CHEMICAL COMPONENTS AND AROMATIC PROFILES OF CITRUS AND COFFEE IN ASIA

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2013

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A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

NATIONAL UNIVERSITY OF SINGAPORE

2013

ACKNOWLEDGEMENTS

This study would not have been completed without the constant support from many people who have helped me through this challenging period of my life.

Gratitude must first go to my supervisor, Asst. Prof. Dr. Liu Shao Quan for entrusting this collaboration project to me; and for his advice and support throughout my study. Special thanks must also be made for the influence of my co-supervisor, Dr. Yu Bin, a truly creative and talented scientist, and a mentor for many lessons in life. Other outstanding characters I had had the fortune of learning from includes Prof. Zhou Weibiao who gave me valuable and constructive comments.

Amongst many other sources of motivation and inspiration too numerous to be mentioned, the flavor creation team of Firmenich Asia Pte. Ltd. deserves special mention for their enthusiastic support of the whole project. I am very grateful to Mr. Philip Curran for having the foresight to commence this project; Mr. Kiki Pramudya who has volunteered himself in the sampling expeditions; Ms. Yeo Jinny, Ms. Chionh Hwee Khim and Ms. Yukiko Ando Ovesen for their time and effort.

Special thanks, also, to my comrades, Weng Wai, Shen Siung, Jing Can, Christine, Li Xiao, Li Jie, Xiu Qing, Jingting, Zhi Soon, Danping, Jia Xin, Alena, Jeremy, Justin, Sheng Jie, for their contributions to all aspects of my work as well as other aspects of my life.

In addition, a huge thank you to the FST laboratory staff – Ms. Lee Chooi Lan, Ms. Lew Huey Lee, Ms. Jiang Xiao Hui and Mr. Abdul Rahman who, have always been instrumental in helping me with my experiments.

I am deeply indebted to my family for their endless love and encouragements that allowed me to pursue my dream without fear. Last but not least, I would like to thank the National University of Singapore for granting the research scholarship.

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SUMMARY

This study centered on flavor analysis of indigenous citrus fruits and Arabica coffee in the Asian region. In the search for novel and unique flavor profiles, several cultivars of pomelo (*Citrus grandis* (L.) Osbeck), calamansi (*Citrus microcarpa*) and Arabica coffee (*Coffea arabica* var.) were characterized (volatile and aromatic profiles) using gas chromatography-mass spectrometer/flame ionization detector (GC-MS/FID). As it is of much academic and commercial interest to identify and replicate the authentic aroma, the ultimate aim of this study was to approximate as closely as possible the authentic composition of natural flavors or process flavors. Therefore, different approaches and techniques were adopted as a means to achieve the specific objectives, which were to improve current extraction techniques, data interpretations and to obtain useful insights by correlating instrumental and sensory data. In addition, non-volatile components, which contribute to taste attributes and potential health benefits such as sugars, organic acids and phenolic acids, were examined by ultra-fast liquid chromatography-photodiode array detector/evaporative light scattering detector (UFLC-PDA/ELSD).

Several sample extraction techniques were employed in this study. Solvent extraction was modified to improve the extraction yield, especially when handling complex juice matrices. Headspace-solid phase microextraction (HS-SPME) was employed to extract aroma compounds from the delicate samples such as pomelo blossoms in order to ensure minimal

damage to the plant tissues. In addition, stir bar sorptive extraction (SBSE) coupled with programmable thermal evaporation system (PTV) was developed to quantify volatile compounds in model citrus beverage simultaneously. Pressurized liquid extraction (PLE) demonstrated the feasibility of producing coffee extracts under controllable extraction conditions in correlation with desirable sensory attributes. Further evaluation of pomelo peel extracts using gas chromatography-olfactometry (GC-O) provided more insights into the aroma-active compounds composing the uniqueness of pomelo flavor. These techniques are useful in analyzing different food matrices.

Statistical approaches, i.e. principal component analysis (PCA), canonical discriminant analysis (CDA) and partial least square regression (PLSR) were used to interpret the instrumental data. Hence, the distributions of chemical compounds in different samples were correlated with their geographical origins and aromatic profile. It is believed that these findings provide substantial information on less common citrus varieties and Arabica coffee based on their chemical compositions and aromatic profile. It is also demonstrated the extraction capability of either improved solvent extraction method or relatively new SBSE method on different food matrices. The integration of statistical approaches into flavor analysis also facilitate the data interpretation of huge data set.

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LIST OF ABBREVIATIONS

Abbreviation	Caption
AEDA	Aroma extract dilution analysis
ANOVA	Analysis of variance
CIS	Cooled injection system
CDA	Canonical discriminant analysis
ELSD	Evaporative light scattering detector
FD	Flavor dilution
FID	Flame ionization detector
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GC-O	Gas chromatography-olfactometry
HS	Headspace
LRI	Linear retention index
MS	Mass spectrometry
NIST	National Institute of Standards and Technology
OAV	Odor activity value
PCA	Principal component analysis
PDA	Photodiode array detector
PDMS	Polydimethylsiloxane
PLE	Pressurized liquid extraction
PLSR	Partial least square regression
PTV	Programmed temperature vaporization
RFA	Relative flavor activity
SBSE	Stir bar sorptive extraction
SPME	Solid phase microextraction
TA	Titratable acidity
TDU	Thermal desorption unit
TSS	Total soluble solid
UFLC	Ultra-fast liquid chromatography

LIST OF PUBLICATIONS

1. Refereed Journal Publications

Cheong, M. W.; Loke, X. Q.; Liu, S. Q.; Pramudya, K.; Curran, P.; Yu, B., Characterization of volatile compounds and aroma profiles of Malaysian pomelo (*Citrus grandis* (L.) Osbeck) blossom and peel. *Journal of Essential Oil Research* **2011,** *23*(2), 34-44.

