Cluster Analysis of GC Data on Oxygenated Terpenes of Young Leaf and Green Fruit Samples of Japanese Pepper (*Xanthoxyum piperitum* DC.)

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Cluster analysis was applied to the GC data on oxygenated terpenes of various samples of young leaves and green fruits of Japanese pepper (*Xanthoxyum piperitum* DC.) to select superior cultivars rich in flavoring potency. Of 74 leaf samples, six samples were rich in citronellal, linalool, isopulegol, piperitone, geranyl acetate, citronellol, and geraniol. Of 71 fruit samples, three samples were rich in isopulegol, piperitone, geranyl acetate, methoxycitronellal, and geraniol, and nine samples were rich in citronellal, linalool, citronellyl acetate, γ-terpineol, and citronellol. Similarity in the clusterings of the leaf and fruit samples were not observed. Of 78 plants, only one plant gave young leaves and green fruits rich in citronellal, linalool, isopulegol, geranyl acetate, and citronellol.

Keywords: *Japanese pepper; volatile compounds; cluster analysis*

INTRODUCTION

Japanese pepper (*Xanthoxyum piperitum* DC.), belonging to the *Rutaceae* family, is a native deciduous plant in valleys and fields of the Korean peninsula and Japan Islands. Its green fruits and young leaves are added to various foods and pickles to impart green and fresh notes or to suppress unpleasant fishy or meaty odors. Furthermore, ripe and dry fruits have been used as a spice or medicinal material.

A cultivar without a thorn, Asakura Sanshou (var. inerme *Makino*) (Makino, 1982), and other native varieties are planted in Japan. It is important from a horticultural aspect to select superior cultivars. Combining GC analysis of volatile flavor compounds and multivariate analysis is a useful technique for evaluation of flavor quality and classification of samples.

There are reports concerning wine discrimination by headspace volatiles (Shimoda et al., 1993), objective aroma evaluation of various trade varieties of coffee (Wada et al., 1987), assignment of avocado fruits to horticultural races (King and Knight, 1992), and classification of commercial orange juice by volatile constituents (Shaw et al., 1993), etc.

As reported in our previous paper concerning Japanese pepper (Wu et al., 1996), aroma concentrates from 71 samples of green fruits and 74 samples of young leaves were quantitatively analyzed by GC, and the volatile compounds were identified by GC−MS. Deviations in the concentrations of total volatiles and almost all of the volatile components were larger than 100% as a relative standard deviation.

In this paper, cluster analysis was applied to gas chromatographic data on young leaves and green fruits of Japanese pepper to select superior cultivars rich in flavoring potency.

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MATERIALS AND METHODS

Sample. Young leaves and green fruits, each 10 g/plant, were harvested in almost the same stage of ripening from 76 native plants and two cultivated plants (Asakura Sanshou) in Fukuoka prefecture from May 17 to May 20 and from June 9 to July 3 in 1993, respectively. The leaves and fruits were immersed in purified methanol just after they were harvested and were stored at −20 °C until analyzed. The samples were numbered randomly, and some failed the analyses.

Isolation of Volatile Compounds. Young leaves (1.0 g) and green fruits (2.0 g) were separately homogenized in 40 mL of methanol using a high-speed blender, and 1.0% cyclohexanol aqueous solution was added as an internal standard to both homogenates, 250 mg/kg, with respect to their fresh weights. Deionized water, 200 mL, was added to the filtrate of the homogenate. The methanolic solution was passed through a column (2 cm i.d. × 10 cm) packed with porous polymer beads (Porapak Q), and then the column was washed with 15 mL of deionized water to remove water soluble constituents. After adsorbed volatiles were eluted with 60 mL of diethyl ether, the eluate was dried over anhydrous sodium sulfate and then concentrated to about 150 μL (Wu et al., 1996).

