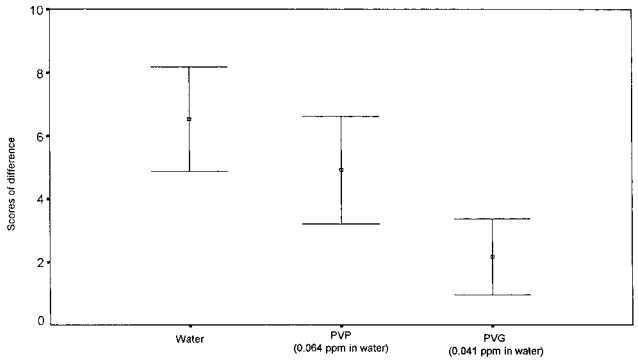

Figure 4. Results of the difference from control test. The vertical lines indicate the intervals for factor means (95% confidence level).

significant quantities at 4 months (Figure 3). As expected, the PVP and PVG quantities were higher in samples stored at 25 °C. Although the thermal concentration treatment the juice undergoes does not modify the original hydroxycinnamic distribution (Table 3), it negatively influences the factors determining stability, with the reconstitued juice showing a greater tendency to form vinylphenols than the freshly squeezed one.

The concentrations of vinylphenols in blood orange juices (Figure 3) have little meaning without the knowledge of the appropriate sensory detection threshold. An off-flavor is a problem only if its concentration exceeds the threshold value. PVG is a well-known off-flavor of the orange juice. Tatum *et al.* (1975) report that this compound, when added to control juice, imparted an "old fruit" or "rotten" taste, with a threshold value of 0.075 ppm ($P < 0.001$) and 0.050 ppm ($P < 0.01$). However the effects of PVP on the juice flavor are unknown to date, and the threshold concentrations of PVP and PVG have not yet been evaluated in blood orange juice. Therefore, some sensory measurements were performed by a 20-member experienced panel.

Preliminarily, the differences from control test were measured in aqueous solution. The results pointed out differences between PVG and PVP, and between PVG and water ($P < 0.05$), whereas no significant difference was found between PVP and water (Figure 4). This suggested that PVP is a less powerful off-flavor than PVG in aqueous solution.

Successively, the distribution of frequency of the flavor detection threshold was evaluated in aqueous solution and juice (Table 4). Threshold values were remarkably lower in water than in juice; however, in the latter they appeared to be more leveled, probably because of the flavor juice overlap. In all cases both substances have been evaluated as off-flavors having similar sensory characteristcs (medicine, mold, overripe).

Table 4. Sensory Detection Threshold of *p*-Vinylphenol (PVP) and *p*-Vinylguaicol (PVG) in Aqueous Solution and in Blood Orange Juice

	threshold value (ppm)	P^a
PVP(water)	0.022	0.005
	0.034	0.001
PVP(juice)	0.045	0.01
	0.067	0.005
PVG(water)	0.012	0.01
	0.023	0.001
PVG(juice)	0.033	0.03
	0.046	0.01

[a] Probability level (Stone and Sidel, 1993).

Table 5. Off-Flavor Units (U.F.) of *p*-Vinylphenol (PVP) and *p*-Vinylguaiacol (PVG) in the 4 Months Stored Juices

	PVP[a]		PVG[b]	
juices stored at	U.F.	%	U.F.	%
Freshly Squeezed				
4 °C	3.33	43.4	4.35	56.6
25 °C	5.55	41.5	7.83	58.5
Reconstituted from Concentrate				
4 °C	9.11	41.5	12.83	58.5
25 °C	10.44	33.3	20.87	66.7

[a] Threshold: 0.045 ppm, $P < 0.01$. [b] Threshold: 0.046 ppm, $P < 0.01$.

Threshold value of PVG in blood orange juice is almost equal to that measured in the not pigmented orange juice at the same probability level (Tatum *et al.*, 1975). PVP and PVG produced a predominant alteration in the taste of aqueous solutions and juices; only a few assessors remarked upon odor. However, alteration of both odor and taste were detected by most assessors during some preliminary trials carried out at concentrations higher than threshold values.

The actual concentration of each vinylphenol in the blood orange juices after 4 months of storage exceeded threshold concentration in all cases, especially in the juice reconstituted from concentrate. Table 5 reports the contribution of PVP and PVG to overall flavor alteration of the stored juices, in terms of off-flavor units (Guadagni *et al.*, 1966).

$$\text{off-flavor units} = \text{actual concentration (ppm)}/\text{threshold concentration (ppm)}$$

In conclusion, in the judgments of panelists, PVP is a "less concentrated" PVG in aqueous solution; but the two off-flavors have similar effects in the juice. Therefore, determination of both vinylphenols might provide a more definite index of blood orange juice quality.

LITERATURE CITED

Amerine, M. A.; Pangborn, R. M.; Roessler, E. B. *Principles of Sensory Evaluation of Food*; Academic Press: New York and London, 1965.

Fernandez de Simon, B.; Perez-Ilzarbe, J.; Hernandez, T.; Gomez-Cordoves, C.; Estrella, I. Importance of phenolic compounds for the characterization of fruit juices. *J. Agric. Food Chem.* **1992**, *40*, 1531−1535.

Guadagni, D. G.; Buttery, R. G.; Harris, J. Odour intensities of hop oil components. *J. Sci. Food Agric.* **1966**, *17*, 142−144.

