Physicochemical Properties, Flavor Characteristics, and Biological Functions of the Chili Pepper Shimatogarashi (*Capsicum frutescens*)

島トウガラシ (*Capsicum frutescens*) の物理化学的特性, フレーバー特性および機能性

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Table of Contents

List of Figures

List of Tables

List of Appendices

ABSTRACT

Chili peppers (*Capsicum* spp.) play a significant role in the culinary world as popular ingredients with their typical spiciness. In Japan, Okinawa has an endemic hot chili pepper cultivar, known as Shimatogarashi, which belongs to the *Capsicum frutescens* species. Shimatogarashi is used in exotic condiments that hold potential economic value, but very few studies have explored their properties. This study aimed to evaluate the physicochemical properties, flavor characteristics, and biological functions of the Shimatogarashi chili pepper.

The hotness or spiciness of chilies has received much attention in chili pepper breeding, whereas their flavor, which is associated with aroma and taste compounds, differs between chili varieties. Therefore, comparative studies evaluating the physical properties, flavor characteristics, and antioxidant capacity had been conducted between the chilies Shimatogarashi and Takanotsume (*Capsicum annuum*), which is the most popular chili pepper in Japan. Shimatogarashi was brightly red colored and highly pungent with a fresh and fruity aroma, whereas Takanotsume was dark red in color and moderately pungent with a warm and herbaceous aroma. Shimatogarashi also showed higher antioxidant activity than Takanotsume in terms of their total phenolic content and oxygen radical absorption capacity. The results demonstrated that Shimatogarashi had unique physical, flavor, and antioxidant properties compared to Takanotsume.

Shimatogarashi undergoes several color changes from green to orange and then to red during fruit maturation. This is accompanied by changes in the fruit metabolites, which affect the aroma, taste, and antioxidant property of the fruit. The evaluation of color, organic acid contents, capsaicinoids, aroma compounds, and antioxidant activity during maturation of Shimatogarashi fruit revealed that its profile developed towards a more pungent and citrus-like aroma, and higher antioxidant capacity at the mature red stage than the immature green stage. On the other hand, utilization of the fruit at its immature green stage is beneficial owing to its pleasant fruity aroma, which may be useful in condiments.

The potential of Shimatogarashi, as a healthy food to preventing obesity which is closely linked to metabolic syndrome was also investigated using 3T3-L1 preadipocytes. Interestingly, the methanolic extract of mature red Shimatogarashi fruit reduced the accumulation of intracellular lipids in a dose-dependent manner during differentiation of 3T3-L1 cells into adipocytes. Moreover, it was shown that the extract attenuated adipogenesis and expression of the peroxisome proliferator-activated receptor γ as an adipogenic marker in differentiated 3T3-L1 adipocytes. Therefore, it was concluded that Shimatogarashi is a promising source of natural antioxidants with potential biological influence in adipogenesis.

トウガラシ（*Capsicum* spp.）は独特の辛味をもつ一般的な香辛料として、調理において重要な役割を果たしている。一方、沖縄には辛味をもつ固有のトウガラシ品種として、*Capsicum frutescens* 種に属する島トウガラシが存在する。島トウガラシは沖縄独自の香辛料として利用され、高い経済価値をもっていると考えられるが、その特性についての研究はほとんどない。そこで本研究では、島トウガラシの物理化学的特性、フレーバー特性および機能性を評価することを目的とする。

トウガラシの辛味はトウガラシの育種において非常に注目されているが、香りや味の成分によって形成されるフレーバー（風味）も品種によって大きく異なる。そこで、島トウガラシと日本で最も一般的なタカノツメ（*Capsicum annuum*）の物理的特性、フレーバー特性および抗酸化活性を比較し、評価した。島トウガラシ果実は鮮やかな赤色で、新鮮な柑橘様の香りとともに強い辛味を呈した。一方、タカノツメ果実は暗赤色で、やわらかなハーブ様の香りをもち、辛味は中程度であった。また、島トウガラシは総フェノール化合物量とラジカル消去活性の分析結果から、タカノツメよりも高い抗酸化活性をもつことが示された。以上のことから、島トウガラシはタカノツメとは物理的特性、フレーバー特性および抗酸化活性が異なることが明らかとなった。

島トウガラシ果実は成熟中に緑色から橙色、赤色と色が変化する。これらの色の変化は果実の代謝物によるが、香り、味、さらに抗酸化活性にも影響があると考えられる。そこで、島トウガラシの果実の成熟期間の色、有機酸、カプサイシノイドおよび抗酸化活性の変化を分析した結果、

未熟な緑色の果実に比べて、熟した赤色の果実ではより強い辛味や柑橘様の香りをもち、抗酸化活性も高いことが明らかとなった。一方、未熟な緑色の果実は穏やかな果実様の香りをもっていたことから、薬味として利用することに適していると考えられた。

島トウガラシがメタボリックシンドロームと密接に関連する肥満を予防する健康食品としての可能性について、前駆脂肪細胞 3T3-L1 を用いて検討した。その結果、3T3-L1 細胞が脂肪細胞へ分化する過程において、赤色の島トウガラシ成熟果のメタノール抽出物が濃度依存的に細胞内脂質の蓄積を減少させるという興味深い知見を得た。さらに、本抽出物は 3T3-L1 細胞が分化した脂肪細胞において、脂質生成や脂質生成マーカーとして知られるペルオキシソーム増殖因子活性化受容体 γ の発現を抑制することを示した。したがって、島トウガラシは生物学的に脂質生成の抑制に関与する天然酸化防止剤の素材として有効であると考えられた。

Chapter I

General Introduction

1.1. Background

Chili peppers (*Capsicum* spp.) with their unique pungent taste are an important ingredient in the culinary world. Endowed with bright attractive color and appealing aroma, these fruits are gastronomically challenging, yet they are extensively used by people from around the world in their cuisine (Bosland and Votava 2012; Russo 2012). Based on widespread geographic and temporal distribution of archaeological remnants, ethnobotanical studies on chili peppers suggest that once the locals adopt chili peppers in their cuisine, the incorporation is sustained (Perry 2012).

Nowadays, with the developments in food science and technology, chili fruits are not only consumed fresh, but also transformed to other forms including dehydrated products, sauces, infusions, and additives. Besides the culinary purpose, the chili plant is also used in the preparation of cosmetics and medicines, and for coloring flamingo feathers and koi fishes. It is also used as an ornamental plant (Bosland and Votava 2012). Genetic manipulation of plants has increased the form and use of chili plants further. Such manipulations have increased the production of compounds related to pungency and other flavor attributes in the plant to fit the market demands (Russo 2012).

The increased fascination for pepper plants is also reflected in a large variety of seeds offered in markets (Bosland and Votava 2012). The fruit of the genus *Capsicum* is diverse in terms of color, size, shape, level of pungency, and nutritional property. The metabolites, especially those with nutritional value, in chili fruit are largely affected by genotype, growing condition, geographical area, and agricultural practices. Owing to a wide variation in each compound among chili plants of different species and genotypes, the improvement of fruit quality through manipulation at the metabolic level using conventional breeding and nonconventional approaches is highly recommended. Chili plants are reported to have an array of nutritive and functional components, such as antioxidants, phenolic compounds, carotenoids, capsaicinoids, and vitamin C. Therefore, the future of research in this direction is promising (Frary and Frary 2012).

Okinawa has its own chili pepper cultivar known as Shimatogarashi or Shima tongarashi, which belongs to the species *C. frutescens*. It is one of the economically important species of the genus *Capsicum* (Forero et al. 2009). The fruit of the species is conical in shape, around 2 cm in length, and about 0.5–1 cm in diameter. In spite of the frequent typhoons, this cultivar has survived in Okinawa. However, Shimatogarashi is not consumed by the local people as much as other vegetables. In Hawaii, chili pepper infusion is famous, where the chili is allowed to be soaked in hot water; used to add flavor and spicy heat to dishes. Furthermore, some people also prepare and use chili pepper-infused olive oil. In Thailand, the fruits of *C. frutescens*—from green to red stages—have been popularly used in pickles owing to their likeable aroma. The Okinawan people produce a unique dipping sauce known as *koregusu* by soaking Shimatogarashi in a local liquor

awamori. Some studies have investigated the flavor and nutritional components of tabasco (*C. frutescens*) peppers (Haymond and Aurand 1971; Howard et al. 2000; Burruezo et al. 2010). However, very few studies have explored Shimatogarashi peppers. As *C. frutescens* is also used in exotic condiments, it is attractive to study this species from flavor and functional benefits perspective.

Furthermore, in the *C. frutescens* accessions in Ryukyu Island, Japan, Shimatogarashi exhibited an atypical isozyme pattern when compared with that of other *C. frutescens* accessions in Southeast and East Asia (Yamamoto and Nawata 2005). Similar to other hot pepper cultivars, Shimatogarashi is also spicy owing to the presence of capsaicinoids, such as capsaicin and dihydrocapsaicin. Some studies have evaluated the role of capsaicin as an antiobesity agent, whereby they increase thermogenesis (Watanabe et al. 1987) and energy expenditure, and inhibit body fat accumulation (Ahuja et al. 2006; Lejeune et al. 2003). Studies have also reported the potential of capsaicin in inhibiting cell differentiation and inducing apoptosis in 3T3-L1 adipocyte (Hsu and Yen 2007). Obesity, which is linked to several diseases, is a consequence of modern sedentary lifestyle. Therefore, investigating the effect of Shimatogarashi extract on obesity will provide an insight on the utilization of its fruit in everyday diet. The developing Okinawa products and increasing consumer interest in them have increased the value of Shimatogarashi in the market. Therefore, understanding the functional property and applicability of Shimatogarashi is essential.

Scientific knowledge on the characteristics of fruits can add value to the products and retain their originality. It would also offer consumers a better understanding of the best way to utilize or consume the product. Further, knowledge on the content of chili fruit can increase its potential use in other sectors of science. Chili has become a popular commodity globally. The area of cultivation affects the properties of chili fruits and makes them differ from one variety to another. Therefore, it is important to explore local cultivars to gain insight about their unique characteristics in order to compare them with other cultivars around the world.

1.2. Thesis outline

Chapter I of this thesis presents a general introduction to the study. Chapter II provides a complimentary literature review related to the study. Chapter III presents the flavor-related compound profile of Shimatogarashi and its antioxidant property. Chili peppers, in addition to their functional benefits, are mostly used for their flavor. The flavor of the fruit depends on aroma- and taste-related compounds, which depend on many factors, especially the genotype. Therefore, a better understanding can be obtained by comparing the compound profile of Shimatogarashi with that of Takanotsume (*Capsicum annuum*), another commercial chili pepper in Japan. Chapter IV focuses on the alteration in flavor, chemical compounds, and antioxidant property during ripening in Shimatogarashi, in order to gain insight on fruit characteristics at each stage and their potential utilization. Because fruits undergo chemical changes during maturation, which alter their property at different stages of growth. Chapter V describes the functional aspect of Shimatogarashi in preventing obesity. The data were obtained by

observing the effects of Shimatogarashi extract on the differentiation of 3T3-L1 adipocyte cells in vitro. The data obtained regarding the characteristic of chili fruit in the present study might benefit the local growers, especially those in Okinawa Prefecture, and also the global community.

Chapter II

Literature Review

2. 1. Origin of *Capsicum* species

The genus *Capsicum* is assumed to have originated from Central America, the Andes Mountains in South America, and the Galapagos Islands, before spreading to the tropical lowland regions of America (Walsh and Hoot 2001). Archeologists have reported that wild chili peppers were being consumed by humans as early as in 7000 B.C.E. It is assumed that the cultivation of chili plants started between 5200 B.C.E. and 3400 B.C.E. (Heiser 1976). Preserved peppers have provided evidence that South Americans consumed and planted chili in 2500 B.C.E (Mortensen and Mortensen 2009). Subsequently, chili peppers were introduced to Europe, and then to Africa, India, China, and Korea; it was brought to Japan by the Europeans (Yamamoto 2013). Although there are different theories on how chili was introduced to Japan, there is one record describing that chili peppers were first brought to Japan by the Portuguese in the mid–16th century (Itoh 2015). Mistaken for black pepper (*Piper nigrum*) by Christopher Columbus, the term pepper is still used to address chili in English (Basu and De 2003). In Japan, the fruits were formerly called as *nanban kosho*, which means peppercorns from southern foreign lands. Later, the chili peppers were collectively known as *tongarashi* or *togarashi*, which means exotic (to) mustard (karashi). However, some people mistook it for mustard (karashi) from China (Tang dynasty), because

they are homophones (Itoh 2015).

Approximately, 27 species have been included in the genus *Capsicum* (Zachariah and Gobinath 2008; Mortensen and Mortensen 2009). Currently, five species of *Capsicum*—*C. annuum*, *C. frutescens*, *C. chinense*, *C. pubescens*, and *C. baccatum*—are domesticated, among which *C. annuum* and *C. frutescens* have been cultivated in Japan. Around the 17th century, Takanotsume (*C. annuum*), which literally means hawk's nails, was the most popular variety of hot pepper cultivated in Japan, and was used in everyday cuisine (Itoh 2015). The taste of the fruit is very hot, however the heat fades away quickly (Bosland and Votava 2012). Later, a non-pungent variety of pepper, green immature bell pepper *C. annuum*, from the US was introduced to Japan in the early Meiji Era (1868–1912) and was called *piman* (from the French word *piment* or *poivron*). Since the Netherlands started exporting the bright yellow- and red-colored sweet bell peppers to Japan, people wrongly thought that they were totally different peppers. Therefore, they called the colored bell pepper as *papurika* (from the Dutch word *paprika*) (Itoh 2015).

In the tropical region of Southern Japan, chili peppers are called *koregusu* in Okinawan language. In Japanese the term *koregusu* means *awamori* (liquor) hot sauce, which consists of Shimatogarashi (*C. frutescens*) fruit soaked in local liquor (*awamori*). Although its precise origin is undetermined, it is speculated that Okinawans who migrated to Hawaii brought the hot sauce to their homeland. Shimatogarashi grows well in the tropical climate of Southern Japan. This cultivar is usually planted in backyards and occasionally used. The fruit of Shimatogarashi is consumed in sashimi dipping sauce as an alternative of wasabi (*Wasabia japonica*) or in soumen (Japanese thin noodles) soup. Interestingly, in the *C.*

frutescens accessions in Ryukyu Island, Japan, Shimatogarashi exhibited an atypical isozyme pattern when compared with that of other *C. frutescens* accessions in Southeast and East Asia (Yamamoto and Nawata 2005).

2.2. Economic importance

Ethnohistorical reports and the current distribution pattern support the fact that interest in chili peppers as a spice in human history is unwavering (Perry 2012). The fruits of the genus *Capsicum* have influenced the diet of people, and also the medicinal uses of the genus globally. Mortensen and Mortensen (2009) estimated that as many as three−quarters of the population around the world use chili peppers in their regular cuisine. The use of red chili pepper in Japan started around the mid to late 17th century as a condiment in shichimi togarashi—a mixture of dried ground chili peppers, sesame seeds, and orange peel (Itoh 2015). In Amami-Oshima Island, Japan, miso soup is consumed with chili fruit in it. A mixture of chili fruit soaked in shochu, a Japanese liquor, is used both as a condiment and medicine (Yamamoto 2013). Consuming egg cooked along with pepper is believed to cure stomach ache. In India and China, chili peppers are used as a principal spice (Bosland and Votava 2012).

Owing to their culinary, nutritional, and medicinal benefits (Kouassi et al. 2012), the chili peppers are of great economic value (Ghasemnezad et al. 2011). Their value is also supported by their availability in diverse range of color, flavor, and shape (Contreras-Padilla and Yahia 1998). Further, the typical pungency of chili peppers contributes to their extensive utilization in the food industry. The flavor attributes and health benefits of chili peppers play a crucial role in their

popularity as a consumer product. These fruits are alternatively used as a food preservative owing to their antimicrobial activity (Mortensen and Mortensen 2009).

Furthermore, the uses of *Capsicum* fruits are diverse. Initially, in Japan, these spicy fruits were not consumed as a food. The plant was used for decorating gardens or warming the toes by placing them inside foot covering (tabi). In Tibet, chili fruit was used to warm stomach, treat hemorrhoids and leprosy, and extend lifespan. In Europe, the fruit was used to punish children while educating them and in warfare to produce smoke by burning them (Basu and De 2003; Bosland and Votava 2012). Nowadays, chili fruits have diverse uses, such as pepper spray for self-defense, rat repellent, and animal coloring. They are also used in cosmetics (Bosland and Votava 2012).

It is estimated that the production of chili is about 2.5 million tons globally (Zachariah and Gobinath 2008). The consumption of chili is relatively high in India, Thailand, and South Korea (Thampi 2003; Pinto et al. 2016). As an integral part of home cooking and food industry, the popularity of chili peppers is expanding (Aizat 2013). Further, the chili fruit also affected the political condition in Korea in 1978–1979. The shortage of chili created an anxiety that the Korean government might fall if the demand for chili was not met. In response, Korea imported 50,000 tons of chili from all over the world (Thampi 2003). In 2014, Japan imported 185 tons of hot peppers (excluding the non-pungent peppers) worth 108.34 million yen from South Korea, 115 tons from China, and 97.2 tons from New Zealand. However, the amount of hot peppers imported to Japan decreased sharply from 10,326 tons in 2000 to 399 tons in 2014. This correlated positively with a steady increase in chili cultivation area, and also the crop yield from 206 tons in 2000 to 305 tons in 2010

(Japanese Ministry of Agriculture, Forestry and Fishery 2010; Japanese Ministry of Finance Trade Statistics 2014).

2.3. Nutritional components

Pepper fruits might be famous for their characteristic pungency due to capsaicinoids and bright color due to carotenoids, but the fruits also contain thousands of other metabolites, including water, fatty oils, carotenoids, resin, protein, fiber, minerals, and volatile compounds, which influence their nutritional value, taste, color, and aroma. The fruits of *Capsicum* are an important source of vitamins and antioxidant compounds (Bosland and Votava 2012; Wahyuni et al. 2013; Kantar et al. 2016).

Water is the major component of pepper fruit in terms of quantity (Bosland and Votava 2012). However, the proportion might vary depending on the age and type of pod harvested. It has been observed that as the fruit matured, the water content decreased. Other nutritional components, such as carbohydrates, lipid, protein, vitamins, and microelements, that are essential for humans are also available in pepper fruits. The carbohydrates in the pepper fruits are mainly sugars, pentosans, and raw fiber. Although the lipid content is relatively low, it consists of palmitic, palmitoleic, linoleic, oleic, vaccenic, alpha linolenic, lauric, myristic, arachidic, and stearic acids (Guil-Guerrero et al. 2006; Japan Food Standard Ingredient Table 2015). A low ratio of unsaturated to saturated fatty acids in the chili fruits suggests that they are sensitive to relatively low temperatures (Bosland and Votava 2012). The amino acids found in pepper include glutamic acid, aspartic acid, lysine, isoleucine, leucine, cysteine, methionine, phenylalanine, tyrosine,

threonine, valine, arginine, histidine, alanine, proline, glycine, and serine (Japan Food Standard Ingredient Table 2015). The macroelements include potassium, calcium, sodium, magnesium, phosphor, and sulfur, while the microelements include iron, zinc, manganese, copper, and selenium (Guil-Guerrero et al. 2006). Kantar et al. (2016) reported that the fruits contain a high amount of vitamin C, E, and folate. Other compounds with health benefits in chili are carotenoids, phenolic compounds, flavonoids, and capsaicinoids (Shahidi and Naczk 2003; Frary and Frary 2012; Giuffrida et al. 2013).

2.4. Botanical aspect

The botanical or scientific name for chili is *Capsicum*, derived from the Greek word *Kapto* meaning to bite (Basu and De 2003) or from the Latin *Capsa* meaning bag with shoulder straps (satchel) (Bosland and Votava 2012). Taxonomically, the members of *Capsicum* species belong to the family Solanaceae (night shade), order Solanales, class Magnoliopsida, and division Magnoliophyta (Nwakchukwu et al. 2007). The family Solanaceae also includes tomato, potato, tobacco, eggplant, and the deadly nightshade (Mortensen and Mortensen 2009). The genus *Capsicum*, as described by Basu and De (2003), is a glabrous, perennial, woody subshrub or shrub, dicotyledonous group of flowering plants. The pods are globose, elongated, or irregularly shaped with many seeds **(Figure 2-1)**. A wide variation in the morphology of *Capsicum* has been highlighted in the shape, color, and size of the fruits (Walsh and Hoot 2001; Basu and De 2003).

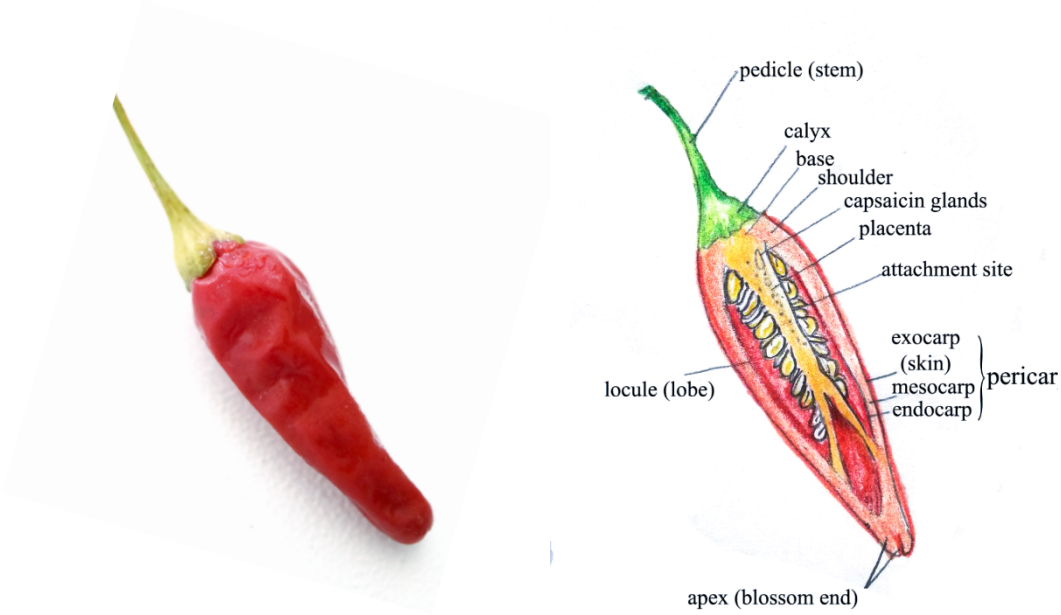

Figure 2-1. A diagrammatic representation of chili pepper.
Left, mature fruit of Shimatogarashi (*Capsicum frutescens*); right, diagram describing the parts of fruit.

Chili peppers propagate under suitable climatic condition as a perennial shrub, some as vines and rarely as herbs. The climatic conditions are of importance to chili plants, because as a tropical plant they require high temperatures (ideally between 21–30°C). The plant is sensitive to relatively low temperatures and intolerant to frost. At lower temperatures, growth of the plant is delayed, younger parts wilt, and germination decreases. Furthermore, temperature affects fruit metabolites such as ascorbic acid, and also pungency and color (Pinto et al. 2016). The fruit color of the genus *Capsicum* ranges from green, yellow, red, and brown to even darker shades depending on the cultivar and ripeness (Ha et al. 2007).

The change in skin color during ripening is due to carotenogenic process, whereby during maturation, chloroplast is converted to chromoplast. Therefore, the chlorophyll content gradually decreases as the carotenoid content increases (Giuffrida et al. 2003). The attractive color of *Capsicum* fruit has been exploited as a natural colorant in various foods.

Apart from its color, the most intriguing aspect of this genus is the pungency of fruits due to the presence of capsaicinoids. Capsaicin is the major capsaicinoid of chili pepper fruit, followed by dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin, and homocapsaicin (**Figure 2-2**). Capsaicin and dihydrocapsaicin account for 90% of the capsaicinoids in the fruit (Reyes-Escogido et al. 2011; Barbero et al. 2014). These compounds are synthesized in the placenta of the fruit (**Figure 2-1**) from fatty acid and vanillylamine by capsaicin synthase (Reyes-Escogido et al. 2011). Further, there are non-pungent varieties of *Capsicum*, as well. The color and pungency of these fruits are the two appealing aspects that support the survival of the plant. The seed dispersers, i.e., birds, are attracted by the color (Zachariah and Gobinath 2008). Furthermore, the pungency of the fruit repels rodents from eating them, because it irritates their nerves. However, the pungency does not have a significant painful effect on birds (Bosland and Votava 1999).

