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Characteristic Odor Components of *Citrus sphaerocarpa* Tanaka (Kabosu) Cold-Pressed Peel Oil

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The volatile components of *Citrus sphaerocarpa* Tanaka (Kabosu) cold-pressed peel oil were investigated by chemical and sensory analyses. Monoterpene hydrocarbons (more than 94.6%) were predominant in Kabosu peel oil, with limonene and myrcene accounting for the major proportions (70.5% and 20.2%, respectively). The Kabosu oxygenated fraction was characterized by quantitative abundance in aldehydes and a relatively wide variety of alcohols. The weight percentages of aldehydes, alcohols, and esters in Kabosu cold-pressed oil were 1.3%, 0.1%, and 0.1%, respectively. Aroma extract dilution analysis was employed for determination of the odors of Kabosu volatile components, flavor dilution factors, and relative flavor activities. Gas chromatography/olfactometry using Kabosu cold-pressed oil and its oxygenated fraction completed by a chiral analysis revealed that (*R*)-(+)-citronellal is a characteristic element of Kabosu peel oil odor. Careful sniff testing demonstrated that aqueous solutions of both 0.25% and 0.016% (*R*)-(+)-citronellal gave an odor similar to that of Kabosu.

KEYWORDS: ***Citrus sphaerocarpa*; Tanaka Kabosu; citrus essential oil; gas chromatography/olfactometry; aroma extraction dilution analysis; characteristic odor components; (*R*)-(+)-citronellal**

INTRODUCTION

Citrus sphaerocarpa Hort. ex Tanaka, called Kabosu in Japan, is now one of the most popular sour citrus fruits in Japan. It is grown in Oita Prefecture on Kyushu Island, southern Japan. The fruit has an attractive and pleasant flavor, which has culinary uses in salad dressings and seasonings, especially in grilled seafood and hotpots. The taste and aroma of Kabosu have been commercially applied to liquor, jelly, and cosmetics.

The composition of Kabosu peel essential oil has been investigated by many researchers. Sixty-five of 70 separated compounds were identified by Njoroge et al. (*1*). Kabosu essential oil and its oxygenated compound fraction were studied by Inataba (*2*). An overview on the composition of Japanese spices including Kabosu has also been reported (*3*). Cluster analysis of Kabosu and other citrus peel essential oils has been investigated in terms of chemotaxonomy (*4*, *5*). Sawamura et al. also reported the inhibitory effects of Kabosu oil and many other kinds of citrus essential oils on the formation of *N*-dimethylnitrosamine, which has been recognized as a carcinogen (*6*). The content of auraptene, a coumarin, in Kabosu peel was determined as well (*7*). Despite the volume of these studies, there still remains a question about key aroma compounds. Although citrus fruit can be distinguished from other kinds of fruits by the characteristic "citrus-like" odor, each citrus fruit clearly differs in cultivars or species according to its characteristic aroma attribute. This points to the necessity of determining the active odor components that contribute most to such attributes.

Aroma extract dilution analysis (AEDA) is a useful application of gas chromatography/olfactometry (GC/O) analysis (*8*), which provides a good first approximation of the chemical nature of an aroma (*9*). It has been used for the detection and identification of potent odorants in various food systems (*9*−*14*), including many kinds of essential oils (*8*, *15*−*17*), as well for the quantification of the sensory activity of chemicals (*18*).

In this study, the main goal was to evaluate the most important odor compounds of Kabosu peel essential oil. AEDA was chosen to obtain a hierarchical list of odorants of Kabosu oil, complemented by a sensory study of the most odor-characteristic compound detected.

MATERIALS AND METHODS

Materials. Kabosu fruit samples, fresh and of the best commercial quality, were obtained in October 2000 from Oita Midori Agriculture Cooperative, Takeda City, Oita Prefecture. The peel oil was extracted by hand pressing the flavedo and was collected in a brine solution on ice in the same manner as previously reported (*19*). The essential oil was stored at −25 °C until analyzed. Standard chemical compounds

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purchased from Wako Pure Chemical Industries (Osaka), Aldrich Chemical Co. (Milwaukee, WI), and Fluka Fine Chemicals (Switzerland), were used for identification of the oil components and for chiral analysis and sensory studies of the most characteristic compound. Smelling-strips were provided by Nagaoka Perfumery Co., Ltd., Osaka.

