

Studies on the Key Aroma Compounds in Raw (Unheated) and Heated Japanese Soy Sauce

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ABSTRACT: An investigation using the aroma extract dilution analysis (AEDA) technique of the aroma concentrate from a raw Japanese soy sauce and the heated soy sauce revealed 40 key aroma compounds including 7 newly identified compounds. Among them, 5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2H)-furanone and 3-hydroxy-4,5-dimethyl-2(5H)-furanone exhibited the highest flavor dilution (FD) factor of 2048, followed by 3-(methylthio)propanal, 4-ethyl-2-methoxyphenol, and 4-hydroxy-2,5-dimethyl-3(2H)-furanone having FD factors from 128 to 512 in the raw soy sauce. Furthermore, comparative AEDAs, a quantitative analysis, and a sensory analysis demonstrated that whereas most of the key aroma compounds in the raw soy sauce were common in the heated soy sauce, some of the Strecker aldehydes and 4-vinylphenols contributed less to the raw soy sauce aroma. The model decarboxylation reactions of the phenolic acids during heating of the raw soy sauce revealed that although all reactions resulted in low yields, the hydroxycinnamic acid derivatives were much more reactive than the hydroxybenzoic acid derivatives due to the stable reaction intermediates. Besides the quantitative analyses of the soy sauces, the estimation of the reaction yields of the phenolic compounds in the heated soy sauce revealed that although only the 4-vinylphenols increased during heating of the raw soy sauce, they might not mainly be formed as decarboxylation products from the corresponding hydroxycinnamic acids but from the other proposed precursors, such as lignin, shakuchirin, and esters with arabinoxylan.

KEYWORDS: raw soy sauce, aroma extract dilution analysis, standard addition method, heating process, phenolic compounds

INTRODUCTION

Traditionally brewed soy sauce has been one of the most popular seasonings in Japan for hundreds of years. Especially the Koikuchi-type soy sauce that accounts for about 85% of total soy sauce consumption in Japan is widely consumed all over the world.¹ Soy sauce is used not only as a sauce for food but as a seasoning in cooking. Nearly an equal amount of soybeans boiled to denature the protein and wheat comminuted after roasting are used as starting materials for brewing the soy sauce, followed by the addition of a small amount of seed mold of *Aspergillus oryzae* or *Aspergillus sojae* for cultivation over several days under high-humidity conditions.¹ This cultured mold is then mixed in an approximate 20% saline solution, and the obtained mash is traditionally fermented for 6–8 months. At first, a salt-tolerant lactic acid bacterium (*Tetragenococcus halophilus*) and salt-tolerant yeasts (*Candida versatilis* and *Candida etchellsii*) grow in the mash, followed by growth of the main fermentation yeast (*Zygosaccharomyces rouxii*). After maturation, the brew is compressed to separate the filtrate, called the raw soy sauce, and the residue. Soy sauce is generally produced as a dark-red liquid by heating the raw soy sauce to deactivate the enzymes and to bring about a complex flavor. On the contrary, the raw soy sauce has been widely marketed in recent years as a new type of soy sauce having fresh aroma characteristics with a short expiration date.

Many investigations concerning the many volatile compounds in soy sauce have been reported, and most of them were generated during the manufacturing processes as described above.¹ In recent years, the key aroma compounds of the Koikuchi-type soy sauce were reported by combining the screening of the soy sauce aroma concentrate with the aroma extract dilution analysis (AEDA), a quantitative analysis

of the key aroma compounds exhibiting high flavor dilution (FD) factors by a stable isotope dilution assay, and the sensory evaluation of their reconstituted solution.² This study suggested that 3-methylbutanal, 3-hydroxy-4,5-dimethyl-2(5H)-furanone, 5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2H)-furanone, 2-methylbutanal, and 3-(methylthio)propanal were the most important aroma components in the soy sauce. More recently, the key aroma compounds in five different types of Japanese soy sauces categorized according to the Japan Agricultural Standard were investigated by AEDA.³ This study suggested that the contributions of 5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2H)-furanone and 4-hydroxy-2,5-dimethyl-3(2H)-furanone to the respective soy sauce aromas were widely different among the varieties despite the significant contributions of sotolon and methional in all of the varieties. On the contrary, the key aroma compounds in the raw soy sauce are thought to be slightly different from those in the common soy sauce because of the lack of the final heating process, which is generally conducted at 80–85 °C for 30 min or at 110–130 °C for several minutes and at 60–65 °C for several days.^{4,5} Although previous studies demonstrated that furanones, such as 4-hydroxy-2,5-dimethyl-3(2H)-furanone and 4-hydroxy-5-methyl-3(2H)-furanone, Strecker aldehydes, such as 3-methylbutanal and phenylacetaldehyde, α -dicarbonyl compounds, such as 2,3-butanedione, pyrazines, and sulfur compounds, such as dimethyl sulfide and dimethyl disulfide, increased during this process,^{4,6–9} the key aroma compounds contribu-

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ting to the raw soy sauce aroma had not yet been fully identified.

