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Influence of Manufacturing Conditions and Crop Season on the Formation of 4-Mercapto-4-methyl-2-pentanone in Japanese Green Tea (Sen-cha)

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4-Mercapto-4-methyl-2-pentanone is one of the most strongly contributing odorants in the volatile fraction of a Japanese green tea (sen-cha) infusion, and on the basis of the results of an aroma extract dilution analysis, the contribution of this compound to the flavor of the sen-cha infusion varied according to the degree of heating of the tea leaves during the roasting process. The concentration of this odorant in the sen-cha infusion, as with other roasty odorants, increased with the increasing roasting temperature. However, the slope of the increase curve differed with the odor compound, and even if roasting was done at a low temperature, at which the other roasty odorants hardly increased, 4-mercapto-4-methyl-2-pentanone still increased and reached a maximum at 112 °C. On the other hand, the amount of 4-mercapto-4-methyl-2-pentanone in sen-cha was a maximum in the first crop, then decreasing in the order of the second and third crops. These results suggested that the amount of 4-mercapto-4-methyl-2-pentanone was closely involved with the quality of sen-cha and that the concentration was dependent on the roasting conditions for the green tea leaves, which might be accompanied by an enzymatic reaction.

KEYWORDS: Green tea; sen-cha; 4-mercapto-4-methyl-2-pentanone; manufacturing process; crop number

INTRODUCTION

Green tea (sen-cha) is one of the most widely consumed beverages in Japan. The high acceptability of sen-cha is due to many factors, one of the most important being its flavor. The sen-cha flavor from the first crop (first flush) is particularly valued as the highest quality with a characteristic green note. The potent odorants in the sen-cha flavor have consequently been the subject of considerable research, and 4-mercapto-4-methyl-2-pentanone has been found to be one of the most important odorants in the high-quality flavor of Japanese green tea (sen-cha) (*1*−*3*). This compound is also an important odorant in the flavor of wine (*4*, *5*) and hand-squeezed grapefruit juice (*6*), and it is known to have a very low odor threshold (*7*). It is well-known that the sen-cha flavor is generally produced during the manufacturing process and that suitable manufacturing conditions result in a high-quality flavor. The manufacturing procedure for sen-cha consists of a process that produces ara-cha (unrefined sen-cha) from the freshly picked leaves and a process that refines the ara-cha by further roasting. Ara-cha is therefore the starting material for sen-cha. The final roasting process is known to be the most important step for generating the characteristic sen-cha flavor (*8*−*11*). It is also well-known that the quality of the sen-cha flavor deteriorates with increasing crop number (*12*). Thus, the roasting conditions and crop number are both important factors for the quality of the sen-cha flavor, and the relationship between these two factors and the volatile components has been mainly investigated by gas chromatography (GC) and GC−mass spectrometry (MS) (*8*, *13*). However, no details have been presented in these investigations about the importance of the recognized compounds, because the GC analysis was not coupled with an aroma extract dilution analysis (AEDA). The actual significance of each compound to the quality of the sen-cha flavor has therefore not yet been determined, and the participation of 4-mercapto-4-methyl-2-pentanone has been unclear.

The objective of the present investigation was to elucidate the participation of 4-mercapto-4-methyl-2-pentanone in the quality of the sen-cha flavor by an AEDA of sen-cha leaves prepared under different roasting conditions from different crop numbers.

MATERIALS AND METHODS

Materials. *Green Tea Samples.* The influence of the roasting conditions on the flavor of sen-cha was investigated using ara-cha (unrefined sen-cha) products (first crop) produced in Shizuoka prefecture (Japan) in 2003. Ara-cha, which had initially been dried by microwaves, was roasted at five different temperatures (80, 90, 102, 112, and 122 °C) in a gas dram direct roaster. The influence of the crop number on the sen-cha flavor was investigated using ara-cha