Silica Gel Column Chromatography. Kabosu essential oil (about 3 g) was fractionated into hydrocarbon and oxygenated compound fractions by silica gel column chromatography (25 cm × 2 cm i.d.) on Wako gel Q-23 (*20*). The hydrocarbon and oxygenated compounds were eluted with *n*-hexane and diethyl ether, respectively. Each fraction was carefully concentrated under reduced pressure at room temperature.

GC and GC/MS. A Shimadzu gas chromatograph GC-14A equipped with a DB-Wax column (60 m × 0.25 mm i.d., film thickness of 0.25 μm; J & W Scientific, Folsom, CA) and a flame ionization detector (FID) were 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 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) were used as internal standards (*19*). The weight percent of each peak was calculated according to the correlation factor to FID (*8*, *16*). An oil sample of 1 μL was injected, with the split ratio of the injector being 1:50.

A Shimadzu GC-17A coupled with a Shimadzu QP-5000 was used for GC−MS analysis. The GC condition was the same as that described above. The MS condition was as follows: ionization voltage, 70 eV; ion source temperature, 250 °C. Individual components were identified by comparison of both mass spectra and their GC retention times with those of authentic compounds previously analyzed and stored in the data system, and also by peak enrichment on co-injection with authentic standards wherever possible. The retention indices were also determined for all constituents by using a homologous series of *n*-alkanes (C_7−C_{27}).

GC/Olfactometry (GC/O). A Shimadzu GC-14A gas chromatograph equipped with a DB-Wax fused silica capillary column (60 m × 0.53 mm i. d.; film thickness of 1 μm; J & W Scientific) and an FID was used. The column oven and other operating conditions were the same as those given above for the GC-14A. The flow rate of nitrogen carrier gas was 9 mL/min and the split ratio was 1:5. An oil sample of 0.5 μL was injected. At the exit of the capillary column, the effluent was split into the FID and a sniffing port. Humid air was constantly added to the effluent at the sniffing port.

AEDA. The cold-pressed Kabosu oil was stepwise diluted 3-fold with acetone until the sniffers could not detect any significant odor in a run (*10*, *21*). Analyses were performed by 4 trained assessors in duplicates. A description in addition to the FD factor was assigned only if the odorant was detected for at least 5 out of 8 GC/O evaluations for a particular dilution test. The highest dilution at which an individual component could be detected was defined as the flavor dilution (FD) factor for that odorant. On the basis of the AEDA results, relative flavor activity (RFA) was calculated for each detected odorant, using the following equation: $RFA = \log_3 3^n/S^{0.5}$, where 3^n is the FD factor and *S* is the weight % of a component (*16*).

Chiral GC Analysis. A Shimadzu GC-14A equipped with a simple on-line coupled DB-5 fused silica capillary column (30 m × 0.25 mm i.d., film thickness of 0.25 μm; J & W Scientific), and a main column consisting of a (2,3-di-*O*-acetyl-6-*tert*-butyldimethylsilyl)-β-CD stationary phase (30 m × 0.25 mm i.d., film thickness of 0.25 μm; Supelco, Bellefonte, PA) was used for enantioselective GC analysis (*22*, *23*). The column temperature was programmed from 70 °C (2 min) to 100 °C (60 min) at 2 °C/min. The injector and detector temperatures, the carrier gas, the amount of sample injected, and the split ratio were the same as described above for the GC-14A.

