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Isotope Ratio by HRGC-MS of *Citrus junos* Tanaka (Yuzu) Essential Oils: *m/z* 137/136 of Terpene Hydrocarbons

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The isotope ratios of monoterpene hydrocarbons in *Citrus junos* Tanaka (yuzu) essential oils from different origins were determined by ordinary high-resolution gas chromatography-mass spectrometry (HRGC-MS). Both intensities of the molecular mass peaks (*m/z* 136) and of the isotope peaks (*m/z* 137) of monoterpene hydrocarbons were measured by single-ion monitoring with an MS analysis. The isotope ratios (*m/z* 137/136) of the ten monoterpene hydrocarbons commonly contained in citrus essential oils, α-pinene, β-pinene, sabinene, myrcene, α-phellandrene, α-terpinene, limonene, γ-terpinene, β-phellandrene and terpinolene, were determined in yuzu samples of the highest commercial quality from 42 different production districts. Statistical treatment of these data by the *t*-test and sign test revealed significant differences of the isotope effects in each yuzu sample. It is suggested that this technique will be applicable for evaluating the quality, genuineness and origin of citrus fruits and their products. The isotope fingerprints were also demonstrated in several citrus fruits other than the yuzu samples.

Key words: *Citrus junos*; yuzu; isotope effect; HRGC/MS; monoterpene hydrocarbon

The natural abundance of isotopes of each element is distributed in a given ratio. Plants on the earth first convert solar energy into biochemical energy; the food chain starts from plants. Higher plants fix CO_2 by the Calvin-Benson cycle to biosynthesize various organic compounds for their constituents.[1] It is known that the enzyme, ribulose-1,5-diphosphate carboxylase, differentiates a small mass difference between $^{12}CO_2$ and $^{13}CO_2$, when it fixes CO_2 in the atmosphere. This function is the so-called "isotope effect." It is also thought that the isotope effect could be achieved by every enzyme involved in biosynthetic and metabolic pathways.[2] We can thus see that this effect should be also applicable to the essential oils comprising terpene compounds. Every species, variety or strain of a plant has some substantially distinct characteristics. Even among the same cultivars, different growing conditions such as the annual atmosphere and moisture, or soil and fertilizers would bring about small but appreciable differences in their compositions.

Citrus fruits have been widely cultivated between the tropical and the temperate zones in both the northern and southern hemispheres. The citrus fruit is one of the most important commercial crops, since it provides us with a pleasant taste, flavor and fragrance. It is said that there are thousands of varieties of citrus in the world. The extensive research on citrus flavor has been reviewed.[3,4] It has recently become commercially important to properly evaluate the quality, origin or genuineness of raw and processed products. A flavor analysis is a good means of revealing the characteristics of a product. It may be, however, difficult to make sufficient discrimination among the same or close cultivars by means of a general flavor compositional analysis by GC and GC-MS.

Faulharber *et al.*[5,6] and other researchers[7–9] have reported that a determination of the isotope values of constituents is gaining increasing importance, especially in view of the increased demand for the authenticity control and origin determination of essential oils and foods. To determine isotope values, gas chromatography-isotope ratio mass spectrometry (GC-IRMS) has been used. It does not seem, however, to be widely used. The authors have explored the possibility of a more convenient and common analysis of isotope values. We examine here the effectiveness of essential oils based on the isotope peak on the mass spectra of compounds.

Citrus junos Tanaka (yuzu), a sour citrus fruit, has been cultivated mainly in Japan and Korea for more than a thousand years, the annual production in each country amounting to approximately 18,000 and 20,000 tons in 1998, respectively. Yuzu has a strongly

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characteristic aroma in comparison with any other citrus fruits, and the volatile components of yuzu have been studied by many researchers.[10-15] In Japan, yuzu juice is popularly utilized in a Japanese dressing called *ponzu* in Japanese, and as a flavoring in various dishes and foods. Yuzu tea is popular among Korean people. It is known that the cultivation of other popular sour citrus fruits, *C. sudachi* Hort. ex Shirai (sudachi) and *C. sphaerocarpa* Hort. ex Tanaka (kabosu), is localized in Tokushima and Oita prefectures, respectively. However, yuzu can be cultivated throughout Japan, except in Hokkaido, although it is principally produced in Kochi prefecture, which accounts for more than 40% of the total production. The second largest producing area is Tokushima prefecture neighboring Kochi prefecture. Yuzu fruit from Kochi have long been judged to be of the best quality in every domestic fruit market. Consumers are increasingly demanding that the origin of agricultural and fishery fresh and processed commercial products should be designated on all products. This designation has been legislated to some extent. Thus, the demand for special techniques to evaluate or judge commercial yuzu fruit will be increased in the future.

The present study focuses on the development of a new analytical method for differentiating the quality of commercial yuzu fruits from various growing districts.

