Analysis of Volatile Compounds from Chili Peppers and Characterization of Habanero (Capsicum chinense) Volatiles

Yusuke Murakami¹*, Hisakatsu Iwabuchi¹, Yukie Ohba², and Harukazu Fukami³

¹ San-Ei Gen F.F.I., Inc. 1-1-11 Sanwa-cho, Toyonaka, Osaka 561-8588, JAPAN
² Suntory Foundation for Life Sciences, Bioorganic Research Institute, 8-1-1, Seikadai, Seika-cho, Soraku-gun, Kyoto 619-0284, JAPAN
³ Department of Bioscience and Biotechnology, Faculty of Bioenvironmental Science, Kyoto University of Advanced Science, 1-1 Nanjyo, Sogabe-cho, Kameoka-city, Kyoto 621-8555, JAPAN

Abstract: The Habanero pepper is characterized by its strong pungency and fruity aroma. The aim of the present study was to extract the volatile compounds of Habanero peppers, using solvent extraction and solvent-assisted flavor evaporation (SAFE) methods, and to analyze them using gas chromatography-mass spectrometry (GC-MS). The analysis detected 66 volatile compounds, including 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1, which was reported previously, and 6-methyl-(E)-4-heptenyl 2-methylpropanoate 2, the corresponding butanoate 3, 2-methylbutanoate 4, and 6-methyl-(E)-4-heptenol 5, which were found in both Habanero and other peppers. 6-Methyl-(E)-4-heptenyl 3-methylbutanoate 1 and related compounds were synthesized. Furthermore, principal component analysis (PCA) and hierarchical cluster analysis (HCA) of the volatile profiles generally grouped the pepper samples by species and indicated that Habanero peppers are characterized by the presence of 6-methyl-(E)-4-heptenyl esters.

Key words: Habanero peppers, Capsicum chinense, volatile compounds, solvent-assisted flavor evaporation (SAFE), 6-methyl-(E)-4-heptenyl 3-methylbutanoate, Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA)

1 Introduction
The Habanero pepper (Capsicum chinense) is one of the main cultivated Capsicum species and is characterized by its strong pungency and fruity aroma. Previous analysis of Habanero fruit resulted in the isolation of 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1—a major component and a potential source of the Habanero pepper’s fruity aroma—and the characterization of the compound’s structure, including its double-bond configuration. There are five major cultivated species of the pepper, and the volatile profile of C. chinense has been compared to those of C. annuum and C. frutescens. However, the volatile profile of C. chinense has yet to be fully reported. Accordingly, the aim of the present study was to analyze the volatile profiles of 11 pepper varieties from all five of the major cultivated species and to determine the specific compound(s) responsible for the Habanero pepper’s unique aromatic characteristics.

2 Experimental Procedures
2.1 Plant materials and volatile extraction
Habanero peppers, Red Savina, and Habanero Orange, were purchased from Shino Farm Limited Co. in 2017, and all other samples were purchased from retail stores (Table 1). The samples were prepared for GC-MS analysis using a modified version of Manikharda’s method. The peduncle, seeds, placenta, and dissepiment were removed from each pepper, and the remaining tissues were frozen and pulverized. A portion of each prepared sample (100 g) was transferred to a 1-L beaker and slowly mixed with 1 mL 3-heptanol (100 μg/mL in methanol), as an internal standard, 200 mL saturated aqueous NaCl (200 mL), and 200 mL dichloromethane (DCM). The resulting mixture was gently stirred using a magnetic stirrer for 1 h at 25°C, and the DCM layer was dried over anhydrous Na₂SO₄. Finally, volatile compounds were separated from non-volatile compounds in the DCM extract using solvent-assisted flavor
evaporation (SAFE)\(^5\), distilled, and then concentrated to a final volume of \(\sim 1\) mL.

### 2.2 Volatile analysis

GC-MS and GC-time-of-flight MS (GC-TOF MS) analysis were performed using a 5975C inert XL MSD (Agilent Technologies Inc., Santa Clara, CA, USA) or GCT Premier (Waters Corporation, Milford, MA, USA) by chemical ionization (reaction gas: methane), respectively. GC was conducted using either a fused silica capillary column (DB-WAX UI, 60 m \(\times\) 0.25 mm, 0.25-\(\mu\)m film thickness; Agilent Technologies) or a fused silica capillary column (DB-5MS, 30 m \(\times\) 0.25 mm, 0.25-\(\mu\)m film thickness; Agilent Technologies). When using the DB-WAX UI and DB-5MS columns, the oven temperature was programmed as from 50\(^\circ\)C (2 min isothermal) to 220\(^\circ\)C at 3\(^\circ\)/min and from 60 to 246\(^\circ\)C at 3\(^\circ\)/min, respectively, and the injection temperature was maintained at 250 and 220\(^\circ\)C, respectively, whereas the flow of the helium carrier gas was maintained at 1.6 and 1.0 mL/min.

