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Identification of the Medicinal Off-Flavor Compound Formed from Ascorbic Acid and (*E*)-Hex-2-enal

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ABSTRACT: A test apple beverage made up of apple juice (20%), high-fructose corn syrup (11.5%), citric acid (0.43%), trisodium citrate (0.02%), apple-odor flavor (0.1%), and ascorbic acid (0.02%) was stored at 40 °C and then analyzed for the change of odor in the beverage. Although no thermoacidophilic bacteria (TAB) were detected, a medicinal off-flavor was perceived after the 8 weeks of storage. Model experiments on the ingredients of the test apple beverage revealed that the off-flavor compound had been formed by ascorbic acid and (*E*)-hex-2-enal. Synthesis and NMR (^{1}H, ^{13}C, HMQC, and HMBC) analyses identified the compound as 6-propylbenzofuran-7-ol. The odor quality, retention index (RI), and mass spectrum of synthetic 6-propylbenzofuran-7-ol were identical with those of the medicinal odor compound from the test apple beverage. Sensory evaluation revealed the recognition thresholds for medicinal odor were 31.4 ppb in water and 24.0 ppb in apple beverage, and the detection thresholds were 19.6 ppb in water and 8.6 ppb in apple beverage, respectively. The quantified concentration of 6-propylbenzofuran-7-ol formed in test apple beverage was 90 ppb, approximately. This concentration was well above the odor threshold, so it was concluded that the compound was the source of the medicinal off-flavor.

KEYWORDS: apple beverage, off-flavor, ascorbic acid, (*E*)-hex-2-enal, 6-propylbenzofuran-7-ol

INTRODUCTION

Public interest in food safety has rapidly grown over the past decade. Consumer complaints about the off-flavors of food products have become one of the most frequent problems confronting the food industry. An off-flavor may arise from the incidental contamination of a food from environmental sources (e.g., air, water, packaging materials), degradation of food components (e.g., nonenzymatic browning, enzymatic action, lipid oxidation), loss of key odorants, or changes in the concentrations of individual aroma substances. Apple juice is particularly susceptible to the appearance of off-flavor, and there are various causes for off-flavor formation.

Off-flavor derived from the so-called "thermoacidophilic" bacteria (TAB) is one of the major problems in apple juice. Many studies on apple juice have identified the source of the off-flavor as *Alicyclobacillus acidoterrestris*.[1−10] Guaiacol[3] and 2,6-dibromophenol have been identified as the off-flavor compounds produced by *A. acidoterrestris*.[4] Guaiacol, a decomposition product of ferulic acid via vanillin, is recognized as the predominant metabolite.[8]

The flavor of apple juice can be changed during processing. Perédi et al. reported that apple juices and concentrates contained less than half of the volatile components present in original fruit.[11] Su and Wiley reported that the flavor profile changed at different stages of production.[12] In a similar vein, Steinhaus et al. reported that key aroma compounds in apple juice changed during juice concentration.[13]

Hashizume et al., meanwhile, reported that exposure to light induced a metallic off-flavor in apple juice.[14] Their experiments identified 1-octen-3-one as the major contributor to the off-flavor and six volatile compounds as secondary contributors (pentanal, 2-methyl-1-penten-3-one, hexanal, (*E*)-hept-2-enal, 6-methyl-5-hepten-2-one, and (*E*)-oct-2-enal).

Our group has often observed these reported phenomena in our own investigations on the stabilities of various types of apple beverages. Recently we encountered a new type of medicinal off-flavor in one of our test apple beverages stored at 40 °C for 8 weeks. Yet in our analyses of samples with this off-flavor, we detected no traces of the microorganisms or compounds mentioned. In this study, we describe the formation and the structure of this off-flavor compound.