The objectives of the present investigation were to clarify the key aroma compounds in the raw Japanese soy sauce by AEDA; to clarify the differences in the aroma characteristics of the raw soy sauce and the heated soy sauce by the combination of AEDA, quantitative analysis, and sensory analysis; and to propose formation mechanisms of the phenolic compounds by the model reactions of hydroxybenzoic acid derivatives and hydroxycinnamic acid derivatives under the same heating conditions of the raw soy sauce.

MATERIALS AND METHODS

Materials. Soy Sauce Sample. The Koikuchi-type raw soy sauce was purchased from Kikkoman Co., Ltd. (Noda, Japan). The heated soy sauce was prepared by heating the vacuum-packed raw soy sauce in a water bath at 80 °C for 30 min.

Chemicals. Ethyl acetate, 2-methylbutanal, 3-methylbutanal, ethyl 2-methylpropanoate, 2,3-butanedione, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, dimethyl disulfide, 3-hydroxy-2-butanone, 2-methyl-3-furanthiol, 2-ethyl-3,5-dimethylpyrazine, dimethyl trisulfide, 2-isopropyl-3-methoxy-pyrazine, 3-(methylthio)propanal, 2-isobutyl-3-methoxy-pyrazine, phenylacetaldehyde, 3-methylbutanoic acid, methyl 2-methyl-3-furyl disulfide, 3-(methylthio)propanol, 3-methyl-2(5H)-furanone, 3-methyl-1,2-cyclopentanedione, 2-methoxyphenol, 3-ethyl-1,2-cyclopentanedione, 3-hydroxy-2-methyl-4-pyrone, 4-ethyl-2-methoxyphenol, 5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2H)-furanone, 4-hydroxy-5-methyl-3(2H)-furanone, 4-decanolide, 5-decanolide, 2'-aminoacetophenone, 2,6-dimethoxyphenol, 3-methylindole, 4-hydroxy-3-methoxybenzaldehyde, 3,5-dimethoxy-4-hydroxybenzaldehyde, malonic acid, piperidine, vanillic acid, syringic acid, ferulic acid, and sinapic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 1-Octen-3-one, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 2-methoxy-4-vinylphenol, 3-hydroxy-4,5-dimethyl-2(5H)-furanone, and phenylacetic acid were purchased from Sigma-Aldrich Japan Co., Ltd. (Tokyo, Japan). *trans*-4,5-Epoxy-(*E*)-2-decenal was synthesized according to the literature.¹⁰ 2,6-Dimethoxy-4-vinylphenol was synthesized according to the literature with a slight modification.¹¹

2,6-Dimethoxy-4-vinylphenol. Malonic acid (2.00 g, 19.2 mmol) was dissolved in 20 mL of pyridine in a 50 mL two-neck round-bottom flask. 3,5-Dimethoxy-4-hydroxybenzaldehyde (2.34 g, 12.8 mmol) and piperidine (0.48 mL, 4.8 mmol) were added to the flask, and the solution was then stirred at 70 °C for 60 min. After cooling to room temperature, the reaction mixture was added to cold water, and then the pH was adjusted to 3.0. The mixture was extracted with ethyl acetate (20 mL × 5) and dried with an excess amount of anhydrous sodium sulfate. After replacement of the solvent by a *n*-hexane/ethyl acetate (8:2) mixture, the mixture was separated by silica gel chromatography using a glass column (500 × 20 mm i.d.) filled with a *n*-hexane slurry of 50 g of Wakogel C-300 (40–64 μm, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Separation was performed with 500 mL of a *n*-hexane/ethyl acetate (8:2) mixture (fractions I–V). The eluates of fractions III and IV were combined and dried in vacuo. The isolated yield was 19%. ¹H and ¹³C {¹H} NMR data agreed with the data reported in the literature.^{12,13} MS-EI: *m/z* (%) 180 (100), 165 (40), 137 (27), 77 (15), 122 (12), 91 (12).

Preparation of the Aroma Concentrates of the Raw Soy Sauce and the Heated Soy Sauce for GC-O and AEDA. The aroma concentrates of the raw soy sauce and the heated soy sauce were obtained according to the concentrating method described in the literature.³ An aliquot (5 mL) of the soy sauce was passed through the glass column (100 mm × 10 mm i.d.) filled with 5 mL of SP 700 resin (Mitsubishi Chemical Co., Ltd.), which was conditioned with distilled water before use, followed by washing with distilled water (5 mL × 4) and eluting with 20 mL of dichloromethane. The dichloromethane fraction was dried with an excess amount of anhydrous sodium sulfate and then distilled by solvent-assisted flavor evaporation (SAFE) (40

°C, <5.0 × 10⁻³ Pa).¹⁴ The soy sauce aroma concentrate was obtained by concentrating the dichloromethane layer by rotary evaporation, followed by nitrogen steam evaporation to about 100 μL. The respective aroma concentrates were used in gas chromatography–olfactometry (GC-O) and AEDA.