Meanwhile, the \(^1\)H and \(^13\)C NMR spectra were recorded in CDCl\(_3\) using a JEOL JNM-ECA600 spectrometer at 600 and 150 MHz, respectively, and tetramethylsilane as an internal standard.

### 2.3 Identification of volatile compounds and standard compounds

#### 2.3.1 Identification and chemicals

A 66 volatile compounds from each sample, except for \(\alpha\)-copaene, \(\beta\)-elemene, \(\alpha\)-himachalene, \(\gamma\)-himachalene, \(\delta\)-cadinene, were identified by comparing the mass spectra and linear retention indices (LRI, using C6-C28 n-alkanes) of the samples to those of standard compounds (Table 2). The standard compounds, (\(Z\))-3-hexenyl 4-methylpentanoate 6, 4-methylpentyl 2-methylpropanoate 7, 4-methylpentyl butanoate 8, 4-methylpentyl 2-methylbutanoate 9, 3-methylbutyl 4-methylpentanoate 10, heptyl 2-methylbutanoate 11, hexyl 4-methylpentanoate 12, 6-methyl-(\(E\))-4-heptenyl 3-methylbutanoate 1, 6-methyl-(\(E\))-4-heptenyl 2-methylbutanoate 4, 6-methyl-(\(E\))-4-heptenyl butanoate 3, and 6-methyl-(\(E\))-4-heptenyl 2-methylpropanoate 2 were synthesized as described below, and the remaining were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Sigma-Aldrich Japan Co., LLC (Tokyo, Japan), Inoue Perfumery MFG. Co., Ltd. (Tokyo, Japan), International Flavors & Fragrances Inc. (New York, USA), and R. C. Treatt & Co. Ltd. (Bury St. Edmunds, UK).

#### 2.3.2 Standard compound preparation

(Z)-3-hexenyl 4-methylpentanoate 6

4-Methylpentanoic acid (1.8 mmol) and \(p\)-toluenesulfonic acid monohydrate (0.15 mmol) were added to (\(Z\))-3-hexeno (1.5 mmol) in toluene (7.5 mL), and the mixture was warmed up to 120\(^\circ\)C. After stirred at 120\(^\circ\)C for 5 hr, then

### Table 1

Name, abbreviation color and average weight of pepper samples.

| Name                                | abbreviation | color | average weight (g) |
|-------------------------------------|--------------|-------|-------------------|
| **C. chinense**                     |              |       |                   |
| Red Savina                          | RS           | red   | 10.1              |
| Habanero Orange                     | HO           | orange| 9.5               |
| Aji Limo                            | AL           | red   | 9.9               |
| **C. chinense \(\times\) C. frutescens** | BJ           | red   | 5.2               |
| Blut Jolokia                        |              |       |                   |
| **C. frutescens**                   |              |       |                   |
| Shima Tougarashi                    | ST           | red   | 1.3               |
| Tabasco                             | TB           | red   | 1.3               |
| **C. annum**                        |              |       |                   |
| Jalapeño                            | JA           | red   | 18.4              |
| Takanotsume                         | TA           | red   | 1.4               |
| HotParasol EX                       | HP           | red   | 4.0               |
| **C. baccatum**                     |              |       |                   |
| Aji Amarillo                        | AA           | orange| 31.6              |
| **C. pubescens**                    |              |       |                   |
| Rocoto                              | RO           | red   | 130.0             |
**Table 2** Volatile components in the solvent extracts of the pepper samples.