MATERIALS AND METHODS

Test Apple Beverage Sample. The test apple beverage used in the experiments was made up of apple juice (20%), high-fructose corn syrup (11.5%), citric acid (0.43%), trisodium citrate (0.02%), apple-odor flavor (0.1%), and ascorbic acid (0.02%). Concentrated juice was purchased from Alps Co. (Nagano, Japan) (ascorbic acid = 0.2−0.3%). The apple-odor flavor contained (*E*)-hex-2-enal, (*E*)-hex-2-enyl acetate, ethyl butanoate, 3-methylbutyl acetate, hexyl acetate, 2-methylbutanoic acid, and ethanol. The pH was controlled (pH 2.8). The test apple beverage was stored at 4 and 40 °C in darkness for 8 weeks in plastic bottles and then analyzed. Also, the test apple beverage without apple-odor flavor was stored in the same conditions and evaluated organoleptically.

Microbiological Methods. TAB were detected with a yeast-extract starch glucose (YSG) agar medium. The YSG agar medium was composed of two solutions mixed after sterilization at 121 °C for 15 min: (a) yeast extract, 2 g; glucose, 1 g; soluble starch, 2 g; and distilled water, 500 mL, adjusted to pH 3.7 with HCl; (b) agar, 15 g; distilled water, 500 mL. After a heat-shock of the test apple beverage at 70 °C for 20 min, the sample (1 mL) was incorporated directly into the YSG agar medium by the pour plate technique and incubated at 45 and 55 °C for 5 days. No colony was observed after the incubation.

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Chemicals. The following chemicals were obtained commercially: (*E*)-hex-2-enal (PFW Aroma Chemicals B.V., Barneveld, Netherlands), ascorbic acid (DSM Nutrition Japan K.K., Tokyo, Japan), and sodium dihydrogen phosphate dihydrate (Junsei Chemical Co., Ltd., Tokyo, Japan). All other reagents and solvents were of analytical grade.

Isolation of Aroma Compounds. Twelve kilograms of test apple beverage sample was distilled under reduced pressure (30 °C/6.5 kPa) until 6 kg of distillate was collected in three cooling traps at low temperature (ice−water/dry ice−ethyl alcohol/liquid nitrogen). After the addition of 1.2 kg of sodium chloride, the distillate was extracted with diethyl ether (3 × 1.2 L). The organic layers were combined, dried over anhydrous sodium sulfate, filtered, and concentrated with a Vigreux column under atmospheric pressure to yield 1.35 g of volatile concentrate.

Apple Beverages Added Each Flavor Constituent. These apple beverages were made up with the same formula as used for the test apple beverage except flavor. Six apple beverages were prepared, and to each beverage was added one of the constituents of apple-odor flavor (Table 1). The beverages were stored at 55 °C for 13 days and then evaluated organoleptically.

Model Solutions. Five model solutions were prepared (Table 2). Model solutions of cloudy apple juice and clear apple juice were reconstituted from concentrate. Concentrated juices were purchased from Alps Co. A model solution of hand-squeezed juice was reconstituted from fresh apples. Solution of citric acid and syrup was adjusted to °Brix 10.0 with high-fructose corn syrup. All reconstituted model solutions were adjusted to pH 2.8 with citric acid and formulated with (*E*)-hex-2-enal at 0.03% concentrations. The model solutions were stored at 55 °C for 13 days and then analyzed.

Gas Chromatography−Mass Spectrometry (GC-MS). The GC-MS analyses were performed with an Agilent 6890 gas chromatograph (GC) combined with an Agilent MSD5973 quadrupole mass spectrometer equipped with a TC-WAX capillary column (0.25 mm i.d. × 60 m, 0.25 μm film thickness; GL Sciences Co., Tokyo, Japan). The injection port was kept at 250 °C. The split ratio was 50:1 with 0.2 μL of sample injected. The oven temperature was held at 40 °C for the initial 3 min and then increased to 230 °C at a rate of 3 °C/min, with a constant carrier helium gas flow of 1.8 mL/min. Mass spectra in the electron impact (EI) mode were recorded at 70 eV ionization energy. The linear retention indices (RI) of the compounds were calculated from the retention times of *n*-alkanes.