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Table 1. Selected Ion and Calibration Factors for Mass Chromatography (SIM)

compound	selected ion (*m/z*)	calibration factor	linear range[a] (ppb)	R^2
2-acetyl-1-pyrroline	111	0.320	1–5000	0.9999
4-mercapto-4-methyl-2-pentanone	132	0.263	1–5000	0.9999
2-ethyl-3,5-dimethylpyrazine	135	0.019	1–20000	0.9791
2,3-diethyl-5-methylpyrazine	149	0.037	1–20000	0.9987
2-acetyl-2-thiazoline	129	0.090	1–20000	0.9993
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	128	0.272	1–20000	0.9960
methyl undecanoate[b]	200	1.000		

[a] Linear range was detemined by the concentration of standard solutions. [b] Internal standard.

products from the first flush (end of April), second flush (end of June), and third flush (beginning of August) produced in the Sagara area of Shizuoka prefecture (Japan) in 2003. The ara-cha leaves were roasted at 110 °C for 15 min in a laboratory hot-air oven. These green tea leaves were stored at −80 °C until needed.

Chemicals. 2-Acetyl-1-pyrroline (*14*), 2-acetyl-2-thiazoline (*15*), and 4-mercapto-4-methyl-2-pentanone (*5*) were synthesized according to the literature procedures. (*E*)-2-Nonenal, 2-ethyl-3,5-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine, 2-isobutyl-3-methoxypyrazine, linalool, (*E*,*E*)-2,4-heptadienal, and (*E*,*E*)-2,4-decadienal were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). (*Z*)-4-Heptenal and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone were obtained from Sigma-Aldrich (Tokyo, Japan), and (*E*,*Z*)-2,6-nonadienal was obtained from Wako Pure Chemical Industries (Osaka, Japan).

Green Tea Infusion. Deionized hot water (4 L) at 70−75 °C was added to 200 g (for the AEDA and quantitative analysis sample) or 40 g (for the sensory evaluation sample) of green tea, and the leaves were removed using coarse filter paper after standing for 5 min. The filtrate (3 L) was immediately cooled to ∼20 °C in tap water.

Sensory Evaluation. Each green tea (sen-cha and ara-cha) infusion (30 mL) was put into a glass beaker at ∼20 °C. The sample was immediately sniffed by 15 panelists between the ages of 25 and 36. Each of these panelists had previously received extensive training in the descriptive sensory analysis of a sen-cha infusion and had experience in the sensory profiling of various food samples. The intensity of each attribute of a sen-cha infusion was scored on a scale of 1 (weak)−7 (strong). The sen-cha aroma from the different roasting conditions was compared with the corresponding ara-cha aroma, and both of the attribute scales (green and roasty) of the ara-cha aroma were defined as 2. The sen-cha and ara-cha aromas from the different crop numbers were compared with the ara-cha aroma from the first flush, and both of the attribute scales (green and hay-like) of the ara-cha aroma from the first flush were defined as 2. The results obtained from all of the panelists were then averaged.

Isolation of the Volatiles. Each green tea (sen-cha and ara-cha) infusion (3 L) was distilled under reduced pressure (40 °C at 20 mmHg). The steam distillate (1 L) was passed through a column packed with 10 g of Porapak Q (Waters). The adsorbed compounds were then eluted with methylene chloride (100 mL). The eluate was dried over anhydrous sodium sulfate, and the solvent was removed by rotary evaporation to leave ∼5 mL. Further concentration was conducted in a nitrogen stream to ∼100 μL. For the quantitative analysis, an internal standard solution (10 μL) prepared from methyl undecanoate (0.508 mg) in methylene chloride (10 mL) was added to the eluate before the solvent was removed by the evaporator. The resulting concentrate was used as the sample for AEDA and the GC-MS analysis.

Gas Chromatography−Olfactometry (GC-O). A Hewlett-Packard (HP) 5890 series II gas chromatograph equipped with a thermal conductivity detector (TCD) and fused silica column (30 m × 0.25 mm i.d., coated with a 0.25 μm film of DB-Wax; J&W Scientific) was used in the splitless injection mode. The column temperature was programmed from 40 to 210 °C at the rate of 5 °C/min for all runs. The injector and detector temperatures were 250 and 230 °C, respectively. Helium was used as the carrier gas at the 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 being pumped into the sniffing port at ∼100 mL/min to quickly remove from the sniffing port the odorant that had been eluted for the TCD.