Materials and Methods

Materials. Yuzu fruits were collected from 41 local wholesale markets from northern to southern Japan in November 1999, in addition to Korean yuzu fruits produced in Chindo, as shown in Fig. 1 and Table 1. All the yuzu samples obtained were ranked as the highest quality by the wholesaler at each market. The cold-pressed peel oil (CPO) of between 5 and 10 kg of each sample was prepared according to the usual method.[16] Each CPO sample was stored at $-25°C$ until needed for analysis. Experimentation with isotope fingerprints was conducted on the following additional citrus fruits: *Citrus unshiu* Marcov. (unshu), *C. natsudaidai* Hayata (natsudaidai), *C. limon* Burmann (lemon) and *C. hanaju* Hort. ex Shirai (hanayu). All of these additional samples were harvested during the same season as yuzu. Authentic chemicals for co-injection during gas chromatography and mass spectrometry were obtained from the commercial sources mentioned previously.[16]

Common evaluation of yuzu fruit. The average of the common analytical evaluation of each yuzu fruit was determined. The juice was extracted by hand-pressing, filtered through a plankton net and centrifuged at 4000 rpm for 15 min. One portion of the

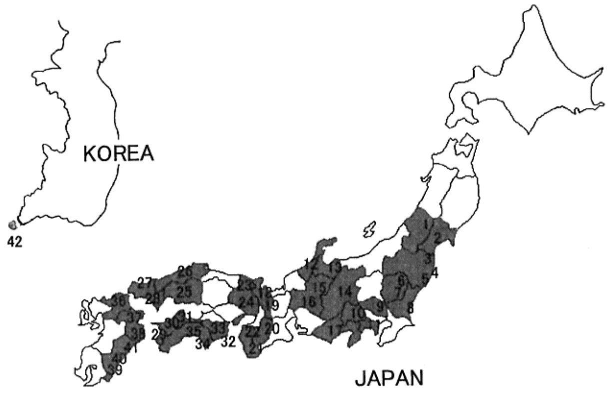

Fig. 1. Sample Origins of *Citrus junos* Tanaka (yuzu) in Japan and Korea.

supernatant was used for measuring of the soluble-solid content with an Atago A-50 refractometer. The other portion was used for determinating free acid by the usual method of neutral titration. The L-a-b values of the peel color were measured with a Minolta CR-200 color meter.

Gas chromatography-mass spectrometry (GC-MS). Gas chromatography combined with mass spectrometry was used for identifying the volatile components which had been detected. The analysis was carried out with a Shimadzu GC-17A linked to a Shimadzu QP-5000 at an MS ionization voltage of 70 eV, accelerating voltage of 1500 V, and ion source temperature of 250°C. The GC column was a DB-Wax fused-silica capillary ($60\ m \times 0.25\ mm$ i.d., 0.25 μm film thickness; J & W Scientific, Folsom, CA, U.S.A.). The column temperature was programmed from 70°C (2 min) to 100°C at a rate of 2°C/min. The column was cleaned before each analysis by heating to 230°C. The injector temperature was 250°C, and helium was used as the carrier gas at a flow rate of 1 ml/min. An oil sample of 0.2 μl was injected at a split ratio of 1:50.

Identification of the components. Each component was initially identified by its GC retention index, gas-co-chromatography with an authentic compound and the NIST library connected to the QP-5000 mass spectrometer as described in the previous paper.[16]

Determination of the isotope ratio. The following 10 monoterpene compounds were examined in the determination of the isotope ratio: α-pinene, β-pinene, sabinene, myrcene, α-phellandrene, α-terpinene, limonene, γ-terpinene, β-phellandrene and terpinolene. Single-ion monitoring (SIM) by GC-MS was performed in order to estimate the intensities of both the ion peak of each molecule (m/z 136) and of its isotope (m/z 137). The total intensity of each compound was regulated to achieve about 8.0×10^7 in MS values, the scanning interval for mass spectrometry

Table 1. Sample Origins of *Citrus junos* Tanaka (yuzu) and Common Analytical Evaluations

