Metabolite Profiling of Ginger (*Zingiber officinale* Roscoe) Using GC-MS and Multivariate Statistical Analysis

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Abstract:
Ginger (*Zingiber officinale* Roscoe) is a perennial plant belonging to the family Zingiberaceae, and its rhizomes have been widely used as a spice, a flavoring agent, and a medicinal plant since ancient times. There are many cultivars of ginger around the world, so discrimination between and characterization of different ginger cultivars are essential for the effective use of ginger. In this study, metabolite profiling of eight samples of ginger from Indonesia and Japan was carried out using gas chromatography-mass spectrometry (GC-MS) and multivariate statistical analysis. The results of principal component analysis (PCA) showed clear differences in composition between the ginger cultivars. It was clarified that oxygenated sesquiterpenes were detected at higher levels in ginger from Indonesia, whereas sesquiterpenes were more abundant in ginger native to Japan. In addition, a heatmap obtained based on the results of hierarchical cluster analysis showed that ginger was clustered according to the properties of each cultivar. These results are helpful for understanding the different traditional uses of ginger. Metabolite profiling using GC-MS coupled with multivariate analysis is an effective way to evaluate the overall chemical differences between the different kinds of ginger.

Keywords: *Zingiber officinale Roscoe*, *Zingiberaceae, Metabolite profiling*, *Gas chromatography-mass spectrometry (GC-MS)*, *Principal component analysis (PCA).*

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1. Introduction

Ginger (*Zingiber officinale* Roscoe) is a perennial plant belonging to the family Zingiberaceae, and its rhizomes have been widely used as a spice, a flavoring agent, and a medicinal plant since ancient times (Ravindran and Babu, 2005). Especially in traditional Asian medicine, it has been used for the treatment of catarrh, rheumatism, nervous diseases, gingivitis, toothache, asthma, stroke, constipation and diabetes (Ali et al., 2008). The medicinal value of ginger is mainly determined by the aroma and the pungency of the rhizomes (Mahboubi, 2019; Semwal et al., 2015). Essential oils responsible for the aroma have anti-bacterial, antifungal, analgesic, anti-inflammatory, anti-ulcer, immunomodulatory, and relaxing effects (Juliani et al., 2007; Mahboubi, 2019). The main pungent compounds are gingerols, and [6]-gingerol is one of the major bioactive compounds found to have anticancer, antioxidant, analgesic, antipyretic, anti-inflammatory, antidiabetic, anti-obesity, anti-allergic, antimicrobial, and antinausea activities (Juliani et al., 2007; Semwal et al., 2015).

The history of the domestication of ginger is not definitely known. However, it is known to have been cultivated and used in India and China for at least the last 2,000 years. This long period of domestication may have played a significant role in the evolution of ginger that is sterile and propagates only by vegetative reproduction (Ravindran and Babu, 2005). Currently, ginger has a rich cultivar diversity, and the main growing areas have their own cultivars. In Indonesia, there are three varieties of ginger including *Jahe merah* (small red ginger, *Z. officinale* var. *rubrum*), *Jahe emprit* (small white ginger, *Z. officinale* var. *amarum*) and *Jahe gajah* (giant ginger, *Z. officinale* var. *officinale*). These three varieties are used for different purposes. The giant ginger is usually used as a spice in cooking or flavoring for food and beverages, while the other gingers are mostly used for medicinal purposes (Supu et al., 2018). In Japan, ginger is usually classified into three groups according to the size of the rhizome: small, medium, and large. In the small group, there are ′Kintoki′, ′Yanaka′, and ′Sanshu′. The dried rhizomes of ginger ′Kintoki′ (*Z. officinale* var. *rubens* MAKINO) are native to Japan and have been used for medicinal purposes for a long time. In the medium group, there are ′Boshu′ and ′Rakuda′. In the large group, there are ′Tosa′, ′Otafuku′, and ′Oumi′. Whereas ginger with small rhizomes is usually used for medicinal purposes, the medium and large types are used in food and beverages (Iijima and Joh, 2014). These different usages of ginger imply that the overall properties are different between the cultivars. Therefore, discrimination and characterization of ginger cultivars are important for the effective use of ginger. However, discrimination between or characterization of each ginger cultivar is not possible when the classical method using only the [6]-gingerol content is applied. In addition, ginger contains a large number of compounds, and no single active ingredient is responsible for the overall efficacy. Thus, a systemic method including various metabolites (metabolite profiling) is desirable in order to distinguish between the different ginger cultivars and evaluate the overall efficacy of each of them.

Metabolite profiling requires an analytical system that can generate useful datasets and identify the compounds of interest. To date, many techniques, such as liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) have been widely employed for metabolite profiling (Wolfender et al., 2015). Among these techniques, GC-MS has the advantages of low cost compared to the other analytical methods, high reproducibility, high resolution, highly repeatable mass spectral fragmentation, and few matrix effects (Kopka, 2006). The obtained chromatographic data is often a multidimensional set, so multivariate statistical analyses are usually performed to process the complex data sets. Multivariate

statistical analyses can clarify the identification of trends between phenotype and the metabolite profile. Metabolite profiles have been obtained from various medicinal plants, including *Artemisia princeps* (Nishidono et al., 2019), *Cinnamomum cassia* (Deng et al., 2014), *Curucuma* species (Xiang et al., 2011), and *Rehmannia glutinosa* (Chang et al., 2006), using GC-MS combined with multivariate statistical analysis.

In this study, we prepared the ethyl acetate (EtOAc)-soluble fractions of eight ginger samples (Table 1) and applied GC-MS in combination with multivariate statistical analysis.

2. Experimental

(1) General Experimental Procedures

Nuclear magnetic resonance (NMR) spectra were recorded using a JNM-ECS400 NMR spectrometer (JEOL Ltd., Tokyo, Japan) with tetramethylsilane (TMS) as an internal standard. Chloroform-*d* ($CDCl_3$) was used as the solvent for sample dissolution. Gas chromatograph-mass spectrometry (GC-MS) analyses were performed using a Shimadzu QP2010 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) equipped with a Shimadzu GC2010 gas chromatography system. High-performance liquid chromatography (HPLC) analyses were performed using a Shimadzu LC-20AD pump equipped with a Shimadzu SPD-M20A photodiode array detector (Shimadzu Corporation).