Identification of the Key Aroma Compounds in the Aroma Concentrates of the Raw Soy Sauce and the Heated Soy Sauce. Each key aroma compound in the respective aroma concentrates was identified by comparing its Kovats GC retention index (RI) and mass spectrum with the authentic compound by gas chromatography–mass spectrometry (GC-MS), in addition to comparison of its RI and odor quality with the authentic compound by GC-O.

Quantitative Analysis of the Key Aroma Compounds in the Raw and the Heated Soy Sauces by Standard Addition Method. An aliquot (5 mL) of the soy sauce with added 2-octanol (4150 μg/L) and the standard solution (0, 100, or 200 μL) containing the chemicals listed in Table 1 was concentrated to about 100 μL using

Table 1. Selected Ions and the Concentration of the Standard Solution of the Key Aroma Compounds for Quantitative Analysis by the Standard Addition Method

compound	selected ion <i>m/z</i>	concentration, mg/L	
		for the raw soy sauce	for the heated soy sauce
3-methylbutanal	44	94.6	94.6
2-methylbutanal	57	92.8	92.8
3-(methylthio)propanal	48	20.4	20.4
2-ethyl-3,5-dimethylpyrazine	135	0.602	0.602
phenylacetaldehyde	91	479	479
3-methylbutanoic acid	60	97.4	97.4
3-(methylthio)propanol	106	101	101
2-methoxyphenol	109	1.91	1.91
4-ethyl-2-methoxyphenol	137	5.32	5.32
4-hydroxy-2,5-dimethyl-3(2H)-furanone	128	103	103
5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2H)-furanone	142	1370	1370
2-methoxy-4-vinylphenol	150	3.37	40.4
3-hydroxy-4,5-dimethyl-2(5H)-furanone	83	4.02	4.02
2,6-dimethoxyphenol	154	3.06	3.06
phenylacetic acid	91	98.1	98.1
2,6-dimethoxy-4-vinylphenol	180	0.511	9.30

the same concentration method described above. The respective aroma concentrates were analyzed by GC-MS in the synchronous selected ion monitoring and full scan (SIM/scan) mode. On the basis of the relationship between the ratio of the total area count of each key aroma compound to that of 2-octanol and the added concentration of each compound, the quantitative analysis of the key aroma compounds was performed by calculation from the approximated curve using the linear least-squares method. The respective quantitative values of the key aroma compounds were determined by averaging the triplicate experiments.

Model Decarboxylation Reactions of Phenolic Acids. An aliquot (100.0 ppm) of phenolic acid was dissolved in an aqueous solution with the pH adjusted to 4.7 with citric acid and sodium citrate and then packed in a glass bottle under a nitrogen atmosphere. The packed solution was heated in a water bath at 80 °C for 30 min. After the heating, the sample was rapidly cooled to lower than room temperature. Quantitative analysis of the generated phenolic compounds was performed by reverse phase high-performance liquid chromatography (RP-HPLC). The quantitative values of the respective compounds were determined by the absolute calibration method by averaging the triplicate experiments.