Table 1. Sensory Evaluation of Apple Beverages Added Each Flavor Constituent and Stored at 55 °C for 13 Days

flavor constituent	off-flavor
(*E*)-hex-2-enal	strongly detected
(*E*)-hex-2-enyl acetate	not detected
ethyl butanoate	not detected
3-methylbutyl acetate	not detected
hexyl acetate	not detected
2-methylbutanoic acid	not detected

Gas Chromatography−Olfactometry (GC-O). The GC-O analyses were performed with an Agilent 6890 GC equipped with a Gerstel ODP2 sniffing port and a flame ionization detector (FID). The effluent of the column at the end of the capillary was divided into two branches and routed by deactivated fused silica capillaries to t he sniffing port and FID, respectively. The column, sample volume, split ratio, injection temperature, oven temperature program, carrier gas, and flow rate were all the same as those set for the GC-MS analysis described above.

Infrared Absorption (IR) Spectra. IR spectra were recorded on a GC-FT-IR, a Hewlett-Packard 5890 GC connected to a Hewlett-Packard 5965 IR equipped with a TC-WAX capillary column (0.32 mm i.d. × 60 m, 0.25 μm film thickness; GL Sciences Co.). Sample was injected in 1 μL volumes in a split mode (50:1) at a constant temperature of 250 °C. The oven temperature was kept at 40 °C for the initial 3 min and then increased to 230 °C at a rate of 3 °C/min, with a constant carrier helium gas flow of 1.8 mL/min.

Nuclear Magnetic Resonance (NMR) Spectra. ^{1}H, ^{13}C, heteronuclear multiple quantum correlations (HMQC), and heteronuclear multiple bond correlation (HMBC) experiments were performed on a JEOL JNM-LA400 spectrometer. Using $CDCl_3$ as solvent, chemical shifts were measured using tetramethylsilane as internal standard. The chemical shifts (δ) and coupling constants (J) are expressed in parts per million (ppm) and hertz (Hz), respectively.

Synthesis of 6-Propylbenzofuran-7-ol. Ascorbic acid (4.0 g, 22.7 mmol) and sodium dihydrogen phosphate dihydrate (4.0 g, 25.6 mmol) were dissolved in deionized water (400 g) under nitrogen atmosphere, and (*E*)-hex-2-enal (4.0 g, 40.8 mmol) was then added. The mixture was stirred and heated to 100 °C for 48 h. After cooling to room temperature, the reaction mixture was extracted with diethyl ether (3 × 50 mL). The organic layers were combined, washed with brine, dried over $MgSO_4$, and concentrated under high vacuum. The residue (4.8 g) was purified by silica gel chromatography using *n*-hexane/ethyl acetate (20:1, v/v) into a yellow oil of 6-propylbenzofuran-7-ol (0.2 g, yield = 5.0%, purity = 97.9%): ^{1}H NMR (400 MHz) δ 1.02 (t, J = 7.4, 3H), 1.72 (tq, J = 7.4, 2H), 2.77 (t, J = 7.4, 2H), 5.55 (br, 1H), 6.75 (d, J = 2.0, 1H), 7.04 (d, J = 8.0, 1H), 7.11 (d, J = 8.0, 1H), 7.57 (d, J = 2.0, 1H); ^{13}C NMR (100 MHz) δ 14.0, 23.7, 31.5, 107.3, 112.5, 124.4, 125.4, 126.7, 138.8, 143.8, 144.4; MS-EI, 176 (M^+, 22), 148 (10), 147 (100), 91 (14), 65 (6); GC-IR, 3629, 3057, 2963, 2882, 1433, 1219, 799 cm^{-1}.