(2) Plant Materials and Reagents

Eight samples of crude drugs and plant specimens (Table 1) were collected in Japan and Indonesia. All the materials in Table 1 were deposited in the Museum of Materia Medica, College of Pharmaceutical Sciences, Ritsumeikan University, under code numbers RIN-190101 (MK Red), 190102 (MK White), 180101 (SR Red), 180102 (SR Giant), 180103 (TK Oumi), 180104 (TK Kintoki), 180105 (TK Sanshu), 180106 (TK Tosa). In addition, ginger ′Kintoki′ (RIN-170101) was purchased from Kimura farm (Aichi, Japan) to isolate the standard compound. Analytical grade chemicals and chromatographic solvents of liquid chromatography-mass spectrometry (LC-MS) grade were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Table 1. Materials Used in Present Study

Local name	English name	Original plant	Production area	Rhizome size	Purchased (collected) from	Label
Jahe merah	Red ginger	*Zingiber officinale* var. *rubrum*	Indonesia	Small	M&K Laboratories Co., Ltd., Nagano, Japan	MK Red
Jahe emprit	Small white ginger	*Z. officinale* var. *amarum*	Indonesia	Small	M&K Laboratories Co., Ltd., Nagano, Japan	MK White
Jahe merah	Red ginger	*Z. officinale* var. *rubrum*	Indonesia	Small	Crude drug market, Surakarta, Indonesia	SR Red
Jahe gajah	Giant ginger	*Z. officinale* var. *officinale*	Indonesia	Large	Crude drug market, Surakarta, Indonesia	SR Giant
Kintoki shoga	Kintoki ginger	*Z. officinale* var. *rubens* MAKINO	Japan	Small	Takii & Co., Ltd., Kyoto, Japan	TK Kintoki
Sanshu shoga	Sanshu ginger	*Z. officinale* var. *rubens* MAKINO	Japan	Small / Middle	Takii & Co., Ltd., Kyoto, Japan	TK Sanshu
Oumi shoga	Oumi ginger	*Z.officinale* var. *macrorhizomum* MAKINO	Japan	Large	Takii & Co., Ltd., Kyoto, Japan	TK Oumi
Tosa shoga	Tosa ginger	*Z. officinale* var. *macrorhizomum* MAKINO	Japan	Large	Takii & Co., Ltd., Kyoto, Japan	TK Tosa

(3) Isolation of Standard Compounds

Fresh rhizomes of Kintoki ginger purchased from Kimura farm (2 kg) were pulverized and extracted with acetone (1.5 L, 1h × 3) under reflux, then the solvent was evaporated *in vacuo* to yield the acetone extract (45.4 g). The acetone extract was suspended in water and extracted with ethyl acetate (EtOAc) to give the EtOAc-soluble fraction (14.8 g) and the water-soluble fraction (27.1 g). The EtOAc-soluble fraction (12 g) was subjected to a Wakogel C-200 column chromatography (FUJIFILM Wako Pure Chemical Corporation) using a solvent gradient elution [EtOAc−*n*-hexane (20:80 → 30:70 → 40:60 → 30:70 (v/v)) → EtOAc] to yield thirteen fractions [fr. 1: 240 mg, fr. 2: 220 mg, fr. 3: 400 mg, fr. 4: 380 mg, fr. 5: 280 mg, fr. 6: 2780 mg, fr. 7: 780 mg, fr. 8: 2130 mg, fr. 9: 430 mg, fr. 10: 880 mg, fr. 11: 460 mg, fr. 12: 510 mg, fr. 13: 2110 mg]. Fraction 6 (2.76 g) was further purified by a Wakogel C-200 column chromatography with chloroform to yield six fractions [fr. 6-1: 18 mg, fr. 6-2: 58 mg, fr. 6-3: 218 mg, fr. 6-4: 667 mg, fr. 6-5: 990 mg, fr. 6-6: 686 mg]. Fr. 6-3 was identified as galanolactone by comparing their NMR spectral data with those reported (Morita and Itokawa, 1988). Standard gingerols ([6]-, [8]-, [10]-gingerol) have previously been isolated from the rhizomes of *Z. officinale* Roscoe (Nishidono et al., 2018).

(4) Analytical Sample Preparation

The samples were individually pulverized. Fifteen grams of fine powder from each sample was extracted with methanol (140 mL) under reflux for 100 min using an Extraction System B-811 LSV (BUCHI, Flawil, Switzerland) to give methanol extracts. The resulting extracts were suspended in water and extracted with ethyl acetate (EtOAc). These layers were concentrated to prepare the EtOAc-soluble fraction and the water-soluble fraction. The respective EtOAc-soluble fractions were dissolved in ethyl acetate at a concentration of 1 mg/mL, and 1 μL samples were injected into the GC-MS. In addition, the respective EtOAc-soluble fractions were dissolved in methanol at a concentration of 1 mg/mL and filtered through a 0.45 μm Millipore filter unit (Advantec, Tokyo, Japan). Five microliters of each filtrate were injected into the HPLC.

(5) GC-MS Analysis

GC-MS analyses were performed using a DB-5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm, Agilent Technologies, Santa Clara, CA, USA). The GC parameters were as follows: the injector and transfer line were maintained at 270 °C. The oven temperature was programmed as follows: initial temperature, 50 °C; initial hold time, 3 min; temperature ramp-up rate, 10 °C/min; final temperature, 300 °C; final hold time, 5 min. The flow rate of the carrier gas (helium) was 1 mL/min. The mass spectrometry conditions were as follows: ionization mode, electron ionization (EI) mode; ionization current, 60 μA; ionization voltage, 70 eV. Each sample was analyzed in triplicate. Eluted compounds were identified using the NIST (http://www.nist.gov/srd/nist1a.cfm), the Wiley NBS, the mass spectral data book (Adams, 2007), and by referring to publications (Arze et al., 2008; Chen and Ho, 1988; Halim and Collins, 1975; Hong and Oh, 2012; Jolad et al., 2004; Jolad et al., 2005; Scott et al., 1987). The retention index (RI) of each compound was calculated using the standard alkane mixture (GL Sciences, Tokyo, Japan) as a reference.

