Characterization of the Odor-Active Volatiles in Citrus Hyuganatsu (*Citrus tamurana* Hort. ex Tanaka)

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The volatile components of Hyuganatsu (*Citrus tamurana* Hort. ex Tanaka) peel oil, isolated by cold-pressing, were investigated by chemical and sensory analyses. According to chemical analysis by GC and GC-MS, limonene (84.0%) was the most abundant compound, followed by γ-terpinene (6.9%), myrcene (2.2%), α-pinene (1.2%), and linalool (1.0%). Monoterpene hydrocarbons were predominant in Hyuganatsu peel oil. The odor-active volatiles in Hyuganatsu flavor were studied by GC−olfactometry and omission tests. The characteristic flavor was present in the oxygenated fraction. Flavor dilution (FD) factors of the volatile flavor components of the Hyuganatsu cold-pressed oil were determined by aroma extraction dilution analysis (AEDA). Furthermore, relative flavor activity was investigated by means of FD factor and weight percent. Ten kinds of odor compounds having Hyuganatsu-like aroma were detected by AEDA: limonene, linalool, octanol, neral, neryl acetate, tridecanal, *trans*-carveol, *cis*-nerolidol, *trans*,*trans*-farnesyl acetate, and *trans*,*trans*-farnesol. Linalool and octanol were regarded as the most odor-active or key compounds of Hyuganatsu aroma. Diluted solutions of linalool and octanol of ∼2 ppm gave a fresh and fruity aroma note similar to Hyuganatsu flavor.

Keywords: *Citrus Hyuganatsu (Citrus tamurana Hort. ex Tanaka); gas chromatography−olfactometry; odor-active volatiles; aroma extraction dilution analysis; omission test; linalool; octanol*

INTRODUCTION

Gas chromatography−olfactometry (GC-O) analysis, including aroma extraction dilution analysis (AEDA) and CharmAnalysis, has been a useful method for estimating the contribution of the most odor-active compounds in an overall odor and has been successfully used to characterize key aroma compounds in foods (*1*, *2*). It is known that in many cases only a limited number of flavor components contribute to the character of an aroma (*3*). AEDA is a useful method for obtaining desirable results on the odor-active compounds through sniffing analysis. By means of sniffing serial dilutions of an essential oil, each volatile in the food flavor can be ranked according to odor potency (*4*). The odor potency or intensity is expressed as the flavor dilution (FD) factor, which is applicable for the comparison of intensities of odor-active compounds in food flavors. Therefore, the FD factor is the ratio of the concentration of a compound in the initial concentration to that in the most diluted concentration in which the odor could be detected by GC-O (*5*). GC-O techniques such as AEDA and CharmAnalysis are based on the determination of odor-threshold values of the volatile components eluted from the GC column (*6*).

Hyuganatsu is one of the predominant citrus crops in Japan from spring to early summer. In 1999 the production of Hyuganatsu in Japan was estimated to be 5500 tons. This fruit has been regarded as a citrus fruit with potential commercial value in Japan because of its attractive and pleasant flavor. The composition of Hyuganatsu peel oil has been quantitatively and qualitatively determined (*7*). In the present study, we aimed to elucidate the odor-active volatiles in Hyuganatsu flavor by using the AEDA GC-O technique. We also performed the omission test using a synthetic aroma model of Hyuganatsu flavor.

MATERIALS AND METHODS

Materials. Fresh Hyuganatsu (*Citrus tamurana* Hort. ex Tanaka), which was harvested in May 2000, was provided by the Kochi Prefectural Fruit Tree Experimental Station, Kochi, Japan. The essential oil sample was prepared according to the cold-pressing method described by Sawamura and Kuriyama (*8*) within 24 h of harvest and stored at −25 °C until analyzed. Authentic chemicals for co-injection in gas chromatography (GC) and mass spectrometry (MS) were obtained from reliable commercial sources as follows: Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan); Wako Pure Chemical Industries (Osaka, Japan); Aldrich Chemical Co. (Milwaukee, WI); Sigma Chemical Co. (St. Louis, MO); and Extrasynthèse S.A. (Genay, France). Some chemicals were provided by Ogawa and Co., Ltd. (Tokyo, Japan).

