EVALUATION OF AROMA VOLATIES AND THEIR ODOR ACTIVITY IN A POPULATION OF TANGERINE HYBRIDS

By

TAKAYUKI MIYAZAKI

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To my family, friends, girlfriend

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Takayuki Miyazaki

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Tangerine (*Citrus reticulata* Blanco) is well known for its pleasant aroma, flavor and ease of consumption. With the desirable combination of sugars and acids, volatile compounds contribute to the essential organoleptic attributes for consumer acceptance. While aroma volatiles in orange juice have been well studied, little information is available on those found in fresh tangerine. There is a growing need to evaluate tangerine aroma components and their odor characteristics for improvement in fruit quality.

Twenty tangerine hybrids were harvested from November 2007 to March 2008, and five commercial cultivars were used as reference. Aroma volatiles were sampled from hand-squeezed juice by the headspace solid phase microextraction (SPME) method, and analyzed by gas chromatography-mass spectrometry (GC-MS). In total, more than 200 volatiles were identified in all samples. Two principal component analyses (PCA), based on relative peak areas (content) of all volatiles and 11 chemical classes, clearly separated the samples with richness in volatiles, such as sesquiterpenes and esters, and genetic background from sweet orange. Furthermore, a cluster analysis grouped these hybrids using qualitative (presence and absence) volatile composition. While ‘Murcott’ contained high levels of carotenoid derived volatiles, those

compounds were absent in its progenies issued from the cross with 8-9. 9-4 × Blood4x and two unknown samples were grouped in the same cluster due to their peculiar terpene profiles.

In addition, aroma active compounds in five selected samples were evaluated by three panelists using gas chromatography-olfactometry (GC-O) and time intensity (Osme) method. Forty nine odorants were found in a consensus and comprised of monoterpenes, aldehydes, esters, alcohols, ketones, phenol and ether. Hexanal, ethyl 2-methylbutanoate, unknown peak (No. 9), 1-octen-3-one, β-myrcene, 1,8-cineole, linalool, (*E*,*E*)-2,4-nonadienal with descriptors of green/grassy, fruity, metallic, mushroom, metallic, green, floral and fatty, respectively, were intense aroma compounds in all five samples. Differences between sample aroma profiles were found for compounds having descriptors of fruity, green/metallic/fatty, terpeney and green/grassy notes as the top notes, and five minor aroma categories. Perceived aroma intensity determined the specific aroma character of each sample. This is the first time that the aroma volatile profile and sensory quality of fruit from fresh tangerine hybrids with various genetic origins were investigated.

CHAPTER 1
INTRODUCTION

Citrus is one of the most economically important and widely grown fruit crops in the world. Citrus is believed to have originated in tropical and subtropical regions of Southeast Asia, where it was domesticated at least 4,000 years B.C. and then spread to other continents (Webber, 1967). Citrus production has exceeded other fruit crops such as grapes, bananas and apples (FAO, 2009). Mandarin and tangerine (*Citrus reticulata* Blanco) production is the second largest market of citrus fruit following oranges (*C. sinensis* L. Osb.) in the world. China is the largest tangerine producing country, followed by Spain and Brazil (Table 1-1). Spain has been significantly successful in the trade of seedless 'Clementine' varieties, accounting for over 50 % of world's exports of fresh mandarin/tangerine fruits.

The tangerine fruit is well known for its pleasant aroma and flavor, desirable combination of sugar and acid and ease of consumption. For human benefits, citrus fruits are a major source of vitamin C. Carotenoids known as antioxidants are more abundant in tangerine than orange (Mayne, 1996; U.S. Department of Agriculture-Nutrition Coordinating Center, 1998; Goodner et al., 2001). Other antioxidants such as flavonoids and phenolics, having a role in disease prevention, could also benefit consumer health. In addition, most tangerines are much more tolerant of citrus canker than are grapefruit and could be an alternative or complement to grapefruit for fresh market production in Florida (Gottwald et al., 2002).

Food flavors are commonly characterized by a combination of aroma, basic tastes (e.g., sweetness, sourness, bitterness, saltiness and umami) and mouth sensation such as astringency. Volatile compounds are responsible for aroma and flavor, and are analyzed by isolation, identification and quantification. Citrus aroma volatiles have usually been extracted by solvent, distillation, sorptive and headspace methods. Solid phase microextraction (SPME) is a

solventless headspace sampling method (Arthur and Pawliszyn, 1990). It is a rapid and relatively inexpensive technique, thereby having been applied to many studies on aroma volatiles in citrus juice (Steffen and Pawliszyn, 1996; Rega et al., 2003). The development of gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) has enabled flavor researchers to identify and quantify volatiles in food and beverages. To date, volatiles in citrus juice and peel oil have been widely investigated. Most aroma compounds arise as a result of degradation reactions from carbohydrates, fatty acids and amino acids (Schwab et al., 2008). Above all, esters play an important role in overall aroma and flavor of climacteric horticultural crops such as apples (Defilippi et al., 2005) and bananas (Shiota, 1993). In non-climacteric citrus, monoterpenes ($C_{10}H_{16}$) and sesquiterpenes ($C_{15}H_{24}$) are the major volatile compounds produced, and citrus aroma is the result of a combination of volatile components such as terpenes, esters, aldehydes, ketones, and alcohols (Nisperos-Carriedo et al., 1990).

Gas chromatography-olfactometry (GC-O) is the most appropriate method to detect and evaluate odor active components from a complex mixture of volatiles, using a human nose as a detector (van Ruth, 2001). The various GC-O techniques, measuring odor activity, can be classified into three categories: time intensity, detection frequency and dilution to threshold methods. A time-intensity method, Osme (Greek word for ‘smell’), takes into consideration a psychophysical law, estimating the magnitude of an odorant (McDaniel et al., 1990). So far, the sensory properties of volatiles in orange juice have been well investigated (Hinterholzer and Schieberle, 1998; Tønder et al., 1998; Buettner et al., 2001b; Rouseff and Pérez-Cacho, 2007). Some major aroma active compounds were known to impart odor notes such as citrus, fruity, floral and green to orange juice aroma. While there have been several reports on aroma volatiles

in tangerine peel and essence oil (Moshonas and Shaw, 1974; Shaw, 1979, Wilson and Shaw, 1981), very little is known regarding those in fresh tangerine fruits.

Citrus fruits with desirable flavor associated with aroma volatiles, as well as sugars and acids, could increase consumption and market value, thereby resulting in greater economic returns to growers, shippers and packers. Improvements in fruit quality and characteristics, as well as improvements in pest and disease resistance, tolerance to various environmental stress factors, horticultural performance, and productivity are the primary goals of citrus breeding (Gmitter et al., 2007). However, the conventional citrus breeding based on hybridization and selection has been hampered by the characteristics of long juvenility, high heterozygosity, gametophytic self- and cross-incompatibility and apomixis. Most of the grown citrus scion and rootstock cultivars at present arose as chance seedlings or limb and bud sport mutations, rather than from organized breeding programs (Hodgson, 1967). Fruit aroma and flavor are affected by a complex system of genes, and environmental (temperature, irrigation, soil condition, illumination, etc.) and postharvest (storage, processing, etc.) factors. Understanding plant genetics and genomics, as well as food science, will enhance the efficiency to improve fruit quality in breeding programs. The fundamental data of fruit aroma and flavor components will provide the basis for developing methods for marker-assisted selection (MAS), to efficiently select at an early stage the superior individuals with high fruit quality from tangerine breeding programs.

The main objectives of this study were:

1. To identify the aroma volatile compounds in juice samples of tangerine hybrids and commercial cultivars by GC-MS,
2. To analyze intervarietal relationships from volatile profiles by using multivariate statistics, principal component analysis (PCA) and cluster analysis (CA),

3. To characterize aroma active compounds of some selected hybrids by GC-O and investigate the contribution of each volatile to overall fresh fruit aroma.

Table 1-1. Total production of tangerine and mandarin fruits in 2007 (FAO, 2009)

Country	Production (tons)
China	14,152,000
Spain	2,080,700
Brazil	1,271,000
Japan	853,000
Turkey	738,786
Italy	702,732
Iran	702,000
Thailand	670,000
Egypt	660,000
Pakistan	640,000
Others*	4,043,768
Total (in the world)	26,513,986

* Others include 328,000 tons of United States of America.

CHAPTER 2
REVIEW OF LITERATURE

Origin and Taxonomy of Citrus

The center of origin and diversity of *Citrus* and its related genera is still unclear, but most researchers have considered that *Citrus* is native to Southeast Asia, especially to east India, north Burma and southwest China, extending to Malay archipelago and the East Indies, northeastern Asia, and Japan (Scora, 1975; Gmitter and Hu, 1990). The genus *Citrus* belongs to the Rutaceae family, subfamily Aurantioideae. The true citrus fruit trees include six closely related genera: *Fortunella* (kumquat), *Eremocitrus* (Australian desert lime), *Poncirus* (trifoliate orange), *Clymenia*, *Microcitrus* (Australian wild lime) and *Citrus*. The taxonomic classification of *Citrus* species is very complicated due to nucellar embryony, self-compatibility and incompatibility, many natural and artificial hybridization, wide dispersion and long history of cultivation (Cameron and Frost, 1968; Moore, 2001; Cornélio et al., 2003). In the past, studies on taxonomy and phylogeny were concluded based on morphological and geographical information. According to Swingle and Reece (1967) and Tanaka (1977), the genus *Citrus* consists of 16 and 162 species, respectively; indeed, these two taxonomic system differ widely. Swingle and Reece (1967) recognized three mandarin species, *C. reticulata*, *C. tachibana* (a wild species from Japan) and *C. indica* (a wild species from India), whereas Tanaka (1977) separated the mandarins into 36 species. Hodgson (1967) recognized 36 *Citrus* species and classified the mandarins into five classes: the satsuma mandarins (*C. unshiu*), the King mandarins (*C. noblis*), the Mediterranean mandarins (*C. deliciosa*), the common mandarins (*C. reticulata*) and the small-fruited mandarins.

Tangerine is grouped into the mandarin species *C. reticulata*, and usually characterized by deep orange and red color, sweet flavor and easiness of peeling. Swingle and Reece (1967) noted

that mandarins include monoembryonic and polyembryonic cultivars, as well as self-fertile and self-incompatible types. Mandarins are the most phenotypically heterogeneous group in *Citrus* (Hodgson, 1967; Moore, 2001). Although mandarins had been cultivated in China and Japan from ancient times, mandarins were introduced to the West (England) from China for the first time in 1805 by Sir Abraham Hume, and they only subsequently spread to the Mediterranean region (Webber, 1967; Soost and Roose, 1996).

Biochemical studies focusing on various types of compounds such as limonoids (Dreyer et al., 1972) and flavonoids (Albach and Redman, 1969) have been conducted to assess taxonomic relationship of *Citrus*. Esen and Scora (1975) widely analyzed the enzymatic browning capacity of young shoot homogenates in 428 accessions of the Aurantioideae including 10 citrus species, and confirmed its species-specificity. Barrett and Rhodes (1976) performed a comprehensive phylogenetic study of 146 morphological and biochemical tree, leaf, flower and fruit characteristics. These two studies suggested that citron (*C. medica*), mandarin (*C. reticulata*) and pummelo (*C. grandis*) are the primary species of *Citrus*.

DNA-based molecular markers and DNA sequences have been applied in many research fields such as phylogeny, species identification, association studies and breeding during the last few decades. A molecular marker may be found at a particular position of genome, and then associated with a gene or trait of interest. Molecular marker techniques are more accurate, reproducible and time-saving than conventional survey only based on phenotypic characteristics of samples, to elucidate genetic relationship or to select interesting genotypes. So far, phylogenetic relationships in *Citrus* have been studied by several marker techniques: e.g., random amplified polymorphic DNA (RAPDs) marker (Coletta Filho et al., 1998; Federici et al., 1998), inter-simple sequence repeat markers (ISSRs) (Fang and Roose, 1997) and sequence-

characterized amplified regions (SCARS) (Nicolosi et al., 2000). These molecular marker analyses support the proposition that there are three primary species in *Citrus* (Federici et al., 1988; Nicolosi et al., 2000). More recently, Bayer et al. (2009) showed the pummelo and all it derivatives (grapefruit, sweet orange, sour orange, lemon, Tahitian lime) were grouped in the same clade, using maternally inherited chloroplast DNA sequences. The three primary species, with other genera of tribe Citreae, were clearly separated into different phylogenetic groups. It is generally accepted that the wide variety of current citrus species were originated from the hybridization between and among these true species, as well as from their crossing with closely related genera and perhaps extinct ancestral species. For instance, sweet oranges are thought to be predominantly of mandarin germplasm introgressed with pummelo (Barrett and Rhode, 1976; Nicolosi et al., 2000). Although some studies revealed a large molecular heterogeneity in mandarin accessions, there were nonetheless high genetic similarities among the samples. Thus, it has been concluded that mandarin is a single species, consisting of a large number of hybrids (Coletta Filho et al., 1998).

In relation to the present study, variety classification based on volatile compounds has been conducted in lemon essential oil components (Scora and Malik, 1970), mandarin essential oils (Merle et al., 2004), tangerine juice (Kerbiriou et al., 2007) and yuzu (*C. junos*) peel oil (Lan-Phi et al., 2009). Since many volatiles have been found in citrus fruits, the multivariate statistical analyses such as principal component analysis (PCA) and cluster analysis (CA) have been valuable tools to differentiate individual varieties.

Comparisons of citrus hybrids with their parents have been conducted based on aroma volatile composition in juice of interspecific hybrids between orange and pummelo and between tangelo (i.e., a cross between tangerine and grapefruit) and grapefruit (Shaw et al., 2001), leaves

and peels of a somatic tetraploid hybrid between ‘Mexican lime’ and ‘Star Ruby’ grapefruit (Gancel et al., 2002), leaves of 13 interpecific and intergeneric somatic tetraploid hybrids (Gancel et al., 2005). Shaw et al. (2001) quantitatively compared juice volatile compounds of ‘Shamouti’ orange, ‘Nakon’ pummelo and their hybrid. The same 39 volatile components were quantified among them, and 13 volatiles of the hybrid showed intermediate levels between the parents. The other compounds were about equally divided between values higher than those present in either parent, and those equal to or lower than those present in either parent. Although allotetraploids contain the diploid sets of chromosomes from each parent, their qualitative (presence/absence) and quantitative (content) aroma composition were widely different from that of parents (Gancel et al., 2002, 2005). The somatic tetraploids from mandarin and non-mandarin (lime, lemon, sweet orange, kumquat or *Poncirus*) parents significantly lowered the production of most aroma volatiles of the latter parent (Gancel et al., 2005). The same author also reported the quantitative and qualitative levels of proteomes in the tetraploids (mandarin + lime, mandarin + kumquat) were closer to mandarin parents, suggesting that the mandarin parent was highly dominant (Gancel et al., 2006). The study on volatile composition in the hybrids and its parents can lead perhaps to the basic knowledge of inheritance mechanisms of genes involved in aroma volatile synthesis.

Analysis of Aroma Volatiles

Gas Chromatography-Olfactometry (GC-O)

Food flavor is commonly divided into the subsets of smell and taste, which are perceived in the nose and mouth, respectively. Odor perception can be considered the response to aroma active volatiles that enter through the nostrils (orthonasal), and aroma perception is caused by volatiles entering from the mouth and respiratory system (retoronasal) (van Ruth, 2001).

Over the last decades, the rapid progress in flavor research has been made due to improvement in instruments and analytical chemistry. Since modern gas chromatography was invented in 1952 (Bartle and Myers, 2002), it has allowed researchers to separate, identify and quantify volatile compounds. The application of GC to evaluate sensory quality of food aroma was first published by Fuller and coworkers in 1964 (Zellner et al., 2008). Later, Dravnieks and O'Donnella (1971) reported more sophisticated GC-O system with humidified air to reduce nasal dehydration. Until now, GC-olfactometry is the most appropriate analytical solution that uses human assessors to detect odor active compounds eluting from a GC separation.

An individual volatile compound has essentially three properties related to its odor potential or activity for humans: absolute threshold, intensity as a function of concentration and quality (Delahunty, 2006). It is essential to estimate the sensorial contribution of each component to overall food aroma by using its odor detection, threshold, perceived odor intensity and duration of odor activity as well as odor quality. Therefore, various GC-O methods have been developed in the past two decades: detection frequency method, dilution to threshold analysis (AEDA) and CharmAnalysis^{TM}, and direct intensity (Osme) and posterior intensity methods.

GC-O Methods

The traditional threshold analysis of food flavor is measured in water or in air (Plotto et al., 2004). The ratio of concentration to its odor threshold is calculated to estimate the importance of each aroma active compound, and this value is called aroma value, odor unit, odor value, flavor unit or odor active value (Delahunty et al., 2006). In dilution analysis, an aroma extract is diluted, usually as a series of 1:2 or 1:3 dilutions, and each dilution is analyzed by GC-O until no odor is perceived by panelists (Acree et al., 1984). Panelists record individual response (yes or no) to a dilution and usually record an odor description. In AEDA first proposed by Ulrich and Grosh (1987), the panelists evaluate samples in increasing dilution order. The maximum dilution

at which odor can still be perceived is expressed as dilution factor (FD) value. Another type of method, CharmAnalysis™ (Acree et al., 1984), presents the dilutions in randomized order to avoid bias introduced by knowledge of the samples. Dilution analysis is the most widely applied method (Zellner et al., 2008), however, this method does not consider the actual odor intensity of aroma volatiles in intact samples. Moreover, dilution procedures are time-consuming and therefore the number of assessors is very limited.

The detection frequency method proposed by Linssen et al. (1993) requires a panel of 6 to 12 assessors that smell the GC effluents once, whereas dilution analyses require only1 to 3 panelists that perform the olfactory test multiple times. Pollien et al. (1997) developed this method for more consistent results and no training for the panelists. The number of assessors detecting an odorant at a particular retention time is used as an estimate of the odor intensity. Thus, this method also does not present actual intensities of odor in food samples. As concentration increases, odor intensity may also contribute to increase; however, detection frequency can not increase. This method is time-saving and easy to handle, and therefore it has been used to determine aroma impact components in several food products: guava fruit puree (Jordán et al., 2003), hand-squeezed juices of four orange varieties (Arena et al., 2006), and wine (Falcão et al., 2008).

The direct intensity or Osme method was developed by McDaniel et al. (1990) to directly measure the perceived odor intensity using trained panelists. Therefore, this method is differentiated from the others due to its consideration of psychophysical law. The odor intensity, duration of odor activity and verbal descriptor of each compound are recorded with variable scales and computer-based devices. The generated figure of retention time (or index) vs. average maximum odor intensity is called an Osmegram, representing the significance of each compound

in food aroma. Although this method is time-saving and requires a small number of panelists, it has not been applied to many studies: apple (Plotto et al., 2000), cooked mussels (Guen et al., 2000), cashew apple (Garruti et al., 2003) and grapefruit oil (Lin and Rouseff, 2001). The comparison of global analysis based on detection frequency, Osme and AEDA showed that their results were well correlated, especially for the most potent odorants in cooked mussels (Guen et al., 2000). Also, van Ruth (2004) compared the detection frequency and time intensity method for 6 volatile compounds, and concluded higher repeatability of the former method and higher discrimination of the latter method. Thus, method selection depends on the study objective, performance of panelists and time availability.