(6) HPLC Analysis

HPLC analyses were performed using a Waters Atlantis T3 column (2.1mm i.d. × 150 mm, 5 μm). The column temperature was maintained at 40 °C and eluted compounds were detected by

monitoring the UV absorbance at 280 nm. The mobile phase was a binary eluent of (A) 5 mM CH_3COONH_4 solution and (B) CH_3CN under the following gradient conditions: 0–30 min, linear gradient from 10 % to 100 % B, 30–40 min, isocratic at 100 % B. The flow rate was 0.2 mL/min.

(7) Quantitative Analysis

Standard gingerols ([6]-, [8]-, [10]-gingerol) were accurately weighed and dissolved in methanol to make stock solutions of 1.0 mg/mL. These stock solutions were serially diluted to obtain calibration standard solutions. Calibration was performed in the range of 0.2–0.8 mg/mL using dilutions of the respective stock solutions. A calibration curve for each standard compound was prepared by plotting the respective peak area *vs* the injection amounts (ng) at three different concentrations.

(8) Multivariate Data Analysis

In this study, many metabolites in the rhizomes of ginger were analyzed. In a nontargeted metabolomics analysis like this study, finding suitable internal standards for all the metabolites and creating calibration curves is impractical. Thus, the use of raw signal intensity data has been accepted in metabolomics analysis (Matsuda, 2016). Each sample is represented by a GC-MS total ion current (TIC). The peaks of TIC were detected (slope =10,000 min^{-1}; width = 3 s; minimal area =100,000; no smoothing), and the absolute peak areas were determined using the Shimadzu GCMS Solution software (Shimadzu corporation). The original data set was constructed using the data regarding the sample names, the metabolite numbers (variable indices), and the absolute peak areas (variables). Then, variable reduction of the original data set was carried out by calculation of the Fisher ratio (Pierce et al., 2006). The Fisher ratio is calculated by the square of the difference of the average areas of the analyte present in the different classes divided by the sum of the variance of the analyte area inside the same class (Fisher, 1936). Variables with a small Fisher ratio (less than one hundred) were removed from the original data set to create a new analytical data set. Statistical analysis was applied to this new analytical data set to clarify the characteristic properties in the chemical profiles of eight ginger samples objectively. Principle component analysis (PCA) and hierarchical cluster analysis (HCA) were carried out with R software (Version 3.6.1, https://www.r-project.org). Prior to the statistical analysis, the absolute peak area values were auto-scaled (the mean area value of each peak throughout all samples was subtracted from each individual absolute peak area value and the result divided by the standard deviation).

3. Results and Discussion

(1) GC-MS Peak Identification

Each EtOAc-soluble fraction derived from the ginger rhizomes was prepared and analyzed by GC-MS. The GC-MS total ion current (TIC) chromatograms of the eight ginger samples are shown in Figure 1. Ninety-eight volatile compounds detected by GC-MS are listed in Table 2. Of these, ninety-two volatile compounds were identified. The identification of peaks **1**-**38**, **40**-**45**, **48**-**51**, **53** and **58** was performed by using a library search program (the NIST and the Wiley Mass Spectral library) and verified by comparing the retention index (RI) and mass spectral data with those given in the literature (Adams, 2007). Peak **52** was identified by comparing the molecular ion peak and fragmentation patterns with those reported (Hong and Oh, 2012; Scott et al., 1987). The identification of peaks **55** and **56** was performed based on the fragmentation patterns and RI (Arze et al., 2008; Chen and Ho,

1988; Halim and Collins, 1975). The gingerol-related compounds (peaks **59**, **60**, **62-92**, **94-98**) were identified by comparing the molecular ion peak and the fragmentation patterns with those reported (Jolad et al., 2004; Jolad et al., 2005). Peak **93** was identified from the retention time and mass fragmentation patterns using an authentic compound. Most of the peaks detected by GC-MS were readily identified whereas several peaks found at high intensity could not be identified. Ma and Gang have identified the peak with a molecular ion at 180 (*m*/*z*) eluted after germacrene B, peak **39** in this study, as coniferyl alcohol based on the NIST library search program (Ma and Gang, 2006). Although the similarity between the mass spectral data for peak **39** and those of (*E*)-coniferyl alcohol was 82% in this study, the RI of peak **39** did not match that of (*E*)-coniferyl alcohol. The RI of peak **39** in this study was 1577, whereas that of (*E*)-coniferyl alcohol given in the literature is 1733. Therefore, peak **39** was left as an unknown compound. Peaks **46** and **47** could not be identified. Ma and Gang also could not identify these peaks, and they labeled these peaks as DRG-GM1-N1-29.99-222-119-17 and DRG-GM1-N1-30.19-220-91-69, respectively (Ma and Gang, 2006). In this study, we used these labels to annotate peaks **46** and **47**.

(2) Analysis of Gingerols by GC-MS and HPLC

GC-MS provides the advantage of both chromatography as a separation method and mass spectrometry as an identification method and is the most preferable method for the analysis of volatile components in crude drugs (Sermakkani and Thangapandian, 2012). On the other hand, there are several disadvantages for analyzing gingerols in ginger. One of the disadvantages is that [6]-gingerol analysis by GC-MS has been shown to produce [6]-shogaol, zingerone, and hexanal as artifacts (Chen and Ho, 1987; Harvey, 1981). No degradation of other compounds in ginger extracts by GC-MS has been reported. In this study, GC-MS analysis was applied to a [6]-gingerol standard, the purity of which was greater than 95% determined by HPLC, and five peaks were detected (Fig. 2). Of these, [6]-isoshogaol was newly identified as a degradation product of [6]-gingerol. The other gingerols ([8]-, [10]-gingerol) also produced corresponding artifacts. These results indicate that GC-MS analysis does not give an accurate representation of the actual gingerol contents in the samples. Therefore, the gingerols ([6]-, [8]-, [10]-gingerols) in each EtOAc-soluble fraction were quantitatively analyzed by HPLC. Schwertner and Rios showed that HPLC analysis does not lead to degradation of any of the gingerols, unlike GC-MS analysis (Schwertner and Rios, 2007). The gingerol content in each EtOAc-soluble fraction is shown in Table 3. The amount of total gingerols varies from 113 to 299 mg/g and the amounts of [6]-, [8]-, and [10]-gingerols vary from 68 to 200, 15 to 36, and 17 to 63 mg/g, respectively. [6]-gingerol was identified as the major gingerol in all samples, and [8]- and [10]-gingerols were found at lower concentrations. The same cultivar, MK Red and SR Red, had different gingerol contents. This implies that the amount of gingerol may depend on other factors (geographical origin, harvest time, cultivation conditions, and post-harvest processes) in addition to the cultivar. As there are large within-cultivar differences of gingerol contents, we were unable to discriminate among the ginger cultivars based only on the amount of one compound such as [6]-gingerol. Therefore, chromatographic data obtained by GC-MS was applied to multivariate statistical analyses in order to clarify the overall properties of each ginger cultivar.