Analysis of Volatile Compounds. Capillary GC analysis was carried out on a Shimadzu GC 14A gas chromatograph equipped with a flame ionization detector (FID) and connected to a Shimadzu Chromatopak C-R5A integrator. Separation was achieved on a 60 m × 0.25 mm i.d. fused silica capillary column coated with cross-linked polyethylene glycol 20M (PEG 20M), film thickness 0.25 μm (DB-Wax; J&W Scientific, Folsom, CA). The oven temperature was programmed from 60 to 230 °C at 3 °C/min. The injector and detector temperatures were 200 and 250 °C, respectively. The helium carrier gas flow rate was 23 cm/s with an injection splitter at a split ratio of 30:1.

Electron impact mass spectrometric data were collected on a JEOL Automass 50 mass spectrometer interfaced to a Hewlett-Packard 5890 series II gas chromatograph. The column and chromatographic conditions were the same as described for GC analysis. Identification of volatile compounds was described in previous paper (Wu et al., 1996).

Statistical Analysis. Deviation was assessed by a calculation of the standard deviation for quantitative value of each peak using Microsoft Excel (Microsoft Corp., Redmond, WA). Cluster analysis was performed on the quantitative values of oxygenated terpenes of 74 samples on young leaves and of 71 samples on green fruits using SPSS for Windows Release 6.0

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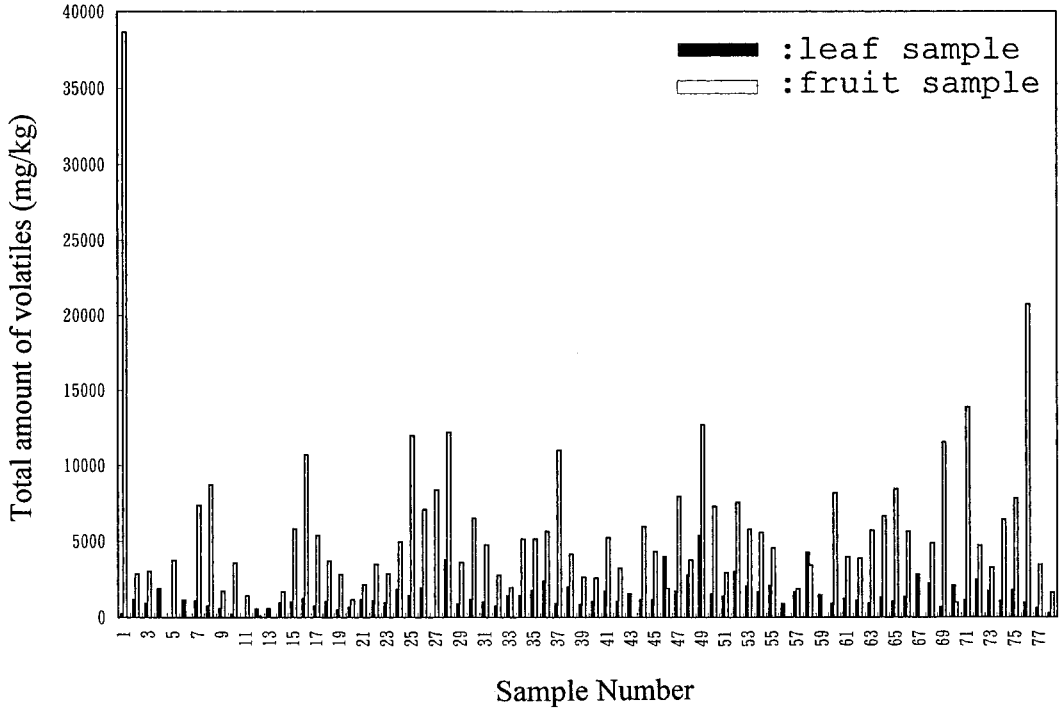

Figure 1. Distributions of total amounts of volatiles in leaf and fruit samples of Japanese papper. The numbers on the abscissa indicate sample number.