SPME Method

Aroma volatiles tend to reside in the headspace above solid or liquid, being less soluble in the presence of water. The concentration of volatiles above food products ranges from about 10^{-4} to 10^{-10} g/L (Reineccius, 2005), and hence extraction of aroma volatiles is a critical factor for the following analyses using GC-MS, GC-O, etc. Volatile compounds in citrus fruits can be extracted by several different methods: distillation (Kirchner et al., 1953; Radford et al., 1974), static headspace (Nisperos-Carriedo and Shaw., 1990), solvent extraction (Buettner and Schiertle, 1999; Chisholm et al., 2003), and a purge and trap method (Cadwallader and Xu, 1994; Shaw et al., 2000). However, disadvantages of these methods are artifact production, the limited number of detected volatiles, and compositional changes as well as time-consuming extraction procedures.

The SPME method, first developed by Pawliszyn's group (Arthur and Pawliszyn, 1990), is a solvent-free, rapid, simple and relatively inexpensive technique that can reduce or eliminate the above problems. SPME involves exposing a fused silica with a polymeric coating to a sample or its headspace. The absorbed volatiles are thermally desorbed in the injector of a GC, GC-MS or

GC-O. So far, this method has been widely applied to the studies on citrus juice volatiles. Rega et al. (2003) reported the optimized SPME conditions for orange juice flavor by sniffing the overall SPME extracts and evaluating their odor representativeness. This study provided the valuable information regarding to fiber selection and sample equilibrium time for SPME of citrus aroma volatiles.

Citrus Aroma and Flavor

Among 6 closely related genera as described earlier, most flavors of commercial value are found in the genus *Citrus* and subgenus *Eucitrus* (Rouseff and Pérez-Cacho, 2007). Citrus flavor is the result of complex combinations of aroma volatiles and soluble solids. An odor is usually elicited by a combination of volatile compounds each of which imparts its own smell. Soluble solids are mainly comprised of sugars (e.g., sucrose, fructose and glucose), organic acids (e.g., citric acid and malic acid), and the flavonoid subgroup. These components are responsible for three basic tastes, sweetness, sourness, and bitterness, respectively.

Most of our knowledge about citrus volatiles has been gained from studies of processed juices and the peel essential oils, essence oils, and aqueous essences used to flavor juice products (Shaw, 1991). Conversely, studies on aroma volatiles in fresh citrus fruit have not much been reported. Most citrus aroma volatiles are mainly classified into terpene hydrocarbons, aldehydes, esters, alcohols and ketones, and many are commonly found within citrus species. Some citrus species, such as lemon and grapefruit, contain one or two major aroma impact compounds. For instance, a terpene aldehyde citral is considered to be one major aroma impact volatile in lemon. However, no single volatile in tangerine/mandarin and orange can be considered a single characteristic impact compound (Shaw, 1991). Aroma and flavor of commercially important citrus fruits, tangerine/mandarin, orange and grapefruit/pummelo, are described below.

Tangerine/Mandarin Aroma

Compared to orange fruit, very little information about tangerine/mandarin aroma and flavor is available, perhaps because tangerine is mostly consumed as fresh fruit and hence its juice and peel is less used for the beverage and fragrance industry. Further, tangerine/mandarin juice products have been more difficult to market because of undesirable off-flavors during processing and storage (Moshonas and Shaw, 1997).

The aroma volatiles in mandarin (tangerine) peel oil have been relatively well studied. The major volatiles in tangerine essence and peel oil are d-limonene, myrcene and α-pinene, together accounting for more than 90% of all detected compounds (Moshonas and Shaw, 1974; Coleman and Shaw, 1972). However, these volatiles may not be major aroma active compounds due to their high thresholds (Plotto et al., 2004). The characteristic smell of mandarin peel oil has been believed to be caused by methyl N-methylanthranilate and thymol. Wilson and Shaw (1981) reported that it was necessary to add β-pinene and γ-terpinene with methyl N-methylanthranilate and thymol, in order to give the tangerine peel oil an aroma similar to that of Sicilian mandarin peel oil. Chisholm et al. (2003) found almost 50 odor active compounds in 'Clementine' peel oil using CharmAnalysis^{TM}, and approximately 80% of its aroma was due to the aldehydes. Buettner et al. (2003) also analyzed aroma volatiles in 'Clementine' peel oil by AEDA. Of 42 odor active compounds detected, linalool, (*E*,*E*)-2,4-decadienal, wine lactone, α-pinene, myrcene and octanal showed high FD factors. Interestingly, many aroma active compounds in these studies impart non-citrus odor notes, described as 'green', 'metallic', 'floral', etc. Sawamura et al. (2004) identified 39 volatile compounds in cold-pressed oil of 'Ponkan' (*C. reticulata*) and characterized their odors by AEDA technique. Coupled with sensory evaluation, it was concluded that two aldehydes, octanal and decanal, played an important role as 'Ponkan-like' odor along with (R)-(+)-limonene as a background component for the overall aroma.

The studies on tangerine/mandarin juice aroma seem to have been conducted mostly in the main producing countries such as Japan and Spain. Yajima et al. (1979) identified 68 and 72 aroma volatiles in aroma concentrate from juice and in peel oil of Satsuma mandarin (*C. unshiu*), respectively. Although most of the same hydrocarbons were identified in both samples, they consisted of 60.5 and 98.0% of total content, respectively. Therefore, the qualitative (presence/absence) and quantitative (content) composition of the oxygenated compounds differed widely. More recently, Pérez-López and collaborators (2006a) quantified volatile compounds in two Spanish mandarin ('Fortuna' and 'Clemenules') juices by GC-MS. This study focused on how pasteurization and storage of juice affected on the volatile compounds, and only five compounds were quantified.

Orange Aroma

Orange juice is the most widely consumed fruit juice in the world. Many aroma volatiles of freshly squeezed and processing orange juices have been reported. Up to now, more than 300 volatiles have been reported in fresh orange juice. However, less than 25 appear to have significant odor activity (Pérez-Cacho and Rouseff, 2008). These compounds mainly consist of terpene hydrocarbons, aldehydes, esters and alcohols.

Terpenes are one of the most abundant groups of volatiles in freshly squeezed orange juice (Nisperos-Carriedo and Shaw, 1990; Bylaite and Meyer, 2006). Although limonene usually accounts for 90% of all terpenes in orange, the role of limonene for overall aroma has not been clearly proven (Shaw, 1991; Bazemore et al., 1999). It might be possible that limonene functions as a "lifting agent" for other volatiles in a similar way as ethanol does in wine (Pérez-Cacho and Rouseff, 2008). The odor qualities of major terpenes are usually characterized as 'citrus-like' and 'minty' (limonene), 'ethereal' and 'piney' (α-pinene), 'wet soil' and 'mossy' (myrcene) and 'terpene-like' and 'pungent' (β-pinene). A sesquiterpene valencene is also present in orange

juice in high quantity (Nisperos-Carriedo and Shaw, 1990; Bylaite and Meyer, 2006), however, its contribution to orange juice aroma has not yet been verified in GC-O studies (Hinterholzer and Schieberle, 1998; Buettner and Schiebertle, 2001b).

Aroma active aldehydes are found in several different forms: saturated aliphatic, unsaturated aliphatic, terpenic and phenolic aldehydes (Pérez-Cacho and Rouseff, 2008). Most aldehydes are major contributors to the fresh, pungent odor quality of orange juice (Buettner and Schieberle, 2001b). Shaw (1991) reported that the three homologous straight-chain aldehydes, octanal, nonanal, and decanal were implicated as important contributors to orange juice aroma. Hinterholzer and Schieberle (1998) characterized their odor qualities as 'green', 'citrus-like' and 'soapy', respectively, and found (*Z*)-hex-3-enal ('green' and 'leaf-like' odor) had the highest FD factor among all the detected aldehydes.

Esters are well known to impart a fruity note to orange juice. Of the identified esters, ethyl butanoate is present in orange juice at 500 to 10,000 times its flavor threshold value in water (Shaw, 1991) and it has been therefore been receiving attention. Indeed, dilution analysis (Hinterholzer and Schieberle, 1998; Buttner and Schieberle, 2001b) and detection frequency methods (Rega et al., 2003) showed that this compound was one of the most potent odorants in orange juice. Ethyl acetate is found at relatively high concentration in orange juice (Moshonas and Shaw, 1987; Nisperos-Carriedo and Shaw, 1990; Moshonas and Shaw, 1994). However, the orthonasal threshold of ethyl acetate (6,038 μg/L) in reconstituted pump-out (deodorized orange juice concentrate) is significantly higher than those of ethyl butanoate (1.71 μg/L) (Plotto et al., 2008). Therefore, ethyl acetate does not seem to make a direct contribution to orange flavor (Shaw, 1991). It is clear that esters are important to fresh orange flavor, but further research on

their balance and interaction is needed to understand the precise role in orange flavor (Shaw, 1991).

Alcohol is also major aroma component in orange juice. The quantity of ethanol is usually the highest among all alcohols in fresh orange juice. Although a large number of alcohols are isolated from orange juice, only a few are considered to contribute to orange juice aroma (Shaw, 1991). Three aliphatic alcohols, 1-hexanol, (*Z*)-3-Hexen-1-ol and 1-octanol, were reported as aroma active compounds in orange juice (Rega et al., 2003), as well as terpene alcohols such as linalool and geraniol (Mahattanatawee et al., 2005). Linalool is the most potent alcohol in orange juice, characterized as 'floral' and 'sweety' (Shaw, 1991; Hinterholzer and Schieberle, 1998; Buttner and Schieberle, 2001b). Also, a terpene ketone β-ionone is characterized as strong 'floral' odor. Mahattanatawee et al. (2005) reported that linalool and β-ionone each contribute 22% of the floral note in orange juice aroma.

Grapefruit and Pummelo Aroma

Grapefruit is thought to be derived from a backcross between pummelo and sweet orange (Nicolosi et al., 2000). Grapefruit flavor is usually distinguished from mandarin/tangerine and sweet orange due to its characteristic sourness and bitterness. The bitterness is caused by the accumulation of flavanone-glycosides, mostly flavanone neohesperidosides such as naringin (Frydman et al, 2004). On the other hand, non-bitter citrus fruits contain mostly tasteless flavanone rutinosides. The major pummelo varieties lack the bitterness characteristic of grapefruit, but bitter pummelos do exist (Hodgson, 1967).

The volatile composition in grapefruit is similar to that of mandarin/tangerine and orange (Cadwallader and Xu, 1994; Shaw et al., 2000). However, nootkatone (MacLeod and Buigues, 1964) and 1-p-menthene-8-thiol (Demole et al., 1982) have been considered as the primary flavor-impact compounds in grapefruit. Nootkatone is used commercially in artificially flavored

grapefruit beverages and perfumes (Wilson and Shaw, 1978). The high level of nootkatone is generally associated with pummelo and some pummelo hybrids including grapefruit. For instance, the high content was detected in cold-pressed oils from 8 pummelo varieties (Sawamura et al., 1991) and peels of 10 grapefruit varieties (Ortuño et al., 1995). However, the threshold of nootkatone is high as previously determined, about 1 ppm in water (Berry et al., 1967; Haring et al., 1972). Its contribution to overall grapefruit aroma is still unclear. Since nootkatone levels of grapefruit increases during fruit maturation (del Río et al., 1992) and postharvest treatment (Biolatto et al., 2002), it is proposed as an indicator of fruit quality.

1-p-Menthene-8-thiol has been proposed as a character impact compound due to its low odor threshold and high content in grapefruit juice. Demole et al. (1982) determined the threshold of 1.0×10^{-7} ppm in water and found 200-fold higher concentration in the juice. Using the AEDA method, Buettner and Schieberle (1999) have reported that 1-p-menthene-8-thiol significantly contributed to grapefruit aroma, along with several other compounds such as ethyl butanoate, (*Z*)-3-hexenal and 1-hepten-3-one.

Aroma Volatile Biosynthetic Pathways

A large number of aroma volatiles have already been found in plants, but most of the enzymes and genes involved in volatile production are still unknown (Schwab et al., 2008). Various aroma volatile compounds with saturated, unsaturated, single-chain, branched-chain and cyclic structures are derived from carbohydrate, fatty acid and amino acid pools. The characteristic flavor of fruits, such as bananas, peaches, pears (all climacteric fruits) and cherries (non-climacteric fruit), develops entirely during a rather brief ripening period (Reineccius, 2005). Several studies on apple aroma synthesis showed that ethylene played an important role in fruit ripening and also expression of genes related to volatile synthesis (Schaffer et al., 2007). Since esters contribute greatly to aroma of many fruits (e.g., apple, melon and banana), most of the

studies on aroma synthesis have focused on the last step of ester formation with alcohol acyltransferase (AAT). As mentioned earlier, it seems that several terpenes, esters, aldehydes, alcohols, etc, contribute to citrus aroma. These compounds are mainly derived from terpenoids, fatty acids and amino acids.

Terpenoid Aroma Volatiles

Terpenoids are the largest and most diverse family of natural products, consisting over 40,000 individual compounds (Aharoni et al., 2005). The terpenoids play diverse functional roles in plants as hormones (gibberellins, abcisic acid), photosynthetic pigments (phytol, carotenoids) and electron carriers (ubiquinone, plastoquinone) (McGarvey and Croteau, 1995). In addition, monoterpenoid ($C_{10}H_{16}$) and sesquiterpenoids ($C_{15}H_{24}$) commonly found in citrus species serve as attractants for seed-dispersing animals, components of desirable aroma/flavor, and flavorings and fragrances for human beings.

In *Citrus*, terpenes are produced especially in leaves, fruit epidermis (flavedo) and juice (Dornelas and Mazzafera, 2007). Terpenoids are derived from common terpene building units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphospate (DMAPP). These two compounds are *de novo* synthesized from acetyl CoA via the acetate-mevalonate pathway in cytoplasm, and pyruvate with gyceraldehydes-3-phosphate via the non-mevalonate (methylerythritol phosphate, MEP) pathway in plastids (Tholl, 2006) (Figure 2-1). The cytosolic pathway is responsible for the synthesis of sesquiterpenes, whereas monoterpenes and carotenoids are produced in plastids. Following the isomerization of IPP to DMAPP by isopentenyl diphosphate (IPP) isomerase, both compounds are condensed to form geranyl diphosphate (GPP; C_{10}). Subsequently, farnesyl diphosphate (FPP; C_{15}) and geranylgeranyl diphosphate (GGPP; C_{20}) are formed by sequential addition of IPP. GPP, FPP and GGPP are the direct precursors of terpenoids, which are synthesized to monoterpenes, sesquiterpenes and

diterpenes, respectively. Moreover, several aroma volatiles such as β-ionone and β-damacenone are derived from carotenoids which precursor is GGPP (Baldwin et al., 2000).

Recently, several studies on terpene synthases have been reported in *Citrus*. Maruyama et al. (2001) reported the cloning and functional expression of the (*E*)-β-farnesene (sesquiterpene) synthase gene from young leaves of 'Yuzu' (*C. junos*). This is the first report of the cloning of a terpene synthase from a Rutaceous plant. Lücker et al. (2002) isolated four monoterpene synthase cDNAs by random sequencing of flavedo-derived cDNA library of lemon (*C. limon*). These cDNAs were functionally expressed in *Escherichia coli*, and three different major products, (+)-limonene (two cDNAs), β-pinene and γ-terpinene were identified by GC-MS using the cDNA-encoded enzyme and GPP. Sharon-Asa et al. (2003) and Shimada et al. (2004) also conducted similar studies with valencene synthase of 'Valencia' orange and monoterpene synthases of Satsuma mandarin, respectively. The former study showed valencene accumulation and the gene expression was responsive to ethylene accumulated toward fruit maturation, suggesting its importance for aroma production even in non-climacteric citrus fruit.

Fatty Acid Derived Aroma Volatiles

The most common fatty acids in citrus juices are palmitic, palmitoleic, oleic, linoleic and linolenic acids (Nordby and Nagy, 1969). Galactolipids and phospholipids are rich in lipoprotein membranes of fruit cells and sub-cellular organelles such as mitochondria and lamellae of chloroplast (Goldschmidt, 1977). During the fruit ripening stage, membrane degradation occurs, resulting in the release of free fatty acids.

Many aliphatic straight-chain alcohols, aldehydes, ketones, acids and esters are formed from fatty acids via three processes: α-oxidation, β-oxidation and the lipoxygenase pathway. The oxidation of unsaturated fatty acids forms aldehydes and their subsequent reduction to alcohols. Rowan et al. (1999) demonstrated that straight chain ester volatiles were derived from fatty acids

via the above three processes in apples, and several C_6 aldehydes and alcohols were intermediate products from linolenic and linoleic acids to esters (Figure 2-2). For instance, (*Z*)-hex-3-enal ($C_6H_{10}O$), derived from linolenic acid, is an odor-active volatile in hand-squeezed juices of 'Valencia' and 'Navel' oranges (Buettner and Schieberle, 2001b).

Amino Acid Derived Aroma Volatiles

Branched-chain and aromatic volatiles (alcohols, aldehydes, acids, esters, sulfur-containing volatiles) are derived from amino acid (Schwab et al., 2008). Although their biosynthetic pathways are still unclear, synthesis of branched-chain esters originated from leucine, isoleucine and valine have been relatively well studied, probably due to their characteristic fruity odors in banana (Tressel and Drawert, 1973), strawberry (Pérez et al., 2002) and apple (Matich and Rowan, 2007). Branched-chain esters, as well as straight-chain esters derived from fatty acids, are formed from the same reaction; alcohol acyltransferase (AAT) catalyzes alcohol and acyl CoA, following the reduction of aldehyde to alcohol by alcohol dehydrogenase (ADH). In fresh orange juice, many esters have already been found by several researchers; however, only a few of these are known as aroma active volatiles: methyl butanoate, ethyl acetate, ethyl butanoate, ethyl-2-methylpropanoate, ethyl-2-methylbutanoate, ethyl hexanoate and ethyl octanoate (Pérez-Cacho and Rouseff, 2008).

Sulfur-containing volatiles derived from methionine and cysteine contribute to the odor of garlic and onions (Jones et al., 2004). These volatiles are also important to some fruits such as durian (Weenen et al., 1996), passion fruit (Tominaga and Dubourdien, 2000) and pineapple (Umano et al., 1992). Several hydrogen sulfides were found in the headspace above fresh orange, grapefruit, tangerine, lemon, lime, tangelo and tangor juices (Shaw and Wilson, 1982). In processed mandarin (Araki and Sakakibara, 1991) and orange juice (Pérez-Cacho et al., 2007),

dimethyl sulfide was found and its odor quality was characterized as ‘sulfur’, giving undesirable smell to juice aroma.