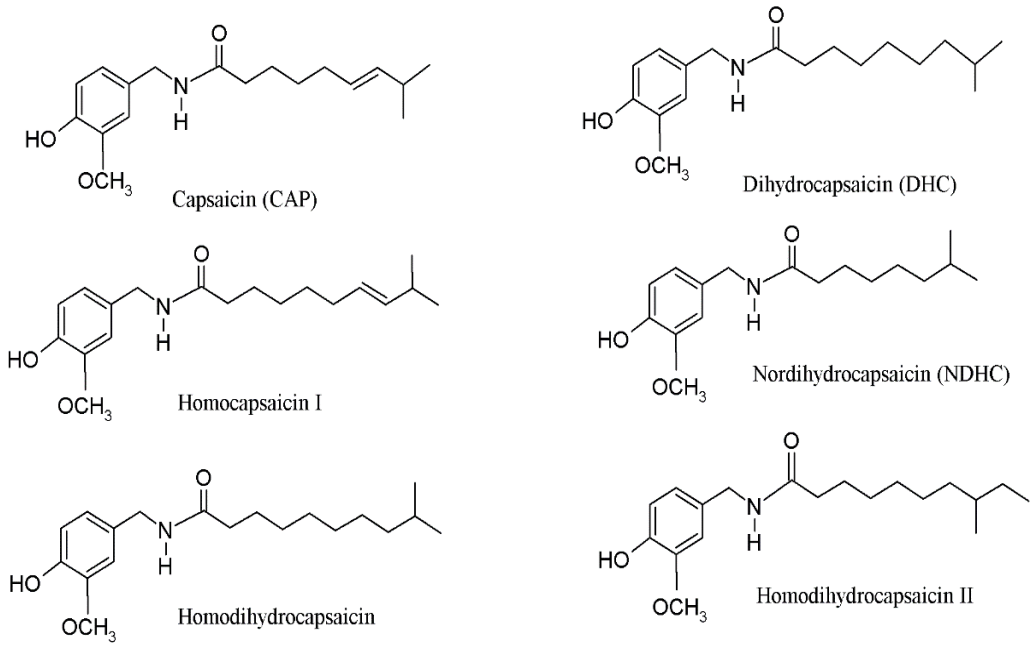

Figure 2-2. Chemical structure of some capsaicinoids

The genus *Capsicum* consists of a large number of domesticated and cultivated species (Basu and De 2003), including 32 native/wild species and 5 domesticated species (Mortensen and Mortensen 2009; Bosland and Votava 2012). Numerous species have a similar morphology, which overlaps with one another. Therefore, careful identification of species, including a combination of diagnostic characters (Walsh and Hoot 2001) is essential. Esbaugh (2012) stated that the genus *Capsicum* has remained a puzzle with unresolved problems, such as defining limit of the genus and allocation of species to the genus (Walsh and Hoot 2001).

Phenotypic as well as genetic and molecular characterization of Capsicum accessions was still one big work for the scientist in order to be able to utilize the potency of the genetic diversity. As recent methods of high throughput genotyping provide larger opportunities for germplasm characterization and knowledge of inherited traits (Stommel and Albrecht 2012). The combination plant genetics

information about inherited traits and environmental factors offers breeders and farmers to have higher chance in developing and manipulating the characteristics of new variant of plant with desired properties and more profitable yields.

Widely used commercial chili pepper cultivars commonly belong to *C. annuum* and *C. frutescens*, mostly the former (Basu and De 2003). The following few sections will focus on the two commonly commercialized cultivars, *C. annuum* and *C. frutescens*, providing a brief description of the two species, while the difference between the two species is summarized in **Table 2-1**.

2.4.1. Capsicum annuum

Basically, all the *Capsicum* cultivars domesticated commercially belong to the species *C. annuum* (Bosland and Votava 2012). *C. annuum* is believed to have spread from Colombia to Southern United States (Esbaugh 2012). This species includes a wide variety of cultivars, such as Jalapeno, Poblano, Pimiento, Mirasol, Chiltepin, Chilhuacle, Anaheim, Serrano, Bell pepper, Cayenne, and Takanotsume. The fruit of these cultivars vary in color, shape, pungency, and size (Walsh and Hoot 2001; Basu and De 2003; Bosland and Votava 2012). In Japan, Takanotsume (**Figure 2-3**) is the most popular cultivar of hot pepper, followed by Hontaka and Santaka (Bosland and Votava 2012). The plant is an annual herb, with solitary white flower, rarely seen in pairs (Basu and De 2003; Nwakchukwu et al. 2007). The leaves are oblong and glabrous.

2.4.2. Capsicum frutescens

C. frutescens consists of relatively few cultivars when compared with that of *C. annuum*. The most popular cultivar is Tabasco (Bosland and Votava 2012). The plant is a finely pubescent, perennial shrub growing up to height of 2 m (Basu and De 2003; Nwakchukwu et al. 2007), with greenish white flower, rarely erect and without constrictions between the base of the calyx and pedicel. The fruit is globose or subconical, erect with calyx embracing the base of the fruit, and the length is less than 2.5 cm (Walsh and Hoot 2001; Basu and De 2003).

Considered a native of Mexico (Esbaugh 2012), *C. frutescens* spread through Caribbean and northern region of South America. The fruits are often preserved as pickles, which are then used to add flavor to food. In India, dried powder of the fruit is commonly available in the market for instant use (Basu and De 2003). In Southern Japan, Shimatogarashi (*C. frutescens*) (**Figure 2-3**) is soaked in a liquor and used to add flavor to dishes.

Figure 2-3. Takanotsume (*C. annuum*) and Shimatogarashi (*C. frutescens*).
Left, Takanotsume pepper; Right, Shimatogarashi pepper.

Table 2-1. Morphological description of *C. annuum* L. and *C. frutescens* L.

Properties	Takanotsume (*Capsicum annuum*)	Shimatogarashi (*Capsicum frutescens*)
Height	▪ 0.3-0.76 m	▪ 0.46-0.76 m
Plant description	▪ Early to medium-late maturing ▪ Glabrous to highly pubescent ▪ Single pedicel or rarely paired ▪ Slender to thick ▪ Erect or pendent	▪ Late maturing ▪ Glabrous to lightly pubescent ▪ Single pedicels or frequently in pairs, sometimes 3-6 at a node ▪ Slender ▪ Erect or pendent
Flower	▪ Dentate calyx ▪ Clear or dingy white corolla ▪ Rarely purple (5-11 mm long)	▪ Truncate or slightly dentate calyx ▪ Light greenish yellow to greenish white corolla ▪ Waxy or shiny (6-10 mm long)
Fruit	▪ Varied pungency from non pungent to pungent ▪ Fruit shape and size extremely variable, generally over 0.8 cm wide and 0.8-25 cm long ▪ Green or yellow when immature ▪ Red, yellow, or brown at maturity	▪ Pungent ▪ Globose, subconical to long and slender, pointed or blunt (0.6-3 cm in width and 1-8 cm in length) ▪ Green or yellow when immature ▪ Red at maturity
Seed diameter	▪ 3-5 mm	▪ 2.5-3.5 mm

Source: Smith and Heiser (1951)

The shape, apex, base, and type of leaf of *C. frutescens* are comparable to those of *C. annuum*, except for the arrangement, which was alternate in *C. annuum* and opposite in *C. frutescens*. Similar observation was made in the flower of *C. annuum* and *C. frutescens*. The type, floral symmetry, pedicel, calyx color, shape, and corolla color of the flower were all similar in both the species, except for the floral arrangement, which was opposite in *C. annuum* and alternate in *C. frutescens* (Nwakchukwu et al. 2007). Both the species have the same haploid chromosome number of 12. Further, genetically, both *C. annuum* and *C. frutescens* are highly cross-sterile (Smith and Heiser 1951).

2.5. Consumer preference on chili

The heat and pronounced flavor of chili fruit correlate positively with the hedonic value of the cuisine (Kostyra et al. 2010). However, chili peppers are more popular for their flavor than their heat (Bosland and Votava 2012). Mortensen and Mortensen (2009) mentioned that "chili peppers were the most widely used seasoning in the world" in relation to their strong flavor. They not only enrich the flavor of food, but also overcome the off-flavor of the food. In their contribution to overall flavor, the taste and aroma of chili fruit are closely related. Furthermore, aroma is considered to play a more significant role in terms of flavor. In this milieu, both non-volatile and volatile constituents that contribute to the taste and aroma of food should be included in the future research on the flavor of chili fruit (Kader 2008). As the market for premium fruits with relatively better aroma is growing, the number of studies on characterization and regulation of aroma volatiles has increased (Zhang and Chen 2014).

The flavor of chili peppers is associated with the compounds related to aroma and taste contained in the fruits. The production of these compounds depends on many factors, such as plant variety, stage of harvest, growing conditions, and geographical origin (Defilippi et al. 2009; Junior et al. 2012). The genetic diversity of the genus *Capsicum* and edaphoclimatic effects on their diversity necessitates studies on phytochemical characteristics of chili peppers (Junior et al. 2012). The subtle difference in flavor among different types of pepper can be recognized by pepper connoisseurs, similar to wine connoisseurs (Bosland and Votava 2012).

Consumer preference for chili peppers depends on many factors, such as flavor, aroma, pungency, and appearance. The perception of flavor is affected by a combination of these factors, which have a potential to increase appetite. Thus, flavor additives in food products increase the value of food (Eggink et al. 2012). This increasing attention to the flavor of chili peppers has led to studies aiming to understand their flavor chemistry (Eggink et al. 2012; Junior et al. 2012).

2.6. Chili pepper maturation

As mentioned earlier, there are many factors that affect the flavor quality of fruits and vegetables. Kader (2008) suggested that the stage of harvest is the second most important factor, with genotype being the first, that affects the flavor of fruits and vegetables. Ripening, a complex developmental process that makes the fruit more tender and flavorful, influences various nutritional and quality characteristics of the fruit as a food commodity (Giovannoni 2001; Defilippi et al. 2009).

Usually harvesting at a fully ripe stage produces the best tasting fruit vegetables and fruits. On the contrary, non-fruit vegetables taste better when harvested at an immature stage. Economically, harvesting fruits before they attain optimum ripening stage is common, because their prices tend to be relatively high at the start of the harvest season (Kader 2008). Besides price, other circumstances also force the farmers to harvest the fruits prematurely, that is during typhoon season. Therefore, characterization of aroma- and taste-related compounds of the fruit and understanding their functional properties are essential. The fruit of Shimatogarashi also undergoes various changes during fruit maturation, including a change in color from green to red (**Figure 2-4**).

Figure 2-4. Shimatogarashi (*C. frutescens*) at different maturing stages

In general, there are two types of fruits based on their physiological response to changes in ethylene level and respiration rate (Aizat 2013): (1) Climacteric fruits that exhibit relatively high ethylene and carbon dioxide (CO_2) levels, and are sensitive to ethylene treatment. The members of climacteric fruit include banana, apple, and tomato. The presence of ethylene allows the fruits to ripen even when harvested at an immature stage; (2) Non-climacteric fruits, such as chili pepper, orange, and strawberry, that cannot ripen naturally when harvested prematurely have relatively low level of ethylene. Further, they exhibit a continuous decrease in CO_2 level (Bapat et al. 2010). The mechanism of fruit ripening in climacteric fruits has been studied extensively, however non-climacteric fruits have not received a similar attention (Aizat 2013).

2.7. Chili pepper as a part of food functionality

The desire for increased lifespan by humans and improved quality of life during the later years coupled with increase in the cost of healthcare have triggered a demand for functional foods. Therefore, in order to stand out from competitors and meet consumer demands, especially for functional foods, research and innovation have been recognized as essential aspects by the food processing and manufacturing companies (Bigliardi and Galati 2013). Therefore, studies on the metabolites of pepper plant have been increasing rapidly (Frary and Frary 2012). The fruit of the species *Capsicum* has been reported to possess various metabolites exhibiting antioxidant, hypoglycemic, antiobesity, immunogenic, antihypertensive, anticholesterol, anti-inflammatory, and antimutagenic properties (Kwon et al. 2007; Menichini et al. 2009; Jeon et al. 2010). Much attention has been directed toward

the compounds that impart the spicy flavor (pungency), which are known as capsaicinoids. These compounds play a significant role in the food and pharmaceutical industries, although their potential application has been limited by the burning sensation that they impart (Reyes-Escogido et al. 2011).

Studies have revealed that capsaicinoids, especially capsaicin, have beneficial biological and physiological activities, such as antioxidant (Howard et al. 2000; Materska and Perucka 2005), anticarcinogenic (Macho et al. 2003), and anti-inflammatory activities (Sancho 2002), promotion of energy metabolism, and suppression of fat accumulation (Ohnuki et al. 2001). Furthermore, capsaicin has also been used topically to relieve neuropathic pain syndromes associated with diabetic neuropathy, post-herpetic neuralgia, musculoskeletal pain, osteoarthritis, and rheumatoid arthritis (Tesfaye 2009; Backonja et al. 2010).

The antioxidant and antiobesity properties of chili fruit have been promising in the prevention of metabolic syndrome through dietary manipulation. The metabolic syndrome and lifestyle-related diseases are prevalent in industrialized countries. This medical and socioeconomic issue is of concern to the modern society (Nagao and Yanagita 2008; Nugara 2016). Previous studies have revealed the potential carcinogenic effect of capsaicin (Surh and Lee 1995). However, a review by Bley et al. (2012) clarified that the reported potential carcinogenic effect of capsaicin might have been due to the presence of cancer-causing contaminants in the extract. Recent investigations employing high-purity capsaicin and standardized protocols have deduced that the genotoxic and carcinogenic potential of capsaicin is significantly low. This indicates that there are many aspects of chili peppers that are yet to be explored.

Chapter III

Physical Properties, Flavor Characteristics and Antioxidant Capacity of Shimatogarashi (*Capsicum frutescens*)

3.1. Introduction

Generally, there are large varieties of chilies and each of them has their own niche markets. Among others, color, pungency, and aroma are key parameters looked by consumer in selecting chili. The huge diversity of chili provides extensive range of sizes, colors, pungency and aroma. This diversity attributed to genotype and also environmental factors, for instance soil, climate and land cultivation practices. Therefore, the chili peppers originated from different regions might possess different characteristics. The edaphoclimatic nature and large genetic diversity of chili peppers also necessitate an unceasing efforts investigation of chili peppers' composition. Flavor characteristics and functional properties are also helpful for differentiating between cultivars and their place of origins, as well as to assess their authenticity (Junior et al. 2012). Characterization of chili peppers, especially in term of flavour and biological function, allows consumers to be better informed about their attributes in order to optimize the utilization of the fruits, prevent fraud, and select the appropriate variants for particular purposes.

People in Japan also include chili pepper in their cuisines, although not as much as their neighboring country, Korea. Yamamoto (2013) reported that two species of chili peppers had been introduced to Japan: *C. annuum* and *C. frutescens*, namely Takanotsume and Shimatogarashi, respectively (**Figure 3-1**). Takanotsume

is commonly used peppers in mainland Japan. It was the most popular variety and has been included in daily cuisine since 17th century (Itoh 2015). Among Capsicum group, *C. annuum* is the most extensively cultivated and commercially important (Stommel and Albercht 2012). On the other hand, Shimatogarashi is mostly cultivated in tropical region of southern Japan, namely Okinawa. In this region, Shimatogarashi peppers are traditionally used in a spice called *koregusu,* which has a unique flavor. Interestingly, Yamamoto and Nawata (2005) reported that Shimatogarashi in Okinawa possessed an atypical isozyme pattern, in contrast to other *C. frutescens accessions* from Southeast and East Asia.

The flavor characteristic (particularly volatiles composition) and functional properties (particularly antioxidant activity) of chili peppers, especially *C. annuum*, including Takanotsume, have been studied (Eggink et al. 2012; Meckelmann et al. 2013), as they are one of the best known and the most widely used spice crop in the world. On the contrary, *C. frutescens* domesticated in subtropical Asian regions, particularly Shimatogarashi peppers from Okinawa, did not receive as much attention. Accordingly, it is of great interest to determine food properties in term of the flavor characteristic and functional properties of Shimatogarashi. It was also noteworthy to mention that based on molecular study *C. annuum* and *C. frutescens* were phylogenetically close to each other that there was a dispute whether to put them into one species or separately (Esbaugh 2012). However, they were treated as different taxa (Walsh and Hoot 2001). Confusion in species designation of *C. annuum* with *C. frutescens* in scientific literature sometimes occurred (Zachariah and Gobinath 2008).

Therefore, the aim of this study was to characterize the physical properties, flavor quality attributes particularly organic acids, capsaicinoids composition and volatile components in Shimatogarashi compared to those of Takanotsume as control. Evaluation of the functional properties of the peppers by analyzing total phenolic content and antioxidant activity using oxygen radical absorption capacity (ORAC) was also conducted. The information obtained would offer supplementary information on differences of the two species grown under the same condition including soil, climate and agricultural practices.

3.2. Materials and methods

3.2.1. Plant materials

Shimatogarashi (*C. frutescens*) seed was obtained from Miyako Island, courtesy of the Okinawa Prefectural Agricultural Research Center. As for Takanotsume (*C. annuum*), the seeds were purchased from commercially available seed packages. The plants were cultivated at the same time in the Subtropical Field Science Center, University of the Ryukyus, Okinawa, Japan (**Figure 3-1**). Germination began on March 21, 2013, by planting the seeds in a tray filled with pumice sand. Once emerged, the seedlings were provided with nutrient solution (NO_3-N: PO_4-P: K: Ca: Mg = 18.6: 5.1: 8.6: 8.2: 3.0 mg/L) and kept in a greenhouse for two months.

Figure 3-1. Shimatogarashi and Takanotsume plants.

Picture showing the plant of Takanotsume (*C. annuum*) (left) and Shimatogarashi (*C. frutescens*) (right) at the Subtropical Field Science Center, University of the Ryukyus.

After two months in a greenhouse, Shimatogarashi and Takanotsume were transplanted and cultivated in the field plot consisted of a 24 m × 1 m field of mixed soil (gray soil and dark-red soil, pH 6.84) with compost (N:P:K = 1.0:2.3:2.1%) until they bear an abundant fruit. The fruits were randomly picked from ten different plants and stored at –30°C in a freezer until they were analyzed, unless otherwise indicated.

3.2.2. Chemicals

Standard Organic acids (malic acid, citric acid, and *L*-ascorbic acid) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Standard capsaicinoids (capsaicin and dihydrocapsaicin) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemicals used as standards to identify the volatile components were obtained from Tokyo Chemical Industry (Tokyo, Japan), Wako Pure Chemical Industries, Kanto Chemical Industry (Tokyo, Japan), ACROS (New Jersey, USA), Fluka (Buschs, Switzerland), and Sigma-Aldrich. 2,2′-azobis

(2-methylpropionamidine) dihydrochloride (AAPH) and Gallic acid were obtained from Wako Pure Chemical Industries. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Calbiochem (San Diego, CA, USA). Folin-Ciocalteu reagent was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade.

3.2.3. Analysis of surface color

Reflective color information was obtained using a handy NF 333 spectrophotometer (Nippon Denshoku Industries Co., Tokyo, Japan) with an 8 mm diameter sensor and standard calibration plate (No. 99067). The observed color was mapped in the CIE color space using the $L^* a^* b^*$ coordinates.

3.2.4. Analysis of organic acids

The organic acids, particularly malic and citric acids, were extracted from the fruit according to the following method. Briefly, sliced fruit (5 g) was mixed in ultra-pure water (20 mL) using a homogenizer (Ultra Turrax T25 basic, Labortechnik, Malaysia), and the mixture was then centrifuged (CR20 GIII, Hitachi, Japan) at 32,300 × *g* at 4°C for 30 min. The supernatant was collected and filtered through Sep-Pak C18 cartridge (Waters, USA) and an Advantec 0.45 μm cellulose acetate membrane filter (Toyo Roshi Kaisha, Ltd, Tokyo, Japan). The organic acids in the filtrate were analyzed using high-performance liquid chromatography (HPLC) according to the method of Ji et al. (2006) with slight modifications. The column used was a Shim-pack SCR-102H column (300 mm × 8 mm i.d., Shimadzu Corp., Kyoto, Japan) connected to a guard column (50 mm × 6 mm i.d.). Two

Shimadzu LC-10 AD pumps were used to flow the mobile phase containing 5 mM *p*-toluenesulfonic acid and post-column detection reagent containing 5 mM *p*-toluenesulfonic acid, 100 μM ethylenediaminetetraacetic acid (EDTA) disodium salt, and 20 mM Bis-Tris buffer in isocratic mode at a flow rate of 0.7 mL/min. The mobile phase and post-column detection solvent were streamed to a post-column reactor and mixed at a ratio of 1:1 before detection with a Shimadzu CDD-6A conductivity detector. The column, guard column, and post-column reactor were maintained at a constant temperature of 40°C using a Shimadzu CTO-10 AC oven, and the injection volume was 10 μL. The concentrations of citric and malic acids were calibrated by plotting peak area against concentration for the respective acid standards and expressed as mg/100 g fresh weight (FW). All assays were performed in triplicate.

L-Ascorbic acid concentration was determined by the method of Topuz and Ozdemir (2007) with slight modifications. Briefly, sliced fruit (5 g) was mixed in 20 mL of 30 g/L metaphosphoric acid aqueous solution including 1 μM EDTA, and 10 μM diethyldithiocarbamic acid using a homogenizer, and the mixture was then centrifuged at 32,300 × *g* at 4°C for 30 min. The supernatant was collected and filtered through Sep-Pak C18 cartridge and a 0.45 μm cellulose acetate membrane filter. Ascorbic acid in the filtrate were analyzed using HPLC on a Cosmosil 5C_{18}-AR-II (250 mm × 4.6 mm i.d., Nacalai Tesque, Kyoto, Japan) with Shimadzu SPD-M20A diode array detector using a mobile phase of 0.2 M potassium dihydrogen phosphate solution (pH 2.2 adjusted with *o*-phosphoric acid). The flow rate and oven temperature were 0.7 mL/min and 28°C, respectively. The injection volume of samples and standards was 5 μL. Ascorbic acid peak was monitored at

254 nm, and its concentration was calibrated by plotting peak area against standard concentrations and expressed as mg/100 g FW. All assays were performed in triplicate.

3.2.5. Analysis of capsaicinoids

Capsaicinoids, particularly capsaicin and dihydrocapsaicin, were extracted from the fruit using a procedure reported by Minami et al. (1998) with the following modifications. Ground freeze-dried fruit without peduncles (400 mg) was mixed in 20 mL of an extraction solvent consisting of equal volume of acetone and ethyl acetate, and the mixture was shaken for 1 h at room temperature and centrifuged at 1292 × *g* at 4°C for 10 min. The extraction using the solvent was repeated twice, and the volume of the supernatant was adjusted to 50 mL using the solvent.

The capsaicinoids in the solution were analyzed using HPLC system with fluorescence detection. A Develosil ODS-SR-3 column (150 mm × 3 mm i.d., Nomura Chemical, Aichi, Japan) was maintained at 40°C in a Shimadzu CTO-20 AC oven. A Shimadzu LC-20AB pump was operated in isocratic mode with a mobile phase containing equal volume of 1% acetic acid aqueous solution and acetonitrile at a flow rate of 0.4 mL/min. The injection volume of samples and standards was 5 μL. The respective capsaicinoids peak was monitored at 485 nm (excitation) and 530 nm (emission) with a Shimadzu RF-20Axs fluorescence detector, and its concentration was calibrated by plotting peak area against standard concentrations and expressed as mg/100 g FW. All assays were performed in triplicate.

The Scoville heat unit (SHU) as index of chili pepper pungency is related to the concentrations of the capsaicinoids including capsaicin, dihydrocapsaicin, and nordihydrocapsaicin in the fruit (Gonzales-Zamora et al. 2013) and was calculated according to Equation 3.1:

$$SHU = [(CAP + DHC) \times 16.1 + nDHC \times 9.3] \quad \ldots (3.1)$$

where *CAP* is the value of concentration (ppm) of capsaicin, *DHC* is the value of concentration (ppm) of dihydrocapsaicin, and *nDHC* is the value of concentration (ppm) of nordihydrocapsaicin.