AEDA. The original odor concentrate of the green tea infusion was stepwise diluted with methylene chloride to 4^n ($n = 1-6$) or 2^n ($n = 3-11$), and aliquots (1 μL) of each fraction were analyzed by capillary GC in the DB-Wax column. The odorants were then detected by GC eluate sniffing (GC-O). The flavor dilution (FD) factors of the odorants were determined by AEDA (*16*). Before the FD factor measurement, two panelists repeatedly checked the retention time and odor quality of the odorants using each diluted sample (1:16), and then the FD factor of the odorants was determined to be detectable at the dilution step by both panelists.

GC-MS. An Agilent 6890 N gas chromatograph coupled to an Agilent 5973 N series mass spectrometer was used. The column was a 60 m × 0.25 mm i.d. DB-Wax fused silica capillary type (J&W Scientific) with a film thickness of 0.25 μm. The column temperature was programmed from 80 to 210 °C or from 40 to 210 °C at the rate of 3 °C/min. The injector temperature was 250 °C, and the flow rate of the helium carrier gas was 1 mL/min. An injection volume of 1 or 4 μL was applied using the split (the split ratio was 1:30) or pulsed splitless technique. The mass spectrometer was used with an ionization voltage of 70 eV (EI) and an ion source temperature of 150 °C. The quantity of the odorants in each volatile fraction of a green tea infusion was determined from the extracted ion peak areas obtained by mass chromatography. The GC-MS utilized the selected ion mode (SIM) coupled with the pulsed splitless injection technique. The conditions of the pulsed splitless injection were as follows: the injector pressure (48 psi) of the pulsed mode was 3 times higher than that of the measurement mode, the pulse time was 1.1 min, the purge time was 1.0 min, and the injection volume was 4 μL. The extracted ions were monitored in the ranges listed in **Table 1**. The calibration factors were determined in a mixture of equal amounts by weight of an odorant and internal standard compound and were calculated as the ratio of the extracted ion peak area of the internal standard to the extracted ion peak area of the odorant. These extracted ion peak areas were the mean values of the triplicate results. The calibration factors were used to calculate the amount of each odorant on the basis of the internal standard. The linearity of the method was estimated on seven levels of concentration by the injection of a series of standard solutions, which was prepared by serial dilutions of the target odorants with methylene chloride in the 1−20 000 ppb range.

Identification of the Components. Each component was identified by comparing its Kovats GC retention index (RI), mass spectrum, and odor quality with those of an authentic compound.

RESULTS AND DISCUSSION

Influence of Roasting on the Green Tea Flavor. The roasting process changed the flavor of the green tea, and the characteristic sen-cha flavor was developed with its unique green note. Flavor extracts of the green tea infusions were prepared by steam distillation under reduced pressure, and each steam distillate was concentrated using an adsorptive column. The flavor extracts well reproduced the characteristic odor of the green tea, and each extract clearly showed a flavor change of the green tea by roasting. Comparative AEDA applied to the volatile fraction of each freshly filtered green tea infusion, which had been prepared from ara-cha (unrefined) and sen-cha (roasted to 102 °C), revealed 47 and 50 odor-active peaks, respectively,