(3) Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA)

Principal component analysis (PCA) is an unsupervised method widely used to reduce high dimensional data to a smaller set using new variables (principal components, PCs). PCA has been used

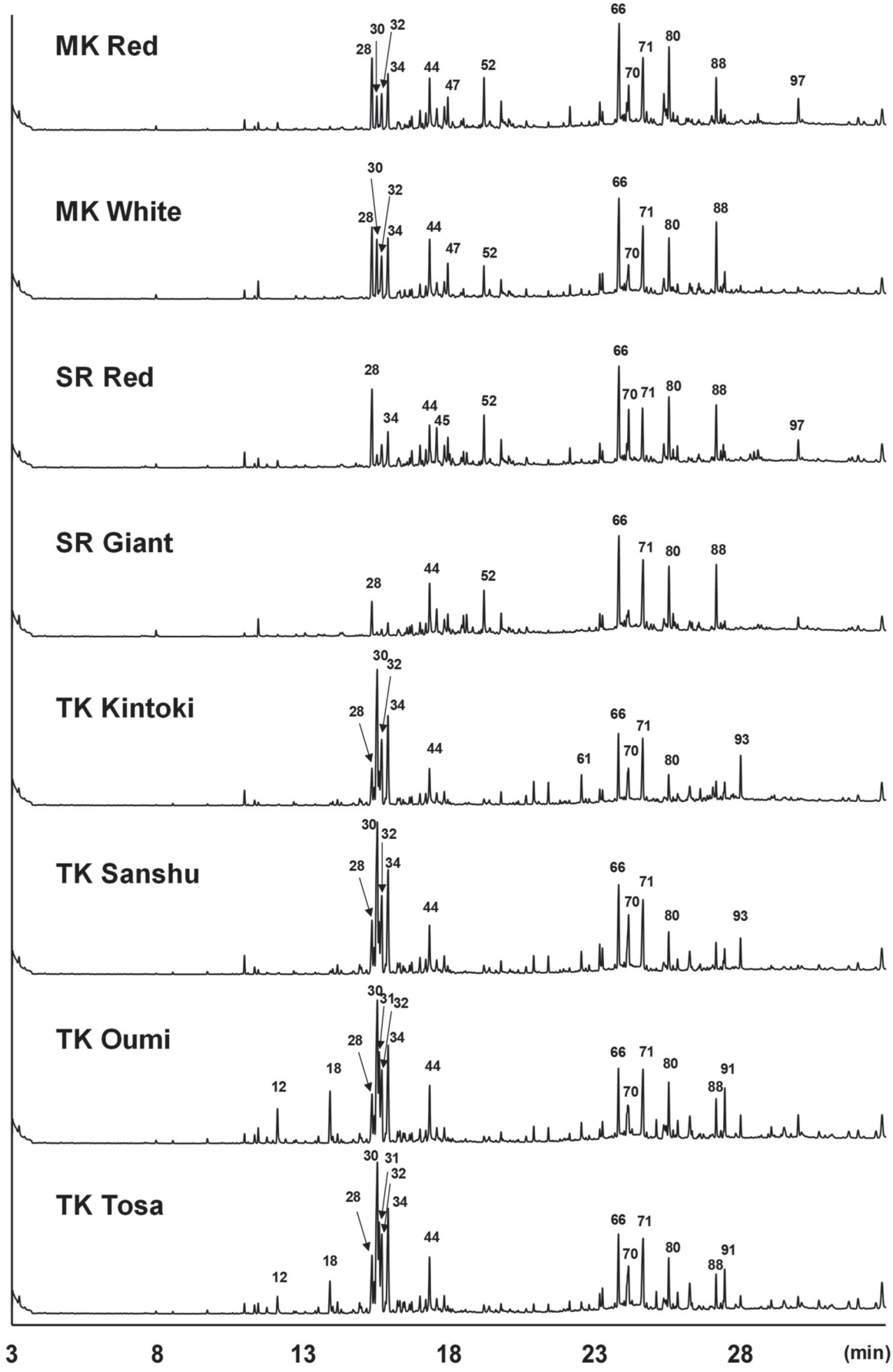

Figure 1. Total Ion Current (TIC) Chromatograms of EtOAc-soluble Fractions of Eight Ginger Samples