Silica Gel Column Chromatography. The whole volatile concentrate was fractionated into hydrocarbon and oxygenated compound fractions on a silica gel column (25 cm × 2 cm i.d.) packed with Wako gel Q-23 (Wako Pure Chemical Industries) (*9*). About 3 g of Hyuganatsu peel oil was applied. The hydrocarbon and oxygenated compound fractions were eluated with *n*-hexane (1 L) and diethyl ether (0.5 L), respectively. Each fraction was concentrated under reduced pressure at room temperature.

GC and GC-MS. A Shimadzu GC-14A gas chromatograph equipped with a DB-Wax fused-silica capillary column (60 m

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× 0.25 mm i.d., film thickness = 0.25 μm, J&W Scientific, Folsom, CA), and a flame ionization detector (FID) was used. Peak areas were integrated with a Shimadzu C-R6A Chromatopack integrator. The column temperature was programmed from 70 °C (2 min) to 230 °C (20 min) at a program rate of 2 °C/min. The injector and detector temperatures were 250 °C. Nitrogen was the carrier gas at a flow rate of 2 mL/min. Authentic compounds of 1-heptanol and methyl myristate (Wako Pure Chemical Industries) were used as internal standards. The ratio of cold-pressed oil to the two internal standards was 150:1:1. The weight percent of each peak was calculated according to the correlation factor to FID (*10*). An oil sample of 1 μL was injected, and the split ratio was 1:50. Kovats retention indices (RI) were calculated for all volatile components using a homologous series of *n*-alkanes (C_7−C_{29}) under the same GC conditions. A nonpolar column was also used for GC analysis: a DB-5 fused silica column (30 m × 0.25 mm i.d., film thickness = 0.25 μm, J&W Scientific Inc.).

GC-MS was used for identification of the volatile flavor components detected. The analysis was carried out on a Shimadzu GC-17A linked with a Shimadzu QP-5000 at an MS ionization voltage of 70 eV, an accelerating voltage of 1500 V, and an ion source temperature of 250 °C. The injector and interface temperatures were 250 °C. The GC column and oven conditions were the same as those given above for the GC-14A instrument. An oil sample of 0.2 μL was injected, and the split ratio was 1:34. The carrier gas was helium at a constant flow of 1.0 mL/min.

Identification of Components. Components were identified by comparing their RI and matching their mass spectra with those of reference compounds in the data system of Compaq-ProLinea (Compaq Co., class 5K software), connected to a QP-5000 mass spectrometer. Whenever possible, the volatile flavor components were matched by co-injection with authentic compounds.

GC—Olfactometry (GC-O). A Shimadzu GC-8A gas chromatograph equipped with a DB-Wax fused-silica capillary column (60 m × 0.53 mm i.d., film thickness = 1 μm, J&W Scientific) and FID was used. The oven condition and injector and detector temperatures were the same as those given above for the GC-14A instrument. The flow rate of nitrogen carrier gas was 5 mL/min, and the split ratio was 1:10. GC-O was performed with aliquots (1 μL) of the diluted essential oils, which were evaluated by sniffing. At the exit of the capillary, the effluent was split into an FID and a sniffing port. Humid air was added to the effluent at the sniffing port.

AEDA. The cold-pressed Hyuganatsu oil was stepwise 3-fold diluted with acetone until the sniffers could not detect any significant odor in a run (*11*). Odor potencies of each volatile in the essential oil of Hyuganatsu were evaluated (*12*), together with the odor description. The highest dilution at which an individual component was detected was given as the FD factor for that odorant. Analyses were performed by two trained assessors in duplicate. On the basis of the AEDA results, relative flavor activity was calculated using the following equation (*13*): relative flavor activity = $\log 3^n/S^{0.5}$, where 3^n is the FD factor and *S* is the weight percent of a component.

Omission Tests. The base for the synthetic model of Hyuganatsu aroma was examined before omission tests. The volume of stock solutions for the aroma model of Hyuganatsu (detailed in Table 2) were added to the 70 mL of medium. Both water and alcohol were used as media for the aroma model (Table 3). The aroma model using water as the base was tested by three formulas: water I, in which the stock solution of each component was prepared with water, and then each component was added to a water medium; water II, in which the stock solution was prepared with 50% ethanol, and then each component was added to water; and water III, in which water added to 20 μL of emulsifier (polyoxyethylene sorbitan monolaurate) was used as the medium and the base for stock solution had the same composition. The aroma model as the alcohol base was tested by two formulas: 10% ethanol and 10% methanol solution, respectively, were used, and the base for the stock solution had the same alcohol concentration.