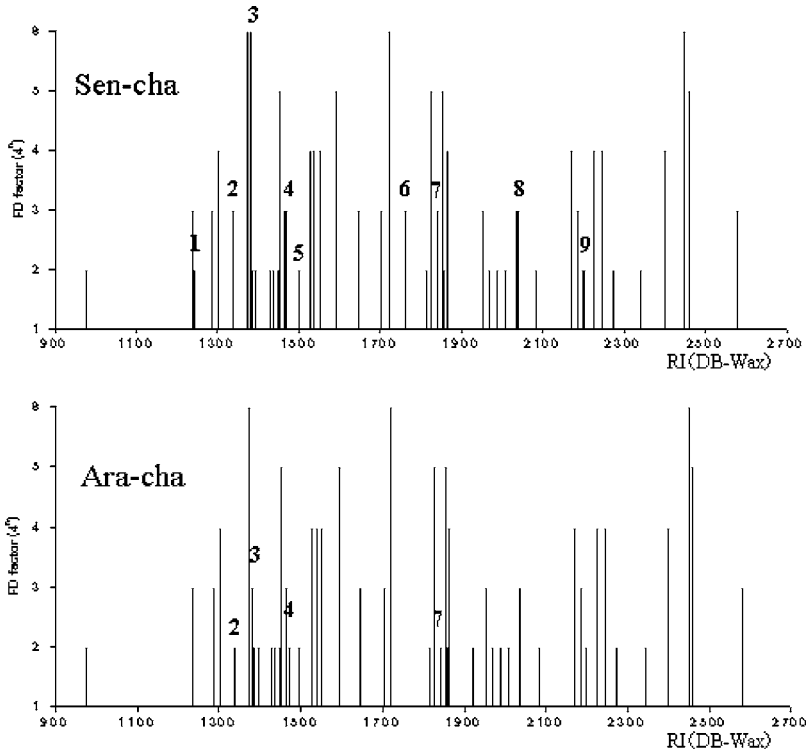

Figure 1. Flavor dilution chromatograms of the sen-cha and ara-cha infusions. (See **Table 2** for details of the numbering.)

Table 2. Comparison of Potent Odorants in the Sen-cha Infusions Prepared from Ara-cha and Sen-cha Leaves That Showed an Increase in FD Factor

				FD factor	
no.	RI[a]	compound	odor quality[b]	ara-cha (unroasted)	sen-cha[c] (roasted)
1	1241	unknown	nutty	<16	16
2	1338	2-acetyl-1-pyrroline	roasty	16	64
3	1383	4-mercapto-4-methyl-2-pentanone	green, meaty	64	4096
4	1470	2-ethyl-3,5-dimethylpyrazine	nutty	16	64
5	1499	2,3-diethyl-5-methylpyrazine	nutty	<16	16
6	1764	2-acetyl-2-thiazoline	roasty	<16	64
7	1842	unknown	burnt, hay-like	16	64
8	2041	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	sweet	<16	64
9	2204	unknown	caramel-like	<16	16

[a] Retention index in the DB-Wax column (30 m × 0.25 mm i.d., coated with a 0.25 μm film) observed for GC-O. [b] Odor quality assigned during AEDA. [c] Leaves were roasted until the temperature of the ara-cha leaves reached 102 °C to produce sen-cha.

with FD factors between 4^2 and 4^6 (**Figure 1**). Among the perceived odorants were nine peaks for which the FD factors had increased during the roasting process, and these were identified by comparing their Kovats indices, mass spectra, and odor quality with those of authentic compounds, as detailed in **Table 2**. Six compounds were identified from the nine peaks by GC-MS, some of these odorants [2-acetyl-1-pyrroline (**2**), 2-ethyl-3,5-dimethylpyrazine (**4**), 2,3-diethyl-5-methylpyrazine (**5**), 2-acetyl-2-thiazoline (**6**), and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (**8**)] being known as thermally generated compounds related to the roasted flavor. These roasty odorants have been shown to be formed during the heating process from the reaction of amino acids with sugar degradation products (*17*−*20*). However, the difference in the FD factor of each of these compounds between ara-cha and sen-cha was comparatively small. On the other hand, there was a marked difference in the FD factor of 4-mercapto-4-methyl-2-pentanone (**3**) between ara-cha and sen-cha. The AEDA results for the sen-cha infusion revealed 4-mercapto-4-methyl-2-pentanone as the most potent odorant with an FD factor of 4^6. This finding suggested that 4-mercapto-4-methyl-2-pentanone, which had been generated during the roasting process as with the other roasty odorants, was related to the difference in the characteristic flavor between ara-cha and sen-cha, especially the unique green note in the overall flavor of sen-cha. Furthermore, because the increase in the FD factor of 4-mercapto-4-methyl-2-pentanone was much greater than that of the other roasty odorants, it can be presumed that the influence of the roasting on the generated amount of 4-mercapto-4-methyl-2-pentanone differed from that of the other roasty odorants.

