JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Chemistry and Biology of Aroma and Taste

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.8b04333 • Publication Date (Web): 21 Sep 2018

Identification and Quantitation of Four New 2-Alkylthiazolidine-4-carboxylic Acids Formed in Orange Juice by a Reaction of Saturated Aldehydes with Cysteine

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ABSTRACT

Despite several technological efforts to maximize the quality and shelf life of chilled stored not-from-concentrate (NFC) orange juice, changes in the overall aroma profile might occur during storage. Besides the degradation of terpenoids, a loss of the aroma-active aldehydes, hexanal, octanal, nonanal and decanal as well-as of 1-penten-3-one were recently confirmed as a major cause for the changes in the aroma profile of orange juice even during storage under aseptic conditions at 0 °C. To unravel the fate of the aroma-active aldehydes, model experiments were carried out considering the oxidation into the corresponding acids as well as a reaction with free amino acids present in orange juice. The oxidation into the acids could be confirmed by isotope labeling experiments, additionally the reaction of the four aldehydes mentioned above with L-cysteine yielded four new compounds identified as 2-alkylsubstituted thiazolidine-4-carboxylic acids. Their quantitation in orange juice samples by newly developed stable isotope dilution assays revealed that these acids were already present in the fresh samples, but were considerably increased after storage. Labeling experiments in orange juice administered with either labeled octanal or labeled cysteine confirmed that the reaction quickly occurs in the juice. The data contribute another puzzle piece to the loss of aroma-active aldehydes during orange juice storage, which may also be relevant in other foods.

KEY WORDS: Aldehydes, chilled storage, 2-alkylsubstituted thiazolidine-4-carboxylic acids, stable isotope dilution assays, L-cysteine

INTRODUCTION

Many consumers enjoy a glass of orange juice as a healthy and tasty part of their daily diet. To meet their demand of a premium orange juice throughout the year, several technological processes have been developed to maximize the quality and shelf life in particular for premium not-from-concentrate (NFC) orange juice, such as chilled storage under aseptic conditions.

But, despite the high technological standards, in particular extended storage time may lead to changes in the overall aroma.[1] In the past, depending on time, temperature, oxygen content and light exposure, the loss of ascorbic acid and juice darkening were the first observed reactions during orange juice storage. Additionally, systematic model studies using forced storage showed the formation of off-flavor compounds, such as carvone, α-terpineol, 2-methoxy-4-vinylphenol or 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone.[1-3] Averbeck and Schieberle[4,5] for the first time applied a comprehensive approach, the molecular sensory concept, to characterize the effect of storage on the overall aroma of orange juice. Next to the formation of off-flavor compounds, also a rapid degradation of terpenoids and saturated aldehydes were confirmed as a function of storage temperature and time. While other studies[6-8] also reported a decline in concentration of saturated aldehydes during storage, Peterson et al.[9] were the first to propose an oxidation to their corresponding acids. However, in model trials, Averbeck and Schieberle[5] could not confirm such oxidation reactions for juice from concentrate.

Although it is commonly known that the degradation of saturated aldehydes mostly occurs at elevated temperatures, losses in the aldehydes also occurred in aseptically storage juice at 0 °C as recently shown by us[10] using the Sensomics approach. Especially, saturated aldehydes, such as hexanal, octanal, nonanal and decanal as well as 1-penten-3-one and acetaldehyde showed a significant decrease already after a

short time of chilled storage.[10] Sensory experiments, i.e. aroma recombinates, confirmed their importance in the aroma of the unstored juice.[11]

Besides the assumed oxidation of the aldehydes to their corresponding acids, no other degradation pathways of these impact orange juice aroma compounds have yet been proposed.

Thus, to unravel the fate of the saturated aldehydes during the storage of orange juice, several model experiments should be carried out as isotope labeling experiments considering a possible oxidation.

Additionally, as already reported in the literature, L-cysteine serves as a nucleophile in many reactions, for example in a reaction with 2,3-butanedione to 5-acetyl-2,3-dihydro-1,4-thiazine[12] or in the reaction of 2-oxopropanal.[13] Hence a reaction of the aldehydes with free amino acids or peptides present in orange juice, such as L-lysine, L-cysteine and L-glutathione should to be studied as well. Using UPLC-TOF/MS, LC-MS/MS and GC×GC-TOF/MS newly formed compounds should be identified and quantitated in NFC orange juice.

MATERIALS AND METHODS

Materials. Orange juice not from concentrate (NFC) was produced by Tropicana Products Inc. from Hamlin oranges, and the pasteurized juice was aseptically stored. in tanks at 0 °C. The fresh juice (on the day of storage) as well as samples after one, two, four and six months of chilled storage were frozen and send to Germany by air freight. The samples were further stored at -80 °C prior to analysis.

Reference Compounds and Chemicals. Reference compounds and chemicals were obtained from the sources given in parentheses: hexanal, decanal, octanal, acetaldehyde, decanoic acid, nonanoic acid, octanoic acid, hexanoic acid, ethanol, citric acid, hydroxycinnamic acid, ethyl 3,4,5-trihydroxybenzoate, potassium acetate, disodium hydrogen phosphate, L-cysteine, L-lysine, L-glutathione, dansyl chloride, and iodoacetic acid (Sigma-Aldrich Chemie, Taufkirchen, Germany); nonanal (Roth, Karlsruhe, Germany); acetonitrile LiChrosolv ®, methanol LiChrosolv ®, formic acid, hydrochloric acid, 2-propanol, sodium sulfate, ethyl acetate and sulfuric acid (Merck, Darmstadt). Diethyl ether and dichloromethane (Merck) were freshly distilled before use.

Isotopically Labeled Standards. [$^{13}C_8$]-Octanoic acid, [$^{2}H_{12}$]-hexanal were purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany). [$^{13}C_3{}^{15}N$]-L-cysteine and [$^{2}H_2$]-hexanoic acid were from Cambridge Isotope Laboratories Inc. (Andover, Massachusetts). [$^{2}H_6$]-Dimethyl sulfoxide (DMSO-d_6) and [$^{2}H_3$]-acetonitrile (acetonitrile-d_3) were from Euroisotop (Saarbrücken, Germany).

[$^{13}C_8$]-octanal,[10] [$^{2}H_4$]-octanal,[14] [$^{2}H_4$]-nonanal,[15] [$^{2}H_2$]-decanal,[4] [$^{2}H_{2\text{-}4}$]-decanoic acid[16] and [$^{2}H_2$]-nonanoic acid[17] were synthesized according to the references given in superscript.

Syntheses of 2-Alkylsubstituted Thiazolidine-4-Carboxylic Acids. The 2-alkylsubstituted thiazolidine-4-carboxylic acids (**1 – 4**, Figure 1) were prepared by condensation of L-cysteine with the respective aldehyde as previously described.[18]

A solution of the respective unlabeled and labeled aldehyde (hexanal, octanal, nonanal, decanal, [$^{13}C_8$]-octanal, [$^{2}H_4$]-octanal, [$^{2}H_{12}$]-hexanal, [$^{2}H_4$]-nonanal and [$^{2}H_2$]-decanal (each 0.67 mmol) in 2.5 mL of 95% ethanol was added to either a solution of L-cysteine or [$^{13}C_3{}^{15}N$]-L-cysteine (0.57 mmol each) and potassium acetate (0.7 mmol) in 2.5 mL of water. Precipitation of the reaction product occurred after a few minutes of agitation. The reaction mixture was stirred for 1 h at room temperature and frozen overnight. The precipitate was filtered with gentle suction and washed with water, cold ethanol and finally diethyl ether before lyophilization. The dry compound was recrystallized from isopropyl alcohol.

For the preparation of labeled standards, lower amounts of the reactants were used and the purification step was changed. In such cases, the reaction mixture was extracted with ethyl acetate (total volume 25 mL), and the ethyl acetate layer was vaporized. The residue was dissolved in acetonitrile containing a few drops of formic acid.

The concentrations of the unlabeled reference compounds were determined by qNMR, while the concentrations of the labeled standards were determined by means of LC-MS/MS.

Characterization of 2-Alkylsubstituted Thiazolidine-4-Carboxylic Acids. Characterization of the synthesized compounds, was carried out by UPLC-TOF/MS, LC-MS and one and two-dimensional NMR experiments. The ring closure during the formation of the 2-alkylsubstituted thiazoline-4-carboxylic acids created a new chiral center at position 2 (Figure 1) yielding two diastereomers, here assigned as *a* and *b*, found in a ratio of 1:1 (Table 1 and 2, Supporting Information Table S1 – S6)

2-Pentylthiazolidine-4-carboxylic acid (2-PT-4-CA), (**1**, Figure 1); UPLC-TOF (ESI^+), *m/z* 204.1061 (measured), *m/z* 204.1058 (calculated for $[C_9H_{17}O_2NS+H]^+$); MS (ESI^+), *m/z* 204 (100, $[M+H]^+$; Supporting Information Figure S1 a).