Table 1. Most Odor-Active Volatiles (FD ≥ 5) in Hyuganatsu Cold-Pressed Oil As Detected by GC-O

peak	compound	concn (μg/kg of fresh wt)	FD factor (3^n)	relative flavor activity
2	α-pinene	2870	6	2.6
4	camphene	13	6	40.1
6	*β*-pinene	1500	7	4.3
9	myrcene	5414	7	2.2
10	α-phellandrene	225	6	9.4
12	limonene	206000	7	0.4
15	*γ*-terpinene	16830	5	0.9
18	terpinolene	837	7	5.7
21	tetradecane	18	6	33.4
22	α-thujone	8	6	48.9
24	*cis*-linalol furanoxide	5	6	61.4
29	citronellal	606	7	6.7
36	linalool	2420	7	3.4
37	octanol	122	8	17.1
38	linalyl acetate	49	7	23.7
40	nonyl acetate	17	6	34.0
41	bornyl acetate	210	5	8.2
43	*β*-caryophyllene	123	6	12.8
49	*trans*-*β*-farnesene	1770	6	3.4
50	α-humullene	45	7	24.5
56	dodecanal	57	6	18.8
60	*l*-carvone	1010	7	5.2
62	*trans*-2-undecenal	57	5	15.7
63	geranyl acetate	103	5	11.6
69	tridecanal	1	5	118.7
73	*trans*-2-dodecenal	72	6	16.7
82	*trans*-nerolidol	116	7	15.3
83	globulol	143	5	9.9
93	isoeugenol	12	5	34.7
94	*trans*,*trans*-farnesyl acetate	59	7	21.5
99	*trans*,*trans*-farnesol	20	7	36.9

Table 2. Composition of the Odorants in the Aroma Model of Hyuganatsu

compound[a]	concn in Hyuganatsu CPO (wt %)	FD factor (3^n)	stock solution (mg/mL)	vol[b] (μL)
limonene (12)	84.0	7	500	15
linalool (36)	0.99	7	395	10
octanol (37)	0.05	8	10	20
neral (53)	0.005	4	3.9	70
neryl acetate (59)	0.04	4	31.7	15
tridecanal (69)	0.0004	5	0.2	10
trans-carveol	0.004	3	4.2	60
cis-nerolidol (80)	0.04	4	28	10
trans,*trans*-farnesyl acetate (94)	0.02	7	9.6	20
trans,*trans*-farnesol (99)	0.008	7	3.3	10

[a] Numbers in parentheses correspond with peak numbers in Table 1. [b] Volume of the stock solution used for the preparation of the aroma model solution.

Sensory evaluations were performed by a selected sensory panel of 10 trained people.

The model solution of Hyuganatsu aroma was prepared on the basis of the results achieved by AEDA, then one or more components were omitted (*14*). In the omission test, we used the hedonic scale (*15*). Assessors compared each omitted model with the complete model by sniffing to report the degree of similarity to, or difference from, the omitted and complete models in reaction to the nine-point hedonic scale statements. The results were tested by one-way analysis of variance ($p < 0.05$) using the Statistical System (*16*) software package. Significant differences between means were determined by Duncan's multiple-range test.

RESULTS AND DISCUSSION

Volatile Components of Hyuganatsu Peel Oil. The cold-pressed oil (CPO) from Hyuganatsu fruits was

Table 3. Flavor Profiles of the Hyuganatsu Sample and Five Aroma Models Differing in Base

attribute[a]	Hyuganatsu sample	aroma model				
		water I[b]	water II[c]	water II[d]	10% ethanol	10% methanol
sweet	5.0	5.6	5.4	6.2	4.5	5.3
fruity	7.9[f]	4.2[g]	4.6[g]	5.2[g]	4.8[g]	5.4[g]
green, herbaceous	4.4	5.4	5.2	5.4	6.1	5.8
similarity[e]		6.7[h]	6.8[h]	6.7[h]	5.0[i]	6.0[h,i]

[a] The intensity of the attributes was scored on a scale of 1 (weak extremely) to 9 (strong extremely). [b] The stock solutions were prepared with water, and then each component was added to water base (70 mL). [c] The stock solutions were prepared with 50% ethanol, and then each component was added to water base (70 mL). [d] The water (70 mL) with added 20 μL of emulsifier was used for the medium, and the base for stock solution was composed of the same composition. [e] Similarity was scored with a scale system of 1 (extremely different from Hyuganatsu) to 9 (extremely similar to Hyuganatsu). [f−i]Values with the same superscripts are not significantly different ($p < 0.05$).