Table 2. Key Aroma Compounds in Raw Soy Sauce and Heated Soy Sauce

no.	RI ^a		odor qualities	compound ^b	FD factor ^c		
	DB-WAX	DB-1			raw soy sauce	heated soy sauce	ref ^d
1	937	559	stimulus	ethyl acetate	1	1	22
2	947	599	malty	2- and 3-methylbutanal	16	32	22, 29
3	975	746	fruity	ethyl 2-methylpropanoate	4	2	2
4	979	558	milky	2,3-butanedione	4	4	24
5	1045	819	fruity	ethyl 2-methylbutanoate	4	4	25
6	1061	831	fruity	ethyl 3-methylbutanoate	4	2	26
7	1074	729	rancid	dimethyl disulfide	1	1	27
8	1278	665	milky	3-hydroxy-2-butanone	8	8	22
9	1295	849	roasty	2-methyl-3-furanthiol ^e	4	4	
10	1352	952	rancid	dimethyl trisulfide	1	1	23
11	1428	1078	earthy, pea-like	2-isopropyl-3-methoxypyrazine ^e	1	1	
12	1449	860	cooked potato-like	3-(methylthio)propanal	512	1024	28
13	1463	1061	nutty, roasty	2-ethyl-3,5-dimethylpyrazine	64	64	28
14	1515	1158	earthy	2-isobutyl-3-methoxypyrazine ^e	1	1	
15	1631	1009	honey-like	phenylacetaldehyde	32	32	29
16	1657	833	sour, cheese-like	3-methylbutanoic acid	64	64	30
17	1682	1171	meaty	methyl 2-methyl-3-furyl disulfide ^e	nd ^f	1	
18	1709	944	cooked potato-like	3-(methylthio)propanol	32	32	31
19	1718		floral	unknown	4	8	
20	1723	940	caramel-like	3-methyl-2(5H)-furanone	nd ^f	2	32
21	1831	1002	caramel-like	3-methyl-1,2-cyclopentanedione	8	8	27
22	1851	1071	burnt	2-methoxyphenol	64	64	33
23	1877	1092	caramel-like	3-ethyl-1,2-cyclopentanedione	8	8	3
24	1960	1077	caramel-like	3-hydroxy-2-methyl-4-pyranone	16	16	34
25	1995	1342	metallic	<i>trans</i> -4,5-epoxy-(<i>E</i>)-2-decenal ^e	8	8	2
26	2016	1265	spicy, burnt	4-ethyl-2-methoxyphenol	256	256	35
27	2024	1041	caramel-like, sweet	4-hydroxy-2,5-dimethyl-3(2H)-furanone	128	256	27
28	2053	1107	caramel-like, sweet	5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2H)-furanone	2048	2048	36
29	2123	1013	caramel-like, sweet	4-hydroxy-5-methyl-3(2H)-furanone	4	8	37
30	2123	1425	fatty, milky	4-decanolide	1	nd ^f	38
31	2181	1287	spicy, burnt	2-methoxy-4-vinylphenol	16	128	39
32	2182	1084	caramel-like, seasoning-like	3-hydroxy-4,5-dimethyl-2(5H)-furanone	2048	2048	40
33	2190	1456	milky	5-decanolide	8	1	
34	2223	1276	grape-like	2'-aminoacetophenone ^e	8	16	3
35	2257	1311	spicy, burnt	2,6-dimethoxyphenol	64	64	29
36	2471	1365	animal-like	3-methylindole ^e	1	1	
37	2546	1232	honey-like	phenylacetic acid	32	32	27
38	2560	1352	vanilla-like	4-hydroxy-3-methoxybenzaldehyde	16	16	41
39	2569	1525	spicy	2,6-dimethoxy-4-vinylphenol	4	64	

^aRI, retention index. ^bThe compound was identified by comparing its RI and mass spectrum with the authentic compound by GC-MS in addition to comparison of its RI and odor quality with the authentic compound by GC-O. ^cFD factor, flavor dilution factor. ^dPrevious papers identified as volatile components in soy sauce. ^eThe compound was tentatively identified by GC-O analysis using DB-WAX and DB-1 columns by comparison to the authentic compound, but no unequivocal mass spectrum was available by GC-MS. ^fnd, not detected.

Quantitative Analysis of Phenolic Acids in Raw Soy Sauce.

Vanillic acid, syringic acid, ferulic acid, and sinapic acid in the raw soy sauce were quantitated by RP-HPLC. The quantitative values of the respective compounds were determined by the absolute calibration method by averaging the triplicate experiments.

GC-O. An Agilent model 6850 series gas chromatograph equipped with a thermal conductivity detector (TCD) was used for the GC-O analysis. A fused silica column (30 m × 0.25 mm i.d. coated with a 0.25 μm film of DB-Wax, J&W Scientific; or 30 m × 0.25 mm i.d. coated with a 0.25 μm film of DB-1, J&W Scientific) was used with a 1.0 μL splitless injection. Helium was used as the carrier gas at the flow rate of 1 mL/min. The injector temperature was set to 250 °C and the purge valve time was set to 60 s. The chromatography was performed at the oven temperature of 40 °C, which was increased to 210 °C at the rate of 5 °C/min. The detector temperature was set to 230 °C. A glass sniffing port was connected to the outlet of the TCD and heated to

>210 °C by a ribbon heater. Moist air was pumped into the sniffing port at 100 mL/min to quickly remove the odorant eluted from the TCD out of the sniffing port. The soy sauce aroma concentrates underwent a GC-O analysis by three subjects. Determination of the odor qualities detected by sniffing was achieved by triplicate experiments for each subject.

AEDA. The original odor concentrate of the soy sauce aroma concentrate was stepwise diluted with dichloromethane from 1:2 to 1:4096, and aliquots (1 μL) of each fraction were analyzed by capillary GC on a DB-WAX column. AEDA was performed three times with respect to each sample by three subjects.¹⁵ The detection of each compound was defined as not less than two detections by all subjects, and the FD factor of each compound was determined as the maximum dilution degree of detection.