*Diastereomer **1a**:* ^{1}H-NMR (400 MHz, DMSO-d_6, COSY), δ 0.86 [t, 3H, *J*= 6.76, H-C(5')], δ 1.18 – 1.47 [m, 8H, H-C(2', 3', 4')], δ 1.61 – 1.83 [m, 1H, Hα-C(1')], δ 1.83 – 1.96 [m, 1H, Hβ-C(1')], δ 2.75 [dd, 1H, *J*= 9.24; 9.84, Hα-C(5)], δ 3.18 [dd, 1H, *J*=6.93; 9.84, Hβ-C(5)], δ 3.70 [dd, 1 H, *J*=6.96; 9.24, H-C(4)], δ 4.40 [dd, 1H, *J*=5.80; 7.40, H-C(2)]; ^{13}C-NMR (100 MHz, DMSO-d_6, HSQC, HMBC), δ 14.3 [C(5')], δ 22.4/ 22.5 [C(4')], δ 27.5/ 27.7 [C(2')], δ 31.5 [C(3')], δ 35.3 [C(1')], δ 37.5 [C(5)], δ 65.7 [C(4)], δ 71.6 [C(2)], δ 172.8 [C(6)].

*Diastereomer **1b***: ^{1}H-NMR (400 MHz, DMSO-d_6, COSY), δ 0.83 [t, 3H, *J*= 6.76, H-C(5')], δ 1.18 – 1.47 [m, 8H, H-C(2', 3', 4')], δ 1.47 – 1.57 [m, 1H, Hα-C(1')], δ 1.61 – 1.83 [m, 1H, Hβ-C(1')], δ 2.93 [dd, 1H, *J*= 5.12; 10.24, Hα-C(5)], δ 3.08 [dd, 1H, *J*=7.04; 10.24, Hβ-C(5)], δ 4.06 [dd, 1 H, *J*=5.16; 6.96, H-C(4)], δ 4.50 [dd, 1H, *J*=6.28; 7.44, H-C(2)]; ^{13}C-NMR (100 MHz, DMSO-d_6, HSQC, HMBC), δ 14.3 [C(5')], δ 22.4/ 22.5 [C(4')], δ 27.5/ 27.7 [C(2')], δ 31.5 [C(3')], δ 37.1 [C(1')], δ 37.1 [C(5)], δ 64.6 [C(4)], δ 70.9 [C(2)], δ 173.4 [C(6)].

2-Heptylthiazolidine-4-carboxylic acid (2-HT-4-CA) (**2**, Figure 1) UPLC-TOF (ESI^+), *m/z* 232.1377 (measured), *m/z* 232.1371 (calculated for $[C_{11}H_{21}O_2NS+H]^+$); MS (ESI^+), *m/z* 232 (100, $[M+H]^+$; Supporting Information Figure S1 b).

*Diastereomer **2a**:* ^{1}H-NMR (400 MHz, DMSO-d_6, COSY), δ 0.86 [t, 3H, *J*= 6.76, H-C(7')], δ 1.15 – 1.47 [m, 10H, H-C(2', 3', 4', 5', 6')], δ 1.60 – 1.83 [m, 1H, Hα-C(1')], δ 1.83 – 1.96 [m, 1H, Hβ-C(1')], δ 2.75 [dd, 1H, *J*= 9.20; 9.84, Hα-C(5)], δ 3.18 [dd, 1H, *J*=6.97; 9.84, Hβ-C(5)], δ 3.69 [dd, 1 H, *J*=6.96; 9.20, H-C(4)], δ 4.40 [dd, 1H, *J*=5.80; 7.40, H-C(2)]; ^{13}C-NMR (100 MHz, DMSO-d_6, HSQC, HMBC), δ 14.4 [C(7')], δ 22.5

[C(6')], δ 27.9/ 28.1/ 29.0/ 29.1/ 29.2/ 29.3 [C(2')], δ 27.9/ 28.1/ 29.0/ 29.1/ 29.2/ 29.3 [C(3')], δ 27.9/ 28.1/ 29.0/ 29.1/ 29.2/ 29.3 [C(4')], δ 27.9/ 28.1/ 29.0/ 29.1/ 29.2/ 29.3 [C(5')], δ 35.3 [C(1')], δ 37.5 [C(5)], δ 65.7 [C(4)], δ 71.6 [C(2)], δ 172.8 [C(6)].

Diastereomer ***2b***: ^{1}H-NMR (400 MHz, DMSO-d_6, COSY), δ 0.83 [t, 3H, *J*= 6.76, H-C(5')], δ 1.15 – 1.47 [m, 10H, H-C(2', 3', 4', 5', 6')], δ 1.47 – 1.60 [m, 1H, Hα-C(1')], δ 1.60 – 1.83 [m, 1H, Hβ-C(1')], δ 2.93 [dd, 1H, *J*= 5.12; 10.24, Hα-C(5)], δ 3.08 [dd, 1H, *J*=7.00; 10.24, Hβ-C(5)], δ 4.06 [dd, 1 H, *J*=5.12; 6.97, H-C(4)], δ 4.50 [dd, 1H, *J*=6.24; 7.44, H-C(2)]; ^{13}C-NMR (100 MHz, DMSO-d_6, HSQC, HMBC), δ 14.4 [C(7')], δ 22.5 [C(6')], δ 27.9/ 28.1/ 29.0/ 29.1/ 29.2/ 29.3 [C(2')], δ 27.9/ 28.1/ 29.0/ 29.1/ 29.2/ 29.3 [C(3')], δ 27.9/ 28.1/ 29.0/ 29.1/ 29.2/ 29.3 [C(4')], δ 27.9/ 28.1/ 29.0/ 29.1/ 29.2/ 29.3 [C(5')], δ 37.1 [C(1')], δ 37.1 [C(5)], δ 64.6 [C(4)], δ 70.9 [C(2)], δ 173.4 [C(6)].

2-Octylthiazolidine-4-carboxylic acid (2-OT-4-CA), (**3**, Figure 1) UPLC-TOF (ESI$^+$), *m/z* 246.1528 (measured), *m/z* 246.1528 (calculated for $[C_{12}H_{23}O_2NS+H]^+$); MS (ESI$^+$), *m/z* 246 (100, $[M+H]^+$; Supporting Information Figure S1 c).

Diastereomer ***3a****:* ^{1}H-NMR (400 MHz, DMSO-d_6, COSY), δ 0.86 [t, 3H, *J*= 7.07, H-C(8')], δ 1.15 – 1.45 [m, 12H, H-C(2', 3', 4', 5', 6', 7')], δ 1.63 – 1.78 [m, 1H, Hα-C(1')], δ 1.83 – 1.94 [m, 1H, Hβ-C(1')], δ 2.70 [dd, 1H, *J*= 9.20; 9.77, Hα-C(5)], δ 3.16 [dd, 1H, *J*=6.97; 9.77, Hβ-C(5)], δ 3.60 [dd, 1 H, *J*=6.97; 9.20, H-C(4)], δ 4.38 [dd, 1H, *J*=5.75; 7.36, H-C(2)]; ^{13}C-NMR (100 MHz, DMSO-d_6, HSQC, HMBC), δ 14.4 [C(8')], δ 22.5 [C(7')], δ 27.8/ 28.1/ 29.3/ 29.4 [C(2')], δ 27.8/ 28.1/ 29.3/ 29.4 [C(3')], δ 27.8/ 28.1/ 29.3/ 29.4 [C(4')], δ 27.8/ 28.1/ 29.3/ 29.4 [C(5')], δ 31.7 [C(6')], δ 35.4 [C(1')], δ 37.8 [C(5)], δ 66.5 [C(4)], δ 71.7 [C(2)], δ 173.0 [C(6)].

Diastereomer ***3b***: ^{1}H-NMR (400 MHz, DMSO-d_6, COSY), δ 0.86 [t, 3H, *J*= 7.07, H-C(8')], δ 1.15 – 1.45 [m, 12H, H-C(2', 3', 4', 5', 6', 7')], δ 1.45 – 1.57 [m, 1H, Hα-

C(1')], δ 1.63 – 1.78 [m, 1H, Hβ-C(1')], δ 2.88 [dd, 1H, *J*= 5.52; 10.18, Hα-C(5)], δ 3.08 [dd, 1H, *J*=6.97; 10.18, Hβ-C(5)], δ 3.99 [dd, 1 H, *J*=5.52; 6.97, H-C(4)], δ 4.55 [dd, 1H, *J*=6.50; 7.22, H-C(2)]; ^{13}C-NMR (100 MHz, DMSO-d_6, HSQC, HMBC), δ 14.4 [C(8')], δ 22.5 [C(7')], δ 27.8/ 28.1/ 29.3/ 29.4 [C(2')], δ 27.8/ 28.1/ 29.3/ 29.4 [C(3')], δ 27.8/ 28.1/ 29.3/ 29.4 [C(4')], δ 27.8/ 28.1/ 29.3/ 29.4 [C(5')], δ 31.7 [C(6')], δ 37.5 [C(1')], δ 37.5 [C(5)], δ 64.9 [C(4)], δ 70.9 [C(2)], δ 173.4 [C(6)].