No.	Sample in prefecture	Fruit weight	Juice		Peel color index		
		g	Soluble-solid %	Acidity %	L	a	b
1	Yamagata	61.4	9.7	4.3	70.2	10.1	73.4
2	Miyagi	114.0	10.3	3.9	67.8	15.1	71.6
3	Fukushima-1	74.4	7.9	3.5	54.5	−13.3	47.5
4	Fukushima-2	111.9	8.1	4.5	70.8	12.2	74.8
5	Fukushima-3	120.2	8.6	2.7	71.5	7.6	75.9
6	Tochigi-1	123.4	7.6	4.1	72.9	11.3	78.7
7	Tochigi-2	95.5	8.5	3.9	71.9	9.1	74.5
8	Ibaragi	176.5	7.6	2.9	71.2	5.9	71.1
9	Saitama	121.9	9.3	4.3	69.7	10.6	73.9
10	Yamanashi	124.2	9.0	5.5	73.6	7.7	76.2
11	Kanagawa	87.1	8.4	3.6	75.1	2.7	78.0
12	Ishikawa	97.5	7.4	4.4	72.3	7.4	78.3
13	Toyama	143.9	8.0	3.4	73.8	5.6	74.2
14	Nagano	131.8	9.3	4.6	72.5	10.0	73.0
15	Gifu-1	78.0	8.2	5.2	66.6	13.2	71.4
16	Gifu-2	112.1	8.1	3.8	68.9	8.9	70.1
17	Shizuoka	108.1	7.4	2.9	71.6	−6.9	66.4
18	Kyoto-1	121.2	7.8	4.1	75.1	5.8	76.9
19	Kyoto-2	108.4	7.6	4.3	72.4	7.2	77.5
20	Nara	100.9	8.2	2.9	69.1	14.9	74.4
21	Wakayama-1	102.5	7.0	3.8	72.1	5.0	74.4
22	Wakayama-2	152.6	6.9	3.1	73.1	6.2	77.7
23	Hyogo-1	122.4	7.4	2.9	71.4	9.9	78.0
24	Hyogo-2	134.4	7.8	2.7	74.2	6.9	78.7
25	Hiroshima	120.3	7.5	2.6	73.2	8.6	75.3
26	Shimane	122.9	7.5	3.0	70.9	3.4	74.9
27	Yamaguchi-1	148.7	8.4	3.2	73.0	3.5	72.8
28	Yamaguchi-2	173.2	7.1	2.7	73.9	9.1	77.0
29	Ehime-1	132.0	7.8	4.6	74.6	5.5	77.1
30	Ehime-2	123.1	7.1	2.8	71.6	9.2	77.2
31	Ehime-3	139.0	8.2	3.6	72.2	7.4	77.0
32	Tokushima-1	132.4	7.9	2.3	71.5	7.2	78.3
33	Tokushima-2	139.6	7.8	3.5	75.5	5.4	78.7
34	Kochi-1	126.5	7.4	3.9	72.3	3.0	77.4
35	Kochi-2	167.1	7.4	3.2	76.8	1.8	78.9
36	Fukuoka	165.3	7.8	3.1	74.7	4.5	75.6
37	Oita-1	154.8	7.2	3.2	71.5	6.5	74.0
38	Oita-2	159.0	8.8	3.6	72.1	6.7	74.4
39	Miyazaki-1	91.5	7.1	4.1	68.9	9.1	75.5
40	Miyazaki-2	161.6	7.3	4.3	73.9	8.1	81.0
41	Miyazaki-3	147.3	8.2	4.2	71.4	8.2	78.1
42	Chindo (Korea)	125.8	8.1	4.2	70.5	6.2	70.2

being 0.1 sec. The isotope ratio (Ir) of each peak was calculated by using the following equation:

$$\text{Ir} = (\text{intensity of an isotope peak of } m/z \text{ 137}) / (\text{intensity of a molecular peak of } m/z \text{ 136}) \times 100$$

where each intensity is the mean value from triplicate determinations.

Statistical treatment. The significant difference between the two Ir values of each compound from different yuzu samples was checked by the t-test ($P < 0.05$). The sign test was then performed on the following basis: The results of each sample were classified as positive, negative or no significant difference. If one Ir value was greater than the other, the former was given a plus and the latter a minus. If there was no significant difference, both were given zero. A database of these values was constructed for the ten compounds of each of the 42 samples.

The sign test is explained briefly as follows. In comparing, for example, two samples A and B, sample A is given ten ratings, each either plus, minus or zero, in comparison with sample B. The smaller absolute value of the number of plus and minus ratings for A and for B is selected. One plus rating or one minus rating is assigned the value of $+1$, and a zero rating is assigned the value of $+0.5$. We assign a sample its score if it has more plus ratings than minus ratings, and assign the other sample a minus, as shown in Table 3. When the score does not exceed 2 in the case of 10 samples, this paired sample is judged to be significantly different ($P<0.05$). Such a designation constitutes an active-passive discrimination between the two samples.