Table 2. Volatile Compounds in EtOAc-soluble Fractions of Eight Ginger Samples

Peak No.	Rt (min)	RI	M^+ (*m*/*z*)	Base peak (*m*/*z*)	Compounds
1	3.24		100	57	Hexanal
2	7.96	1007	128	57	Octanal
3	8.03	1011	136	93	β-Phellandrene
4	8.53	1040	154	81	1,8-Cineol
5	9.72	1101	154	71	Linalool
6	10.99	1179	154	95	Borneol
7	11.11	1186	154	71	Terpinen-4-ol
8	11.33	1199	154	59	α-Terpineol
9	11.46	1207	156	57	Decanal
10	11.76	1228	156	69	β-Citronellol
11	11.96	1242	152	69	Neral
12	12.12	1253	154	69	Geraniol
13	12.39	1270	152	69	Citral
14	12.68	1289	196	95	Bornyl acetate
15	13.07	1316	150	150	4-Vinylguaiacol
16	13.42	1341	204	121	δ-Elemene
17	13.53	1349	198	81	Citronelly acetate
18	13.92	1377	196	69	Geranyl acetate
19	14.02	1384	204	105	α-Copaene
20	14.18	1395	204	93	β-Elemene
21	14.28	1402	152	151	Vanilin
22	14.31	1405	204	119	Sesquithujene
23	14.72	1437	204	121	γ-Elemene
24	14.75	1440	204	93	*trans*-α-Bergamotene
25	14.94	1454	204	69	(*E*)-β-Farnesene
26	15.01	1459	204	69	Sesquisabiene
27	15.18	1472	204	91	Alloaromadendrene
28	15.36	1486	202	132	ar-Curcumene
29	15.44	1492	204	161	Germacrene-D
30	15.54	1499	204	93	α-Zingiberene
31	15.60	1504	204	93	(*E*,*E*)-α-Farnesene
32	15.69	1512	204	69	β-Bisabolene
33	15.83	1523	204	161	γ-Cadinene
34	15.91	1530	204	69	β-Sesquiphellandrene
35	16.24	1557	222	59	Elemol
36	16.28	1560	222	69	(*Z*)-Nerolidol
37	16.32	1563	222	69	(*E*)-Nerolidol
38	16.43	1572	204	121	Germacrene B
39	16.48	1577	180	137	Not identified[1]
40	16.56	1583	218	119	ar-Turmerol
41	16.73	1596	222	69	*cis*-Sesquisabinene hydrate
42	17.01	1620	222	69	Zingiberenol
43	17.21	1638	222	69	*trans*-Sesquisabinene hydrate
44	17.34	1649	194	137	Zingerone
45	17.59	1670	216	83	ar-Turmerone
46	17.84	1692	222	137	RG-GM1-N1-29.99-222-119-137[2]
47	17.97	1702	220	69	RG-GM1-N1-30.19-220-91-69[3]
48	18.03	1708	218	120	β-Turmerone
49	18.50	1751	218	136	Xanthorrhizol
50	18.61	1761	206	161	Ethyl-*p*-methoxycinnamate
51	18.83	1780	218	119	(*E*)-Nuciferol
52	19.20	1815	238	69	4,5-Dihydroxybisabola-2,10-diene
53	19.39	1834	240	149	Cryptomeridiol
54	19.79	1872	238	109	Not identified[4]
55	20.63	1953	272	119	Pentyl curcumene
56	20.65	1955	270	119	Pentenyl curcumene
57	20.91	1981	272	69	Not identified[5]
58	21.41	2033	290	69	(*E*,*E*)-Geranyl linalool
59	21.93	2088	248	137	[4]-Shogaol
60	22.16	2112	274	137	1-(4-Hydroxy-3-methoxyphenyl)-2,4-dehydro-6-decanone
61	22.55	2154	290	69	Not identified[6]
62	22.81	2183	266	137	[4]-Gingerol
63	23.18	2224	276	137	[6]-Isoshogaol
64	23.28	2235	278	137	[6]-Paradol
65	23.70	2284	280	137	[6]-Dihydroparadol
66	23.82	2297	276	137	[6]-Shogaol
67	24.02	2321	322	137	Acetoxy-[6]-dihydroparadol
68	24.10	2331	352	137	Diacetoxy-[4]-gingerdiol
69	24.13	2335	292	137	[6]-gingerdione
70	24.17	2339	308	137	5-Methoxy-[6]-gingerol
71	24.66	2397	294	137	[6]-gingerol
72	24.93	2431	304	137	[8]-isoshogaol
73	25.07	2447	306	137	[8]-paradol
74	25.12	2454	336	137	Acetoxy-[6]-gingerol
75	25.34	2481	296	137	[6]-gingerdiol
76	25.38	2485	296	137	Stereoisomer of **75**
77	25.43	2491	338	137	5-Acetoxy-[6]-gingerdiol
78	25.48	2497	338	137	Stereoisomer of **77**
79	25.54	2506	380	137	Diacetoxy-[6]-gingerdiol
80	25.55	2507	304	137	[8]-Shogaol
81	25.69	2524	394	151	4'*O*-Methyl-diacetoxy-[6]-gingerdiol
82	25.85	2543	336	137	5-Methoxy-[8]-gingerol
83	26.27	2592	290	177	1-Dehydro-[6]-gingerdione
84	26.34	2609	322	137	[8]-Gingerol
85	26.58	2640	332	137	[10]-Isoshogaol
86	26.73	2660	334	137	[10]-Paradol
87	27.03	2700	408	137	Diacetoxy-[8]-gingerdiol
88	27.17	2720	332	137	[10]-Shogaol
89	27.33	2742	408	137	[10]-Gingerdiol, cyclic methyl orthoester
90	27.42	2754	364	137	5-Methoxy-[10]-gingerol
91	27.47	2762	348	137	[10]-Gingerdione
92	27.85	2814	318	177	1-Dehydro-[8]-gingerdione
93	28.01	2836	318	81	Galanolactone
94	28.73	2931	360	137	[12]-Shogaol
95	29.06	2971	376	137	[12]-Gingerdione
96	29.50	3023	346	177	1-Dehydro-[10]-gingerdione
97	29.98	3077	430	137	(2*E*)-Geranial acetal of **75**
98	32.66	3359	460	137	Diacetoxy-1,7-bis-(4*O*-hydroxy-3*O*-methoxyphenyl)heptane

[1]Peak **39**, EI-MS *m*/*z* (%): 180 (M^+, 42), 137 (100), 124 (39), 122 (23), 109 (14), 94 (12), 91 (18), 77 (14).
[2]Peak **46**, EI-MS *m*/*z* (%): 222 (M^+, 0.2), 137 (100), 119 (90), 109, (52), 93 (48),84 (82), 69 (72), 55 (54).
[3]Peak **47**, EI-MS *m*/*z* (%): 220 (M^+, 0.9), 118 (29), 109 (18), 91 (19), 81 (15), 79 (15), 69 (100), 55 (26).
[4]Peak **54**, EI-MS *m*/*z* (%): 238 (M^+, 0.9), 135 (52), 109 (100), 100 (35), 95 (35), 71 (63), 69 (69), 55 (47).
[5]Peak **57**, EI-MS *m*/*z* (%): 272 (M^+, 0.6), 132 (44), 119 (86), 105 (44), 93 (36), 91 (29), 69 (100), 55 (30).
[6]Peak **61**, EI-MS *m*/*z* (%): 290 (M^+, 0.1), 135 (20), 121 (32), 107 (19), 93 (36), 79 (15), 69 (100), 55 (26).