| Component | Red Serrano | Habanero Orange | Orange Ají | Limo | Bhut Jolokia | Shima | Tougarashi | Tabasco | Jalapeño | Takanotsume | HotParasol EX | Ají Amarillo | Rocoto |
|-----------|-------------|-----------------|------------|------|--------------|-------|------------|---------|----------|-------------|----------------|-------------|--------|
| 2-methylpentanal | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| hexanal | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| (E)-2-pentanal | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| (Z)-3-hexenal | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 1-penten-3-ol | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 3-methylbutanol | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| (E)-2-hexenal | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| γ-terpinene | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| (E)-β-ocimene | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 3-methylbutyl 2-methylbutanoate | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 4-methylpentyl 2-methylpropanoate | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 4-methylpentanol | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| (E)-2-pentenol | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 3-methylbutyl 4-methylpentanoate | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| hexyl 2-methylbutanoate | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| hexyl 3-methylbutanoate | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 4-methylpentyl pentanoate | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 6-methyl-(Z)-3-hexenyl 2-methylbutanoate | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 6-methyl-(E)-4-heptenyl 2-methylpropanoate | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 6-methyl-(E)-4-heptenol | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 3-methylbutyl 4-methylpentanoate | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| hexyl 2-methylbutanoate | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |
| 2-isobutyl-3-methoxypyrazine | 240.2 | 230.1 | 250.3 | 260.4 | 270.5 | 280.6 | 290.7 | 300.8 | 310.9 | 321.0 | 331.1 | 341.2 | 351.3 |

*RI = retention index*
### Table 2 Continued.

| Component | ID | R1 | C. chinense | C. chinense × C. frutescens | C. annuum | C. baccatum | C. pubescens |
|------------|----|----|-------------|-----------------------------|-----------|-------------|-------------|
|            |    |    | Red Sorumia | Habanero Orange | Ají Limo | Bhut Jolokia | Shima Tongari | Tabasco | Jalapeño | Takanotsume | HotPeanolEX | Ají Amarillo | Rocoto |
| 6-methyl-(E)-4-heptenyl 3-methylbutanoate | A 1596 | 1380 | 1.43 | 1.10 | 0.23 | 0.07 | 0.13 | 0.02 |
| β-caryophyllene | A 1600 | 1416 | 0.38 | 0.10 | 0.12 | 0.32 | 0.01 |
| (E)-3-hexenyl 4-methyl pentaanoate | A 1608 | 1345 | 0.48 | 4.03 | 0.33 | 1.05 | 0.39 | 0.60 | 0.18 | 6.57 |
| 6-methyl-(E)-4-heptenyl 3-methylbutanoate | A 1618 | 1386 | 7.35 | 9.56 | 0.77 | 0.60 | 0.08 | 0.03 | 1.19 | 0.29 | 0.33 |
| octyl 3-methylbutanoate | B | A 1645 | 1438 | 1.53 | 1.03 | 0.01 | 0.05 |
| α-thimichalene | B 1647 | 1448 | 2.04 | 0.40 | 0.14 | 0.09 | 0.02 | 2.10 | 5.69 | 0.22 |
| (E)-β-farnesene | A 1663 | 1491 | 2.35 | 0.57 | 0.22 | 0.16 | 1.48 | 0.34 |
| 3-methylbutanoic acid | B 1665 | 828 | 6.19 | 4.90 | 0.32 | 0.09 | 0.20 | 0.92 | 0.24 | 0.27 | 0.20 | 2.72 | 1.50 |
| 2-methylbutanoic acid | A 1666 | 832 | 0.54 | 0.19 | 0.19 | 0.01 | 0.17 | 0.82 | 3.45 | 8.52 | 2.43 | 1.19 | 0.23 |
| α-bisabolene | A 1673 | 1453 | 0.45 | 0.13 | 0.14 | 0.28 | 0.05 | 12.04 |
| γ-thimichalene | B 1699 | 1476 | 19.31 | 4.47 | 1.20 | 1.04 | 0.21 | 6.50 | 29.03 | 0.87 |
| γ-myrcalene | A 1718 | 1478 | 0.79 | 0.08 | 2.10 | 5.95 | 0.01 | 0.11 | 0.13 | 3.89 |
| pentalactone | A 1732 | 0.22 | 0.03 | 0.06 | 0.46 | 0.27 | 0.09 | 0.06 | 0.15 |
| δ-cadinene | B 1759 | 1515 | 3.35 | 0.34 | 0.46 | 0.70 | 0.01 | 0.07 | 0.90 |
| methyl salicylate | A 1775 | 1190 | 3.50 | 1.26 | 1.21 | 0.73 | 0.06 | 1.19 | 0.49 | 3.36 | 2.69 | 1.16 | 5.95 |
| 4-methylpentanoic acid | A 1800 | 941 | 1.31 | 0.41 | 0.27 | 0.19 | 1.57 | 0.33 |
| 3-methylbutyl(3-methyl)6-octanoate | A 1835 | 1588 | 1.37 | 1.77 | 0.32 | 0.28 | 0.02 | 0.01 | 0.10 | 0.97 | 1.28 | 0.32 |
| heptanoic acid | A 1841 | 969 | 1.20 | 0.20 | 0.32 | 0.14 | 0.51 | 4.48 | 0.36 | 0.97 | 4.67 | 23.13 |
| guaiacol | A 1856 | 1083 | 1.05 | 0.16 | 0.12 | 0.11 | 0.09 | 0.25 | 0.78 |
| butyl 2-methylbutanoate | A 1867 | 1383 | 0.14 | 0.05 | 0.61 | 0.08 | 0.29 |
| β-phenethyl alcohol | A 1910 | 1111 | 6.19 | 0.09 | 0.24 | 0.04 | 0.11 | 7.28 | 0.09 | 4.20 | 0.99 | 0.21 |
| β-sitosterol | A 1943 | 1475 | 1.19 | 1.13 | 1.41 | 0.50 | 0.06 | 0.07 | 0.35 | 11.18 | 2.89 | 0.05 | 0.12 |
| heptanoic acid 17 | A 1949 | 1076 | 13.17 | 1.22 | 0.01 | 0.03 | 0.11 | 0.43 | 0.19 | 0.21 | 0.19 | 3.85 |
| (E)-2-heptenoic acid | A 1966 | 1099 | 0.48 | 0.95 | 0.14 | 0.09 | 0.14 | 3.64 | 0.40 | 0.95 | 5.80 | 34.28 |
| trans-geranyl | A 2036 | 1599 | 0.15 | 0.01 | 0.72 | 0.61 | 2.10 | 0.09 |
| octanoic acid | A 2038 | 1170 | 4.15 | 0.33 | 0.02 | 0.03 | 0.06 | 0.42 | 0.08 | 0.12 | 0.09 | 1.75 |
| vanillin | A 2561 | 1391 | 2.01 | 0.90 | 0.04 | 0.02 | 0.22 | 0.02 | 3.72 | 0.75 | 0.31 | 0.13 | 0.24 |
| acetovanillone | A 2635 | 1478 | 0.85 | 0.31 | 0.56 | 0.01 | 0.05 | 7.34 | 0.14 | 0.14 | 0.33 | 0.16 |

