3-Hydroxy-4,5-dimethyl-2(5*H*)-furanone (Sotolon) Causing an Off-Flavor: Elucidation of Its Formation Pathways during Storage of Citrus Soft Drinks

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Gas chromatography/olfactometry (GCO) and gas chromatography−mass spectrometry (HRGC−MS) revealed 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolon) to be responsible for the "burnt" and "spicy" off-flavor observed in citrus soft drinks during storage. Among the ingredients of citrus soft drinks, ethanol and ascorbic acid were identified as the essential precursors of sotolon. Two formation pathways were postulated by studies using ^{2}H (D)- and ^{13}C-labeled ethanol and ascorbic acid; i.e., sotolon is formed from two molecules of ethanol and carbons 2 and 3 of ascorbic acid (pathway 1), or it is generated from one molecule of ethanol and carbons 3−6 of ascorbic acid (pathway 2).

Keywords: *3-Hydroxy-4,5-dimethyl-2(5H)-furanone; sotolon; off-flavor; ascorbic acid; ethanol; stable isotopes; soft drink*

INTRODUCTION

3-Hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolon) is an impressive flavor compound with a very low threshold value of 0.02 ng/L of air (Blank et al., 1996). It contributes significantly to the characteristic sensorical impression of several foods, e.g., stewed beef (Guth and Grosch, 1994), roasted coffee (Blank et al., 1992a; Semmelroch et al., 1995), and bread crust (Schieberle and Grosch, 1994), as well as flor-sherry and botrytized wine (Guichard et al., 1992; Martin et al., 1992). Sotolon has been described first as a degradation product of threonine (Sulser et al., 1967). Later, Kobayashi (1989) reported on the formation of sotolon in sake and wine by an aldol condensation of acetaldehyde and α-ketobutyric acid followed by lactonization. The α-ketobutyric acid can be either derived from threonine or formed by the condensation of two molecules of acetaldehyde (Takahashi et al., 1976; Pisarnitskii et al., 1988). Thermally induced oxidative deamination of 4-hydroxyisoleucine yielded also sotolon (Sauvaire et al., 1984; Blank et al., 1992b, 1996), and recently, Hofmann and Schieberle (1996) detected sotolon after heating of an aqueous solution containing hydroxyacetaldehyde and butane-2,3-dione at pH 5.

The aim of our study was to elucidate the formation pathways of sotolon which has been recognized to cause an off-flavor in citrus soft drinks during storage. In this paper, the results of our investigations using isotopically labeled precursors are described.

EXPERIMENTAL PROCEDURES

Chemicals. The chemicals obtained from Aldrich (Steinheim, Germany) and Fluka (Neu-Ulm, Germany) were of high purity at purchase. Solvents were redistilled before use. [1,1-D_2]Ethanol, [2,2,2-D_3]ethanol, [1,1,2,2,2-D_5]ethanol, and [$^{13}C_2$]-ethanol were purchased from Aldrich; [1-^{13}C]ascorbic acid, [2-^{13}C]ascorbic acid, and [3-^{13}C]ascorbic acid were obtained from Omicron Biochemicals (South Bend, IN). Lemon oil, lime oil, and citral were kindly provided by Doehler GmbH (Darmstadt, Germany).

Preparation of Model Soft Drinks. Various model soft drinks were prepared from the individual essential oils (lemon and lime), citral, or individual essences with or without the addition of ascorbic acid according to a recipe provided by Doehler GmbH. The model soft drinks were stored at 70 °C for 2 weeks and analyzed by HRGC−MS.

Model Solutions. Solutions consisting of 250 μL of ethanol, 83 mg of ascorbic acid, and 42 mL of water were stored for 2 weeks at 70 °C. The solution was extracted and subsequently analyzed by HRGC−MS. Several modifications were applied: (i) addition of EDTA (50 mg), (ii) storage under nitrogen, and (iii) substitution of ascorbic acid with dehydroascorbic acid.