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2. Conferences/ proceedings

Cheong, M. W., Chong, Z. S., Zhou, W., Liu, S. Q., Curran, P. and Yu, B. Characterisation of volatile compounds in calamansi (*Citrus microcarpa*) from Southeast Asia. 11th ASEAN Food Conference held in Bangkok, Thailand on 16-18 June 2011.

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CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1. Background

Flavor has been part of the quest in preparing food and beverage in our daily life. In fact, food is a complex system which provides a multimodal stimulus and flavor is a multimodal sensory experience (*1*). In a scientific context, flavor can be defined as a biological sensation which combines the perceptions of taste, aroma and trigeminal (*2, 3*). These perceptions are the aggregate of the characteristics of the material that produces the sensation of flavor, which is one of prior sensory perceptions for consumers in choosing food products (*2-5*). With the development of commercial food processing, quality consistency of food products has become an important issue. Thus, a more science-based route has been taken to create flavor ingredients that could be incorporated into the mass production of food in order to ensure quality consistency.

Flavor science is a multidisciplinary field that focuses on the interplay of physical and chemical properties of food with physiological taste and smell receptors (*6*). Flavor compounds are comprised of essential oils, oleoresins,

protein hydrolysates, or any product of pyrolysis or enzymolysis derived from a plant or animal source, whose significant function in food is flavoring rather than nutritional (*7*). Though flavor compounds are usually present in trace amounts in a food system (less than 0.1% of total weight), they are one of the important elements in a food system. Thus, flavor research is essential in providing substantive understanding and information of flavor compounds. Progress in flavor research has been an evolutionary process along with the growing demands in the flavor industry (*8*). Today, flavor research is expanding from analytical and synthetic chemistry (*9-11*) into areas including biotechnology (*12-14*), psychophysics (*15-17*), encapsulation (*18-20*), and addressing flavor problems of functional foods (*6, 21-23*). Nevertheless, flavor analytical chemistry continues to play a key role in flavor research (*1*).

From an analytical perspective, the main challenges in flavor analysis are to obtain the genuine chemical profile and correlate the identified compounds with their flavor attributes (*24*). The presence of most potent odors is usually in trace amounts and/or reactive and unstable, making their profiling much more complicated (*25, 26*). Therefore, systematic flavor analysis is required to justify the findings from various aspects, especially when dealing with specific food matrices. Flavor compounds could exhibit different rates of flavor release when incorporated into different food matrices, e.g. in the presence of fats, proteins or carbohydrates (*27-29*). The interaction among flavor compounds in a particular food matrix might lead to an enhancement, synergy or suppression of their relative volatility that could change the way of aroma is perceived.

Conscientious flavor analysis enhances the identification and quantification of potent volatiles from different food sources and matrices. This is mainly due to the recent developments in analytical techniques with improved accuracy and enhanced limits of detection. Furthermore, sensory evaluation is necessary in order to correlate potent key odorants with their aroma profiles, to integrate the science and art of flavor creation and also to provide insights of flavor delivery systems. Among numerous studies in flavor chemistry, analysis of natural flavor (e.g. flavor/aroma emission from the fruit or blossoms) and process flavor generated during roasting of coffee beans are of major interest but yet to be fully understood. Analysis of citrus fruit and coffee flavor could be very different. Even analyses of different parts of plants (i.e. blossoms, peels and juices) require much effort in developing appropriate analytical methods. Hence, citrus and coffee analyses could be the models in developing flavor analytical methods for other complex food systems.

The subsequent sections provide more detailed discussions on the developments of flavor science, analytical techniques and their implications. Furthermore, aroma evaluation techniques and applications of statistical analysis of analytical data in understanding flavor compositions will be discussed.

1.2. Recent developments of flavor science

"The knowledge and use of plants as flavoring and seasoning to enhance the quality of foods, beverages and drugs is as old as the history of mankind" (*12*). However, the use of essential oil was continuously expanding without deeper understanding on molecular knowledge of these ingredients

until the evolution of organic chemistry in the early 1800s. By the turn of the 20th century, the progress of organic chemistry and scientific methodology has embarked much groundbreaking research in flavor industry. In the 1950's, there were about 500 compounds that had been characterized for their flavor attributes (*30, 31*). Due to the astonishing development of instrumentations (e.g. gas and liquid chromatography, mass spectrometry, nuclear magnetic resonance) in the late 1950s, the progress of flavor science in deciphering the novel molecules of flavor compounds was fostered (*7*). The importance of analytical chemistry in supporting the development of flavor research was also established.