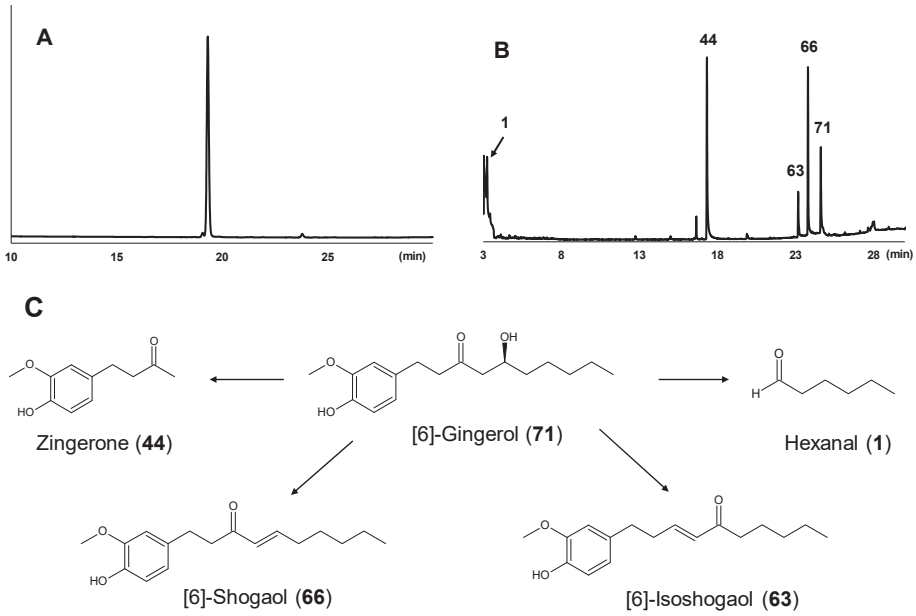

Figure 2. HPLC and GC-MS Analysis of [6]-gingerol
A: HPLC chromatogram of [6]-gingerol standard. B: GC-MS/TIC chromatogram of [6]-gingerol standard.
C: Scheme of degradation of [6]-gingerol standard induced by GC-MS analysis.

Table 3. Concentration of the Gingerols (mg/g) in the Dried EtOAc-soluble Fractions of Eight Ginger Samples

	Indonesia				Japan			
	Small			Large	Small		Large	
	MK Red	MK White	SR Red	SR Giant	TK Kintoki	TK Sanshu	TK Oumi	TK Tosa
[6]-Gingerol	110	129	68	138	110	150	127	200
[8]-Gingerol	29	22	21	41	15	17	29	36
[10]-Gingerol	47	43	24	61	17	21	60	63
Total content	186	195	113	240	142	188	216	299

as an effective method for the differentiation of many ginger samples. Kumar et al. applied PCA to clarify the genetic diversity of ginger collected from the North-Eastern region of India using morphological characters as variables (Kumar et al., 2016). In the present study, to clarify the differences between the volatile constituents in the eight ginger samples, PCA was carried out using forty-one of the ninety-eight peaks obtained by GC-MS analysis. The PCA score plots are shown in Figures 3A and 3B, where the first three PCs account for 81.5 % of the total variance (PC1, 52.1 %; PC2, 16.3 %, PC3, 13.1). PC1 describes the differences between the cultivars from Indonesia and Japan (Fig. 3A). One group located in the positive region of PC1 is composed of ginger samples produced in Indonesia, and the other group located in the negative region of PC1 is represented by ginger samples from Japan. Fig. 3C shows the metabolites that contributed to the separation of PC1. In the chemometric analysis, the peaks with high loading values can be considered as markers that strongly contribute to the classification of the samples by PCA, so compounds with higher loading on PC1 (both positive and negative) are shown in Table 4-1 and Figure 4. These results show that oxygenated sesquiterpenes were present at higher levels in ginger from Indonesia, whereas sesquiterpenes were more abundant in ginger native to Japan. It has been reported that sesquiterpenes such as α-zingiberene, β-bisabolene, and β-sesquiphellandrene, which are abundant in cultivars produced in Japan, exhibit anti-rhinovirus activity (Denyer et al., 1994). Rhinoviruses are the most common cause of common colds in all age groups (Heikkinen and Jarvinen, 2003). Therefore, these

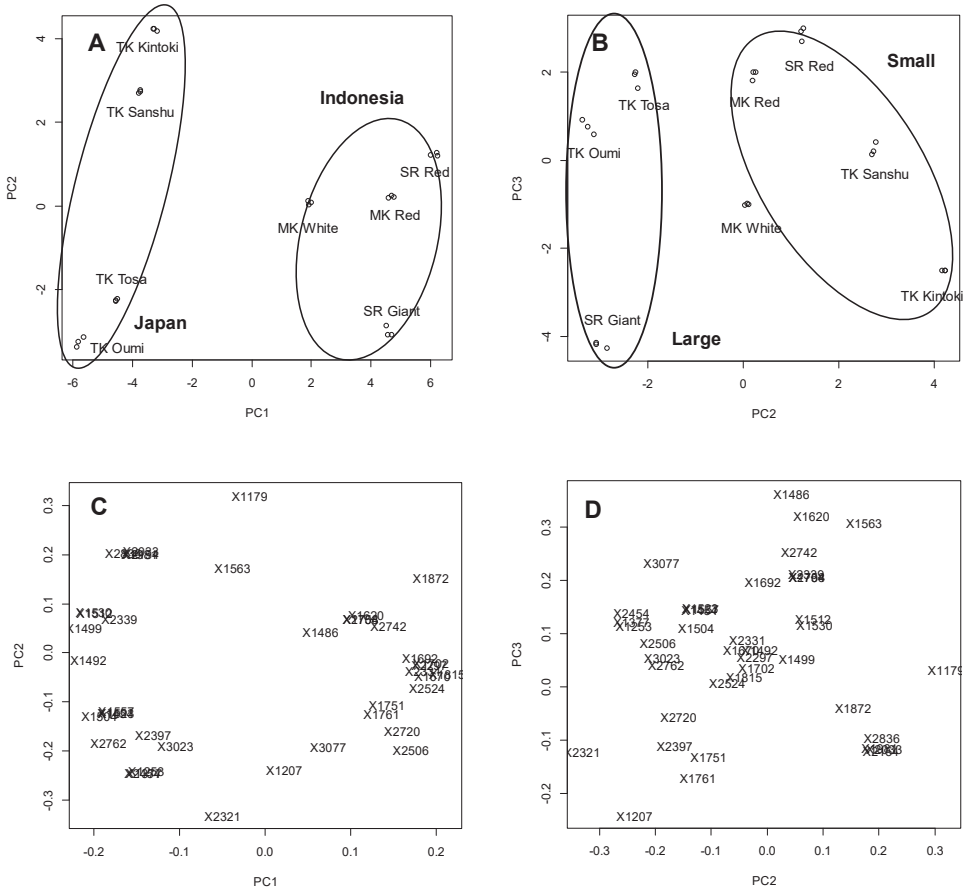

Figure 3. Results of Principal Component Analysis (PCA)