Results and Discussion

The characteristics and quality of citrus flavors have commonly been evaluated on the basis of their essential oil components. Citrus flavors or their essential oils, on the other hand, are apt to vary in composition as a result of maturity, storage or cultivation conditions. Moreover, it may be difficult to clearly distinguish characteristic differences among the same cultivars from different production districts solely by means of an essential oil component analysis, since their compositions are quite similar. The isotope analysis by HRGC-MS will provide new information about these matters. The principle of GC-IRMS involves a compound separated through the column being burned to form CO_2, and the amount of ^{13}C and ^{12}C in CO_2 is measured. Thus, the result yields only information about the carbon skeleton of the compound. According to the method presented here, on the other hand, the result yields complete information about the total intensity, including the amount of carbon and hydrogen in the compound. The isotope effect, which is dependent on the enzyme reaction, should occur not only in the discrimination between ^{12}C and ^{13}C, but also in that between ^{1}H and ^{2}H. Therefore, measurement of the isotope peaks by HRGC-MS will yield complete information about the presence of hydrocarbons. Furthermore, this method is applicable to oxygenated compounds with ^{16}O, ^{17}O and ^{18}O, for which the mass spectra will indicate $M^+ + 1$ and $M^+ + 2$, as well as M^+.

Several analytical estimates regarding yuzu fruit

Several common analytical evaluations of yuzu fruit, *i.e.*, fruit weight, soluble-solid content, acidity and peel color index are shown in Table 1. These evaluations varied considerably for samples from different origins, even though every sample fruit was ranked as the highest quality at the individual wholesale markets. The smallest fruit was 61.4 g of sample No. 1, and the largest was 173.2 g of No. 28. The soluble-solid content and acidity ranged between 6.9% and 10.3%, and between 2.3% and 5.5%, respectively. The peel color index based on the L-a-b analysis respectively ranged between 54.5 and 76.8, -13.3 and 15.1, and 47.5 and 81.0. It should be noted that, in general, these evaluations will not always be correlated with the quality, because they are apt to vary considerably with the degree of maturity or storage conditions for fruits from the same origin. Therefore, no scientific criteria for the quality of yuzu fruit have been established. The subjective judgment of the wholesalers and consumers has been the only means of evaluating the commercial quality.

Ir values for monoterpene hydrocarbons of yuzu peel oil

The 10 selected monoterpene hydrocarbons were completely separated on a gas chromatogram (Fig. 2). The result of the SIM analysis of all essential oils is summarized by the Ir ratios of m/z 137 to 136, as shown in Fig. 3. The Ir data given as the mean of triplicate values were so stable and reliable that the coefficient of variance was less than 0.5% overall. This demonstrates that the Ir values were different among the 10 monoterpene compounds with the same molecular weight of 136. All the $C_{10}H_{16}$ monoterpene hydrocarbons should theoretically show an Ir ratio of 11.42 from a calculation of the natural abundance ratio of the hydrogen and carbon isotopes. Even though there would be a considerable deviation from the theoretical value as a result of mechanical factors, the low coefficient of variance in-

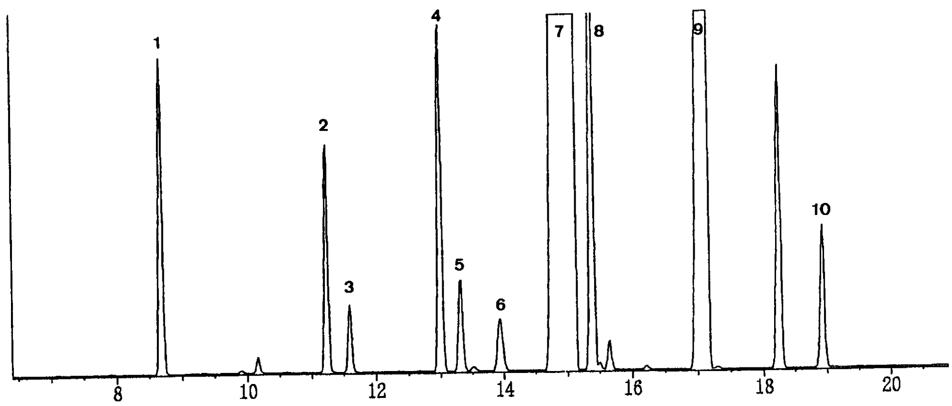

Fig. 2. Gas Chromatogram of Yuzu Essential Oil.
1: α-pinene, 2: β-pinene, 3: sabinene, 4: myrcene, 5: α-phellandrene, 6: α-terpinene, 7: limonene, 8: β-phellandrene, 9: γ-terpinene, 10: terpinolene.