As flavor science continuously developed, investigations have evolved from the mere identification of volatiles to studies of other essential aspects of flavor chemistry. Detailed chemical characterization of aroma compounds and the assessment of their sensorial significance could distinguish and quantify those aroma-active compounds from the complex spectrum of flavor compounds (*32*). As will be seen below, several main aspects will be further elaborated.

1.2.1. The search for novel flavor compounds

It remains important for flavor companies to own their captive (proprietary) collections to create unique flavor blends that are suitable for mainstream acceptance, yet which have an authentic appeal. Hence, new sources of aroma and flavor compounds are consistently being sought (*3*). Flavor compounds are mainly derived from a wide range of natural sources

with very varied organoleptic characteristics such as fruit, dairy, cereal and vegetable sources of flavor (*2, 3*).

Many of these flavors rely on one of more functional groups in exhibiting their characteristic flavors, which are known as odor/aroma-active compounds (*2*). In many cases, particular compounds are essential flavor components and, without them, a distinctive flavor of the particular fruit or vegetable cannot be achieved (*3*). Even the flavors of citrus varieties within a family are composed by a diverse array of volatile compounds with disparate concentration. An artificial citrus flavor, for example, could contain from 70 to 80 critical aroma-active compounds; collectively mimicking the taste and aroma of a real citrus, which contains hundreds of flavor compounds (*33*). Nevertheless, there can be a single predominant flavor chemical in some food responsible for the flavor quality; also known as character-impact compound such as benzaldehyde for cherry flavors and vanillin for vanilla flavors (*3*).

Grapefruit from citrus family provides a very interesting example. It has been recognized that (*R)*-nootkatone, a sesquiterpene with a potent grapefruit flavor character and a low odor threshold of 1 μg/L, was also found to be important in pomelo (*34, 35*). More recently, it was discovered that a chemically different compound, *ρ*-menthene-8-thiol also gives grapefruit character at considerably low concentration (below 10 μg/L) with a remarkably low threshold of 0.00002 μg/L (*3, 36*). This demonstrates that a great variety and range of flavor compounds still remains undiscovered, even in seemingly familiar food. As the identification work on unique potential new flavor components with desired performance attributes continues to increase the range of innovative flavors, developing new improved analytical methods

becomes a key aspect as well. Hence, there are long-established international organizations such as International Organization of the Flavor Industry (IOFI), which are actively involved in developing analytical methods and provide guidelines (*37*).

1.2.2. Biogenesis of fruit aroma

Fruit aroma varies widely though all fruits share a very high proportion of the same volatile compounds. Most volatile compounds in fruits contain aliphatic hydrocarbon chains, or their derivatives (esters, alcohols, acids, aldehydes, ketones, lactones). For instance, citrus fruits are rich in terpenoids whereas most non-citrus fruits, such as apple, raspberry, cranberry and banana, are characterized by esters and aldehydes (*2*). Fruit aroma compounds are mainly secondary products of various metabolic pathways as a result of degradation reaction during ripening (*38*). They are derived from an array of compounds including phytonutrients such as fatty acids, amino acids, carotenoids, phenolics and terpenoids (*39*).

Many of the terpenoids are stored in fruits as non-volatile glycosides. When a glycosidase enzyme cleaves the sugar off the glycoside precursor, aromatic terpenoids will be released (*40*). Rearrangements and dehydrations of terpenoid compounds could occur under very mild conditions. The formation of a cation will easily rearrange non-cyclic terpenes into many different bicyclic species. Only a small amount of acid or base is needed to initiate double-bond shifts, cyclizations, and the loss of water. Thus, artifact

formations are a problem during flavor isolation as well as during the processing and storage of food products (*2, 41*).

Aldehydes, alcohols and esters arise from the enzymatic degradation of lipids and/or are produced from free fatty acids, e.g. linoleic and linolenic acids via lipoxygenase activity or amino acids (such as acetaldehyde that comes from alanine) (*39*). The volatile esters are formed during the esterification (alcoholysis) of alcohols by alcohol acetyltransferase as the acyl donor during the ripening of many fruits including apples, citrus and melons (*39, 42*). When the lipid oxidation forms 4- or 5-hydroxy acids, lactones are usually formed which stabilize the hydroxyl fatty acid so further oxidation does not occur (*2*).

Each type of plant has its own set of enzymes, pH and medium conditions (*2*). Apart from varietal differences, environmental factors, such as variations in growing temperatures, rainfall, irrigation and soil nutrients, can influence the compositions of flavor compounds present in similar varieties.