Extraction. Soft drinks (1000 mL), model soft drinks (250 mL), and model solutions (42 mL) were subjected to continuous liquid−liquid extraction using 250 mL of a pentane/dichloromethane mixture (2/1, v/v) for 24 h. The organic extract was dried over anhydrous sodium sulfate, concentrated to approximately 0.5 mL using a Vigreux column, and analyzed by HRGC−MS.

High-Resolution Gas Chromatography−Mass Spectrometry (HRGC−MS). HRGC−MS analysis was performed with a Fisons Instrument (Egelsbach, Germany) GC 8000 Series system fitted with a split injector (1:20) at 230 °C coupled with a Fisons Instrument MD800 quadrupole mass detector. A J&W DB-Wax fused silica capillary column (30 m × 0.25 mm inside diameter; df = 0.25 μm) which was programmed from 50 °C for 3 min and then to 220 °C (for 10 min) at a rate of 4 °C/min, was used with helium at a flow rate of 2 mL/min. The software Masslab for windows was used for data aquisition. The MS operating parameters were as follows: 70 eV; ion source temperature, 220 °C; interface temperature, 250 °C; scan range, 40−250 units (mass units); and scan duration, 0.69 s. Identification was carried out by comparison of mass spectral data and retention indices with those of authentic reference compounds.

Gas Chromatography/Olfactometry (GCO). The GCO analyses were performed with a Dani 6500 HRGC system equipped with a special nose adapter as detector. The chromatographically separated compounds were sensorically characterized by the operator using the nose adapter, and the retention times were recorded. The chromatographic conditions were the same as described for HRGC−MS analysis.

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High-Performance Liquid Chromatography (HPLC). Quantification was achieved by HPLC analysis. Reverse-phase HPLC was carried out on an Eurospher 100 C-18 column (250 mm × 4 mm, 5 μm, Knauer, Berlin, Germany) using a flow rate of 1 mL/min, employing a Knauer HPLC pump MaxiStar coupled with a Knauer multiwavelength UV/VIS detector at 235 nm. The following gradient with solvent A (acetonitrile) and sovent B (H_2O adjusted to pH 2.5 with H_2SO_4) was used: from 0 to 5 min, 100% B; from 5 to 20 min, 75% B; and from 20 to 30 min, 10% B.

Multidimensional Gas Chromatography−Mass Spectrometry (MDGC−MS). MDGC−MS anaylsis was performed with two Fisons gas chromatographs (8160 and 8130) fitted with a split injector (1:10) at 230 °C, a FID at 250 °C, and a Fisons Instrument MD800 quadrupole mass detector (as decribed above). A J&W DB-Wax fused silica capillary column (30 m × 0.25 mm inside diameter; df = 0.25 μm) was used in the first GC run for the preseparation of volatiles. Separation of enantiomers was achieved in the second GC run using a fused silica capillary column coated with 30% heptakis(2,6-di-*O*-methyl-3-*O*-pentyldimethylsilyl)-β-cyclodextrin/OV1701 (30 m × 0.25 mm; df = 0.25 μm). The column for GC 1 was connected to a moving column stream switch (MCSS) (Fisons) to the column for GC 2. The following temperature programs were applied: GC 1, from 100 to 240 °C at a rate of 10 °C/min; and GC 2, 80 °C, 20 min at the isothermal temperature, and then to 200 °C at a rate of 5 °C/min.

RESULTS

During the storage of citrus soft drinks, a "burnt" and "spicy" off-flavor was observed. An extract obtained by liquid−liquid extraction of the citrus soft drinks was analyzed by gas chromatography/olfactometry (GCO) and gas chromatography−mass spectrometry (HRGC−MS). A trace constituent of the extract showed the same odor impression as the off-flavor exhibited by the soft drink. This compound was identified as 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolon) by HRGC−MS analysis by comparison with the authentic reference.