3.2.6. Analysis of volatile compounds

Freshly harvested whole fruits without peduncles were frozen using liquid nitrogen and ground in a mortar. The ground sample (1 g) in the vial was stored in a freezer (–20°C) until analysis. Following methods reported by Eggink et al. (2012), the stored sample was incubated at 30°C for 10 min, and 100 mM EDTA-NaOH (1 mL, pH adjusted to 7.5 with NaOH) solution and ethyl nonanoate (40 µL, 0.11 mg/mL in ethanol) as an internal standard were added. Calcium chloride (2 g) was added to halt the enzymatic reaction and the vial was sonicated for 5 min. The solution (1 mL) was pipetted to a 10 mL crimp cap vial (Agilent) and used for the SPME fiber exposure according to methods reported by Junior et al. (2012). As advised in the optimized result performed by Junior et al (2012), the fiber employed was Supelco 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS).

The previously prepared sample was put in waterbath for 15 min at 40°C for headspace equilibration. As for extraction, the fiber was exposed to the vial headspace for 80 min at 40°C. The volatile compounds were desorbed from the fiber in the GC injector (split ratio 1:1) at 250°C for 1 min. The fiber was reconditioned after each analysis for 5 min. A GC 6890N (Agilent Technologies, USA) equipped with a DB-wax (60 m × 0.25 mm × 0.25 μm) column was used for quantification. The injector and flame ionization detector (FID) temperatures were set to 250°C.

Initially, the oven was set to 40°C, held for 2 min, then ramped to 200°C (2°C/min, 38 min). The resulting peak was calibrated by FID response of the internal standard, and the content of aroma compounds was expressed as milligrams per 1 kg of fresh weight. GC-MS analyzes were performed on a GC 7890N (Agilent Technologies, USA) coupled to a 5975C inert XL Mass Selective Detector and the separations were performed with a DB-wax (60 m × 0.25 mm × 0.25 μm) column with similar conditions as described for GC-FID. The injector and transfer line temperatures were set at 250°C, the carrier gas (helium) flow rate was 32 cm/s, the detector was operated in EI mode (70 eV, mass range = m/z 29–450), and the temperature for electron ion source and the interface were set at 230°C. The compounds were identified by matching the mass spectra fragmentation pattern with the NIST 2008 library, and by comparing the linear retention indices (RIs) of *n*-alkanes (C_7–C_{28}) with literature data. The identities were further confirmed by co-elution using reference standards.

3.2.7. Analysis of Oxygen Radical Absorption Capacity (ORAC)

The antioxidant activity of the fruit sample was evaluated in terms of their ORAC value. Briefly, Ground freeze-dried fruit without peduncles (100 mg) was added to 1 mL of 75% methanol, and the mixture was sonicated for 5 min and centrifuged at 1631× *g* for 5 min. The extraction using the methanol was repeated twice, and the supernatant was assayed for ORAC. A diluted supernatant (25 μL) and 90 nM fluorescein solution (150 μL) were transferred to a black 96-well microplate. The microplate was immediately placed and agitated in a fluorescence microplate reader (Synergy™ HT, Bio Tek, Winooski, VT, USA) and then left to stand at 37°C for 7 min. Further, 25 μL of 160 mM AAPH as the peroxyl radical generator was immediately added to the well. The reaction temperature was maintained at 37°C, and the fluorescence was monitored kinetically with data taken every minute for 90 min with fluorescent filters set at 485 nm (excitation) and 530 nm (emission).

A Trolox curve was plotted and used as external standard and the area under the curve (AUC) of relative fluorescence value was calculated according to Equation 3.2:

$$AUC = 0.5 + f_1/f_0 + ... + ... + f_{89}/f_0 + 0.5\ (f_{90}/f_0) \ldots (3.2)$$

where f_0 is the initial relative fluorescence reading at 0 min and f_i is the relative fluorescence reading at time *i*. The ORAC value was calculated by using a quadratic regression equation relating the Trolox or sample concentration and AUC, and was expressed as μmol of Trolox equivalents (TE) per 100 g FW. All assays were performed in triplicate.

3.2.8. Analysis of total phenolic content

The sample for evaluation of total phenolic content was prepared by the same condition of ORAC assay as above. The total phenol content of the sample was evaluated using the Folin-Ciocalteu method of Singleton and Rossi (1965) with slight modifications. Briefly, various concentrations of diluted sample (20 μL), distilled water (60 μL), and Folin-Ciocalteu reagent (15 μL, previously diluted 2-fold with distilled water) were transferred to a 96-well microplate (Nunc, Denmark) and mixed well. The microplate was immediately placed in a microplate reader (PowerWave™ XS2, BioTek, USA), agitated, and then allowed to stand for 15 min until stable absorption values were obtained. The absorbance was then measured at 750 nm. The TPC was calculated from a linear gallic acid calibration curve and expressed as mg of gallic acid equivalents (GAE)/g FW. All assays were performed in triplicate.

3.2.9. Statistical analysis

The value of physical properties (n = 30), color analysis (n = 6), and the other measurements (n = 3) were expressed as mean ± standard deviation (SD). The statistical difference was determined by Student's *t*-test using BellCurve for Excel 2012 (Social Survey Research Information Co. Ltd., Tokyo, Japan), a Microsoft Excel extension program. Differences were considered significant at $p < 0.05$ or 0.01.

3.3. Results and discussion

3.3.1. Physical properties

Shimatogarashi had smaller size and lighter weight compared to Takanotsume (**Figure 3-2**). Shimatogarashi's physical properties (**Table 3-1**) were within the ranges for *C. frutescens* reported by Jarret et al. (2007). The ratio of flesh to seed weights ratio was also lower in Shimatogarashi, indicating that Shimatogarashi had a thinner flesh and less placental tissue compared to Takanotsume. This observation was in agreement with *C. frutescens* description by Basu and De (2003), which portrayed the fruit pods as having thin flesh.

The color of mature fruits of each species was studied and the L^*, a^*, and b^* values were different with an 11.18 difference in magnitude ($\Delta E ab$) (**Table 3-2**). The chroma and tone (hue angle) were also different. Shimatogarashi showed a slightly higher lightness (L^*) compared to Takanotsume. The a^* and b^* values showed that Shimatogarashi were lighter, and their yellow and red colors were more saturated compared to Takanotsume. Compared to red Habanero (*C. chinense*) cultivars, the a^* and b^* values of both chilies were within the ranges reported by Pino et al. (2007). Moreover, Shimatogarashi had more color turning stages, from green, yellow, orange, and red compared to Takanotsume, which only ranged from green to red.

Figure 3-2. Size comparison of Shimatogarashi and Takanotsume.

Picture showing the red mature fruits of Shimatogarashi (on the left) and Takanotsume (on the right) compared to ruler with a centimeters scale.

Table 3-1. Physical properties of Takanotsume and Shimatogarashi peppers

Parameter	**Shimatogarashi (*C. frutescens*)**	**Takanotsume (*C. annuum*)**
Length (cm)	2.31±0.53	3.88±0.98
Diameter (cm)	0.77±0.14	0.64±0.13
Whole weight (g)	0.63±0.29	0.87±0.43
Number of seed	22±12	26±15
Seed weight (g)	0.19±0.10	0.16±0.11
Flesh weight (g)	0.43±0.18	0.70±0.32
Moisture content	74.26%	65.03%

Data are expressed as means ± SD (n = 30)

Table 3-2. Color analysis of Takanotsume and Shimatogarashi peppers

Parameter	Shimatogarashi (*C. frutescens*)	Takanotsume (*C. annuum*)
*L**	48.71±2.11	41.92±1.97
*a**	41.29±1.60	38.30±2.58
*b**	39.12±2.76	30.75±3.50
Δ*Eab*	11.18	
C	56.89±2.87	49.13±4.09
h°	43.40±1.41	38.66±1.72

Data are expressed as means ± standard deviation (n = 6)

3.3.2. Organic acid

The predominant organic acids in Shimatogarashi and Takanotsume were malic acid and citric acid, respectively (**Figure 3-3**). The organic acid in Shimatogarashi was dominated by malic acid, whereas in Takanotsume, it was citric acid. Hence, the appealing difference between Shimatogarashi and Takanotsume in organic acid composition can be seen by their dominating acids. Luning et al. (1994) reported malic, citric and ascorbic acids as the prevalent organic acids in fresh bell peppers (*C. annuum*). They also stated that the total citric acid content and to a lesser extent *L*-ascorbic acid contributed to sourness, while malic acid contributed negatively to sourness. Furthermore, Eggink et al. (2012) discovered that among the organic acids in chilies, the citric acid content correlated well with sourness; however, the relation between organic acids and sourness perception might not be significant owing to possible interferences from volatile and non-volatile compounds, or texture difference of the fruit.

The citric acid content in Shimatogarashi (154.8 ± 7.4 mg/g FW) was within the range for *C. annuum* L. (155–393 mg/g FW) reported by Matsufuji et al. (2007). The *L*-ascorbic acid content (**Figure 3-3**) reported for Shimatogarashi and Takanotsume (65.8 and 105.6 mg/100 g FW, respectively) were slightly higher than the range reported in the study conducted by Topuz and Ozdemir (2007) for *C. annuum* L. (15.2–64.9 mg/100 g FW).

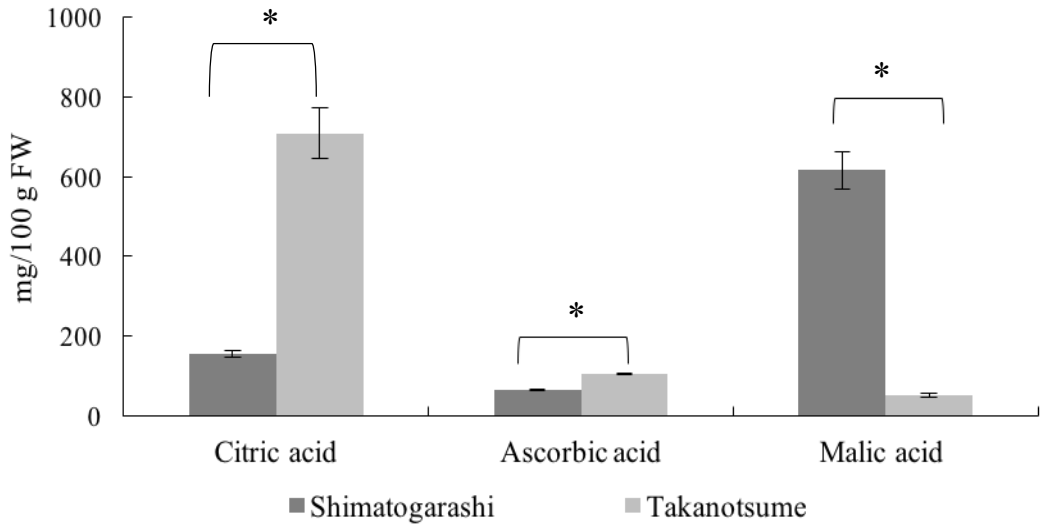

Figure 3-3. Organic acid content in Takanotsume and Shimatogarashi.

Data expressed as means ± SD (n = 3). Asterisk (*) indicated significantly difference between the two cultivars analyzed by student *t*-test at $p < 0.05$.

3.3.3. Pungency

The pungency of chili pepper, an essential parameter from sensorial point of view, is expressed in Scoville Heat Units (SHU). The SHU values expressing the heat levels for Shimatogarashi and Takanotsume, as shown in **Table 3-3**, were 29,732 and 11,902 SHU, respectively. Based on classification of pungency levels by Weiss (2002), Shimatogarashi can be classified as highly pungent (25,000–

70,000 SHU) and Takanotsume as moderately pungent (3,000–25,000 SHU). The heat level of Shimatogarashi was almost three times higher than that of Takanotsume. The heat level is also proportional to capsaicinoids content.

Shimatogarashi had a nearly three-fold higher capsaicin and dihydrocapsaicin content than Takanotsume. The capsaicinoids content reported for Shimatogarashi and Takanotsume was within the range observed in previous reports for *C. frutescens* (Jarret et al. 2007) and *C. annuum* (Minami et al. 1994). As major compounds, capsaicin and dihydrocapsaicin comprised about 79–90% of the total capsaicinoid content during maturation (Barbero et al. 2014). Interestingly, the capsaicin to dihydrocapsaicin ratios in Shimatogarashi and Takanotsume were 1.15 and 0.82, respectively. The biosynthesis gene of capsaicinoids had been elucidated as *CS* and *Pun1* and the compounds synthesized by capsaicin synthase through condensing vanillylamine from phenylpropanoid pathway with 8-methyl-6-nonenoyl-CoA. However, the biochemical reactions, evolution and regulation of capsaicinoid biosynthesis were barely understood (Kim et al. 2014).

Chili pepper pungency is expressed as SHU and the calculated values are shown in **Table 3-3**. SHU is related to the concentrations of the capsaicinoids including capsaicin, dihydrocapsaicin, and nordihydrocapsaicin in a chili pepper fruit (Gonzales-Zamora et al. 2013). Since nordihydrocapsaicin in Takanotsume and Shimatogarashi was detected in trace quantities (data not shown), the SHU was also assessed by the concentration of only capsaicin and dihydrocapsaicin obtained in this study. Based on the SHU classification of pungency levels by Weiss (2002), Shimatogarashi can be classified as highly pungent and Takanotsume as moderately pungent. Another study by Al-Othman et al. (2011) categorized five varieties of

C. annuum L.: hot chilies as highly pungent, red chilies as moderately pungent, green chilies as mildly pungent, and green, yellow and red bell peppers as non-pungent.

Table 3-3. Capsaicinoid contents and Scoville Heat Unit (SHU) of Takanotsume and Shimatogarashi

Variety	Capsaicinoids		Scoville Heat Unit (SHU)
	Capsaicin (mg/100 g FW)	Dihydrocapsaicin (mg/100g FW)	
Shimatogarashi	289.46±14.76*	238.68±12.09*	29731.63±1511.18*
Takanotsume	133.40±7.11	153.88±10.39	11901.75± 722.03

Data are expressed as means ± SD (n = 3). Asterisk (*) denotes significant difference analyzed by student *t*-test at $p < 0.05$.

3.3.4. Volatile compounds

Shimatogarashi and Takanotsume had 48 volatile compounds in common. 2-Hexenal, which had a strong impact on the aroma attribute of sweet peppers (*C. annuum*) according to Eggink et al. (2012), was prevalent in Shimatogarashi, while in Takanotsume, it was the second most common volatile compound (**Table 3-4**). In Shimatogarashi, the total content of hexanal, having fresh fruity notes, was higher than in Takanotsume, suggesting that Shimatogarashi had fresher notes compared to Takanotsume. Hexanal, 2-hexenal, and hexanol were also reported by Ziino et al. (2009) as major compounds in Calabrian hot peppers (*C. annuum* L.).

Mazida et al. (2005) attributed hexanal and 2-hexenal as the typical aroma compounds in chilies as well as linalool, 2,3-butanedione, 2-isobutyl 3-methoxypyrazine and 3-carene. Eventhough the study conducted by Takahashi et al. (2008) detected small amount of 2-isobutyl 3-methoxypyrazine in

Shimatogarashi using SPME method, however, 2,3-butanedione, 2-isobutyl 3-methoxypyrazine, and 3-carene were not detected in either Shimatogarashi or Takanotsume peppers in this study. We found 2-penten-1-ol in both chilies, which was described by Eggink et al. (2012) as a significant contributor to pepper flavor by imparting them with fruity/apple and sweetness attributes, however, since it was detected in minute amount, the influence of the compound presence on the overall flavor of Shimatogarashi and Takanotsume was still unclear.

Aldehydes were prevalent volatile compounds in Shimatogarashi, while in Takanotsume, terpenoids were the major constituents (**Figure 3-4**) with 10*s*,11*s*-himachala-3(12),4-diene in highest concentration. The existence of this compound evidently distinguished the volatile profile of Shimatogarashi from Takanotsume. In contrast, Rodriguez-Burruezo et al. (2010) reported a relatively small amount of this volatile compound in one *C. frutescens* and one *C. annuum* studied. Kollmannsberger et al. (2011) mentioned that himachalenes are typical compounds found in more pungent *C. frutescens* and *C. annuum* varieties. However, α-himachalene was only detected in Takanotsume peppers.

Octadecanal, heptanol, *p*-cymene, 2-methylhexyl propanoate, (*Z*)-3-hexenyl 2-methyl butanoate and pentanoic acid were found in Shimatogarashi but not in Takanotsume. Both 2-methyl hexylpropanoate and (*Z*)-3-hexenyl 2-methyl butanoate were described as having a strong fruity odor (Burdock 2010). Bauer et al. (2001) mentioned that dehydrogenation of limonene led to the formation of *p*-cymene, thus the absence of *p*-cymene in Takanotsume peppers' volatile compound profile might be related to the low level of limonene. On the other hand, *p*-menth-1-en-9-al, β-ocimene, ylangene, (*Z*)-β-farnesene,

(*E*)-nerolidol, and butanoic acid were present in volatile profile of Takanotsume but not in Shimatogarashi (**Table 3-4**). Therefore, *p*-menth-1-en-9-al (fruity notes) and β-ocimene (sweet, warm and herbaceous notes) might be key compounds in differentiating between the flavor characteristic of Shimatogarashi and Takanotsume peppers. In their study, Eggink et al. (2012) also predicted that *p*-menth-1-en-9-al and β-ocimene were important metabolites in determining flavor differences between genotypes and harvests of sweet peppers.

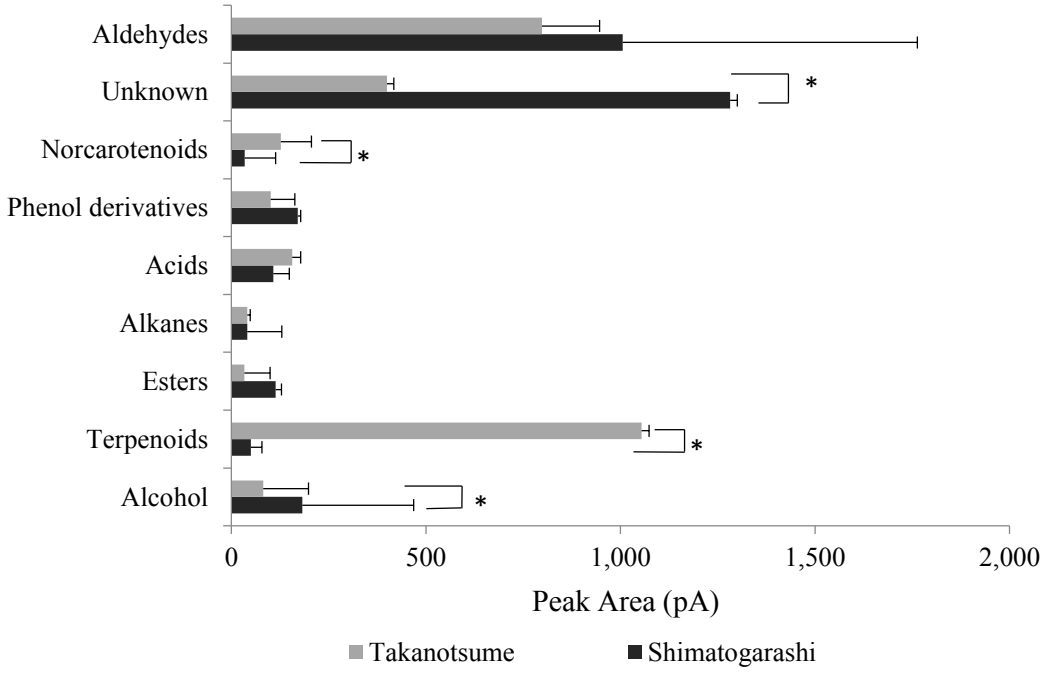

Figure 3-4. The profile of volatile compound groups of Shimatogarashi and Takanotsume.

Data expressed as means ± SD (n = 3). Asterisk (*) indicated significantly higher amount between the two cultivars analyzed by student *t*-test at $p < 0.05$.

Table 3-4. Characterization of the volatile compounds in Takanotsume (as control) and Shimatogarashi peppers

Compound	RI[a]	TAK	SHI	Identification	Aroma description
Esters					
3-methylbutyl 2-methylbutanoate	1278	0.06±0.01	0.16±0.09	RI, MS	fruity[d]
hexyl-2 methylpropanoate	1340	nd	0.64±0.29	RI, MS	fruity[d]
hexyl-2 methylbutanoate	1425	0.03±0.01	2.19±1.15*	RI, MS	fruity,[b] sweet, exotic[h]
hexyl-3 methylbutanoate	1443	0.05±0.01	0.70±0.35*	RI, MS	fruity,[b] sweet, exotic[h]
(*Z*)-3-hexenyl 2-methylbutanoate	1470	nd	1.54±1.11	RI, MS, STD	herb, sweet,[c] unripe apple, pineapple-like[d]
ethyl hexanoate	1952	0.15±0.07	0.13±0.07	RI, MS, STD	powerful, fruity, pineapple-banana note[d]
Terpenoids					
limonene	1194	0.13±0.04	0.37±0.14	RI, MS, STD	citrus, mint,[c] lemon-like[f]
γ-terpinene	1241	0.04±0.01	0.10±0.06	RI, MS, STD	gasoline, turpentine,[c] herbaceous, citrus[f]
β-ocimene	1247	0.30±0.20	nd	RI, MS	sweet, herb, [c]warm, herbaceous,[d] rancid, sweaty[e]
p-cymene	1265	nd	0.06±0.03	RI, MS, STD	solvent, gasoline, citrus,[c] lemon-like[d]
terpinolene	1275	0.26±0.08	0.25±0.07	RI, MS, STD	pleasant, sweet-piney[d]
ylangene	1477	0.22±0.05	nd	RI, MS	-
linalool	1548	0.27±0.15	0.34±0.04	RI, MS, STD	citrus, fruity, floral,[b] lavender[c]
1-terpinen-4-ol	1599	0.04±0.01	0.07±0.04	RI, MS, STD	turpentine, nutmeg, must,[c] herbaceous, lavender[f]
α-himachalene	1635	0.53±0.13	nd	MS	-
(*Z*)-β-farnesene	1663	0.18±0.04	nd	RI, MS	citrus, green[c], fruity, herbaceous[d]
α-terpineol	1704	0.58±0.33	0.49±0.15	RI, MS, STD	oil, anise, mint[c],peach-like[d], lilac[f]
10s,11s-himachala-3(12),4-diene	1719	29.62±7.55	nd	MS	-
geraniol	1850	0.12±0.06	0.11±0.06	RI, MS, STD	rose, geranium,[c] citrus[f]
(*E*)-nerolidol	2000	0.10±0.07	nd	RI, MS, STD	wood, flower, wax[c], fresh, rose and apple-like[d]
Alkanes					
2-methyltetradecane	1453	0.37±0.05	0.85±0.48	RI, MS	-

pentadecane	1499	0.14±0.03	0.40±0.13*	RI, MS, STD	alkane[c]
hexadecane	1598	0.04±0.01	0.10±0.05	RI, MS, STD	alkane[c]
heptadecane	1697	0.62±0.29	0.86±0.16	RI, MS, STD	alkane[c]
Aldehydes					
hexanal	1078	1.32±0.31	2.67±0.88 *	RI, MS, STD	grassy,[b] grass, tallow[c] fruity odor characteristic[d]
2-pentenal	1124	0.21±0.05	0.86±0.13*	RI, MS, STD	tomato, green, apple, orange, pungent[d], strawberry[e]
2-hexenal	1213	22.10±6.78	45.27±5.20*	RI, MS, STD	green, leaf,[c] fruity, almond, spicy, sweet[e]
benzaldehyde	1514	0.13±0.02	0.27±0.13	RI, MS, STD	almond, burnt sugar,[c] bitter almond[f]
p-menth-1-en-9-al	1607	0.26±0.07	nd	RI, MS	spicy, herbal[g]
pentadecanal	1976	0.57±0.12	1.31±0.35*	RI, MS	fresh[c]
octadecanal	2080	nd	1.73±0.89	RI, MS	-
Alcohols					
butanol	1143	0.10±0.04	0.14±0.09	RI, MS, STD	medicinal, fruit[c], fusel-like, sweet and pleasant[d]
1-penten-3-ol	1159	0.13±0.04	0.37±0.21	RI, MS, STD	mild, grassy-green[d]
pentanol	1250	0.04±0.01	0.16±0.11	RI, MS, STD	balsamic[c], fusel-like, sweet and pleasant[d]
4-methyl 1-pentanol	1315	0.23±0.14	1.99±0.93*	RI, MS, STD	-
2-penten-1-ol	1320	0.20±0.12	0.49±0.24	RI, MS	plastic, rubber[c], green-diffusive[d]
hexanol	1354	0.07±0.02	0.74±0.16*	RI, MS, STD	resin, flower, green[c], fruity, bell pepper, herbal[e]
(*Z*)-3-hexen-1-ol	1384	0.05±0.02	0.37±0.12	RI, MS, STD	grass,[c] herbal, leafy[d]
heptanol	1457	nd	0.12±0.07	RI, MS, STD	mushroom[c], woody, heavy, oily, aromatic, fatty[d]
2-ethyl-1-hexanol	1491	0.48±0.06	0.81±0.18*	RI, MS, STD	oily, sweet, slightly floral, rose-like[d]
benzyl alcohol	1875	0.20±0.09	0.24±0.13	RI, MS, STD	flower,[c] pleasant, fruity,[d] sweet[f]
2,6-dimethyl 3,7-octadiene-2,6-diol	1962	0.18±0.24	1.36±0.75	MS	-
dodecanol	1972	0.22±0.18	0.78±0.08*	RI, MS, STD	fatty, waxy[d]
tetradecanol	2178	0.07±0.02	0.13±0.08	RI, MS, STD	coconut[c]

Phenol derivatives					
methyl salicylate	1768	1.60±0.93	8.38±0.94*	RI, MS, STD	green, sweet, phenolic,[b] peppermint[c]
guaiacol	1856	1.79±2.45	0.33±0.11	RI, MS, STD	smoke, sweet, medicinal,[c] phenolic, hot[j]
Norcarotenoids					
α-ionone	1850	0.05±0.02	0.88±0.47*	RI, MS, STD	floral,[b] wood, violet,[c] sweet[f]
β-ionone	1936	3.89±1.25*	0.83±0.19	RI, MS, STD	fruity, floral,[b] seaweed, violet, raspberry,[c] cedarwood[f]
Acids					
acetic acid	1446	0.03±0.01	tr	RI, MS, STD	sour,[c] pungent, cider vinegar-like[d]
2,2-dimethylpropanoic acid	1585	2.07±1.00	3.56±1.93	RI, MS, STD	-
butanoic acid	1625	0.06±0.02	nd	RI, MS, STD	rancid, butter-like[d]
isovaleric acid	1668	0.20±0.10	0.12±0.07	RI, MS, STD	sweat, acid, rancid,[c] cheese-like[d]
3,3-dimethylbutanoic acid	1687	0.59±0.20	0.61±0.07	RI, MS, STD	-
pentanoic acid	1737	nd	tr	RI, MS, STD	sweat,[c] unpleasant[d]
4-methylpentanoic acid	1805	nd	0.35±0.07	RI, MS	unpleasant, sour[d]
hexanoic acid	1846	0.15±0.08	0.10±0.02	RI, MS, STD	sweaty, rancid, sour, pungent, cheesy[d]
2-hexenoic acid	1967	1.35±1.81	1.36±0.75	RI, MS, STD	must, fat,[c] green, earthy, sweet, fruity odor[d]
octanoic acid	2056	0.26±0.20	0.58±0.22	RI, MS, STD	sweat, cheese,[c] mildly unpleasant, fruity-acid[d]
nonanoic acid	2170	0.06±0.03	nd	RI, MS, STD	green, fat,[c] cheesy, waxy[d]
n-decanoic acid	2277	0.07±0.01	nd	RI, MS, STD	rancid, fat,[c] unpleasant[d]

Data expressed as mean values±standard deviation in mg/kg FW (n = 3). Asterisk (*) indicated significantly higher amount between the two cultivars analyzed by student *t*-test at $p < 0.05$.