1.2.3. Thermal generation of flavors

Process flavors, generated from the Maillard reactions (non-enzymatic browning) (*43*), can range from the major reaction flavors in nuts and chocolate to chicken and beef (*44, 45*). Other reactions such as the decomposition of fats and oils or caramelization also play an important role in the development of process flavors. Coffee flavor is one of the most studied process flavors with great commercial potential. Although most of the flavor compounds that characterize the coffee flavor are already known, there are many that needs to be discovered with the potential of some emerging new

technologies (*25, 46-48*). Roasted coffee flavors are mainly results from the thermal decomposition of carbohydrates and phenols, especially chlorogenic acids during roasting (*3, 49*). There are marked differences in flavor character caused by variations in composition of flavor compounds. This is due to the different varieties of coffee plants, ways of roasting and different brewing methods (*26, 50, 51*). With the understanding of these factors, insights on important aroma-active compounds in coffee could be gained. Semmelroch and Grosch (*52*) include the following chemicals as contributing to coffee flavor and aroma, i.e. acetaldehyde, propanal, methylpropanal, 2- and 3-methylbutanals, 2-methyl-3-furanthiol, methanethiol, dimethyl trisulfide and 2-ethenyl-3,5-dimethyl- and 2-ethenyl-3-ethyl-5-methylpyrazine which explain the complexity and individual variations of coffee flavors. However, coffee flavors are known to be extremely unstable. Much work has been done on isolation, separation and identification of these flavor compounds and will be discussed in the following sections (*21*).

1.2.4. Flavor release in complex food systems

Flavor compounds could exhibit different rates of flavor release when incorporating into different food matrices, i.e. in the presence of fat, protein or carbohydrates (*53*). In fact, the interaction between flavor compounds in a food matrix might lead to an enhancement, synergy or suppression of their relative volatility that could change the way an aroma is perceived (*54*). For instance, changing the fat content can modify the overall perception of a mixture of flavor compounds from different chemical classes, especially hydrophobic flavor compounds resulting in noticeable effects on flavor

perception. As a result, a drastic shift of the overall flavor profile can result in different odor sensation, even if the changes in the fat content are small (*27*). In general, the retention of volatiles by protein is much lower than that by fat. In emulsions, however, the presence of protein at the oil/water interface induces a significant effect on flavor release and flavor perception of hydrophobic flavor compounds. Emulsification and droplet size also affect flavor release and perception (*28*). For starch, an extensively studied hydrocolloid, amylose has been shown to form complexes with aroma compounds (*20*). The physical state of carbohydrates is one parameter influencing flavor retention. However, the major effect of hydrocolloids seems to be a limitation for the diffusion of aroma compounds due to changes in viscosity (*53*). Studies proved that flavor compounds are delivered at different rates to the aroma receptors in a wide range of foods, e.g. sugar confectionery (*55-57*), strawberries (*58*) and tomatoes (*59*). More research is required on the effects of real food samples containing mixtures of different flavor compounds. Precise measurement therefore is an essential tool in understanding the matrix effect on flavor performance.

1.3. Flavor isolation techniques

One of the challenges in flavor analysis is the sheer number and range of chemical compounds present in a flavor. To date, there are about 2,500 known odorants and complex flavors. Coffee can contain up to 800 compounds (*60-63*). Most flavors contain a smaller number of character-impact compounds which, when combined give a recognizable, if not perfect, flavor. Among these compounds, aroma-active compounds typically have low thresholds or

are relatively unstable. Thus, careful extractions are required to obtain genuine volatile profiles.

The first few steps of flavor analysis usually involve isolation and concentration of volatiles and semi-volatiles from their original food matrices. These techniques are numerous and have been extensively review (*64-68*). Traditionally, volatile plant components are obtained as essential oil through hydrodistillation of leaves, flowers, stems, roots, the bark of aromatic plants, or by cold expression of the peel in the case of citrus fruits (*69*). However, hydrodistillation may cause partial decomposition and rearrangement processes in the case of labile compounds. On the other hand, cold expression will extract not only volatiles but also plant waxes, fatty oils and high boiling lipids that tend to contaminate the GC column (*70*). Although the fundamentals of modern organic chemical methods used in flavor chemistry have been established, details of modifications and extensions of existing methods must be worked out in order to solve specific problems (*30*). Isolation techniques such as solvent extraction methods and the relatively new sorptive extraction techniques are discussed in the following subsections (*67, 71*).

1.3.1. Solvent extraction techniques

With many flavor analytical methods, solvent extraction is the traditional method used, with direct contact between an extraction solvent and a sample. This is a complicated task as the isolation procedures require multiple steps and are time consuming (*72*). The chemical compounds in a food sample are pulled into the organic solvent, corresponding to a reflection of the amount of substances present in the sample. Further sensory evaluation

by sniffing through a smelling strip or olfactometer is also made possible with solvent extraction to correlate the instrumental data with its aroma profile.

The choice of an organic solvent is the most critical element. An ideal solvent should have a maximal solubility for the analytes of interest and a minimal solubility for the matrix (*73*). Due to solvent polarity, which affects extraction efficiency on different groups of volatile compounds, the composition might be altered, resulting in discrimination towards different groups of volatile compounds during extraction. Common organic solvents include methanol, ethanol, dichloromethane, diethyl ether and hexane.