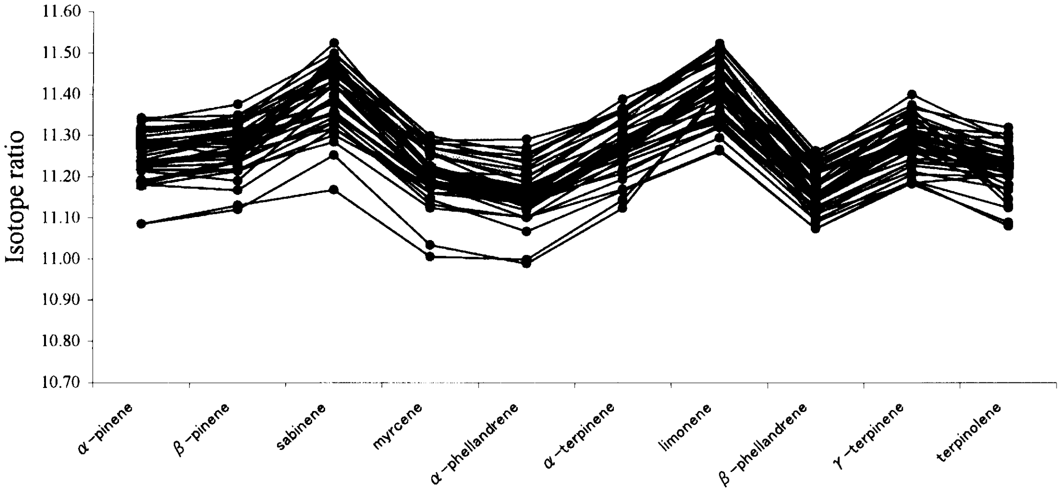

Fig. 3. Isotope Ratios of Monoterpene Hydrocarbons in Yuzu Essential Oils from 41 Origins.

dicates high reliability. This result also suggests that a Q-mass instrument like the QP-5000, which is relatively common, is applicable to these analyses.

The 10 monoterpene hydrocarbons in yuzu peel oil had unique Ir values. The range of Ir variation of compounds in yuzu oil was as follows: 11.08–11.34 for α-pinene; 11.13–11.35 for β-pinene; 11.47–11.52 for sabinene; 11.01–11.30 for myrcene; 10.99–11.29 for α-phellandrene; 11.17–11.39 for α-terpinene; 11.26–11.52 for limonene; 11.07–11.26 for β-phellandrene; 11.18–11.37 for γ-terpinene; and 11.08–11.32 for terpinolene. Most of these Ir values are lower than the theoretical Ir value for a monoterpene hydrocarbon (11.42). This is likely to have been the result of the isotope effect in those yuzu plants in which differences from the theoretical Ir value occurred. All the Ir values of the standard chemicals, except for β-phellandrene can be seen in Table 2. It is assumed that those chemicals would have been chemically synthesized, but the initial materials for chemical synthesis are often biosynthesized products. Thus, those Ir values are not identical to the theoretical values.

Table 2. Isotope Ratios of Authentic Compounds

Compound	Isotope ratio
α-Pinene	11.26
β-Pinene	11.18
Sabinene	11.23
Myrcene	11.02
α-Phellandrene	11.36
α-Terpinene	11.36
Limonene	11.26
γ-Terpinene	11.21
Terpinolene	11.23

Sign test for Ir values of the yuzu samples

Table 3 shows the results of sign testing of the 41 yuzu fruit samples. The shaded values indicate the occurrence of a significant difference between two sample origins. It is to be noticed that a greater number of shaded values in one row indicates greater variance from the other samples. An examination of the table reveals several predominant cultivation regions. Only 8 of the 41 samples have not less than 20 shaded value points. The Kochi-2 sample is the most distinct with 33 points. The Yamagata, Miyagi and Fukuoka samples are in the second category, ranging from 25 to 29 points. The third category, between 20 and 24 points, includes the Kochi-1, Tokushima-2 and Shizuoka samples. The Korea sample received 21 points. This is the first time that a substantial difference among yuzu fruits from different production areas has been detected.

There appears to be little correlation between the common analytical evaluation (Table 1) and the result of the sign test (Table 2). Of the 8 samples (Nos. 1, 2, 17, 33, 34, 35, 36 and 42) which were rated over 20, the fruit weight, soluble-solid, acidity, L, a and b values were in the range of 61.4 to 165.3, 7.4 to 10.3, 2.9 to 4.3, 67.8 to 76.8, −6.9 to 15.1 and 66.4 to 78.9, respectively. On the other hand, those of the 7 samples which were rated zero (Nos. 13, 19, 22, 23, 24, 25, 28 and 38) were 108.4 to 159.0, 6.9 to 8.8, 2.6 to 4.3, 76.4 to 74.2, 5.6 to 9.9, and 74.2 to 78.7. There is overlap between the high-scoring and low-scoring samples. This means that these common factors may not be relevant to a determination of the quality and characterization of the production area. If producers wish to establish a relationship between the quality and place of origin, a more reliable and less variable evaluation is required. These considerations suggest that the statistically treated isotope ratio can be used as an evaluation tool. Further investigations are being carried out confirm the reliability of the isotope ratio during maturation, among different trees and among different positions of fruit on the