TAK (Takanotsume); SHI (Shimatogarashi); tr (traces, content less than 0.01); nd (not detectable); MS (mass spectrum was in agreement with NIST 08 library); RI (retention index was in agreement with literature data); and STD (retention index was in agreement with standard co-elution).

[a]Retention Index on DB-Wax column (60 m × 0.25 mm × 0.25 μm) with homologous series of *n*-alkanes (C_7–C_{23});

Description based on [b]Rodriguez-Burruezo et al. (2010), [c]www.flavornet.org, [d]Burdock (2010), [e]Luning et al. (1994), [f]Bauer et al. (2001), [g]thegoodscentcompany.com, [i]Kollsmannberger et al. (2011), and [j]Shahidi and Naczk (2003).

The ester content in Shimatogarashi was higher than in Takanotsume (**Figure 3-4**). Many esters contribute to fruity flavors (Bauer et al. 2001). Namely, 3-methylbutyl 2-methylbutanoate, hexyl 2-methylpropanoate, (*Z*)-3-hexenyl 2-methylbutanoate, hexyl 2-methylbutanoate, hexyl 3-methylbutanoate, and 2-ethyl hexanoate were associated with fruity notes (Rodriguez-Burruezo et al. 2010 and Burdock 2010). While 3-methylbutyl-2-methylbutanoate, hexyl 2-methylbutanoate and hexyl 3-methylbutanoate, and 2-ethylhexanoate were found in both chilies, hexyl 2-methylpropanoate and (*Z*)-3-hexenyl 2-methylbutanoate were only found in Shimatogarashi. Shimatogarashi had higher hexyl 2-methylbutanoate and hexyl 3-methylbutanoate contents than Takanotsume. These compounds were attributed to fruity and sweet notes (Kollsmanberger et al. 2011).

Methyl salicylate was also found to be a major component in the volatile compound profile of both chilies and was more prevalent in Shimatogarashi than in Takanotsume. This supported the results of a study by Rodriguez-Burruezo et al. (2010), indicating that methyl salicylate was detected in *C. frutescens* through smell, but not noticeable in the *C. annuum* and *C. chinense* varieties. Conversely, guaiacol, one of the trace compounds discovered in capsaicin's thermal degradation products (Henderson and Henderson 1992), was found in larger concentration in Takanotsume than in Shimatogarashi. Among other phenolic volatiles, benzyl alcohol was found in small amount in both chilies. The floral-fruity aroma compounds in *C. frutescens* and *C. annuum*, α-ionone and β-ionone (Rodriguez-Burruezo et al. 2010) were classified as norcarotenoids (**Table 3-4**). Both α-ionone and β-ionone were prevalent in Shimatogarashi and Takanotsume.

3.3.5. Antioxidant properties

Some bioactive compounds found in foods were known to have health benefit by performing cellular and physiological functions (Kris-Etherton et al. 2004). Peppers were reported to possess excellent antioxidant activity owing to its phytochemicals, for instance flavonoids, capsaicinoids, phenolic compounds, carotenes, and ascorbic acid (Howard et al. 2000; Bae et al. 2014). Those compounds were described as having strong antioxidant activity, as a means to protect cells from oxidative stress thus prevent chronic human ailments (Imran et al. 2018). The oxygen radical absorbance capacity (ORAC) is a method that quantify antioxidant capacity by measuring inhibition time and degree of inhibition of the compounds and combine them into a single quantity (Cao and Prior 1999). The ORAC value of Shimatogarashi was significantly higher than that of Takanotsume (**Figure 3-5A**). This might correlate with the total phenolic content in Shimatogarashi (3.28 ± 0.28 mg GAE/g FW), which was higher than in Takanotsume (2.54 ± 0.07 mg GAE/g FW) (**Figure 3-5B**).

Higher ORAC value in Shimatogarashi compared to Takanotsume might showed positive correlation with its phenolic content. A positive correlation between antioxidant activity and phenolic content of chili peppers was also reported by Meckelmann et al. (2013) in *C. annuum*, *C. baccatum*, *C. chinense,* and *C. frutescens*. Capsaicinoids (**Table 3-3**) might also influence the phenolic content and ORAC due to their antioxidant properties (Li-E et al. 2008). The phenol derivatives in the volatile profile of Shimatogarashi also had a larger peak area (**Figure 3-4**). Chili peppers were also reported to contain several flavonoids, for instance quercetin, myricetin, luteolin, kaempferol, and apigenin in form of glycosides and

aglycones (Imran et al. 2018).

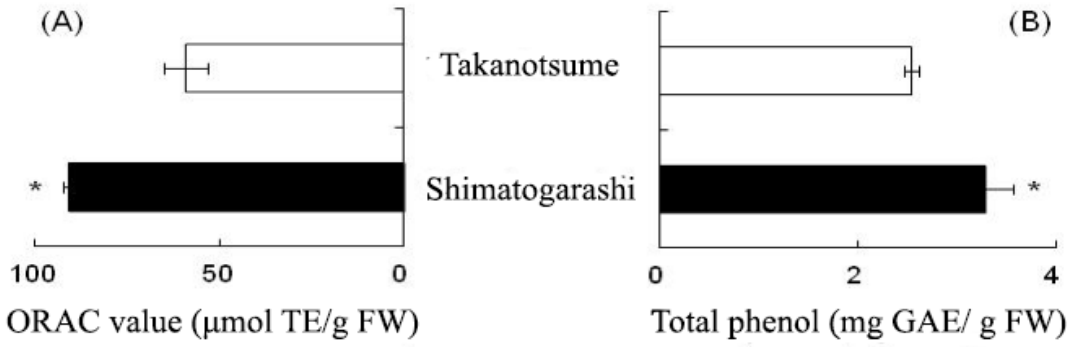

Figure. 3-5. Antioxidant properties of Takanotsume and Shimatogarashi peppers. (A) ORAC value and (B) total phenolic content. Data are expressed as means ± standard deviation (n = 3). Asterisk (*) denotes significant difference analyzed by student *t*-test at $p < 0.05$.

Overall, the ORAC value and total phenolic in Shimatogarashi (**Figure 3-5B**) were higher than the value ranges of *C. annuum* cultivars (16.31–32.90 μmol TE/g FW and 0.74–1.28 mg GAE/g FW, respectively) investigated by Mikami-Konishide et al. (2013). However, *L*-ascorbic acid did not appear to have correlation with ORAC value, which was in agreement with the study conducted by Alvares-Parrilla et al. (2011). Since phenolic compounds and the capsaicinoid content are important factors affecting the antioxidant activity of chilies, this information might lead to practical product development and quality control of chili products.

3.4. Conclusion

The physical properties, flavor characteristics and antioxidant properties of red mature peppers of Shimatogarashi were compared to those of Takanotsume. Physically, Shimatogarashi was smaller and had brighter color than Takanotsume. These peppers had different dominant organic acids. Shimatogarashi was more pungent than Takanotsume, owing to higher concentration of capsaicinoids.

Shimatogarashi had fresh and fruity aroma owing to be rich in aldehyde and ester compounds, while Takanotsume was warm and herbaceous owing to be rich in terpenoid compounds. Shimatogarashi also exhibited higher antioxidant capacity compared to Takanotsume, owing to higher phenolic and capsaicinoid contents. From the current result, it was demonstrated that Shimatogarashi had unique characteristics in terms of physical, flavor and functional properties compared to Takanotsume. Thus, this information might provide the function as a basis for differentiating between cultivars and their place of origins, as well as to attest their authenticity.

Chapter IV

Influence of Fruit Ripening on Color, Organic Acid Contents, Capsaicinoids, Aroma Compounds, and Antioxidant Capacity of Shimatogarashi (*Capsicum frutescens*)

4.1. Introduction

Two most important factors affecting fruit's flavor quality are genotype and stage of maturity (Kader et al. 2008). In Chapter III, the influence of genotype on fruit's flavor and biological function had been studied by comparing Shimatogarashi (*C. frutescens*) with Takanotsume (*C. annuum*). Since it was discovered that Shimatogarashi showed distinct flavor as well as higher antioxidant activity compared to Takanotsume, the next step of the study focused on investigating the effect of ripening on the fruit properties of Shimatogarashi. During maturation, the fruit of Shimatogarashi undergoes several color changes from green to orange and then to red. Although considered horticulturally ripe, the green stage is physiologically immature. It means that once harvested, the green fruits are incapable of normal ripening. Thus, some of *Capsicum* fruits are categorized as having non-climacteric ripening (Bosland and Votava 2012).

Characterizing fruit components with respect to the different stages of harvest is essential, because of the numerous changes occurring during maturation, including changes in the synthesis, transport, and degradation of fruit metabolites that affect aroma and taste of the fruit (Defilippi et al. 2009). Thus, aroma and taste

compounds of the fruits as well as functional attributes depend on stage of harvest (Howard et al. 2000; Defilippi et al. 2009). There are also several circumstances that might force farmer to harvest the fruits before their optimum maturity stage, namely the economic consideration, in which the price of chili tends to be higher at the start of the harvest season (Kader 2008).

Many studies have investigated the effect of ripening in climacteric fruits, particularly apples (Liu et al. 2016), bananas (Sonmezdag et al. 2014), and tomatoes (Tohge et al. 2014). On the other hand, the aroma evolution of non-climacteric fruits especially chili pepper had not well understood (Defilippi et al. 2009). Liu et al. (2009) has investigated the changes in volatile profiles during ripening in *C. frutescens*. Meanwhile, the content of capsaicinoids in the fruits was also reported to vary depend on the growing conditions, cultivars, and growth stages in *C. annuum* (Barbero et al. 2014). The study on bioactive molecules and antioxidant changes in *Capsicum chinense* has also been reported (Castro-Concha et al. 2014). So it is also interesting to see the chemical changes during fruit development of Shimatogarashi, in terms of their flavor components and food functional properties.

The aim of the study in this chapter is to characterize the physical properties, flavor quality attributes particularly organic acids, capsaicinoids composition and volatile components in three different stages of fruit maturation. Therefore, this study may also contribute to our understanding of the origin of aroma and taste as well as the functional properties concerning the utilization of both mature and immature chili pepper fruits.

4.2. Materials and methods

4.2.1. Plant materials

The plant materials used were the same as described in Chapter III (**section 3.2.1.**). The fruits were randomly harvested, classified based on their skin color (green, orange, red) and stored at –30°C in a freezer until they were analyzed (**Figure 4-1**), unless otherwise explained. The size of the fruits at different maturation stages are listed in **Table 4-1**.

Figure 4-1. Sample materials of Shimatogarashi fruits in different maturing stages. From left to right: green stages, orange stage, and red stage.

4.2.2. Chemicals

The chemicals used were the similar with the previous experiment as described in Chapter III (**section 3.2.2.**), except for 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2.3. Analysis of surface color

Reflective color was measured with a Handy NF 333 spectrophotometer (Nippon Denshoku Industries Co., Tokyo, Japan) with a sensor (8 mm diameter) and standard calibration plate (No. 99067) on two different skin zones of three fruits. The measured color was plotted in the CIE color space using L^* a^* b^* coordinates.

4.2.4. Analysis of organic acids

The composition of the organic acids, including ascorbic acid was measured according to a previous study as described in Chapter III (**section 3.2.4.**). The concentrations of linear regressions of peak area and the concentration of citric and malic acid standards were used for quantification purposes. The data are expressed in mg/100 g fresh weight (FW). All assays were performed in triplicate.

4.2.5. Analysis of capsaicinoids

Capsaicinoids were extracted from the fruit using a procedure reported by Minami et al. (1998) with the following modifications. The powder of freeze-dried fruit without peduncles (400 mg) was mixed with 20 mL of an extraction solvent comprising an equal volume of acetone and ethyl acetate. The mixture was shaken for 1 h at room temperature and centrifuged at 1,292 × *g* at 4°C for 10 min. The extraction was repeated twice using fresh solvent, and the volume of the combined supernatant was adjusted to 50 mL. Capsaicinoids content including capsaicin, dihydrocapsaicin and nordihydrocapsaicin was measured using an HPLC system with a fluorescence detector. A COSMOSIL $_5C_{18}$-AR-II column (250 mm × 4.6 mm i.d., Nacalai Tesque, Kyoto, Japan) was kept at 40°C in a Shimadzu CTO-20 AC

oven. A Shimadzu LC-20AB pump was operated in isocratic mode with a mobile phase containing equal volumes of 1% acetic acid aqueous solution and acetonitrile at a flow rate of 0.7 mL/min.

The injection volume of samples and standards was 5 µL. The respective capsaicinoids peaks were monitored at 485 nm (excitation) and 530 nm (emission) using a Shimadzu RF-20Axs fluorescence detector, and the concentration was calibrated by plotting peak area against standard concentrations and expressed as mg/100 g FW. Quantification of nordihydrocapsaicin was done using the calibration curve of dihydrocapsaicin, since there were no commercial standards available for nordihydrocapsaicin and the two molecules share structural similarities. All assays were performed in triplicate.

The Scoville heat unit (SHU) was used as an indicator of chili pepper pungency and is related to the concentrations of the capsaicinoids including capsaicin, dihydrocapsaicin, and nordihydrocapsaicin in the fruit. The SHU calculated according to Equation 3.1 (refer to **section 3.2.5.**).

4.2.6. Analysis of volatile compounds

Freshly harvested whole fruits without peduncles were frozen using liquid nitrogen and stored in a freezer (–80°C). At the time of analysis, the frozen fruit were soaked in liquid nitrogen and ground in a mortar. The ground sample (1 g) was transferred to a 10 mL crimp cap vial (Agilent). To halt the enzymatic reaction, 2 mL of 100 mg/mL NaCl in double distilled water (ddw) was added and 10 µL of 2 mg/mL in 3-pentanol in ddw was introduced as an internal standard. The vial was then sealed and sonicated for 5 min.

The method involving exposure of the headspace (HS) of analyte mixtures to a solid phase microextraction (SPME) fiber reported by Junior et al. (2012) was used with some modifications as follows. The volatile compounds were desorbed from the fiber in the GC injector at 250°C for 1 min (split ratio 1:1). The vial was pre-incubated in a water bath for 15 min before conducting HS-SPME volatile compounds absorption for 1 h. The Supelco 50/30μm DVB/CAR/PDMS (divinylbenzene/carboxen/ polydimethylsiloxane) SPME fiber was reconditioned after each analysis for 5 min. A GC 6890N (Agilent Technologies, USA) equipped with a DB-wax (60 m × 0.25 mm × 0.25 μm) column was used for quantification.

The injector and flame ionization detector (FID) temperatures were set to 250°C. Initially, the oven was set to 40°C, held for 2 min, and then ramped to 200°C at a heating rate of 2°C/min for 38 min. The resultant peak was calibrated using the FID response of the internal standard, and the content of aromatic compounds was expressed as mg/kg FW. GC-MS analyses were performed on a GC 7890N (Agilent Technologies, USA) linked to a 5975C inert XL Mass Selective Detector and the separations were performed with a DB-wax (60 m × i.d. 0.25 mm, 0.25 μm film thickness) column as well as a DB5-MS (60 m × i.d. 0.25 mm, 0.25 μm film thickness) column with similar conditions as described for GC-FID measurements. The carrier gas (helium) flow rate was 32 cm/s, the injector and transfer line temperatures were set at 250°C, the detector was operated in EI mode (70 eV, mass range = 29–450 m/z with quadrupole analyzer), and the temperature for the electron ion source and the interface were set at 230°C. The compounds were identified by matching the mass spectra fragmentation patterns with the NIST 2008 library, and by comparing the linear retention indices (RIs) of *n*-alkanes (C_7–C_{28})

with literature data. The identities were further confirmed by co-elution using reference standards.

4.2.7. Evaluation of antioxidant properties

Analysis of oxygen radical absorption capacity (ORAC) and total phenolic content (TPC) were performed following a procedure described in Chapter III (refer to **section 3.2.7.**). Analysis of DPPH free radical scavenging activity was assessed according to Barros et al. (2012) with some modifications. A 50 µL aliquot of the prepared extract, 50 µL of MES buffer (pH 6.0) and 50 µL of DPPH (0.4 mM) were pipetted to a clear 96-well microplate (Nunc, Denmark) and gently mixed for 10 seconds.

After incubation for 20 min, the absorbance was measured at 520 nm using a microplate reader (PowerWave™ XS2, BioTek, USA). The TPC was calculated using a linear gallic acid calibration curve and expressed as µg of gallic acid equivalents (GAE)/g FW while ORAC and DPPH were expressed as µmol of Trolox equivalents (TE)/g FW. All assays were performed in triplicate.

4.2.8. Statistical analysis

The value of physical properties (n = 30), color analysis (n = 6), and the other measurements (n = 3) was expressed as the mean ± standard deviation (SD). The statistical difference was determined by the Tukey test using BellCurve for Excel 2012 (Social Survey Research Information Co. Ltd., Tokyo, Japan). Differences were considered significant at $p < 0.05$ or 0.01.

4.3. Results and discussion

4.3.1. Physical properties

The dimensions of Shimatogarashi measured fell on the range of study on *C. frutescens* cultivars reported by Jarret et al. (2007). The size of Shimatogarashi pepper was relatively small (**Figure 4-2**), The fruits would grow bigger and heavier from green stage to red stage, however due to dehydration, the weight of red mature stage would slightly lighter compared to the orange stage (**Table 4-1**).

Table 4-1. Physical properties of Shimatogarashi in three maturing stages

Properties	Green	Orange	Red
Length (cm)	1.91±0.41	2.51±0.31	24.10±3.98
Diameter (cm)	0.62±0.11	0.92±0.11	8.60±1.43
Whole weight (g)	0.38±0.15	0.95±0.24	0.76±0.24
Flesh weight (mg)	307.83±105.29	711.74±159.61	557.31±155.60
Seeds weight	68.17±44.49	236.26±87.21	206.69±96.83
Moisture content	77.83%	69.23%	61.96%

Data are expressed as means ± SD (n = 6).

The moisture content decreased from 77.83% in green stage to 61.96% (**Table 4-1**). According to Bosland and Votava (2012), water is the most plentiful constituent in chili pods, which depends on the age of the fruits. The green stage holds around 90%, which gradually decreased to about 70% as the fruit matures on plant.

Figure 4-2. Size comparison of three stages of Shimatogarashi fruits.

4.3.2. Color

The surface color of Shimatogarashi were studied at three different stages of maturation (**Figure 4-2**), yielding different a^* and b^* values, tone or hue angles ($h°$) and chroma (C) for each stage (**Table 4-2**). In the green stage the mean value of a^* was –4.18, while in the orange and red stages it was 37.43 and 40.36, respectively. The b^* parameter had a mean of 35.99 in the green stage, 65.45 in the orange stage and 36.70 in the red stage. An $h°$ of 0° corresponds to pure red, and 90° indicates pure yellow, hence orange will have values between red and yellow (Meckelmann et al. 2013). **Table 4-2** shows that the value of $h°$ in the green stage was 89.72° which indicates a color closer to pure yellow, while the orange and red stages had lower angles of 60.89° and 43.40°, respectively. Simple observation on the fruit showed that the green stage would turn into yellowish in 40 to 50 days since the fertilization took place. The fruit turned pale orange color around day 60 and finally fully ripe in 80 to 95 days from the fertilization.

The measured value of $h°$ from the green stage to red stage showed the gradual inclination to 0° which demonstrating that the color turned to red as the fruit ripened. This can be explained by the transformation of chloroplasts into chromoplasts, followed by the disappearance of chlorophylls and the new formation of carotenoids such as capsanthin and capsorubin (Giuffrida et al. 2013). Therefore, skin $h°$ was considered a good parameter to measure the maturity index for peppers (Barrera et al. 2008). Meanwhile, the C value showed that the orange stage had the brightest color (60.79), followed by the red stage (56.89) and the green stage (45.56).

Table 4-2. Surface color of Shimatogarashi at three maturing stages.

Parameters	Green	Orange	Red
L^*	58.08±3.37^{b}	61.86± 6.34^{a}	46.24±2.20^{c}
a^*	–4.18±4.37^{c}	37.43± 6.94^{b}	40.36±1.66^{a}
b^*	35.99±8.21^{ab}	65.45± 10.77^{a}	36.70±2.24^{b}
h^o	89.72±6.36^{a}	60.89± 1.82^{b}	43.40±1.41^{c}
C	45.56±7.33^{b}	60.79±11.65^{a}	56.89±2.87^{ab}

Data are expressed as means ± SD (n = 3). Different letters in the same row denote a significant difference as analyzed by the Tukey test at $p < 0.05$.

4.3.3. Organic acids

In peppers, organic acids are essential components that influence the taste and nutritional value of the fruit (Goff and Klee 2006; Jarret et al. 2009). The major organic acids found in Shimatogarashi, citric, ascorbic and malic acid, are well perceived by human sour taste receptors (Goff and Klee 2006). The content of all three organic acids significantly increased from the green stage to the red stage (**Figure 4-3A** and **Figure 4-3B**), as the fruits generally accumulate citric, ascorbic

and malic acid during maturation (Cherian et al. 2014). Compared to Takanotsume which had citric acid as its most dominant organic acid (Manikharda et al. 2017), among the organic acids in Shimatogarashi, malic acid was present in the largest quantities (**Figure 4-3B**), followed by citric acid and ascorbic acid (**Figure 4-3A**).