Improvement of Citrus Aroma and Flavor

In conventional citrus breeding, breeders put a lot of efforts and time toward their breeding programs, in order to identify superior individual plants with desirable traits. Citrus breeding has been impeded by several reproductive characteristics such as long juvenility, gametophytic self- and cross-incompatibility, and nucellar embryony (Talon and Gmitter, 2008). The juvenile periods of citrus seedlings range from one to twenty years, though typically most will flower and fruit within 3 to 7 years (Talon and Gmitter, 2008). The sources of citrus genetic variation can currently arise not only from seed/bud introduction, natural mutants and sexual hybrids, but from irradiated budlines, somaclones, somatic hybrids/cybrids and molecular genetics (Gmitter et al., 2007). However, genetic improvement of aroma and flavor of mandarin fruits still highly depends on the traditional breeding strategy from sexual hybridization (pollination) to selection. Therefore, the development of molecular markers that can associate the genotype with the phenotype of interest can greatly accelerate citrus breeding.

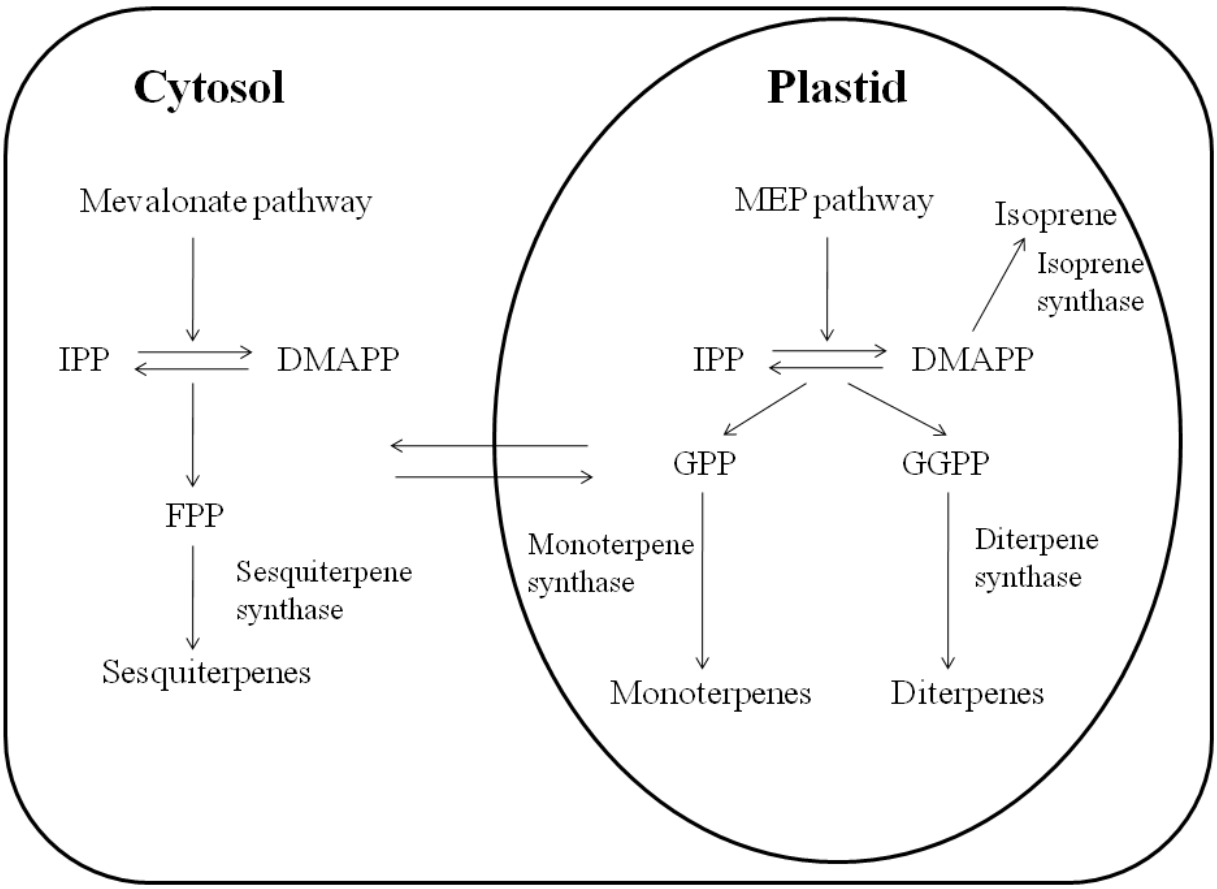

Figure 2-1. Terpenoid aroma volatiles (Reproduced with permission from Elsevier. Tholl, D. 2006. Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. Curr. Opin. Plant Biol. 9: 297-304.)

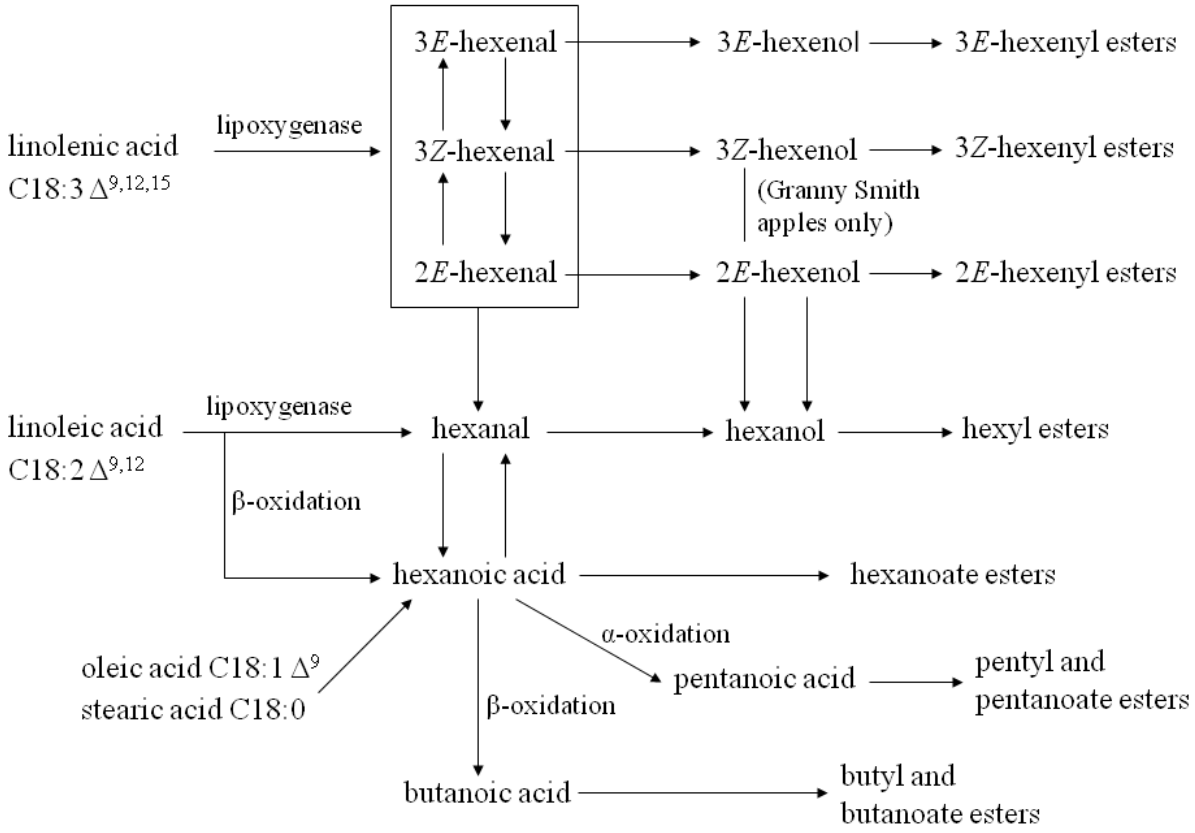

Figure 2-2. Fatty acid derived aroma volatiles (Reproduced with permission from American Chemical Society. Rowan, D. D., A. J. M., S. Fielder, and M. B. Hunt. 1999. Biosynthesis of straight-chain ester volatiles in red delicious and granny smith apples using deuterium-labeled precusors. J. Agr. Food Chem. 47: 2553-2562.)

CHAPTER 3
DISTRIBUTION OF AROMA VOLATILE COMPOUNDS IN TANGERINE HYBRIDS AND PROPOSED INHERITANCE

Introduction

Florida fresh tangerines (*Citrus reticulata* Blanco) as well as oranges (*C. sinensis* L. Osb.) and grapefruits (*C. paradisi* Macf.) are one of the largest agricultural commodities in the U.S. citrus market. While grapefruits are very sensitive to endemic citrus canker disease, tangerines are fairly tolerant and could provide an alternative to grapefruit production for the future fresh fruit market (Gottwald et al., 2002). The fresh tangerine fruit is widely consumed due to its desirable fruit quality of aroma/flavor and ease of peeling. In addition, the high nutritional content from vitamin C and carotenoids in tangerines can benefit human health. From 2007 to 2008, five and a half million 95-lb boxes of all Florida tangerines (early tangerines, 'Fallglo' and 'Sunburst'; late tangerine, 'Murcott') were produced, with the value of production of $37.8 million (U.S. Department of Agriculture, 2009). Approximately 60 % of Florida tangerines were utilized for the fresh fruit market.

Improvement in fruit quality is one of the primary goals of fresh tangerine breeding programs in Florida. The commercial tangerine fruits are currently graded by inspectors based on only fruit appearance such as color, size and damage (U.S. Department of Agriculture, 1997). Therefore, new quality standards that describe flavor attributes should be created to be used in the selection of high quality fruits. The conventional citrus breeding based on crossing and selection requires a large amount of space, and considerable time and labor during the long juvenile period of citrus trees; such efforts are rather costly to conduct. In the last decades, the advancement of plant genetics and genomics has enhanced the efficiency to improve fruit quality in breeding programs. The application of molecular markers to citrus breeding may allow breeders to select efficiently superior recombinants improved for multiple traits by conventional

breeding efforts, using marker-assisted selection (MAS) (Gmitter et al., 2007). Molecular markers associated with citrus fruit quality can be a valuable tool for genetic improvement leading to the faster release of new superior scions and rootstocks. High-value fruits would result in greater economic impact on the Florida citrus industry.

Aroma, as well as taste, color and texture, is one of the most important quality attributes of citrus fruits. So far, the aroma volatiles of the major processing orange and grapefruit cultivars have been well investigated. Over 300 aroma volatiles have been already reported from gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses on fresh orange juices (Pérez-Cacho and Rouseff, 2008). It is well known that citrus aroma components are a mixture of monoterpenes, sesquiterpenes, alcohols, aldehydes, acids, esters, ketones, etc. Although there have been several reports on aroma volatiles in tangerine essence and peel oil (Coleman and Shaw, 1972; Moshonas and Shaw, 1974; Buettner et al., 2003, Chisholm et al., 2003; Sawamura et al., 2004), very little information is available regarding those in fresh tangerine fruits (Elmaci and Altug, 2005, Pérez-López and Carbonell-Barranchina, 2006a; Kerbiriou et al., 2007; Barboni et al., 2009). The information on volatile quality and quantity can be useful to evaluate tangerine fruit quality. In addition, peel and juice volatile composition have been analyzed for classification of different citrus fruits: yuzu (*C. junos* Sieb.) cultivars (Lan-Phi et al., 2009), lemon (*C. limon* Burm.) cultivars (Allegrone et al., 2006), grapefruit hybrids (Shaw et al., 2001) and tangerine/mandarin hybrids (Kerbiriou et al., 2007; Barboni et al., 2009). The differentiation of tangerine hybrids based on their aroma profiles may lead to better understanding of genetic control of aroma production.

The main objectives of this study were to investigate aroma volatile compounds in a population of tangerine hybrids, and analyze inter-varietal relationships from volatile profiles by

using multivariate statistics, principal component analysis (PCA) and cluster analysis (CA). It was hypothesized that if similarities were present, they could be due to common genetic background (Kerbiriou et al., 2007). The study on aroma volatiles present among tangerine hybrids of diverse origins would provide fundamental information on fruit quality, maturity and development of early DNA-based MAS of interesting individuals.

Materials and Methods

Plant Materials

All tangerine hybrids were originated from the University of Florida Citrus Research and Education Center (UF-CREC) breeding program. The trees were grown under the same environmental conditions of soil, irrigation, and illumination at the CREC groves. Fruit were harvested from November 2007 until March 2008 (Table 3-1). Twelve of the 56 tangerine hybrids evaluated in 2006 to 2007 (Kerbiriou et al., 2007) were selected for re-evaluation: sample a, c and d (three seedlings issued from a 8-9 × 'Murcott' cross); sample b and g (two seedlings of a 'Robinson' × 'Fairchild' cross); sample i ('Fallglo' × 'Fairchild'); sample l (8-9); sample m (8-10); sample n (8-9 × 'Orlando'); sample p and r (two seedlings from unknown parentage); and sample y (8-9 × Val4x). Eight new hybrids and 5 named commercial cultivars as references were also included for the present study. These hybrids have various genetic backgrounds from tangerines, oranges and grapefruits (Figure 3-1) (Hodgson, 1967; Wutscher et al., 1973; Futch and Jackson, 2003a, 2003b; Jackson and Futch, 2003a, 2003b, 2003c, 2003d, 2003e). Each sample was a juice composite from approximately 50 to 60 fruits harvested from one tree. A total of 25 samples were prepared at the USDA/ARS Citrus and Subtropical Products Laboratory.

Sample Preparation

Fruit were soaked in warm water with 200 mL detergent (DECCO 241 Fruit and Vegetable Kleen, Monrovia, CA, USA) in a 16 L bucket, washed for about 30 s, and rinsed. Fruits were then sanitized in 10 L water at 30 to 35 °C using a 100 ppm peroxyacetic acid solution (Biosafe Systems, East Hartford, CT, USA) for 3 min prior to processing in the lab. Individual fruits were cut in half on sterile foil, and juiced manually with an electric juicer (Model 3183; Oster, Rye, NY, USA). The fruit were juiced carefully for 3 s, avoiding any scraping of the albedo or squeezing of the flavedo to prevent potential peel components from contaminating the juice. Most of the seeds were removed, and aliquots (2.5 mL) of tangerine juice were placed in 20 mL glass vials (Gerstel, Inc., Baltimore, MD, USA) along with saturated sodium chloride solution (2.5 mL) to help drive volatiles into the headspace and stabilize any potential enzymatic activity. 3-Hexanone (1 ppm) was also added into the vials as an internal standard. The vials were capped with magnetic crimp caps containing Teflon-coated septa and stored at -20 °C until analyzed.

Optimization of Volatile Sampling

Direct gas chromatography-olfactometry (D-GC-O) is a technique to evaluate odor from headspace solid phase microextraction (SPME) extracts (Lecanu et al., 2002). It was used to determine the optimum extraction conditions so that the odor of the headspace extract was the most representative of that of the reference tangerine juice samples. A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber (Supelco, Bellefonte, PA, USA) was used due to its superior extraction performance for orange juice (Rega et al., 2003). After a sample equilibration time of 30 min at 40 °C in a water-bath (Baxter Scientific Products, Cincinnati, OH, USA), the fiber was exposed to the juice headspace for 3, 30 or 60 min at 40 °C. The fiber was then introduced into the injector of the gas chromatography (GC) (Perkin Elmer, Autosystem XL, Waltham, MA, USA) for 3 min at 240 °C for desorption of

volatiles. The GC was equipped with a sniffing port (Olfactory Detector Port ODP 2, Gerstel), and a 15-cm deactivated silica column (0.32-mm internal diameter) to assess global odor without chromatographic separation (Jouquand et al., 2008). The flow rate of the carrier gas (He) was 18 $mL \cdot min^{-1}$, and the oven temperature was kept at 70 °C. A similarity test was performed in triplicate for the different extracts (3, 30 and 60 min of fiber exposure times) with three panelists. Each panelist smelled the odor of the extract from the GC sniff port and then rated their similarity to the sample using a 10-cm scale ranging from 0 (far from the sample) to 10 (close to the sample). As a result, the condition of 60 min fiber exposure time generated the most representative tangerine odor and therefore was applied to further volatile analyses (Table A-1).

Headspace Sampling and GC-MS analysis

The extraction of aroma volatiles was performed by a SPME method with an MPS-2 autosampler (Gerstel). The vials were incubated at 40 °C for 30 min and then a 2-cm SPME fiber (50/30 μm DVB/CAR/PDMS) was inserted into the headspace of the sample vial and exposed for 60 min. The analytes were thermally desorbed in the GC injector (splitless mode) port for 3 min at 250 °C. The separation of volatile compounds was accomplished using an Agilent 6890 GC (Agilent Technologies, Santa Clara, CA, USA) equipped with DB-5 (60 m length, 0.25 mm i.d., 1.00 μm film thickness; J&W Scientific, Folsom, CA, USA) and DB-Wax (60 m length, 0.25 mm i.d., 0.50 μm film thickness; Agilent Technologies, Santa Clara, CA, USA) columns, coupled with a 5973N MS detector (Agilent Technologies). The column oven was programmed to increase at 4 $°C \cdot min^{-1}$ from the initial 40 °C to 230 °C, then ramped at 100 $°C \cdot min^{-1}$ to 260 °C and held for 11.70 min for a total run time of 60 min. Helium was used as carrier gas at flow rate of 1.5 $mL \cdot min^{-1}$. Inlet, ionizing source, and transfer line were kept at 250, 230, and 280 °C, respectively. Mass units were monitored from 40 to 250 *m/z* and ionized at 70 eV. Data were

collected using the ChemStation G1701 AA data system (Hewlett-Packard, Palo Alto, CA, USA). Samples were run in triplicate on a DB-5 column, with a blank run between each hybrid to assure fiber cleanness between samples. A mixture of C-5 to C-15 n-alkanes was run at the end of each day to calculate retention indices (RIs) (Goodner, 2008). Samples were also analyzed (one run per sample) on a DB-Wax column to identify potential co-eluting compounds on the DB-5 column.

Volatile Compound Identification

Volatile compounds were identified by the comparison of their retention indices (RI) and mass spectra with library entries (NIST/EPA/NIH Mass Spectral Library, version 2.0d; National Institute of Standards and Technology, Gaithersburg, MA, USA). Chemical authentic standards, when available, were run on both columns and their RIs and spectra confirmed compound identities.

Statistical Analyses

Volatile compounds were semi-quantified by calculating each peak area relative to the peak area of the internal standard. A principal component analysis (PCA) was performed to differentiate individual samples based on their volatile composition using XLSTAT software (Addinsoft, Paris, France). With the data transformed into presence/absence (1/0) of volatiles, a cluster analysis was performed to find correlations between volatile composition and sample origin (Kerbiriou et al., 2007). Clusters were formed using the unweighted pair-group average method with arithmetic mean (UPGMA) and the Kulczynski coefficient, to measure similarities between samples (XLSTAT, Addinsoft).