Influence of the Degree of Roasting on the Concentration of 4-Mercapto-4-methyl-2-pentanone and Other Roasty Odorants. The concentrations of 4-mercapto-4-methyl-2-pentanone and the other roasty odorants (**2**, **4**, **5**, **6**, and **8**) in the green tea infusions prepared from the leaves with different degrees of roasting were investigated. The results in **Figure 2** show that the concentration of these compounds significantly increased with the increasing roasting temperature. However, a difference was apparent in the rate of increase of these compounds with different degrees of roasting in each green tea. Namely, the amount of 4-mercapto-4-methyl-2-pentanone increased even at a comparatively low roasting temperature and

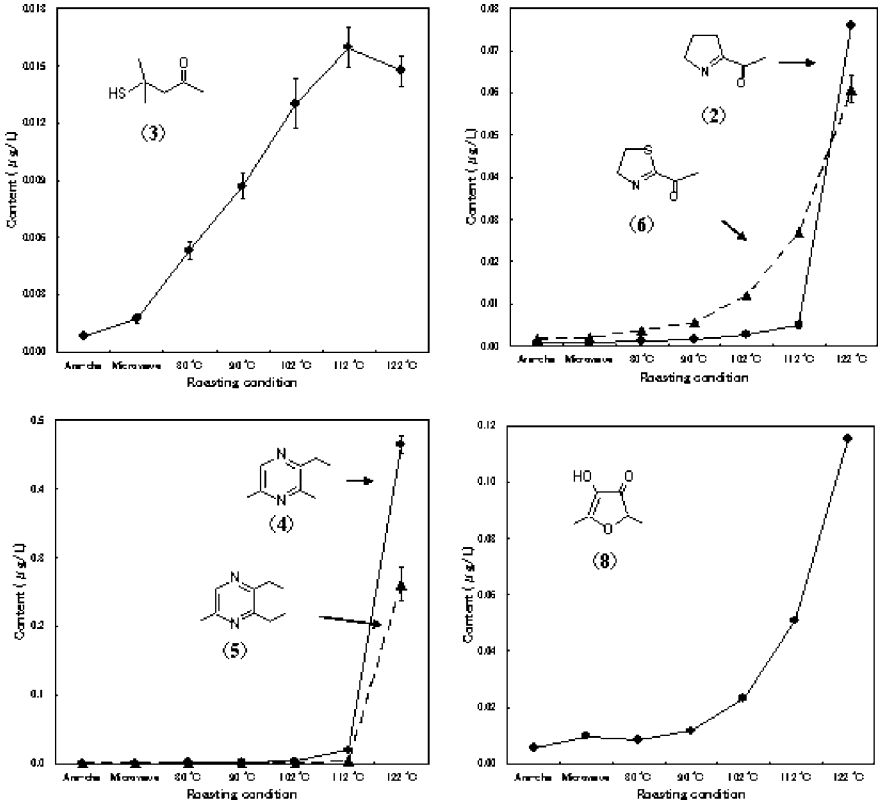

Figure 2. Quantitative changes in potent odorants during the roasting process of green tea.

Table 3. Change in Attribute of the Green Tea Aroma during the Roasting Process[a]

	roasting condition						
attribute	ara-cha	microwave	80 °C	90 °C	102 °C	112 °C	122 °C
green	2.0[b]	2.8	3.3	4.0	4.2	3.7	1.4
roasty	2.0[b]	2.3	2.5	3.3	4.1	4.9	6.7

[a] The intensity of the attribute was scored on a scale of 1–7. [b] The aroma of each sample was compared with the ara-cha aroma, the scale of ara-cha being defined as 2.