RESULTS AND DISCUSSION

Volatile Components of Kabosu Peel Oil. As shown in **Table 1**, 67 compounds were identified and quantified in the Kabosu peel oil. For GC and GC/MS analysis, the cold-pressed oil was used. As recommended for an undiluted or concentrated sample (*24*), as well as in this experiment's condition, injection of 1 μL at the split ratio of 1:50 gave the most favorable result. The components are listed in order of their elution on the DB-Wax column. In agreement with earlier reports (*1*, *3*), the monoterpene hydrocarbons (more than 94.6%) were found to make up of most of the oil, with limonene (70.5%) and myrcene (20.2%) accounting for the major proportions; while β-farnesene was the most abundant sesquiterpene hydrocarbon, accounting for about 33.3% of the total sesquiterpene. Among the oxygenated compounds, aldehydes including octanal and decanal were relatively rich in the peel oil. Linalool was principal among the fourteen alcohols identified. Neryl acetate was the major ester. One ketone, carvone, and 3 oxides were detected in Kabosu peel oil. The percentages by weight of aldehydes, alcohols, esters, ketones, and oxides were 1.3%, 0.1%, 0.1%, 0.005%, and 0.005%, respectively.

GC/O and AEDA. Sniff analysis of Kabosu oil was carried out using AEDA. Regarding the wider bore column (0.53 mm) used in GC/O than that (0.25 mm) used in GC, an oil sample of 0.5 μL was injected, at which peaks' separation was checked in comparing to that of GC. A slight overlap between limonene (peak **10**) and β-phellandrene (peak **11**) occurred when the original cold-pressed oil was used, however their odor difference was detectable. As from the first diluted oil sample they were fully separated. The highest dilution of individual components detected by assessors was defined as its FD factor. The FD factor was expressed as a power of 3. **Table 2** shows the odor description of volatile components of Kabosu peel oil as detected at the sniffing port. An aromagram and a gas chromatogram are shown in **Figure 1**. The range of the FD factor of each peak was between 4 and 9. The following components had an FD factor greater than 7: limonene and myrcene as monoterpenes; β-caryophyllene and α-caryophyllene as sesquiterpenes; nonanal as an aldehyde; and neryl acetate as an ester. Among these, limonene and myrcene were the most odor-active. Although both limonene and myrcene were abundant, they are of minor importance in Kabosu odor, because the FD factor often depends on the concentration of the component. Recently, the concept of the RFA has been employed in a wide range of flavor investigations (*8*, *15*−*17*). The compounds of high FD factors do not always present high RFA values (less than 30), as shown in **Table 2**.

As previously reported, citronellol, (*E*)-2-decenal, and geranyl acetate were the characteristic oxygenated compounds of Kabosu (*3*). However, (*E*)-2-decenal was not detected in our study, whereas octyl acetate (peak **22**), citronellal (peak **23**), linalyl acetate (peak **28**), and neral (peak **39**) were noted because they were described as having Kabosu-like odor at the GC sniffing port (**Table 2**). Their power of 3 FD factors and RFAs were 5 and 37.3; 4 and 10.4; 4 and 16.8; and 5 and 17.6, respectively.

Analysis of Oxygenated Fractions of Kabosu Cold-Pressed Oil. Citrus oils are characterized by a high percentage of terpenes (*25*). Oxygenated compounds comprising alcohols, aldehydes, ketones, acids, and esters occur in relatively small amounts, but they are responsible for the characteristic odor or flavor profiles of citrus fruits (*25*). When the hydrocarbon fraction has been removed from a citrus oil, the oxygenated fraction will be concentrated to become more odorous. The results from the analysis of Kabosu cold-pressed oil showed that all 4 compounds judged as being Kabosu-like odors belonged to the oxygenated group. Analysis of the Kabosu oxygenated fraction led to a focus on those compounds and confirmed the importance of their contribution to the odor.

Forty-seven compounds were identified in the Kabosu oxygenated fraction: 12 aldehydes, 19 alcohols, 11 esters, 1