Results and Discussion

A total of 203 compounds were identified by GC-MS in the 25 samples (Table 3-2 and 3-3). The average number of volatiles per sample was 77, ranging from 52 to 118, and more than

77 compounds were detected in 12 samples. The volatiles were widely distributed among samples, however, only a small number of volatiles (5 to 26 volatiles) contributed to more than 90% of the total content. Many volatile compounds in Table 3-2 and 3-3 have already been reported in tangerine essence, peel oil and juice (Moshonas and Shaw; 1972, 1974, 1997; Coleman and Shaw, 1972; Minh Tu et al., 2002; Elmaci and Altug, 2005; Njoroge et al., 2005; Pérez-López et al., 2006a, 2006b; Dharmawan et al., 2007; Kerbiriou et al., 2007; Barboni et al., 2009). All samples contained 15 volatiles in common: ethanol, hexanal, α-pinene, β-myrcene, octanal, α-terpinene, p-cymene, d-limonene, terpinolene, dehydro-p-cymene, linalool, nonanal, decanal, α-terpineol and d-carvone (Table 3-2). Although the samples have various genetic backgrounds from mandarin/tangerine, orange and grapefruit (Figure 3-1), the same seven monoterpenes (Table 3-2) were detected in all samples. d-Limonene was the most abundant compound, representing 40.6 to 82.5% of the total aroma volatiles. Among 22 identified monoterpene hydrocarbons, four monoterpenes were also present in relatively high amount in most samples: β-myrcene (0.5–4.9% of relative peak area), p-cymene (0.3–7.0%), β-phellandrene (0–3.5%) and dehydro-p-cymene (0.5–9.5%). The other major compounds present in large amount in samples were valencene (0–24.9%), hexanal (0.1–14.1%) and linalool (0.5–9.5%). Conversely, fifty five volatiles were present in only one or two samples (1–8%, Table 3-3) and therefore might be more cultivar specific.

Most volatiles were classified into 6 chemical classes: monoterpenes, sesquiterpenes, aldehydes, esters, alcohols and ketones (Table 3-4 and 3-5). The number of volatiles in these classes alone accounts for 76.8% of the total (203 volatiles). In addition, terpene-derived aroma volatiles were grouped into several classes: eg., neral and geranial in aldehyde, linalool and citronellol acetates in ester, linalool and citronellol in alcohol and α- and β-ionones in ketone

(Table 3-4). Most volatiles are originated from enzymatic synthesis in terpenoid and fatty acid metabolisms, thereby playing an important role in aroma production in samples. Only a few volatiles were detected in other chemical classes: phenols, ethers, acids and epoxides (Table 3-4 and 3-5).

Principal Component Analysis (PCA)

Principal component analysis is a multivariate statistical method to reduce the dimensionality of the data sets into fewer components when there are large amounts of variables, and identify patterns of similarities and dissimilarities in data (Iezzoni and Pritts, 1991; Ringnér, 2008). The relative peak areas (volatile relative content) were semi-quantified by dividing the chromatographic peak areas of compounds with that of the internal standard. These variables were used in the Pearson's correlations, the basis of this analysis.

The first and second principal components (F1 and F2) represented 39.80% of the total variance (Figure 3-2A). The third component explained an additional 11.69% of the variance (Figure 3-2B). The plot of scores in the PCA analysis illustrates that samples q (9-4 × Blood orange4x), t ('Temple') and u ('Sanguinelli' orange) were clearly different from the others due to their volatile profile. These samples were much richer in volatiles than the average (77 volatiles), since they contained 118, 109 and 111 volatiles, respectively. According to the first two components biplot data of this PCA analysis (Figure 3-2A), many vectors representing sesquiterpenes were in the direction of the first quadrant (upper right), along with some on the fourth quadrants (lower right), and most vectors representing esters were located on the fourth quadrant (lower right) (data not shown). Therefore, sample q (9-4 × Blood4x) may be distinguished by its high number and amount of sesquiterpenes, and samples t ('Temple') and u ('Sanguinelli') by their abundance in sesquiterpenes and esters, as can be seen in Table 3-5.

Another type of PCA analysis was performed using relative peak areas of total volatiles in each 11 chemical classes (Figure 3-3). Ketones and alcohols were highly correlated with each other, as well as correlated with aldehydes. Samples u ('Sanguinelli') and t ('Temple') had high scores on PC1 (esters, other, ketones and alcohols), sample y (8-9 × Val4x) had a high score on PC2, and samples s ('Murcott') and v ('Fortune' × 'Murcott') had relatively high negative scores on PC2. In general, this graph shows that the samples on the right side, q (9-4 × Blood4x), s ('Murcott'), t ('Temple'), u ('Sanguinelli'), v ('Fortune' × 'Murcott'), w ('Ortanique') and y (8-9 × Val4x) showed high total content of volatiles except of ethers and phenols. Indeed, sample t ('Temple') produced 3.1, 6.9 and 5.3 times more sesquiterpenes, esters and ketones than the averages of all samples, respectively (Table 3-5). Sample u also contained high amounts of sesquiterpenes, esters and alcohols. On the left side of Figure 3-3, the samples with more mandarin/tangerine genetic components had less volatiles.

Cluster Analysis (CA) Based on Qualitative Volatile Composition

The main goal of cluster analysis is to assign individual samples into groups. This analysis is based on distance measures between groups of variables (clusters), being often applied with molecular marker techniques to assess genetic relationship among samples based on presence/absence of certain genes or alleles (Coletta Filho et al., 1998; Chao et al., 2004). In the present study, a dendrogram provides information on volatile qualitative differences among samples as well as relationships of their volatile composition and genetic background (Figure 3-4).

Cluster 1 (C1)

Cluster 1 grouped samples a (8-9 × 'Murcott') and b ('Robinson' × 'Fairchild') that contain less volatiles than the average of all samples. These samples are characterized by having less aldehydes (except sample b), esters and ketones (Table 3-6). The aliphatic aldehydes,

pentanal, (*E*)-2-hexenal, heptanal and (*E*)-2-heptenal were not detected in sample a (8-9 × 'Murcott'), but were in all other samples. Sample b ('Robinson' × 'Fairchild') was the only sample without any esters; esters usually impart a fruity note to orange juice (Plotto et al., 2008). On the other hand, these samples were the richest in monoterpenes among the 5 clusters both in quality (Table 3-6) and amount (Table 3- 5). Above all, the highest amounts of β-myrcene, d-limonene and γ-terpinene contribute to the distinctive volatile profile of sample a (8-9 × 'Murcott').

Since samples a (8-9 × 'Murcott') and b ('Robinson' × 'Fairchild') are grouped in the same cluster as determined by their volatile profile, it is hypothesized that they have similar genetic background. Indeed, they both have 'Clementine' as a grandparent: 8-9 is a cross between 'Clementine' and 'Minneola'. 'Robinson' and 'Fairchild' are both hybrids from 'Clementine' and 'Orlando' (Figure 3-1).

Cluster 2 (C2)

Cluster 2 grouped samples c (8-9 × 'Murcott') and l (8-9), and had less volatiles than the average samples, especially less sesquitepenes and aldehydes, like cluster 1 (Table 3-6). According to the relationship between volatile composition and parentage, sample c (8-9× 'Murcott') as well as sample a (8-9 × 'Murcott') in C1 was more similar to sample l (8-9) than s ('Murcott') in terms of qualitative volatile composition, indicating that the seed parent might be a dominant parent for its aroma volatile production. However, it is not a rule since sample d (8-9× 'Murcott') is in the same cluster 3 as its parent 'Murcott (sample s). It has been observed that some volatiles in parents are not synthesized in progenies, and conversely some new volatiles are synthesized in progenies (Kerbiriou et al., 2007; Barboni et al., 2009). In the current study, 1-octen-3-one was found in both parents (sample l, 8-9; sample s, 'Murcott'), whereas it was absent in the progenies, samples c, a and d (all are 8-9 × 'Murcott'). This volatile is the most

intense odor active aliphatic ketone, imparting a mushroom-like odor to orange juice (Pérez-Cacho and Rouseff, 2008). The content of 1-octen-3-one in sample s (‘Murcott’) was the highest and more than twice the average of all samples. Thus, its production was likely to be suppressed in the progenies. Moreover, samples c, a and d (all are 8-9 × ‘Murcott’) do not contain aroma volatiles from carotenoids, including neral and geranial, which are also synthesized from geraniol (Lewinsohn et al., 2005). Their seed parent (sample l, 8-9) contains 6-methyl-5-hepten-2-one and geranyl acetone, degraded from lycopene and ξ-carotene, respectively. In addition, β-cyclocitral, β-ionone and dihydroactinidiolide, degradation products from β-carotene, were only present in the pollen parent (sample s, ‘Murcott’). For example, β-ionone is well known as an aroma active compound, and its odor is usually described as ‘floral’ in orange juice (Hinterholzer and Schieberle, 1998; Mahattanatawee et al., 2005). This suggests that the carotenoid degradations to aroma volatiles might be regulated by that specific hybridization.

Some sesquiterpenes (α-cubebene, α-caryophyllene, α-muurolene, calamenene) were detected in the progenies, but not in their parents (sample l, 8-9; sample s, ‘Murcott’). These compounds might be inherited from the parents with additive genetic effects.

Cluster 3 (C3)

Cluster 3 grouped 12 samples equally divided into ones that contain more or less volatiles than the average of all samples (77 volatiles). Most samples share common genetic background with others in this cluster. Samples d (8-9 × ‘Murcott’), v (‘Fortune’ × ‘Murcott’) and x (8-8 × ‘Murcott’) have the common pollen parent ‘Murcott’. Also, samples j and k are siblings of the same parents, ‘Fallglo’ and ‘Fairchild’. Samples e, g and h are siblings originated from the same cross between ‘Robinson’ and ‘Fairchild’. Sample e as well as b in C1 (both are ‘Robinson’ and ‘Fairchild’) were harvested on November 16th, 2007, which had 61 and 66 volatiles, respectively. Samples g and h (both are ‘Robinson’ and ‘Fairchild’), harvested on December

14th, 2007, contained 84 and 82 volatiles, respectively. These samples are originated from different trees (siblings) of the same parents, though their volatile composition of early and late harvests was quite different, highlighting the relationship between aroma volatile production and fruit maturity.

Cluster 4 (C4)

Cluster 4 contained samples with higher than average number of volatiles except sample n (8-9 × 'Orlando'). Samples in this cluster contain a distinctly higher number of volatiles in the sesquiterpenes and esters categories (Table 3-6). Samples t (Temple') and u ('Sanguinelli'), the outliers in the PCA analysis, are grouped in this cluster due to their distinctive volatile composition, as previously mentioned. Sample n (8-9 × 'Orlando') is also characterized by its richness in esters, and samples i ('Fallglo' × 'Fairchid'), w ('Ortanique') and y (8-9 × Val4x) are characterized by their high content of sesquiterpenes and esters. With regard to samples u ('Sanguinelli'), w ('Ortanique') and y (8-9 × Val4x), the sesquiterpene valencene was the second most abundant volatile, accounting for 12.5, 24.8 and 11.9% of relative peak area, respectively (data not shown). Nootkatone, a putative derivative from valencene, was also detected in the three samples and sample t ('Temple'). This compound is a major aroma impact compound that contributes to characteristic aroma and flavor of grapefruit and pummelo (MacLeod and Buigues, 1964; Sawamura and Kuriyama, 1988). These samples are grouped in the same cluster, probably because they have some orange genetic background that brings the production of esters. Kerbiriou et al. (2007) had found a similar clustering in 2006-2007. 'Temple' and 'Ortanique' are believed to be tangors, originated in Jamaica (Hodgson; 1967; Blazquez, 1967; Jackson and Futch, 2003e). 'Sanguinelli' is a blood orange. Val4x is a tetraploid Valencia orange. Sample i ('Fallglo' × 'Fairchild') does not have a direct orange parent, however, its orange background may be originated from grandparent 'Temple' (Figure 3-1).

Cluster 5 (C5)

Cluster 5 grouped samples that are outliers based on their volatile composition. Samples p and r are themselves in a sub-cluster (Figure 3-4). The parentage of samples p and r are unknown, though they may be originated from the same parents due to their very similar volatile composition. The marked characteristics of these samples are significantly lower amounts of total volatiles and an absence of sesquiterpenes. In addition, aldehydes are rich in samples p and r, accounting for 31.0% and 33.9% of relative peak area, respectively. 2-Methyl-2-hexenal, (*E*,*Z*)-2,4-heptadienal and (*E*,*E*)-2,4-heptadienal were detected only in the three samples in this cluster. Most aldehydes present in samples have already been found in many citrus fruits (Sawamura and Kuriyama, 1988; Shaw, 1991; Shaw et al., 2000, Allegrone et al., 2006; Kerbiriou et al., 2007; Pérez-Cacho and Rouseff, 2008), and they are well known to impart 'green' and 'fatty' notes. Therefore, the high level of aldehyde content might negatively influence the overall aroma of samples p and r as well as q (9-4 × Blood4x).

As shown in Figure 3-2, Table 3-5 and Table 3-6, sample q (9-4 × Blood4x) is also clearly characterized by a high number and quantity of sesquiterpenes. Sample q (9-4 × Blood4x) contained 33 compounds of the 39 different sesquiterpenes detected in the 25 samples. This sample and sample y (8-9 × Val4x) in C4 have two-thirds chromosome complement from Blood4x (tetraploid 'Ruby' blood orange) or Val4x, respectively. While cluster 4 grouped samples that have some orange in their background, this sample is differentiated from them due to its lower amount of esters. Interestingly, although sample q (9-4 × Blood4x) contained the highest number of volatiles (118 volatiles) (Table 3-6), their amount was only slightly higher than the average of the 25 samples and similar to those of the siblings of 9-4, or 8-9 and 8-10 (all are 'Clementine'×'Minneola') (Table 3-5). Sample y (8-9 × Val4x) in C4 contained higher

number of volatiles (93) than its parent '8-9' (65) (Table 3-6), whereas the total content is similar to that of the parent (Table 3-5).

From these results, it may be deduced that samples q (9-4 × Blood4x) and y (8-9 × Val4x) can synthesize aroma volatiles from the pollen parents, though the total volatile content is regulated by the seed parents. So far, inheritance of genes involved in volatile synthesis and their expression in sexual citrus progenies are still unknown. Gancel et al. (2005) showed that leaf volatile composition of a somatic hybrid (2n = 4x = 36) between Willow leaf mandarin (*C. deliciosa* Ten.) with sweet orange was similar to that of the mandarin parent. Since the parents 9-4 and 8-9 have background from mandarin, its genetic components might play an important role in the control of aroma production.

The volatile difference among hybrids provides fundamental information for future improvement in tangerine aroma and flavor. Further research is needed to understand the relationship between aroma volatiles and sample aroma/flavor, as described in Chapter 4.

Table 3-1. List of samples and corresponding selection names or parentage, hybrid numbers, harvest dates and sample codes

Selection name or parentage	Hybrid number	Harvest date	Sample code
8-9 × 'Murcott'	1	16 Nov. 2007	a
8-9 × 'Murcott'	2	16 Nov. 2007	c
8-9 × 'Murcott'	3	16 Nov. 2007	d
'Robinson' × 'Fairchild'	1	16 Nov. 2007	b
'Robinson' × 'Fairchild'	2	16 Nov. 2007	e
'Robinson' × 'Fairchild'	3	14 Dec. 2007	g
'Robinson' × 'Fairchild'	4	14 Dec. 2007	h
'Fallglo'	–	16 Nov. 2007	f
'Fallglo' × 'Fairchild'	1	14 Dec. 2007	i
'Fallglo' × 'Fairchild'	2	14 Dec. 2007	j
'Fallglo' × 'Fairchild'	3	14 Dec. 2007	k
8-9	1	14 Dec. 2007	l
8-10	1	14 Dec. 2007	m
Unknown	–	11 Jan. 2008	o
Unknown	–	11 Jan. 2008	p
Unknown	–	11 Jan. 2008	r
8-9 × 'Orlando'	1	11 Jan. 2008	n
9-4 × Blood4x	1	11 Jan. 2008	q
'Murcott'	–	14 Feb. 2008	s
'Temple'	–	14 Feb. 2008	t
'Sanguinelli'	–	14 Feb. 2008	u
'Fortune' × 'Murcott'	1	14 Feb. 2008	v
'Ortanique'	–	25 Mar. 2008	w
8-8 × 'Murcott'	1	25 Mar. 2008	x
8-9 × VAL4x	1	25 Mar. 2008	y

Each sample is a juice composite from fruits harvested from one tree of individual hybrid or commercial cultivar.

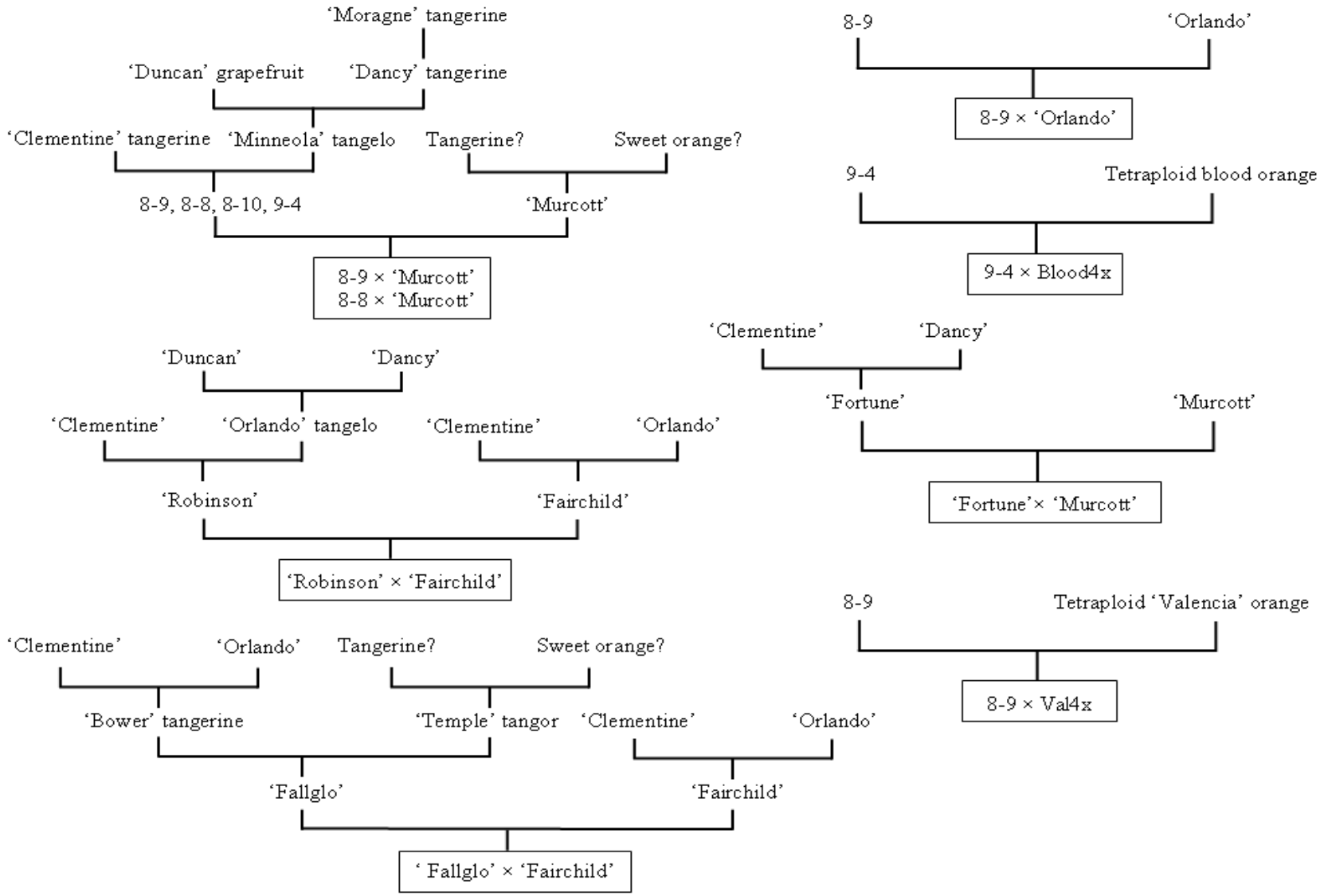

Figure 3-1. Pedigree of tangerine hybrids.