Table 3. Sign Test of Yuzu Essential Oils from 41 Origins

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
1		4.5	2	1	2	0.5	1.5	4.5	1	4	2.5	0.5	1.5	0	1.5	1.5	3	2	0.5	2	2	1	0.5	0.5	0.5	1	2	2	1.5	1.5	1	0.5	4.5	4.5	—	5	4.5	0	3.5	2	4.5
2	—		1	0.5	2	0.5	1	4.5	1.5	4.5	2	0.5	2	1	1.5	1.5	3	2.5	0.5	2.5	2	1	1	1	0.5	1	2.5	2	2	1.5	1	0.5	4.5	5	—	5	3.5	0	4	1.5	4.5
3	—	—		3.5	5	5	—	—	3	—	4.5	2	5	4	4.5	4.5	—	5	1	—	5	4.5	0.5	2	3	4.5	3.5	5	—	4.5	3	2	—	—	—	—	—	1.5	—	—	—
4	—	—	—		5	—	—	—	4	—	—	3.5	5	—	4.5	—	—	—	2	—	—	5	2.5	3	4.5	—	—	—	—	5	3.5	4	—	—	—	—	—	3.5	—	—	—
5	—	—	5	5		5	5	—	4.5	—	—	4.5	5	—	5	5	—	5	2	—	—	5	2	3	5	5	—	5	—	5	4.5	4.5	—	—	—	—	—	3	—	—	—
6	—	—	5	4.5	5		5	—	4	—	—	2	4.5	—	4.5	4.5	—	—	1.5	—	—	4.5	1.5	2.5	4.5	—	—	4.5	—	—	3	4	—	—	—	—	—	3	—	—	—
7	—	—	4.5	4	5	5		—	3	—	—	2	4.5	4	4	4	—	5	1.5	—	—	4.5	1.5	2	4	4	—	4.5	5	4.5	2.5	3	—	—	—	—	—	2	—	4.5	—
8	—	—	2	1	2	1	1.5		1	4	2.5	0.5	1.5	1.5	2	2	3.5	3	0.5	3	2	1	0.5	0.5	1	1.5	2.5	2.5	2	2	1	1	4	—	—	—	4	0.5	3.5	2.5	4.5
9	—	—	—	—	—	—	—	—		—	—	4	—	—	—	—	—	—	2	—	—	5	2	3	—	—	—	—	—	—	5	5	—	—	—	—	—	2.5	—	—	—
10	—	—	2.5	1.5	2	1.5	1.5	—	1		2.5	0.5	3	1	2.5	2.5	4.5	3.5	0.5	3.5	3	2	1	1.5	1	2.5	3	2.5	2	2	1	0.5	5	—	—	4.5	4	0.5	5	2.5	5
11	—	—	4.5	3.5	4	4	4.5	—	2.5	—		1.5	4.5	3.5	3.5	4.5	—	4.5	1.5	—	—	4	1.5	2	4	5	—	5	5	4.5	2	3	—	—	—	—	—	1.5	—	5	—
12	—	—	—	—	—	—	—	—	—	—	—		—	—	—	—	—	—	2	—	—	5	2	4.5	—	—	—	—	—	—	5	—	—	—	—	—	—	—	—	—	—
13	—	—	5	5	5	—	—	—	4.5	—	—	4.5		—	5	5	—	5	3	—	5	5	2.5	4.5	5	—	—	—	5	5	5	5	—	—	—	—	—	5	—	—	—
14	—	—	5	4.5	4.5	4.5	—	—	3.5	—	—	2	4.5		—	—	—	—	1.5	—	—	4	1.5	2	4	—	—	—	—	—	3	4	—	—	—	—	—	2	—	—	—
15	—	—	—	4.5	5	—	—	—	2.5	—	—	2.5	5	4.5		5	—	5	1.5	—	—	5	2	2.5	4	—	—	—	—	—	3	3	—	—	—	—	—	2	—	—	—
16	—	—	—	4.5	5	—	—	—	2.5	—	—	2.5	5	4.5	5		—	5	1.5	—	—	5	2	2.5	4	—	—	—	—	—	3	3	—	—	—	—	—	2	—	—	—
17	—	—	2	0	2	0.5	1	—	0.5	—	3.5	0	2.5	1	1	1		3.5	0	3.5	2.5	0.5	0	1	0.5	1	2.5	2.5	2	2.5	0.5	0	4.5	—	—	—	5	0	5	2.5	—
18	—	—	5	4.5	5	4	5	—	4.5	—	—	3.5	5	4.5	5	5	—		1	—	4.5	4.5	1	3	4.5	4.5	5	4.5	5	5	4.5	4	—	—	—	—	—	2.5	—	5	—
19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	—	—	4.5	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20	—	—	4	2.5	3	2.5	3.5	—	0.5	—	4.5	0	4	2	3	3	—	4	0.5		4	2	0	1	2.5	2.5	3.5	3	3.5	3.5	0.5	0	—	—	—	—	—	0	—	4.5	—