1. **ID = identification by A = mass spectrum and linear retention indices agreed with the standards; B = mass spectrum and linear retention indices agreed with the literature data.**
2. **RI = Linear Retention Indices on DB-wax and DB5-MS column (60 m × 0.25 mm × 0.25 mm) as a homologous series of n-alkanes (C7-C25).**
the reaction mixture was allowed to cool to room temperature. The mixture was neutralized by saturated NaHCO₃ aqueous solution. The mixture was extracted with toluene and washed with brine. The extract was concentrated to dryness in vacuo. The crude product was purified by flash chromatography (silica gel, n-hexane-ethyl acetate (50:1 v/v) as an eluent) to afford (Z)-3-hexenyl 4-methylpentanoate 6.

4-Methylpentyl 2-methylpropanoate 7, 4-methylpentyl butanoate 8, 4-methylpentyl 2-methylbutanoate 9, 3-methylbutyl 4-methylpentanoate 10, heptyl 2-methylbutanoate 11, and hexyl 4-methylpentanoate 12 were prepared in the same manner as above.

6-methyl-(E)-4-heptenyl 3-methylbutanoate 1

Isopropylmagnesium bromide (1.0 M in tetrahydrofuran (THF), 254 mmol) was added dropwise to a solution of acrolein 13 (231 mmol) in anhydrous THF (450 mL) at −78°C under nitrogen atmosphere. After stirring at −78°C for 30 min, the reaction mixture was warmed up to room temperature. The mixture was quenched by adding saturated NH₄Cl aqueous solution, slightly acidified with HCl (1.0 M) solution, and then extracted with Et₂O. The extract was washed with brine, dried over Na₂SO₄, and then concentrated under reduced pressure. The crude product was purified by flash chromatography (silica gel, n-hexane-ethyl acetate (20:1 v/v) as an eluent) to afford 4-methyl-1-penten-3-ol 14 in 20% yield. MS: m/z %: 100 (M⁺) (0.2), 85 (3), 58 (17), 57 (100), 55 (6), 43 (15), 41 (11), 39 (7). ¹H NMR (600 MHz, CDCl₃): δ 3.91 (3H, d, J = 6.9 Hz), 0.93 (3H, d, J = 6.9 Hz), 1.74 (1H, dd, J = 6.9, 6.2 Hz), 3.86 (1H, dd, J = 6.2, 6.2 Hz), 5.16 (1H, dd, J = 10.3, 1.4 Hz), 5.23 (1H, dd, J = 17.2, 1.4 Hz), 5.87 (1H, dd, J = 17.2, 10.3, 6.9 Hz). ¹³C NMR (150 MHz, CDCl₃): δ 17.9, 18.2, 39.6, 78.4, 115.7, 139.5.