Table 3-2. List of aroma volatiles detected by GC-MS among samples and identified by linear retention index (LRI) on DB-5, DB-wax column and confirmed with chemical standards. Volatiles are listed according to their frequency of appearance, 48% to 100%, in samples

48% to 71%[a]			72% to 95%[b]			96% to 100%[c]		
	LRI			LRI			LRI	
	DB-5	DB-wax		DB-5	DB-wax		DB-5	DB-wax
(*E*)-2-Pentenal[d]	755	1167	Acetaldehyde	469	659	Ethanol[d]	489	929
Benzaldehyde[d]	985	1604	(*E*)-2-Decenal	1262	1868	Hexanal[d]	805	1123
Allo-ocimene	1132	1384	α-Phellandrene[d]	1025	1195	α-Pinene[d]	956	1061
α-Selinene	1545	2228	γ-Terpinene[d]	1073	1253	β-Myrcene[d]	999	1189
α-Cubebene[d]	1363	1496	Perillaldehyde[d]	1293	2447	Octanal[d]	1013	1283
α-Caryophyllene[d]	1499	2013	Cadinene	1557	2309	α-Terpinene[d]	1035	1239
γ-Selinene	1530		Propanal[d]	511	747	p-Cymene[d]	1042	1272
(-)-α-Panasinsen	1577	2393	Terpinene-4-ol[d]	1196	1765	d-Limonene[d]	1049	1223
β-Cyclocitral	1234	1854	p-Menth-1-en-9-al	1231	1839	Terpinolene[d]	1100	1282
2-Undecenal[d]	1366	2255	Copaene[d]	1401	1551	Dehydro-p-cymene[d]	1105	1446
(*Z*)-p-Mentha-2,8-dien-1-ol	1150	1933	Caryophyllene[d]	1458	1801	Linalool[d]	1105	1600
β-Terpineol[d]	1162	1796	1-Octen-3-one	988		Nonanal[d]	1109	1384
Octyl acetate[d]	1202	1491	6-Methyl-5-hepten-2-one	994	1316	Decanal[d]	1205	1538
β-Elemene	1409	1754	p-Menth-1-en-9-al isomer	1234		α-Terpineol[d]	1208	2026
Calamenene[d]	1566	2631	1-Penten-3-one[d]	676	1057	d-Carvone[d]	1257	2225
α-Calacorene[d]	1593	3117	(*E*)-2-Nonenal[d]	1163	1615	Acetone	509	775
1 Unknown monoterpene[e]			Geranyl acetone[d]	1454	2680	Ethyl acetate[d]	599	870
2 Unknown sesquiterpenes[f]			Valencene[d]	1539	2191	Pentanal[d]	690	1005
4 Unknowns[g]			β-Ionone	1506	3187	(*E*)-2-Hexenal[d]	864	1233
			2 Unknown monoterpenes[e]			Heptanal[d]	913	1208
			2 Unknowns[g]			(*E*)-2-Heptenal[d]	970	1315
						β-Phellandrene[d]	1052	1229
						(*E*)-2-Octenal[d]	1067	1433
						1,3,8-p-Menthatriene	1127	1392

Table 3-2. Continued

48% to 71%[a]			72% to 95%[b]			96% to 100%[c]		
	LRI			LRI			LRI	
	DB-5	DB-wax		DB-5	DB-wax		DB-5	DB-wax
						(+/-)-4-Acetyl-1 methylcyclohexene	1144	1670
						Dihydrocarvone[d]	1212	1819

[a] Volatiles detected in 12 to 17 out of 25 samples
[b] Volatiles detected in 18 to 23 out of 25 samples
[c] Volatiles detected in 24 to 25 samples
[d] Volatiles confirmed with chemical standards
[e] Unknown monoterpenes unidentified by GC-MS
[f] Unknown sesquiterpenes unidentified by GC-MS
[g] Unknown volatiles unidentified by GC-MS except monoterpenes and sesquiterpenes

Table 3-3. List of aroma volatiles detected by GC-MS among samples and identified by linear retention index (LRI) on DB-5, DB-wax column and confirmed with chemical standards. Volatiles are listed according to their frequency of appearance, 1 % to 47%, in samples

1% to 11%[a]			12% to 23%[b]			24% to 47%[c]		
	LRI			LRI			LRI	
	DB-5	DB-wax		DB-5	DB-wax		DB-5	DB-wax
2-Methyl-2-propanol	530		Ethyl propanoate[d]	699	974	Butanal[d]	583	861
2-Butanone	583	894	Ethyl butanoate[d]	798	1075	Neral[d]	1241	2004
(*E*)-2-Butenal	639	1084	Methyl hexanoate[d]	929	1208	Geranial[d]	1268	2005
(*Z*)-3-Hexenal	799		Ethyl hexanoate[d]	1001	1240	Methyl acetate	531	784
1-Octen-3-ol	987	1436	Ethyl 3-hydroxyhexanoate[d]	1128	1967	3-Pentanone	685	1002
p-Mentha-3,8-diene	1084	1271	Ethyl octanoate[d]	1188	1427	4-Heptanone	878	1162
Octanoic Acid	1152		α-Muurolene	1534	2160	4-Methyl-hexanal	889	
Camphor[d]	1171	2076	Caryophyllene oxide[d]	1658	3584	Styrene[d]	911	1262
Bornyl acetate	1294		Nootkatone[d]	1881	7433	α-Thujene[d]	942	1063
(*E*,*E*)-2,4-Decadienal[d]	1320		Dimethyl sulfide	529		1,8-Cineole[d]	1054	1228
(*Z*)-Carvyl acetate[d]	1332		Methyl butanoate[d]	712	1004	Dodecanal	1411	2076
δ-Elemene[d]	1351		Ethyl 2-methylbutanoate[d]	856	1054	β-Ionone epoxide	1511	3500
(S)-Perillyl acetate	1442		Sabinene[d]	991	1191	Undecanal[d]	1305	1782
α-Guaiene[d]	1459	2212	Hexyl ethanoate	1018	1266	Citronellol acetate[d]	1341	1913
α-Farnesene	1518	2200	3-Carene[d]	1031	1187	β-Pinene[d]	1003	1149
(*E*,*E*)-2,4-Hexadienal[d]	923	1397	(*E*)-Ocimene	1056	1241	Thymol[d]	1289	4594
Ethyl tiglate	944	1245	β-Cubebene	1463	1788	Neryl acetate[d]	1350	2126
1-Methylbutyl butanoate	1025		1-Penten-3-ol[d]	671	1150	Geranyl acetate[d]	1370	2261
Methyl octanoate[d]	1120	1373	2-Pentanone	674	1005	Selina-3,7(11)-diene	1600	2137
Hexyl butanoate[d]	1188		2,3-Pentanedione[d]	683		Juniper camphor	1741	5244
(*Z*)-Carveol[d]	1229	2536	3-Methyl-butanol[d]	729	1164	2 Unknown sesquiterpenes[f]		
Citronellol[d]	1236	2186	Ethyl 2-butenoate	848	1194	7 Unknowns[g]		
(*E*)-Carveol[d]	1243	2664	2-Methyl-2-hexenal	884				
Nonanoic acid	1248		Hexanoic acid[d]	965				

Table 3-3. Continued

1% to 11%[a]			12% to 23%[b]			24% to 47%[c]		
	LRI			LRI			LRI	
	DB-5	DB-wax		DB-5	DB-wax		DB-5	DB-wax
Nonyl acetate[d]	1300	1689	2,3-Octanedione	991				
Methyl geranate	1315		(*E*,*Z*)-2,4-Heptadienal	1007	1517			
exo-2-Hydroxycineole acetate	1343		(*E*,*E*)-2,4-Heptadienal[d]	1022	1532			
α-Terpinyl acetate[d]	1350	2063	Thymol methyl ether	1231	1766			
Ylangene	1396		Linalool acetate[d]	1242	1638			
Decyl acetate[d]	1403	1973	α-Ionone	1439	2735			
β-Farnesene	1460	1918	γ-Elemene	1455	1890			
1 Unknown monoterpene[e]			2,6-Bis(1,1-dimethylethyl)-phenol	1523	5439			
11 Unknown sesquitepenes[f]			Nerolidol[d]	1585	3646			
12 Unknowns[g]			Dihydroactinidiolide	1592				
			3 Unknown sesquitepenes[f]					
			10 Unknowns[g]					

[a] Volatiles detected in 1 to 2 out of 25 samples
[b] Volatiles detected in 3 to 5 out of 25 samples
[c] Volatiles detected in 6 to 11 out of 25 samples
[d] Volatiles confirmed with chemical standards
[e] Unknown monoterpene unidentified by GC-MS
[f] Unknown sesquiterpenes unidentified by GC-MS
[g] Unknown volatiles unidentified by GC-MS except monoterpene and sesquiterpenes

Table 3-4. Tangerine aroma volatiles among 11 chemical classes

Monoterpenes	Sesquiterpenes	Aldehydes	Esters	Alcohols
α-Thujene	δ-Elemene	Acetaldehyde	Methyl acetate	Ethanol
α-Pinene	α-Cubebene	Propanal	Ethyl acetate	2-Methyl-2-propanol
Sabinene	Ylangene	Butanal	Ethyl propanoate	1-Penten-3-ol
β-Myrcene	Copaene	(*E*)-2-Butenal	Methyl butanoate	3-Methylbutanol
β-Pinene	β-Elemene	Pentanal	Ethyl butanoate	1-Octen-3-ol
α-Phellandrene	γ-Elemene	(*E*)-2-Pentenal	Ethyl 2-butenoate	Linalool
3-Carene	Caryophyllene	(*Z*)-3-Hexenal	Ethyl 2-methylbutanoate	cis-p-Mentha-2,8-dien-1-ol
α-Terpinene	β-Farnesene	Hexanal	Methyl hexanoate	β-Terpineol
p-Cymene	α-Guaiene	(*E*)-2-Hexenal	Ethyl tiglate	Terpinene-4-ol
d-Limonene	β-Cubebene	2-Methyl-2-hexenal	Ethyl hexanoate	α-Terpineol
β-Phellandrene	α-Caryophyllene	4-Methyl-hexanal	Hexyl ethanoate	(*Z*)-Carveol
(*E*)-Ocimene	α-Farnesene	Heptanal	1-Methyl butyl butanoate	Citronellol
γ-Terpinene	γ-Selinene	(*E*,*E*)-2,4-Hexadienal	Methyl octanoate	(*E*)-Carveol
p-Mentha-3,8-diene	α-Muurolene	(*E*)-2-Heptenal	Ethyl 3-hydroxyhexanoate	Nerolidol
Terpinolene	Valencene	Benzaldehyde	Hexyl butanoate	Juniper camphor
Dehydro-p-cymene	α-Selinene	(*E*,*Z*)-2,4-Heptadienal	Ethyl octanoate	
1,3,8-p-Menthatriene	Cadinene	Octanal	Octyl acetate	
Allo-ocimene	Calamenene	(*E*,*E*)-2,4-Heptadienal	Linalool acetate	
4 Unknown monoterpenes	(-)-α-Panasinsen	(*E*)-2-Octenal	Bornyl acetate	
	α-Calacorene	Nonanal	Nonyl acetate	
	Selina-3,7(11)-diene	(*E*)-2-Nonenal	Methyl geranate	
	18 Unknown sesquiterpenes	Decanal	(*Z*)-Carvyl acetate	
		p-Menth-1-en-9-al	Citronellol acetate	
		p-Menth-1-en-9-al isomer	Exo-2-hydroxycineole acetate	
		β-Cyclocitral	Neryl acetate	
		Neral	α-Terpinyl acetate	
		(*E*)-2-Decenal	Geranyl acetate	
		Geranial	Decyl acetate	
		Perillaldehyde	(S)-Perillyl acetate	
		Undecanal		
		(*E*,*E*)-2,4-Decadienal		
		2-Undecenal		
		Dodecanal		

Table 3-4. Continued

Ketones	Phenols	Ethers	Acids	Epoxides	Others
Acetone	Thymol	1,8-Cineole	Hexanoic acid	β-Ionone epoxide	Dimethyl sulfide
2-Butanone	2,6-Bis(1,1-dimethylethyl)-phenol	Thymol methyl ether	Octanoic Acid	Caryophyllene oxide	Styrene
1-Penten-3-one			Nonanoic acid		(+/-)-4-Acetyl-1-methylcyclohexene
2-Pentanone					35 Unknowns
2,3-Pentanedione					
3-Pentanone					
4-Heptanone					
1-Octen-3-one					
2,3-Octanedione					
6-Methyl-5-hepten-2-one					
Camphor					
Dihydrocarvone					
d-Carvone					
α-Ionone					
Geranyl acetone					
β-Ionone					
Dihydroactinidiolide					
Nootkatone					

Table 3-5. Amount (relative peak area) of aroma volatiles arranged by 11 chemical classes in 25 samples. M='Murcott'; R='Robinson'; FC='Fairchild'; FG='Fallglo'; F='Fortune'; O='Orlando'; T= 'Temple'; SANG= 'Sanguinelli'; ORT='Ortanique'

hybrid	sample code	Monoterpenes	Sesquiterpenes	Aldehydes	Esters	Alcohols	Ketones	Phenols	Ethers	Acids	Epoxides	Others	Total
8-9 × M	a	27.58	0.09	0.61	0.01	0.80	0.02	0.002	0	0	0	0.01	29.13
R × FC	b	16.05	0.18	0.42	0	0.87	0.20	0	0	0	0	0.03	17.76
8-9 × M	c	9.04	0.08	0.41	0.01	0.48	0.12	0.015	0.198	0	0	0.02	10.39
8-9 × M	d	13.74	0.05	0.50	0.03	0.37	0.18	0	0.130	0	0	0.09	15.09
R × FC	e	10.36	0.03	0.83	0.06	0.36	0.25	0	0.114	0	0	0.11	12.12
FG	f	3.69	0.46	0.78	0.03	0.27	0.13	0	0	0	0.005	0.06	5.43
R × FC	g	6.22	0.66	0.80	0.03	0.47	0.16	0	0.08	0	0.004	0.06	8.49
R × FC	h	10.61	0.05	0.96	0.05	0.74	0.36	0	0.17	0	0.003	0.13	13.08
FG × FC	i	17.01	1.73	0.46	0.24	0.73	0.19	0	0	0	0	0.11	20.46
FG × FC	j	7.76	0.04	0.59	0.00	0.66	0.19	0	0	0	0.005	0.05	9.30
FG × FC	k	4.88	0.67	0.71	0.01	0.46	0.14	0	0.105	0	0.006	0.05	7.03
8-9	l	10.03	0.11	0.32	0.05	0.63	0.04	0.064	0.003	0	0	0.15	11.40
8-10	m	5.88	2.45	0.72	0.02	0.76	0.07	0.043	0.024	0	0	0.10	10.05
8-9 × O	n	8.55	0.32	0.43	0.23	0.42	0.09	0	0.157	0	0	0.03	10.22
Unknown	o	5.64	0.13	0.98	0.01	0.31	0.22	0	0.068	0.004	0	0.06	7.43
Unknown	p	1.75	0	0.93	0.03	0.10	0.14	0	0	0	0.003	0.03	2.98
9-4 × Blood4x	q	7.62	3.99	1.14	0.02	0.49	0.35	0	0	0.003	0	0.34	13.94
Unknown	r	1.80	0	1.11	0.03	0.11	0.18	0	0	0	0.005	0.04	3.27
M	s	11.78	0.03	1.57	0.07	0.65	0.48	0	0	0	0.003	0.12	14.69
T	t	19.44	3.30	2.03	1.32	3.43	0.48	0	0	0	0	0.71	30.70
SANG	u	19.05	5.56	0.99	1.11	1.35	0.63	0	0	0	0	0.35	29.03
F × M	v	17.63	0.03	1.07	0.35	0.72	0.35	0	0	0.003	0	0.11	20.26
ORT	w	8.35	2.13	0.65	0.44	0.27	0.37	0	0	0	0	0.15	12.36
8-8 × M	x	4.95	0.03	0.73	0.04	0.18	0.10	0.030	0	0.003	0.002	0.05	6.10
8-9 × Val4x	y	6.12	4.31	0.58	0.59	0.47	0.24	0.022	0.044	0.051	0	0.33	12.76

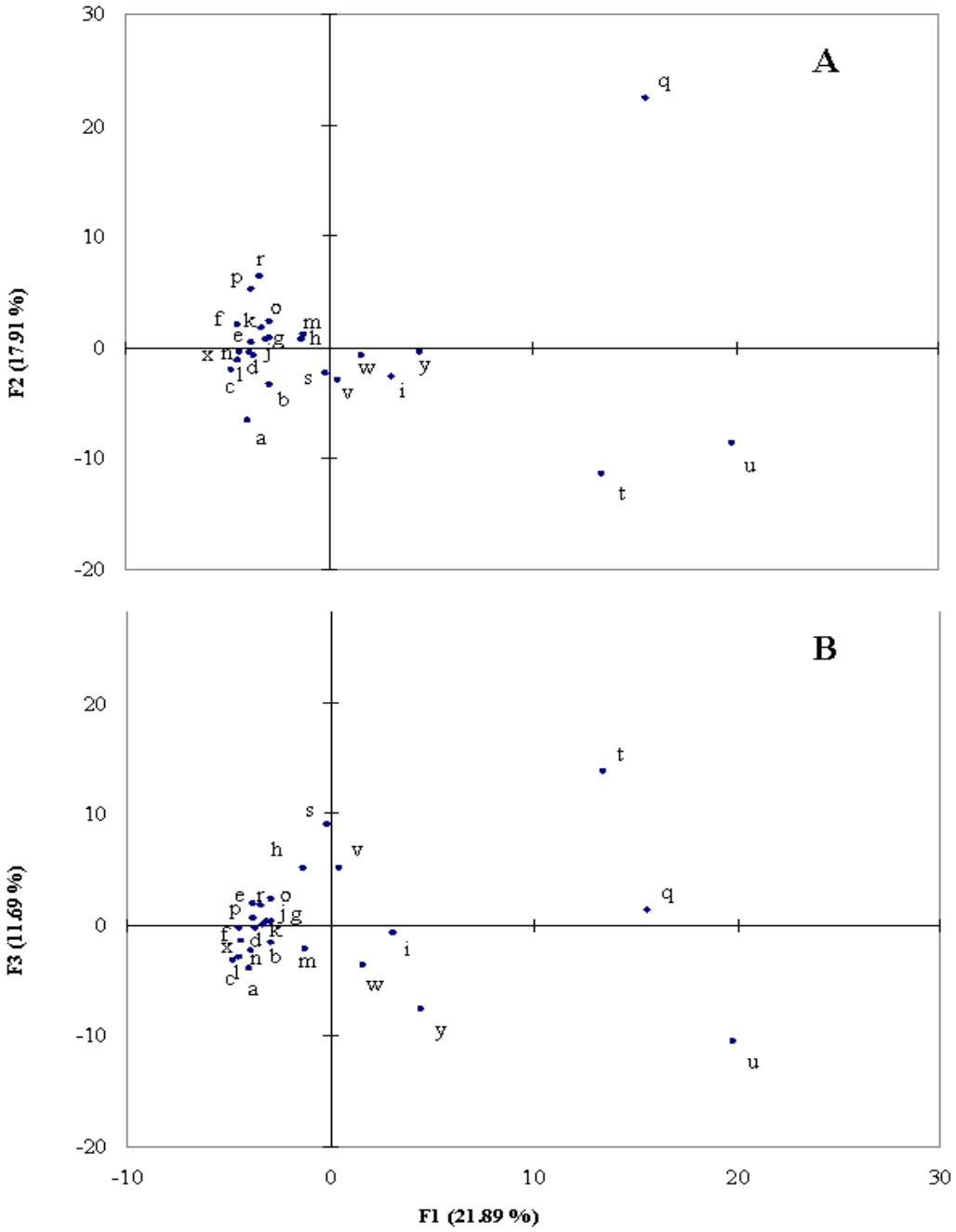

Figure 3-2. Principal component analysis by using volatile relative peak areas among samples. Each percent of variance explained by the three factors is shown in parenthesis. Letters refer to sample codes (Table 3-1).