Table 1. Composition of Oxygenated Terpenes in Young Leaves of Japanese Pepper Grouped by Cluster Analysis

		cluster[a]			
compounds	total sample	1[b]	2[c]	3[d]	4[e]
citronellal	44 ± 80[e]	8.3[j] ± 4.1[f]	48 ± 26[g]	47 ± 25[h]	330[k] ± 155[i]
linalool	6.0 ± 9.5	0.70 ± 0.99	4.4 ± 6.2	1.6 ± 2.2	13 ± 6.0
linalyl acetate	2.6 ± 5.1	1.6 ± 0.30	2.2 ± 3.1	3.3 ± 4.7	2.6 ± 3.7
isopulegol	100 ± 62	73 ± 44	59[j] ± 16	165 ± 56	142[k] ± 11
neral	10 ± 7.7	7.9 ± 4.3	7.0[j] ± 0.95	18 ± 6.5	15[k] ± 0.71
geranial	37 ± 34	12[j] ± 10	41 ± 41	67 ± 68	45[k] ± 6.3
α-terpineol	74 ± 53	14[j] ± 11	184 ± 135	88[k] ± 1.1	155[l] ± 56
piperitone	8.9 ± 7.3	1.0[j] ± 1.4	7.3 ± 10	12[k] ± 1.8	22[l] ± 0.57
geranyl acetate	18 ± 28	3.8[j] ± 0.32	1.3[j] ± 1.8	27[k] ± 11	97 ± 72
citronellol	18 ± 35	8.1[j] ± 9.6	88 ± 124	3.6[j] ± 5.1	126[k] ± 56
geraniol	5.7 ± 14	5.1 ± 7.1	31 ± 43	5.0 ± 7.1	35 ± 20

[a] Values represent the average concentrations (mg/kg) ± standard deviations (mg/kg). Means with different roman superscript letters (e−l) in the same row are significantly different at $p < 0.05$. [b] Cluster 1 was composed of samples 1, 6, 8−10, 13, 19−21, 23, 27, 28, 31, 33, 36, 37, 39, 51, 56, 70, 72, 76, and 78. [c] Cluster 2 was composed of samples 4, 7, 12, 14−18, 22, 26, 30, 35, 38, 40, 45, 46, 53, 57, 58, 60, 65, and 77. [d] Cluster 3 was composed of samples 2, 3, 24, 25, 29, 32, 34, 41, 42, 44, 47, 48, 50, 52, 59, 61, 62, 66, 67, 71, and 73−75. [e] Cluster 4 was composed of samples 43, 49, 54, 55, 68, and 69. [f] Standard deviation ($n = 74$). [g] Standard deviation ($n = 23$). [h] Standard deviation ($n = 22$). [i] Standard deviation ($n = 23$). [j] Standard deviation ($n = 6$).

statistical package for a personal computer system (SPSS Inc., Chicago, IL). The cluster analysis was carried out by Ward's clustering method in a measure of Euclidean distance.

RESULTS AND DISCUSSION

Total amounts of 25 volatile compounds in young leaves and of 43 volatile compounds in green fruits are shown in Figure 1. Wide variations from 200 to 5200 ppm for young leaves and from 100 to 39 000 ppm for green fruits were observed. The correlation coefficient between the total amounts on the leaf and fruit samples was −0.0076. The amount of terpene hydrocarbons comprised 45%−92% of the total amounts of volatiles for leaf samples and 37%−96% for fruit samples. The average percentages of *β*-phellandrene and *d*-limonene, major components of the hydrocarbons, were 33% and 14% for leaf samples and 42% and 23% for fruit samples, respectively (Wu et al., 1996).

On oxygenated terpenes of young leaves, average concentrations and standard deviations are listed in the second column of Table 1. Deviations in the concentrations of citronellal, linalool, linalyl acetate, geranyl acetate, citronellol, and geraniol were larger than 100% as a relative standard deviation. In a similar manner, average concentrations and standard deviations on green fruits are listed in Table 2. Deviations in their concentrations were much larger than 100% except for isopulegol. Figure 2 shows the distributions of citronellal in young leaves and green fruits, which had been reported to be the main contributor to the odor of Japanese pepper (Sakai et al., 1968; Kusumoto et al., 1968). The distribution ranged from 0 to 440 ppm on young leaves and from 0 to 1200 ppm on green fruits. The correlation coefficient between the concentrations in the leaf and fruit samples was 0.242. Large deviations in total amounts of volatiles and concentrations of individual compounds indicated that the odor attributes of each leaf and fruit sample could be different from another.