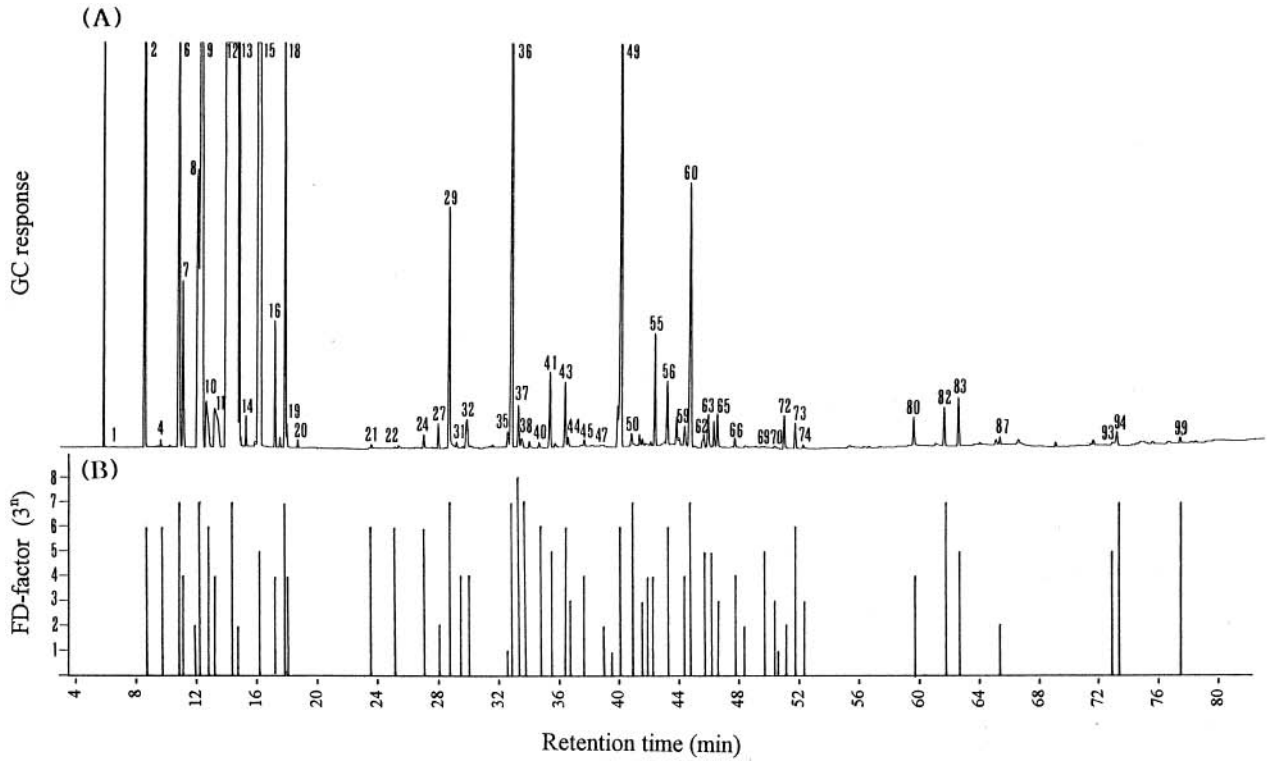

Figure 1. Gas chromatogram (A) and aromagram (B) of odor-active volatiles of Hyuganatsu peel oil.

obtained in a yield of 0.24 g/kg based on fresh weight. The gas chromatogram of Hyuganatsu oil is shown in Figure 1A. One hundred and two volatile flavor components were identified by GC-MS, RIs on polar and apolar columns, and co-injection with authentic compounds, and 58 peaks were confirmed by sniffing with GC-O. The components are listed in order of their elution on the DB-Wax column. Limonene (84.0%) was the most abundant compound, followed by γ-terpinene (6.9%), myrcene (2.2%), α-pinene (1.2%), and linalool (1.0%). In a previous study, we reported 126 compounds from Hyuganatsu CPO. However, here we did not deal with some trace amount compounds, which were considered to be unimportant compounds with regard to characteristic Hyuganatsu flavor by chemical and sensory analyses. Monoterpene hydrocarbons were predominant in Hyuganatsu peel oil. The contents of *trans*-β-farnesene (0.7%), which was a principal sesquiterpene hydrocarbon, and *l*-carvone (0.4%), which was a principal ketone, have been shown to be higher in Hyuganatsu oil than in other citrus essential oils (*17*, *18*).