21	—	—	5	3	4	3	4	—	1.5	—	4	1	5	3	4.5	4.5	—	—	1	—		3	1	2	2.5	4	4	4.5	5	5	1.5	1.5	—	—	—	—	—	0.5	—	4.5	—
22	—	—	—	5	5	—	—	—	5	—	—	5	5	—	5	5	—	—	3	—	—		3	4.5	4.5	5	—	5	—	—	5	5	—	—	—	—	—	5	—	—	—
23	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
24	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	—	—	—	4		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
25	—	—	—	—	5	—	—	—	4	—	—	3	5	—	—	—	—	—	1.5	—	—	—	2	3		—	—	—	—	—	3.5	4	—	—	—	—	—	1.5	—	—	—
26	—	—	—	3.5	5	4.5	—	—	3	—	5	2.5	4.5	4	4	4	—	—	1.5	—	—	5	1.5	3	4		—	5	—	5	2.5	2.5	—	—	—	—	—	2	—	—	—
27	—	—	—	4	4	4	4.5	—	3	—	4.5	1.5	4.5	3.5	4.5	4.5	—	5	1	—	—	4.5	1.5	2.5	4	4.5		—	5	5	2.5	3	—	—	—	—	—	1	—	4.5	—
28	—	—	5	4.5	5	—	—	—	3	—	5	2.5	4.5	4	4	4	—	—	1.5	—	—	5	1.5	2.5	4	5	4.5		5	5	3	4	—	—	—	—	—	2.5	—	5	—
29	—	—	4.5	4	4	4.5	5	—	1	—	5	0.5	5	4	4	4	—	5	1	—	5	4	1	1.5	3.5	4.5	5	5		4.5	2	1.5	—	—	—	—	—	0.5	—	5	—
30	—	—	—	5	5	4	—	—	1	—	—	1.5	5	3	4.5	4.5	—	5	1	—	5	4	1	2.5	4.5	5	5	5	—		2	2	—	—	—	—	—	1	—	5	—
31	—	—	—	—	—	—	—	—	5	—	—	5	5	—	—	—	—	—	2	—	—	5	2	3.5	—	—	—	—	—	—		—	—	—	—	—	—	2.5	—	—	—
32	—	—	—	—	—	—	—	—	5	—	—	3.5	5	—	—	—	—	—	1.5	—	—	5	1	3	—	—	—	—	—	—	4.5		—	—	—	—	—	2.5	—	—	—
33	—	—	2	1	4	1	2	—	2	5	2.5	1	2.5	1.5	1.5	1.5	—	2.5	1	4	3	1.5	1	1	1	1	2	2	1.5	2	1.5	1		5	—	—	4	0.5	5	2.5	—
34	—	5	2	1.5	2	1	2	4.5	1.5	4	2.5	1	2	0.5	2	2	3.5	2.5	1	2.5	1.5	2	1	1	1.5	1.5	2	1.5	2	2.5	1.5	1	4.5		5	5	3	0.5	2.5	2.5	3
35	—	3	1	0.5	1	0	1	4.5	1	2	1.5	0.5	1	0	1	1	1.5	1	0.5	1	1	1	0.5	0.5	0.5	0.5	1.5	1	1.5	1	1	0.5	2	5		5	2	0	2	1	2.5
36	5	5	2	0	1.5	0.5	1	4.5	0.5	5	2.5	0	1.5	0	1.5	1.5	3.5	3	0	2	2	1	0	0.5	0.5	0.5	2	2	2.5	2	0.5	0.5	3.5	5	5		3.5	0	4	2	4
37	—	—	3	2	3.5	1.5	2	—	1	—	3	0.5	3	1	2.5	2.5	5	3.5	0.5	4.5	4	2	0.5	1.5	2	2.5	3.5	3.5	3.5	2.5	1	0.5	—	—	—	—		0	5	3.5	—
38	—	—	—	—	—	—	—	—	—	—	—	4.5	5	—	—	—	—	—	3.5	—	—	5	2.5	4.5	—	—	—	—	—	—	—	—	—	—	—	—	—		—	—	—
39	—	—	2	1	2.5	1.5	2	—	1.5	5	2.5	0.5	2.5	1.5	1.5	1.5	5	3.5	0.5	3.5	3	2	1	0.5	1	1.5	3.5	3	2	2.5	1	1.5	5	—	—	—	5	0.5		3	—
40	—	—	4.5	4.5	4.5	4	—	—	2	—	5	1	4.5	4.5	4.5	4.5	—	5	1	—	—	4.5	1	2	3.5	4.5	—	5	5	5	2	2	—	—	—	—	—	1	—		—
41	—	—	2	1	3	1	1.5	—	1	5	2.5	0.5	2.5	1.5	2.5	2.5	4	3.5	1	3.5	3	2	1	1.5	1	2	3.5	2.5	2.5	3.5	1	1	4.5	—	—	—	4.5	1	4.5	2.5	

Shaded cells show significant difference between two samples.