Klaren-De Wit, M.; Frost, D. J.; Ward, J. P. Formation of p-vinylguaiacol oligomers in the thermal decarboxylation of ferulic acid. *Recl. Trav. Chim. Pays-Bas* **1971**, *90*, 906−911.

Lanza, C. M.; Pagliarini, E.; Tomaselli, F. Sensory and chemical evaluation of frozen blood orange juice. *Agric. Med.* **1995**, *125*, 421−426.

Lee, C. Y.; Jaworski, A. W. Phenolic compounds in white grapes grown in New York. *Am. J. Enol. Vitic.* **1987**, *38*, 277−280.

Lee, H. S.; Nagy, S. Formation of 4-vinylguaiacol in adversely stored orange juice measured by an improved HPLC method. *J. Food Sci.* **1990**, *55*, 162−163, 166.

Maccarone, E.; Maccarrone, A.; Rapisarda, P. Acylated anthocyanins from oranges. *Ann. Chim., Rome* **1985a**, *75*, 79−86.

Maccarone, E.; Maccarrone, A.; Rapisarda, P. Stabilization of anthocyanins of blood orange fruit juice. *J. Food Sci.* **1985b**, *50*, 901−904.

Maccarone, E.; Maccarrone, A.; Rapisarda, P. Color Stabilization of orange fruit juice by tannic acid. *Int. J. Food Sci. Technol.* **1987**, *22*, 159−162.

Maccarone, E.; Longo, M. L.; Leuzzi, U.; Maccarrone, A.; Passerini, A. Stabilizzazione del succo d'arancia pigmentata con trattamenti fisici e additivi fenolici. *Chim. Ind., Milan* **1988**, *70* (11), 95−98.

Naim, M.; Striem, B. J.; Kanner, J.; Peleg, H. Potential of ferulic acid as a precursor to off-flavors in stored orange juice. *J. Food Sci.* **1988**, *53*, 500−503, 512.

Naim, M.; Zehavi, U.; Nagy, S.; Rouseff, R. L. Hydroxycinnamic acids as off-flavor precursors in citrus fruits and their products. In *Phenolic Compounds in Food and Their Effects in Health*; Ho, I. C. T., Lee, C. Y., Huang, M. T., Eds.; ACS Symposium Series 506; American Chemical Society: Washington, DC, 1992.

Naim, M.; Zuker, I.; Zehavi, U.; Rouseff, R. L. Inhibition by thiol compounds of off-flavor formation in stored orange juice. II. Effect of L-cysteine and *N*-acetyl-L-cysteine on *p*-vinylguaiacol formation. *J. Agric. Food Chem.* **1993**, *41*, 1359−1361.

Naim, M.; Zehavi, U.; Zuker, I.; Rouseff, R. L.; Nagy, S. Effect of L-cysteine and *N*-acetyl-L-apteine on off-flavor formation in stored citrus products. In *Sulfur Compounds in Food*; Mussinan, C. J., Keelen, M. E., Eds.; ACS Symposium Series 564; American Chemical Society: Washington, DC, 1994.

Norusis, M. J. *SPSS for Windows. Version 6.1.2*; SPSS *Inc.:* Chicago, 1995.

Peleg, H.; Naim, M.; Rouseff, R. L.; Zehavi, U. Distribution of bound and free phenolic acids in oranges (*Citrus sinensis)* and grapefruits (*Citrus paradisi*). *J. Sci. Food Agric.* **1991**, *57*, 417−426.

Peleg, H.; Naim, M.; Zehavi, U.;Rouseff, R. L.; Nagy, S. Pathways of 4-vinylguaiacol formation from ferulic acid in model solution of orange juice. *J. Agric. Food Chem.* **1992**, *40*, 764−767.

Rapisarda, P.; Fallico, B.; Izzo, R.; Maccarone, E. A simple and reliable method for determining anthocyanins in blood orange juices. *Agrochimica* **1994**, *38*, 147−164.

Risch, B.; Herrmann, K. Hydroxycinnamic acid derivatives in citrus fruits. *Z. Lebensm. Unters. Forsch.* **1988**, *187*, 530−534.

Rouseff, R. L.; Seetharaman, K.; Naim, M.; Nagy, S.; Zehavi, U. Improved HPLC determination of hydroxycinnamic acids in orange juice using solvents containing THF. *J. Agric. Food Chem.* **1992**, *40*, 1139−1143.

Spanos, G. A.; Wrostald, R. E. Phenolics of apple, pear and white grape juices and their changes with processing and storage. A review. *J. Agric. Food Chem.* **1992**, *40*, 1478−1487.

Stone, H.; Sidel, J. L. *Sensory Evaluation Practices*, 2nd ed.; Academic Press: New York and London, 1993.

Tatum, J. H.; Nagy, S.; Berry, R. E. Degradation products formed in canned single-strength orange juice during storage. *J. Food Sci.* **1975**, *40*, 707−709.

Received for review June 1, 1995. Revised manuscript received April 29, 1996. Accepted June 6, 1996.[⊗] Research supported by the National Research Council of Italy, Special Project RAISA, Subproject No. 4, Paper No. 1875.

JF9503319

[⊗] Abstract published in *Advance ACS Abstracts*, August 1, 1996.