Extraction of the process flavors or thermally generated compounds is challenging as many potent odorants are present in trace amounts and/or unstable become lost during the procedures (*25, 26*). This is valid, particularly for volatile sulfur compounds present in coffee flavor such as thiols, due to their susceptibility to oxidative degradation reactions (*26*). Moon and Shibamoto (*44*) identified volatile compounds in roasted ground coffee with dichloromethane and the results suggested that the liquid extraction method allowed the differentiation of different roasting conditions, with the identification of different major compounds. With a polarity index of 3.1, dichloromethane is the suitable solvent for flavor isolation, allowing more polar potent components to be dissolved. There is innovative work to reduce the time required by the isolation step, to automate the process with a programmed sequence, and to reduce consumption of organic solvents (*72*). The improved or newly developed methods are supercritical fluid extraction (SFE), pressurized liquid extraction (PLE).

PLE and SFE apply external pressure and/or heating to speed up the extraction process, especially when dealing with solid materials (*73*). Based on the use of compressed fluids as extracting agents, PLE and SFE are useful for sample preparation for food analyses, including fats, pesticide residues and toxins (*72, 74-77*). PLE, also referred to as accelerated solvent extraction, is performed at elevated pressure (1500-2000 p.s.i.) and temperature (50-200 °C) above the boiling point of the organic solvent (*77*). It was modified according to Soxhlet extraction but with the use of higher temperature, thus, increasing the ability of solvent to solubilize the analyte, decreasing the viscosity of liquid solvents and allowing better penetration of the solvent into the matrix (*78-80*). The use of higher pressure facilitates the extraction of the analytes from samples by improving the solvent accessibility to the analytes that is trapped in the matrix (*81*), thereby the extraction time and solvent consumption are significantly minimized compared to a typical Soxhlet extraction. Manipulation of isolation parameters could result in differences in the relative composition of the extracts because the extraction power of the applied solvents and the applied pressure and temperature parameters have a strong influence on the yield of each compound of the essential oil (*70*).

SFE uses a variety of fluids (typically CO_2, possibly modified with organic solvents), at higher pressure (2000-4000 p.s.i.) and temperature (50-150 °C) than PLE (*72*). It has been promoted as an effective and virtually solvent-free sample pretreatment technique (*74*). Under certain conditions, supercritical CO_2 is comparable to *n*-hexane in its polarity. Therefore, it may preferentially extract nonpolar compounds (*70*). Nevertheless, SFE is heavily

matrix-dependent and even much more prominent than PLE, and hence, detailed method development is always required (*82*).

1.3.2. Sorptive extraction techniques

To enhance the identification and quantitation of potent volatiles from different food matrices, sorptive extraction techniques have been developed (*64*). Sorptive extraction is a solventless extraction and enrichment method based on sorption mechanisms for extracting the analytes from a liquid or gaseous matrix into a non-miscible liquid phase (*83, 84*). Nongonierma and coworkers (*85*) have extensively reviewed the effect of various parameters on the extraction of aroma compounds from foods using sorbents. The choice of an adsorbent is an important factor in determining the efficiency of extraction, including hydrophobicity of the analyte and the adsorbent, adsorbent structure, traps and fiber size. It is known that lipophilic volatiles have a higher affinity to the polymer (polysiloxanes of different polarities) coasted fused-silica fiber (*70*). These techniques can be categorized according to the types of adsorbent namely, open-tubular trapping (OTT), solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE) (*83, 84*).

The advent of static headspace (e.g. HS-SPME) and dynamic headspace (e.g. purge and trap) analyses have provided methodologies for understanding the relationships between the relative volatility of flavor compounds and the aroma perceived in different food matrices (*54, 86, 87*). Among these techniques, SPME has become a valuable tool in capturing volatiles from aqueous solutions or directly from the headspace (*70*). Furthermore, the HS-SPME technique can directly be combined with gas chromatography without

any modification of injection port. This is very direct and rapid as the SPME fiber is directly transferred and desorbed into the hot injection port.

Food matrix applications of HS-SPME include flavor analysis from a large variety of foods (*88, 89*); aroma emissions from plant branches or blossoms (*90, 91*); and pesticides from fruits and vegetables (*92*). For example, HS-SPME had been used to successfully identify potent aromatic chemicals by coffee origins and varieties (*93-95*). However, due to the mass transfer between SPME fibers and sample matrices that complicates the quantification and causes poor reproducibility of the measurements, major challenges remain in quantifying the amount of analytes extracted from complex sample matrices.

Dynamic headspace with trapping on a solid sorbent or in a cold trap is an alternative method for analysis of volatile compounds in foods (*86, 87*), airborne pollutants (*96*), and volatile organic compounds in water (*97, 98*). A purge-and-trap technique involves an inert carrier gas that is bubbled through a liquid sample while solid samples can be warmed by an electrical heater or microwave to increase the fugacity of volatile compounds. The stripped volatiles are then trapped on a solid or liquid sorbent, in a cold-trap or in a solvent. This step can be carried out in an open- or closed-loop. In an open-loop configuration, the non-trapped molecules are eliminated. In the closed-loop method, the gaseous phase flows through the sample and the trap in a closed circuit (*67*). After desorption onto a sorbent, the trapped compounds are desorbed by heating and then cryofocused at the head of the GC-column (*99*). Detailed reviews on every aspects of dynamic headspace have been reported (*67, 68, 100*).