2-Nonylthiazolidine-4-carboxylic acid (2-NT-4-CA) (**4**, Figure 1; UPLC-TOF (ESI$^+$), *m/z* 260.1684 (measured), *m/z* 260.1684 (calculated for $[C_{13}H_{25}O_2NS+H]^+$); MS (ESI$^+$), *m/z* 260 (100, $[M+H]^+$; Supporting Information Figure S1 d).

*Diastereomer **4a**:* ^{1}H-NMR (400 MHz, DMSO-d_6, COSY), δ 0.86 [t, 3H, *J*= 6.98, H-C(9')], δ 1.15 – 1.46 [m, 12H, H-C(2', 3', 4', 5', 6', 7', 8')], δ 1.62 – 1.81 [m, 1H, Hα-C(1')], δ 1.84 – 1.95 [m, 1H, Hβ-C(1')], δ 2.74 [dd, 1H, *J*= 9.20; 9.83, Hα-C(5)], δ 3.18 [dd, 1H, *J*=6.93; 9.83, Hβ-C(5)], δ 3.69 [dd, 1 H, *J*=6.93; 9.20, H-C(4)], δ 4.40 [dd, 1H, *J*=5.86; 7.46, H-C(2)]; ^{13}C-NMR (100 MHz, DMSO-d_6, HSQC, HMBC), δ 14.4 [C(9')], δ 22.6 [C(8')], δ 27.9/ 28.1/ 29.1/ 29.2/ 29.3/ 29.4 [C(2')], δ 27.9/ 28.1/ 29.1/ 29.2/ 29.3/ 29.4 [C(3')], δ 27.9/ 28.1/ 29.1/ 29.2/ 29.3/ 29.4 [C(4')], δ 27.9/ 28.1/ 29.1/ 29.2/ 29.3/ 29.4 [C(5')], δ 27.9/ 28.1/ 29.1/ 29.2/ 29.3/ 29.4 [C(6')], δ 31.8 [C(7')], δ 35.3 [C(1')], δ 37.5 [C(5)], δ 66.7 [C(4)], δ 71.6 [C(2)], δ 172.8 [C(6)].

*Diastereomer **4b**:* ^{1}H-NMR (400 MHz, DMSO-d_6, COSY), δ 0.86 [t, 3H, *J*= 6.98, H-C(9')], δ 1.15 – 1.46 [m, 12H, H-C(2', 3', 4', 5', 6', 7', 8')], δ 1.48 – 1.58 [m, 1H, Hα-C(1')], δ 1.62 – 1.81 [m, 1H, Hβ-C(1')], δ 2.93 [dd, 1H, *J*= 5.09; 10.22, Hα-C(5)], δ 3.07 [dd, 1H, *J*=7.02; 10.22, Hβ-C(5)], δ 4.06 [dd, 1 H, *J*=5.09; 7.02, H-C(4)], δ 4.53 [dd, 1H, *J*=6.30; 7.41, H-C(2)]; ^{13}C-NMR (100 MHz, DMSO-d_6, HSQC, HMBC), δ 14.4 [C(9')], δ 22.6 [C(8')], δ 27.9/ 28.1/ 29.1/ 29.2/ 29.3/ 29.4 [C(2')], δ 27.9/ 28.1/ 29.1/ 29.2/ 29.3/ 29.4 [C(3')], δ 27.9/ 28.1/ 29.1/ 29.2/ 29.3/ 29.4 [C(4')], δ 27.9/ 28.1/ 29.1/

29.2/ 29.3/ 29.4 [C(5')], δ 27.9/ 28.1/ 29.1/ 29.2/ 29.3/ 29.4 [C(6')], δ 31.8 [C(7')], δ 35.3 [C(1')], δ 37.1 [C(5)], δ 64.6 [C(4)], δ 70.7 [C(2)], δ 173.4 [C(6)].

Model Reactions.

Stability of Aldehydes under Nitrogen or Oxygen. An aqueous solution of hexanal, octanal, nonanal and decanal (~5 µmol/L), respectively, adjusted to the natural pH of orange juice (pH 3.6) with citric acid, was stored in gas tight brown glass vessels at 37 °C (in an warming cabinet) for 4 weeks either under nitrogen or an air atmosphere. In further trials, hydroxycinnamic acid or ethyl 3,4,5-trihydroxybenzoate (~0.05 µmol/L) were added as antioxidants. The stored solutions were extracted with diethyl ether, subjected to distillation by Solvent Assistant Flavor Evaporation (SAFE) and the volatiles formed were analyzed by GC/MS.

Reaction of Aldehydes with Free Amino Acids and Glutathione. First, an aqueous solution of octanal and the free amino acid, L-lysine, L-cysteine, and L-glutathione, respectively, was adjusted to pH 3.6 with citric acid and stored in gas tight brown glass bottles at 37 °C (in an warming cabinet) for 14 days. After dilution with doubly distilled water (1:1000) and subsequent filtration (0.45 µm, Schleicher & Schuell, Dassel, Germany), the solutions were subjected to a screening by UPLC-TOF/MS. For hexanal, nonanal and decanal only the model trial with L-cysteine was carried out. For NMR characterization the precipitate of the aldehyde/ L-cysteine-trials was washed with water, cold ethanol and finally diethyl ether, followed by lyophilization and recrystallization. The purified crystals were dissolved in deuterated dimethyl sulfoxide (DMSO-d_6) and subjected to NMR analysis.

Formation of 2-Alkylsubstituted Thiazolidine-4-Carboxylic Acids in Orange Juice. [$^{13}C_8$]-Octanal was spiked to a sample of fresh orange juice. The sample in a gas tight brown glass bottle was stored either for 14 d at 37 °C (in a warming cabinet) or for 14 d at 7 °C (in a refrigerator), respectively. In a third trial, [$^{15}N^{13}C_3$]-L-cysteine

was spiked to the juice and stored for 14 d at 7 °C. Several samples were taken during the storage period and the formation of the formed, differently labeled 2-heptylthiazolidine-4-carboxylic acids was quantitated using either [$^{2}H_{4}$]- or [$^{13}C_{8}$]-labeled 2-heptylthiazolidine-4-carboxylic acid as internal standard.

Isolation of the Volatiles from Orange Juice. The work-up procedure was carried out as described previously.[10] After the separation of neutral/ basic (NBF) and acidic fraction (AF), the aldehydes were recovered in the NBF, while the acids were found in AF.

Quantitation of Aldehydes and Their Corresponding Acids in Orange Juice and Model Solutions.

Stable isotope dilution assays of the four aldehydes and four acids under consideration were carried out as described previously.[10]

Quantitation of L-Cysteine. For the quantitation, a stable isotope dilution assay was carried out as described previously.[18]

Quantitation of 2-Alkylsubstituted Thiazolidine-4-Carboxylic Acids. An aliquot of the isotopically labeled standards was added to the orange juice (10 g). After 30 minutes of equilibration, the sample was centrifuged (4600 g, 20 min, 6 °C), and the supernatant was filtered (0.45 µm, Schleicher & Schuell) and finally analyzed by means of HPLC-MS/MS. A response factor was determined by monitoring the most abundant transitions (Table 3) of analyte/ standard mixtures in different ratios (1:20, 1:10, 1:5, 1:3, 1:1, 3:1, 5:1, 10:1, 20:1) in water/acetonitrile (80:20).

High Resolution Gas Chromatography–Mass Spectrometry (HRGC/MS). HRGC/MS was performed by using a Hewlett-Packard gas chromatograph 5890 series II (Waldbronn, Germany) connected to a Finnigan sector field mass spectrometer type MAT 95 S (Bremen, Germany). 0.5 µL were injected on-column on an Agilent DB-FFAP (30 m, 0.25 mm i.d., 0.25 µm film thickness) (Waldbronn, Germany) with

the following temperature program: 40 °C held for 2 min, 20 °C/min rate up to 60 °C (held for 2 min), 6 °C/min rate up to 180 °C, followed by 20 °C/min rate up to 230 °C (held for 5 min). Mass spectra were generated in the electron impact mode (MS-EI) at 70 eV with a scan range from 35 – 250 m/z (scan rate 0.8 s/d) and a resolution of 5000 with an accuracy error < 5 ppm.