The citrus essence used for the production of the soft drinks consisted of ethanolic extracts of essential oils such as lemon oil (lemon essence), lime oil (lime essence), and citral. To gain insight into the formation of sotolon, we prepared various model soft drinks composed of the individual essential oils or individual essences with or without the addition of ascorbic acid. Model soft drinks were stored at 70 °C for 2 weeks and analyzed by HRGC−MS. Sotolon was only detected in model soft drinks based on essences and supplemented with ascorbic acid. As the essences are ethanolic extracts of essential oils, the major difference is the occurrence of ethanol in the essences. Therefore, we assumed that ethanol and ascorbic acid are important constituents of the soft drinks responsible for the formation of sotolon. Model soft drinks prepared from methanolic or propanolic extracts of essential oils were devoid of the furanone.

Final evidence was provided by a model solution consisting of only ethanol and ascorbic acid. After storage at 70 °C for 2 weeks, this solution exhibited the typical burnt and spicy off-flavor and sotolon was detected by HRGC−MS. Storage of the same solution under nitrogen or addition of EDTA inhibited the formation of the furanone, indicating the importance of oxygen and metal ions such as iron, respectively, for the production of the off-flavor. Quantitative HPLC analysis revealed that the amount of sotolon gradually increased from 58 to 2380 and 8560 ng after storage periods of 1, 2, and 3 weeks, respectively. MDGC−MS analysis using

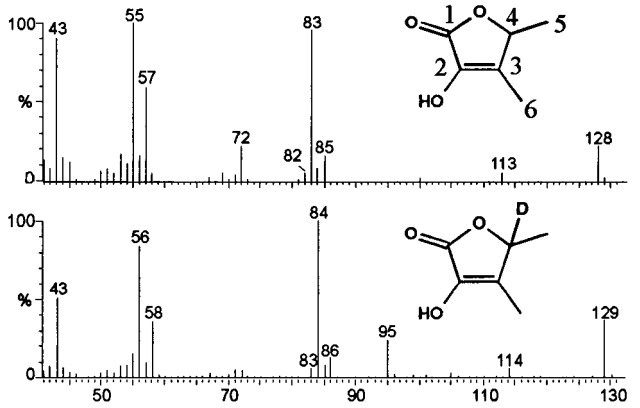

Figure 1. Mass spectra of unlabeled sotolone (upper trace) and sotolone formed from [1,1-D_2]ethanol and ascorbic acid (lower trace).

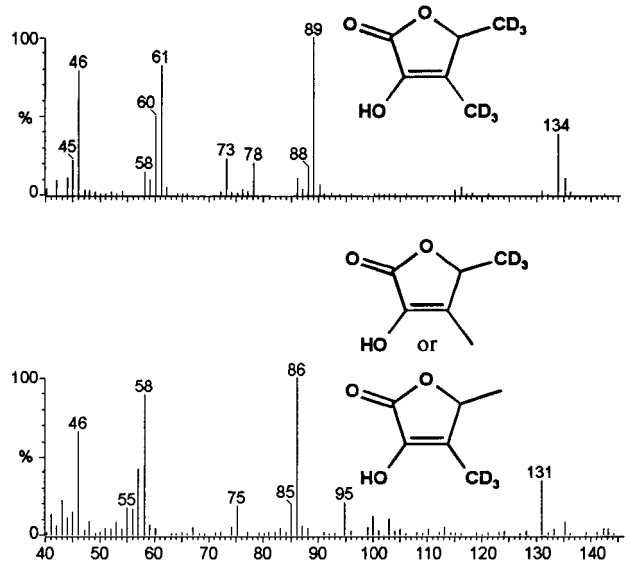

Figure 2. Mass spectra of sotolone isotopomer 1 (upper trace) and isotopomer 2 (lower trace) formed from [2,2,2-D_3]ethanol and ascorbic acid.

the chiral phase demonstrated the presence of racemic sotolon. The furanone was also produced from dehydroascorbic acid and ethanol. Dehydroascorbic acid was as effective as ascorbic acid. We observed no pH dependency for sotolon formation in the pH range of 5−9. However, it was very difficult to quantify nanogram amounts of sotolon. Therefore, minor change cannot be observed.