reached a maximum at 112 °C, whereas the other roasty compounds hardly increased at low temperatures, but sharply increased in the temperature range of 112−122 °C. These results clearly indicate that 4-mercapto-4-methyl-2-pentanone was specifically generated at a lower roasting temperature than that required for the other roasty odorants. The influence on the green tea flavor of the change in the amounts of 4-mercapto-4-methyl-2-pentanone and the other roasty odorants was verified by a sensory evaluation of the green tea infusions prepared from leaves with different degrees of roasting. **Table 3** shows the change in the odor attributes (green and roasty) of the green tea infusions. The roasty odor of the green tea infusions increased with the increasing roasting temperature, and the score reached a maximum at 122 °C. On the other hand, the temperature at which the score for the characteristic green odor began to increase was lower than that for the roasty odor, showing a maximum at 102 °C. The results of the sensory evaluation therefore agree well with the change in the amounts of 4-mercapto-4-methyl-2-pentanone and the other roasty odorants, and it can be assumed that 4-mercapto-4-methyl-2-pentanone is closely related to the characteristic green odor of sen-cha.

Influence of Crop Number on Green Tea Flavor. The quality of sen-cha is generally dependent on the crop number, green tea leaves with a high crop number late in the plucking season having a low quality and low grade on the market (*12*). The flavor of green tea infusions made from leaves plucked as the first, second, and third crops were compared by a sensory evaluation of sen-cha and ara-cha samples. **Table 4** shows the odor attributes (green and hay-like) of the green tea infusions. It was recognized that the roasting process influenced the green odor; the green odor of sen-cha was strongest from the first crop, decreasing in the order of the second and third crops, whereas that of ara-cha had a low evaluation for all three crops. On the other hand, the hay-like odor of sen-cha and ara-cha increased with the later crops and was only slightly affected by the roasting process. In further experiments, the volatile fraction of each green tea infusion, which had been prepared from leaves from the first and third crops (ara-cha and sen-cha), was isolated and then evaluated by AEDA (**Figure 3**). Nine peaks appeared for which the FD factors had changed between the first and the third crops. The results of the AEDA experiments are listed in **Table 5**. Seven compounds from the green tea of the third crop showed a higher FD factor than that from the first crop and were only slightly affected by the roasting process. It was therefore assumed that these odorants have a close relationship with the hay-like odor recognized by the sensory evaluation. Among these odorants, five were unsaturated aldehydes (**10**, **11**, **13**, **15**, and **16**), these being well-known as the oxidization products of lipids (*21*). However, it has been reported that the lipid content of tea leaves was only slightly influenced by the crop number (*22*); other factors by which the generation of these unsaturated aldehydes from lipids promoted oxidation may therefore have been involved. The FD factor for linalool (**14**) in green tea from the third crop was observed to be much higher than that from the first crop. This result is consistent with that of the previous study in which a large amount of **14** was contained in the low-grade sen-cha (*13*). 2-Isobutyl-3-methoxypyrazine (**12**), which has an earthy and musty odor, was detected in the high FD factor of green tea from the third crop.

Table 4. Comparison of the Flavor Attributes of the Different Green Tea Crops[a]

	ara-cha (unroasted)			sen-cha (roasted)[b]		
attribute	first flush	second flush	third flush	first flush	second flush	third flush
green	2.0[c]	2.4	2.3	5.7	4.8	3.6
hay-like	2.0[c]	2.9	3.8	2.5	3.1	3.9

[a] The intensity of the attribute was scored on a scale of 1–7. [b] Samples were roasted at 110 °C for 15 min. [c] The aroma of each sample was compared with the ara-cha aroma from the first flush, and the scale for ara-cha from the first flush was defined as 2.