Quantification of 6-Propylbenzofuran-7-ol in Test Apple Beverage. 1-Naphthol was used as internal standard. An aqueous solution of 1-naphthol (0.5 g/L, 1 mL) was added to 500 g of the test apple beverage. The sample was distilled under reduced pressure (40 °C at 3 × 10^{-3} Pa) by solvent-assisted flavor evaporation (SAFE).[15] The SAFE distillate was extracted with diethyl ether (3 × 100 mL). The organic layers were combined, dried over anhydrous sodium salfate, filtered, and concentrated with a Vigreux column under atmospheric pressure to yield 19.8 and 18.8 mg of volatile concentrates from the test apple beverage after 8 weeks of storage at 40 and 4 °C, respectively. The volatile concentrates were analyzed by GC-MS.

Sensory Evaluation. The evaluation was conducted in a quiet room kept at 23 °C. Two booths were set up in the room, and each

Table 2. Sensory Evaluation of Model Solutions Stored at 55 °C for 13 Days

substrate[a]	°Brix	pH	juice (%)	ascorbic acid (%)	off-flavor
cloudy juice[b]	3.6	2.8	30	0.02	detected
clear juice[b]	3.7	2.8	30		slightly detected
hand-squeezed juice[b]	3.6	2.8	30		slightly detected
solution of citric acid and syrup	10.0	2.8			not detected
ascorbic acid	1.1	2.8		1.00	strongly detected

[a] Each substrate contains 0.03% of (*E*)-hex-2-enal. [b] Apple juice.

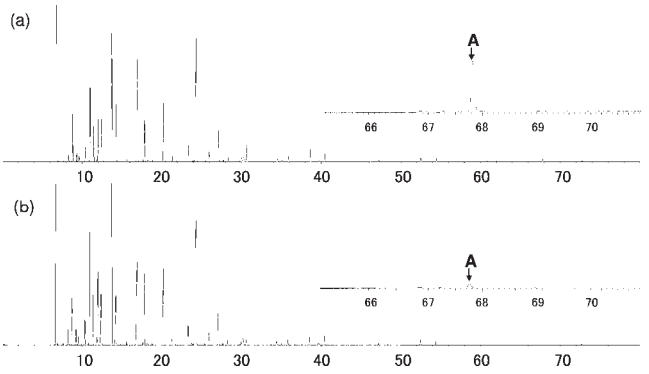

Figure 1. Total ion chromatogram of aroma concentration from (a) model apple juice stored at 40 °C for 8 weeks and (b) model apple juice stored at 4 °C for 8 weeks. (**A**) Off-flavor compound.

panelist performed the assessment alone in one booth to ensure that the panelists would not influence each other.

Sensory evaluation of model solutions or test apple beverages with each flavor constituent added was performed by three well-trained panelists. A 20 mL portion of each sample was put into a closed sensory vial (total volume = 30 mL). Each panelist was presented samples with instructions to sniff each sample and rate the intensity of off-flavor on the categorized scale from "not detected" to "slightly detected" to "detected" to "strongly detected".

Determination of Orthonasal Odor Threshold of 6-Propylbenzofuran-7-ol. Each evaluation was performed by 14−15 well-trained panelists. Orthonasal odor thresholds were determined in water or in test apple beverage without apple-odor flavor by means of a triangle test using the method of Czerny et al.[16]

RESULTS AND DISCUSSION

The test apple beverages were stored at 4 and 40 °C for 8 weeks in plastic bottles. After storage, medicinal off-flavor was detected organoleptically from the beverage stored at 40 °C, but not from the beverage stored at 4 °C. According to earlier studies,[3,6−8] the medicinal off-flavor in apple beverage originates from guaiacol formed as a metabolite of TAB. To determine whether TAB were present in our test apple beverage stored at 40 °C, we performed a microbiological test by the pour plate technique using a YSG agar medium. Upon finding no traces of TAB by this method, we isolated the volatiles from the samples by distillation under reduced pressure and extraction with diethyl ether and then analyzed the volatile concentrate by GC-MS. No traces of guaiacol were found.