Table 1. Volatile Components of Kabosu (*Citrus sphaerocarpa* Tanaka) Peel Oil

peak	compound	retention index DB-Wax	w/w (%)[a]	identification[b]	reference[c]
1	ethyl acetate	889	*	RI	*1, 2*
2	α-pinene	1025	0.7	RI, MS	*1, 2*
3	camphene	1071	*	RI, Co-GC	*1, 2*
4	undecane	1096	*	RI, MS, Co-GC	
5	β-pinene	1115	0.2	RI, MS	*1, 2*
6	sabinene	1123	0.1	RI, MS	*1, 2*
7	myrcene	1162	20.2	RI, MS	*1, 2*
8	α-phellandrene	1169	**	RI, MS	*1, 2*
9	α-terpinene	1183	0.1	RI, MS	*1, 2*
10	limonene	1215	70.5	RI, MS	*1, 2*
11	β-phellandrene	1225	nq	RI, MS	*1*
12	(*Z*)-β-ocimene	1230	**	RI	*1, 2*
13	γ-terpinene	1250	2.6	RI, MS	*1, 2*
14	*p*-cymene	1271	0.1	RI, MS	*1, 2*
15	terpinolene	1284	0.1	RI, MS	*1, 2*
16	octanal	1288	0.7	RI, MS	*1, 2*
17	tetradecane	1299	*	RI, Co-GC	*1*
18	nonanal	1391	0.1	RI, Co-GC	*1, 2*
19	(*Z*)-limonene oxide	1435	**	RI	*1, 2*
20	(*E*)-limonene oxide	1458	*	RI	*1, 2*
21	(*E*)-linalool oxide	1464	**	RI	*1*
22	octyl acetate	1470	*	RI, Co-GC	*1, 2*
23	citronellal	1476	**	RI, Co-GC	*1, 2*
24	α-copaene	1490	nq	RI, MS	*1, 2*
25	decanal	1493	0.5	RI, MS	*1, 2*
26	β-cubebene	1539	0.1	RI, MS, Co-GC	*1*
27	linalool	1542	0.1	RI, MS, Co-GC	*1*
28	linalyl acetate	1551	**	RI, Co-GC	*1*
29	octanol	1555	*	RI, MS, Co-GC	*2*
30	longifolene	1576	*	RI	*1*
31	β-elemene	1585	**	RI	*1*
32	β-caryophyllene	1595	0.2	RI, MS	*1, 2*
33	undecanal	1597	nq	RI, MS	*1, 2*
34	thujyl alcohol	1638	*	RI	*1*
35	citronellyl acetate	1652	*	RI, MS	*1, 2*
36	β-farnesene[d]	1655	0.4	RI, MS	*1, 2*
37	α-caryophyllene	1666	**	RI	*1, 2*
38	decyl acetate	1671	**	RI, Co-GC	*1, 2*
39	neral	1675	**	RI, MS	*1, 2*
40	geranyl formate	1681	**	RI, Co-GC	*1*
41	α-terpineol	1690	**	RI, MS	*1, 2*
42	dodecanal	1699	nq	RI, MS	*1, 2*
43	germacrene-D	1702	0.2	RI	*1, 2*
44	valencene	1707	*	RI	*1*
45	neryl acetate	1714	0.1	RI, Co-GC	*1, 2*
46	α-muurolene	1724	nq	RI	*1*
47	bicyclogermacrene	1728	0.1	RI	*1*
48	carvone	1734	**	RI	*1*
49	geranyl acetate	1745	nq	RI, Co-GC	*1, 2*
50	δ-cadinene	1749	0.2	RI	*1, 2*
51	citronellol	1757	nq	RI, MS	*1, 2*
52	sesquiphellandrene	1760	**	RI	*1*
53	cumin aldehyde	1776	*	RI	*1*
54	perillaldehyde	1780	**	RI, Co-GC	*1, 2*
55	octadecane	1791	0.1	RI, Co-GC	*1*
56	tridecanal	1805	*	RI, MS	*1, 2*
57	(*Z*)-carveol	1819	**	RI	*1, 2*
58	nerol	1837	*	RI, Co-GC	*1, 2*
59	geraniol	1845	*	RI, Co-GC	*1*
60	unknown	1888	*		
61	tetradecanal	1935	**	RI, MS	*1, 2*
62	perillyl alcohol	1985	**	RI, MS	*1, 2*
63	nerolidol	2025	**	RI, MS	*1, 2*
64	β-elemol	2070	**	RI	*1, 2*
65	cedrol	2097	*	RI	*1*
66	eugenol	2178	**	RI, Co-GC	*1, 2*
67	myrcene dimer	2237	**	RI	*1*
68	isoeugenol	2255	*	RI	*1*