The concentration of malic acid in three different Shimatogarashi maturation stages (0.8–1.2 g/100 g FW) fell within the range of malic acid concentrations reported in a study of *C. frutescens* accessions (0.62–2.07 g/100 g FW) by Jarret et al. (2007). Levels of citric and malic acid over time are important in the study of fruit ripening (Osorio et al. 2013), because in analogy with tomatoes, the changes in acids can alter the perceived flavor of the fruits (Baldwin et al. 2008).

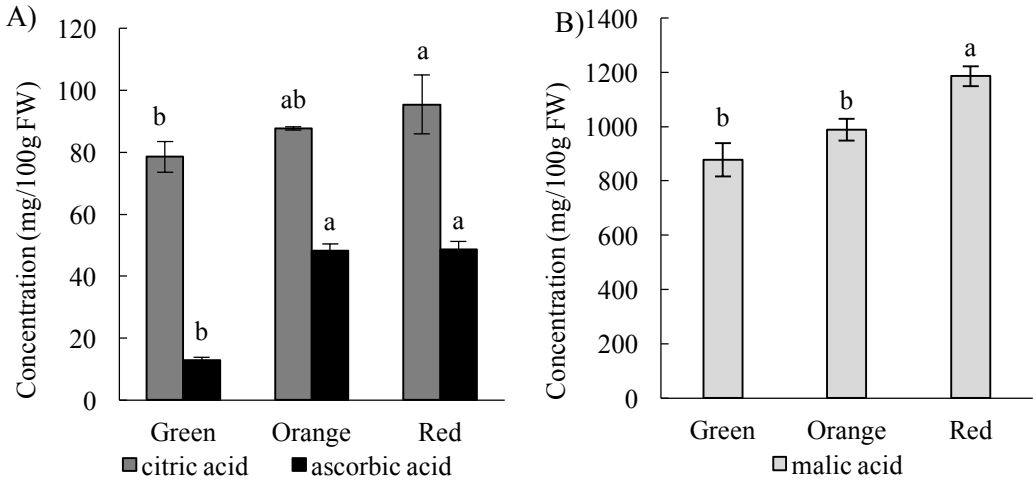

Figure 4-3. Organic acid content at three different stages of Shimatogarashi maturation.

(A) Citric and ascorbic acid content and (B) malic acid content. Data are expressed as the mean ± SD (n = 3). Different letters in the same category denote significant differences as analyzed by a Tukey test at $p < 0.05$.

The ascorbic acid concentration increased four-fold in Shimatogarashi from the immature (green) stage to the mature (orange and red) stages. The increase of ascorbic acid was also observed in other investigations on *Capsicum* spp. (Howard et al. 2000; Navarro et al. 2006; Bae *et al.* 2014). The accumulation of ascorbic acid in the mature stages is attributed to higher light intensities and the necessity to prevent damage due to oxidative stress in fruits (Defilippi et al. 2009; Bae et al. 2014; Wahyuni et al. 2014). However, the ascorbic acid content is also influenced by growth conditions and genetic. Among the genes involved in ascorbate biosynthesis such as ascorbate oxidases (APXs), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), DHAR was found to be highly expressed in hot pepper during fruit maturation (Kim et al. 2014).

In parallel with the amount of ascorbic acid in Tabasco (15–74 mg/100 g FW) reported by Howard et al. (2000), the concentration in Shimatogarashi was 12–48 mg/100 g FW. Thus, both peppers might not serve as an excellent source of ascorbic acid compared to *C. annuum* (63–202 mg/100 g FW) or mature *C. chinense* (115–122 mg/100 g FW) (Howard et al. 2000).

4.3.4. Capsaicinoids

Capsaicinoids are responsible for the pungent taste of hot peppers. Capsaicin, dihydrocapsaicin and nordihydrocapsaicin were found to be the major capsaicinoids in Shimatogarashi (**Table 4-3**). With respect to capsaicinoid content, capsaicin was detected with the highest concentration, followed by dihydrocapsaicin and then a small amount of nordihydrocapsaicin. Capsaicin and dihydrocapsaicin are considered the typical pungent compounds in chili peppers, inducing a sensation of

heat in the mid mouth, palate, throat and back of the tongue, while nordihydrocapsaicin is considered less spicy and yields a short-lived and mildly hot sensation (Krajewska and Powers 1988). Other capsaicinoids, homocapsaicin and homodihydrocapsaicin, were detected in minute amounts (data not shown). Both compounds induce strong irritation and piercing heat with slow development and long lasting effects on the throat, palate and back of the tongue (Krajewska and Powers 1988). These compounds can be extracted by ethanol, therefore the pungency of Shimatogarashi as a spice can be intensified in koregusu.

According to Scoville heat units (SHU), Shimatogarashi (44,552–57,995 SHU) is comparable to hot chili (67,984 SHU) (Al-Othman et al. 2011) and is considered highly pungent. As the fruits ripened, the heat level of Shimatogarashi fruit intensified as indicated by significant increases in capsaicin (from 207 mg/100 g FW to 273 mg/100 g FW) and dihydrocapsaicin (from 79 mg/100g FW to 81 mg/100 g FW). The increase of capsaicinoids during maturation was also reported by Bae et al. (2014) in Cayenne pepper with increases from 14.95 to 21.17 mg/100 g FW and 7.20 to 11.46 mg/ 100 g FW, for capsaicin and dihydrocapsaicin respectively.

There are many factors affecting capsaicinoids, particularly capsaicin and dihydrocapsaicin content. As well as depending on cultivar type, the evolution of capsaicinoids, which is affected by the activity of capsaicin synthase (Bae et al. 2014) and peroxidase (a capsaicin degrading enzyme), depends on the growing conditions (Estrada et al. 2000).

Table 4-3. Capsaicinoid profile of Shimatogarashi peppers at three different maturing stages.

Stages	Capsaicin (mg/100 g FW)	Dihydrocapsaicin (mg/100 g FW)	Nordihydrocapsaicin (mg/100 g FW)	Scoville Heat Unit
Green	207.11±4.46[b]	79.65±10.04	6.10±0.21[b]	46,736±1202
Orange	207.00±32.78[b]	65.87±13.42	6.67±0.69[b]	44,552±7500
Red	273.40±24.39[a]	81.40±11.05	9.38±0.11[a]	57,995±5703

Data are expressed as means ± standard deviation (n = 3). Different letters in the same column denote a significant difference as analyzed by the Tukey test at $p < 0.05$.

4.3.5. Volatile compounds

Using SPME method, there were 77 volatiles compounds that could be detected in the volatile fractions obtained from the three maturing stages of Shimatogarashi (**Table 4-4**). The main compounds comprised of esters (87.5%), alkanes (5.1%), terpenes (3.1%), and norcarotenoids (0.5%) which were lipid soluble compounds. Esters group was the largest, not only in term of amount, but also varieties. The high amount and diverse varieties of esters in the volatile fraction in *C. frutescens* was also reported by other investigators (Haymond and Aurand 1971; Rodríguez-Burruezo et al. 2010; Junior et al. 2012). For instance, high levels of esters significantly distinguished Malagueta peppers (*C. frutescens*) from green peppers and paprika (*C. annuum*) (Junior et al. 2012). Rodriguez-Burruezo et al. (2010) also reported that similar ester compounds (**Table 4-4.**) were found in Tabasco, Laotian, and Pebrera peppers (*C. frutescens*) and *C. chinense* accessions. Similar to Tabasco, using Shimatogarashi as a spice might complement dishes due to the pleasant and fruity aromas arising from its ester content.

During ripening, significant changes in the lipid soluble volatile compounds were observed in Shimatogarashi, especially as ester content decreased, while terpene content increased. This is in contrast to characteristics found in climacteric fruit, such as apples and bananas, where the concentration of the fruit's aroma, especially esters develop during ripening (Defilippi et al. 2009). A decrease in the volatile aroma in mature Capsicum fruit was also observed in *C. frutescens* by Liu et al. (2009), and in *C. frutescens*, *C. baccatum* var pendulum and *C. chinense* Jacq. by Junior et al. (2012).

The changes in the volatile profile with respect to ripening stages is further elaborated by the PCA scores plot (**Figure 4-4A**) showing the major types of differences between maturing stages: discriminating red stages (R) from the orange (O) and green (G) stages along the horizontal axis (PC 1, 95.8% explained variance) and the orange and green stages variation along the vertical axis (PC 2, 2.6% explained variance). The green and orange stages were grouped at negative PC 1 values, while the red stage was positioned at positive values. This was further shown by the PCA loading plot (**Figure 4-4B**), as a noticeable difference in the red stage, marked by the presence of terpene groups.

In agreement with the PCA description of the terpene groups, which underlined the differences of the red stage from the green and orange stages, the amount of limonene, *p*-cymene and γ-terpinene were elevated significantly (**Table 4-4**). These compounds having a citrus-like aroma increased in concentration at the red stage, while compounds imparting sweet and fruity flavors such as hexyl 2-methylbutanoate, hexyl 3-methylbutanoate, 4-methylpentyl 3-methylbutanoate, 4-methylpentyl 2-methylpropanoate, 3-methylbutyl 3-methylbutanoate, β-ocimene,

α-ionone, and β-ionone decreased. The increase in the proportion of compounds with citrus-like flavors, along with the accumulation of organic acids, may indicate the change in the perceived flavor of Shimatogarashi from immature green stage to the mature red stage.

On the other hand, the presence of alkane and phenol derivatives were highlighted in the orange and green stages in PC 2, respectively. The alkane group separates the orange fruit from the green in PC 2. Aliphatic alkanes as well as 2-methyl branched alkanes can be found in Shimatogarashi, while identical alkanes were also found in the *C. frutescens* domesticated in China reported by Liu et al. (2009). Rodriguez-Burruezo et al. (2010) considered aliphatic and methyl branched alkanes to be associated to capsaicin biosynthesis as they were found in pungent species of chili peppers. Furthermore, based on the PCA loading score (**Figure 4-4B**), the presence of phenol derivatives was prominent in the green stage in PC 2. This group was marked by the amount of methyl salicylate, which is mostly found in green (unripe) tomatoes (Lewinsonh et al. 2005). These results reveal the groups of volatile compounds that distinguish the fruits in different stages of maturation. Thus the data provide valuable information on the further utilization of Shimatogarashi in food flavoring.

Table 4-4. Volatile compounds in Shimatogarashi peppers

Compounds	RI		Green	Orange	Red	ID	Descriptions*
	DBwax	DB-5					
Esters							
ethyl acetate	915		19±8^{c}	28±16^{b}	131±16^{a}	A	ethereal, fruity, sweet
methyl pentanoate	1090		31±2^{a}	11±2^{b}	5±0^{c}	B	pungent, green-fruity, apple, pineapple-like
methyl 4-methylpentanoate	1147	939	41±3^{a}	18±2^{b}	11±1^{c}	B	sweet, pineapple-like
methyl hexanoate	1186	992	23±3^{a}	4±0^{b}	3±0^{b}	B	ether-like odor reminiscent of pineapple
3-methylbutyl 2-methylbutanoate	1281	1174	50±6^{c}	212±14^{a}	136±25^{b}	B	fruity
3-methylbutyl 3-methylbutanoate	1294	1180	112±33	118±13	67±16	B	fruity, apricot, mango and sweet-apple-like
4-methylpentyl 2-methylpropanoate	1298	1186	5742±691^{a}	3969±701^{b}	1661±452^{c}	B	
pentyl 2-methylbutanoate	1328	906	194±24^{a}	152±11^{a}	63±23^{b}	B	
hexyl 2-methylpropanoate	1343	1211	636±118^{a}	446±54^{a}	201±91^{b}	A	powerful, fruity
pentyl 3-methylbutanoate	1350	1222	77±11^{a}	102±14^{a}	37±11^{b}	B	apple, fresh, fruity
4-methylpentyl butanoate	1371	1232	206±27^{a}	69±40^{b}	100±38^{b}	B	
4-methylpentyl 2-methylbutanoate	1384	1278	11106±2619^{a}	9806±429^{a}	5111±1246^{b}	B	sweet green
(*Z*)-3-hexenyl butanoate	1387	1216	374±160^{a}	33±10^{b}	69±55^{b}	B	fresh, green apple, fruity
4-methylpentyl 3-methylbutanoate	1401	1285	5052±1448^{ab}	6849±310^{a}	3449±1047^{b}	B	fruity, peach
heptyl 2-methylpropanoate	1409	1324	80±19^{a}	48±21^{ab}	35±8^{b}	B	woody odor with distinctly herbaceous, sweet undernotes, fruity
hexyl butanoate	1413		263±89^{a}	177±48^{ab}	99±20^{b}	A	fruity, apricot, pineapple-like
hexyl 2-methylbutanoate	1428	1314	1867±454^{ab}	1998±369^{a}	981±380^{b}	B	fruity, sweet, exotic, green, unripe strawberry
hexyl 3-methylbutanoate	1446	1305	802±209	833±234	479±144	A	fruity, sweet, exotic, unripe fruit
4-methylpentyl pentanoate	1469	1333	3033±540^{a}	3171±99^{a}	1391±381^{b}	B	

(*Z*)-3-hexen-1-yl 2-methylbutanoate	1473	1308	2064±550[a]	1123±76[b]	723±276[b]	A	herb, sweet, unripe apple, pineapple-like
5-methylhexyl 2-methylbutanoate	1479	1364	114±23[a]	91±11[ab]	65±13[b]	B	
(*Z*)-3-hexenyl 3-methylbutanoate	1487		588±231	277±21	287±93	A	sweet, green odor of apple and buttery
4-methylhexyl 3-methylbutanoate	1511	1380	205±42[a]	153±15[ab]	89±16[b]	B	
4-methylpentyl 5-methylpentanoate	1524	1384	13738±2196[a][b]	14037±713[a]	9673±1740[b]	B	
heptyl 2-methylbutanoate	1528		498±152	336±13	305±76	B	fruity, apple
methyl 8-methyl nonanoate	1543		817±85[a]	66±21[b]	152±40[b]	C	
heptyl 3-methylbutanoate	1545	1417	173±78[a]	166±9[a]	5±1[b]	B	
(*Z*)-3-hexen-1-yl pentanoate	1560	1311	40±4[a]	31±5[a]	13±3[b]	A	heavy green with a fresh fruity nuance of apple, pear, grape, banana and kiwi
hexyl 4-methylpentanoate	1566	1419	3443±512[a]	1788±205[b]	1173±281[b]	B	
(*Z*)-3-hexenyl 4-methylpentanoate	1611	1407	356±53	334±29	248±58	B	
methyl decanoate	1616		106±11	102±10	118±22	B	oily, wine, fruity, floral
phenylmethyl 2-methylbutanoate	1863		185±24[a]	57±18[b]	63±25[b]	B	
benzyl 3-methylbutanoate	1886		66±12[a]	35±11[b]	39±11[ab]	B	fruity apple
total			52100±8439[a]	46462±1616[a]	26981±6417[b]		
Terpenes							
α-pinene	1031		6±0[b]	6±1[b]	8±0[a]	A	pine, turpentine-like
β-myrcene	1164		3±0[b]	4±4[b]	36±4[a]	A	pleasant, sweet, balsamic, plastic odor
(R)-(+)-limonene	1194	1101	124±38[a]	89±99[a]	1321±111[b]	A	citrus, mint, lemon-like
β-phellandrene	1199		3±1	3±4	8±3	B	mint terpentine
γ-terpinene	1243		63±9[b]	37±23[b]	520±53[a]	A	gasoline, turpentine, herbaceous, citrus
(*E*)-β-ocimene	1257		20±1[a]	11±1[b]	13±2[b]	A	sweet, herb, warm, herbaceous, rancid, sweaty

p-cymene	1267		22±6[b]	17±19[b]	171±12[a]	A	solvent, gasoline, citrus, lemon-like
α-terpinolene	1278		308±101[a]	2±1[b]	2±0[b]	A	pleasant, sweet-piney
ectocarpene	1363		260±40[a]	223±44[a]	tr	C	green, sweet
β-farnesene	1665		346±61[a]	219±17[a]	261±68[a]	B	citrus, green, fruity, herbaceous
alloaromadendrene	1687		159±63[a]	124±25[ab]	41±11[b]	B	woody
10*s*,11*s*-Himachala-3(12),4-diene	1715		58±19[a]	27±2[b]	25±4[b]	B	
total			1374±224[b]	762±175[c]	2406±134[a]		
Alkanes							
2-methyl tridecane	1358		204±26[a]	128±11[b]	170±26[ab]	C	mild waxy
2-methyl tetradecane	1461	1538	702±90[a]	774±30[a]	300±69[b]	C	
pentadecane	1499		259±18[a]	317±5[a]	160±53[b]	A	alkane
2-methyl pentadecane	1558	1642	321±38[b]	421±19[a]	199±42[c]	C	
2-methyl pentadecene	1572		121±27[a]	162±7[a]	53±11[b]	C	
hexadecane	1600	1681	581±16	632±56	483±154	A	mild waxy
(*Z*)-7-hexadecene	1623		84±6[b]	149±8[a]	53±14[c]	C	
2-methyl hexadecane	1658		218±10[a]	189±6[a]	116±18[b]	C	
heptadecane	1701	1698	196±39[a]	132±12[ab]	117±29[b]	A	alkane
2-methyl (*Z*)-7-hexadecene	1734		42±3[a]	22±2[b]	24±4[b]	C	
(*Z*)-3-heptadecene	1748		64±8[a]	32±4[b]	31±7[b]	C	
octadecane	1800		36±8[a]	15±2[b]	15±3[b]	A	
nonadecane	1899		26±6[a]	13±1[b]	13±3[b]	A	
total			2853±146[a]	2985±112[a]	1734±389[b]		
Ketones							
acetone	861		52±4[b]	59±16[b]	94±3[a]	A	pungent, somewhat sweet taste
3-pentanone	992	784	67±4	80±1	89±24	B	ethereal acetone
total			119±2[b]	139±17[ab]	183±27[a]		

Aldehydes							
(E)-2-hexenal	1217		43±3[a]	6±1[c]	19±6[b]	A	green, fruity, almond, spicy, sweet
pentadecanal	1975		57±6[b]	62±11[b]	118±13[a]	B	fresh
total			100±7[b]	68±12[c]	137±9[a]		
Alcohols							
1-pentanol	1255		5±2[b]	4±2[b]	11±1[a]	A	balsamic, fusel-like, sweet and pleasant
4-methyl 1-pentanol	1319	906	538±36[b]	351±75[b]	649±113[a]	A	nutty
1-hexanol	1362	939	283±22[b]	413±11[a]	107±28[b]	A	resin, flower, green, fruity, green bell pepper, herbal
(*Z*)-3-hexen-1-ol	1387	873	146±12[a]	37±3[b]	120±27[a]	A	grass, herbaceous, leafy
4-methyl 3-penten-1-ol	1389	928	106±48	68±26	31±5	B	
4-methyl 1-hexanol	1443	1321	251±67	124±50	132±43	B	sweaty
1-heptanol	1464	1041	tr	tr	tr	A	mushroom, woody, fatty
2-ethyl 1-hexanol	1496	1096	32±10	32±2	28±2	A	oily, sweet, slightly floral, rose-like
benzyl alcohol	1874	1105	27±6[a]	5±0[b]	10±3[b]	A	flower, pleasant, fruity, sweet
phenethyl alcohol	1919		178±44	110±6	136±15	B	fruity, sweet, floral, honey note with a cocoa and balsamic nuance.
total			1566±48[a]	1146±130[b]	1224±63[b]		
Phenol derivatives							
toluene	1048		6±0[b]	8±2[b]	12±1[a]	A	sweet
methyl salicylate	1768	1272	467±205[a]	96±4[b]	89±12[b]	A	green, sweet, phenolic, peppermint
total			473±205[a]	104±2[b]	101±10[b]		
Norcarotenoids							
α-ionone	1846	1555	43±28	15±1	15±3	A	floral, wood, violet, sweet

β-ionone	1937	285±44[a]	164±22[b]	184±15[b]	A	fruity, floral, seaweed, violet, raspberry, cedarwood
total		328±46[a]	179±23[b]	199±17[b]		
Acids						
hexanoic acid	1832	70±18[a]	18±1[b]	19±3[b]	A	sweaty, rancid, sour, sharp, pungent, cheesy

ID = identification by A = mass spectrum and linear retention index agreed with the standards; B = mass spectrum and linear retention index agreed with the literature data C = mass spectrum and NIST 08. RI = Retention Index on DB-wax and DB5-MS column (60 m × 0.25 mm × 0.25 μm) as a homologous series of *n*-alkanes (C_7–C_{25}). tr = trace level (area less than 0.01); different letters in the same row indicate a significant difference by Tukey test at $p \leq 0.05$. Data expressed as the mean value of triplicate measurements ± SD in the peak area obtained by GC-FID.

[*]Description based on Rodriquez-Burruezo et al. (2010), Burdock (2010), www.goodscentcompany.com and www.flavornet.org.

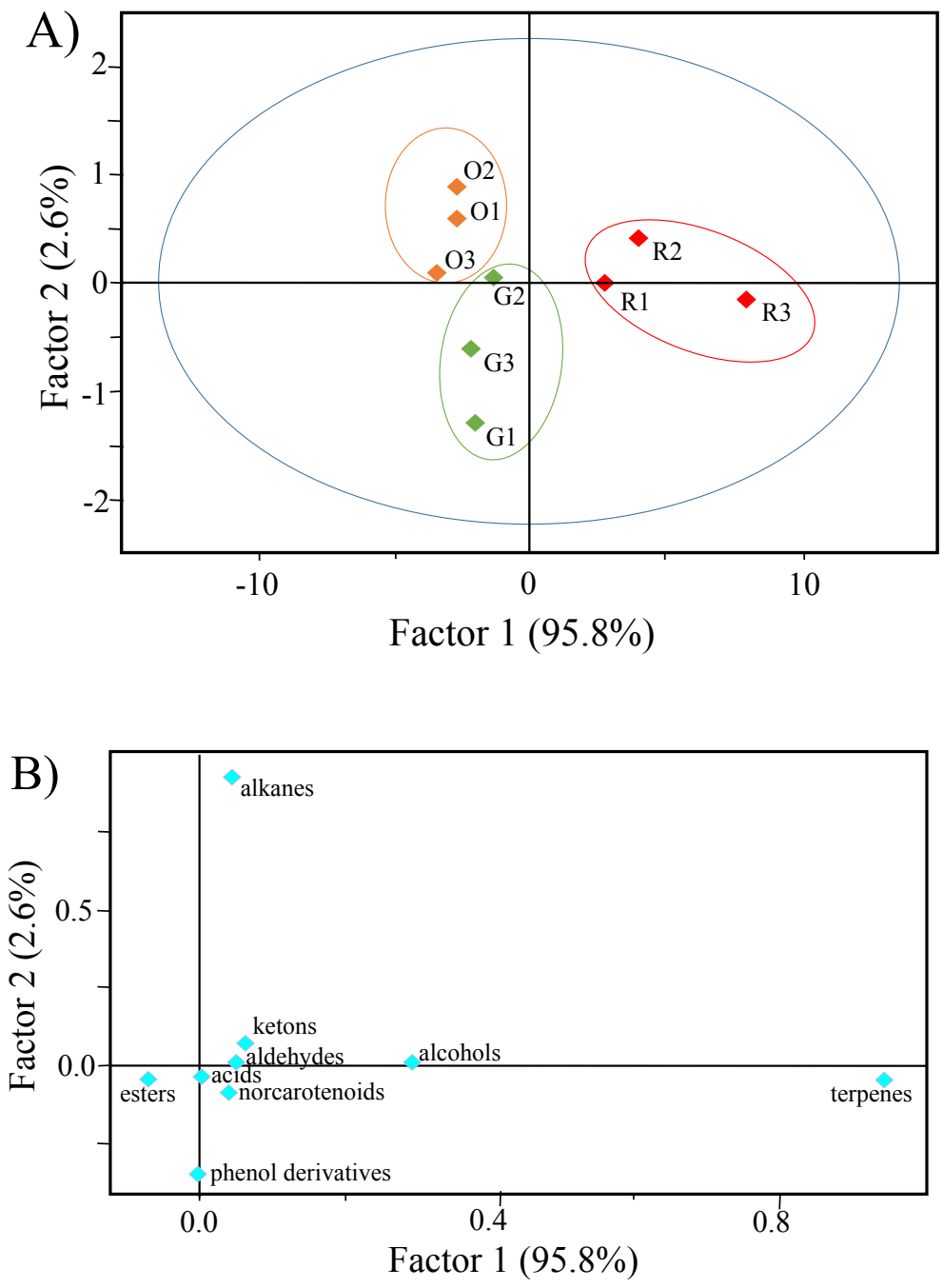

Figure 4-4. Principal component analysis (PCA) plots of volatile aroma components of Shimatogarashi peppers in three maturing stages.