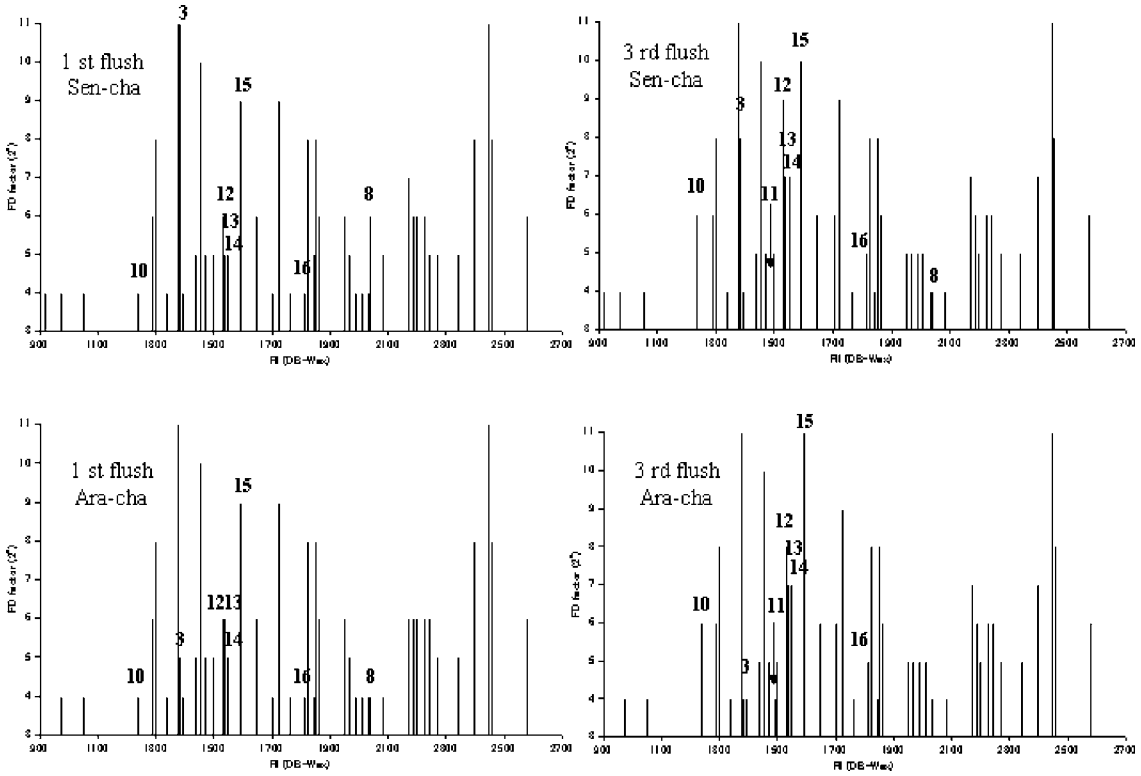

Figure 3. Flavor dilution chromatograms of the sen-cha (first and third flushes) and ara-cha (first and third flushes) infusions. (See **Table 5** for details of the numbering.)

Table 5. Comparison of Potent Odorants in Green Tea from Different Flushes and Effect of Roasting Process

				FD factor			
				first flush		third flush	
no.	RI[a]	coumpound	odor quality[b]	ara-cha (unroasted	sen-cha (roasted)	ara-cha (unroasted)	sen-cha (roasted)
10	1237	(*Z*)-4-heptenal	metallic, hay-like	16	16	64	64
3	1383	4-mercapto-4-methyl-2-pentanone	green, meaty	32	2048	16	256
11	1495	(*E*,*E*)-2,4-heptadienal	fatty, hay-like	<16	<16	16	16
12	1529	2-isobutyl-3-methoxypyrazine	earthy, musty	64	64	256	512
13	1537	(*E*)-2-nonenal	green, sweet	64	32	128	128
14	1550	linalool	floral, green	32	32	128	128
15	1593	(*E*,*Z*)-2,6-nonadienal	green, cucumber-like	512	512	2048	1024
16	1815	(*E*,*E*)-2,4-decadienal	fatty	16	16	32	32
8	2041	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	sweet	16	64	<16	16

[a] Retention index in the DB-Wax column (30 m × 0.25 mm i.d., coated with a 0.25 μm film) observed for GC-O. [b] Odor quality assigned during AEDA.