[a] *: Peak area detected less than 0.005%. **: Peak area detected not less than 0.005% and less than 0.05%. nq: not quantified. [b] RI: Identification based on retention index. MS: Identification based on comparison of mass spectra. Co-GC: Identification based on co-injection with authentic standards. [c] Reference number where identified earlier. [d] Correct isomer not identified.

ketone, and 3 oxides. Among them, hexanol, 3-hexenol, sabinene hydrate, nonyl acetate, terpinyl acetate, geranial, 10-dodecyl-1-ol, *p*-menth-8-en-2-ol, dihydrocarvyl acetate, muurolol, hexadecanal, farnesol, and bisabolol were not detected by injection

Table 2. Odor Description of Volatile Components of Kabosu Peel Oil

peak	compound	odor description[a]	$\log_3$(FD factor)[b]	relative flavor activity[c]
2	α-pinene	resinous, pine-like	6	3.4
3	camphene	warm, herbaceous	5	41.5
4	undecane	green	5	42.8
5	β-pinene	fresh, pine-like	4	4.3
6	sabinene	green	6	9.0
7	myrcene	pungent green, sour	9	1.0
8	α-phellandrene	fresh, green	6	15.8
9	α-terpinene	resinous	5	7.6
10	limonene	pungent green, lemon-like	8	0.5
11	β-phellandrene	pine-like	4	nq
13	γ-terpinene	waxy	6	1.8
14	*p*-cymene	green	6	10.6
15	terpinolene	fresh, green	4	6.0
16	octanal	sweet, citrusy	5	2.9
18	nonanal	sweet, citrusy	7	10.6
19	(*Z*)-limonene oxide	floral, citrusy	5	33.1
20	(*E*)-limonene oxide	floral, soapy	5	53.3
21	(*E*)-linalool oxide	fruity, citrusy	6	35.0
22	octyl acetate	green, kabosu-like	5	37.3
23	citronellal	fresh, kabosu-like	4	10.4
24	α-copaene	fresh, fragrant	5	nq
25	decanal	sour, metallic	5	3.4
26	β-cubebene	sweet, floral	4	6
27	linalool	sweet, floral, citrusy	6	9.1
28	linalyl acetate	floral, kabosu-like	4	16.8
29	octanol	fruity, citrusy	7	52.8
31	β-elemene	fresh, green	5	25.1
32	caryophyllene	fresh, fruity	7	7.5
33	undecanal	sour, metallic	5	nq
34	thujyl alcohol	citrusy	4	31.0
36	β-farnesene	fresh	6	4.5
37	α-humulene	flowery	7	19.0
39	neral	fresh, kabosu-like	5	17.6
41	α-terpineol	oily	4	11.4
42	dodecanal	green, sour	4	nq
45	neryl acetate	green	7	10.6
49	geranyl acetate	fresh, citrusy	5	nq
50	δ-cadinene	green, floral	4	4.3
51	citronellol	floral	4	nq
52	sesquiphellandrene	fruity	5	11.2
54	perillaldehyde	green, nutty	4	13.5
55	octadecane	fresh, fragrant	5	7.5
56	tridecanal	citrusy, oily	4	42.7
57	(*Z*)-carveol	citrusy	6	37.0
58	nerol	sweet, floral	5	34.8
59	geraniol	sweet, citrusy	4	60.4
61	tetradecanal	sweet, herbaceous	4	24.6
62	perillyl alcohol	green	4	24.6
63	nerolidol	dry grass-like	4	21.3
64	β-elemol	green	5	30.8
65	cedrol	herbaceous	4	30.2
66	eugenol	citrusy, spicy	6	17.8
67	myrcene dimer	burnt	4	19.1
68	isoeugenol	spicy	5	53.3

[a] Odor description at the GC sniffing port during GC/O. [b] FD factor: flavor dilution factor on DB-Wax column. [c] Relative flavor activity = $\log_3$ (FD factor)/$S^{0.5}$, where S is the weight percentage.

of the cold-pressed oil. GC/O analysis of the oxygenated fraction confirmed the presence of the 4 Kabosu-odor characteristic compounds. Their identification in both the cold-pressed oil and the oxygenated fraction was based on either the retention index and mass spectra or on the retention index and co-injection with authentic standards.