GC-MS. An Agilent 7890A gas chromatograph coupled to an Agilent model 5975C inert XL series mass spectrometer was used. A

Table 3. Quantitative Analysis of the Key Aroma Compounds in Raw Soy Sauce and Heated Soy Sauce by the Standard Addition Method

no.	compound	raw soy sauce		heated soy sauce		ratio
		concentration, ^a $\mu\text{g/L}$ (SD, ^b $\mu\text{g/L}$)	R^{2c}	concentration, ^a $\mu\text{g/L}$ (SD, ^b $\mu\text{g/L}$)	R^{2c}	
2a	3-methylbutanal	2640 (± 20)	0.944–0.995	3220 (± 60)	0.982–0.988	1.22 ^d
2b	2-methylbutanal	2670 (± 70)	0.962–0.997	3160 (± 200)	0.932–0.989	1.18 ^d
12	3-(methylthio)propanal	388 (± 4)	0.994–1.000	513 (± 16)	0.968–1.000	1.32 ^d
13	2-ethyl-3,5-dimethylpyrazine	15.2 (± 0.7)	0.987–1.000	16.4 (± 0.6)	0.997–1.000	1.08
15	phenylacetaldehyde	10000 (± 700)	0.994–0.997	13100 (± 600)	0.988–0.995	1.31 ^d
16	3-methylbutanoic acid	2340 (± 120)	0.998–0.999	2300 (± 120)	0.990–1.000	0.98
18	3-(methylthio)propanol	3390 (± 120)	0.981–0.995	3690 (± 140)	0.959–0.997	1.09
22	2-methoxyphenol	51.9 (± 1.6)	0.996–1.000	52.6 (± 3.0)	0.975–0.994	1.02
26	4-ethyl-2-methoxyphenol	280 (± 31)	0.995–1.000	266 (± 12)	0.965–1.000	0.95
27	4-hydroxy-2,5-dimethyl-3(2H)-furanone	1650 (± 60)	0.999–1.000	2440 (± 160)	0.984–1.000	1.48 ^d
28	5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2H)-furanone	42800 (± 1700)	0.989–0.998	40400 (± 2400)	0.982–1.000	0.94
31	2-methoxy-4-vinylphenol	98.6 (± 7.5)	0.988–0.998	1120 (± 62)	0.969–0.996	11.3 ^d
32	3-hydroxy-4,5-dimethyl-2(5H)-furanone	105 (± 4)	0.991–0.999	113 (± 6)	0.985–0.998	1.08
35	2,6-dimethoxyphenol	104 (± 6)	0.984–1.000	114 (± 10)	0.948–0.998	1.09
37	phenylacetic acid	2480 (± 100)	0.998–1.000	2520 (± 120)	0.996–0.998	1.02
39	2,6-dimethoxy-4-vinylphenol	13.6 (± 0.8)	0.985–0.996	235 (± 11)	0.989–0.997	17.2 ^d

^aMean value in triplicate experiments. ^bSD, standard deviation. ^c R^2 , coefficient of determination obtained by the linear least-squares method. ^dThere were significant differences between the raw soy sauce and the heated soy sauce by two-sample *t* test ($p < 0.05$).

fused silica column (60 m \times 0.25 mm i.d. coated with a 0.25 μm film of DB-Wax, J&W Scientific; or 60 m \times 0.25 mm i.d. coated with a 0.25 μm film of DB-1, J&W Scientific) was used for the analyses. Helium was used as the carrier gas at the flow rate of 1 mL/min, and the injector temperature was set to 250 $^{\circ}\text{C}$. Aliquots (1 μL) of the sample were injected with the split ratio of 1:30 or splitless. The chromatography was performed from an oven temperature of 80 to 210 $^{\circ}\text{C}$ at the rate of 3 $^{\circ}\text{C}/\text{min}$ for the split injections and from an oven temperature of 40 to 210 $^{\circ}\text{C}$ at the rate of 3 $^{\circ}\text{C}/\text{min}$ for the splitless injections. The mass spectrometer was used under the following conditions: ionization voltage, 70 eV (EI); ion source temperature, 150 $^{\circ}\text{C}$.

RP-HPLC. An Agilent model 1200 series HPLC apparatus equipped with a photodiode array detector was used for quantitative analysis. The phenolic acids were analyzed using an ODS RP18 column (10 μm , 250 mm \times 4.6 mm i.d., Shiseido Co., Ltd., Tokyo, Japan) under the following conditions. Monitoring the effluent at 280 nm for vanillic acid and syringic acid and at 324 nm for ferulic acid and sinapic acid, chromatography was performed with a 3% acetonitrile solution containing 0.1% phosphoric acid at the flow rate of 1.0 mL/min, then increasing the acetonitrile content to 25% over 29 min, and finally increasing the acetonitrile content to 100% over an additional 1 min. The phenolic compounds of 2-methoxyphenol, 2-methoxy-4-vinylphenol, 2,6-dimethoxyphenol, and 2,6-dimethoxy-4-vinylphenol were analyzed using the same ODS column but with a slightly modified condition. Monitoring the effluent at 280 nm, chromatography was performed with a 10% acetonitrile solution containing 0.1% phosphoric acid at the flow rate of 1.0 mL and then increasing the acetonitrile content to 100% over 25 min.