In order to obtain typical flavor patterns of young leaves of Japanese pepper, multivariate analysis was applied. However, the analyses on all volatiles except for minor components did not give significant results. Therefore, cluster analysis was done on the basis of the

Table 2. Composition of Oxygenated Terpenes in Green Fruits of Japanese Pepper Grouped by Cluster Analysis

compounds	total sample	cluster[a] 1[b]	2[c]	3[d]	4[e]
citronellal	44 ± 79[e]	28 ± 66[f]	81 ± 97[g]	115 ± 146[h]	227 ± 476[i]
2-methyl-6-methylene-1,7-octadien-3-one	2.2 ± 6.1	2.5 ± 6.9	0.3 ± 1.2	2.4 ± 2.1	4.1 ± 6.7
linalool	52 ± 140	21 ± 28	64 ± 50	158 ± 219	231 ± 420
linalyl acetate	19 ± 23	18 ± 26	18 ± 17	27 ± 26	21 ± 18
methyl citronellate	20 ± 23	17 ± 20	24 ± 23	60 ± 46	25 ± 18
isopulegol	48 ± 37	40 ± 24	44 ± 31	143 ± 95	76 ± 27
terpinen-4-ol	5.0 ± 6.1	4.8 ± 6.5	4.1 ± 3.1	7.4 ± 2.1	6.6 ± 9.2
(*E*)-2-decenal	2.4 ± 3.8	2.5 ± 4.3	2.4 ± 2.7	1.6 ± 2.8	2.2 ± 3.3
citronellyl acetate	18 ± 22	14 ± 21	27 ± 21	31 ± 23	53 ± 83
neral	14 ± 36	16 ± 43	8.3 ± 4.8	20 ± 18	18 ± 35
geranial	9.3 ± 14	9.2 ± 15	5.8 ± 6.8	19 ± 34	11 ± 14
γ-terpineol	34 ± 68	35 ± 75	20 ± 27	45 ± 77	87 ± 105
α-terpineol	70 ± 77	73 ± 79	70 ± 86	32 ± 35	76 ± 75
(*E*,*E*)-farnesal	2.5 ± 6.3	1.9 ± 5.9	4.0 ± 8.2	2.4 ± 4.1	2.0 ± 5.0
piperitone	13 ± 17	12 ± 18	9.9 ± 8.6	35 ± 31	12 ± 18
geranyl acetate	320 ± 540	75[j] ± 71	375[j] ± 97	2360[k] ± 732	1009[k] ± 262
citronellol	24 ± 55	16[j] ± 32	34[j] ± 33	0[k]	72[j] ± 162
methoxycitronellal	8.2 ± 14	4.1 ± 5.5	16 ± 14	34 ± 48	6.4 ± 10
methoxycitronellal	6.1 ± 9.3	4.8 ± 8.5	8.9 ± 7.8	19 ± 23	1.2 ± 1.8
geraniol	72 ± 110	19 ± 21	140 ± 118	272 ± 177	252 ± 214
carveol	7.9 ± 36	10 ± 44	3.4 ± 3.4	4.8 ± 5.9	2.5 ± 2.4

[a] Values represent the average concentrations (mg/kg) ± standard deviations (mg/kg). Means with different rom superscript letters (e−k) in the same row are significantly different at $p < 0.05$. [b] Cluster 1 was composed of samples 3, 7−12, 15−19, 21−24, 29, 30, 32−35, 39, 40, 42, 44, 46, 48, 49, 51, 52, 57, 58, 61−66, 69, 70, 72, 73, and 75−78. [c] Cluster 2 was composed of samples 2, 14, 20, 26, 31, 36, 38, 41, 45, 53, 55, and 68. [d] Cluster 3 was composed of samples 25, 50, and 54. [e] Cluster 4 was composed of samples 1, 5, 27, 28, 37, 47, 60, 71, and 74. [f] Standard deviation ($n = 71$). [g] Standard deviation ($n = 47$). [h] Standard deviation ($n = 12$). [i] Standard deviation ($n = 3$). [j] Standard deviation ($n = 9$).