Comprehensive Two-Dimensional Gas Chromatography–Time of Flight/Mass Spectrometry (GC×GC-TOF/MS). A Lego Pegasus 3D GC×GC-TOF-MS instrument (St. Joseph, MI, USA) was used consisting of an Agilent GC model 6890N (Wald-bronn, Germany) equipped with a dual stage quad-jet thermal modulator and a sec-ondary oven coupled to the mass spectrometer providing unit mass resolution. A PTV-inlet was used for cold-on-column injection, operated by a CTC-Analytics GC-PAL auto sampler (Zwingen, Switzerland). In the first dimension an Agilent DB-FFAP (30 m, 0.25 i.d., 0.25 µm film thickness) equipped with a deactivated precolumn (5 m, 0.53 mm i.d.) was connected to an Agilent DB-1701 column (2 m, 0.1 i.d., 0.1 µm film thickness) in the second dimension. The primary oven temperature was held for 2 min at 40 °C, then raised with 9 °C/min to 60 °C, held for 2 min, then raised with 9 °C/min to 180 °C and then with 6 °C/min to the final temperature 230 °C, held for 5 min. The secondary oven started at 47 °C, held for 2 min, was then raised with 9 °C/min to 60 °C, held isothermal for 2 min, then raised with 6 °C/min to 187 °C, fol-lowed by a rise of 9 °C/min to the final temperature of 250 °C, held for 1 min. Modula-tion period (4 s) and temperature programming rate were adjusted to obtain a mini-mum of three modulations per peak at the concentration level equal to the limit of quantitation (LoQ). The detector voltage was optimized and set to 1750 V. Mass spectra were acquired within *m/z* 30 − 350 at a rate of 100 spectra/s. Data were elaborated using GC Image (GC-Image, Lincoln, NE, USA).

Ultra-Performance Liquid Chromatography/ Time of Flight–Mass Spectrometry (UPLC/TOF-MS). High resolution mass spectra were measured on a Waters Synapt G2 HDMS mass spectrometer (Manchester, UK) coupled to a Waters Acquity UPLC core system (Milford, MA, USA) consisting of a binary solvent manager, a sample manager and column oven. All compounds were dissolved in 1 mL water/methanol (9:1, v/v) and aliquots (1-5 µL) were injected onto a 2.1 x 150 mm i.d., 1.7 µm, Waters ACQUITY CSHT C18 column operated with a flow rate of 0.4 mL/min at a temperature of 40 °C. For chromatography the following gradient was used: starting with 100% water, increasing the acetonitrile concentration until 100% within 4 min and then kept constant for 30 s. Scan time for the MS^E method (centroid) was set to 0.1 s. Analyses were performed in the positive ESI and in resolution mode using the following ion source parameters: capillary voltage, +2.5 kV; sampling cone, 50 V; extraction cone, 4.0 V; source temperature, 120 °C; desolvation temperature, 450 °C; gone gas, 10 L/h; and desolvation gas, 850 L/h. Data processing was performed by using Waters MassLynx 4.1 SCN 779 and the elemental composition tool for determining the accurate mass. All data were lock mass corrected to the pentapeptide leucine enkephaline (Tyr-Gly-Gly-Phe-Leu, *m/z* 556.2771, $[M+H]^+$) in a solution (2 ng/µL) of acetonitrile/ 0.1% formic acid (1:1, v/v). Scan time for the lock mass was set to 0.3 s, an interval of 15 and 3 scans to average with a mass window of ±0.3 Da. Calibration of the Synapt G2 in the range form *m/z* 50 to 1300 was performed using a solution of sodium formate (0.5 mmol/L) in 2-propanol/water (90:10, v/v). The UPLC and Synapt G2 systems were operated with the Waters MassLnyx software.

High Performance Liquid Chromatography–Tandem Mass Spectrometry (HPLC-MS/MS). A Finnigan TSQ Quantum Discovery quadrupole mass spectrometer coupled with a Finnigan Surveyor Plus HPLC system (Thermo Electron Corporation, Waltham, MA) was used for LC-MS/MS analysis. The stationary phase was a Phe-

292 nomenex Synergi HydroRP C18 column (2.0 mm × 150 mm, 4 µm particle size, 8 nm
293 pores) (Aschaffenburg, Germany), which was equipped with a Phenomenex C18
294 guard column. For the separation of the 2-substituted thiazolidine-4-carboxylic acids,
295 gradient elution (flow rate: 0.2 ml/min) was employed with aqueous 0.1% formic acid
296 (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The initial condition of
297 80% A and 20% B were held for 2 min, then the proportion of solvent B was raised to
298 90% within the next 16 min and held for 5 min. Before each injection, the column was
299 equilibrated for 15 min. Injection was carried out at full loop mode, and injection vol-
300 ume was set at 10 µL. The mass spectrometer was operated in ESI^+ mode with the
301 following conditions: spray needle voltage, 3.2 kV; capillary temperature, 300 °C; ca-
302 pillary offset, 35 V; sheath gas, 30 arbitrary units (arb); auxiliary gas, 15 arb; collision
303 gas (argon) pressure, 1.2 mTorr; scan time, 0.2 s, peak width of both quadrupole fil-
304 ters 1 and 3, 0.7 amu. During method development, the unlabeled compounds were
305 subjected to MS/MS using a full scan in the product mode to find the two most in-
306 tense and specific product ions for selected reaction monitoring (SRM). To optimize
307 the intensity of these product ions, different collision energies in quadrupole 2 were
308 tested using SRM mode (Table 3).

309 **Nuclear Magnetic Resonance Spectroscopy (NMR).** All NMR spectra were pro-
310 duced with a Bruker Avance III 400 spectrometer (400MHz) equipped with a
311 BBFOplus probe (298 K; Rheinstetten, Germany). The samples were dissolved in
312 deuterated dimethyl sulfoxide (DMSO-d_6) and filled in NMR-tubes (178 x 5 mm;
313 Schott E, Mainz, Germany). The chemical shift was referenced to TMS. The Bruker
314 software TopSpin (Version 3.0, Rheinstetten, Germany) was used for data pro-
315 cessing. The appropriate standard pulse sequences for ^{1}H- and ^{13}C-NMR measure-
316 ments, as well as for the standard 2D NMR experiments gs-COSY ($^{2,3}J_{H,H}$ correla-
317 tion), gs-HSQC ($^1J_{H,C}$ correlation) and gs-HMBC ($^{2,3}J_{H,C}$ correlation) were taken from

318 the library of the TopSpin software. Data interpretation was then carried out with
319 MestReNova V.5.1.0-2940 (Mestrelab Research, La Coruña, Spain).

320 *Quantitative NMR (qNMR).* Determination of the concentrations of newly synthe-
321 sized compounds was carried out by means of qNMR with L-tyrosine as reference as
322 described previously.[20]

RESULTS AND DISCUSSION

Oxidation of Aldehydes into Corresponding Acids. Quantitative data obtained in our recent study on the storage of NFC orange juice, indicated considerable losses of saturated and unsaturated aldehydes.[10] With a series of sensory trials, it was confirmed that these aldehydes have a positive impact on the overall aroma of the unstored orange juice.[10,11] But, although the degradation of aldehydes during storage of orange juice has already been observed in several former studies,[5-9] the reason for the losses has not yet been clarified. In order to get to the bottom of this phenomenon, [$^{13}C_8$]-octanal was spiked to an authentic fresh orange juice and the sample was stored for four weeks at 37 °C. The volatiles were then extracted and isolated according to the previously described protocol[10] and subjected to GC×GC-TOF/MS analysis. For comparison, a non-spiked juice was stored under the same conditions. Monitoring the volatiles present in the spiked juice for isotopical shifts, indicated traces of [$^{13}C_8$]-octanol next to greater amounts of [$^{13}C_8$]-octanoic acid (data not shown). These findings confirmed an oxidation of octanal to its corresponding acid as it was previously suggested by Peterson et al.[9] To get more information on the responsible mechanism of this oxidation, aqueous solutions of octanal (pH 3.6) were stored either under nitrogen or an air atmosphere for 14 days at 37 °C. In a third trial, water-soluble antioxidants were added to the octanal solutions. The concentrations of the remaining aldehyde and its corresponding acids were monitored by means of stable isotope dilution assays using HRGC-MS. Only 47% or 27% of the initial octanal concentration were recovered in the samples stored under nitrogen or air, respectively. However, adding the antioxidants resulted in a stable solution of octanal. In this trail, octanoic acid was not detectable after storage (data not shown). This can only be explained by a radical autoxidation of octanal to its corresponding acid via a peroxyacid as intermediate (Figure 2).[21–23]

However, the quantitative data showed an imbalance in their relationship. While the increase of hexanoic acid as well as decanoic acid were lower than the losses in the aldehydes, the concentrations of octanoic acid and nonanoic acid were even higher than the decrease in both aldehydes (Table 4). These observations led to the conclusion that also other mechanisms may lead to the formation of the organic acids during storage, and that there must be another degradation pathway besides the radical induced oxidation of the aldehydes.

Because the experiment with labeled octanal (see above) did not hint at any other possible degradation product in sufficient concentrations, possible reactions with the non-volatile fraction of the juice were investigated.