Sensory Evaluations of Raw Soy Sauce and Heated Soy Sauce. Eighteen subjects were recruited from Ogawa & Co., Ltd., and trained for 6 months with in-house programs of recognition, description, and discrimination tests involving about 300 odorants. The respective soy sauce samples (5 mL) were poured into a plastic cup at 25 $^{\circ}\text{C}$ for the sensory panelists, who were asked to score the strengths of the malty, cooked potato-like, nutty, honey-like, sour, caramel-like, and spicy/burnt aroma notes on a scale from 1 (weak) to 7 (strong). The evaluation terms were defined as the following aromas: 3-methylbutanal for malty aroma, 3-(methylthio)propanal for cooked potato-like aroma, 2-ethyl-3,5-dimethylpyrazine for nutty aroma, phenylacetaldehyde for honey-like aroma, 3-methylbutanoic acid for sour aroma, 5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2H)-furanone

for caramel-like aroma, and 4-ethyl-2-methoxyphenol for spicy/burnt aroma. A two-way layout analysis of variance without replication was applied to the sensory evaluation results performed with a seven-point scoring method.

RESULTS AND DISCUSSION

Identification of the Key Aroma Compounds in Raw Soy Sauce. Raw Japanese soy sauce had caramel-like, burnt/spicy, and malty aromas, and the aroma concentrate of it had the same characteristics when it was dipped in a filter paper for sensory evaluation and sniffed.

To identify the key aroma compounds in the raw soy sauce, AEDA was used for the aroma concentrate of the raw soy sauce, and 37 compounds were detected with FD factors of ≥ 1 (Table 2). Among them, 2-methyl-3-furanthiol (9), 2-isopropyl-3-methoxypyrazine (11), 2-isobutyl-3-methoxypyrazine (14), 5-decanolide (33), 3-methylindole (36), and 2,6-dimethoxy-4-vinylphenol (39) were identified as the key aroma compounds in the soy sauce for the first time.

5(or 2)-Ethyl-4-hydroxy-2(or 5)-methyl-3(2H)-furanone (28), exhibiting the caramel-like/sweet note, and 3-hydroxy-4,5-dimethyl-2(5H)-furanone (32), exhibiting the caramel-like/seasoning-like note, were detected as having the highest FD factor of 2048, followed by 3-(methylthio)propanal (12) (cooked potato-like), 4-ethyl-2-methoxyphenol (26) (spicy/burnt), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (27) (caramel-like), 2-ethyl-3,5-dimethylpyrazine (13) (nutty), 3-methylbutanoic acid (16) (sour/cheese-like), 2-methoxyphenol (22) (spicy/burnt), and 2,6-dimethoxyphenol (35) (spicy/burnt) as having FD factors from 64 to 512. Because most of these compounds have already been reported as the key aroma compounds in the common heated Koikuchi-type Japanese soy sauce,^{2,3} they might also be important in the raw soy sauce aroma.

Characterization of Raw Soy Sauce Aroma by Comparative AEDAs, Quantitative Analysis, and Sensory Analysis. To compare the aroma characteristics of the raw soy sauce with those of the heated soy sauce, AEDA was applied to

Table 4. Sensory Evaluation of Raw Soy Sauce and Heated Soy Sauce

sample	aroma intensities ^a (SD ^b)						
	malty	cooked potato-like	nutty	honey-like	sour	caramel-like	spicy/burnt
raw soy sauce	3.6a (±0.9)	4.0c (±0.7)	3.1 (±1.0)	3.7 (±1.0)	3.2 (±1.0)	3.8 (±0.9)	3.6e (±0.7)
heated soy sauce	4.8b (±0.9)	4.7d (±1.0)	3.4 (±0.7)	3.8 (±1.1)	3.6 (±1.1)	4.4 (±0.8)	4.8f (±0.6)
raw soy sauce + increased chemicals ^c	4.8b (±1.0)	4.7d (±0.9)	3.7 (±0.9)	4.3 (±1.3)	3.6 (±1.0)	4.7 (±1.0)	5.3f (±1.0)

^aDifferent letters indicate significant differences within the column ($p < 0.05$). ^bSD, standard deviation. ^c3-Methylbutanal (580 $\mu\text{g/L}$), 2-methylbutanal (490 $\mu\text{g/L}$), 3-(methylthio)propanal (125 $\mu\text{g/L}$), phenylacetaldehyde (3100 $\mu\text{g/L}$), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (790 $\mu\text{g/L}$), 2-methoxy-4-vinylphenol (1020 $\mu\text{g/L}$), and 2,6-dimethoxy-4-vinylphenol (221 $\mu\text{g/L}$) were added to the raw soy sauce.