A: PCA score plot with respect to PC1 and PC2. B: PCA score plot with respect to PC2 and PC3.
C: PCA loading plot with respect to PC1 and PC2. D: PCA loading plot with respect to PC2 and PC3.
The numbers on the loading plot correspond to the RI of each compound.

results suggest that ginger produced in Japan is more suitable than ginger from Indonesia when used to treat common colds. Actually, in traditional Japanese medicine, Kampo, the formulas used in the early stages of a common cold contain ginger. In contrast, in traditional Indonesian medicine, Jamu, ginger is not included in the formulas used in the early stages of common colds and was not used for the treatment of common cold (Takahashi, 1988).

PC2 contains information on the differences between the sizes of the rhizomes. One group located in the positive region of PC2 is composed of small type ginger, and the other group located in the negative region of PC2 is represented by ginger with large rhizomes. The metabolites that contributed to the separation in PC2 are shown in Figure 3D, and compounds with higher loading on PC2 (both positive and negative) are shown in Table 4-2 and Figure 4. Higher levels of borneol and diterpenoids were found in cultivars with small rhizomes, and acetylated compounds were more abundant in large type ginger. In both Indonesia and Japan, ginger with large rhizomes are usually used for food and beverages, while ginger with small rhizomes are mostly used for medicinal purposes. Galanolactone which was determined to be a characteristic compound of the small type ginger has many biological activities including antifungal (Morita and Itokawa, 1988), antiplasmodial (Duker-Eshun et al., 2002), anti-HIV-1 (Chareonkla et al., 2011), anti-obesity (Ahn and Oh, 2012), and 5-HT_3 antagonistic activities (Huang et al., 1991). Therefore, it is suggested that galanolactone is one of the important compounds in ginger that bestows it with medicinal qualities. These PCA results are beneficial for understanding the different usages of different ginger cultivars.

Table 4-1. Compounds with Higher Loading of PC1

Loading on PC1	RI	Compounds	Absolute peak areas							
			Indonesia				Japan			
			MK Red	MK White	SR Red	SR Giant	TK Oumi	TK Kintoki	TK Sanshu	TK Tosa
0.213	1815	4,5-Dihydroxy bisabola-2,10-diene (**52**) [1]	5853415	3418900	5840035	5439123	0	686536	0	0
0.196	1670	ar-Turmerone (**45**) [1]	2947905	2137060	4660810	3555459	1333690	1528755	1433413	1685716
0.194	1702	RG-GM1-N1-30.19-220-91-69 (**47**) [1]	3375153	3947526	3253483	2252718	0	0	0	0
-0.200	1530	β-Sesquiphellandrene (**34**) [2]	7667675	8728757	4833690	1094664	13896766	16859497	15038749	16349100
-0.206	1492	Germacrene-D (**29**) [2]	0	0	0	0	2302832	3316523	3407001	3947037
-0.211	1499	α-Zingiberene (**30**) [2]	4429393	8379772	1884146	0	23518576	28410826	26218744	28194737

[1] Oxygenated sesquiterpenes. [2] Sesquiterpenes.

Table 4-2. Compounds with Higher Loading of PC2

Loading on PC2	RI	Compounds	Absolute peak areas							
			Small					Large		
			MK Red	MK White	SR Red	TK Kintoki	TK Sanshu	SR Giant	TK Tosa	TK Oumi
0.320	1179	Borneol (**6**) [1]	0	0	1456819	1454311	1718555	0	0	0
0.210	2033	(*E*,*E*)-Geranyl linalool (**58**) [2]	0	0	0	2213363	1592592	0	1309256	0
0.205	2836	Galanolactone (**93**) [2]	0	0	0	4709270	3133835	0	2244875	1346375
-0.242	1377	Geranyl acetate (**18**) [1, 3]	0	0	0	0	0	0	7155938	4208380
-0.243	2454	Acetoxy-[6]-gingerol (**74**) [3, 4]	0	0	0	0	0	0	1712368	1556880
-0.331	2321	Acetoxy-[6]-dihydroparadol (**67**) [3,4]	0	0	0	0	0	1501858	1145806	1196477

[1] Monoterpenes. [2] Diterpenes. [3]Acetylated compounds. [4] Gingerol-related compounds.

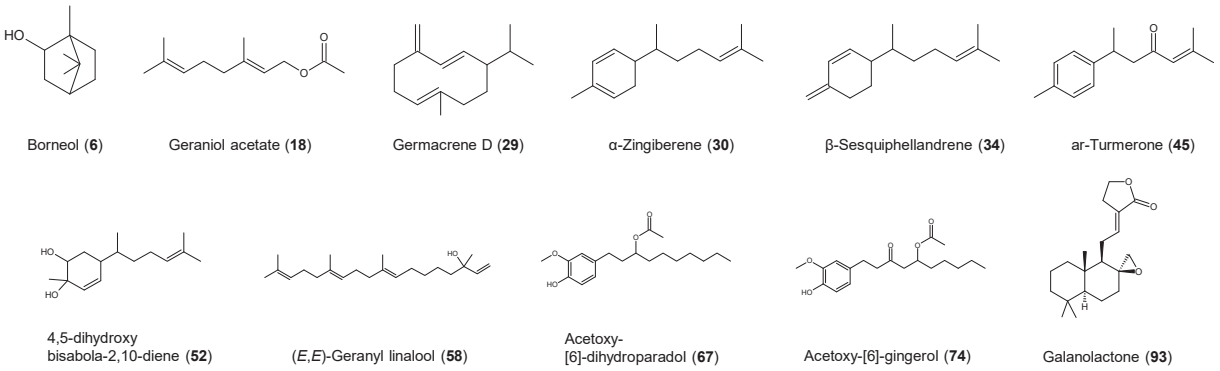

Figure 4. Chemical Structures of Identified Compounds with Higher Loading on PC1 and PC2