(A) PCA scores plot showing the major types of differences between maturing stages with G, O, and R represent the Green, Orange and Red stages, respectively, and (B) PCA loadings plot showing the distribution of the volatile groups.

4.3.6. Antioxidant properties

The antioxidant properties at different maturation stages were determined on the basis of the peroxyl radical quenching properties of compounds soluble in methanol/water (3:1) can be seen in **Figure 4-5**. Applying a universal isolate from the fruits over a purified one confers the benefit of gaining an overall estimation of the soluble compounds influencing the fruit's antioxidant properties (Howard et al. 2000).

From green to red stages, the fruit extract exhibited high antioxidant properties based on ORAC and DPPH tests (**Figure 4-5**). Both the ORAC test and the DPPH quenching ability significantly increased as the fruit matured. The TPC in Shimatogarashi fruit also significantly increased during maturation from 260.3 to 397.4 mg/100 g FW sample (**Figure 4-6**), which was comparable to Bell peppers (284.6–308.5 mg/100 g FW), but lower than Tabasco (513.6–524.4 mg/100 g FW) (Howard et al. 2000).

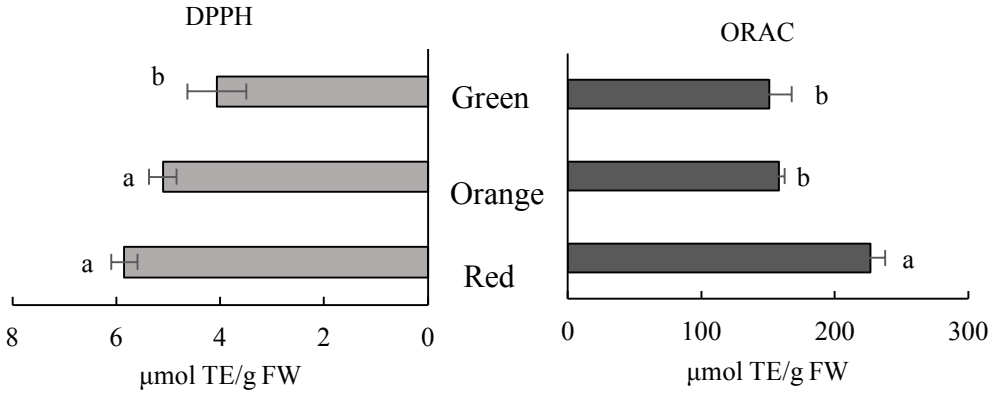

Figure 4-5. Antioxidant activity of Shimatogarashi peppers at three different maturing stages measured by ORAC and DPPH methods.

Data are expressed as the mean ± SD (n=3). Different letters in the same category denote significant differences as analyzed by a Tukey test at $p < 0.05$.

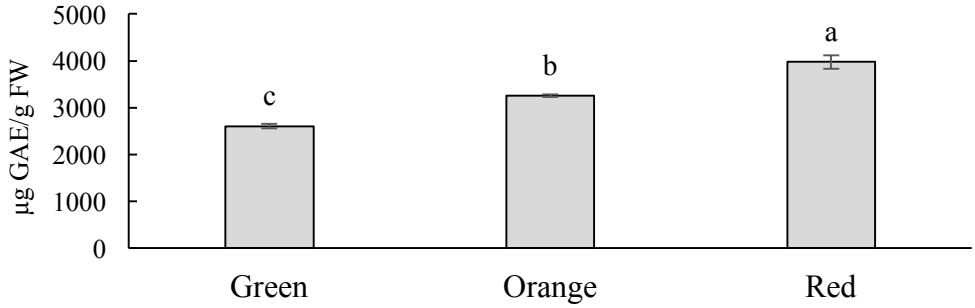

Figure 4-6. Total phenolic content of Shimatogarashi peppers at three different maturing stages.

Data are expressed as the mean ± SD (n=3). Different letters in the same category denote significant differences as analyzed by a Tukey test at $p < 0.05$.

There are various bioactive compounds that influence the antioxidant properties of peppers, including, lipid soluble compound such as capsaicinoids and hydrophilic compounds such as polyphenols, flavonoids and ascorbic acid (Howard et al. 2000; Bae et al. 2014). Phenolic compounds belong to a group of secondary plant metabolites which are synthesized as a response to biotic and abiotic stresses (Wahyuni et al. 2014). As such, dietary supplements of phenolic compounds are recommended for human consumption as they cannot be synthesized by the human body and may confer potential health benefits in preventing oxidative stress (Oboh and Rocha 2007).

Our results show a strong correlation between TPC and capsaicin content with DPPH quenching activity (r = 0.992 and 0.812, respectively) and ORAC (r = 0.917 and 0.996, respectively). Based on a Pearson correlation analysis, a correlation coefficient (r) greater than 0.8 was considered strong, while a value less than 0.5 was considered weak (Bolboaca and Jäntschi 2006). Ascorbic acid content also had a good correlation with DPPH (r = 0.914), although a lower correlation

with ORAC (r = 0.579) was found. The same phenomena were observed in another study (Alvares-Parilla et al. 2011). In other words, the content of capsaicinoids as lipid soluble antioxidant, which found in higher amount in the red stage compared to the green stage, also showed good correlation with antioxidant activity on both DPPH and ORAC assays. The increase in antioxidant activity in mature pepper fruits was also described in previous studies on *Capsicum* spp. (Howard et al. 2000; Navarro et al. 2006; Ghasemnezad et al. 2011; Bae et al. 2014).

Materska and Perucka (2005) reported that red stage of the chili pepper fruit possessed highest quantities of flavonoids, while the green ones were contained high amount of quercetin 3-*O*-α-L-rhamnopyranoside, though its quantity decreased in later stage. Eventhough the methanolic extract in this study might not contain the non polar carotenoids compounds, the presence of carotenoids was reported to influence the antioxidant activity of the fruit. Carotenoids present in red stages of chili peppers, for example, capsanthin, cryptocapsin, and capsorubin, were reported to exhibit strong antioxidant activity and capsanthin's degradation was found to be slower than other carotenoids, thus having longer radical scavenging effect (Matsufuji et al. 1998). These findings might support the enhanced nutritional benefit in consuming the fruits at the mature red stage.

4.4. Conclusion

The changes occurred in color, organic acids, capsaicinoids, and volatile compounds content as well as antioxidant activity during Shimatogarashi's fruit ripening had been observed. The data on fruit's maturation revealed that the flavor changes towards a more pungent, citrus-like aroma owing to the increase on organic acids, especially malic and citric acid, as well as the increase in volatiles with citrus notes like limonene and γ-terpinene. The red mature stage also showed the highest antioxidant activity. On the other hand, the utilization of the fruit at the immature green stage is still beneficial owing to its pleasant fruity smell, which may be useful in condiments.

Chapter V

Antiobesity potential of Shimatogarashi (*Capsicum frutescens*) extract

5.1. Introduction

In the ease of modern living that might promote sedentary lifestyle, the prevalence of obesity, oxidative stress and its related diseases had largely become a growing problem. It necessitates the development of successful treatment or prevention strategies, namely the changes in one's diet or diet intervention (Nagao and Yanagita 2008; Matsuda and Shimomura 2013). In regards to capsaicin, some studies had been reported that this compounds had several functional properties, particularly antioxidative as well as antiobesity (Kawada et al. 1986; Dairam et al. 2008; Galano and Martinez 2012; Baboota et al. 2014; Imran et al. 2018). In Chapter III and IV, we learned that Shimatogarashi fruits contained high capsaicin as well as potent antioxidant properties. Therefore, this chapter was dedicated on describing the antiobesity potential of Shimatogarashi.

In Chapter IV, it was described that the extract of red mature stage of Shimatogarashi had the highest antioxidant capacity based on ORAC and DPPH, as well as high content of total phenol and high level of total capsaicinoids. As a follow up, an investigation of the effect of the extract of the red mature stage on Shimatogarashi on adipogenesis was conducted. The study might signify whether the extract has the potential of antiobesity in order to be purposefully included in

dietary intervention to prevent obesity. Overall estimation of soluble compounds's potential, as well as the sum of their interaction in preventing obesity can be obtained by utilizing a crude extract or universal isolate, instead of a purified isolate.

In the study of adipogenesis and adipocytes functions, 3T3-L1 cells, which cell line is derived from non-clonal Swiss 3T3 cells and already committed to adipocytic lineage, are often used as cell models in vitro (Green and Kehinde 1975; Fu et al. 2005). Differentiated 3T3-L1 cells grown *in vitro* simulate characteristics of adipocytes from animal tissue (Nugara 2016). Therefore, this study aimed to investigate the effect of red mature stage Shimatogarashi extract in different stages of differentiation of 3T3-L1 preadipocyte to mature adipocytes.

5.2. Materials and methods

5.2.1. Plant materials

The plant materials used were the same as described in Chapter III (refer to **section 3.2.1.**). The fruits were randomly picked from ten different plants based on their surface color (mature red), freeze dried, grounded and stored at –30°C in a freezer until they were analyzed, unless otherwise indicated.

5.2.2. Chemicals

Penicillin-streptomycin solution, ethyl alcohol, methanol, 3-isobutyl-1-methylxanthine (IBMX), 0.25 w/v% trypsin-1 mmol/L. EDTA·4Na solution, phosphate buffer solution, dimethyl sulfoxide (DMSO) and 2-propanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). 3T3-L1 fibroblasts

were obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). Insulin human United States pharmacopeia (USP) Reference Standard, capsaicin standard and Oil Red O (certified by Biological Stain commission) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified eagle medium (DMEM) high glucose, with HEPES, but without sodium pyruvate, were obtained from Thermo Fisher Scientific (Waltham, MA, USA). PPAR-gamma Transcription Factor assay kit (ab133101) and nuclear extraction kit (ab113474) were ordered from Abcam (Cambridge, UK). Protein quantification kit-rapid was obtained from Dojindo (Kumamoto, Japan). CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was procured from Promega (Madison, WI, USA). Dexamethasone (DEX) were obtained from Funakoshi (Tokyo, Japan). Dithiothreitol (DTT) and sodium hydrogen carbonate were purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade.

5.2.3. Preparation of Shimatogarashi extract

Ground freeze-dried red mature fruit without peduncles (3 g) was added to 30 mL of 75% methanol, and the mixture was sonicated for 5 min, put on shaker for 1 h and centrifuged at 10,000 rpm for 10 min using CR20 GIII (Hitachi, Tokyo, Japan). The extraction was repeated twice. Supernatant was collected and evaporated.

5.2.4. Cell culture maintenance

Maintenance of 3T3-L1 preadipocyte cells were performed by culturing in DMEM supplemented with 10% FBS, sodium hydrogen carbonate and 100 U/mL penicillin-streptomycin solution basal medium I (BMI) inside 60 or 90 mm^2 culture dish, which was put in an incubator with a humidified atmosphere 5% CO_2 at 37°C. Subcultures were performed diligently using trypsin/EDTA to detach the cells before reaching 6×10^4 viable cells/cm^2 of cell population (or 80% confluence) and the passage number was recorded. A mixture of 90% BMI and 10% DMSO was used to freeze the subculture samples, which then kept in −80°C freezer for later use.

5.2.5 Differentiation to adipocyte cells

Utilizing the passage number of the cells as low as possible, the 3T3-L1 preadipocytes were seeded at 4×10^4 cells/well in 24-well plates and cultured in BMI. Two days after reaching 100% confluence, the cells were supplied with inducing medium, particularly BMI supplemented with 10 μg/mL insulin, 0.5 μM IBMX, and 1 μM DEX for 48 h. Then the cells were sustained in BMI containing 10 μg/mL insulin for another 2 days, followed by culturing with BMI for required additional days. To evaluate of Shimatogarashi extracts on adipogenesis, the adipocyte cell cultures were administered with the sample throughout the differentiation process as described in the following paragraph (**section 5.2.6.**), then the accumulation of lipid was measured. Pure capsaicin standard was used as positive control.

5.2.6. Sample treatment

The 3T3-L1 cell cultures were treated with several experimental scheme as depicted in the following charts (**Figure 5-1**, **Figure 5-2** and **Figure 5-3**) to evaluate the effect of the extract on different stages of the cell development. The scheme in **Figure 5-3** describes the effect of continuous administration of Shimatogarashi extract compared to its corresponding capsaicin concentration.

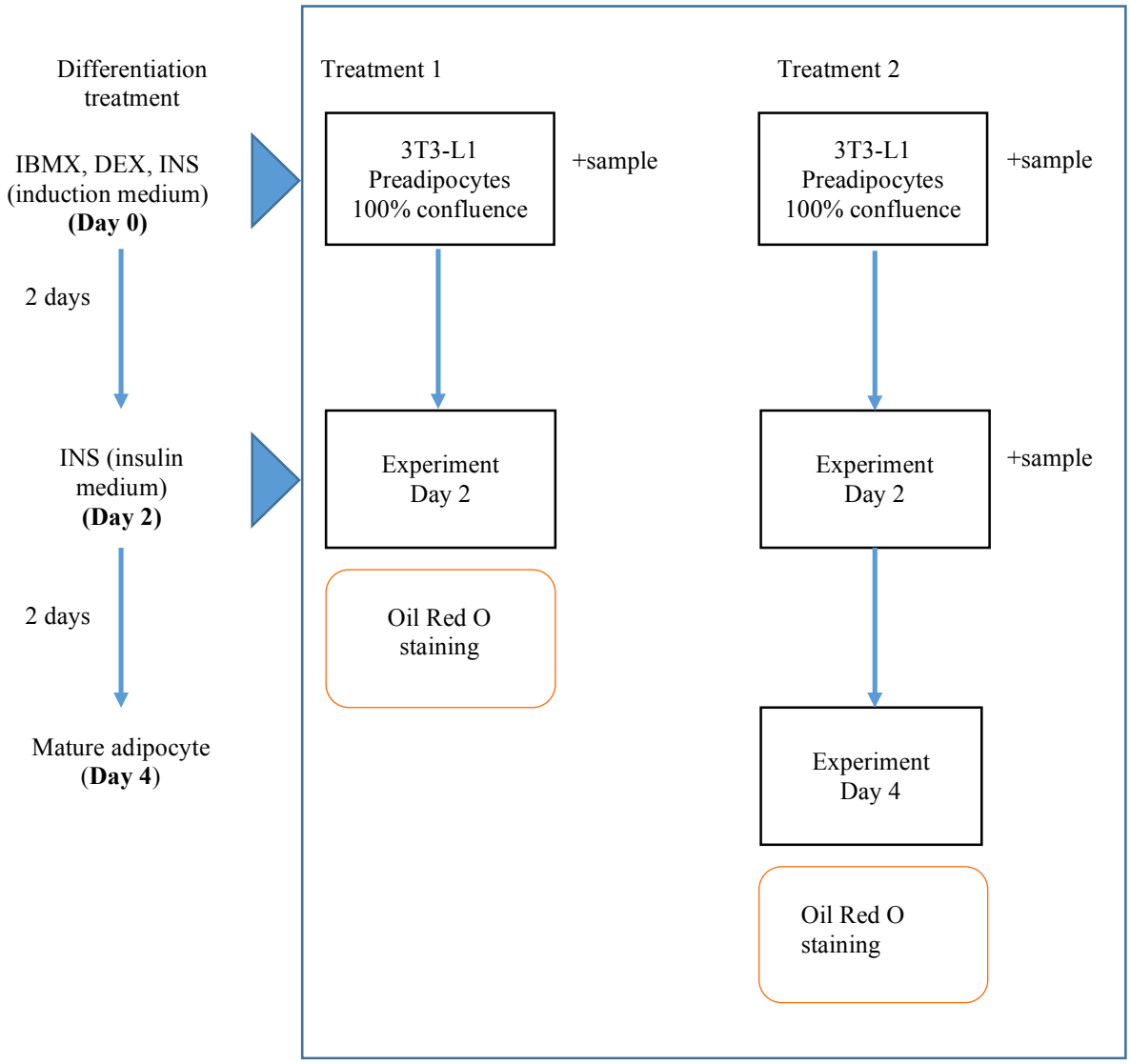

Figure 5-1. Experimental scheme for evaluating the effect of Shimatogarashi at early stage.

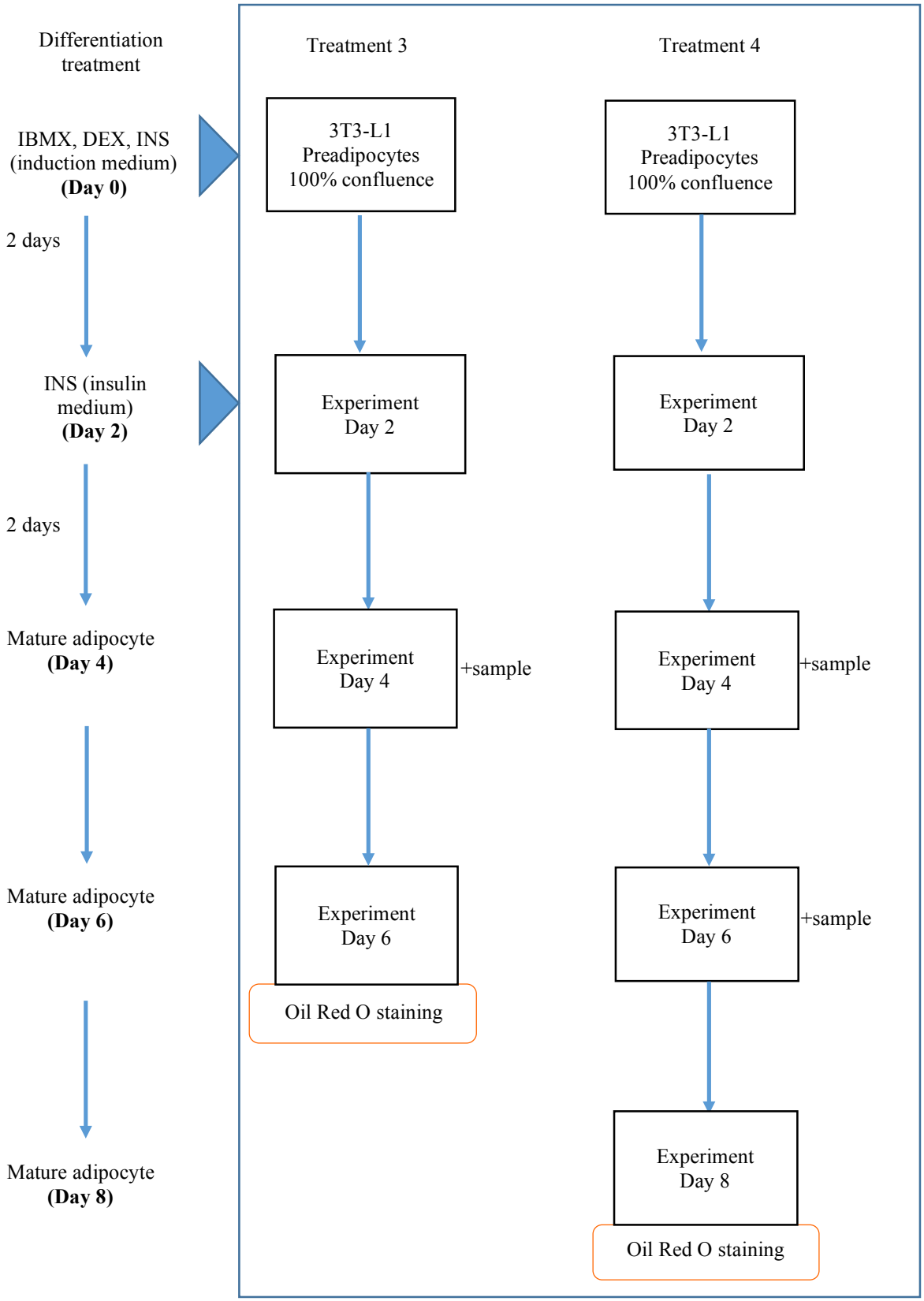

Figure 5-2. Experimental scheme for evaluating the effect of Shimatogarashi at mature stage.

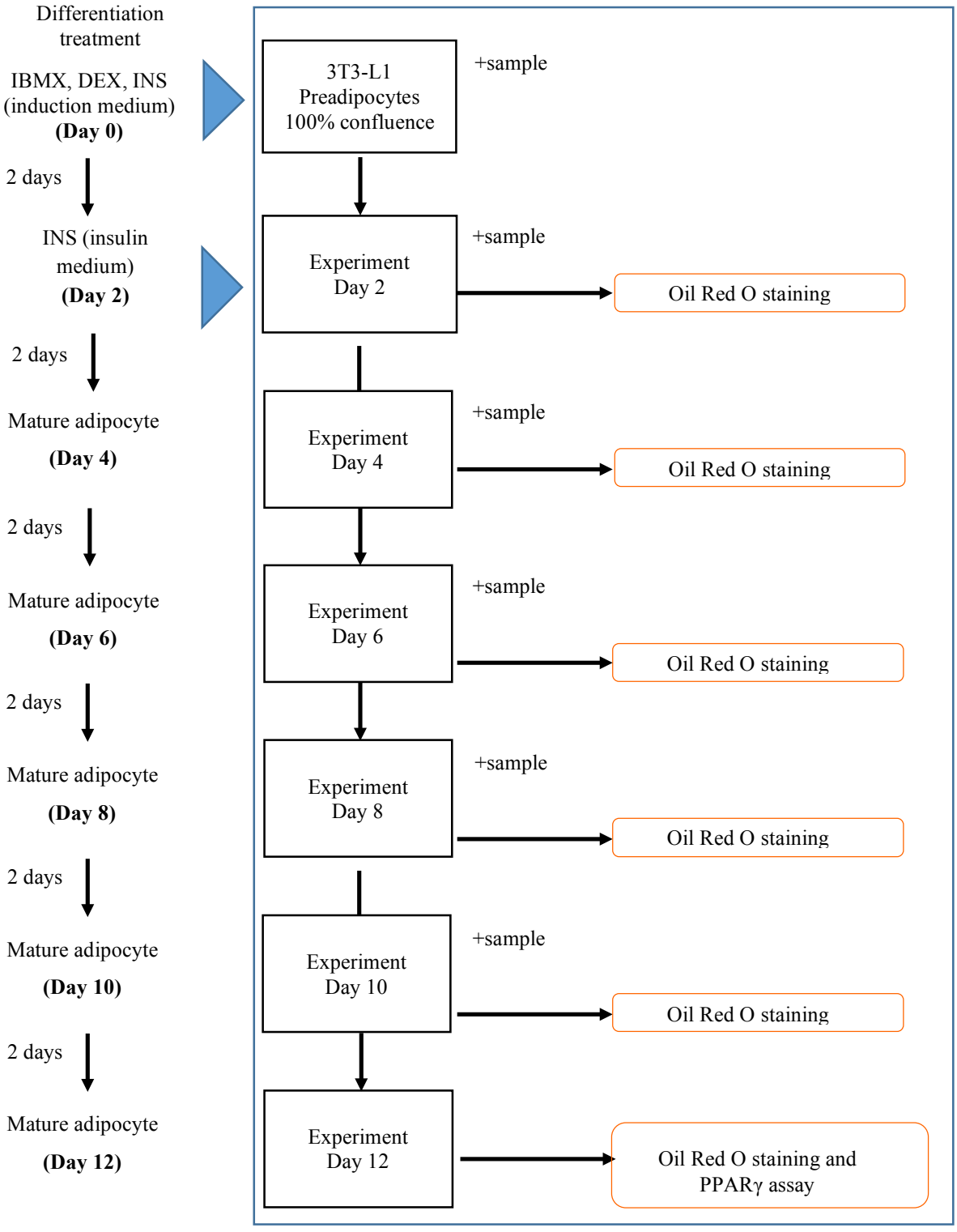

Figure 5-3. Experimental scheme for evaluating the effect of continuous administration of Shimatogarashi extract compared with its corresponding capsaicin concentration.