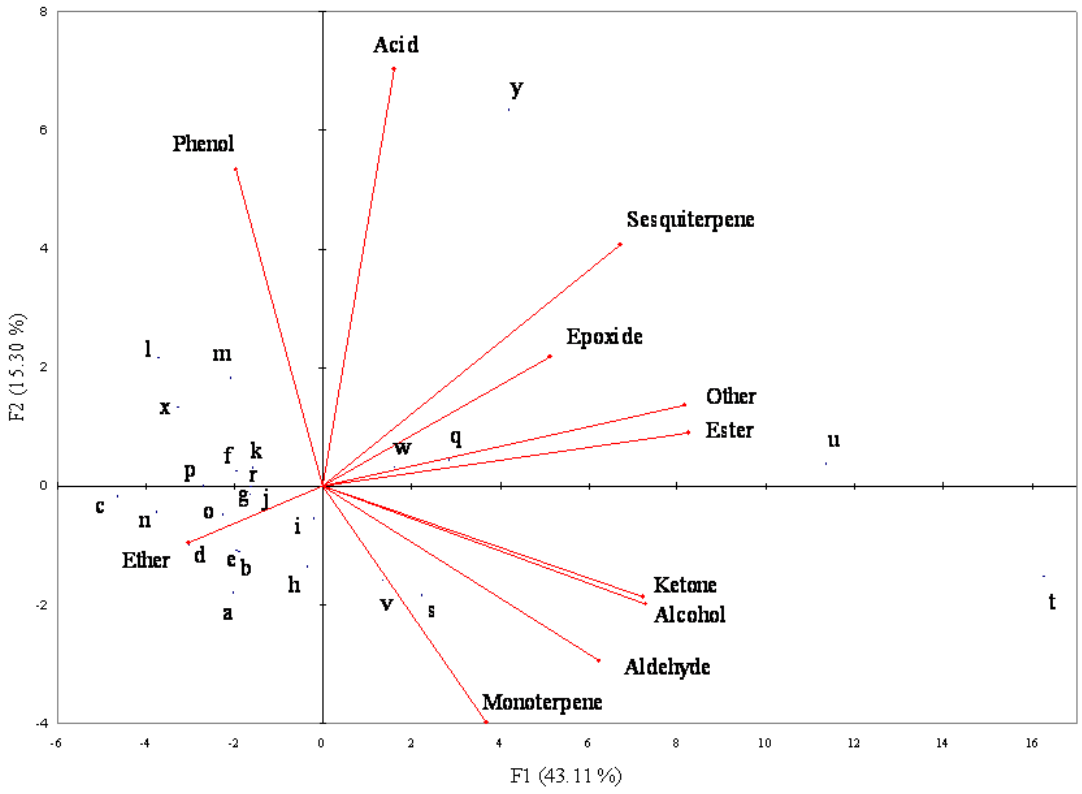

Figure 3-3. Principal component analysis by using volatile peak areas of classified compound categories among samples. Letters refer to sample codes (Table 3-1).

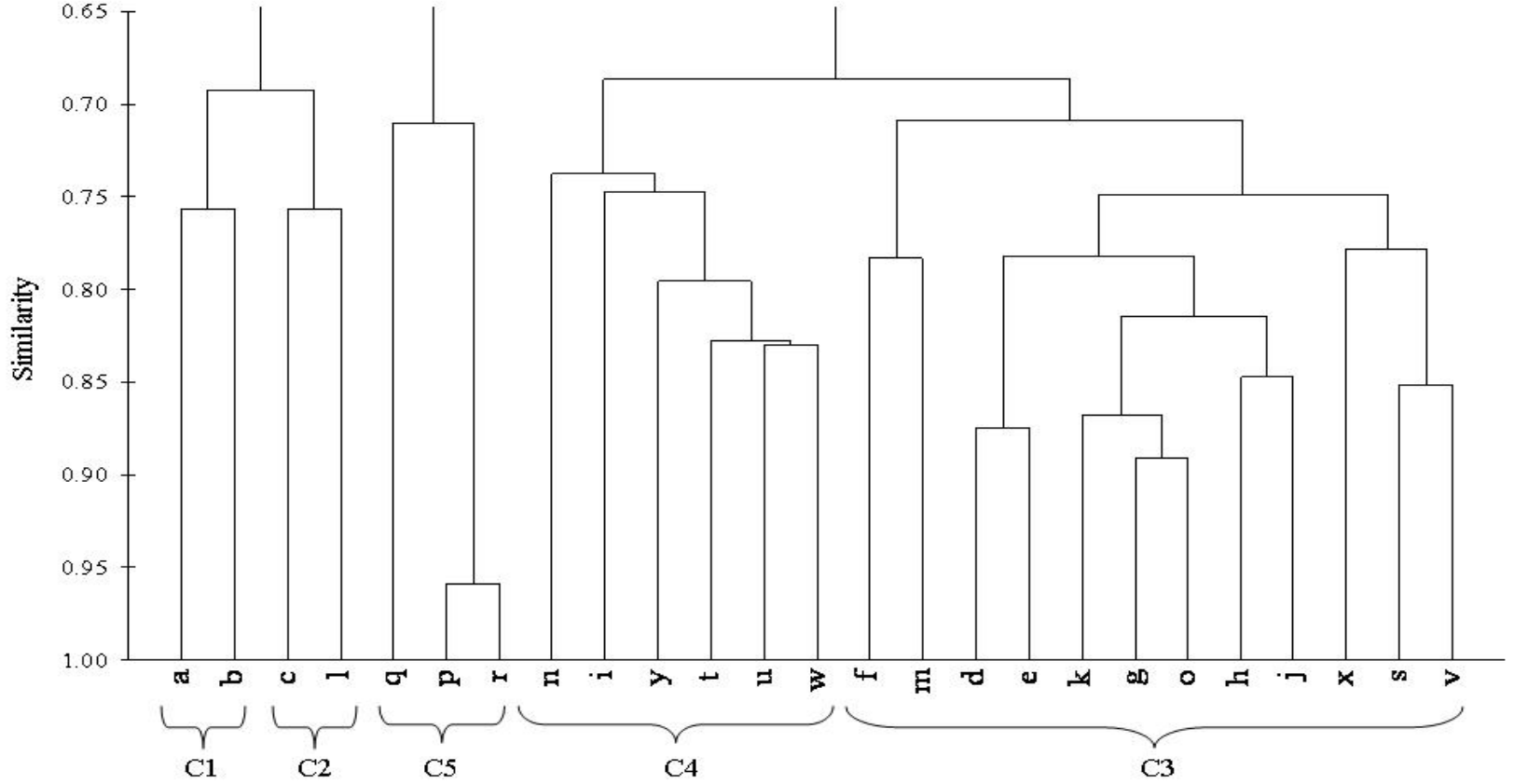

Figure 3-4. Cluster analysis by using volatile presence and absence among samples. Letters indicate sample codes (Table 3-1).

Table 3-6. Samples in five clusters formed by cluster analysis based on presence and absence of volatiles, and their number of volatiles in classified compounds. M='Murcott'; R='Robinson'; FC='Fairchild'; FG='Fallglo'; F='Fortune'; O='Orlando'; T= 'Temple'; SANG= 'Sanguinelli'; ORT='Ortanique'

Cluster no.	Sample code	Sample name	Monoterpenes	Sesquiterpenes	Aldehydes	Esters	Alcohols	Ketones	Phenols	Ethers	Acids	Epoxides	Others	Total
C1	a	8-9 × M	18	10	9	2	5	4	1	0	0	0	3	52
	b	R × FC	19	11	17	0	9	5	0	0	0	0	5	66
C2	c	8-9 × M	17	7	14	2	6	6	2	2	0	0	6	62
	l	8-9	16	8	14	2	6	8	2	1	0	0	8	65
C3	f	FG	13	8	16	2	3	8	0	0	0	1	7	58
	m	8-10	18	17	21	3	6	7	1	1	0	0	15	89
	d	8-9 × M	13	8	16	2	4	4	0	1	0	0	10	58
	e	R × FC	14	5	17	2	5	8	0	1	0	0	9	61
	k	FG × FC	14	11	21	1	5	8	0	1	0	2	9	72
	g	R × FC	14	14	23	5	6	9	0	1	0	1	12	85
	o	Unknown	14	12	24	2	5	9	0	1	1	0	12	80
	h	R × FC	15	8	26	3	6	8	0	1	0	1	14	82
	j	FG × FC	16	8	22	1	5	9	0	0	0	1	8	70
	x	8-8 × M	14	6	20	2	5	11	2	0	1	1	6	68
	s	M	15	4	26	5	7	12	0	0	0	1	12	82
	v	F × M	15	5	24	9	7	10	0	0	1	0	9	80
C4	n	8-9 × O	14	13	16	10	4	6	0	1	0	0	5	69
	i	FG × FC	16	20	19	11	5	8	0	0	0	0	11	90
	y	8-9 × Val 4x	13	19	20	9	6	11	1	1	2	1	10	93
	t	T	14	19	24	17	7	12	0	0	0	1	15	109
	u	SANG	17	23	18	20	8	11	0	0	0	1	13	111
	w	ORT	14	20	19	10	6	12	0	0	0	1	13	95
C5	q	9-4 × Blood 4x	14	33	28	4	7	9	0	0	1	0	22	118
	p	Unknown	10	0	26	2	5	9	0	0	0	1	8	61
	r	Unknown	8	0	25	2	4	9	0	0	0	1	7	56

CHAPTER 4
CHARACTERIZATION OF AROMA VOLATILES IN TANGERINE HYBRIDS BY GAS CHROMATOGRAPHY-OLFACTOMETRY

Introduction

Tangerine is one of the most popular citrus consumed as fresh fruit due to its ease of peeling, delicate and pleasant flavor. Aroma volatiles, as well as sugars and acids, are critical factors to evaluate fruit quality and contribute to the organoleptic quality of fresh tangerine fruit. Since high quality citrus fruit can lead to greater economic return to the industry, improvement in fruit aroma and flavor has been one of the primary goals of fresh fruit breeding programs. Citrus breeders have selected superior genotypes from the segregating populations and released some of them as commercial cultivars, such as 'Fallglo' and 'Sunburst' tangerines (Jackson and Futch, 2003b; Futch and Jackson, 2003c). However, there are no available fruit quality criteria that address tangerine aroma and flavor in Florida-grown fruit that enter commercial channels (U.S. Department of Agriculture, 1997), so the success of breeding programs highly depends on the ability and long experience of the breeders. The evaluation of tangerine aroma profile as well as their chemical composition has become an important research objective in the breeding program, facilitating the process of screening high quality fruit and selection of interesting hybrids with desirable aroma and flavor.

The sensation of smell is triggered by aroma volatiles entering the nostrils, mouths and respiratory system (van Ruth, 2001). The improvement of analytical instruments and techniques has led to a large number of volatiles reported in fruits and vegetables. So far, over 7,000 aroma volatiles have been identified in food (Zellner et al., 2008). However, it is indicated that only a small fraction of many volatiles present in food make a direct contribution to the odor. Gas chromatography-olfactometry (GC-O) is a valuable tool to determine aroma activity using the human nose as a detector. Aroma volatiles extracted from samples are separated by the GC

column and eluted from the sniffing port, from which the panelists can smell and characterize aroma active compounds. Combining this instrumental and sensory analysis, several techniques have been proposed: aroma extraction dilution analysis (AEDA) (Ulrich and Grosh, 1987), CharmAnalysis (Acree et al., 1984) and detection frequency method (Linssen et al., 1993). McDaniel et al. (1990) developed the time intensity (Osme) method to directly measure perceived odor intensity by trained panelists. While AEDA and CharmAnalysis are based on olfactory thresholds, Osme is the only method that considers Steven's law of psychophysics which states that the response to an odor stimulus with increasing concentration follows a sigmoidal curve (Stevens, 1957). This method has been applied to several aroma studies, among which studies on apples (Plotto et al., 2000), cashew apples (Garruti et al., 2003), grapefruit oil (Lin and Rouseff, 2001), and unpasteurized and excessively heated orange juice (Bazemore et al., 1999).

Tangerine aroma is due to a complex combination of several volatile compounds in the proper proportions such as hydrocarbons, aldehydes, esters, alcohols and ketones (Shaw, 1991). Among more than 300 volatile compounds reported from GC-MS studies in fresh orange juice, less than 25 appear to have significant odor activity at levels found (Pérez-Cacho and Rouseff, 2008). Unfortunately, there is very little information available on aroma volatiles and their odor activity in fresh tangerines. So far, GC-O has been applied to characterize aroma volatiles in only a few tangerine peel oils: 'Ponkan' (*C. reticulata*) (Sawamura et al., 2004) and 'Clementine' peel oil (Buettner et al., 2003; Chisholm et al., 2003). Elmaci and Altug (2005) detected 26 volatile compounds in three mandarin cultivars (Satsuma, Bodrum, Clementine) using dynamic headspace analysis and a sensory descriptive panel. Based on the volatile quantitative data, they concluded the key aroma impact compounds were limonene, γ-terpinene, p-cymene, myrcene, α-

pinene, β-pinene, and α-terpinolene in all samples. However, their results do not give information on important aroma active compounds, without consideration of odor threshold. With volatile identification and quantification by GC-MS, the GC-O technique can play an important role for assessing key aroma compounds and later developing molecular markers associated with their synthesis and sensory attributes.

In the previous chapter, volatiles were analyzed in 25 tangerine hybrids or commercial cultivars by GC-MS. This chapter describes the characterization of aroma volatiles in five selected tangerine hybrids using GC-O.

Materials and Methods

Sample Preparation

Based on GC-MS analysis (Figure 3-4) and a trained taste panel (data not shown), five samples were selected for GC-O analysis due to their unique and diverse aromas: sample l (8-9), m (8-10), p (Unknown), v ('Fortune' × 'Murcott') and y (8-9 × Val4x). Fruit received from the field were juiced on the same day and aliquots were frozen at -20 °C. Juicing was done to carefully avoid incorporating any peel oil into the juice. Aliquots of frozen juice were thawed in water, and a volume of 2.5 mL juice was introduced into 20 mL glass vials (Gerstel). Saturated aqueous sodium chloride (2.5 mL) was also added to help drive volatiles into the headspace and stabilize any potential enzymatic activity. The vials were capped with magnetic crimp caps containing Teflon-coated septa and stored at -20 °C until analyzed, within 3 weeks.

Gas Chromatography-Olfactometry

Each sample was equilibrated in a water-bath (Baxter Scientific Products) for 30 min at 40°C. A 2-cm SPME fiber (50/30 μm DVB/Carboxen/PDMS; Supelco) was then exposed to the headspace for 60 min at 40 °C. After exposure, the SPME fiber was inserted into the injector of a gas chromatography (Perkin Elmer) to desorb the extract for 5 min at 250 °C. The GC was

configured with a flame ionization detector (FID) and an olfactory detection port (Gerstel). An HP-5 capillary column (30 m length, 0.32 mm i.d., 0.25 μm film thickness; Agilent Technologies) was installed to separate each volatile compound. The oven temperature was 40 °C for 2 min (0 to 2 min run time), increased to 180 °C at 6 °C·min^{-1} (2 to 25.33 min), then to 250 °C at 10 °C·min^{-1} (25.33 to 32.33 min) and then held for 7 min (32.33 to 39.33 min). The flow rate of the carrier gas (He) was 1.75 mL·min^{-1}. The column effluent was split (3:1) between the sniffing port and the FID, and the sniffing port was connected to a humidified air make-up (8.9 mL·min^{-1}). Linear retention indices (RI) of volatile compounds were calculated using a series of n-alkanes (C-5 to C-15, C-17 and C-18) that was run under the same chromatographic conditions.

Osme Analysis

Training sessions were conducted to familiarize the panelists (1 male and 2 females) with the optimum positioning, time intensity device and verbal descriptors. Test samples were analyzed after panelists demonstrated reliable consistency and reproducibility. Panelists adjusted their seating positions to evaluate comfortably the GC effluents during 30 min sessions. To simultaneously record a chromatogram and an olfactogram, a data acquisition system model NI USB-6210 and a computer program written in LabVIEW 8.5 (National Instruments, Austin, TX, USA) were used to interface the GC-O to the computer and the panelist. Every 200 ms, the program simultaneously collects graphs and saves voltage data from the GC and aroma intensity. Panelists actuate a large slide bar on the front panel (computer screen) with a mouse to conveniently report aroma intensity. The 10-point intensity scale (0 = no aroma perceived, 10 = extremely strong) was used. Aroma descriptors were manually written on a notebook. Each

sample was evaluated three times per panelist, each replicate representing a different GC run. The samples were run in a random order of presentation to avoid introducing bias into the results.

The criteria to develop a consensus was: 1) first identify the aroma active peaks that each panelist detected in two out of three replications, 2) and then compare the peaks of 3 panelists that met in the criteria, 3) select the peaks that two out of three panelists detected and 4) calculate the average retention times, RIs and maximum odor intensities (Imax) of three panelists (Bazemore et al., 1999). Volatile compounds were identified by their RIs from FID and GC-MS analysis. Their aroma descriptors were also compared with published information to confirm the compound identities.

