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Identification of Potent Odorants in Different Green Tea Varieties Using Flavor Dilution Technique

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Two kinds of pan-fired green teas (Japanese Kamairi-cha and Chinese Longing tea) were compared with the common Japanese green tea (Sen-cha). Application of the aroma extract dilution analysis (AEDA) using the volatile fraction of the Sen-cha, Kamairi-cha and Longing tea infusions revealed 32, 51, and 52 odor-active peaks with flavor dilution factors between 16 and 1024, respectively. (*Z*)-1,5-Octadien-3-one (metallic, geranium-like), 4-mercapto-4-methyl-2-pentanone (meaty, black currant-like), methional (potato-like), (*E*,*Z*)-2,6-nonadienal (cucumber-like), and 3-methylnonane-2,4-dione (green, fruity, hay-like) showed high flavor dilution factors in all varieties. In addition, 2-acetyl-1-pyrroline (popcorn-like), 2-ethyl-3,5-dimethylpyrazine (nutty), 2,3-diethyl-5-methylpyrazine (nutty), and 2-acetyl-2-thiazoline (popcorn-like) belonged to the most potent odorants only in the pan-fired green teas. Among these odorants, 2-acetyl-1-pyrroline and 2-acetyl-2-thiazoline were identified for the first time among the tea volatiles.

KEYWORDS: Green tea; pan-fired; aroma extract dilution analysis; gas chromatography−olfactometry; 2-acetyl-1-pyrroline; 2-acetyl-2-thiazoline

INTRODUCTION

Green tea is one of the most widely consumed beverages in Japan and China. The high acceptability of green tea is due to many factors, one of the most contributory factors being its flavor. The characteristic manufacturing process of green tea includes steaming or pan-firing during which the enzyme in the tea leaves is inactivated and the green color of the tea leaves is maintained. The major portion of Japanese green tea is Sen-cha, which includes a steaming process to deactivate the enzyme in the tea leaves. On the other hand, the Japanese green tea (Kamairi-cha) and most of the Chinese green teas (Longing tea is a typical Chinese green tea) includes the pan-firing process. Therefore, despite the same green tea classification, it is known that the characteristic flavors of pan-fired green teas are quite different from that of Sen-cha. A number of volatile components in the Japanese Kamairi-cha and Chinese Longing tea have been identified by gas chromatography (GC) and GC/mass spectrometry (MS) (*1*−*7*). However, in these investigations, the authors gave no details about the importance of the recognized compounds, because the GC analysis was not coupled with the GC−olfactometry technique. Therefore, the actual significance of compounds to the pan-fired aroma has not yet been determined.

The aim of this investigation was to elucidate the most potent odorants involved in the pan-fired aroma of Kamairi-cha and Longing tea by comparison with Sen-cha using the aroma extract dilution analysis (AEDA) technique (*8*).

MATERIALS AND METHODS

Materials. Green tea samples: The Sen-cha and Kamairi-cha products were produced in the Shizuoka and Saga prefectures (Japan) in 2001. The Longing tea product was produced in the Zhejiang province (China) in 2001. These products were high grade based on market price. Chemicals: The following compounds were synthesized according to the literature procedures: 2-acetyl-1-pyrroline (*9*); 2-acetyl-2-thiazoline (*10*); 1-octen-3-one and (*Z*)-1,5-octadien-3-one; (*11*); 4-mercapto-4-methyl-2-pentanone (*12*); and 3-methylnonane-2,4-dione (*13*). Compounds **1**−**3**, **6**, **12**−**13**, **17**, **19**−**23**, **26**−**28**, **31**, **33**−**37**, **39**, **41**, **44**, **46**, and **52**−**54** (**Table 1**) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan); **4**, **16**, **18**, **24**, **42**, and **45** were obtained from Sigma-Aldrich (Tokyo, Japan); **11**, **43**, **47**, and **48** were obtained from Nacalai Tesque (Kyoto, Japan); **25** was obtained from Wako Pure Chemical Industries (Osaka, Japan); **49**−**51** were obtained from Zeon Corporation (Tokyo, Japan); and **32** was obtained from Nihon Firmenich (Tokyo, Japan).

Isolation of the Volatiles. Deionized hot water (70−75 °C, 4 L) was added to 200 g of green tea, and the leaves were filtered using coarse filter paper after standing for 5 min. The filtrate (3 L) was immediately cooled to about 20 °C in tap water, and then steam distillation was performed under reduced pressure (40 °C, 20 mmHg). The steam distillate (1 L) was passed through a column packed with 10 g of Porapak Q (Waters). The adsorbed compounds were eluted with methylene chloride (100 mL). The eluate was dried over anhydrous sodium sulfate, and the solvent was removed by rotary evaporation to about 5 mL in volume. Further concentration to ~100 μL was conducted with a nitrogen stream. The concentrate was used as the AEDA sample.