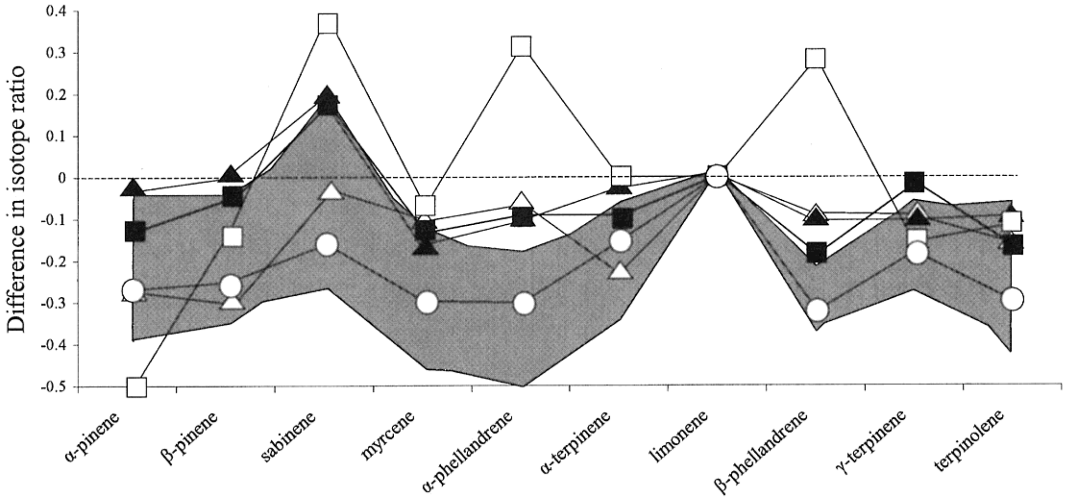

Fig. 4. Isotope Fingerprint of Yuzu Essential Oils and Isotope Ratios of Other Citrus Essential Oils.
The shaded band shows the fingerprint of yuzu essential oils. ○ Korean yuzu, □ natsudaidai, △ unshu, ■ lemon, ▲ hanayu.

tree, and among different years.

Isotope fingerprint of the yuzu oil

The $\delta^{13}C_{PDB}$ value induced from the isotope effect is influenced by such exogenous factors as the location, climate and harvest time.[2,6] The isotope ratio of endogenous biosynthesized compounds is also affected by exogenous factors including differences in cultivars. The isotope ratio of the yuzu essential oil components varied among the yuzu samples to some extent, as shown in Fig. 3. When the Ir value of one compound in a series of the 10 selected compounds was reduced to zero and its difference was taken from the Ir values of the other 9 compounds, the influence of the isotope discrimination on CO_2 fixation was eliminated.[6] This calculation will result in an Ir pattern based on secondary metabolites, showing the specific pattern of each species of plants.

As shown in Fig. 4, a shaded band designates the fingerprint of yuzu with a >95% probability of significance. Several citrus essential oils other than those of yuzu, i. e., unshu, natsudaidai, lemon and hanayu, were also analyzed. All these samples' plotted patterns were clearly distinct from the yuzu fingerprint. These citrus fruits, except for hanayu, are different species from yuzu according to the taxonomy systems of both Swingle[17] and Tanaka.[18] Hanayu is said to be comparatively close to yuzu, but is obviously different from yuzu in Tanaka's taxonomy. Its aroma is similar to that of yuzu, although not as strong. Hanayu is sometimes used as a substitute for or as an imitation of genuine yuzu, and may be confused with yuzu. However, the fingerprint analysis based on the isotope ratio demonstrates a distinct difference between yuzu and hanayu. On the other hand, a yuzu sample from Korea fitted perfectly within the Japanese yuzu fingerprint band for the 10 compounds tested. These results suggested that this method could be applied to evaluate the genuineness of essential oils in addition to an enatiomeric analysis.[19,20] We selected in this experiment 10 monoterpene hydrocarbons as monitoring compounds, because all of them are present in most citrus essential oils. The data for oxygenated compounds in addition to hydrocarbons will yield a more accurate profile. It was expected that the isotope ratios of yuzu from different places of origin would trace the presented yuzu fingerprint, as shown in Fig. 4. However, not all the fruits included in the fingerprint were of the yuzu variety, because only a few kinds of citrus fruit were examined in the study. It is essential to accumulate data for many kinds of citrus essential oils, and this will be done in further research.

In conclusion, the results of this study support the notion that an isotope ratio analysis of citrus essential oils by an ordinary HRGC-MS method will be applicable for evaluating the quality, genuineness and origin of citrus fruits and their products.

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