Sniff Testing of Kabosu Cold-Pressed Oil. Sniff testing is used not only for AEDA, but also for expressing the aroma character of each component. Organoleptic response to a compound, in general, depends on its concentration (*17*). The FD factor or RFA has proven to be a useful criteria for reconstruction of the original aroma from the odor active compounds detected by AEDA (*8*, *15*, *16*). However, the FD factor and RFA often have no relation to the aroma character of each compound (*17*). In other words, even if the FD factor of one compound is not comparatively high, it often contributes primarily to the original odor of a food. As reported earlier (*26*), aldehydes are important keynotes in producing characterizing flavor. Particularly for the citrus family, it has been reported that the characteristic aroma of lemon is citral (*27*) and that 2-dodecenal is a primary characteristic compound of Tosabuntan (*Citrus grandis* Osbeck forma *Tosa*) aroma (*17*). We performed sniff testing (*28*) of Kabosu cold-pressed oil and 4 compounds for which GC/O analysis detected a Kabosu-like odor, and successfully identified one aldehyde, i.e. citronellal, whose odor strongly resembles that of Kabosu.

Chiral GC Analysis. Citronellal is known as a chiral oxygenated compound (**Figure 2**). A GC equipped with 2 on-

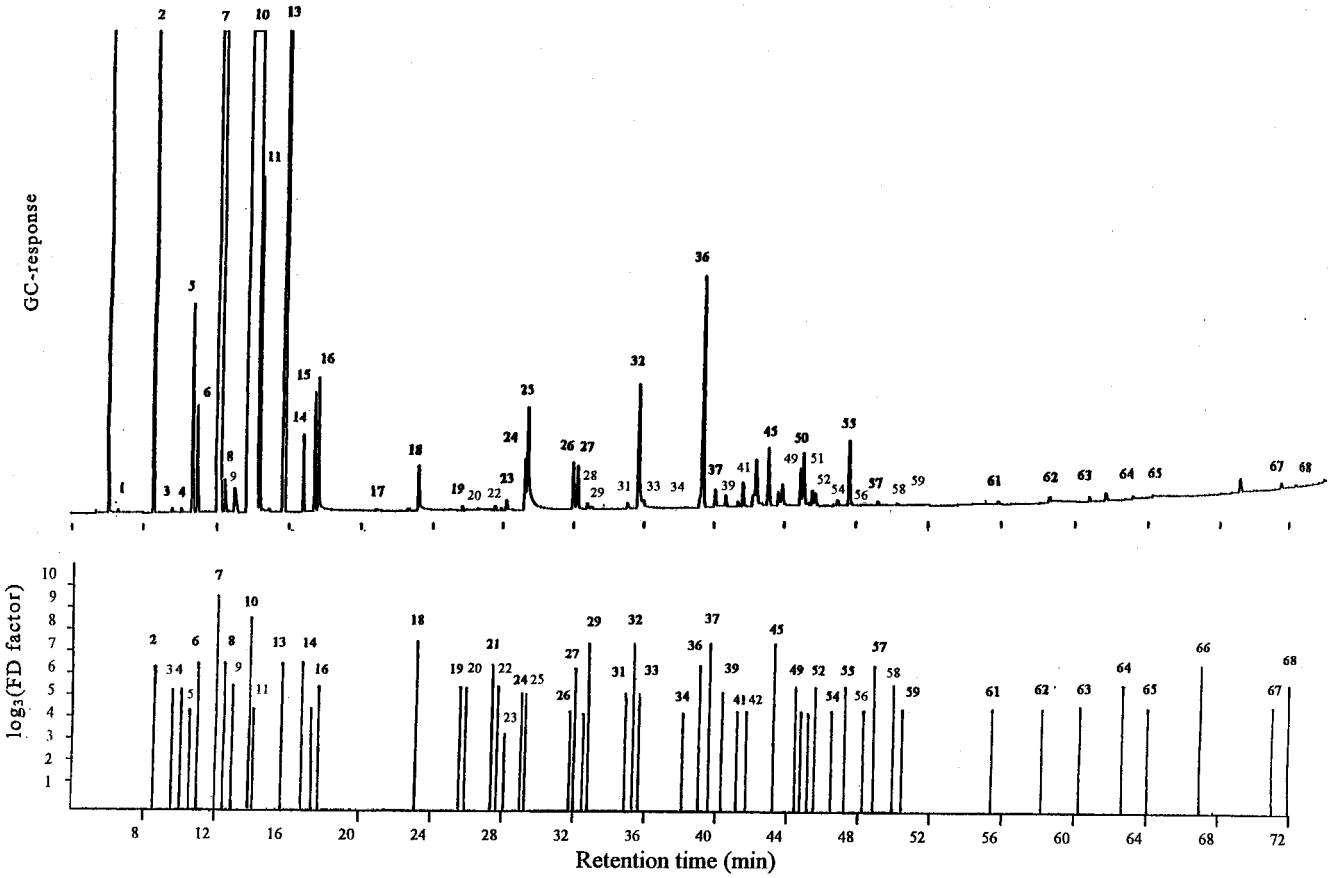

Figure 1. Gas chromatogram (top) and aromagram (bottom) of odor active volatiles of Kabosu peel oil.

Table 3. Chiral Analysis of Citronellal

stereoisomer	retention index (on DB-5 + chiral column)	peak area (%) (in citronellal authentic)	w/w (%) in CPO[a]	odor description[b]