Enrichment of Odorants for Identification. For the identification experiments, the green tea volatiles were isolated from the Kamairi-

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Table 1. Potent Odorants in the Volatile Fraction of Different Green Tea Varieties

no.	RI[a]	RI[b]	RI[c]	fraction[d]	compound	odor quality[g]	FD factor		
							Sen-cha	Kamairi-cha	Longjing tea
1	918	923			3-methylbutanal	stimulus	<16	16	16
2	973	975			2,3-butanedione	buttery	<16	16	16
3	1052	1052			2,3-pentanedione	buttery	<16	16	16
4	1237	1240			(*Z*)-4-heptenal	hay-like	<16	64	64
5	1241				unknown	nutty	<16	64	16
6	1288	1289		N-4	octanal	orange-like	<16	<16	16
7	1301	1302		N-5	1-octen-3-one	mushroom-like	16	16	16
8	1338	1341		B	2-acetyl-1-pyrroline[e]	popcorn-like	16	1024	1024
9	1375	1379			(*Z*)-1,5-octadien-3-one	metallic	256	1024	1024
10	1383	1384			4-mercapto-4-methyl-2-pentanone	meaty	256	256	256
11	1387	1387			(*Z*)-3-hexenol	green	<16	<16	16
12	1395	1396		N-4	nonanal	orange-like	<16	16	16
13	1412	1411			2,3,5-trimethylpyrazine	nutty	nd	<16	16
14	1428				unknown	nutty	<16	16	16
15	1436				unknown	nutty	<16	16	64
16	1449	1452			2-ethyl-3,6-dimethylpyrazine	nutty	<16	64	16
17	1453	1456			methional	potato-like	256	1024	256
18	1470	1470			2-ethyl-3,5-dimethylpyrazine	nutty	<16	1024	1024
19	1495	1497			(*E*,*E*)-2,4-heptadienal	fatty	<16	16	64
20	1499	1499		B	2,3-diethyl-5-methylpyrazine	nutty	<16	256	256
21	1529		1163		2-isobutyl-3-methoxypyrazine[e,f]	earthy, musty	64	256	64
22	1537	1539		N-5	(*E*)-2-nonenal	green	16	16	64
23	1550	1549			linalool	floral	16	64	256
24	1563	1565			4,5-dihydro-3(2*H*)-thiophenone	roasty	nd	64	16
25	1593	1590			(*E*,*Z*)-2,6-nonadienal	cucumber-like	256	256	256
26	1632	1632		B	2-acetylpyrazine	roasty	nd	16	16
27	1646	1646			phenylacetaldehyde	honey-like	16	256	64
28	1703	1705		N-6	(*E*,*E*)-2,4-nonadienal	fatty	64	64	256
29	1722	1723			3-methylnonane-2,4-dione	green	256	256	256
30	1764	1765		N-7	2-acetyl-2-thiazoline[e]	popcorn-like	16	256	256
31	1815	1815			(*E*,*E*)-2,4-decadienal	fatty	16	16	16
32	1825	1829			β-damascenone	honey-like	64	64	256
33	1854	1850			geraniol	floral	64	64	256
34	1859	1858			geranylacetone	floral	64	64	64
35	1864	1863			guaiacol	burnt	64	64	256
36	1923	1924			4-octanolide	sweet	<16	16	64
37	1953	1955			(*Z*)-jasmone	green	16	16	16
38	1968				unknown	sweet	16	16	16
39	1989	1985		N-6	5-octanolide	sweet	16	64	16
40	2009				unknown	sweet	<16	16	16
41	2036	2038			4-nonanolide	sweet	16	16	1024
42	2041	2039			4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	caramel-like	16	64	16
43	2083	2085			*p*-cresol	phenolic	<16	16	64
44	2171	2172			eugenol	spicy	64	16	<16
45	2198	2200			2-methoxy-4-vinylphenol	spicy	<16	64	64
46	2204		1079		3-hydroxy-4,5-dimethyl-2(5*H*)-furanone[f]	caramel-like	nd	16	<16
47	2227	2228		B	2-aminoacetophenone	grape-like	64	64	64
48	2244	2248		B	methyl anthranilate	grape-like	16	16	64
49	2273	2272			jasmine lactone	sweet	16	16	16
50	2342	2343		N	(*E*)-methyl jasmonate	floral	16	16	16
51	2399	2404		N	(*Z*)-methyl jasmonate	floral	256	64	64
52	2448	2451			indole	animal-like	256	256	64
53	2458	2463			coumarin	sweet	64	64	256
54	2578	2585			vanillin	vanilla-like	64	64	64