the aroma concentrate of the heated soy sauce in addition to the raw soy sauce. The comparative AEDAs revealed that 2-methoxy-4-vinylphenol (31) and 2,6-dimethoxy-4-vinylphenol (39) significantly increased and 5-decanolide (33) decreased during the heating process, in addition to the formation of methyl 2-methyl-3-furyl disulfide (17) (Table 2). Methyl 2-methyl-3-furyl disulfide (17) might be formed from 2-methyl-3-furanthiol (9) and methanethiol. Although 2-methoxy-4-vinylphenol (31) and 2,6-dimethoxy-4-vinylphenol (39) might be formed from the corresponding hydroxycinnamic acids, these glycosides, and/or lignin by decarboxylation reactions,^{1,16} there have been no experimental data to support this claim. Model reactions of the phenolic acids under the same heating conditions of the raw soy sauce were investigated, as will be discussed in detail later. Because 5-decanolide (33) is easy to decompose in an aqueous solution, it might have been reduced during heating.

Furthermore, to clarify the precise changes in the key aroma compounds of the raw soy sauce during the heating process, a quantitative analysis of the key aroma compounds exhibiting high FD factors of ≥ 32 in either soy sauce by the standard addition method was performed (Table 3). A comparison of the quantitative data of the respective soy sauces revealed that 3-methylbutanal (2a), 2-methylbutanal (2b), 3-(methylthio)propanal (12), phenylacetaldehyde (15), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (27), 2-methoxy-4-vinylphenol (31), and 2,6-dimethoxy-4-vinylphenol (39) significantly increased during the heating process. On the basis of previous studies concerning the formation mechanisms of these compounds, they all seem to be generated from amino acids, sugars, and phenolic acids by a thermal treatment.^{16–18}

Additionally, to clarify the changes in the aroma characteristics during the heating process, a sensory experiment was performed by comparing both soy sauces as well as the raw soy sauce with the added significantly increased compounds during the heating process listed in Table 3. The evaluation terms were determined according to the aroma descriptions of the key aroma compounds detected as having the FD factor of ≥ 32 (Table 4). The raw soy sauce represented moderate cooked potato-like (4.0) and caramel-like (3.8) notes, followed by honey-like (3.7), malty (3.6), and spicy/burnt (3.6) notes, whereas the heated soy sauce was represented as relatively strong spicy/burnt (4.8), malty (4.8), cooked potato-like (4.7), and caramel-like (4.4) notes. Furthermore, the heated soy sauce as well as the raw soy sauce with the increased chemical compounds exhibited significantly higher scores on malty, cooked potato-like, and spicy/burnt notes than the raw soy sauce. On the contrary, although 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (27) exhibiting a caramel-like note increased during the heating process as shown by quantitative analysis, there were no significant differences in the caramel-like note

among all of the samples by the sensory analysis. Because the caramel-like note might also be contributed by other compounds, such as 5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2*H*)-furanone (28) and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (32), exhibiting the highest FD factor of 2048, no significant differences were observed among the samples. There were also no significant differences in the honey-like note among them. Although phenylacetaldehyde (15) exhibiting the honey-like note increased during the heating process, the relatively low FD factor of 128 might be the cause of no significant differences in the sensory evaluation result.

In summary, comparative AEDAs, a quantitative analysis, and a sensory analysis demonstrated that whereas most of the key aroma compounds in the raw soy sauce were common in the heated soy sauce, some of the Strecker aldehydes (3-methylbutanal (2a), 2-methylbutanal (2b), and 3-(methylthio)propanal (12)), exhibiting a malty note and a cooked potato-like note, and 4-vinylphenols (2-methoxy-4-vinylphenol (31) and 2,6-dimethoxy-4-vinylphenol (39)), exhibiting a spicy/burnt note, contributed less to the raw soy sauce aroma.

Model Reactions of the Phenolic Acids and the Proposed Mechanism of the Phenolic Compounds Formation in Raw Soy Sauce during the Heating Process. Whereas 2-methoxy-4-vinylphenol (31) and 2,6-dimethoxy-4-vinylphenol (39) significantly increased during the heating process in the raw soy sauce by the quantitative analysis, 2-methoxyphenol (22) and 2,6-dimethoxyphenol (35), which could be formed as decarboxylation products from the respective hydroxybenzoic acids, did not increase during the heating process (Table 3). To verify these differences, the model decarboxylation reactions of the phenolic acids were investigated on the basis of the heating conditions of the raw soy sauce (Table 5). Sinapic acid (43) resulted in the highest