The generation of sotolon from ethanol and ascorbic acid was further investigated in model reactions with isotopically labeled precursors. [1,1-D_2]Ethanol, [2,2,2-D_3]ethanol, and [1,1,2,2,2-D_5]ethanol were added to aqueous solutions of unlabeled ascorbic acid, and the mixtures were stored at 70 °C for 2 weeks. HRGC−MS analysis revealed that sotolon formed from [1,1-D_2]-ethanol carried one deuterium atom (Figure 1). The deuterium atom must be attached to carbon 4 of the sotolon molecule as this is the only carbon carrying one proton. Two isotopomers of sotolon were detected by HRGC−MS analysis in the model reaction mixture containing [2,2,2-D_3]ethanol (Figure 2). The mass spectra showed that the first isotopomer contained six and the second carried three deuterium atoms. The separation of the two isotopomers by HRGC is caused by different interactions of the substances with the station-

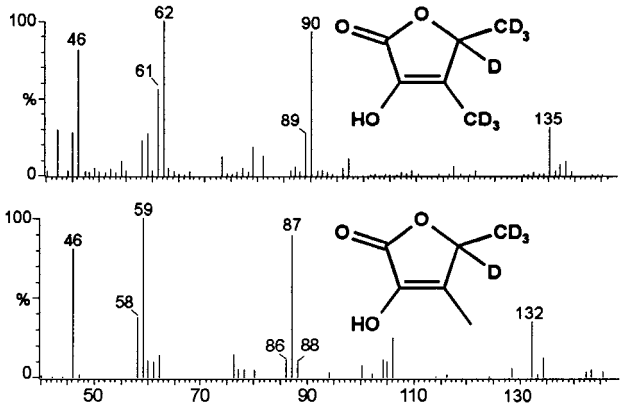

Figure 3. Mass spectra of sotolone isotopomer 1 (upper trace) and isotopomer 2 (lower trace) formed from [1,1,2,2,2-D_5]-ethanol and ascorbic acid.

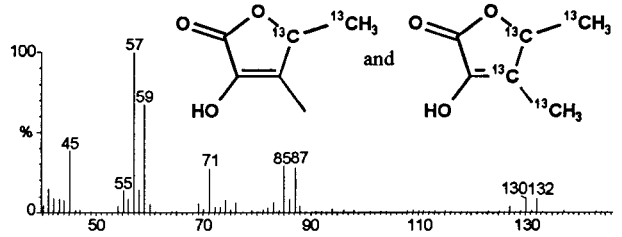

Figure 4. Mass spectrum of sotolone isotopomers formed from [$^{13}C_2$]ethanol and ascorbic acid.

ary phase. The deuterium atoms in the first isotopomer are located in both methyl groups, while only one methyl group contains the deuterium atoms of the second isotopomer. Therefore, two formation pathways for the furanone might exist. In pathway 1, two molecules of ethanol are incorporated into the sotolon molecule, whereas in pathway 2, only one molecule of ethanol is transformed to sotolon.

To localize the deuterium label in the second isotopomer (Figure 2), we analyzed solutions containing unlabeled ascorbic acid and [1,1,2,2,2-D_5]ethanol. HRGC−MS separation showed again the formation of two isotopomers of sotolon, i.e., one carrying seven and the other carrying four deuterium atoms (Figure 3). In the case of the first isotopomer, all the protons are replaced by deuterium atoms. The four deuterium atoms of the second isotopomer are located in methyl group 5 attached to carbon 4 and at carbon 4. The result confirms the hypothesis of two different pathways for the formation of sotolon from ethanol and ascobic acid. It also implies that in pathway 1 two molecules of ethanol form carbon 3 and carbon 4 and the carbons of both methyl groups 5 and 6 in the sotolon molecule. In pathway 2, the carbons of one molecule of ethanol form carbon 4 and the carbon of attached methyl group 5.