It has been suggested that this compound is generated by biosynthesis in plants (*23*), such that the biosynthetic activity of this compound in green tea leaves from the third crop may have been higher than that made from the first crop. On the other hand, the FD factors of 4-mercapto-4-methyl-2-pentanone and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone in sen-cha from the first crop were higher than those from the third crop. In particular, 4-mercapto-4-methyl-2-pentanone was detected with a very high FD factor in green tea made from the first crop, and the FD factors of sen-cha were much higher than those of ara-cha (**Table 5**). The amounts of 4-mercapto-4-methyl-2-pentanone in the infusions of ara-cha and sen-cha prepared from the leaves of different crops (first, second, and third) were also investigated (**Figure 4**). It was found that the later the crop, the more significant was the decrease in the amount of 4-mercapto-4-methyl-2-pentanone in the sen-cha infusion. The lower content of this compound in sen-cha is considered to have been one of the main causes for the lower quality of the second and third crops, because 4-mercapto-4-methyl-2-pentanone was suggested to be one of the most important contributors to the

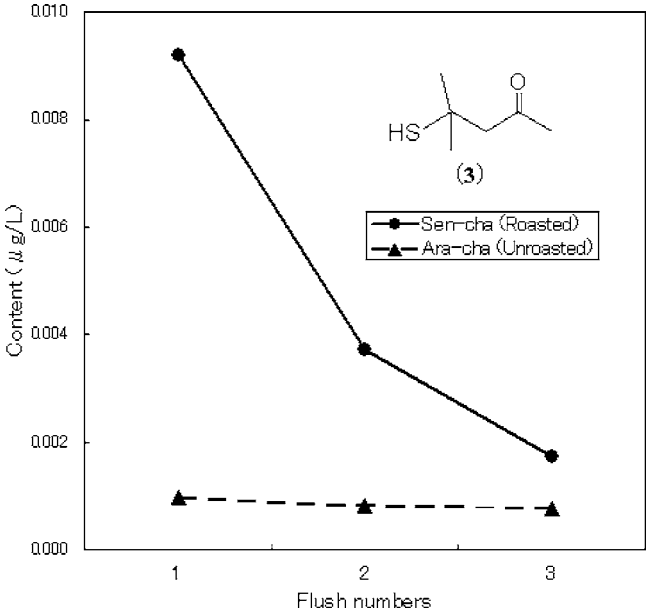

Figure 4. Changes in the content of 4-mercapto-4-methyl-2-pentanone in the green tea infusions prepared from ara-cha (▲) and sen-cha (●) of different crop numbers.

characteristic green odor of high-grade sen-cha. It was further shown that the amount of 4-mercapto-4-methyl-2-pentanone in ara-cha was much less than that in sen-cha and that its concentration in ara-cha was almost constant regardless of the crop number. It has been reported that 4-mercapto-4-methyl-2-pentanone can be released from its *S*-cysteine conjugate (4-*S*-cysteinyl-4-methyl-2-pentanone) by an enzymatic reaction with cysteine-*S*-conjugate β-lyase in a grape or model system (*24*, *25*). Carotenoid degradation enzymes, which have a high optimal temperature, have recently been found in Japanese green tea (*26*). It can be assumed from these results that some precursors are contained in the tea leaves or in ara-cha and that 4-mercapto-4-methyl-2-pentanone is enzymatically generated at the appropriate temperature during the sen-cha manufacturing process.

On the basis of these results, one of the most important odorants involved in the quality of sen-cha with its characteristic green odor was suggested to be 4-mercapto-4-methyl-2-pentanone. Therefore, it is presumed that the investigation of the formation mechanism of 4-mercapto-4-methyl-2-pentanone during the process of manufacturing green tea must be the shortest way to produce the high-quality green tea called sen-cha. However, there is a limit in the comparison of individual odorants with odor attributes because the synergistic and antagonistic effects between the odorants and/or other constituents in the sen-cha infusions are not considered. Therefore, to further clarify that 4-mercapto-4-methyl-2-pentanone is related to the characteristic green note of sen-cha, it would be necessary to investigate in detail the calculation of the odor activity values and flavor recombination experiments.

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