Propionic acid (4 mmol) and triethylorthoacetate (160 mmol) were added to 4-methyl-1-penten-3-ol 14 (40 mmol), and then the mixture was warmed up to 120°C. After stirring at 120°C for 2 h, the reaction mixture was allowed to cool to room temperature. Dichloromethane was added to the reaction mixture, and the resulting solution was washed with saturated NaHCO₃ aqueous solution. The solution was acidified with HCl (1.0 M) aqueous solution and stirred for 30 min to remove the excess triethylorthoacetate. Then, the solution was washed with saturated NaHCO₃ aqueous solution and brine. The DCM extract was dried over Na₂SO₄ and then concentrated in vacuo. The crude product was purified by flash chromatography (silica gel, n-hexane-ethyl acetate (50:1 v/v) as an eluent) to afford 6-methyl-(E)-4-heptenyl-3-methylbutanoate 1 in 85% yield. MS: m/z %: 212 (M⁺) (0.03), 110 (28), 95 (100), 82 (40), 69 (47), 67 (41), 57 (37), 55 (31), 41 (39). ¹H NMR (600 MHz, CDCl₃): δ 3.96 (6H, d, J = 6.9 Hz), 0.96 (6H, d, J = 6.9 Hz) 1.88 (2H, tt, J = 7.6, 6.9 Hz), 2.07 (2H, td, J = 7.6, 6.9 Hz), 2.21 (2H, td, J = 7.6, 6.9 Hz), 2.11 (1H, sept, J = 6.9, 6.9 Hz), 2.18 (2H, d, J = 6.9 Hz), 2.23 (1H, sept, J = 6.9, 6.2 Hz), 4.06 (2H, t, J = 6.9 Hz), 5.33 (1H, td, J = 15.1, 6.2 Hz), 5.41 (1H, dd, J = 15.1, 6.2 Hz). ¹³C NMR (150 MHz, CDCl₃): δ 22.5 (2C), 22.7 (2C), 25.8, 26.8, 28.9, 31.1, 43.6, 63.8, 125.7, 138.8, 173.4. LRI (DB-5MS): 1014, LRI (DB-WAX): 1524.

3-Methylbutanoic acid (1.8 mmol) and p-toluenesulfonic acid monohydrate (0.15 mmol) were added to 6-methyl-(E)-4-heptenyl-3-methylbutanoate 1 (1.5 mmol) in toluene (7.5 mL), and then the mixture was warmed up to 120°C. After stirring at 120°C for 5 h, the reaction mixture was allowed to cool to room temperature. The mixture was neutralized by saturated NaHCO₃ aqueous solution. The mixture was extracted with toluene and washed with brine. The extract was concentrated to dryness in vacuo. The obtained crude product was purified by flash chromatography (silica gel, n-hexane-ethyl acetate (50:1 v/v) as an eluent) to afford 6-methyl-(E)-4-heptenyl 2-methylbutanoate 4, 6-methyl-(E)-4-heptenyl butanoate 3, and 6-methyl-(E)-4-heptenyl 2-methylpropanoate 2 were prepared in the same manner as above.

6-Methyl-(E)-4-heptenyl 2-methylbutanoate 4, 6-methyl-(E)-4-heptenyl butanoate 3, and 6-methyl-(E)-4-heptenyl 2-methylpropanoate 2 were prepared in the same manner as above.
= 6.9 Hz), 1.14 (3H, d, J = 6.9 Hz), 1.47 (1H, m), 1.65-1.71 (3H, m), 2.05 (2H, td, J = 7.6, 6.9 Hz), 2.23 (1H, sep d, J = 6.9, 6.2 Hz), 2.36 (1H, m), 4.07 (2H, d, J = 6.9 Hz), 5.34 (1H, td, J = 15.1, 7.6 Hz), 5.41 (1H, dd, J = 15.1, 6.2 Hz). 13C NMR (150 MHz, CDCl3): δ 11.7, 16.7, 22.7 (2C), 26.9, 28.7, 28.9, 31.1, 41.2, 63.7, 125.7, 138.7, 176.9. LRI (DB-5MS) 1380, LRI (DB-WAX) 1596.