Further evidence was provided by HRGC−MS analysis of sotolon produced from [$^{13}C_2$]ethanol and unlabeled ascorbic acid (Figure 4). Two isotopomers were generated, but separation of the ^{13}C-labeled compounds by HRGC was not achieved. Therefore, the mass spectrum shown in Figure 4 represents the sum of two spectra. However, it is obvious that the isotopomers shown in Figure 4 were formed.

Consequently, either two or four carbons of the formed sotolon molecule originate from ascorbic acid. Hence, we performed model reactions using solutions of [1-^{13}C]-, [2-^{13}C]-, and [3-^{13}C]ascorbic acid as well as unlabeled ethanol to identify the carbons originating from ascorbic

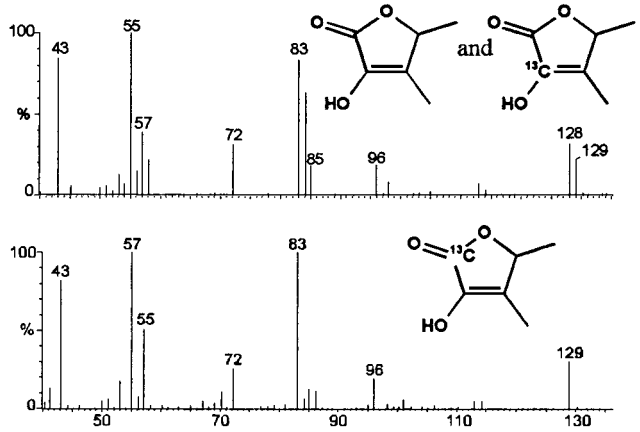

Figure 5. Mass spectra of sotolone isotopomers formed from [2-^{13}C]ascorbic acid and ethanol (upper trace) as well as from [3-^{13}C]ascorbic acid and ethanol (lower trace).

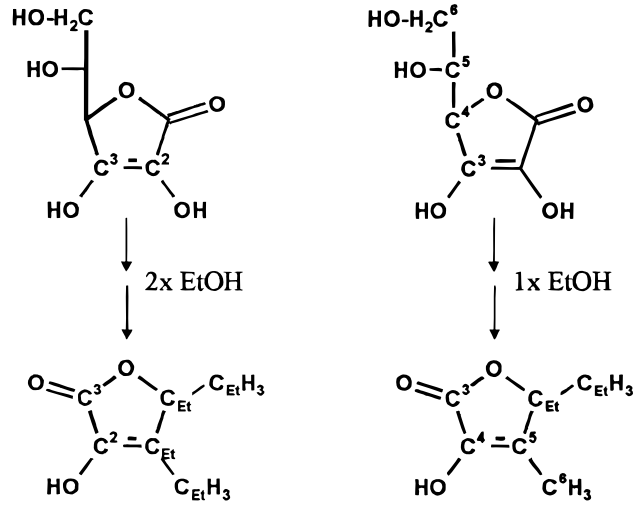

Figure 6. Postulated formation pathways of sotolone from ascorbic acid and ethanol. C^2 and C^3 represent carbons originating from ascorbic acid; C_{Et} represents carbons originating from ethanol.

acid. Unlabeled sotolon was obtained from [1-^{13}C]-ascorbic acid. Therefore, carbon 1 of the acid is not involved in the formation of the furanone. It is probably lost as CO_2 by decarboxylation as recently described (Shin and Feather, 1990). Unlabeled and singly labeled sotolon was produced by [2-^{13}C]ascorbic acid, and only singly labeled sotolon was formed by [3-^{13}C]ascorbic acid (Figure 5).