Table 5. Yields of the Model Decarboxylation Reactions of Phenolic Acids by a Thermal Treatment^a

no.	compound	decarboxylation product	yield, ^b (SD, ^c %)
40	vanillic acid	2-methoxyphenol	0.010 (±0.000)
41	syringic acid	2,6-dimethoxyphenol	0.023 (±0.001)
42	ferulic acid	2-methoxy-4-vinylphenol	0.758 (±0.019)
43	sinapic acid	2,6-dimethoxy-4-vinylphenol	1.656 (±0.033)

^a80 °C × 30 min, pH 4.7. ^bMean value in triplicate experiments. ^cSD, standard deviation.

yield (1.656%) of 2,6-dimethoxy-4-vinylphenol (39), followed by ferulic acid (42), which resulted in the next highest yield (0.758%) of 2-methoxy-4-vinylphenol (31). On the contrary, vanillic acid (40) and syringic acid (41) reacted with low yields of the corresponding phenolic compounds (0.010 and 0.023%, respectively). These results revealed that although all reactions

resulted in low yields, the hydroxycinnamic acid derivatives were more reactive than the hydroxybenzoic acid derivatives. This might be because the hydroxycinnamic acid derivatives react through β -carbonyl ions (protonation to the olefin at first) as the intermediates, but the hydroxybenzoic acid derivatives react through the unstable carbanions.^{19,20} The decarboxylation reaction of the hydroxycinnamic acid derivatives might proceed as a zwitter ion form under the weakly acidic condition (pH 4.7) of the raw soy sauce.¹⁹ Furthermore, the 2,6-dimethoxy skeleton is more reactive than the 2-methoxy skeleton on the basis of the model reaction yields (Table 5). Because the decarboxylation reaction of the phenolic acid is promoted by the increasing stability of the reaction intermediate,²⁰ the 2,6-dimethoxy derivatives are thought to be more reactive due to the stable intermediates provided by the two methoxy substituents at the meta positions of the phenyl group, which have an electron-withdrawing effect (inductive effect).

Furthermore, to verify the increases in the phenolic compounds during the heating process in the raw soy sauce, quantitative analysis of the phenolic acids in the raw soy sauce was performed by RP-HPLC (Table 6). Syringic acid (41) was

Table 6. Quantitative Analysis of Phenolic Acids in Raw Soy Sauce

no.	compound	concentration, ^a mg/L (SD, ^b mg/L)
40	vanillic acid	6.1 (\pm 0.2)
41	syringic acid	36.7 (\pm 0.4)
42	ferulic acid	19.7 (\pm 0.1)
43	sinapic acid	3.8 (\pm 0.1)

^aMean value in triplicate experiments. ^bSD, standard deviation.

contained in the highest amount of 38.4 μ g/L in the raw soy sauce, followed by vanillic acid (40), ferulic acid (42), and sinapic acid (43) (29.2, 22.1, and 4.1 μ g/L, respectively). To discuss the contribution to the increase in the phenolic compounds in the heated soy sauce by the respective phenolic acids, the estimated yields of the phenolic compounds were calculated from the yields of the model reactions (Table 7). Although the estimated yields of 2-methoxyphenol (22) and 2,6-dimethoxyphenol (35) were quite low (0.6 and 8.3 μ g/L), those of 2-methoxy-4-vinylphenol (31) and 2,6-dimethoxy-4-

Table 7. Estimated Yields of Phenolic Compounds by a Thermal Treatment from Raw Soy Sauce

no.	compound	concentration, μ g/L		ratio ^c
		increase during heating ^a	estimated yield formed from phenolic acid ^b	
22	2-methoxyphenol	0.7	0.6	84
35	2,6-dimethoxyphenol	9	8.3	89
31	2-methoxy-4-vinylphenol	1020	150	15
39	2,6-dimethoxy-4-vinylphenol	121	62.9	28

^aDifference between the raw soy sauce and the heated soy sauce calculated from Table 3. ^bThe multiplication of the concentration of the corresponding precursor in the raw soy sauce shown in Table 5 and the yield of the corresponding phenolic compound by the thermal treatment shown in Table 6. ^cThe ratio of the estimated yield to the increased amount during heating of the raw soy sauce.

vinylphenol (39) were comparatively high (150 and 62.9 μ g/L). Furthermore, whereas the ratios of the increased amounts of 2-methoxyphenol (22) and 2,6-dimethoxyphenol (35) by the above reactions in the total increased amounts during the heating process were estimated to be high (84 and 89%, respectively), those of 2-methoxy-4-vinylphenol (31) and 2,6-dimethoxy-4-vinylphenol (39) were estimated to be quite low (15 and 28%, respectively) (Table 7). These results indicated that although only the 4-vinylphenols increased during the heating process in the raw soy sauce, they were not mainly formed from the respective hydroxycinnamic acids, but from the other precursors. Although some components, such as lignin, shakuchirin, and esters with arabinosyl, ^{1,21} are proposed as precursors, it is still unclear.

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Notes

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