6-Methyl-(E)-4-heptenyl butanoate 3 (in 83% yield). MS: m/z (M + H)+ = 199.1634, calcd.; m/z (M + H)+ = 199.1698 for C8H14O2. m/z %: 212 (M+) (0.02), 110 (25), 95 (100), 82 (40), 69 (42), 67 (45), 55 (30), 43 (43), 41 (37). IR (nujol): ν 2963, 1737, 1469, 1193, 1158 cm⁻¹. 1H NMR (600 MHz, CDCl3): δ 1.05-1.15 (3H, t, J = 7.6 Hz), 0.96 (6H, d, J = 6.9 Hz), 1.63-1.71 (4H, m), 2.04 (2H, td, J = 7.6, 6.9 Hz), 2.23 (1H, sep d, J = 6.9, 6.2 Hz), 2.28 (2H, t, J = 6.9 Hz), 4.06 (2H, t, J = 6.9 Hz), 5.33 (1H, td, J = 15.1, 6.9 Hz), 5.41 (1H, dd, J = 15.1, 6.2 Hz). 13C NMR (150 MHz, CDCl3): δ 13.8, 18.6, 22.7 (2C), 28.6, 28.9, 31.1, 36.4, 63.8, 125.7, 138.7, 173.9. LRI (DB-5MS) 1337, LRI (DB-WAX) 1584.

6-Methyl-(E)-4-heptenyl 2-methylpropanoate 2 (in 80% yield). MS: m/z (M + H)+ = 199.1647, calcd.; m/z (M + H)+ = 199.1698 for C8H14O2. m/z %: 212 (M+) (0.03), 110 (28), 95 (100), 82 (36), 69 (41), 67 (43), 55 (28), 43 (52), 41 (36). IR (nujol): ν 2963, 1737, 1469, 1193, 1158 cm⁻¹. 1H NMR (600 MHz, CDCl3): δ 0.96 (6H, d, J = 6.9 Hz), 1.17 (6H, d, J = 6.9 Hz), 1.69 (2H, tt, J = 7.6, 6.9 Hz), 2.05 (2H, td, J = 7.6, 6.9 Hz), 2.23 (1H, sep d, J = 6.9, 6.2 Hz), 2.54 (1H, sep, J = 6.9 Hz), 4.06 (2H, td, J = 6.9 Hz), 5.34 (1H, td, J = 15.1, 6.9 Hz), 5.41 (1H, dd, J = 15.1, 6.2 Hz). 13C NMR (150 MHz, CDCl3): δ 19.1 (2C), 22.7 (2C), 28.6, 28.9, 31.1, 34.1, 63.8, 125.7, 138.7, 177.3. LRI (DB-5MS) 1292, LRI (DB-WAX) 1517.

3 Results and Discussion

3.1 Detection of 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1 in Habanero peppers

In our previous report, we isolated 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1 from Habanero fruit and determined its chemical structure using NMR. In the report, however, we could not compare the data of isolated compound with those of authentic sample, so we synthesized standard sample of it on this paper.

The 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1 was synthesized as below (Fig. 1). Briefly, the ester was synthesized by preparing the corresponding alcohol 5 from acrolein 13, using the Grignard reaction, Johnson-Claisen rearrangement, and reduction10, and then condensing alcohol 5 with 3-methylbutanoic acid. The 1H NMR spectrum of the synthetic compound was corresponded exactly with that of isolated 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1 from Habanero pepper fruit (Fig. 2).