These results can be interpreted with the conclusion drawn from the studies with labeled ethanols. Carbon 4 and its attached methyl group 5 are always generated by ethanol. Carbon 3 and its attached methyl group 6 originate from either ethanol or ascorbic acid. The remaining carbons of the sotolon molecule must be provided by ascorbic acid since there is no further carbon source available. However, carbon 1 of the ascorbic acid molecule is not involved. Summarizing the results, we postulate two formation pathways (Figure 6). Sotolon is either formed from two molecules of ethanol and carbons 2 and 3 of ascorbic acid (pathway 1) or generated from one molecule of ethanol and carbons 3−6 of ascorbic acid (pathway 2).

By comparison of the mass spectra given in Figure 5, it is even possible to locate the fragments of the ascorbic acid in the formed sotolon molecule. In the mass spectra of the furanone formed from [3-^{13}C]ascorbic acid, only

the molecular ion $[M]^+$ contained a ^{13}C isotope (*m/z* 129) (Figure 5). The rest of the mass spectrum is similiar to that of unlabled sotolon (Figure 1). The fragment ion at *m/z* 83 resulting from the loss of CHO_2 (typical for lactones) carried no label. This implies that the ^{13}C label is located in carbon 1 of the sotolon molecule. The mass spectrum of the sotolon formed by [2-^{13}C]ascorbic acid also exhibited a labeled molecule ion $[M]^+$ (*m/z* 129). But after the loss of CHO_2, the resulting fragment still carried ^{13}C (*m/z* 84) (Figure 5). Following pathway 1 in Figure 6, this means that carbon 2 of the ascorbic acid molecule forms carbon 2 of the sotolon molecule.

The theory was tested with model solutions containing [1,1,2,2,2-D_5]ethanol and either [2-^{13}C]- or [3-^{13}C]-ascorbic acid. Two isotopomers of sotolon (*m/z* 132 and 136) were produced from [2-^{13}C]ascorbic acid and [D_5]-ethanol. The furanone with an ion at *m/z* 132 is composed of one molecule of [D_5]ethanol and carbons 3−6 of the [2-^{13}C]ascorbic acid molecule. The other isotopomer is formed from two molecules of [D_5]ethanol and carbons 2 and 3 of the [2-^{13}C]ascorbic acid molecule. Two isotopomers of sotolon (*m/z* 133 and 136) were formed from [3-^{13}C]ascorbic acid and [D_5]ethanol. Furanone (*m/z* 136) contained two molecules of [D_5]ethanol and carbons 2 and 3 of [3-^{13}C]ascorbic acid. Sotolon (*m/z* 133) was composed of one [D_5]ethanol molecule and carbons 3−6 of [3-^{13}C]ascorbic acid.

The results unambiguously demonstrate the formation of sotolon by ascorbic acid and ethanol and provide the first evidence for two different pathways leading to the off-flavor in soft drinks. The ratios of the intensities of $[M]^+$ *m/z* 130 and 132 in Figure 4 and *m/z* 128 and 129 (upper trace) in Figure 5 nicely display the ratio of pathways 1 and 2.

Although various beverages contain ethanol and ascorbic acid, the off-flavor caused by sotolon has been described so far only in citrus soft drinks. It is possible that natural ingredients in beverages other than citrus soft drinks bind reactive intermediates of the sotolon pathways and therefore inhibit the formation of the off-flavor.

ABBREVIATIONS USED

df, thickness of the stationary phase; EDTA, ethylenediaminetetraacetic acid; GCO, gas chromatography/olfactometry; HPLC, high-performance liquid chromatography; HRGC, high-resolution gas chromatography; HRGC-MS, high-resolution gas chromatography−mass spectrometry; MCSS, moving column stream switch; MDGC, multidimensional gas chromatography; MDGC-MS, multidimensional gas chromatography−mass spectrometry.

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