5.2.7. Proliferation assay

The effect of Shimatogarashi on cell proliferation was performed before further assays to determine safe concentration. According to the manufacturer's manual (Promega, Madison, WI) with little modification, CellTiter 96® AQueous One Solution was used to determine the number of viable cells. Differentiated 3T3-L1 cells were incubated in clear 96-wellplate in 100 µL/well of BMI administered with several extract concentrations. After the incubation period of 48 h, the medium was aspirated and the cell was washed with 100 µL/well of PBS. Then a mixture of 20 µL of CellTiter 96® AQueous One Solution or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS) and 100 µL of BMI per well was added. The plate was then further incubated in a 5% CO_2 incubator at 37˚C for 1 h. The reaction is evaluated based on the cleavage of MTS into a formazan product which was soluble in the medium and measured at absorbance of 490 nm in plate reader (PowerWave™ XS2, BioTek, USA). Cell viability was calculated using Equation 5.1:

$$\%\ \text{cell viability} = \frac{\text{Absorbance of treatment at 490 nm}}{\text{Absorbance of control at 490 nm}} \times 100\% \quad \ldots (5.1)$$

5.2.8. Oil Red O assay

Briefly, Oil Red O stock solution was prepared by dissolving 150 mg Oil Red O powder in isopropanol. Stable for 2 h after mixing, Oil Red O working solution was prepared by diluting the stock solution with ddw (6:4 v/v) then allowed to stay for at least 20 min, and filtered through 0.22 µm filter prior to use. Cell cultures in 24-well plate were washed with 400 µL/well of PBS and fixed with

400 μL/well 10% formalin isopropanol aqueous solution (in double distilled water or ddw) for at least 1 h. Then, cells were treated with 60% isopropanol aqueous solution for 1 min, then aspirated. Oil Red O working solution (400 μL/well) was added to stain the culture for 1 h. After Oil Red O lipid staining, cells were treated with 60% isopropanol aqueous solution for 5 min, then washed with ddw two times or until no pink color appeared on the liquid surface. The cells were photographed and then remaining liquid was aspirated. Oil Red O dye was measured by adding 100% isopropanol and then incubating at room temperature for 15 min. The absorbance of the resulting eluent was measured at 490 nm using a PowerWave™ XS2 plate reader (Bio-Tek, Winooski, VT, USA).

5.2.9. Nuclear Extraction

The cells were cultured in 90 mm^2 dish and treated with samples and differentiation procedures (**Figure 5-3**). After twelve days since induction medium administered, the cells were harvested according to the abcam kit (ab113474; abcam, Cambridge, MA, USA) protocol. The cells were washed two times with PBS(-) (2 mL). To detach the cells, 1 mL of trypsin/EDTA was added. Then the cells were collected into 15 mL conical tubes and the cell pellets were centrifuged at 1,200 rpm (25^oC) for 5 min. The cells were counted, re-suspended accordingly with 100 μL of pre-extraction buffer per 1×10^6 cells into microtubes and incubated on ice for 10 min. The collected cells were vortexed vigorously for 10 seconds, and then centrifuged for 1 min at 12,000 rpm. The cytoplasmic extract was removed from the nuclear pellet. The obtained pellet was added with 10 μL of extraction buffer containing dithiothreitol (DTT) and protease inhibitor cocktail (PIC) per

1×10^6 cells. Immediately, the extract was incubated on ice for 15 min with intermittent vortex for 5 seconds every 3 min. Then the mixture was further centrifuged for 10 min at 14,000 rpm at 4°C, then supernatant was transferred to a new vial.

5.2.10. PPARγ assay

The supernatants obtained from the nuclear extraction step was used. PPARγ levels were evaluated according to the abcam PPARγ transcription factor assay kit (ab133101; abcam, Cambridge, MA, USA) protocol. Using 96-well plate provided by the kit, extracted PPARγ samples from the cell cultures were bound to the consensus dsDNA sequence coated on the bottom of the well by letting them stay overnight after applying the samples. The next day, the wells were washed with buffer to eliminate all the unbound reagents. PPARγ primary antibody was allowed to bind to transcription factor and then unbound reagent was washed with buffer. Goat anti-rabbit HRP (secondary antibody) was added to bind with the primary antibody. Washing was performed to eliminate unbound reagent. After developing solution was added, the plate was then incubated for 15 min. Absorbance was measured at 450 nm within 5 min after adding the stop solution.

5.2.11. Statistical analysis

The value of the measurements (n = 3) was expressed as the mean ± standard deviation (SD). The significant difference was determined by one-way analysis of variance and Tukey multiple comparisons using SPSS statistical software for Mac (v.23.0.0; Statistical Package for the Social Sciences, Chicago, IL, USA).

Differences were considered significant at $p < 0.05$ or 0.01.

5.3. Results and discussion

5.3.1. Proliferation assay

The proliferation assay using MTS reagent showed that 500 μg/mL or lower concentration of Shimatogarashi extract, there was no significant toxicity detected compared to the control (**Figure 5-4**). Meanwhile, concentrations of 1600 and 2000 μg/mL were discovered to be toxic to the cells (under 50% of cell viability). The extract of yellow capsicum and capsaicin in the study performed by Feng et al. (2014) also exhibited high toxicity in concentration of 1000 μg/mL and 64 μg/mL, respectively. Consequently, the range concentration of Shimatogarashi extract tested in the next step was 10–500 μg/mL.

Shimatogarashi extract in this study contained 21.48 μg capsaicin and 7.11 μg dihydrocapsaicin per mg extract, that means 1,000 μg/mL of the extract is equal to 21.48 μg/mL capsaicin and 7.11 μg/mL dihydrocapsaicin. Therefore, range concentration of both capsaicin and dihydrocapsaicin tested were 6.25–100 μg/mL. While no significant difference found at concentrations of 6.25–50 μg/mL capsaicin compared to the control (**Figure 5-5**), the concentration of 100 μg/mL capsaicin was found to be lethal that showed under 50% cell viability. This finding is in accordance with research by Baboota et al. (2014) and Lee et al. (2010) which reported safe range from 0.1–10 μM of capsaicin (which equal 0.3–30.5 μg/mL capsaicin).

Capsaicin was reported to stimulate apoptosis in 3T3-L1, through increasing reactive oxygen species (ROS) and the activation of caspase-3 (Hsu and Yen 2007; Jeong et al. 2014). For dihydrocapsaicin extract, even though significant differences

can be found at concentrations of 6.25–50 μg/mL, the cell viability was more than 80% (expressed as % control).

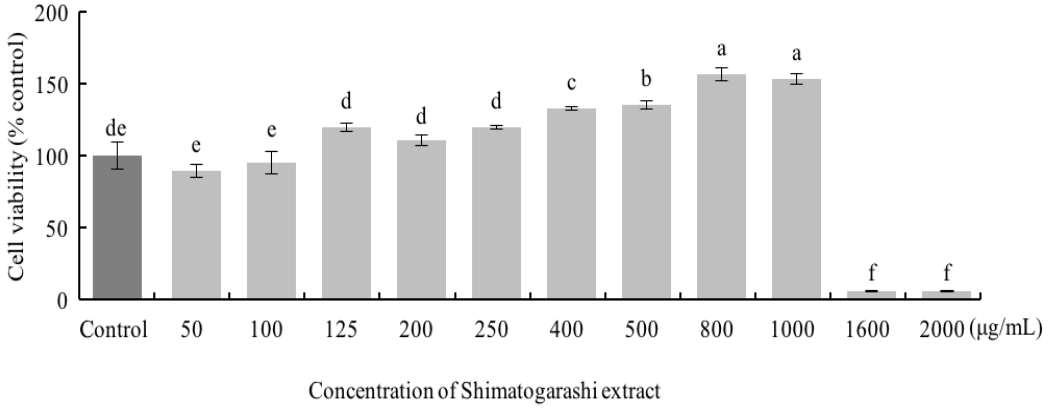

Figure 5-4. The effect of various concentration Shimatogarashi extract on cell viability in 3T3-L1 adipocytes.

Data are expressed as the percent growth rate of cells cultured in the presence of samples compared with control or untreated cells (mean ± SD; n = 3). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) as analyzed by Tukey test.

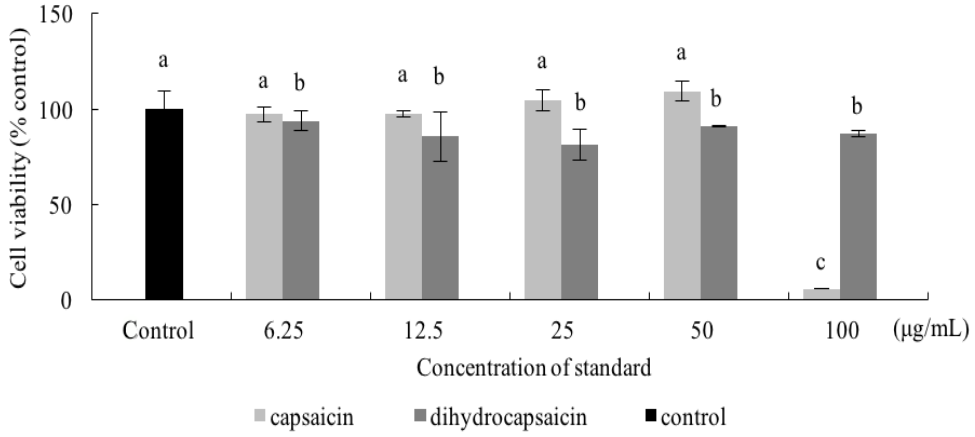

Figure 5-5. The effect of capsaicin and dihydrocapsaicin on cell viability in 3T3-L1 adipocytes. Data are expressed as the percent growth rate of cells cultured in the presence of samples compared with control or untreated cells (mean ± SD; n = 3). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) as analyzed by Tukey test.

5.3.2. Lipid accumulation

During differentiation process of 3T3-L1 cells from preadipocytes into adipocytes, there are two phase of differentiation, which are early and late differentiation, respectively (Kim and Jang 2017). The early differentiation is marked by cell arrest that is regulated by a group of receptors, namely nerve growth factor-induced gene B, nuclear receptor-related factor 1 and vitamin D receptor. The expression of those genes decreased in later stage of differentiation, while the expression of PPARγ and liver X receptor α increased. The degree of differentiation measured using Oil Red O staining technique showed observable trend in relation to sample concentration and cytoplasmic lipid accumulation in Day 4 (four days after receiving induction medium, treatment 2) (**Figure 5-6**).

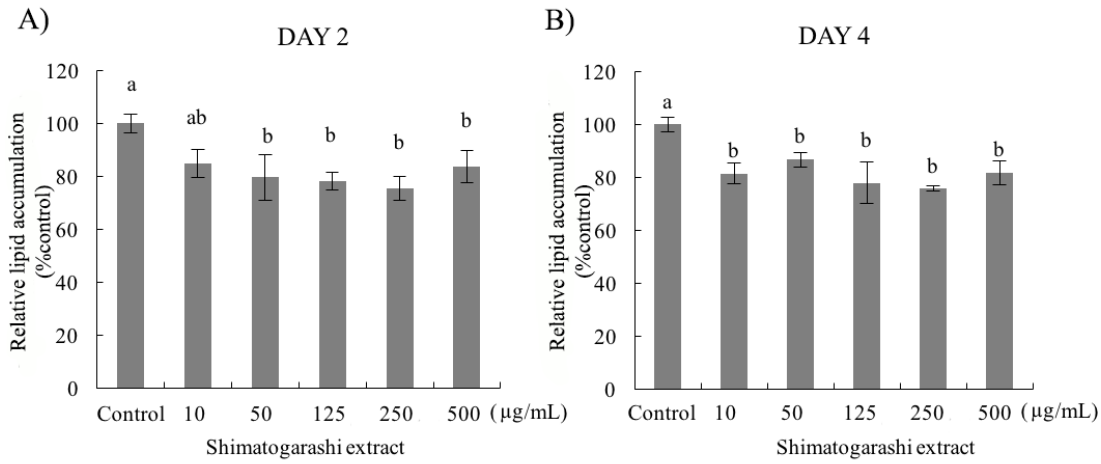

Figure 5-6. The effect of various concentration Shimatogarashi extract on lipid accumulation in early stages 3T3-L1 adipocytes.

(A) Day 2 or two days after treatment with Shimatogarashi extract and induction medium (treatment 1) and (B) Day 4 or four days after treatment with Shimatogarashi extract, induction and insulin medium (treatment 2). The experiment was conducted as designed in **Figure 5-1**. Data are expressed as the percent growth rate of cells cultured in the presence of samples compared with control or untreated cells (mean ± SD; n = 3). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) as analyzed by Tukey test.

In the first treatment (**Figure 5-1**), the lipid was hardly accumulated in the cells from Day 0 to Day 4. Though no significant difference detected among Shimatogarashi extract in various concentrations in Day 4, Shimatogarashi extract and control were significantly different. Therefore, the extract might have inhibition effect on adipogenesis of preadipocytes during differentiation process toward mature fat cells. In the late stage of differentiation, the sample treatment 3 (Day 6) and 4 (Day 8) as described in **Figure 5-2**, showed concentration dependent inhibition of lipid accumulation in the mature adipocyte or late stage of differentiation (**Figure 5-7**). Kim and Jang (2017) reported that PPARγ and C/EBPα was considered as key transcription factors controlling adipogenesis and highly expressed in the late stage of differentiation.

In Day 6, cell morphology under microscope observation showed a tendency to form aggregation which can be seen in control, 10 and 50 μg/mL of sample treatment, while mildly seen in 125 μg/mL of sample treatment. However, in 250 and 500 μg/mL of sample treatment, the similar cell's aggregation was hardly observed. It was assumed that the extract might affect differentiated cell's shape and appearance to a certain degree. In Day 8, the lipid droplets in each wells had been swelling and grew bigger. The culture with 500 μg/mL of sample treatment had been forming a little aggregation just like the cultures treated with 125 μg/mL in Day 6.

Furthermore, cell's aggregation of 125 μg/mL extract sample treatment in Day 8 shaped like that of 50 μg/mL sample treatment in Day 6. In contrast, cells treated with 10 and 50 μg/mL of extract, as well as control, developed more distinct aggregation. The inhibitory effect of Shimatogarashi extract in both early and late

adipogenesis might be owing to the presence of capsaicin. Ibrahim et al. (2014) suggested that capsaicin inhibited adipogenesis in early and late stages which indicated by the repression of PPARγ and C/EBPα.

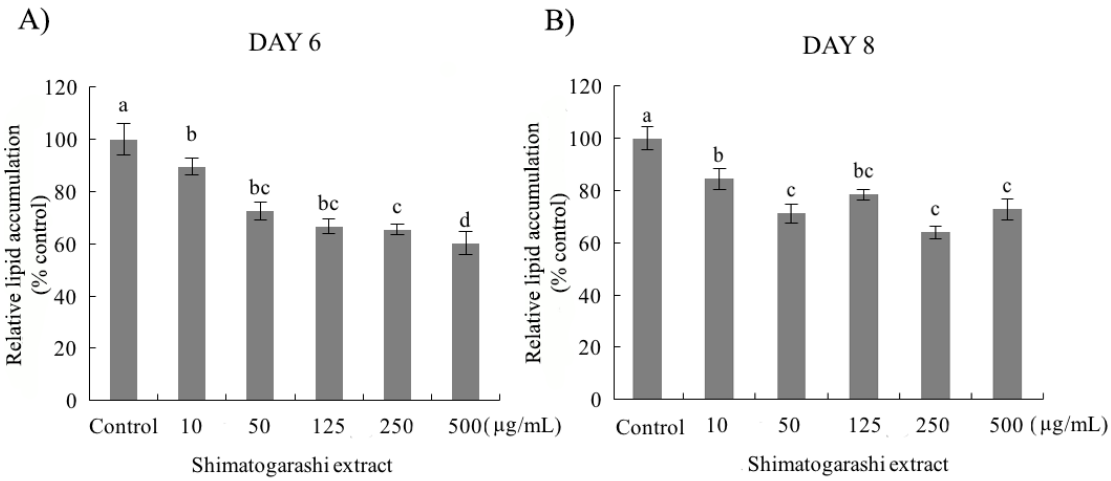

Figure 5-7. The effect of various concentration Shimatogarashi extract on lipid accumulation in late stages 3T3-L1 adipocytes.

(A) Day 6 or two days after mature adipocyte obtained and treatment with sample (treatment 3). (B) Day 8 or four days after mature adipocyte obtained and treatment with sample (treatment 4). The experiment was conducted as designed in **Figure 5-2**. Data are expressed as the percent growth rate of cells cultured in the presence of samples compared with control or untreated cells (mean ± SD; n = 3). Letters with different superscripts indicate samples that are significantly different ($p < 0.05$) as analyzed by Tukey test.

The next step of the study was to investigate the effect of continuous administration of Shimatogarashi extract compared to the corresponding capsaicin concentration as control according to experimental scheme in **Figure 5-3**. The experiment showed concentration dependent inhibition of Shimatogarashi extract (**Figure 5-8**). The extract inhibited lipid accumulation further as the adipocytes reached Day 12. Shimatogarashi extract contained capsaicin that possessed lipolysis effect in adipocytes, as reported by Lee et al. (2010) through measuring the glycerol

released in the medium. As a positive control, the range of capsaicin tested was 0.2, 1.0, 2.5, 5.0, and 10 μg/mL, in corresponding to amount contained in the extract (10, 50, 125, 250, and 500 μg/mL, respectively), since 500 μg/mL extract contained about 11.24 μg/mL capsaicin. The result revealed the inhibition of lipid accumulation in the presence of capsaicin (as positive control) in time and dose dependent manner (**Figure 5-9**). Similar result found in the study conducted by Jeong et al. (2014) using bovine bone marrow mesenchymal stem cells treated with capsaicin. In addition, they also found that the expression levels of adipogenic transcription factor genes, such as *PPARG* and *CEBPA*, as well as adipogenic proteins, fatty acid binding protein 4 and stearoyl-CoA desaturase, were lower in capsaicin treated cells.

By comparing the effect of Shimatogarashi extract with its corresponding capsaicin concentration on 3T3-L1 adipocytes, a strong correlation ($r = 0.886$) can be found between Shimatogarashi extract and its corresponding capsaicin concentration. It can also be observed that red stage of Shimatogarashi extract had a potential as antiobesity, which might be more potent than just capsaicin alone. This result might be owing to the presence of other compounds having synergistic effect in inhibiting lipid accumulation (**Figure 5-8** and **Figure 5-9**).

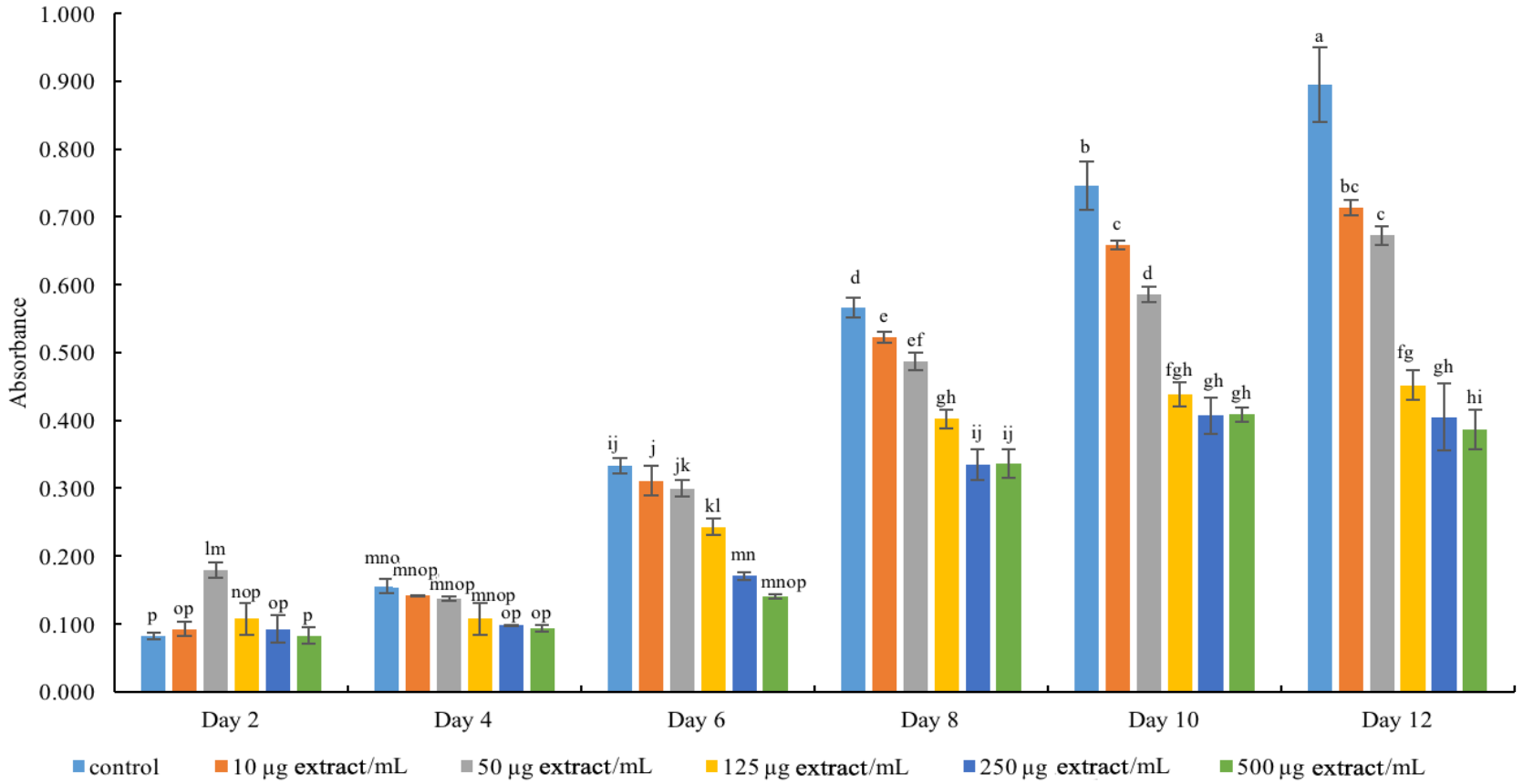

Figure 5-8. The effect of various concentrations of Shimatogarashi extract on lipid accumulation in several stages 3T3-L1 adipocytes.

The cell treatment was performed as designed in **Figure 5-3**. Data are expressed as absorbance of extracted Oil Red O staining in proportion with lipid accumulations in the cells (mean ± SD; n = 3). Letters with different superscripts indicate significant difference ($p < 0.05$).

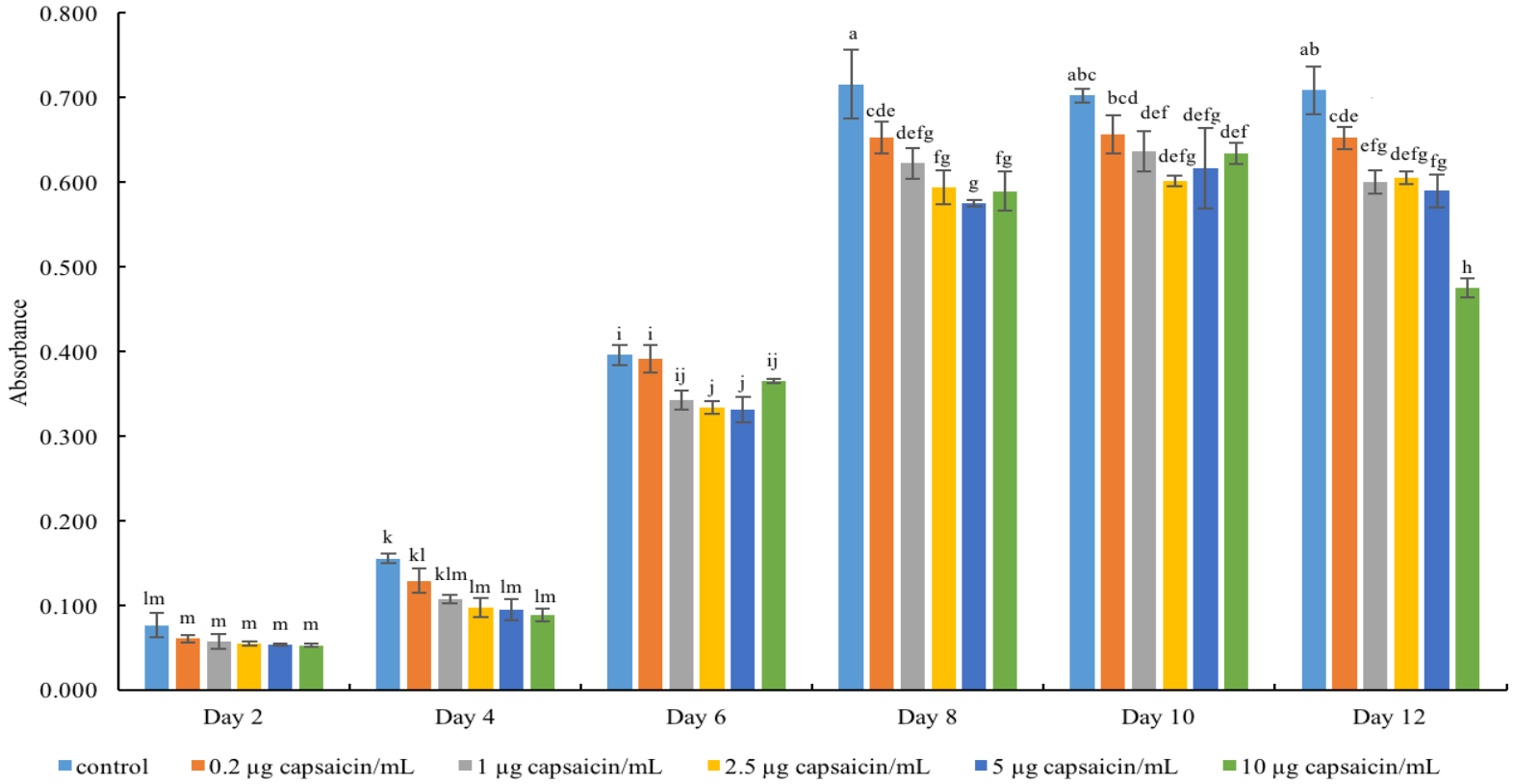

Figure 5-9. The effect of various concentration of capsaicin on lipid accumulation in several stages 3T3-L1 adipocytes.