3.2 GC-MS analysis of pepper samples

A total of 66 volatile compounds were detected and identified from 11 pepper samples. Hydrocarbons, except for terpene hydrocarbons with some aroma, were taken off the list for little aroma (Table 2). The main volatile compounds of Red Savina pepper were y-himachalene 16, heptanoic acid 17, and 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1, in order of decreasing peak area %, whereas the main volatile compounds of Habanero Orange were (E)-2-hexenal 18, 4-methylpentyl 3-methylbutanoate 19, and 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1. As in Habanero peppers, 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1 was the main volatile of these varieties, and related compounds (6-methyl-(E)-4-heptenyl 2-methylbutanoate 4, 6-methyl-(E)-4-heptenyl 2-methylpropanoate 2, 6-methyl-(E)-4-heptenyl butanoate 3, and 6-methyl-(E)-4-heptenol 5) were also found in Red Savina and Habanero Orange as well as in others. The detection of both 6-methyl-(E)-4-heptenyl 2-methylpropanoate 2, in Habanero Orange, Ají Limo, and Ají Amarillo, and 6-methyl-(E)-4-heptenyl 2-methylbutanoate 4, in all C. chinense and C. frutescens varieties, has been reported previously, but previous
studies have identified the compounds by the comparison of mass spectrums and retention indices (RIs) to those of homologues, rather than to those of standard samples. Furthermore, such studies have not mentioned the geometric isomerism of the double bond, whereas the present study identified these compounds, including double bond stereochemistry, by comparison to standard samples. To our knowledge, the present study is the first to isolate 6-methyl-(E)-4-heptenol 5, which was isolated from Habanero Orange, Bhut Jolokia, ShimaTougarashi, and Tabasco, and 6-methyl-(E)-4-heptenyl butanoate 3, which was isolated from Ají Amarillo, from natural products.

3.3 Analysis of pepper volatile profiles

The volatile profiles (i.e., GC-MS data) of the 11 pepper samples were subject to PCA, using the peak areas (%) of each compound (Fig. 3). The pepper samples were roughly categorized by species, and the PC2 scores of the samples increased as follows: C. annuum, C. pubescens, C. frutescens, C. chinense, and C. baccatum. The scores of C. frutescens and C. pubescens overlapped on the PC2 axis but were separated on the PC1 axis. Interestingly, the Bhut Jolokia, which is a hybrid of C. chinense and C. frutescens, was mapped in between the C. chinense and C. frutescens groups, which suggests that the Bhut Jolokia has inherited aroma characteristics from both C. chinense and C. frutescens.

The same data were also subject to HCA to investigate the compounds that characterize Habanero peppers (Fig. 4). Even though the Red Savina and Habanero Orange were grouped together, Ají Limo, which is also a variety of C. chinense, was grouped with Bhut Jolokia. The compounds that characterized in Habanero peppers were 3-methylbutyl 8-methyl-(E)-6-nonenoate 20, (E)-β-farnesene 21, heptyl or octyl 3-methylbutanoate 22, 3-methylbutanoic acid 24 (solid yellow line frame). In addition, the HCA results indicated that these novel compounds (i.e., 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1 and 2-methylbutanoate 4) were also characteristic of Habanero peppers. Ají Limo and Bhut Jolokia were grouped together, owing to the high levels of 4-methylpentyl 3-methylbutanoate 19, pentyl 3-methylbutanoate 25, hexyl 3-methylbutanoate 26, and (Z)-3-hexenyl 3-methylbutanoate 27, which all include the 3-methylbutanoyl moiety (dotted yellow line frame). Interestingly, heptyl or octyl
3-methylbutanoate 22, 23, 3-methylbutanoic acid 24, and 6-methyl-\((E)\)-4-heptenyl 3-methylbutanoate 1, which characterized Habanero peppers, also include 3-methylbutanoyl moiety. Therefore, the 3-methylbutanoyl moiety is apparently characteristic of \textit{C. chinense}.

In contrast, the \textit{C. frutescens} varieties Tabasco and ShimaTougarashi were characterized by the presence of 2-methylbutanoates and 2-methylpropanoates, such as 4-methylpentyl 2-methylbutanoate 9 and 4-methylpentyl 2-methylpropanoate 7 (dotted black line frame). These results suggest that the fatty acid constructing these esters may reflect species.