Next, to screen for the medicinal odor compound, we analyzed the volatile compounds in the beverage by GC-O. The GC-O analysis detected a strong medicinal odor at RI 2520 (TC-WAX) from only the volatile concentration of the test apple beverage stored at 40 °C, thus implicating this compound as the off-flavor compound (**A**) (Figure 1). Compound **A** was also detected from the beverage stored at 4 °C, but the peak was very small. Therefore, it was thought that the generation of compound **A** was accelerated when the storage temperature became higher. To investigate the structure of this unknown compound (**A**), we then analyzed the volatile concentrate by both GC-MS and GC-IR and obtained the spectra. A molecular weight of 176 and the molecular formula $C_{11}H_{12}O_2$ were assumed from the mass spectrum (Figure 2a). Fragment ions at m/z 91 and 65 suggested that this compound contained a benzene ring, and a fragment ion

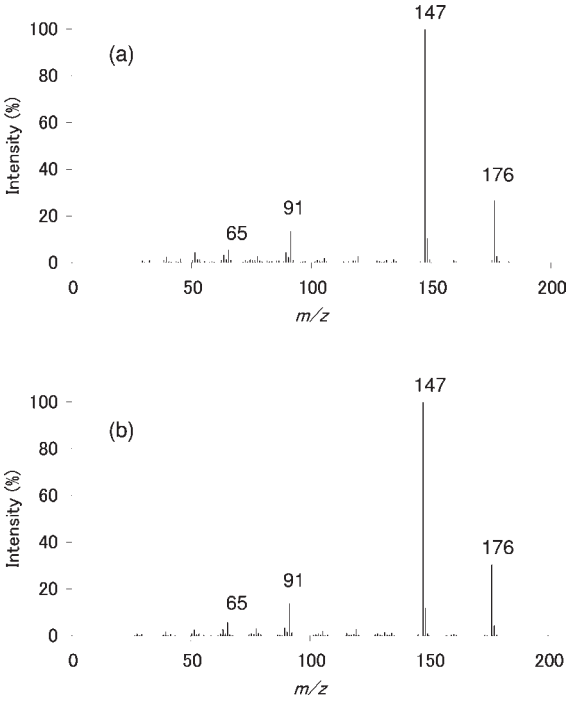

Figure 2. Mass spectra of off-flavor compound obtained from (a) model apple juice stored at 40 °C for 8 weeks and (b) synthesized compound.

at m/z 147 [M − 29] indicated cleavage of an ethyl group. These data suggested that an aromatic group was substituted by a propyl group. In the IR data, we observed a broad band at 3629 cm^{-1} corresponding to a hydroxyl group or, more specifically, to a phenolic hydroxyl group.

To elucidate whether apple-odor flavor took part in the generation of the off-flavor, we stored the test apple beverage without the apple-odor flavor in the same conditions. After storage, medicinal off-flavor was not detected organoleptically from this beverage. Therefore, it was confirmed that the apple-odor flavor was essential for the formation of off-flavor compound. Next, to gain insight into the formation of the off-flavor compound, we prepared apple beverages with each constituent of apple-odor flavor added (Table 1). For the purpose of accelerating the generation of the off-flavor, the beverages were stored at 55 °C. After storing the beverages for 13 days, we organoleptically detected a strong medicinal odor in the beverage containing (*E*)-hex-2-enal (Table 1). Therefore, we assumed that (*E*)-hex-2-enal reacted with other constituents to form the off-flavor compound.

Then we prepared a series of model solutions. Specifically, we added (*E*)-hex-2-enal to cloudy juice, clear juice, hand-squeezed juice, a solution of citric acid and high-fructose corn syrup, and a solution of ascorbic acid. After storing the model solutions at 55 °C for 13 days, we evaluated them organoleptically. Then, upon detecting a strong medicinal odor in the ascorbic acid solution (Table 2), we isolated the volatiles of this ascorbic acid solution by distillation under reduced pressure and extraction with diethyl ether and analyzed them by GC-MS and GC-O. These analyses detected the same off-flavor compound (**A**) from the volatiles. On this basis, we reveal that (*E*)-hex-2-enal and ascorbic acid were important constituents responsible for the formation of the off-flavor compound.

