Inhibition by Thiol Compounds of Off-Flavor Formation in Stored Orange Juice. 1. Effect of L-Cysteine and *N*-Acetyl-L-cysteine on 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone Formation†

Michael Naim,*,‡ Shirley Wainish,‡ Uri Zehavi,‡ Hanna Peleg,‡ Russell L. Rouseff,§ and Steven Nagy‖

Department of Biochemistry, Food Science and Nutrition, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76-100, Israel, Citrus Research and Education Center, University of Florida, Lake Alfred, Florida 33850, and Citrus Research and Education Center, Florida Department of Citrus, Lake Alfred, Florida 33850

Orange juice stored at 45 °C for 7 and 14 days resulted in increased browning and increased concentration of sugar degradation products such as 5-(hydroxymethyl)furfural (HMF) and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF). Addition of L-cysteine (2.5–12.3 mM) significantly reduced browning and HMF and DMHF formation in the 14-day-stored orange juice in a dose-related manner. Ascorbic acid retention increased with increasing L-cysteine concentrations. Low levels (2.5 mM and below) of L-cysteine and *N*-acetyl-L-cysteine reduced browning and DMHF formation, but only L-cysteine reduced ascorbic acid degradation. Sensory similarity analyses indicated that orange juice fortified with 0.5 mM L-cysteine during 14 days of storage at 45 °C produced aroma that was most similar to that of the control juice kept at 4 °C compared to other treatments. The addition of *N*-acetyl-L-cysteine produced juices with aroma that was located in an inferior position in the similarity diagram when compared to that of juices fortified by L-cysteine.

INTRODUCTION

Maillard and other nonenzymic reactions that occur during processing and storage of food products are detrimental to color (browning), aroma, and taste (Handwerk and Coleman, 1988; Meydav and Berk, 1978; Shaw et al., 1977; Tatum et al., 1975). Sodium sulfite has been used to minimize Maillard-type browning in a variety of foods including citrus. However, because some human subjects are sensitive to sulfite (Brown, 1985; Taylor and Bush, 1986), the use of sodium sulfite has been restricted or eliminated in most foods. It is not allowed to be added to citrus products. Early suggestions [e.g., Arnold (1969) and Montgomery (1983)] for the use of L-cysteine to inhibit nonenzymic browning have not attracted much further research since cysteine, in itself, is a source for flavors in some foods (Hurrell, 1982; Shu et al., 1985a) and can be objectional in fruit juice (Molnar-Perl and Friedman, 1990a). Nevertheless, recent studies (Molnar-Perl and Friedman, 1990a,b) suggest that thiols should be considered as additives for browning prevention. Some of these thiols are natural components in human diets and play significant physiological roles in vivo as nucleophiles and scavengers of free radicals (Friedman, 1991). 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), one of the putative degradation products of sugar (Baltes, 1982; Shaw et al., 1967), possesses an intense pineapple flavor. In stored citrus products, DMHF is formed by nonenzymic processes producing a characteristic malodorous, pineapple-like flavor in aged canned orange juice (Tatum et al., 1975). The detection threshold was 0.1 ppm. In addition, it can mask the fresh orange juice aroma at levels above 0.05 ppm (Nagy et al., 1989). When formed, DMHF can be further degraded to additional flavors (Shu et al., 1985b).

The present study investigated the effects of L-cysteine and *N*-acetyl-L-cysteine on the formation of DMHF, browning, and 5-(hydroxymethyl)furfural (HMF) and on ascorbic acid retention in orange juice during accelerated nonenzymic browning. The significance of the thiol effects to the juice aroma was also studied.

MATERIALS AND METHODS

Materials. L-Cysteine, *N*-acetyl-L-cysteine, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), and 5-(hydroxymethyl)furfural (HMF) were purchased from Sigma. Commercial single-strength orange juice (SSOJ) was purchased from Rimon, Givaat Brener, Israel.

Preparation and Storage of Fortified SSOJ Samples. Pasteurized SSOJ (1-L bottles) was preserved with 500 ppm of sodium benzoate. Twenty-milliliter SSOJ samples with and without added thiol compounds were transferred to 20-mL glass vials, sealed, and then stored at 45 °C. During the first series of experiments (conducted for either 7 or 14 days), relatively high doses (2.5–12.3 mM) of L-cysteine were used, whereas in the second series (last 14 days) low levels (0.5–2.5 mM) of either L-cysteine or *N*-acetyl-L-cysteine were applied.