The cell treatment was performed as designed in **Figure 5-3**. Data are expressed as absorbance of extracted Oil Red O staining in proportion with lipid accumulations in the cells (mean ± SD; n = 3). Letters with different superscripts indicate significant difference ($p < 0.05$).

5.3.3. PPARγ expression

In adipose tissue, PPARγ is expressed as a regulator of adipogenesis and plays significant role in 3T3-L1 cell differentiation (Fu et al. 2005). Along with C/EBPs, they involve in fat cell regulations and increase the expression of terminal makers, namely glucose transporter-4 and fatty acid synthase (Kim and Jang 2017). In agreement with Baboota et al. (2014) which mentioned that the expression of PPARγ was higher in adipocytes compared to preadipocytes, in this study, the expression levels of PPARγ using nuclear extracts was significantly increased in 3T3-L1 adipocyte cells treated with the induction medium compared to non-differentiated cells (**Figure 5-10** and **Figure 5-11**).

The expression of PPARγ in the sample treated groups (capsaicin and both low and high level of Shimatogarashi extracts) were significantly decreased at the nuclear protein and mRNA levels compared to the control. The downregulation of PPARγ indicated the inhibition of adipogenesis (Kim and Jang 2017). Ibrahim et al. (2014) proposed the mechanism of suppression of PPARγ expression by capsaicin via transient receptor potential vanilloid type-1 channels which were induced, thus the activation of this channels increases cytosolic calcium (Zhang et al. 2007). The increase might trigger several cascades via oxidative and nitrosative stress. Therefore, it promotes ROS and reactive nitrogen species to participate in the activation of 5'-adenosine monophosphate-activated protein kinase (Hwang et al. 2005) and prevent C/EBP from binding to its promoter region (Rosen et al. 2002). The expression of forkhead box protein O1 and interferon regulatory protein 1 were also suppressed which then might promote direct repression of PPARγ.

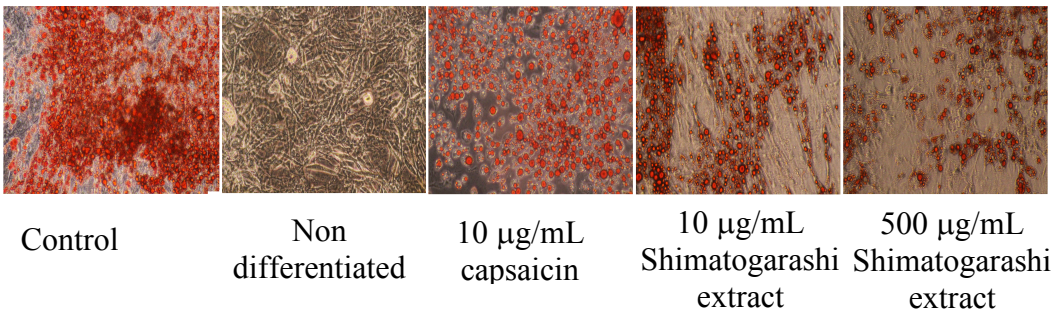

Figure 5-10. The Oil Red O staining of 3T3-L1 treated with various concentration of samples.

The cell treatment was performed as designed in **Figure 5-3**.

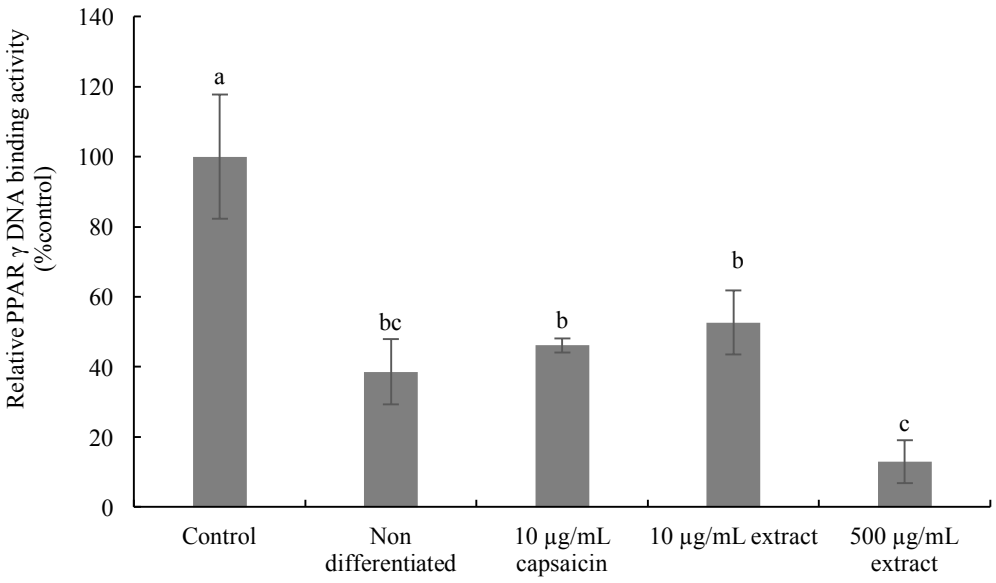

Figure 5-11. Inhibition of nuclear PPARγ expressions in 3T3-L1 adipocyte cells.

The cell treatment was performed as designed in **Figure 5-3**. Data are expressed as PPARγ levels in comparison with control (mean ± SD; n = 3). Letters with different superscripts indicate significant difference ($p < 0.05$).

5.4. Conclusion

Shimatogarashi is a common hot pepper in Southern Japan with a pungent taste that contains significant amounts of capsaicin. In the present study, we conducted an investigation on the inhibitory action of Shimatogarashi extract and capsaicin on the differentiation of 3T3-L1 preadipocytes to mature adipocytes measured by Oil Red O staining and PPARγ levels. The results indicated that both Shimatogarashi extract and capsaicin inhibited lipid accumulation in 3T3-L1 adipocytes in concentration and time dependent manner. Therefore, it was recommended that more elaborate studies on gene expressions as well as further purification need to be performed to explore the potential of extract in the management of obesity.

Chapter VI

General Conclusion

Okinawan has hot pepper cultivar known as Shimatogarashi (*C. frustecens*), which is commonly cultivated in Southern Japan and used in popular condiment called koregusu. Despite the frequent typhoon, this cultivar survives in tropical climate of Okinawa. Consumer preference of chili peppers is dominantly based on their flavor, which consists of aroma and taste compounds. Due to edaphoclimatic nature of *Capsicum* cultivars, the flavor characteristics as well as biological functions of the fruits vary substantially, not only due to genetic but also as environmental aspects particularly soil, climate and cultivation practices. In this study, characterization of Shimatogarashi in regards to its flavor as well as antioxidant capacity, had been conducted.

Through comparison with Takanotsume, the popular cultivar in mainland Japan, Shimatogarashi's distinct characteristics could be better understood. In this study, the physical properties, flavor characteristics and antioxidant properties of both peppers at red mature stage had been investigated. In appearance, Shimatogarashi was smaller and brighter in color than Takanotsume. The taste compounds evaluated in this study included organic acid and pungent compounds. Even though both peppers contained malic, citric and ascorbic acid, the dominant organic acids were different. Shimatogarashi contained high amount of malic acid, while the most dominant acid in Takanotsume was citric acid. In term of pungency, Shimatogarashi had higher hotness level compared to Takanotsume, owing to

higher concentration of capsaicinoids. In regards to aroma compounds, both peppers had different aroma profile. Shimatogarashi had fresh and fruity aroma owing to be high levels aldehyde and ester compounds, while Takanotsume was warm and herbaceous owing to elevated amounts of terpenoid compounds. Shimatogarashi also had higher antioxidant capacity compared to Takanotsume, owing to higher amount of phenolic and capsaicinoid contents. The results of study in **Chapter III** demonstrated that Shimatogarashi possessed distinctive of physical, flavor and functional properties, compared to Takanotsume. Therefore, the findings might serve as a basis for differentiating between two cultivars, as well as to testify their authenticity and their place of origins. Further research on odor contributing volatile compounds of the peppers using Gas Chromatography-Olfactometry (GC-O) with trained panelists would be an interesting study in order to have a better understanding of flavor-related compounds as well as flavor impressions of the peppers, particularly those cultivated in Japan.

Maturity stage is an essential factor for characteristics of fruits in general. Therefore, in this study characterization of the changes in color, organic acids, capsaicinoids, and volatile compound content as well as antioxidant activity during ripening in Shimatogarashi fruits had been conducted. The results of study in **Chapter IV** indicated that the aroma developed towards a more pungent and citrus-like aroma in the late maturing stage of Shimatogarashi. Meanwhile, the utilization of the young green fruits had the benefit of its pleasant fruity aroma, which was favorable to be included as condiment. The observed decrease in volatile compounds, especially esters also contrasted with that of climacteric fruits like apple and banana in which esters were produced during maturation. The antioxidant

capacity of the fruits in the mature red stage also showed the highest compared to the younger stages. Further investigation of the chemical changes under different cooking processes, particularly drying, frying and boiling, as well as different in different infusions medium, particularly oil, vinegar or alcohol, might be an interesting topic in future research.

Utilizing red mature Shimatogarashi extract which had the highest antioxidant capacity as well as capsaicin content, the study continued with the investigation of the fruit potential in the management of obesity prevention through diet intervention. By using 3T3-L1 to simulate adipocyte functions, we conducted the inhibitory action of Shimatogarashi extract and capsaicin. The degree of differentiation in 3T3-L1 cells had been measured by Oil Red O staining and PPARγ levels as a marker for adipogenesis in **Chapter V**. Both Shimatogarashi extract and capsaicin positively inhibited lipid accumulation in 3T3-L1 adipocytes in concentration-dependent manner and in different stages of differentiation. Because the mechanism underlying the phenomena was still unidentified, it necessitates further study to investigate the subject deeper into more elaborate gene expression using mice. Furthermore, the crude antiobesity and antioxidant potent Shimatogarashi extract might contain novel compounds that can be isolated and further identified.

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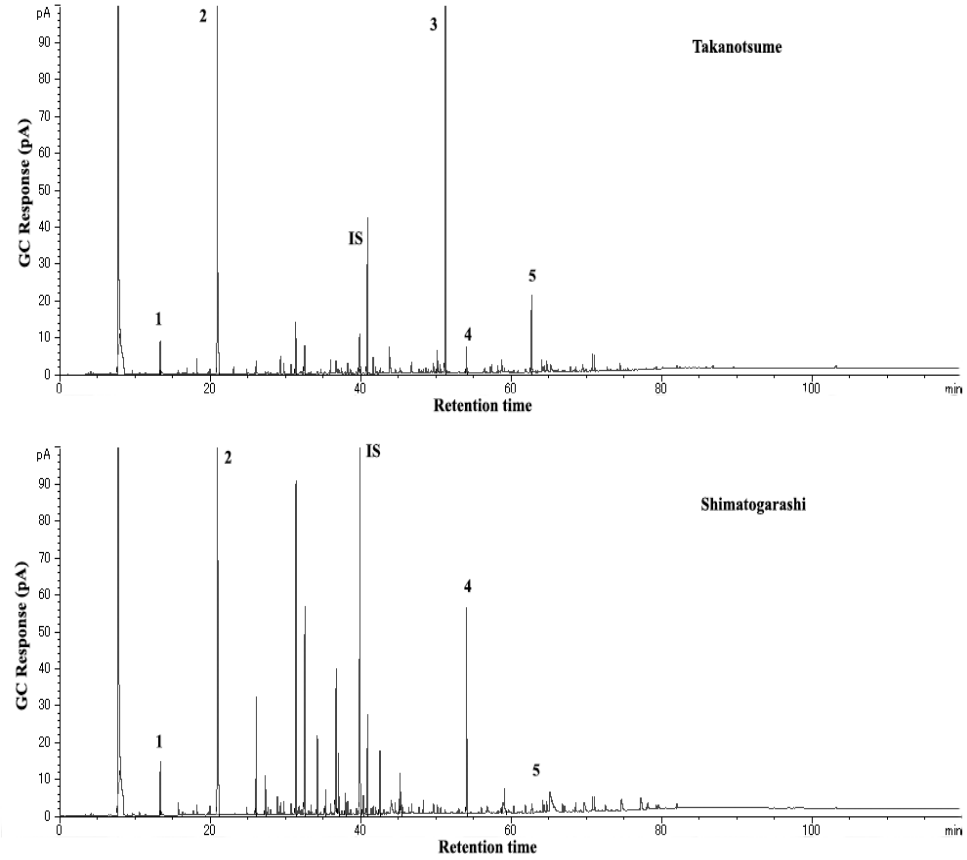
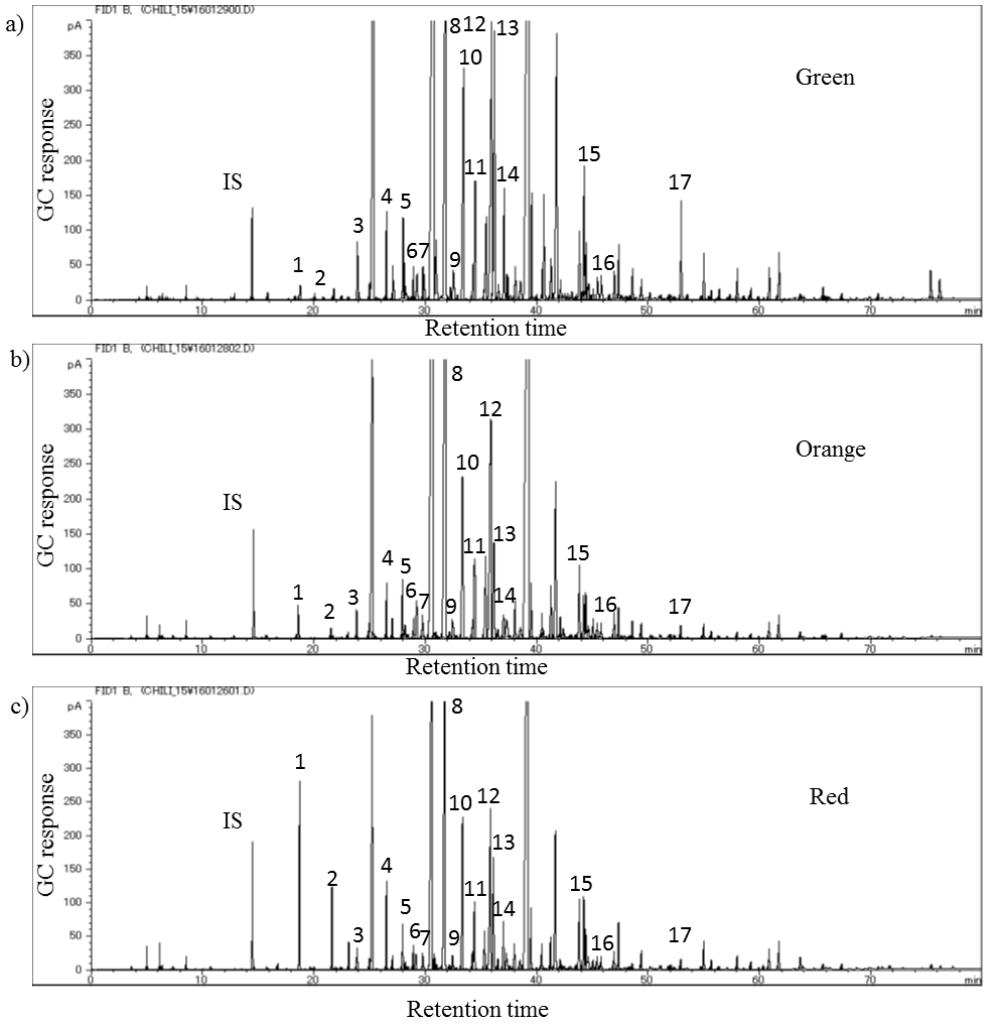
APPENDIX



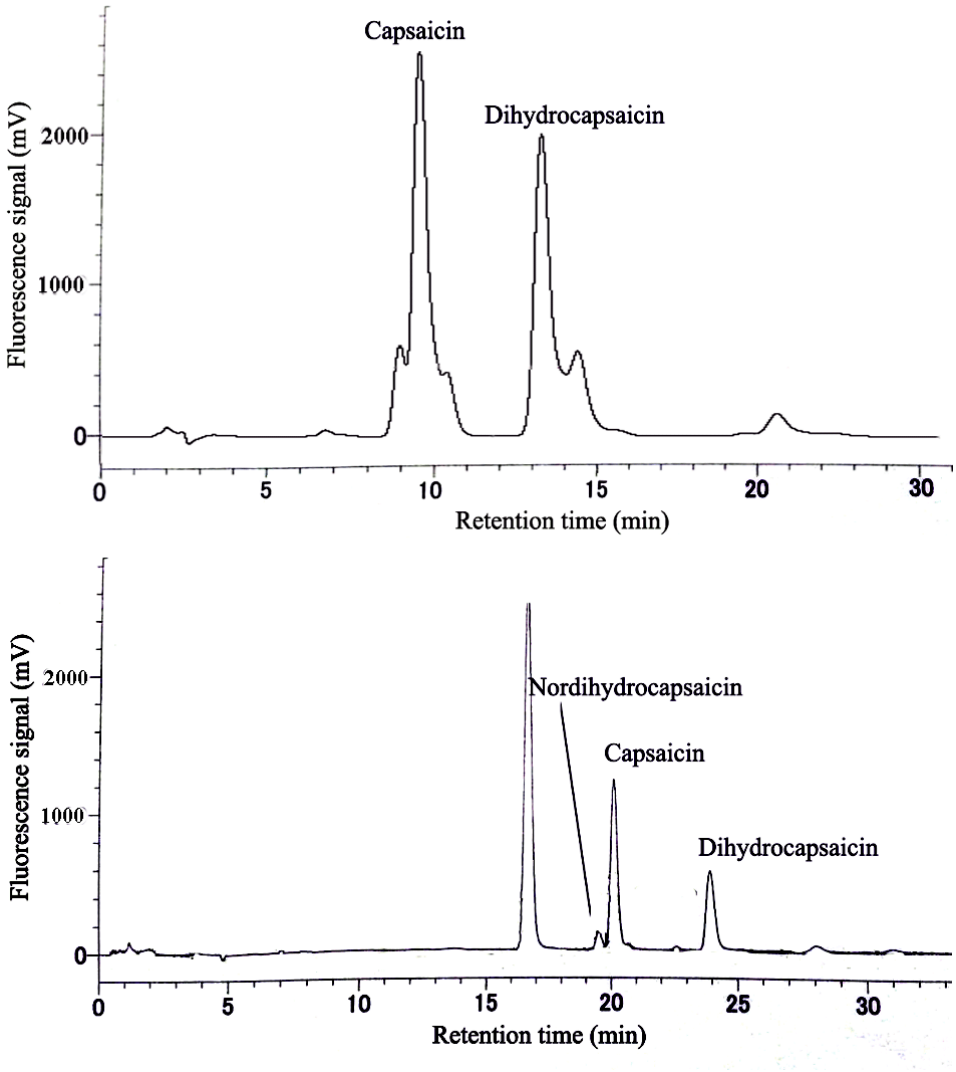
Appendix Figure 1. Gas chromatogram of Takanotsume and Shimatogarashi obtained using GC HS-SPME.

The chromatogram shows volatiles compounds of Takanotsume and Shimatogarashi. Peak Internal standard (IS): ethyl nonanoate; Peak 1: hexanal; Peak 2: 2-hexenal; Peak 3: himachala diene; Peak 4: 4-methyl salicylate; Peak 5: β-ionone. Other abbreviations: pA, picoampere.



Appendix Figure 2. Gas chromatogram of Shimatogarashi fruit's maturing stages obtained using GC HS-SPME.

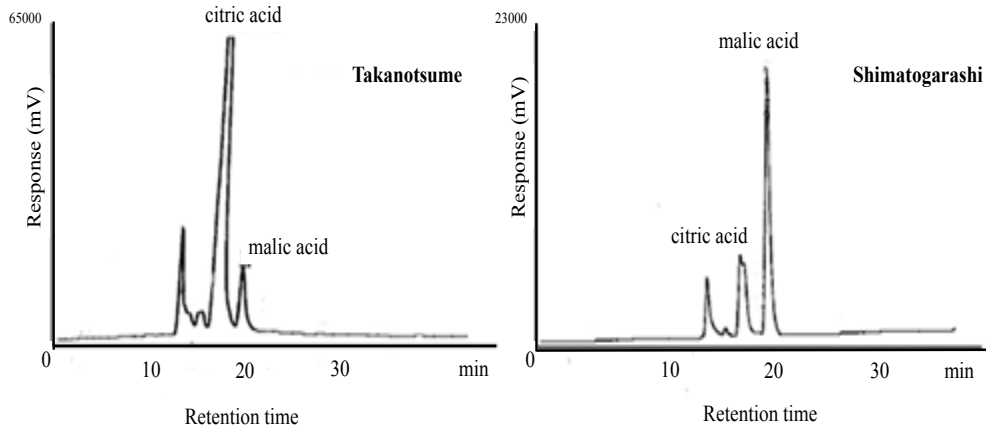
The chromatogram shows olatiles compounds of maturing stages of Shimatogarashi fruit: a) green, b) orange, and c) red stages. Peak Internal standard (IS): 3-pentanol; Peak 1: limonene; Peak 2: γ-terpinene; Peak 3: 4-ethylpentyl 2-methylpropanoate; Peak 4: 4-methyl 1-pentanol; Peak 5: hexyl 2-methylpropanoate; Peak 6: 2-methyl tridecane; Peak 7: 1-hexanol; Peak 8: 4-methylpentyl 3-methylbutanoate; Peak 9: hexyl butanoate; Peak 10: hexyl 2-methyl butanoate; Peak 11: hexyl 3-methyl butanoate; Peak 12: 4-methylpentyl pentanoate; Peak 13: (*Z*)-3-hexenyl 2-methylbutanoate; Peak 14: (*Z*)-3-hexenyl pentanoate; Peak 15: (*Z*)-3-hexenyl 4-methylpentanoate; Peak 16: 2-methyl hexadecane; Peak 17: methyl salicylate. Other abbreviations: pA, picoampere.



Appendix Figure 3. Chromatogram of Shimatogarashi capsaicinoids

(Up) Chromatogram obtained using HPLC equipped with Develosil SR-3 (150x3.0mm) column and fluorescence detector as described in Chapter III.

(Bottom) Chromatogram obtained using HPLC equipped with COSMOSIL 5C18-AR-II (250 mm × 4.6 mm) column and fluorescence detector as described in Chapter IV.



Appendix Figure 4. Chromatogram of Takanotsume and Shimatogarashi malic and citric acids The chromatogram obtained using HPLC equipped with Shim-pack SCR-102H (300 mm × 8 mm) column and conductivity detector as described in Chapter III and IV.