Among the oxygenated compounds, 13 aldehydes were identified; citronellal (0.3%) was the most abundant component. Ketones such as α- and β-thujones, menthone, *d*-camphor, *l*-carvone, isopiperitone, and β-ionone accounted for 0.5%. Twenty-two alcohols were identified in the oxygenated compound fraction of Hyuganatsu peel oil, and linalool (1.0%) and α-terpineol (0.1%) were the principal components. Thirteen ester components and 8 oxides were identified in Hyuganatsu peel oil.

GC-O and AEDA. The Hyuganatsu peel oil was snifg-tested by AEDA. The FD factor was expressed as a power of 3. An aromagram is shown in Figure 1B. As shown in Figure 1, the range of FD factors of each peak was between 1 and 8. Octanol showed the highest FD factor of 8, whereas β-cubebene, *l*-menthol, and isopiperitone had the lowest. Components having an FD factor of 7 were as follows: β-pinene, myrcene, limonene, terpinolene, citronellal, linalol, linalyl acetate, α-humulene, *l*-carvone, *trans*-nerolidol, *trans*,*trans*-farnesyl acetate, and *trans*,*trans*-farnesol. The odor-active volatiles (FD ≥ 5) in Hyuganatsu peel oil are given in Table 1. Ten odor compounds having Hyuganatsu-like aroma were detected by AEDA: limonene (peak 12), linalool (peak 36), octanol (peak 37), neral (peak 53), neryl acetate (peak 59), tridecanal (peak 69), *trans*-carveol (peak 74), *cis*-nerolidol (peak 80), *trans*,*trans*-farnesyl acetate (peak 94), and *trans*,*trans*-farnesol (peak 99) (Table 2). This result was also confirmed by analysis of an oxygenated fraction of Hyuganatsu peel oil. In addition to the Hyuganatsu-like odorant group, several components were perceived as having desirable fruity,

floral, sweet, and citrus-like odors (e.g., peaks 10, 16, 21, 22, 29, 38, 40, 41, 44, 56, 62, 65, and 73). Within this group, citronellal (peak 29) and linalyl acetate (peak 38) had the highest FD factor of 7. A great variety of odor qualities such as green, fruity, floral, herbaceous, or citrus-like were perceived by GC-O.

The FD factor does not always represent a significant contribution to aroma. We determined the relative flavor activity as a more realistic expression (*13*). As shown in Table 1, limonene was the most predominant component (0.206 g/kg of fresh wt) and its FD factor was as high as 7. However, its relative flavor activity was as low as 0.4. In addition to limonene, β-pinene, myrcene, terpinolene, citronellal, linalool, and *l*-carvone showed high FD factors, as high as 7, but they also showed low relative flavor activity of <10.0. The concentration of tridecanal in Hyuganatsu peel oil was very low (1.0 μg/kg of fresh wt), but its relative flavor activity was as high as 118.7 and its FD factor was 5. Occasionally, relative flavor activity had no direct relation to the aroma character of each compound. Thus, we performed the omission test of a synthetic aroma model of Hyuganatsu, which is an effective means of determining the characteristic or odor-active compounds of the aroma.

Omission Tests. Ten odor compounds were regarded as contributing to Hyuganatsu aroma (Table 2). The sensory evaluation for omission tests began with a search for a suitable medium for the aroma model. Ten odorants were added to the water bases (water I, II, and III) and 10% ethanol and 10% methanol solutions (Table 3). The flavor profiles of these models were compared with that of the Hyuganatsu CPO from the aspect of sweet, fruity, and green notes. There were no significant differences in sweet and green notes between the Hyuganatsu sample and aroma models comprising each base, but the fruity impression was much more intense in the genuine Hyuganatsu sample. As shown in Table 3, similarity to the Hyuganatsu sample was highest in the case of the aroma model with water as a base, especially water II. From this result the suitable formula for the aroma model was prepared from a stock solution with 50% ethanol, and then each component was added to 70 mL of water.