Chemical Analyses. Following storage, DMHF and HMF were extracted according to the method of Lee and Nagy (1987). HMF and DMHF were analyzed by HPLC equipped with a Lichrosorb RP-18 column (5 μm, 250 mm × 4 mm, Merck) and with a RP-18 precolumn (25 mm × 4 mm, Merck). The column was eluted isocratically with 1.5% acetic acid in water/methanol (20:80) at a flow rate of 0.5 mL/min, at room temperature. Each sample (20 μL) was injected twice. The separated chromatographic peaks were identified and quantified with known markers by a Chrom-A-Scope UV–visible rapid-scanning detector (Barspec, Rehovot, Israel) set for HMF and DMHF at 290 nm (with similar results at 270 nm). The detection threshold of DMHF by this detector was 7.5 ng. Browning was determined in 10-mL stored SSOJ samples after centrifugation (4 °C, 5 min, 500*g*) and ethanol

* Author to whom correspondence should be addressed.

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‡ The Hebrew University of Jerusalem.
§ University of Florida.
‖ Florida Department of Citrus.

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extraction of the supernatant (Meydav and Berk, 1978). The absorbance at 420 nm was measured. Ascorbic acid content was determined potentiometrically (Speath et al., 1962). The levels of thiols added to juice (up to 12.3 mM) did not affect ascorbic acid determination in the potentiometric procedure. L-Cysteine and *N*-acetyl-L-cysteine were determined in SSOJ according to the procedure of Ellman (1959).

Sensory Analysis. The significance of thiols to aroma characteristics was studied using cluster analysis (Everitt, 1974; Naim et al., 1986; Sattath and Tversky, 1977). Eight students, 20–25 years old, evaluated the aroma similarity for eight orange juice treatments. Panelists were unaware of the objectives or the experimental design and requested to avoid eating or drinking (except water) for 1 h prior to a session. They were trained during a 2-h session with the smelling procedure. Vials containing samples were wrapped with aluminum foil so that panelists could not see the color of the tested samples, which were adjusted to room temperature prior to each aroma test. During each session, three aroma evaluation tests were performed with 30-min intervals. Only one panel member was present in the testing room during each session. On a verbal signal from the experimenter, a panelist opened the vials of two samples at 15-s intervals, smelled them, and was requested to rate the similarity level of aroma of the two samples on a 1–20 scale (1 for no similarity, 20 for identical). Each panelist did so for all 64 treatment combinations presented in coded randomized order, and this procedure was replicated three times. Two sensory evaluation experiments were performed, the first in the spring of 1991 and the second in the spring of 1992. In each experiment different panelists and different juice samples were used.

Data Analyses. Results of chemical analyses were tested by one- and two-way analyses of variance using SAS statistical package programs. Duncan multiple-range test was performed for comparisons among the means. A data matrix representing the similarity results for each of the sensory tests was obtained, where each cell in the matrix represents the mean similarity for all panelists for the corresponding comparison. This proximity matrix was then analyzed by a clustering program (ADDTREE; Sattath and Tversky, 1977), which yields a tree structure of branches and subdivisions with aromas located at the ends of the branches. Sets of aromas that are connected to the same node with relatively short (horizontal) distances from each other represent high similarity.

RESULTS AND DISCUSSION

In line with recent data on browning inhibition by thiol compounds (Molnar-Perl and Friedman, 1990a,b), the present results indicate that the use of a variety of concentrations of L-cysteine inhibited browning and HMF (RT in HPLC = 7.5 ± 0.13 min) formation and reduced ascorbic acid degradation (all at least at $p < 0.01$) in a dose-dependent manner (Figure 1). The effect of cysteine on the formation of DMHF was complex. During the first series of experiments (Figure 2, 7 days of storage), L-cysteine at concentrations of 2.5 and 4.1 mM stimulated and 12.3 mM inhibited the formation of DMHF (RT in HPLC = 12 ± 0.13 min). After 14 days of storage, regardless of concentration, L-cysteine inhibited significantly ($p < 0.001$) the formation of DMHF. The reason for the discrepancy between the effects of low and high doses of cysteine on DMHF formation is unknown at this time. If a free radical mechanism is involved in L-cysteine inhibition of DMHF formation, then the results may be related to the effects of other reducing agents (e.g., phenols, ascorbic acid, and tocopherol). These agents may accelerate free radical formation at low doses while acting as effective scavengers at high concentrations [e.g., Naim et al. (1976) and Kanner et al. (1977)]. Although the mechanism for the inhibition of DMHF formation by L-cysteine remains to be elucidated, the addition of L-cysteine to active carbonyl groups may occur in the early phase of sugar degradation.