Statistical Analysis

Data were analyzed by using the following linear model:

$$X_{ijkl} = \mu + \alpha_i + \beta_j + \alpha_i\beta_j + \gamma_{j(k)} + \varepsilon_{ijkl}$$

where X_{ijkl} is the lth perceived intensity of a compound of the jth hybrid evaluated by the ith panelist in the kth replicate, μ is the overall mean intensity, α_i is the effect of the ith panelist, β_j is the effect of the jth hybrid, $\alpha_i\beta_j$ is the effect of the interaction between the ith panelist and the jth hybrid, $\gamma_{j(k)}$ is the effect of the kth replicate nested within the jth hybrid, and ε_{ijk} is the random residuals (Lea et al., 1997). Panelist and hybrid are considered as fixed effects, and all other terms are considered as random effects. Replicates were nested within hybrids, because panelists evaluated a different vial from the same juice in each replicate. Unlike most sensory data analysis, each panelist is considered as the fixed effect in this study. The reason is that panelists are calibrated like an instrument and do not represent a random section of the population. Statistical analysis was performed by using PROC GLM in SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC). Separation of means was performed with the Fisher's least significant difference (LSD) test with $\alpha < 0.05$.

Results and Discussion

Consensus of Tangerine Aroma Active Compounds

When analyzed by GC-MS, the number of volatiles in 8-9 (sample l), 8-10 (sample m), Unknown (sample p), 'Fortune' × 'Murcott' (sample v) and 8-9 × Val4x (sample y) was 65, 89, 61, 80 and 93, respectively, with a total of 150 different volatiles (Table 3-6). When the same samples were analyzed by GC-O, 119 aroma active peaks were detected, though many generated peaks had low intensity and inconsistent detection among three panelists and replications per sample. Therefore, a consensus among all three replications and three panelists was built, with 49 aroma active compounds that were detected at least in two replications out of three for each panelist, and by at least two of the three panelists (Table 4-1). According to this consensus, eight volatiles showed a strong aroma activity in most samples: hexanal, ethyl 2-methylbutanoate, unknown (No. 9), 1-octen-3-one, β-myrcene, 1,8-cineole, linalool and (*E*,*E*)-2,4-nonadienal (Table 4-1). Their average intensity among the five samples was 5.0 to 7.4 on a 10 point scale, so they may largely contribute to the overall aroma. Moreover, 12 compounds had relatively high aroma intensity greater than 5 in a few samples: ethyl butanoate, heptanal, octanal, β-phellandrene, γ-terpinene, unknown (No. 27 and 30), camphor, d-carvone, (*E*)-2-decenal, 2-undecenal and β-damascenone. As shown in Table 4-2, volatile compounds were classified into monoterpenes (7 volatiles), aldehydes (12), esters (3), alcohols (4), ketones (7), phenol (1), ether (1) and unknowns (14). It is to be noted that even though sesquiterpenes were identified in these samples by GC-MS (chapter 3), none had odor activity in the amount present in the samples. Odor-active peaks were also grouped based on the similarity of their aroma descriptors: fruity, green/metallic/fatty, terpeney (minty/piney), green/grassy, citrus, mushroom, floral and other (Table 4-3).

Terpenes

Among 61 terpene hydrocarbons found by GC-MS, seven monoterpenes exhibited aroma activity. d-Limonene was the most abundant in all samples, accounting for 40.6 to 76.4% of the total content by GC-MS. However, because it has a high odor threshold (13,700 μg/L in a deodorized orange juice matrix (DOJ) (Plotto et al., 2004), its citrus-like odor was not detected by GC-O. β-Myrcene, one of the richest volatiles, had the highest intensity among seven terpenes, with the threshold of 773 μg/L in DOJ (Plotto et al., 2004). This compound possessed a strong green and metallic odor, which may negatively affect the aroma and flavor characteristics. However, its intensity was equally perceived between samples (Table 4-1). Rega et al. (2003) showed all panelists evaluated β-myrcene as an unpleasant odor in fresh orange juice using a GC-O detection frequency method. Although the samples contained high amounts of the remaining terpenes, they had relatively low intensity along with some aroma descriptors: fruity (terpinolene), terpeney (β-pinene, β-phellandrene, γ-terpinene and dehydro-p-cymene) and green/grassy (p-cymene) (Table 4-3). p-Cymene and terpinolene had the highest perceived intensity in 8-10, and γ-terpinene was the lowest in 8-9 x Val4x (Table 4-1). Unlike these aroma active monoterpenes, 39 sesquiterpenes did not produce odor activity. In the GC-MS analysis, valencene was the second most abundant compound that constitutes 18.4% and 24.8% of the total content in 8-10 and 8-9 × Val4x, respectively. However, it was not detected in the olfactometric analysis because the content did not exceed its high threshold (4,756 μg/L in DOJ) (Plotto et al., 2008).

Aldehydes

The number of aldehydes (12 volatiles) was the highest among the seven identified chemical classes (Table 4-2). Heptanal, (*E*,*E*)-2,4-nonadienal, (*E*,*E*)-2,4-decadienal, 2-undecenal and dodecanal were grouped into the green/metallic/fatty aroma category (Table 4-3). (*E*,*E*)-2,4-

Nonadienal and (*E*,*E*)-2,4-decadienal were not identified in the five samples by GC-MS because they were below the MS detection limit, yet, they could be detected by GC-O. On the other hand, hexanal was the richest aldehyde imparting fresh green/grassy odor unlike fatty (cooked vegetable-like) odors given by these dienals. Jensen et al. (1999) demonstrated that the odor threshold for (*E*,*E*)-2,4-nonadienal in water (0.0017 nL/kg) was about 1.47×10^3 times lower than for hexanal (2.5 nL/kg). (*E*,*E*)-2,4-decadienal (0.045 nL/kg) had approximately 56 times lower threshold than hexanal. In the present study, (*E*,*E*)-2,4-nonadienal was the most potent aldehyde in most samples, followed by hexanal, (*E*)-2-decenal and octanal. It is evident that GC-O analysis can be a powerful method to determine the presence of these compounds, using the sensitivity of the human nose, in comparison with GC-MS.

A straight-chain saturated aldehyde, octanal, may be an important contributor to citrus and fruity notes. Its aroma intensity was the highest in 'Fortune' × 'Murcott' and the lowest in the unknown sample (Table 4-1). Although decanal, as well as octanal and nonanal, has been implicated to contribute to orange flavor (Shaw, 1991), its aroma intensity was low in the tangerine samples. The terpenic aldehydes, neral and geranial, which were also believe to be important contributors to orange flavor (Ahmed et al., 1978a, 1978b), were not detected by GC-O analysis, though they were identified in 'Fortune' × 'Murcott' and 8-9 × Val4x by using GC-MS. The unsaturated aldehydes, (*E*)-2-pentenal, (*E*)-2-heptenal, (*E*)-2-nonenal, (*E*)-2-decenal and 2-undecenal had their own characteristic odors, as seen in Table 4-3. All detected aldehydes are degradation products, probably derived from C_{16} and C_{18} fatty acids rich in citrus juice sacs (Nordby and Nagy, 1971). Chisholm et al. (2003) reported that 47 aldehydes were found in 'Clementine' peel oil, which contributed over 80% of its aroma using dilution analysis (CHARM analysis). In the present study where juice samples were processed to avoid any peel oil in the

juice, aldehydes contributed 24 to 28% of fresh tangerine aroma, indicating the role of other aroma components.

Esters

The aroma activity of ethyl butanoate, ethyl 2-methylbutanoate and ethyl hexanoate have also been reported in hand-squeezed orange juice (Tønder et al., 1998; Buettner and Schieberle, 2001b; Arena et al., 2006). The trace amounts of these compounds in 8-9, 8-10, Unknown and 'Fortune' × 'Murcott' did not allow their individual identification by MS, unlike the most abundant ethyl acetate. However, the panelists could detect their fruity odors because the thresholds are significantly low: 1.71, 0.08 and 3.3 μg/L in DOJ, respectively (Plotto et al., 2008). The content of ethyl butanoate was the highest (2.2%) in 8-9 × Val4x, followed by ethyl hexanoate (1.2%), ethyl acetate (0.4%) and ethyl 2-methylbutanoate (0.05%) by GC-MS. Based on the perceived aroma intensity, ethyl butanoate and ethyl 2-methylbutanoate alone contributed to 22 to 29% of the fruity note in the five samples. Likewise, Buettner and Schieberle (2001b) determined these two compounds were potent odorants of 'Valencia' late orange juice. Ethyl-2-methyl butanoate had high fruity/floral aroma intensity in all five samples (Table 4-1). On the other hand, ethyl butanoate and ethyl hexanoate had the highest intensity ratings in 8-9 × Val4x, with ethyl hexanoate the highest among the samples. Since ethyl butanoate is an important contributor to desirable orange juice flavor (Shaw, 1991), the higher level of ethyl butanoate and ethyl hexanoate in 8-9 × Val4x would contribute to the fruity characteristic of this hybrid (unpublished sensory data).

Alcohols

Ethanol was relatively rich in the samples but showed low aroma intensity. Shaw (1991) inferred that it may give a "lift" to other aromas, without contributing to its own aroma. A terpene alcohol, linalool, accounted for 1.2 to 5.3% of the total content by GC-MS, described as

floral with a strong intensity. This compound makes a positive contribution to orange flavor in combination with several other orange volatiles (Shaw 1991). It had the lowest aroma intensity in the unknown sample (Table 4-1). Two isomeric (*Z*)- and (*E*)-carveol, derived from limonene by limonene-6-hydroxylase (Bouwmeester et al., 1998), had aroma activity with different odors: minty/pencil/piney/fruity/floral and green/rubber/sulfury, respectively. (*Z*)-carveol also had the lowest intensity in the unknown sample (Table 4-1). These compounds were found in cold-pressed oils of 'Natsudaidai' (a natural hybrid of pummelo) (Lan-Phi et al., 2006), two mandarin cultivars and 'Minneola' tangelo (Njoroge et al., 2005). With low aroma intensities and multiple descriptors, it is possible that several compounds co-eluted at this time in our samples.

Ketones

1-Octen-3-one was the single most potent aliphatic ketone, having a mushroom odor. Camphor and d-carvone, cyclic terpene ketones with a terpeney odor, also showed aroma activity with relatively high intensity in some samples. Despite the peculiar green buchu oil odor imparted by camphor, it has not been found in many citrus fruits: e.g., two mandarin cultivars (Frizzo et al., 2004) and Pontianak orange peel oil (Dharmawan et al., 2009). The weak spicy/woody odor of nootkatone, which level is usually high in grapefruit and pummelo, was detected in all samples. All of the above ketones were perceived with similar intensities in the five samples (Table 4-1). Alpha- and β-ionone were detected and are important components to contribute to the floral note, in addition to linalool mentioned above (Mahattanattawee et al., 2005). Interestingly, one panelist could not detect β-ionone at all, confirming that some people may have specific anosmia to that compound (Plotto et al., 2006). Beta-damascenone was not identified by GC-MS, but had high aroma intensity in some samples with apple sauce/fruity odor. This compound and β-ionones showed the highest intensity in 8-9 × Val4x (Table 4-1). Since the seed parent 8-9 had low intensity of the ionones, tetraploid 'Valencia' orange may play

an important role in the production of these floral norisoprenoids. The three C_{13} norisoprenoids are degradation products from putative carotenoid precursors, α- and β-carotene, α- and β-cryptoxanthin and neoxanthin (Mahattawanatawee et al., 2005). Thus, their content may be one of the critical factors to control the desirable odor and flavor at fruit maturity. Although the ionones had similar aroma intensities in 'Fortune' × 'Murcott' and in 8-9 × Val4x, that of β-damascenone was significantly lower in 'Fortune' × 'Murcott'. Aroma intensity of α-ionone was very low in 8-9, 8-10 and unknown sample.

Phenol

A high level of thymol was found by GC-MS in 8-9, 8-10 and 8-9 × Val4x sharing common genetic background from 'Clementine' and 'Minneola' (a hybrid between 'Duncan' grapefruit and 'Dancy' tangerine). Thymol was found in 'Dancy' tangerine peel oil and Sicilian mandarin peel oil (Shaw, 1979), and 'Temple' tangor essence oil (Moshonas and Shaw, 1983). Wilson and Shaw (1981) reported that thymol and methyl N-methylanthranilate, in addition to γ-terpinene and β-pinene, were necessary components for mandarin aroma in cold-pressed oil. Furthermore, the elevated levels of thymol and methyl N-methylanthranilate in mandarin peel oil are a discriminative factor from orange peel oil (Rouseff and Pérez-Cacho, 2007). In this study, thymol was found at the highest concentration by GC-MS and aroma intensity (Table 4-1) in 8-9, where it might contribute to specific tangerine aroma.

Ether

1,8-Cineole (eucalyptol), a monoterpene cyclic ether, was a strong aroma active compound with a low threshold (9 μg/L in DOJ - Plotto, unpublished data). This compound may have negative impact on tangerine aroma, since its odor was a characteristic green and sulfury. GC-MS chromatograms showed it coeluted with d-limonene, and its peak area (relative content) was

not quantified. However, because of its characteristic green/minty odor, it was clearly identified from the limonene by GC-O. 1,8-Cineole was found in grapefruit oil (Lin and Rouseff, 2001), 'Clementine' peel oil (Chisholm et al., 2003) and orange essence oil (Högnadóttir and Rouseff, 2003) by using GC-O. However, its aroma activity has not been clearly reported in citrus juice.

Unknown Compounds

Fourteen compounds could not be identified by either FID or GC-MS. Although most compounds had low intensity of less than 5, they are not negligible because they may interact with other aroma volatiles in the juice. Compared with their RIs and aroma descriptors in published data, four compounds can be tentatively identified: methional (No. 9), furaneol (No. 20), sotolon (No. 26), trans-4,5-epoxy-(*E*)-2-nonenal (No. 39) and wine lactone (No. 46) (Hinterholzer and Scieberle, 1998; Buettner and Schieberle, 2001a, 2001b; Chisholm et al., 2003; Högnadóttir and Rouseff et al., 2003; Valim et al., 2003; Pérez-Cacho et al, 2007). Each compound has been described with distinctive odors: methional (potato, cooked potato), furaneol (burnt sugar, caramel), sotolon (geranium, green, metallic, floral), trans-4,5-epoxy-(*E*)-2-nonenal (caramel, spicy, seasoning, mushroom) and wine lactone (sweet, spicy, dill, floral, metallic). In addition, a few aroma-active peaks were described as sulfur or rubber. These are likely to be sulfur compounds with very low odor threshold. Sulfur compounds are not detected by the FID type of detector, and might be in too low concentrations to be detected by GC-MS. It is known that processing and storage easily cause the development of "skunky" (sulfury) off-flavor in mandarin juice (Shaw, 1991). It is possible these compounds resulted from storage of the juice. In citrus juice, they are derived from amino acids such as cystein, cystine and methionine (Pérez-Cacho and Rouseff, 2008).

Tangerine Aroma Profiles

As shown in Figure 4-1, a total intensity of aroma active peaks (119 peaks) was used to represent the relative importance of each aroma category. The total intensity of all categories was 181, 174, 149, 197 and 197 in 8-9, 8-10, Unknown, 'Fortune' × 'Murcott' and 8-9 × Val4x, respectively. All samples presented three aroma categories as top notes: fruity, green/metallic/fatty, and terpeney. Among 30 separate aroma active peaks in the fruity category, ethanol, (*E*)-2-pentenal, ethyl butanoate, ethyl 2-methylbutanoate, ethyl hexanoate, terpinolene, unknown (No. 28 and 48) and β-damascenone (Table 4-3) contribute 73 to 92% of its total intensity. Moreover, the other aroma categories of tangerine aroma can be primarily explained by the compounds in Table 4-3.

8-9 and 8-10, siblings from the same cross, had different aroma volatile composition and aroma profiles. For instance, ethyl butanoate and ethyl 2-methylbutanoate had higher intensity in 8-9 than 8-10, even though not significant (Table 4-1), contributing to a fruitier note to the former. β-Phellandrene and γ-terpinene, described as terpeney, also showed higher intensity in 8-9 than 8-10. On the other hand, green/grassy compounds, (*E*)-2-heptenal and p-cymene, had higher intensity in 8-10 than 8-9 (Figure 4-1 and Table 4-1). In addition, the unknown compound (No. 21) and terpinolene, described as burned/mushroom and fruity/green/toasted, respectively, showed higher intensity in 8-9 and 8-10 than the other samples. Their fruits were grown under the same environmental condition, and harvested at the same date. Thus, genetic effect may play an important role to differentiate their aromas. With respect to the terpeney category, it was higher in 8-9 than in 8-10 (Figure 4-1). More fresh terpeney odors of 8-9 might balance undesirable fatty odor, mainly caused by (*E*,*E*)-2,4-nonadienal and (*E*,*E*)-2,4-decadienal. When juice samples are consumed, aroma perception is affected by the presence of soluble solids (e.g., sugars and acids) and the corresponding change of gas-liquid partition coefficient of volatile

compounds (Friel et al., 2000). Baldwin et al. (2008) reported that the addition of acids with volatiles increased perception of overall tomato aroma using deodorized tomato puree. The higher acidity in 8-9 (1.57% of titratable acidity - TA) might also contribute to the aroma and flavor differences from 8-10 (1.27% of TA) (data not shown).

The unknown sample was the least aromatic among the five tangerine samples. The total volatile content was 3.4 to 6.8 times lower than the other four samples by GC-MS (Chapter 3). Interestingly, three homologous aldehydes, hexanal (C_6), heptanal (C_7) and octanal (C_8) were the most abundant volatiles, following d-limonene. The green/metallic/fatty category, which is mostly the characteristic odor of aldehydes, accounted for 34% of its aroma. The abundance of aldehydes in addition to low fruity/floral compounds could contribute to a peculiar "pumpkin, fatty" aroma and flavor of this sample as described by a sensory trained panel (data not shown). In addition to those aroma volatiles, the highest sugar content (15 °Brix) and lowest acidity (0.71% TA) among the five samples added to its unique flavor (data not shown).

'Fortune' × 'Murcott' shared a similar aroma profile to 8-9, in terms of total intensities of the major aroma categories, fruity and terpeney, but had more floral peaks, mostly from α- and β-ionone. The unknown compound (No. 8) with a citrusy/fruity odor, in addition to β-pinene, was detected only in 8-9 and 'Fortune' × 'Murcott' (Table 4-1), and might contribute to their desirable aroma. 'Fortune' × 'Murcott' contained aldehydes in high quality and quantity (Table 3-5 and 3-6), and hence its overall aroma may smell slightly more green, metallic and/or fattier.