A plausible pathway for capsaicin biosynthesis 7, 8 is proposed (Fig. 5). Capsaicin 28 was biosynthesized by the condensation of vanillylamine 29 and 8-methyl-6-nonenoyl-CoA 30. Vanillylamine 29 was synthesized from phenylalanine 31 via vanillin 32, and 8-methyl-6-nonenoyl-CoA 30 was synthesized from valine 33. The biosynthesis of 8-methyl-6-nonenoyl-CoA 30, which is the branched-chain fatty acid moiety of capsaicin, involved the conversion of valine 33 to 2-methylpropanoyl-CoA 34 via transamination and oxidative decarboxylation. The 2-methylpropanoyl-
CoA 34 was converted to 8-methyl-6-nonenoyl-CoA 30 via carbon chain elongation. During the elongation process, 6-methyl-4-heptenoyl-ACP 35, which includes the same carbon skeleton as the alcohol moiety of 6-methyl-(E)-4-heptenyl esters, was produced. The carbon skeleton of 35 also same as a branched-chain fatty acid moiety of nororcapsaicin, which is a homologue of capsaicin\(^9\). The relationships among these structures suggest that 35 is an intermediate of nororcapsaicin and that the 6-methyl-(E)-4-heptenyl esters detected in Habanero peppers are produced by the nororcapsaicin biosynthesis pathway.

4 Conclusion

To identify the compound(s) responsible for the pungency and fruity aroma of Habanero peppers, the volatile compounds of Habanero and other peppers were extracted and analyzed. A total of 66 volatile compounds, including 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1 and related compounds, were detected, and PCA and HCA of the volatile profiles indicated that 6-methyl-(E)-4-heptenyl 3-methylbutanoate 1 and 6-methyl-(E)-4-heptenyl 2-methylbutanoate 4 are characteristic of Habanero peppers.
Acknowledgments

The author thanks the San-Ei Gen F.F.I. (Peru) S.A. for information of Peruvian pepper samples.

References

1) Murakami, Y.; Iwabuchi, H.; Horikawa, M.; Mori, S.; Ohba, Y.; and Fukami, H. Isolation and identification of volatile compound in Habanero pepper (Capsicum chinense). *J. Oleo Sci.* 67, 1219-1225 (2018).

2) Rodrigez-Burruezo, A.; Kollmannsberger, H.; González-Mas, M. C.; Nitz, S.; and Nuez, F. HS-SPME Comparative analysis of genotypic diversity in the volatile fraction and aroma-contributing compounds of Capsicum fruits from the annuum-chinense-frutescens complex. *J. Agric. Food Chem.* 58, 4388-4400 (2010).

3) http://shinofarm.jp/ Home Page of Shino Farm Limited Co. Accessed 12 June 2019.

4) Manikharda; Takahashi, M.; Arakaki, M.; Yonamine, K.; Hashimoto, F.; Takara, K.; Wada, K. Influence of fruit ripening on color, organic acid contents, capsaicinoids, aroma compounds, and antioxidant capacity of Shimagarashi (Capsicum frutescens). *J. Oleo Sci.* 67, 113-123 (2018).

5) Engel, W.; Bahr, W.; Schieberle, P. Solvent assisted flavour evaporation - a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. *Eur. Food Res. Technol.* 209, 237-241 (1999).

6) Grunanger, C.U.; Breit, B. Remote control of regio- and diastereoselectivity in the hydroformylation of bishomoallylic alcohols with catalytic amounts of a reversibly bound directing group. *Angew. Chem. Int. Ed.* 44, 6110-6127 (2005).

7) Narasimha Prasad, B.C.; Kumar, V.; Gururaj, H.B.; Parimalan, R.; Giridhar, P.; Ravishankar, G.A. Characterization of capsaicin synthase and identification of its gene (csy1) for pungency factor capsaicin in pepper (Capsicum sp.). *Proc. Natl. Acad. Sci. USA* 103, 13315-13320 (2006).

8) Mazourek, M.; Pujar, A.; Borovsky, Y.; Paran, I.; Mueller, L.; Jahn, M.M. A dynamic interface for capsaicinoid systems biology. *Plant Physiol.* 150, 1806-1821 (2009).

9) Gannett, P. M.; Nagel, D. L.; Reilly, P. J.; Lawson, T.; Sharpe, J.; and Toth, B. The Capsaicinoids: Their Separation, Synthesis, and Mutagenicity. *J. Org. Chem.* 53, 1064-1071 (1988).

10) Mookdasanit, J.; Tamura, H.; Yoshizawa, T.; Tokunaga, T.; Nakanish, K. Trace volatile components in essential oil of Citrus sudachi by means of modified solvent extraction method. *Food Sci. Technol. Res.* 9, 54-61 (2003).

11) Babushok, V.I.; Linstrom, P.J.; Zenkevich, I.G. Retention indices for frequently reported compounds of plant essential oils. *J. Phys. Chem.* Ref. Data, 40, 043101-1-47 (2011).