Although the PCA score plot provides some clues for grouping, the available PCs are limited because only three PCs can be displayed graphically. In addition, score plots do not give information on the closeness between groups. On the other hand, Hierarchical Cluster Analysis (HCA) can provide further information on these aspects. HCA is another unsupervised method, and its results can be easily visualized using a tree-based representation, which can clearly show the classification of the samples (Granato et al., 2018). A heatmap developed based on the HCA results is shown in Figure 5. When performing cluster analysis, Ward's method of agglomeration and Euclidean distances were used to evaluate the similarity between the samples. The heatmap data show that all of the ginger samples are divided into two major branches that consist of samples from Indonesia and Japan. High peak intensities for sesquiterpenes were found in the branch consisting of ginger from Japan. In the branch consisting of ginger produced in Indonesia, the peak intensities of oxygenated sesquiterpenes were high. These results were almost the same as those from PCA, showing that classification of the eight ginger samples is according to their country of origin.

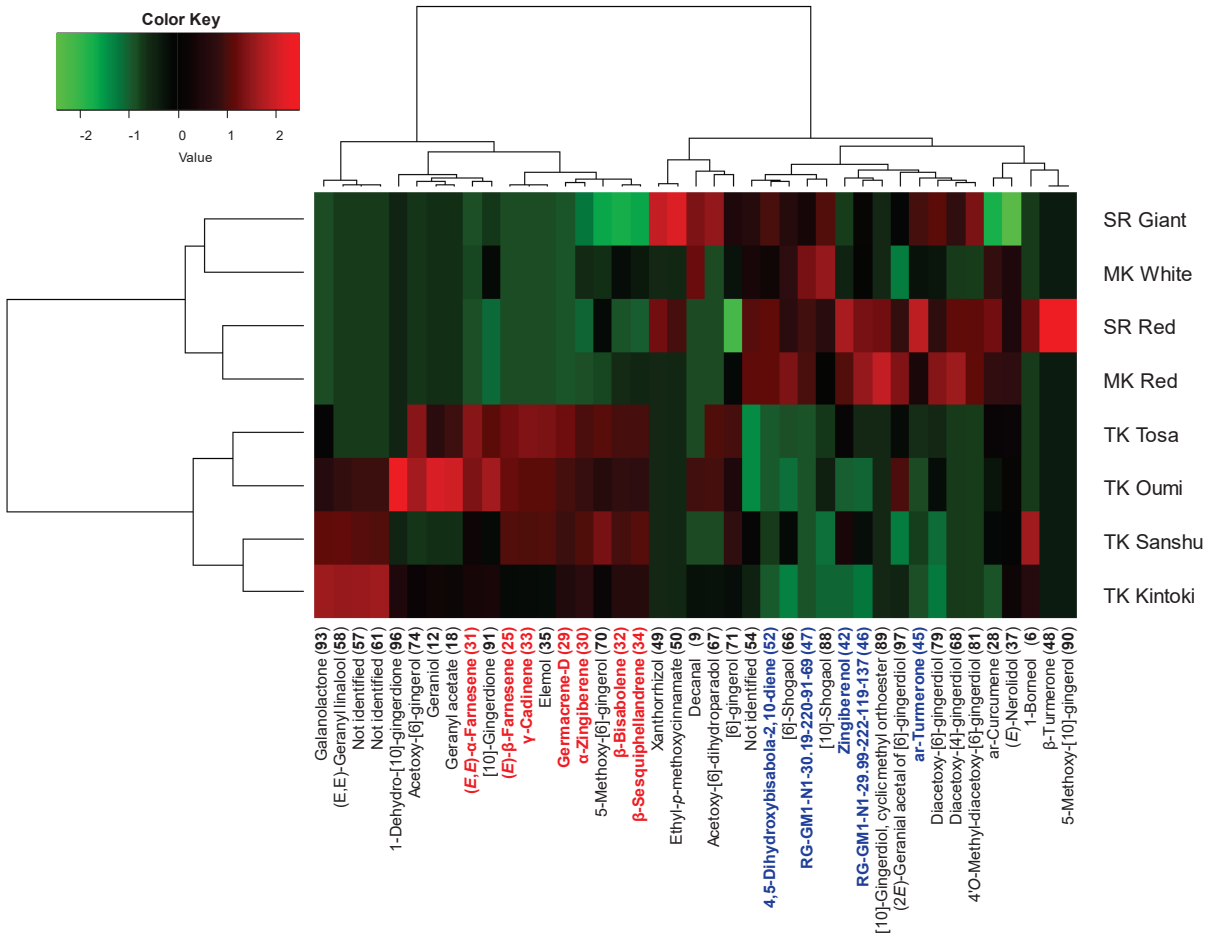

Figure 5. A Heatmap of Eight Ginger Samples Combined with a Hierarchical Clustering Dendrogram
The eight ginger samples are represented by the rows, and forty-one peaks are represented by the columns.
The sesquiterpenes and oxygenated sesquiterpenes are shown in red and blue, respectively.
The color bar shows the relative content, green: lowest, red: highest.

4. Conclusion

Metabolic profiling using GC-MS coupled with multivariate statistical analysis provided chemical differentiation of the various ginger samples. The levels of metabolites in ginger were clearly different between the different cultivars, according to the results of PCA and HCA. The PC loadings were examined in order to obtain information on the chemical basis for the clustering behavior. High levels of oxygenated sesquiterpenes (4,5-dihydroxy bisabola-2,10-diene and ar-turmerone) were found in ginger produced in Indonesia, and the levels of sesquiterpenes (α-zingiberene, germacrene-D, and β-sesquiphellandrene) in samples from Japan were much higher than in those from Indonesia. In addition, it was clarified that ginger with large rhizomes has more acetylated compounds, and ginger with small rhizomes has more diterpenoids (galanolactone and (*E*,*E*)-geranyl linalool) and borneol. The results of HCA were almost the same as those from PCA, and the eight ginger samples could be classified according to where they were from, either Indonesia or Japan. These results are helpful for understanding the different uses of ginger. This study showed that the metabolite profiling strategy using GC-MS in combination with multivariate data analysis can be further applied for investigating the effect of other factors such as storage, harvesting time and/or seasonal variation on the volatile metabolites contained in ginger.

Acknowledgements

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