Table 3. RI on TC-WAX and TC-1 of the Off-Flavor Compound (A) and Synthesized Compound

	RI on	
	TC-WAX	TC-1
off-flavor compound (**A**) in the test apple beverage	2520	1473
synthesized compound	2520	1473

Table 4. Assignment of ^{1}H NMR Signals (400 MHz, $CDCl_3$) of 6-Propylbenzofuran-7-ol

H at relevant C atom[a]	δ	I	multiplicity	J (Hz)
H−C(10)	1.02	3	t	7.4
H−C(9)	1.72	2	tq	7.4
H−C(8)	2.77	2	t	7.4
HO−C(7)	5.55	1	br	
H−C(3)	6.75	1	d	2.0
H−C(5)	7.04	1	d	8.0
H−C(4)	7.11	1	d	8.0
H−C(2)	7.57	1	d	2.0

[a] Arbitrary numbering of carbon atoms refers to structure in Figure 3c.

To determine the chemical structure of unknown compound **A**, it seemed necessary to isolate and analyze the compound by means of NMR. For this analysis, we found a way to isolate the off-flavor compound from a large-scale reaction of (*E*)-hex-2-enal and ascorbic acid. A reaction of ascorbic acid (4.0 g) and (*E*)-hex-2-enal (4.0 g) yielded 0.2 g of medicinal odor compound. The odor quality, RI, and spectral properties of this synthesized compound closely matched those of the off-flavor compound (Table 3; Figure 2). The structure of the off-flavor compound was confirmed by NMR analysis. The ^{1}H NMR spectrum (Table 4) showed signals of four aromatic protons between δH 6.75 and 7.57 and one phenolic hydroxyl group at δH 5.55. The signals at δH 1.02 and 1.72 corresponded to the ethyl group, and the signal at δH 2.77 revealed methylene of benzyl position. In addition, the coupling constant between the signals at δH 6.75 and 7.57 was 2.2 Hz, and the chemical shifts and coupling constant fit well with the protons on the furan ring of benzofuran. Although this was sufficient evidence to identify the off-flavor compound as propylbenzofuranol, it did not allow us to decide the positions of the propyl and hydroxyl groups. The ^{13}C NMR (Table 5) showed three aliphatic carbons and eight aromatic carbons, a structure that fit well with propylbenzofuranol. The structure of the off-flavor compound was finally confirmed from the 2D NMR spectra, including the HMQC and HMBC spectra (Table 5). The compound was identified as 6-propylbenzofuran-7-ol. From the HMBC correlation with protons on the furan ring, the signals at δC 126.7 and 143.8 corresponded with the quaternary carbons of the furan ring, and the downfield signal and upfield signal were 7a carbon and 3a carbon, respectively (Figure 3a). The two signals of the quaternary carbons thus remained, and the downfield signal and the upfield signal were the carbon-bonded hydroxy group and the carbon-bonded propyl group, respectively. From the HMBC correlation with H−C8, the hydroxyl group was confirmed to be the ortho position of the propyl group and the other ortho position of the propyl group was confirmed as hydrogen. Only two structures satisfy these demands (Figure 3b). We also observed the

Table 5. Assignment of ^{13}C NMR Signals (100 MHz, $CDCl_3$) of 6-Propylbenzofuran-7-ol

		heteronuclear ^{1}H, ^{13}C connectivity[a]	
C atom[b]	δ	via $^1J_{C,H}$	via $^{2,3}J_{C,H}$
C(10)	14.0	H−C(10)	H−C(8), H−C(9)
C(9)	23.7	H−C(9)	H−C(10), H−C(8), H−C(5)
C(8)	31.5	H−C(8)	H−C(10), H−C(9), H−C(5)
C(3)	107.3	H−C(3)	H−C(2), H−C(5), H−C(4)
C(4)	112.5	H−C(4)	H−C(3)
C(6)	124.4		H−C(9), H−C(8), H−C(5), H−C(4)
C(5)	125.4	H−C(5)	H−C(8), H−C(4)
C(3a)	126.7		H−C(2), H−C(3), H−C(5), H−C(4)
C(7)	138.8		H−C(8), H−C(5), H−C(4)
C(7a)	143.8		H−C(2), H−C(3)
C(2)	144.4	H−C(2)	H−C(3)

[a] Assignments based on HMQC ($^1J_{C,H}$) and HMBC ($^{2,3}J_{C,H}$) experiments. [b] Arbitrary numbering of carbon atoms refers to structure in Figure 3c.