Characterization of 2-Alkylsubstituted Thiazolidine-4-Carboxylic Acids in Orange Juice. First, model solutions of octanal with different single free amino acids, such as L-cysteine and L-lysine, and glutathione showing a reactive side chain, respectively, were prepared in an acidic aqueous solution (pH 3.6) and stored for 14 days at 37 °C. The samples were diluted 1:1000 with water and subjected to a screening by means of UPLC/TOF-MS. Monitoring of the solutions with L-lysine and L-glutathione resulted only in the identification of the respective amino acid and glutathione disulfide, but no reaction product with octanal was found. However, in the suspension of the octanal/ L-cysteine model solution an unknown reaction product with a *m/z* ratio of 232.136576 was found with the sum formula $[C_{11}H_{21}O_2NS+H]^+$. This formula was predicted to be caused by 2-heptylthiazolidine-4-carboxylic acid, a condensation product of L-cysteine and octanal. The experiment was extended to the other saturated aldehydes hexanal, nonanal and decanal and the corresponding 2-alkylsubstituted thiazolidine-4-carboxylic acids could be predicted on the basis of the UPLC/TOF-MS monitoring (Table 5). For a full characterization of the newly found compounds, NMR experiments were carried out and compared to the synthesized 2-

alkylthiazolidine-4-carboxylic acids. While a synthesis of these acids was already described in the late 1950ies,[18] no full NMR characterization was available in the literature.

Exemplarily the characterization of 2-pentylthiazolidine-4-carboxylic acid (**1**; Figure 1) (Table 1 and 2) will thus be explained in more detail. To characterize the linkage between the L-cysteine moiety and the saturated aldehydes, one- and two dimensional NMR experiments were carried out. In the ^{1}H spectrum, the two doublets of doublets observed at 3.70 ppm (H-C(4a)) and 4.06 ppm (H-C(4b)), both integrating for one proton, were assigned to the L-cysteine-moiety. Application of the HMBC experiment showed a linkage of these protons to two carbon signals at 64.6 ppm (C(4b)) and 65.7 ppm (C(4a)). In the COSY spectrum (Figure 3) these two protons showed correlations with the two diastereotopic methylene groups, H-C(4a) featured ^{1}H, ^{1}H couplings with Hα-C(5a) (J=9.24) and Hβ-C(5a) (J=6.96), while H-C(4b) was correlated with Hα-C(5b) and Hβ-C(5b) with respective coupling constants of J=6.96 and J=5.12. The ^{1}H, ^{1}H connectivity between Hα-C(5) and Hβ-C(5) was found to be J=9.84 (a) or J=10.24 (b), respectively. The identification of two individual proton signals originating from C(4) and the chemical shift of the diastereotopic methylene group into four signals with integrals of each one proton suggested that a new chiral center was introduced to the molecule and that two diastereomers were formed during the reaction. The chemical shift of the carbon signal of C(6) (172.8 ppm (a) and 173.4 ppm (b)) in the ^{13}C spectrum and the DEPT experiment led to the conclusion that the carboxylic acid sits on this position. Furthermore, the doublets of doublet at 4.40 ppm (a) and 4.50 ppm (b) were assigned to the new chiral center at H-C(2). The HMBC experiment showed the correlation to C(2a) (71.6 ppm) and C(2b) (70.9) and the COSY experiment revealed ^{1}H, ^{1}H connectivities to the diastereotopic methylene groups at C(1'). Hence, H-C(2a) showed couplings to Hα-C(1'a) and Hβ-C(1'a) with

401 *J*=5.80 and *J*=7.40, while H-C(2b) featured coupling constants of *J*=6.28 and *J*=7.44
402 to Hα-C(1'b) and Hβ-C(1'b). Additionally, the long range couplings of C(2) to C(5) and
403 C(4) and C(1') proved the new linkage between the carbon chain and the thiazolidine
404 ring (Figure 1). Due to the similar chemical shift of H-C(2'), H-C(3') and H-C(4'), a
405 multiplet at 1.18 to 1.47 ppm with an joint integral of 12 protons for a and b was found
406 in the ^{1}H spectrum. Thus, a correct assignment of the individual carbon atoms to the
407 signals in the ^{13}C spectrum was not possible for C(2'a) and C(2'b) or C(4'a) and
408 C(4'b).
409 The other three 2-alkylsubstituted thiazolidine-4-carboxylic acids formed from hex-
410 anal, nonanal and decanal featured similar NMR patterns. Only the integral of the
411 multiplet for the elongated carbon chain was found for the larger number of protons
412 (Supporting Information Table S1 – S6).
413 To screen the authentic reference orange juices for these newly found 2-
414 alkylsubstituted thiazolidine-4-carboxylic acids, a fresh and a 6 month stored juice
415 were subjected to HPLC-MS/MS. After optimizing the LC-MS/MS conditions as well
416 as the collision energy to monitor the four 2-alkylsubstituted thiazolidine-4-carboxylic
417 acids, all four compounds were found to be present in the stored orange juice.
418 **Quantitation of 2-Substituted Thiazolidine-4-Carboxylic Acids.** To quantitate
419 the four thiazolidine carboxylic acids, stable isotope dilution assays were developed
420 using the newly synthesized deuterated or [^{13}C]-labeled analogues, respectively. All
421 four 2-alkylsubstituted thiazolidine-4-carboxylic acids could be quantitated in one run
422 within less than 25 min. Monitoring the specific mass transitions of analyte and la-
423 beled standards (Table 3), the concentration of the acids could be determined in a
424 fast and precise way (Figure 4).
425 Following this approach, concentrations ranging between 14.2 µg/kg 2-
426 octylthiazolidine-4-carboxylic acid (2-OT-4-AC) and 160 µg/kg 2-heptylthiazolidine-4-

carboxylic acid (2-HT-4-AC) were already found in the fresh orange juice. However, after six months of chilled storage higher concentrations ranging from 20.1 µg/kg (2-OT-4-AC) to 202 µg/kg (2-HT-4-AC) were found, equivalent to an increase of 26% to 55% (Table 6).

Additionally, the free cysteine was determined in the fresh and six month stored juice to see if the storage had an impact on the amount of the free amino acid. For this purpose, the SIDA approach according to Reinbold et al.[19] was followed using a derivatization with dansyl chloride and [$^{13}C_3{}^{15}N$]-L-cysteine as internal standard. After the chilled storage of the juice for six months the concentration of free L-cysteine was decreased from 2.89 mg/kg to 2.03 mg/kg.

Studies on the Formation of 2-Alkylsubstituted Thiazolidine-4-Carboxylic Acids (2-AT-4-CA). While the concentrations of saturated aldehydes and free L-cysteine declined after chilled storage, the concentrations of 2-AT-4-CAs went up. Thus, the formation can be proposed as a condensation reaction between the aldehyde and L-cysteine leading to a ring formation between the carbonyl function of the aldehyde and the amino- and sulfanyl group of L-cysteine by water elimination (Figure 5).

To confirm that the same reaction also takes place in orange juice, spiking experiments with orange juice using stable isotope labeled analogues of L-cysteine and octanal, respectively, were carried out. For this purpose, the juice was either spiked with [$^{13}C_3{}^{15}N$]-L-cysteine or with [$^{13}C_8$]-octanal, and stored for several days at 7 °C or 37 °C, respectively. The concentrations of the unlabeled and labeled 2-heptylthiazolidine-4-carboxylic acid were measured using [$^{2}H_4$]-2-HT-4-CA as internal standard to monitor the formation of [$^{13}C_8$]-2-HT-4-CA formed from the added [$^{13}C_8$]-labeled octanal (Table 7). At 7 °C, the concentration of the labeled [$^{13}C_8$]-2-HT-4-CA quickly raised to 262 µg/kg within the first hour, but then dropped and remained at

about 30 to 35 µg/kg during further storage. But, spiking the juice with [$^{13}C_3{}^{15}N$]-L-cysteine only resulted in lower concentrations of the corresponding [$^{13}C_3{}^{15}N$]-2-HT-4-AC after 6 days of storage (Table 8). A possible reason for the lower conversion rate maybe the lower amount of the available reaction partner octanal compared to the higher amount of free available L-cysteine (2.89 mg/kg), which may quickly react with the added [$^{13}C_8$]-octanal (Table 7). In the storage trial at 37 °C, a drastic decrease of the labeled as well as the unlabeled 2-HT-4-AC following the fast formation of the condensation product in the very beginning. Nevertheless, screening a 2-HT-4-AC solution at higher temperatures did neither result in octanal nor L-cysteine suggesting a formation of yet unknown degradation products. The results confirmed that the formation of the 2-HT-4-AC also occurs in orange juice following the mechanism shown in Figure 5. So, this reaction clearly contributes to the loss of the aroma-active aldehydes during chilled storage of NFC juice.

Although the reaction of L-cysteine with saturated as well as unsaturated aldehydes, ketones or steroids is long-known,[18,24–26] in previous studies the focus was mainly on structural characterization and biochemical functionality. For example, the formation of steroid thiazolidines showed reactivity towards proteins and enzymes proposing steroid hormone functionality as prosthetic groups or co-enzymes.[25] Penicillin, also containing a thiazolidine moiety, aroused the interest in these heterocyclic acids and their bioactivity and pharmaceutical properties.[27] For example, 2-thiazolidine-4-carboxylic acid, the reaction product of formaldehyde and L-cysteine, belonging to the group of reactive carbonyl metabolites in the human body, has been found to have anti-inflammatory, anti-diabetes or anti-cancer properties.[28] However, to our knowledge, this study is the first report on a clear characterization of these heterocyclic acids in a food.