[a] Retention index on DB-Wax column (30 m × 0.25 mm i.d.; coated with a 0.25 μm film) observed for GC–O. [b] Retention index on DB-Wax column (60 m × 0.25 mm i.d.; coated with a 0.25 μm film) observed for GC/MS. [c] Retention index on DB-1 column (30 m × 0.32 mm i.d.; coated with a 0.25 μm film) observed for GC–O. [d] Fraction in which most of the compound appeared after separation in neutral (N) and basic (B) and additionally after column chromatography on silica gel of the neutral fraction. [e] Newly identified compounds of green tea. [f] The MS signals were too weak for an uneqivocal interpretation. The compound was tentatively identified by matching its retention indexes and odor quality with those of standard compounds. [g] Odor quality assigned during AEDA.

cha infusion as described above. These procedures were repeated six times and concentrated to ~50 μL (total, 24 L of deionized water was added to 1.2 kg of Kamairi-cha). The basic volatiles were isolated by treatment of the volatile concentrate, in which the green tea volatiles from above were dissolved in ~20 mL of methylene chloride with 1 M hydrochloric acid (3 × 10 mL). The organic phase containing the neutral and acidic volatiles, which was washed with brine (2 × 5 mL), was dried over anhydrous Na_2SO_4 and finally concentrated to 50 μL (neutral fraction). The combined acid extract was then washed with methylene chloride (2 × 5 mL). The washed acid extract was neutralized with aqueous sodium hydroxide and then extracted with methylene chloride (3 × 10 mL). This extract was washed with brine (2 × 5 mL), dried over anhydrous Na_2SO_4, and finally concentrated to 50 μL (basic fraction). The neutral fraction was applied onto a column (30 × 0.7 cm i.d.) filled with silica gel (Wakogel C-200; Wako Pure Chemical Industries). Elution was performed using the following solvents: hexane (50 mL, fraction N-1), hexane/methylene chloride (50 mL, 90 + 10, v/v, fraction N-2), hexane/methylene chloride (50 mL, 80 + 20, v/v, fraction N-3), hexane/methylene chloride (50 mL, 60 + 40, v/v, fraction N-4), hexane/methylene chloride (50 mL, 40 + 60, v/v, fraction N-5), hexane/methylene chloride (50 mL, 20 + 80, v/v, fraction N-6), methylene chloride (50 mL, fraction N-7), and ethyl acetate (50 mL, fraction N-8). These fractions were concentrated to 50 μL as described above.

Gas Chromatography−Olfactometry (GC−O). A Hewlett-Packard (HP) model 5890 series II gas chromatograph equipped with a thermal conductivity detector (TCD) was used. 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 splitless injection. The column temperature was programmed from 40 °C to 210 °C at the rate of 5 °C/min for all runs. The injector and detector temperatures were 250 °C and 230 °C, respectively. Helium was used as the carrier gas at a flow rate of 1 mL/min. A glass sniffing port was connected to the outlet of the TCD and heated 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.

Aroma Extract Dilution Analysis (AEDA). The original odor concentrate of the green tea infusion was stepwise diluted with methylene chloride to 1:4, 1:16, 1:64, 1:256 and 1:1024, and aliquots (1 μL) of each fraction were analyzed by capillary GC on the DB-Wax column. The odor-active compounds were detected by GC eluate sniffing (GC−O). The flavor dilution (FD) factors of the odorants were determined by AEDA.

Gas Chromatography/Mass Spectrometry (GC/MS). An Agilent 6890 N gas chromatograph coupled to an Agilent model 5973 N series mass spectrometer was used. The column was a 60 m × 0.25 mm i.d. DB-Wax fused-silica capillary column (J & W Scientific) with a film thickness of 0.25 μm. The column temperature was programmed from 80 °C or 40 °C to 210 °C at the rate of 3 °C/min. The injector temperature was 250 °C. The flow rate of the helium carrier gas was 1 mL/min, and the split ratio was 1:30 or splitless. The mass spectrometer was used under the following conditions: ionization voltage, 70 eV (EI); ion source temperature, 150 °C.

Identification of Components. The identification of the components was made by comparison of their Kovats GC retention indices, mass spectra, and odor quality to those of the authentic compounds.