The objective of the second series of experiments was to test the effect of low levels of thiols as high levels may,

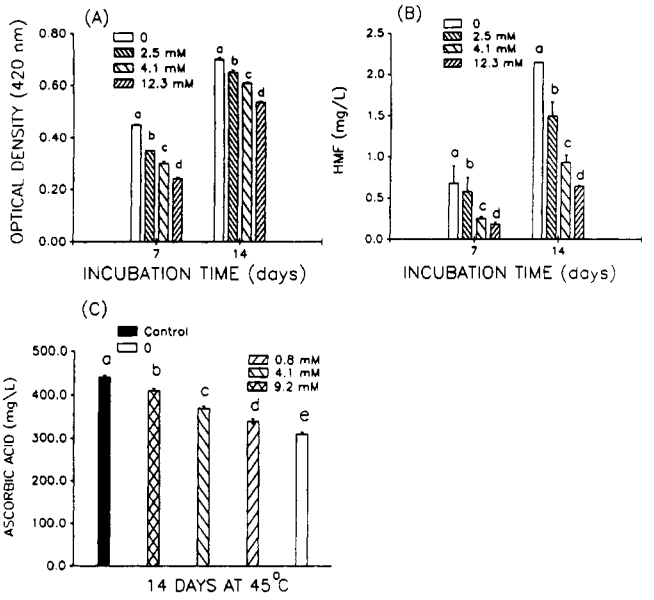

Figure 1. Effects of L-cysteine on browning (A), HMF formation (B), and ascorbic acid retention (C) in SSOJ stored for 7 and 14 days at 45 °C. Values for optical density and ascorbic acid level are the mean and SEM of two samples. Values for HMF formation are the mean and SEM of three samples, each analyzed twice by HPLC. Control juice was stored at 4 °C. Bars not sharing the same superscript letter are different by at least the $p < 0.05$ level. In cases where SEM bars are missing, their values were too small to be shown in the figure.

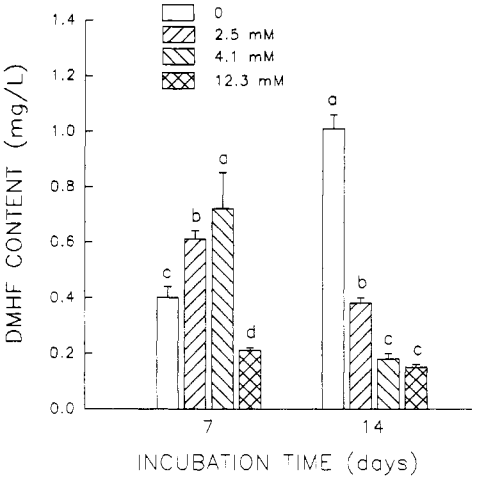

Figure 2. Effects of L-cysteine on DMHF formation in SSOJ. Samples were stored for 7 and 14 days at 45 °C. Values are the mean and SEM of three samples, each analyzed twice by HPLC. Bars not sharing the same superscript letter are different by at least the $p < 0.05$ level.

in themselves, lead to the formation of off-flavor in fruit juices (Molnar-Perl and Friedman, 1990a). *N*-Acetyl-L-cysteine was used since it is believed to be an effective inhibitor of browning with less off-flavor formation (Molnar-Perl and Friedman, 1990a). The results of the second series of experiments demonstrated that low levels of thiols (below 2.5 mM) can significantly reduce browning and DMHF ($p < 0.01$; see Figures 3 and 4) formation in stored SSOJ. A level of 2.5 mM L-cysteine was also effective in reducing HMF content (Figure 5). Furthermore, low levels of L-cysteine reduced ascorbic acid degradation by 40% ($p < 0.001$; Figure 6). In contrast, the same levels of *N*-acetyl-L-cysteine were ineffective in preventing ascorbic acid degradation. At the end of the 14 days of storage, due to the addition of 0.5, 1.0, and 2.5 mM thiols, the resulting residues of L-cysteine and *N*-acetyl-L-cysteine in SSOJ fortified samples were 0.49, 0.55, and 0.90 mM L-cysteine and 0.46, 0.80, and 0.95 mM