Baert et al.[29,30] recently monitored the amounts of some aldehydes in beer during storage. The decrease in aldehyde concentrations after adding L-cysteine led to the assumption on the formation of 2-substituted thiazolidine-4-carboxylic acids. However, the individual condensation products in beer were not characterized on the basis of synthesized reference compounds. Only 2-(furan-2-yl)-1,3-thiazolidine-4-carboxylic acid was characterized by means of UPLC-UV in a pale lager beer in comparison to a reference solution, but no quantitative data were reported.

Kim and Shin[31] developed an LC-MS/MS method with an online derivatization of D-cysteine for the determination of aldehydes in water and alcoholic beverages. For thiazolidine-4-carboxylic acid also a stable isotope dilution assay was developed using [$^2H_2$]-formaldehyde for the synthesis of the labeled standard.[31] In our study, new stable isotope dilution assays were developed for the simultaneous quantitation of four different 2-alkylsubstituted thiazolidine-4-carboxylic acids using individually labeled internal standards. Monitoring not only the $[M+H]^+$ mass traces, but also characteristic mass transitions at an optimized ionization energy, the analysis was made specific and yielded very precise results, even in a complex matrix like orange juice.

In beer, aldehydes contribute to a staling or aging off-flavor during storage. Hence, introducing free L-cysteine into beer was suggested to positively influence the aroma of beer during storage.[29,30] Naim et al.[32,33] had already proposed a similar approach to inhibit the generation of off-flavor compounds during storage of orange juice. They found a significantly lower formation of *p*-vinylguaiacol and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone, next to a decreased degradation of ascorbic acid after adding L-cysteine to orange juice. However, the effect of a L-cysteine administration on other key aroma compounds in orange juice has not been investigated until now. As aldehydes were confirmed to have a positive impact on the desirable aroma of fresh orange juice,[11] this well-meant approach of adding L-cysteine to stabilize the aroma of

orange juice could backfire and result in losses of key aroma compounds, and consequently the loss of freshness in the juice aroma profile.

The results show that an oxidation of aroma-active aldehydes to the corresponding acids also occurs during chilled storage of NFC orange juice. But, in addition the formation of 2-alkylsubstituted thiazolidine-4-carboxylic acids was characterized as a new pathway leading to losses of four saturated aldehydes during juice storage. The previously suggested addition of L-cysteine to stabilize the juice[32,33] was not confirmed as a useful idea for juice stabilization, because 2-AT-4-CAs are immediately formed, four of which were identified for the first time in orange juice. The results clarified a new puzzle piece in the degradation of aroma-active saturated aldehydes during storage of orange juice. Results of studies on the stability and degradation pathways of 1-penten-3-one, recently identified also as important indicator of juice freshness, will be published separately.

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Funding

We thank Tropicana Products Inc./ PepsiCo Global R & D for funding this project.

Notes

The authors declare no competing financial interest.

Please note that Deutsche Forschungsanstalt für Lebensmittelchemie was recently renamed as Leibniz-Institute for Food Systems Biology at the Technical University Munich.

ACKNOWLEDGMENTS.

We are grateful to S. Kaviani and I. Otte for excellent technical assistance with the LC-MS measurements and thanks to C. Dawid for her help with the UPLC-TOF/MS measurements. We also thankfully acknowledge J. Kreissl for the skillful acquisition of the NMR spectra.

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625 off-flavor formation in stored orange juice. 2. Effect of L-cysteine and *N*-acetyl-L-
626 cysteine on *p*-vinylguaiacol formation. *J. Agric. Food Chem.* **1993**, *41*, 1359–
627 1361.

FIGURE CAPTIONS

Figure 1. Chemical structures of 2-pentylthiazolidine-4-carboxylic acid (**1**), 2-heptylthiazolidine-4-carboxylic acid (**2**), 2-octylthiazolidine-4-carboxylic acid (**3**) and 2-nonylthiazolidine-4-carboxylic acid (**4**).

Figure 2. Proposed radical oxidation of octanal to the corresponding octanoic acid via an intermediate peroxyacid.

Figure 3. gs-COSY ($^{2,3}J_{H,H}$ correlation) spectrum of 2-heptylthiazolidine-4-carboxylic acid.

Figure 4. LC-MS/MS run showing the specific mass transitions of 2-heptylthiazolidine-4-carboxylic acid and the respective internal standard [$^{13}C_8$]-2-heptylthiazolidine-4-carboxylic acid in an authentic orange juice sample.

Figure 5. Proposed mechanism of the formation of 2-substituted thiazolidine-4-caboxylic acids.

TABLES

Table 1. Assignment of ^{1}H NMR Signals (400MHz, DMSO-d_6) of 2-Pentylthiazolidine-4-Carboxylic Acid (**1**).

H at relevant C atom[b]	δ[a]		I[c]	M[c]	J[c] (Hz)		connectivity[d] with
	(1)a	(1)b			(1)a	(1)b	
CH3 (5')	0.86	0.83	3	t	6.76	6.76	H-C(2'), H-C(3'), H-C(4')
H-C(2'), H-C(3'), H-C(4')	1.18-1.47	1.18-1.47	6	m			H-C(2'), H-C(3'), H-C(4'), H-C(5')
Hα-C(1')	1.61-1.83	1.47-1.59	1	m			H-C(2)
Hβ-C(1')	1.83-1.96	1.61-1.83	1	m			H-C(2)
Hα-C(5)	2.75	2.93	1	dd	9.24, 9.84	5.12, 10.24	H-C(4), Hβ-C(5)
Hβ-C(5)	3.18	3.08	1	dd	6.93, 9.84	7.04, 10.24	H-C(4), Hα-C(5)
H-C(4)	3.70	4.06	1	dd	6.96, 9.24	5.16, 6.96	Hα-C(5), Hβ-C(5)
H-C(2)	4.40	4.50	1	dd	5.80, 7.40	6.28, 7.44	Hα-C(1'), Hβ-C(1')

[a] The ^{1}H chemical shifts (ppm) are given in relation to $CDCl_3$. [b] Numbering of carbon atoms refers to formula (1) in Figure 1. [c] Determined from 1D spectrum. [d] Observed homonuclear ^{1}H,^{1}H connectivity by COSY.

Table 2. Assignment of ^{13}C NMR Signals (400 MHz, DMSO-d_6) of 2-Pentylthiazolidine-4-Carboxylic Acid (**1**)

relevant C atom[b]	δ[c]			heteronuclear $^{1}H,^{13}C$ multiple-quantum coherence[a]	
	(1)a	(1)b	DEPT[d]	via ^{1}J(C,H)	via $^{2,3}J$(C.H)
C(5')	14.3	14.3	CH_3	H-C(5')	H-C(2'), H-C(3'), H-C(4')
C(4')	22.4/ 22.5	22.4/ 22.5	CH_2	H-C(4')	H-C(2'), H-C(3'), H-C(4'), H-C(5')
C(2')	27.5/ 27.7	27.5/ 27.7	CH_2	H-C(2')	H-C(2'), H-C(3'), H-C(4'), H-C(5')
C(3')	31.5	31.5	CH_2	H-C(3')	H-C(2'), H-C(3'), H-C(4'), H-C(5')
C(1')	35.3	37.1	CH_2	Hα-C(1'), Hβ-C(1')	H-C(2)
C(5)	37.5	37.1	CH_2	Hα-C(5'), Hβ-C(5')	H-C(4)
C(4)	65.7	64.6	CH	H-C(4)	Hα-C(5'), Hβ-C(5')
C(2)	71.6	70.9	CH	H-C(2)	Hα-C(1'), Hβ-C(1'), H-C(4), Hα-C(5), Hβ-C(5)
C(6)	172.8	173.4	COOH	-	H-C(4), Hα-C(5), Hβ-C(5)

[a] Assignments based on HMQC (^{1}J) and HMBC ($^{2,3}J$) experiments. [b] Numbering of carbon atoms refers to formula (1) in Figure 1. [c] The ^{13}C chemical shifts (ppm) are given in relation to DMSO-d_6. [d] DEPT-135 spectroscopy.