Changes in the overall flavor of the aroma model were evaluated by a sensory panel after omission of one or more odorants. The results of the omission tests are summarized in Table 4. In experiments 1 and 4, the model without *trans*-carveol, neral, and neryl acetate was more similar to the genuine Hyuganatsu sample than the Hyuganatsu aroma model. It is suggested that these compounds do not play a significant role in the Hyuganatsu original flavor. The omission of linalool in experiment 9 significantly affected the Hyuganatsu flavor. A comparison of experiments 8−10 indicated that linalool was the important factor of Hyuganatsu flavor. A similar behavior could be also seen in experiments 20 and 21. A comparison of experiments 1 and 17 indicated that octanol was also an important odorant in the Hyuganatsu aroma. In addition to this, the model without octanol (experiment 12) had a significantly lower intensity of Hyuganatsu flavor. The importance of octanol was also shown in experiments 13−16. If both octanol and linalool were missing (experiment 11), the difference from the complete model was outstanding. In the omission test, linalool and octanol were regarded as necessary and important components of the desirable aroma of Hyuganatsu fruit. This result coincided with those of the sniffing test by GC-O. Diluted solutions of linalool and octanol of ~2 ppm gave a fresh and fruity aroma note similar to Hyuganatsu flavor.

Table 4. Sensory Evaluation for the Aroma Model of the Hyuganatsu As Affected by the Absence of Compounds

expt no.	compound(s) omitted	hedonic scale*
	Hyuganatsu aroma model (prepared by water base)	$6.8^{a,b,c,d,e}$
1	*trans*-carveol, neral	7.9^{a}
2	*trans*-carveol	$6.8^{a,b,c,d,e}$
3	neral	$7.7^{a,b}$
4	*trans*-carveol, neral, neryl acetate	7.9^{a}
5	*trans*-carveol, neral, neryl acetate, *cis*-nerolidol	$6.8^{a,b,c,d,e}$
6	*cis*-nerolidol	$7.4^{a,b,c}$
7	tetradecanal	$7.3^{a,b,c,d}$
8	limonene	$7.4^{a,b,c}$
9	linalool	$5.9^{c,d,e}$
10	limonene, linalool	$5.8^{d,e}$
11	linalool, octanol	$5.5^{e,f}$
12	octanol	$6.2^{b,c,d,e}$
13	*trans*,*trans*-farnesyl acetate, *trans*,*trans*-farnesol	$7.7^{a,b}$
14	*trans*,*trans*-farnesyl acetate	$7.2^{a,b,c,d}$
15	*trans*,*trans*-farnesol	$6.5^{a,b,c,d,e}$
16	*trans*,*trans*-farnesyl acetate, *trans*,*trans*-farnesol, octanol	$5.5^{e,f}$
17	*trans*-carveol, neral, octanol	$6.3^{b,c,d,e}$
18	*trans*-carveol, neral, octanol, limonene	$6.2^{b,c,d,e}$
19	*trans*-carveol, neral, *trans*,*trans*-farnesyl acetate, octanol	$5.9^{c,d,e}$
20	*trans*-carveol, neral, neryl acetate, *cis*-nerolidol, tetradecanal, linalool, *trans*,*trans*-farnesyl acetate, *trans*,*trans*-farnesol	3.7^{g}
21	*trans*-carveol, neryl acetate, tetradecanal, linalool, *trans*,*trans*-farnesyl acetate, *trans*,*trans*-farnesol	$4.1^{f,g}$

*The hedonic scale system was scored with a scale of 1 (extremely different from Hyuganatsu) to 9 (extremely similar to Hyuganatsu). $^{a-g}$Significant flavor difference ($p < 0.05$) between the complete and the omitted models.

From these experiments it is concluded that comprehensive evaluation of the flavor should be accomplished by simultaneous chemical and sensory analyses. FD factor or relative flavor activity may be useful means for reconstruction of an original aroma. The use of relative flavor activity is not for the determination of characteristic flavor components but for the consideration of relative contribution in flavor activity. The sniffing test including the omission test is an effective means of determining the characteristic or odor-active compounds of an aroma. In the present study, linalool and octanol are regarded as odor-active compounds of Hyuganatsu flavor.

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