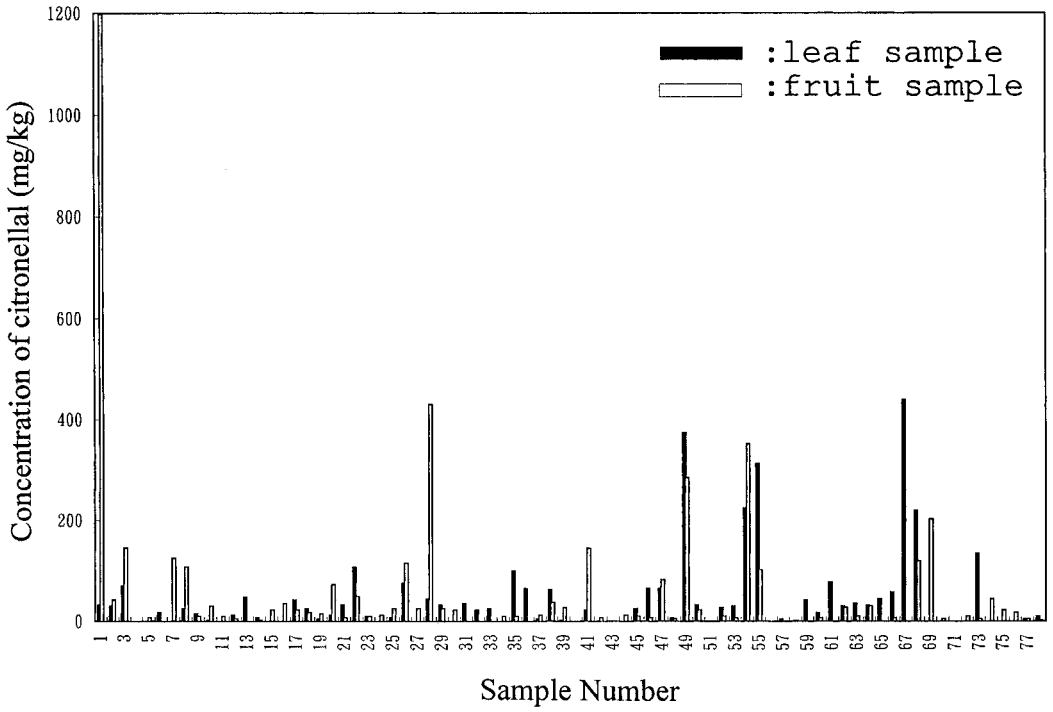

Figure 2. Distributions of the concentrations of citronellal in leaf and fruit samples of Japanese pepper. The numbers on the abscissa indicate sample number.

concentrations of 11 oxygenated terpenes listed in Table 1. These oxygenated terpenes are considered to have larger sensorial contribution than terpene hydrocarbons. The dendrogram obtained could classify 74 samples into four clusters, and the average value and standard deviation of each volatile compound in the samples combined into a cluster are listed in Table 1. Significant differences in concentrations of volatiles between clusters were tested by Welch's method because the deviations were significantly different between the clusters. Cluster 1, which consisted of 23 samples, had the lowest average concentrations of citronellal, linalool, linalyl acetate, geranial, α-terpineol, and piperitone. These samples were considered to be poor in aroma intensity because of the low concentrations of all components in Table 1. In the samples of cluster 2, the average concentrations of isopulegol, neral, and geranyl acetate were the lowest, but α-terpineol was included in the highest level. The samples of this cluster seemed to be rich in the fresh rose-like odor of citronellol and geraniol (Arctander, 1969a) and the floral and sweet odor of α-terpineol (Arctander, 1969b). In cluster 3, linalyl acetate, isopulegol, neral, and geranial were included in the highest levels. On the other hand, the concentrations of citronellol and geraniol were the lowest among four clusters. The samples of this cluster could have