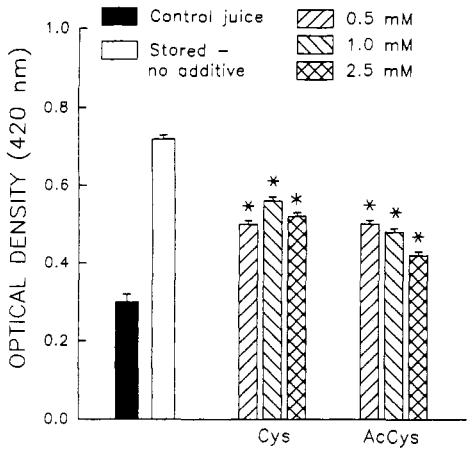

Figure 3. Effects of low levels of L-cysteine (Cys) and *N*-acetyl-L-cysteine (AcCys) on browning in SSOJ samples stored for 14 days at 45 °C. Control juice was stored at 4 °C. Values are the mean and SEM of three samples. Asterisks indicate significantly ($p < 0.05$) lower values than that observed in samples stored with no thiol fortification.

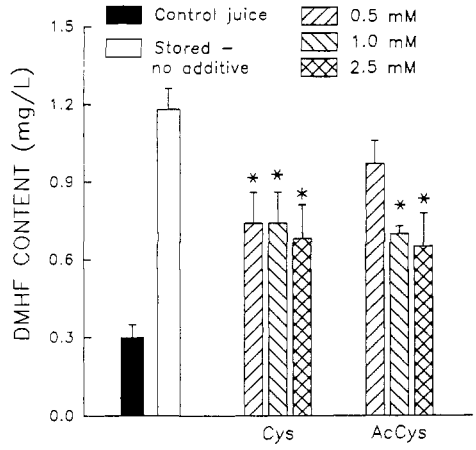

Figure 4. Effects of low levels of L-cysteine (Cys) and *N*-acetyl-L-cysteine (AcCys) on formation of DMHF in SSOJ samples stored for 14 days at 45 °C. Control juice was stored at 4 °C. Values are the mean and SEM of three samples, each analyzed twice by HPLC. Asterisks indicate significantly ($p < 0.05$) lower values than that observed in samples stored with no thiol fortification.

N-acetyl-L-cysteine. It is possible that the lack of *N*-acetyl-L-cysteine effect on ascorbic acid degradation, as compared to that of L-cysteine, is related to the difference between these two compounds in their ability to form complexes with heavy metal ions such as copper ions. Complexes (chelating properties) between copper and amino acids or peptides are well recognized (Liang and Olin, 1984; Thomas and Zacharias, 1984). Chelates (e.g., EDTA) have been reported to reduce browning and ascorbic acid degradation in grapefruit juice (Kanner and Shapira, 1989). Cysteine may be more effective in forming metal complexes when compared to *N*-acetyl-L-cysteine due the presence of a free amino group. Furthermore, L-cysteine and apparently also *N*-acetyl-L-cysteine, being in great excess compared to the content of Cu^{2+}, are expected to reduce Cu^{2+} to Cu^{+}, which in turn forms a particularly stable complex with L-cysteine (Levitzki, 1964). L-Cysteine was also more effective than *N*-acetyl-L-cysteine in reducing HMF (RT in HPLC = 12 ± 0.13 min) formation (Figure 5) during storage.

In conjunction with chemical analyses, the significance of thiols to aroma characteristics was studied in two different tests (Figure 7) using cluster analysis. The proportion of variance explained was 0.677 ($r = 0.84$) and 0.774 ($r = 0.86$) for experiment 1 and 2, respectively,

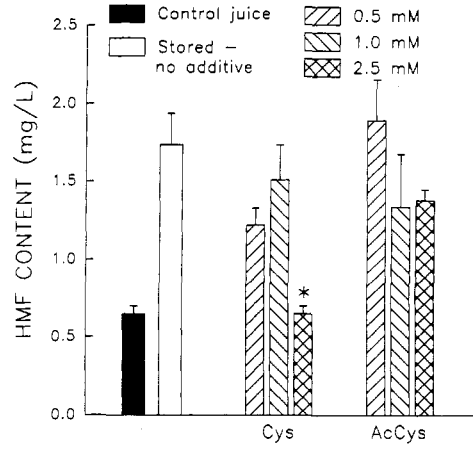

Figure 5. Effects of low levels of L-cysteine (Cys) and *N*-acetyl-L-cysteine (AcCys) on formation of HMF in SSOJ samples stored for 14 days at 45 °C. Control juice was stored at 4 °C. Values are the mean and SEM of three samples, each analyzed twice by HPLC. The asterisk indicates a significantly ($p < 0.05$) lower value than that observed in samples stored with no thiol fortification.