(*R*)-(+)-citronellal	1221	91.9	0.019	fruity, fresh, Kabosu-like
(*S*)-(−)-citronellal	1223	8.1	not detected	sweet, turpentine-like

[a] CPO, Cold-pressed oil. [b] Odor description at the GC-sniffing port of a 0.38% solution in acetone.

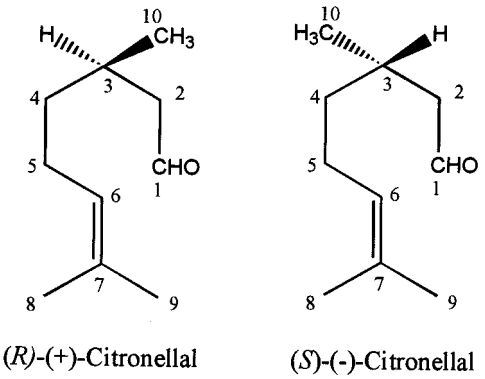

Figure 2. Chemical structures of citronellal isomers.

line coupled columns was used to separate the citronellal enantiomers in Kabosu cold-pressed oil in accordance with the previously described method (*22*, *23*). As shown in **Table 3**, (*R*)-(+)-citronellal and (*S*)-(−)-citronellal were detected in a citronellal authentic; their peak areas were 91.9% and 8.1%, respectively. However, in Kabosu cold-pressed oil, as well as in its oxygenated fraction, (*S*)-(−)-citronellal was not detected. To confirm this result, citronellal, (*R*)-(+)-citronellal, and (*S*)-(−)-citronellal were subjected to GC/O analysis. (*R*)-(+)-Citronellal was described as fruity, fresh, and Kabosu-like, and (*S*)-(−)-citronellal was described as sweet and turpentine-like.

In conclusion, (*R*)-(+)-citronellal was detected at a level of 0.019% (w/w) in Kabosu peel oil. Sniffing of a wide rage of concentrations of (*R*)-(+)-citronellal found that an aqueous solution of 0.25% and of 0.016% (*R*)-(+)-citronellal, by smelling-strip and by headspace-sniff in a 30-mL vial, respectively, showed an odor similar to that of Kabosu. It is therefore suggested that (*R*)-(+)-citronellal plays an important role in the characteristic odor of Kabosu peel oil.

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