In favor of acquiring values on the absolute amount of volatile compounds expressed, a quantitatively-based extraction method is required. HS-SPME might not be sufficiently comprehensive in quantitative analysis due to the selectivity and limited loading capacity of SPME sorbents, respectively (*83, 84*). Hence, this has led to developing more effective and versatile analytical methods to enhance the sensitivity and reproducibility with minimum discrimination of genuine volatile profiles.

With a larger volume of sorbent materials used, the sensitivity of SBSE and sample capacity could be remarkably increased as compared to SPME (*84, 101, 102*). Furthermore, SBSE has been widely applied in environmental (*84, 101-109*) and biomedical analyses (*104, 110, 111*). SBSE is also gaining acceptance in flavor analysis, not only volatile profiling in wine (*112-116*), beer (*117*), fruit juices (*118, 119*), vinegar (*120*) but also elucidation of the changes of volatile metabolites in an intra-oral odor investigation (*121*). Apart from the advanced development of different flavor isolation techniques, instrumental analyses are also important for identification and quantification work.

1.4. Instrumental methods of flavor analysis

As mentioned earlier, flavor analytical research has made giant strides due to the technological developments of analytical instruments in improved sensitivity and selectivity. Most work has focused on volatile flavor compounds using gas chromatography as they give food products their characteristic aroma, whereas the availability of liquid chromatography contributes to narrowing the gap between the knowledge of the spectrum of

volatiles and the structures of the non-volatile conjugates (*122*). The following discussion will focus mainly on chromatographic separation techniques and mass spectrometric techniques as detection techniques (*123*).

1.4.1. Chromatographic techniques

Gas chromatography (GC) has been the most common and established technique in flavor analysis. It involves the separation of volatile analytes, which are subsequently submitted to different kinds of detectors, e.g. flame ionization detector (FID) (*124*). Traditional GC instrumentation has been subjected to a number of advancements over the past years, one of them being the evolution of capillary column technology (*8, 125*). Various polar and nonpolar fused silica capillaries, which are now commercially available and offer exceptional flexibility and higher thermal stability, improved the separation capability (*125*). Single column (one-dimensional) chromatographic analysis has been the method of choice and a standard separation tool in a broad variety of applications including food and environmental analysis. It provides satisfactory separation and rewarding analytical results for samples of low to medium complexity and it has been capable of resolving 100 – 150 peaks in a single run (*126*).

Some terpenoids present in natural plant volatiles are chiral compunds, and either one of the two enantiomers or enantiomeric mixtures or, in case of more than one stereocenter, diastereomeric mixtures of both (*70*). Their proportions can be directly determined even from very complex mixtures by two-dimensional GC by transferring small sections of a GC peak from a conventional capillary column to an enantioselective capillary column (*11*).

Thus, heart-cutting two-dimensional GC-MS (2D GC-MS) can significantly improve the resolution of complex regions. Nevertheless, in certain cases, 2D GC-MS is not able to produce high quality mass spectra for the olfactory detected compounds (no peaks on the second dimensional total ion chromatogram (TIC) at the corresponding retention times), particularly when analyzing highly complex aroma compunds (*127*).

Enantioselective capillary columns with high separation efficiency were introduced in the mid-1960s (*124, 128*). It was intriguing to find that the presence of a certain ratio of enantiomers in the natural oil could exhibit different physiological properties especially in odor and taste (*71, 128*). Reviews have been published regularly in this field (*70, 71, 128*). Studies have shown that the enantiomeric composition of chiral compounds of essential oils may vary considerably depending on origins and processing conditions (*129, 130*).

The hyphenation of the chromatographic techniques to the different detecting instruments has proved highly successful in the resolution and identification of the molecules and further expands the capability of the chemical analysis of highly complex sample matrices (*124*).

1.4.2. Gas chromatography-olfactometry

Gas chromatography-olfactometry (GC-O) is designed to couple the enormous separation power of capillary gas chromatography with the unique selectivity and sensitivity of the human nose (*131-133*). The aroma contribution of each compound to a flavor is estimated with two possible ways, namely odor activity value (OAV) and relative flavor activity (RFA).

OAV is the ratio of concentration to the odor threshold of the compounds. It is generally accepted that the compounds with higher OAV contribute more to the food aroma. Alternatively, RFA is derived from the ratio of log FD factor to the square root of weight percentage of the compound. Several aroma evaluation techniques have been introduced to investigate aroma impact compounds of a food flavor systematically and they have been reviewed extensively (*32, 133-137*).

Generally, these techniques can be classified as dilution methods and intensity methods with the common goal of estimating the contribution of single volatiles to the overall aroma (*138, 139*). Dilution methods refer to the methods that produce quantitative estimates of relative potency for the aroma compounds of the diluted eluent of a gas chromatograph through successive dilutions such as CharmAnalysis™ or aroma extraction dilution analysis (AEDA). The main differences between CharmAnalysis and AEDA is that Charm measures the dilution value over the entire time the compounds elute (dilution value), whereas AEDA determines the dilution factor (FD), which is the last dilution at which an aroma-active compound is detected (*138*). In fact, the dilution value at the peak maximum in a Charm chromatogram is identical to the FD factor calculated on an AEDA basis (*135*). Because of its simplicity of use, AEDA method has been widely used to identify the key aroma components of *Citrus* (*140-144*).