Table 3. Compositions of Oxygenated Terpenes in the Young Leaves and Green Fruits Harvested from Selected Plant (No. 54)[a]

compounds	concn (mg/kg) in leaves	concn (mg/kg) in fruits
citronellal	353	225
2-methyl-6-methylene-1,7-octadien-3-one	4.1	
linalool	137	50
linalyl acetate	72	40
methyl citronellate	63	
isopulegol	35	117
terpinen-4-ol	7.6	
(*E*)-2-decenal	2.3	
citronellyl acetate	78	
neral	13	13
geranial	10	tr[b]
γ-terpineol	15	
α-terpineol	50	105
(*E*,*E*)-farnesal	tr	
piperitone	30	15
geranyl acetate	375	20
citronellol	88	35
methoxycitronellal	43	
methoxycitronellal	38	
geraniol	265	7.5
carveol	5	

[a] The plant (no. 54) gave young leaves and green fruits rich in oxygenated terpenes. [b] tr represents concentration less than 1 mg/kg.

minty and fragrant odors of isopulegol and lemon-like odors of citral (neral + geranial) (Arctander, 1969c). In cluster 4, citronellal and citronellol together with linalool, piperitone, geranyl acetate, and geraniol were included in the highest levels. The volatile composition of cluster 4 indicated that samples of this cluster could have an intense Japanese pepper-like odor.

Two leaf samples of Asakura Sanshou belonged to clusters 2 and 3 and were poor in the concentrations of volatile flavor compounds.

Table 2 lists the four flavor patterns of green fruits which were obtained by the same cluster analysis as young leaves. Significant differences ($p < 0.05$) in concentrations of volatiles between clusters were observed only in two compounds, though for $p < 0.1$ or $p < 0.2$ the differences were observed in many compounds. The samples of cluster 1 had the lowest average concentrations in citronellal, linalool, linalyl acetate, methyl citronellate, isopulegol, citronellyl acetate, (*E*,*E*)-farnesal, geranyl acetate, methoxycitronellal, and geraniol, but (*E*)-2-decenal and carveol were included in the highest levels among four clusters. The samples of cluster 1, which was the largest cluster consisting of 47 samples, were considered to be poor in aroma intensity and to be inferior in odor quality. The samples of cluster 2 showed an average pattern except for a few compounds. Thus, the average concentrations of 2-methyl-6-methylene-1,7-octadien-3-one, linalyl acetate, terpinen-4-ol, neral, geranial, γ-terpineol, and piperitone were the lowest, but that of (*E*,*E*)-farnesal was the highest. In cluster 3, linalyl acetate, methyl citronellate, isopulegol, terpinen-4-ol, neral, geranial, piperitone, geranyl acetate, two isomers of methoxycitronellal, and geraniol were included in the highest levels. Citronellal and linalool were also included in relatively high levels. On the other hand, the concentrations of (*E*)-2-decenal, α-terpineol, and citronellol were the lowest among four clusters. It was noteworthy that citronellol could not be detected in the samples of cluster 3. These samples were considered to be rich in fresh fruit and citrus like odors because they were abundant in citronellal and linalool with citrus odor (Arctander, 1969d), citral (neral + geranial) with typical lemon-like odor, and geranyl acetate with fruity and rose-like odor (Arctander, 1969e). On the other hand, piperitone, which had a spicy odor, was included in the highest level. This might be a significant component which contributes to the spicy odor of Japanese pepper. In cluster 4, citronellal, 2-methyl-6-methylene-1,7-octadien-3-one, linalool, citronellyl acetate, γ-terpineol, α-terpineol, and citronellol were included in the highest levels. The volatile composition of cluster 4 indicated that samples of this cluster could have a typical odor attribute of Japanese pepper that was different from the samples of cluster 3.

Two fruit samples of Asakura Sanshou belonged to cluster 1 and were poor in volatile flavor compounds as well as their leaf samples.

Any compositional correlations between the volatile compounds on the leaf and fruit samples harvested from the same plant were not observed. For example, of 12 plants of green fruit clusters 3 and 4, four plants were classified into cluster 1 of the leaf sample, one plant was classified into cluster 2, five plants were classified into cluster 3, and one plant was classified into cluster 4.

Of the 78 plants analyzed in present paper, only one plant (No. 54) gave young leaves and green fruits rich in oxygenated terpenes such as citronellal, linalool, isopulegol, geranyl acetate, and citronellol (Table 3).

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