The aroma profile of 8-9 × Val4x (2n = 3x = 27) may be distinguished from that of 8-9 because of its additional genetic background from orange. The differences between the two samples mostly stand in the fruity and terpeney categories and may be associated with the content of esters and monotepenes. Indeed, the total intensity of esters (ethyl butanoate, ethyl 2-

methylbutanoate, ethyl hexanoate) in 8-9 × Val4x was about 1.6 times higher, and that of monoterpenes (β-pinene, β-phellandrene, γ-terpinene, dehydro-p-cymene) was about 1.8 times lower than that in 8-9 (Table 4-1). Green/grassy, citrus, mushroom and other categories are responsible for about 34% of their aromas, indicating that they are more likely to contribute to tangerine aroma as background notes.

Table 4-1. Consensus of aroma active compounds in five tangerine hybrids determined by GC-MS and GC-O using Osme analysis

No.*	Compound	RT[a]	LRI[b]	Descriptors	Aroma Intensity[c]				
					8-9	8-10	Unknown[d]	F × M[e]	8-9 × Val4x
1	Ethanol	2.08	535	ethanol, alcohol	1.0	2.4	0.7	1.6	1.9
2*	Unknown	3.69	652	sulfury, metallic, herb	0 b	0 b	2.3 a	0.9 b	0.3 b
3	(*E*)-2-Pentenal	4.91	730	fruity, floral	3.2	2.1	3.8	2.7	3.4
4	Hexanal	5.72	776	green, grassy	5.5	5.1	5.2	5.7	4.9
5*	Ethyl butanoate	5.80	781	fruity	3.5 ab	2.5 b	1.3 b	3.1 ab	6.0 a
6	Ethyl 2-methylbutanoate	7.01	844	fruity, floral	5.3	3.9	5.1	5.3	5.5
7	Heptanal	8.31	904	green, fatty, vegetable	4.5	4.8	5.3	4.4	5.1
8*	Unknown	8.37	906	citrusy, fruity	2.1 a	0 b	0 b	3.5 a	0 b
9	Unknown	8.52	913	metallic, cooked vegetable	4.3	5.2	5.3	3.9	6.5
10*	(*E*)-2-Heptenal	9.57	957	green, grassy, rubber	1.8 bc	3.9 ab	4.6 a	1.7 bc	1.0 c
11	1-Octen-3-one	10.51	993	mushroom	4.3	4.7	6.2	5.4	6.0
12	β-Myrcene	10.65	998	green, metallic	7.3	7.4	7.0	7.4	8.0
13	β-Pinene	10.85	1005	green, piney	1.0	0	0	1.9	0
14*	Ethyl hexanoate	11.07	1013	fruity, floral	1.0 b	0.6 b	0.3 b	0 b	4.8 a
15*	Octanal	11.19	1017	citrus, fruity	5.2 ab	4.4 b	3.3 b	6.6 a	4.7 ab
16*	p-Cymene	11.63	1033	green, rubber	0.3 b	2.6 a	0.5 b	0.5 b	0.2 b
17	β-Phellandrene	12.05	1048	minty, piney, terpene	5.6	4.0	4.1	4.8	4.4
18*	1,8-cineole	12.22	1054	green, herb, rubber	2.8 b	8.6 a	8.4 a	7.8 a	8.3 a
19*	γ-Terpinene	12.78	1072	minty, piney, terpeney, fruity	5.3 a	2.6 bc	3.0 bc	4.5 ab	1.5 c
20	Unknown	13.04	1081	burned sugar, caramel	0.7	2.0	2.0	2.0	2.0
21*	Unknown	13.30	1090	burned, mushroom	3.6 a	2.5 a	0.7 b	0.3 b	0.2 b
22*	Unknown	13.48	1096	burned, musty	3.6 a	0 c	2.3 ab	0.7 bc	3.5 a
23*	Terpinolene	13.63	1101	fruity, green, toasted	2.9 ab	4.2 a	0.3 c	1.9 bc	1.1 bc
24	Dehydro-p-cymene	13.76	1105	green, minty, plant, apple	3.0	1.7	2.1	3.0	2.5
25*	Linalool	13.87	1109	floral	6.7 a	7.4 a	4.4 b	7.0 a	5.9 ab
26	Unknown	14.21	1120	burned sugar, caramel, musty	2.0	2.3	3.3	4.4	3.9
27	Unknown	14.90	1143	green, metallic, rubber, sulfury	3.7	5.3	3.3	5.5	4.0
28	Unknown	15.11	1149	fruity, musty, floral, green	2.5	3.9	2.8	4.2	3.7
29	(*E*)-2-Nonenal	15.32	1156	cucumber, perfumey	0.4	1.6	2.9	1.2	3.4

Table 4-1. Continued

No.*	Compound	RT[a]	LRI[b]	Descriptors	Aroma Intensity[c]				
					8-9	8-10	Unknown[d]	F × M[e]	8-9 × Val4x
30	Unknown	15.44	1160	musty, minty, floral	4.2	5.3	4.6	5.4	5.4
31	Camphor	15.61	1166	buchu oil, green tea, hay, musty	3.3	3.6	3.2	6.3	4.7
32*	Unknown	15.87	1175	musty, scotch tape	0.9 b	0.3 b	0 b	2.1 a	0 b
33	Decanal	16.34	1191	apple, green	2.1	1.8	0.6	1.1	2.0
34	(*E*,*E*)-2,4-Nonadienal	16.88	1209	fatty, vegetable, noodle	6.9	7.4	5.0	6.3	7.3
35*	(*Z*)-Carveol	17.19	1220	floral, lemon, minty, pencil	4.7 a	3.0 ab	1.1 b	3.8 a	3.6 a
36	(*E*)-Carveol	17.53	1232	green, rubber, sulfury	3.4	3.6	4.2	2.5	1.6
37	d-Carvone	17.68	1238	minty	5.4	3.9	4.6	5.8	4.9
38	(*E*)-2-Decenal	17.83	1243	minty, pencil, piney, fruity, floral	5.7 a	4.6 ab	3.6 b	5.1 ab	6.0 a
39*	Unknown	18.64	1274	green, rubber, sulfury	2.4 b	3.0 ab	0.2 c	4.2 a	2.6 ab
40*	Thymol	18.86	1282	medicinal	4.2 a	2.9 ab	0.5 b	1.1 b	1.9 ab
41	(*E*,*E*)-2,4-Decadienal	19.42	1305	fatty, vegetable	4.5	4.2	2.5	4.2	4.3
42	2-Undecenal	20.92	1370	metallic, musty	4.7	2.4	4.1	5.1	5.2
43*	β-Damascenone	21.07	1377	apple sauce, fruity, floral	5.6 ab	5.9 ab	4.0 b	1.4 c	7.3 a
44	Dodecanal	21.43	1395	green, rubber	0.8	1.2	0.5	2.8	1.0
45*	α-Ionone	22.08	1427	floral, perfumey, soap	0.9 b	0.7 b	0.4 b	3.3 a	3.2 a
46*	Unknown	22.82	1467	fermented, musty, butter	0.3 c	2.2 ab	0 c	1.8 b	3.4 a
47*	β-Ionone	23.40	1499	floral, violet	2.1 b	4.1 a	3.9 a	4.2 a	5.0 a
48*	Unknown	24.14	1544	sulfury, fruity, floral	1.2 abc	0 c	1.0 bc	2.7 a	2.3 ab
49	Nootkatone	28.06	1843	spicy, woody	1.8	2.1	1.0	1.8	2.8

* Peaks for which there was a difference between samples for that aroma intensity by ANOVA

[a] Retention time

[b] Linear Retention Index on the HP-5 column

[c] Averages of three panelists by three replications. Numbers followed by the same letter within a row are not significantly different using the LSD test (α = 0.05).

[d] Three samples with unknown parentage were analyzed by GC-MS. Sample p was used for this analysis.

[e] 'Fortune' × 'Murcott'

Table 4-2. Tangerine aroma active compounds in 8 chemical classes

Monoterpenes	Aldehydes	Esters	Alcohols	Ketones	Phenol	Ether	Others
β-Myrcene	(*E*)-2-Pentenal	Ethyl butanoate	Ethanol	1-Octen-3-one	Thymol	1,8-Cineole	14 Unknowns
β-Pinene	Hexanal	Ethyl 2-methylbutanoate	Linalool	Camphor			
p-Cymene	Heptanal	Ethyl hexanoate	(*Z*)-Carveol	d-Carvone			
β-Phellandrene	(*E*)-2-Heptenal		(*E*)-Carveol	β-Damascenone			
γ-Terpinene	Octanal			α-Ionone			
Terpinolene	(*E*)-2-Nonenal			β-Ionone			
Dehydro-p-cymene	Decanal			Nootkatone			
	(*E*,*E*)-2,4-Nonadienal						
	(*E*)-2-Decenal						
	(*E*,*E*)-2,4-Decadienal						
	2-Undecenal						
	Dodecanal						

Table 4-3. List of tangerine aroma active compounds in 8 aroma categories

fruity	green, metallic, fatty	terpeney	green, grassy	citrus	mushroom	floral	other
Ethanol	Unknown (No. 2)	β-Pinene	Hexanal	Unknown (No. 8)	1-Octen-3-one	Linalool	Unknown (No. 20)
(*E*)-2-Pentenal	Heptanal	β-Phellandrene	(*E*)-2-Heptenal	Octanal	Unknown (No. 21)	α-Ionone	Unknown (No. 26)
Ethyl butanoate	Unknown (No. 9)	γ-Terpinene	p-Cymene			β-Ionone	(*E*)-2-Nonenal
Ethyl 2-methylbutanoate	β-Myrcene	Dehydro-p-cymene	1,8-cineole				Thymol
Ethyl hexanoate	Unknown (No. 22)	Camphor	Decanal				Nootkatone
Terpinolene	Unknown (No. 27)	(*Z*)-Carveol					
Unknown (No. 28)	Unknown (No. 30)	d-Carvone					
β-Damascenone	Unknown (No. 32)	(*E*)-2-Decenal					
Unknown (No. 48)	(*E*,*E*)-2,4-Nonadienal						
	(*E*)-Carveol						
	Unknown (No. 39)						
	(*E*,*E*)-2,4-Decadienal						
	2-Undecenal						
	Dodecanal						
	Unknown (No. 46)						

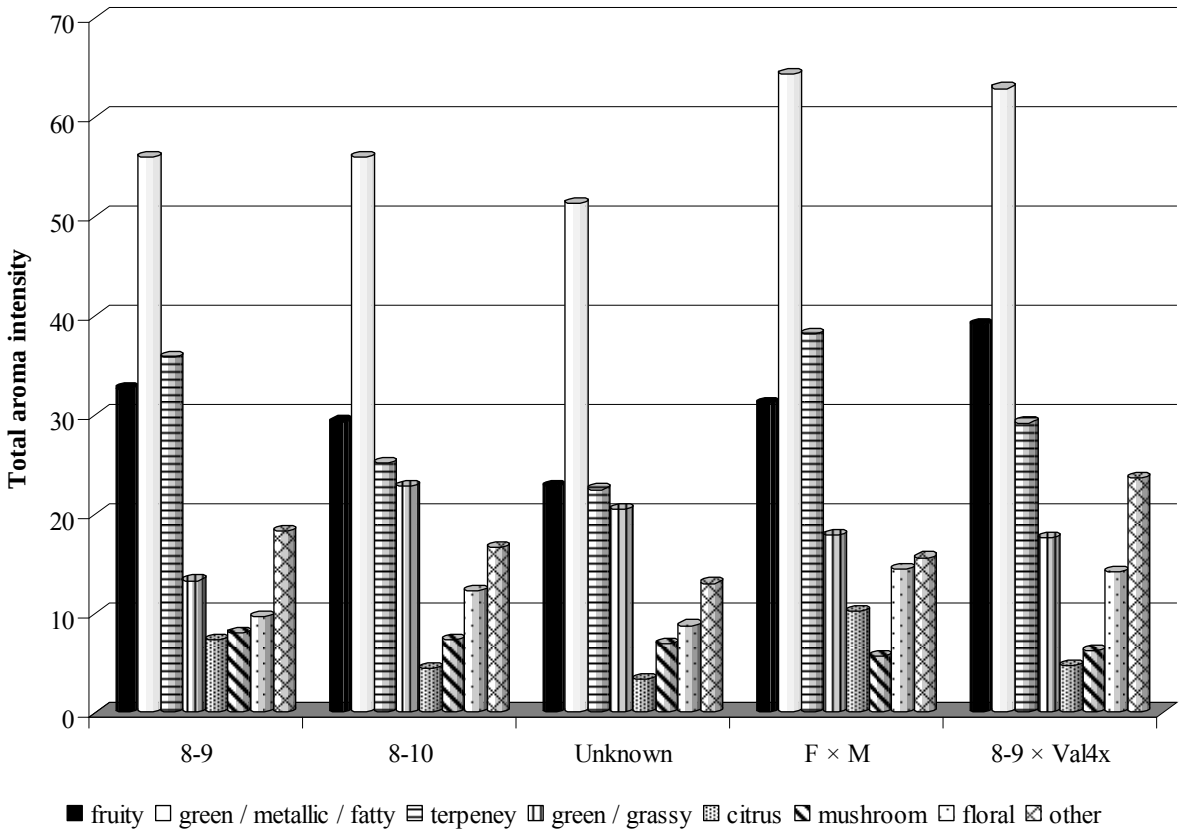

Figure 4-1. Aroma profiles of five tangerine hybrids. Each bar is the sum of intensities in a specific category.

CHAPTER 5
SUMMARY AND CONCLUSIONS

The qualitative and quantitative differences of aroma volatile composition were observed among 20 tangerine hybrids and five commercial cultivars. Among 193 aroma volatiles identified in the hybrids using GC-MS, only a small number of volatiles accounted for more than 90 % of the total content. Volatiles in lower quantity were widely distributed among samples, and were classified mainly as terpene hydrocarbons and oxygenated compounds, such as aldehydes, esters, alcohols and ketones. PCA based on relative peak areas clearly differentiated three selections (9-4 × Blood4x, 'Temple' and 'Sanguinelli') with greater volatile levels; 'Sanguinelli' is a sweet orange and the other two have sweet orange as a direct progenitor. In addition, a second PCA using the content of 11 chemical classes revealed that most hybrids with more mandarin/tangerine genetic contributions were distinguished from the other samples due to their lower volatile quality and/or quantity. The cluster analysis based on volatile presence/absence grouped samples into five clusters, each having effects of genetic background on volatile composition. Different siblings of the same parentage (8-9 × 'Murcott', 'Robinson' × 'Fairchild', 'Fallglo' × 'Fairchild', or 8-9 and 8-10) showed different volatile composition, implying complex genetic controls for aroma volatile production. Nevertheless, hybrids with sweet orange in their background appeared to produce more sesquiterpenes and esters.

Aroma active volatiles in five selected juice samples were analyzed by GC-O using the time intensity (Osme) method. The choice of samples analyzed by GC-O was determined from GC-MS and sensory studies of 20 tangerine hybrids. Although a total of 150 volatiles were identified by GC-MS, only 49 aroma active peaks were found in a consensus of three panelists. Nineteen volatiles were potent aroma compounds with perceived intensity of more than 5 (on a 0 to 10 point scale) in at least one sample. The olfactometric analysis also revealed compounds that

were not detected by GC-MS. These compounds were camphor, (*E*,*E*)-2,4-nonadienal, (*Z*)-carveol, (*E*)-carveol, (*E*,*E*)-2,4-decadienal, β-damascenone and α-ionone, with associated descriptors of buchu oil, fatty, floral, green, fatty, apple sauce and floral, respectively. Moreover, (*E*,*E*)-2,4-nonadienal, (*E*,*E*)-2,4-decadienal and β-damascenone were not identified in any of the other 20 samples by GC-MS. On the other hand, many sesquiterpenes were detected by GC-MS, but none of these had any odor-activity. The top notes of tangerine hybrids were mainly from terpene hydrocarbons, aldehydes and esters. The other chemical classes (alcohol, ketone, phenol and ether), associated with various descriptors, may also contribute to the overall aroma and flavor. Most of these compounds or odor-active peaks were found in all samples, but in different intensity levels, explaining sensory differences that might be found between samples. GC-O results confirm partially the sensory characteristics of some samples (for example, a hybrid of unknown origin with pumpkin/fatty aroma and flavor; 8-9 × Val4x with an orange aroma); however, it is recognized that sugars, acids and other non-volatile compounds also contribute to flavor.

This study provides useful information on aroma volatile profiles and sensory quality for future tangerine breeding efforts. Further research is needed to understand what constitutes a desirable combination of tangerine aroma active compounds. Moreover, further biochemical and genetic research could lead to a better understanding of the metabolic pathways and genes associated with aroma volatile formation. An increasing number of citrus protein and DNA sequence (e.g., expressed sequence tags, EST) entries are available in public databanks. However, only a few citrus terpene synthases and their cDNAs have been reported, and the formation of other major chemical groups (aldehyde, ester, alcohol and ketone) is not yet well understood. Since it is likely that many genes control and influence the complexities of citrus

aroma and flavor, quantitative trait locus (QTL) analysis may be applied to identify particular regions of the genome linked to volatile production. In addition to specific gene coding regions, regulatory regions (*cis*- and *trans*-acting QTLs) might likewise affect quantitative differences of odor active volatiles, resulting in the variety of tangerine overall aromas. Citrus aroma formation occurs as fruits ripen, and hence microarray technology could be used to elucidate chronological changes of gene expression involved with volatile production. With these combined techniques, the development of molecular markers associated with citrus aroma and flavor should accelerate long-term tangerine breeding programs, by enabling selection of individual hybrids at an early stage which possess a greater likelihood of producing high quality fruit.

APPENDIX A
OPTIMIZATION OF SPME FOR GC-MS AND GC-O ANALYSIS

Table A-1. Optimization of volatile sampling

Panelist	Treatment	Rating			Mean	Standard deviation
A	1[a]	3	3	8	4.67	2.89
	2[b]	6	9	7	7.33	1.53
	3[c]	8	7	9	8	1
B	1[a]	3	3	7	4.33	2.31
	2[b]	9	5	7	7	2
	3[c]	7	8	9	8	1
C	1[a]	3	4	5	4	1
	2[b]	2	6	6	4.67	2.31
	3[c]	2	5	4	3.67	1.53

[a] 30 min fiber equilibrium and 3 min fiber exposure
[b] 30 min fiber equilibrium and 30 min fiber exposure
[c] 30 min fiber equilibrium and 60 min fiber exposure

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BIOGRAPHICAL SKETCH

Takayuki Miyazaki was born in Arida city, Wakayama prefecture, Japan. His hometown is well known as a center of citrus production, especially Satsuma mandarin. He attended the Laboratory of Horticultural Crop Physiology in the Department of Life Sciences/Faculty of Bioresources at Mie University and received a Bacheolor of Science in March 2006. After graduation, he attended the Graduate School of Bioresources at Mie University till July 2006.

Takayuki was awarded an ambassadorial scholarship from Rotary Foundation and enrolled in the graduate program of the Horticultural Sciences Department at University of Florida in August 2006. After taking courseworks in Gainesville, he started the research project under the supervision of Dr. Frederick G. Gmitter, Jr., a professor of citrus breeding and genetics, at the Citrus Research and Education Center in Lake Alfred, Florida. Following the completion of his M.S. program, he will go back to Japan and pursue his career in horticulture.