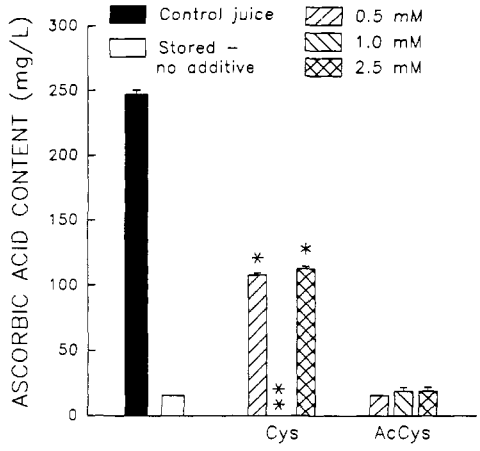

Figure 6. Effects of low levels of L-cysteine (Cys) and *N*-acetyl-L-cysteine (AcCys) on ascorbic acid retention in SSOJ samples stored for 14 days at 45 °C. Control juice was stored at 4 °C. Values are the mean and SEM of three samples. Asterisks indicate significantly ($p < 0.05$) higher values than that observed in stored with no thiol fortification. A double asterisk indicates that no value was available.

suggesting that distances in the clustering structure are highly correlated with the original aroma similarity data. Although these tests were conducted at different times, with different SSOJ samples and with different panelists, the results were almost identical. Two major clusters were obtained in both experiments, and the structures were essentially the same. The only main difference between the two experiments was the location of branch 7. In one experiment (Figure 7A), it joins branch 1 with branches 5 and 6 in between, whereas in the other (Figure 7B), branch 7 joins branch 1 directly. The most intriguing finding in both experiments was that the addition of 0.5 mM L-cysteine (60 ppm) to stored SSOJ resulted in a branch (2) connected to the same node as that of control juice kept at 4 °C (branch 8), indicating that both exhibited similar aroma. This suggests that relatively minor amounts of L-cysteine added to juice stored at an elevated temperature were able either to retain the aroma of the original juice or to reduce off-flavor formation. Other treatments (higher doses of cysteine or the use of *N*-acetyl-L-cysteine) either indicated similar aroma to that of the stored–untreated juice (no. 1) or produced completely different aroma. In both similarity experiments, the aroma of *N*-acetyl-L-cysteine-fortified SSOJ samples (branches 5–7)

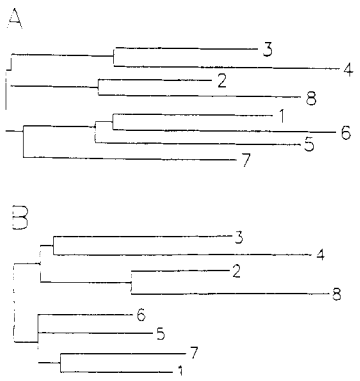

Figure 7. Cluster analysis by ADDTREE of aroma similarity values during experiment 1 (A) and experiment 2 (B). 1, SSOJ stored at 45 °C for 14 days; 2, with added 0.5 mM L-cysteine; 3, with 1 mM L-cysteine; 4, with 2.5 mM L-cysteine; 5, with 0.5 mM *N*-acetyl-L-cysteine; 6, with 1 mM *N*-acetyl-L-cysteine; 7, with 2.5 mM *N*-acetyl-L-cysteine; 8, control SSOJ, stored at 4 °C. Each value in the original matrix represents the mean of eight panelists (each was tested three times).

grouped together, separate from the L-cysteine-fortified samples (branches 2–4) which were always connected to the aroma of control SSOJ samples (branch 8). This indicates that, in line with the chemical analyses, the aroma of *N*-acetyl-L-cysteine-fortified SSOJ was inferior compared with that of L-cysteine-fortified SSOJ.

In conclusion, the present chemical experiments suggest that browning and DMHF formation and ascorbic acid degradation in stored SSOJ can be significantly reduced by low doses (0.5 mM) of L-cysteine, an amino acid found in almost all proteins. The aroma similarity experiments were compatible with the chemical analyses suggesting that such a low dose of L-cysteine may significantly improve the aroma quality of stored orange juice. Furthermore, addition of similar levels of L-cysteine to stored SSOJ and model solutions of orange juice (Naim et al., 1993) inhibited significantly the formation of *p*-vinylguaiacol, another major off-flavor in citrus product. Therefore, the consideration of the use of low levels of L-cysteine (and perhaps similar thiols) to retain the fresh aroma of orange juice should be further tested under practical processing and storage conditions.

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