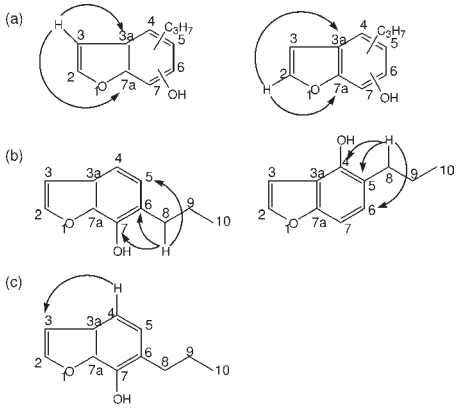

Figure 3. HMBC correlations observed for 6-propylbenzofuran-7-ol: (a) correlation with the proton on the furan ring; (b) correlation with H−C8; (c) correlation with the proton on the benzene ring at the meta position of the propyl group.

HMBC correlations of the proton on the benzene ring at the meta position of the propyl group to C3. Therefore, we identified the compound as 6-propylbenzofuran-7-ol (Figure 3c). To our knowledge, this compound had never been identified in foods or materials.

The sensory property of the synthesized 6-propylbenzofuran-7-ol seemed to be the predominant contributor to the medicinal off-flavor. We determined the orthonasal odor threshold of 6-propylbenzofuran-7-ol in water and in the test apple beverage by means of a triangle test.[16] The human recognition thresholds for medicinal odor were 31.4 ppb in water and 24.0 ppb in apple beverage, and the detection thresholds were 19.6 ppb in water and 8.6 ppb in apple beverage, respectively.

We also quantified 6-propylbenzofuran-7-ol in test apple beverage using 1-naphthol as internal standard to confirm that the concentration was higher than the threshold. As a result, the amount of the compound in test apple beverage stored at 40 °C for 8 weeks was 90 ppb, approximately. This concentration was high enough over the threshold to identify the compound as a major contributor to the medicinal off-flavor. On the other hand,

the amount of the compound in test apple beverage stored at 4 °C for 8 weeks was <1 ppb, and this concentration was considerably lower than the threshold.

In conclusion, we identified 6-propylbenzofuran-7-ol as the medicinal off-flavor compound formed from (*E*)-hex-2-enal and ascorbic acid in test apple beverage, and it was the first time this compound was identified in foods or materials.

(*E*)-Hex-2-enal and ascorbic acid exist widely in natural foods. Ascorbic acid is also widely used in processed foods as an antioxidant or for nutritional supplementation, and (*E*)-hex-2-enal is used as a flavoring material to give freshness. Insight into the mechanisms by which 6-propylbenzofuran-7-ol forms will help us prevent the appearance of off-flavors in foods made with (*E*)-hex-2-enal and ascorbic acid. Studies are underway to clarify the generation conditions and formation mechanisms of 6-propylbenzofuran-7-ol.

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ABBREVIATIONS USED

TAB, thermoacidophilic bacteria; GC, gas chromatography; GC-MS, gas chromatography—mass spectrometry; GC-MS-O, gas chromatography—mass spectrometry—olfactometry; FID, flame ionization detector; IR, infrared absorption; MS, mass spectrum; NMR, nuclear magnetic resonance; RI, retention index; HMQC, heteronuclear multiple quantum correlation; HMBC, heteronuclear multiple bond correlation; SAFE, solvent-assisted flavor evaporation.

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