Table 3. Most Abundant Mass Transitions and Collision Energies for the SRM Detection of 2-Substituted Thiazolidine-4-Carboxylic Acids and the Respective Isotopically Labeled Standards

2-substituted thiazolidine-4-carboxylic acid	ion transition	collision energy (V)
2-pentylthiazolidine-4-carboxylic acid	*m/z* 204 → *m/z* 158	13
	m/z 204 → *m/z* 100*	19
[$^{2}H_{12}$]-2-pentylthiazolidine-4-carboxylic acid	*m/z* 216 → *m/z* 170	13
	m/z 216 → *m/z* 112*	19
2-heptylthiazolidine-4-carboxylic acid	*m/z* 232 → *m/z* 182	18
	m/z 232 → *m/z* 128*	20
[$^{13}C_{8}$]-2-heptylthiazolidine-4-carboxylic acid	*m/z* 240 → *m/z* 194	18
	m/z 240 → *m/z* 136*	20
[$^{13}C_{3}{}^{15}N$]-2-heptylthiazolidine-4-carboxylic acid	*m/z* 236 → *m/z* 189	18
	m/z 236 → *m/z* 129*	20
[$^{2}H_{4}$]-2-heptylthiazolidine-4-carboxylic acid	*m/z* 236 → *m/z* 190	18
	m/z 236 → *m/z* 132*	20
2-octylthiazolidine-4-carboxylic acid	*m/z* 246 → *m/z* 200	14
	m/z 246 → *m/z* 142*	18
[$^{2}H_{4}$]-2-octylthiazolidine-4-carboxylic acid	*m/z* 250 → *m/z* 204	14
	m/z 250 → *m/z* 146*	18
2-nonylthiazolidine-4-carboxylic acid	*m/z* 260 → *m/z* 214	15
	m/z 260 → *m/z* 156*	17
[$^{2}H_{2}$]-2-nonylthiazolidine-4-carboxylic acid	*m/z* 262 → *m/z* 216	15
	m/z 262 → *m/z* 158*	17

* most abundant transition used for quantitation.

Table 4. Concentrations of Aldehydes and Corresponding Acids in Fresh and 6 Month Stored Orange Juice.

	amount of substance [µmol/kg][a]		
aldehyde and corresponding acid	fresh	6 month stored	Δ in amount of substance [µmol/kg]
C6			
hexanal	3.21	1.79	-1.42
hexanoic acid	2.40	3.05	+0.65
C8			
octanal	2.55	1.23	-1.33
octanoic acid	2.31	4.65	+2.34
C9			
nonanal	0.64	0.37	-0.27
nonanoic acid	0.76	2.95	+2.19
C10			
decanal	1.63	0.62	-1.01
decanoic acid	0.12	0.42	+0.30

[a] mean values of triplicates.

Table 5. Aldehydes and Their Corresponding L-Cysteine Reaction Product

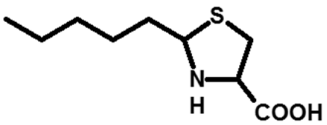
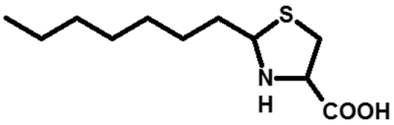
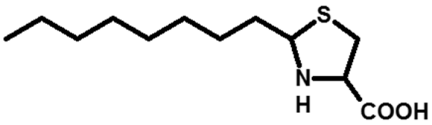
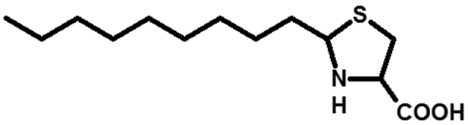
aldehyde	predicted product	sum formula and mass	structure
hexanal	2-pentylthiazolidine-4-carboxylic acid (2-PT-4-CA)	$C_9H_{17}O_2NS$ (209 g/mol)	
octanal	2-heptylthiazolidine-4-carboxylic acid (2-HT-4-CA)	$C_{11}H_{21}O_2NS$ (231 g/mol)	
nonanal	2-octylthiazolidine-4-carboxylic acid (2-OT-4-CA)	$C_{12}H_{23}O_2NS$ (245 g/mol)	
decanal	2-nonylthiazolidine-4-carboxylic acid (2-NT-4-CA)	$C_{13}H_{25}O_2NS$ (259 g/mol)	

Table 6. Concentration of 2-Substituted Thiazolidine-4-Carboxylic Acids and Free L-Cysteine in Fresh and 6 Month Stored Orange Juice.

	concentration [µg/kg][a]		increase
cysteine-adduct	fresh	6 month stored	(%)
2-pentylthiazolidine-4-carboxylic acid	82.6	126	+53 %
2-heptylthiazolidine-4-carboxylic acid	160	202	+26 %
2-octylthiazolidine-4-carboxylic acid	14.2	20.1	+42 %
2-nonylthiazolidine-4-carboxylic acid	64.8	100	+55 %
free L-cysteine	2890	2030	-30 %

[a] mean values of triplicates.

Table 7. Concentration of 2-Heptylthiazolidine-4-Carboxylic Acid and [$^{13}C_8$]-2-Heptylthiazolidine-4-Carboxylic Acid during Storage at 7 °C or 37 °C, respectively, of Orange Juice Spiked with [$^{13}C_8$]-Octanal.

	at 7 °C		at 37 °C	
storage time	2-HT-4CA [µg/kg][a]	[$^{13}C_8$]-2-HT-4CA [µg/kg][a]	2-HT-4CA [µg/kg][a]	[$^{13}C_8$]-2-HT-4CA [µg/kg][a]
initial (0 day)	9.87	262	9.87	262
1 day	11.5	26.1	4.06	117
4 days	11.3	37.4	0.38	9.59
6 days	11.2	42.1	0.23	3.74
8 days	9.87	40.3	0.16	2.18
11 days	8.79	38.2	0.11	2.17
14 days	6.96	32.6	0.10	1.78

[a] mean values of triplicates.

Table 8. Concentration of 2-Heptylthiazolidine-4-Carboxylic Acid and [$^{13}C_3{}^{15}N$]-2-Heptylthiazolidine-4-Carboxylic Acid during Storage at 7 °C of Orange Juice Spiked with [$^{13}C_3{}^{15}N$]-L-Cysteine.

storage time	at 7 °C	
	2-HT-4CA [µg/kg][a]	[$^{13}C_3{}^{15}N$]-2-HT-4CA [µg/kg][a]
10 min	9.12	1.43
1 h	8.90	2.21
6 h	9.54	5.31
1 d	9.37	5.46
2 d	9.86	7.01
3 d	11.2	8.73
6 d	12.3	9.89

[a] mean values of triplicates.

FIGURES

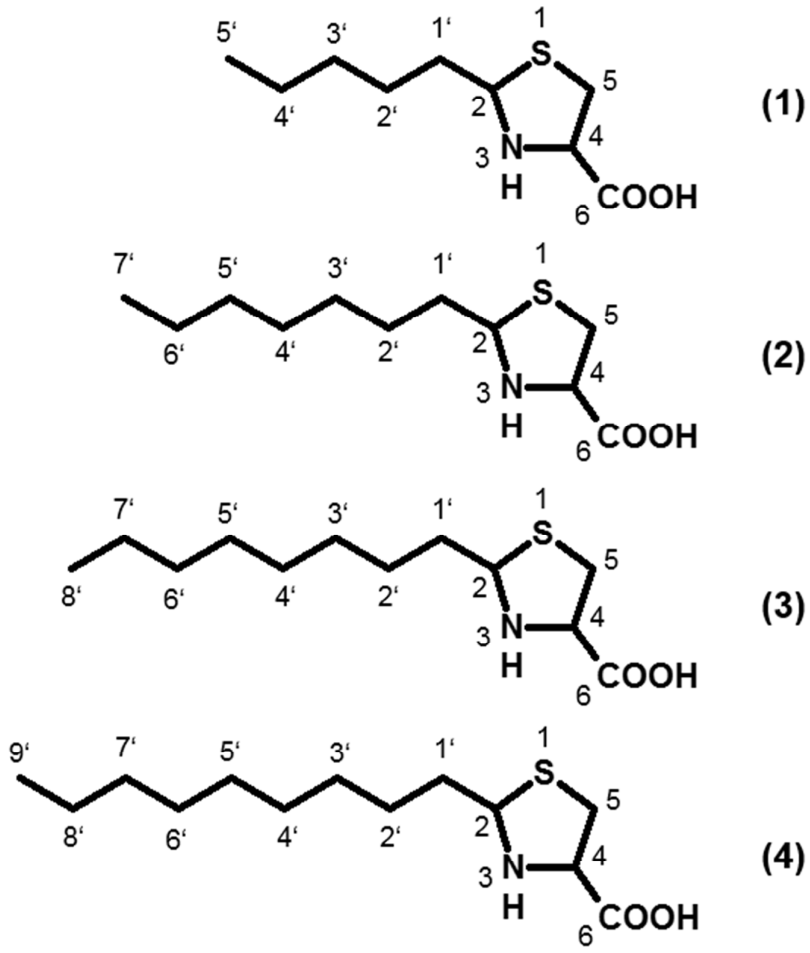
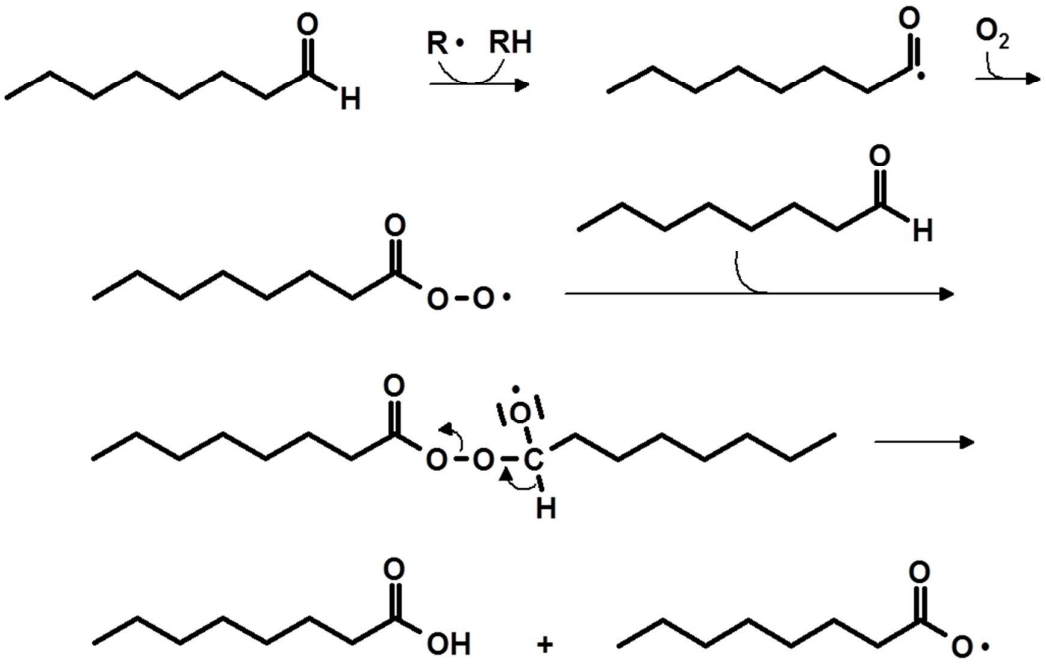