RESULTS AND DISCUSSION

The flavor extracts of the green tea infusions were prepared by steam distillation under reduced pressure, and the steam distillate was concentrated by the adsorptive column method. The volatile fractions isolated from the green tea infusions were screened by AEDA for potent odorants. As summarized in Table 1, the AEDA of the flavor extracts revealed 32, 51, and 52 odor-active compounds with FD factors in the range of 16−1024. Among the perceived odorants, there were two compounds, **8** and **30**, showing high FD factors in both pan-fired green teas (Kamairi-cha and Longing tea). These unknown compounds smelled popcorn-like. However, these compounds were not detectable with GC/MS. This finding suggested that the odor threshold values of these odorants were too low. For identification of these odorants, the volatile fraction was obtained from a large volume of Kamairi-cha infusion (about 20 L), and the odorants were separated into basic and neutral fractions. A GC/MS analysis of this basic fraction made it possible to obtain a mass spectrum of the 2-acetyl-1-pyrroline (**8**), and its retention index on the DB-Wax stationary phase gave 1341. On the other hand, from the GC/MS analysis of the neutral and basic fractions, it was not possible to obtain an unequivocal mass spectrum of 2-acetyl-2-thiazoline (**30**), because this compound could not be satisfactorily separated from the other compounds. The neutral fraction was separated by silica gel column chromatography. As a consequence, the pure mass spectrum of the 2-acetyl-2-thiazoline (**30**) was obtained from the N-7 fraction and showed a retention index on the DB-Wax stationary phase of 1765. The identifications of these odorants, which had not yet been detected in teas, were compared to their retention indexes on DB-Wax and the mass spectra to their synthesis compounds. Of the two popcorn-like odorants, 2-acetyl-1-pyrroline was reported as a character impact compound for the roasty note of cooked rice (*9*), popcorn (*14*), bread crust (*15*), and heated milk (*16*), and 2-acetyl-2-thiazoline was detected as a potent odorant of several processed meat products, chicken broth (*17*), roast beef (*10, 18*), and boiled trout (*19*). According to the literature, the odor threshold values of these odorants were very low and showed 0.02 ng/L (*14*) and 0.02−0.08 ng/L (*20*) in air, respectively. Therefore, these popcorn-like odorants may contribute to the flavor of the green tea infusions in trace amounts. The mass spectra of the remaining compounds **1**−**4**, **6**, **7**, **9**−**13**, **16**−**20**, **22**−**29**, **31**−**37**, **39**, **41**−**45**, and **47**−**54** were detected by GC/MS in the volatile fraction or enriched fractions, as detailed in **Table 1**, of the Kamairi-cha infusion. The mass spectrum signals of 2-isobutyl-3-methoxypyrazine (**21**) and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (**46**) were too weak for an unequivocal interpretation; therefore, these odorants were tentatively identified by matching their retention indexes and odor quality with those of standard compounds on the two different stationary GC column phases (DB-Wax and DB-1).

On the basis of their high FD factors (≥256), (*Z*)-1,5-octadien-3-one (metallic, geranium-like, **9**), 4-mercapto-4-methyl-2-pentanone (meaty, black currant-like, **10**), methional (potato-like, **17**), (*E*,*Z*)-2,6-nonadienal (cucumber-like, **25**), and 3-methylnonane-2,4-dione (green, fruity, hay-like, **29**) were shown as common potent odorants for all varieties of the green tea infusions. These compounds have been identified as important odorants of the Japanese Sen-cha (*21*) and Chinese green tea powder (*22*). However, some potent odorants in the Japanese green tea, which were described in our previous study (*21*), were not detectable at FD factors of 16 or greater. This finding may be assumed to be caused by the slight difference in the condition of the green tea cultivation or production. Therefore, in addition, it would be necessary to investigate the detailed formation mechanism of these odorants and the effect of them on the green tea flavor. On the other hand, significant differences between the Sen-cha and pan-fired green teas (Kamairi-cha and Longing tea) were the absence of the popcorn-like odorants (2-acetyl-1-pyrroline and 2-acetyl-2-thiazoline) and nutty odorants (2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine) in the Sen-cha infusion. This difference was in agreement with the overall odor impression that the pan-fired odor note is absent in the Sen-cha infusion. Therefore, these four popcorn-like and nutty odorants may contribute to the pan-fired aroma of green teas. These compounds were shown to be formed during the heat processing from the reactions of amino acids with the sugar degradation products (*23*−*25*). It seems likely that the pan-firing, which is a characteristic manufacturing process of pan-fired green teas, affords a similar reaction within the tea leaf.

On the basis of these results, the potent odorants involved in different green tea varieties were screened. However, it is know that steam distillation under reduced pressure and the adsorptive column sampling techniques have the problem that the high water solubility and high boiling point compounds suffer losses, and odorants boiling higher than the solvent are partially lost during the concentration of the extract by distilling off the solvent (*26*). Therefore, to reveal potent odorants in the initial form that are involved in the green tea infusion, a further study would be required.

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