On the other hand, odor-specific magnitude estimation (OSME) is the method based on time-intensity, which was developed to measure the perceived odor intensity of a compound eluting from a chromatographic column, with assessors sniffing the non-diluted extracts (*138, 139*). The main

difference between dilution methods and time–intensity methods is that the latter are not based on odor detection thresholds but on odor intensity (*135*). However, high variability within and between panelists could occur with both dilution methods and intensity methods (*139*). In order to verify the correct concentration and intensity of the flavor compounds, aroma models are prepared on the basis of the OAV and omission experiment are essential and validate the analytical results (*145*).

1.4.3. Mass spectrometric techniques

Mass spectrometry (MS) is a powerful analytical technique that measures the mass-to charge ratio of ions. In general, MS is applied to elucidating the composition of a sample by generating a mass spectrum representing the masses of the sample components (*146*). The ability to elucidate structural conformation from collected fragmentation patterns of analytes has been proven for identification of unknown compounds. MS is the most widely applied analytical platform in identifying volatile organic compounds, especially if it is hyphenated to chromatographic instrumentation. To date, thousands of volatile compounds have been discovered and correlated with specific odor attributes. However, GC-MS approach is time-consuming and identification is limited or difficult to interpret when there are several compounds in a single peak of recorded mass spectra (*124*). There are several possible solutions, such as tandem mass spectrometer (MS × MS) to couple with GC and allow the separation of each compound of such complex peaks.

In addition, latest developments in proton transfer reaction-mass spectrometry (PTR-MS) with soft ionization method allows quantitative on-

line monitoring of volatile organic compounds and provides flavor analysis in real-time. Hence, the fragmentation of the analyte molecule is very much reduced and the mass spectra produced are much easier to interpret. Yet, PTR-MS is a one-dimensional technique that characterizes compounds only via their mass, which is not sufficient for positive identification of the individual volatile organic compounds (*146*).

1.5. Sensory evaluation

"Sensory evaluation is the utilization of psychophysical techniques in the food industry for different purposes such as description, discrimination and affective/hedonics" (*2*). Flavors that we perceive is composed of complex volatile compounds that are present in concentrations above the sensitivity threshold (*134*). It should be noted that aroma evaluation techniques using GC-O based on odor threshold detections are functions of the odorants' concentrations in the extract and are not psychophysical measures for perceived odor intensity. This is because a relationship between odorant concentration and odor intensity is not straightforward. To understand the flavor quality of a product, it is no longer the sole aim to identify the concentrations of each individual compounds, but to evaluate the perceptual interactions of aroma-active compounds in mixtures as detected by the human nose (*137*).

Sensory evalutation methods can be classified into discriminative analysis and descriptive descriptive analysis (DA) is used to measure their ability to evaluate qualitative and quantitative characteristics of the product analysis. Discriminative analysis is a technique employed to detect differences

between the control and other products with trained panelists, whereas (*147*). Thus, DA is commonly used to map a product's perceived attributes for the purpose of relating them to instrumental, chemical or physical properties (*2*). It is distinguished from other sensory methods by profiling all of the product aspects or perceived sensory attributes, e.g. for quality control purposes and sensory mapping on market products. A detailed discussion of the descriptive analysis methods is contained in an American Society for Testing and Materials (ASTM) publication (*148*).

Generally, there are several DA methods. Flavor profiling (FP) was first technique to assess the flavor and aroma impression of food products. Profile attribute analysis (PAA) is an extension of FP, which incorportates numerical aspects of sensory description. Quantitative descriptive analysis (QDA) was subsequently developed to address the problem of quantifying sensory description. The spider plot is used as a graphical tool for presenting the QDA results. On the other hand, the spectral descriptive analysis (SDA) method was developed to analyze the data obtained from a line scale anchored on both ends. Unlike other DA, the free-choice profiling is a popular method, which uses untrained judges for evaluating products (*147, 149*). To facilitate the description, the perceived attributes can also be quantified by repeated measures and statistical analysis is generally conducted using analysis of variance (*150*). In addition, statistical approaches like multivariate analysis of the sensory data, with other instrumental data, allow identification and correlation of characteristic compounds with their sensory attributes. Recently, several methods have been offered as alternatives to DA and were reviewed by Valentin *et. al.* (*151*).

1.6. Statistical analysis

Proper experimental designs are important to maximize the information and ensure the validity of an experiment. Conventional approach in experimental design involves identifying various independent factors and levels, and later conducting the experiments by altering "one variable at a time" (OVAT), while keeping all others at a predetermined level is very inefficient as many experiments are required. Moreover, these OVAT designs often overlook the interactions among the variables.

In contrast, multivariate experimental design methods that allow the simultaneous study of several control variables could be useful in optimization (*44*). One of the experimental design tools namely, responses surface methodology (RSM), has been increasingly applied in optimization. RSM, comprising a group of mathematical and statistical techniques, is based on the best fit of empirical models to the experimental data, through which interactions among experimental factors also become evident (*152, 153*). With RSM, the number of test runs is minimized and the interaction among effects, which may influence the experiments, is taken into account.