Figure 1.



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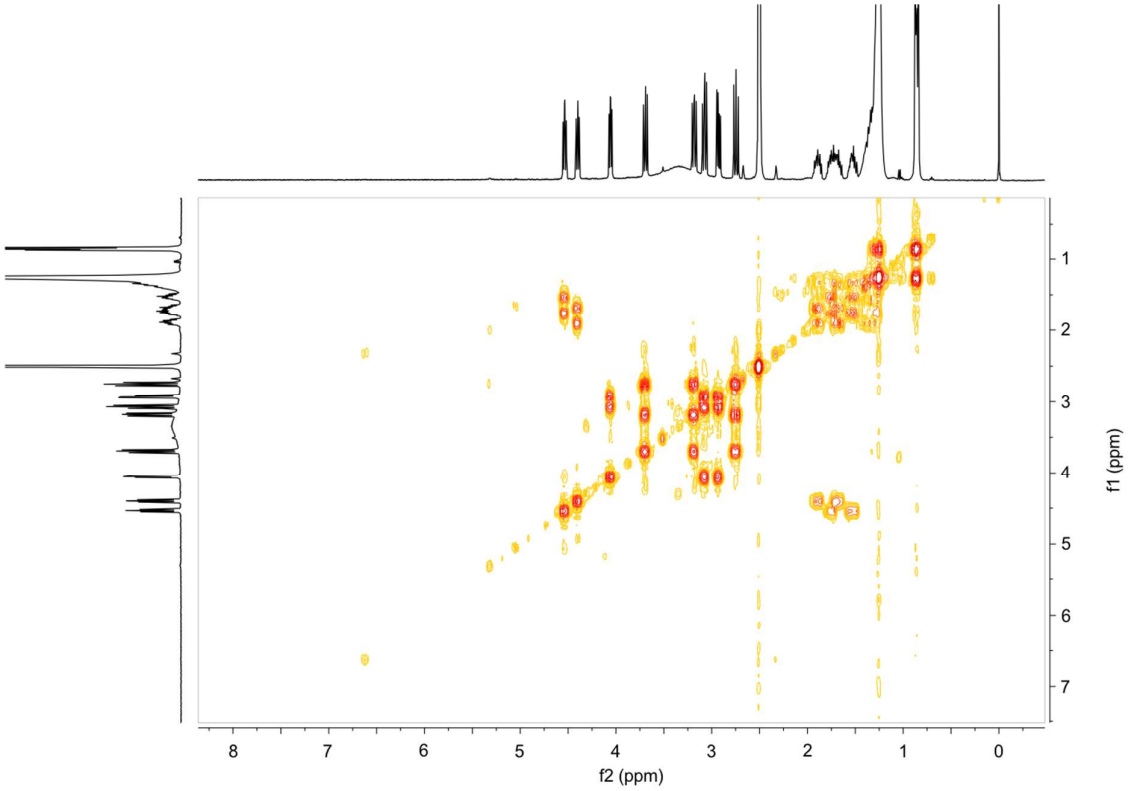

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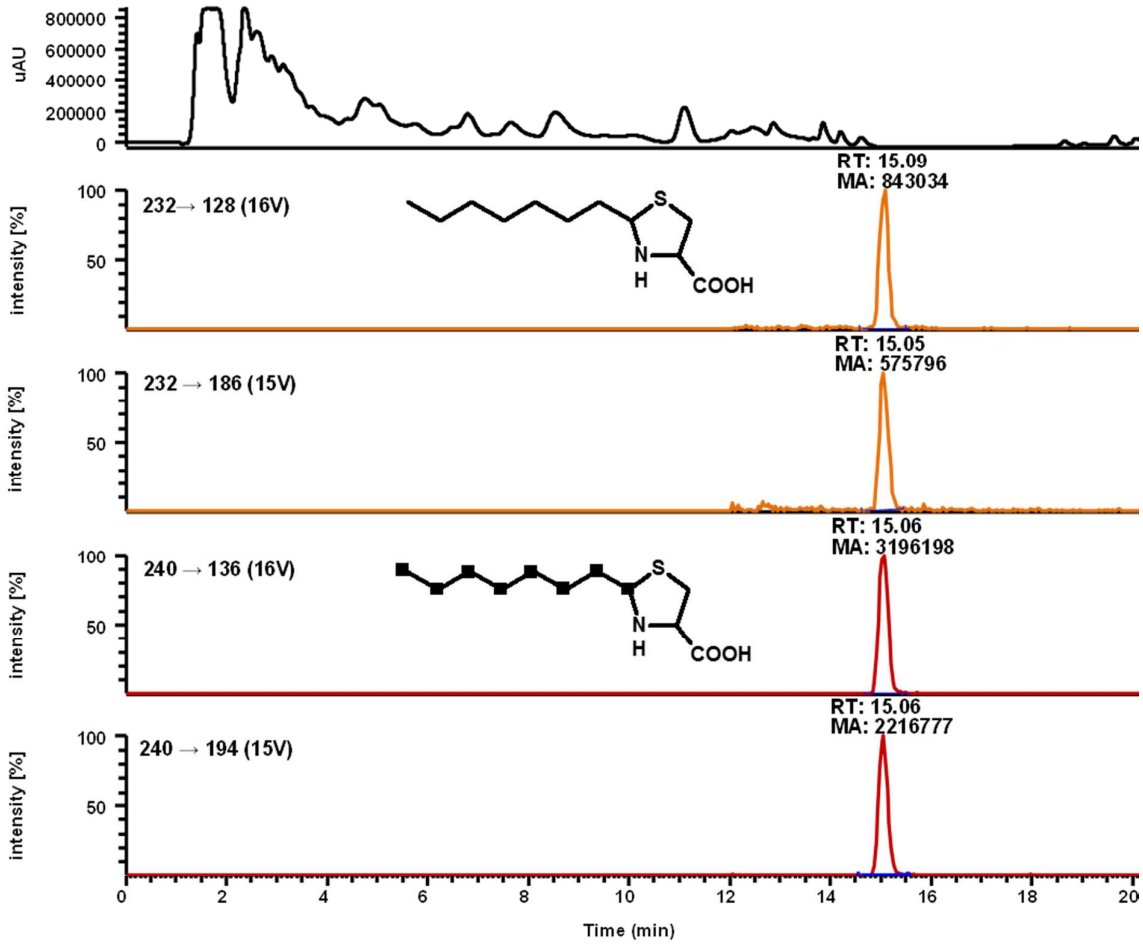
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COOH
RT: 15.05
MA: 575796
232 → 186 (15V)
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240 → 136 (16V)
RT: 15.06
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0
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4
6
8
10
12
14
16
18
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Time (min)

Figure 4.

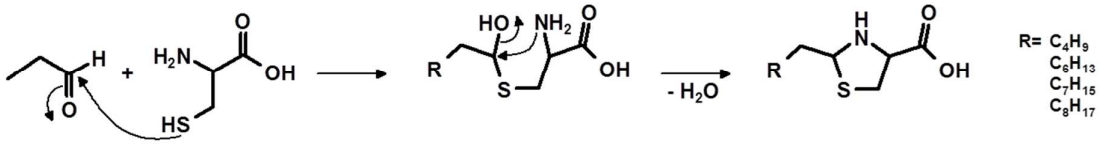

Figure 5.

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