Flavor is a multimodal sensory experience; it has been difficult to relate the concept of flavor with the chemical components of foods (*1*). To interpret complex data of citrus fruits, analysis of variance (ANOVA) is one of the most common statistical methods to assess the significance among various variables (e.g. cultivars and geographical origins), but these variables are normally interdependent and may interact with each other, which unavoidably leads to complications in extracting information (*154*). In order to gain a better insight into flavor perception, especially focusing on the correlation between

the chemical compositions and sensory perceptions (*29, 155-157*), chemometric is often employed to extract information from analytical data (*154*). Common methods are such as multivariate statistical analysis – principal component analysis (PCA), discriminant analysis (DA), partial least square (PLS) (*46, 157-159*).

As a basic multivariate, PCA has been used to describe the data set composed of sample mean scores as observations and chemical components as variables (*160*). PCA has been applied as a data exploration tool, which allows visualizing correlations in datasets by compressing information in a low number of dimensions. PCA has been applied as a data exploration tool, which allows visualizing correlations in datasets by compressing information in a low number of dimensions. An important step in PCA is the determination of the number of latent variables, which contain relevant information (*161*). However, the determination of which components in the PCA can be attributed to noise is not at all straightforward.

To assess the relationships between different origins, an adequate method should focus on between-group variability, while neglecting within-group variation. This is precisely the rationale of discriminant analysis (DA) (*162*). DA defines a model to summarize the origin differentiation between groups, while overlooking within-group variation. The method therefore achieves the best discrimination of individuals into pre-defined groups (*163, 164*). In comparison to PCA, CDA has some unique features especially with regard to its capability to separate classes (*164*), which could compensate for the limitation of PCA as an unsupervised method.

Partial least square regression (PLSR) tends to be used for extensive model building exercises with a flexibility of exploring the best combination of X-variables (chemical components) to produce a good prediction of the Y-data (intensity of flavor attributes) (*165*). It can be considered as a hybrid cross of multiple regression and PCA (*166*). Early references to its use in regard to sensory evaluation of foods include correlating instrument measurements to sensory meat quality data (*167*) and predicting the aged red wine aroma properties from aroma chemical composition (*168*). Furthermore, partial least-squares discriminant analysis (PLS-DA) provided evidence of the ability of the content of volatile compounds to discriminate among the different commercial categories of Sherry Brandies (*169*).

1.7. Exploration of authentic and indigenous citrus and coffee flavors in Asia

1.7.1. Pomelo (*Citrus grandis* (L.) Osbeck) and calamansi (*Citrus microcarpa*)

Citrus fruits have been the source of distinctive flavors that have been esteemed by people for centuries (*33*). In view of their economic importance and varied scope of applications, the composition of citrus fruits has received much attention (*170-172*). Citrus fruits are largely processed for the juice as well as for the essential oil. On the other hand, citrus peel constitutes a major part of the fruit but is of much less importance than juice (*132*). Only peel oil and pectin are important peel products for human consumption, perfumery, and cosmetics.

South and Southeast Asia is believed to be the place of origin of citrus fruits (*173*). Apart from the most traded varieties such as mandarins, oranges and lemons, Southeast Asia produces many uncommon citrus fruits with distinct characteristics that can provide infinite possibilities in the production of innovative and novel flavors. However, consumer exposure to citrus flavors tends to center on the common citrus fruits due to the limited types produced on a large scale for consumption as fresh fruits, or for production of juices and natural aroma chemicals. Hence, common citrus fruits have been extensively studied (*69, 132, 171, 174-177*). Recently, exotic citrus fruits have increasingly attracted attention worldwide due to their unique sensory attributes and health benefits (*178*). Until recently, only limited research has been undertaken to evaluate these exotic citrus such as Australian wild lime (*179*), Pontianak orange (*Citrus nobilis* var *microcarpa*) from Indonesia, Mosambi (*Citrus sinensis* var *mosambi*) from India and Dalandan (*Citrus reticulata*) from the Philippines (*180*). These obscure fruits (hybrids) can be utilized to develop flavors that are signature or differ organoleptically from leading citrus varieties and feature varying qualities depending on the time of year and maturation. The lack of knowledge about the chemical and aromatic profiles of these fruits may hamper their introduction into the flavor industry and subsequent commercialization.

Pomelo (*Citrus grandis* (L.) Osbeck) is a citrus fruit native to Southeast Asia and the Indo-China regions. Other names for pomelo include pummelo, pommelo, Chinese grapefruit, *limau bali* and *shaddock* (*33, 181*). Being the largest citrus fruit, pomelo is classified as one of the basal species of edible citrus and also believed to be an ancestor of grapefruit. The peel of pomelo

may be greenish-yellow or pale-yellow while the pulp varies from greenish-yellow or pale-yellow to pink or red (*33, 181*). The fruit is commonly eaten fresh; its taste varies from mildly sweet and bland to subacid or rather acid, and sometimes with a faint touch of bitterness (*182*). The main production areas of pomelo are southern China, southern Japan, Thailand, Vietnam, Malaysia and Indonesia. It is also cultivated in the United States of America (California and Florida), the Caribbean islands, and Africa (*34, 122, 140, 183, 184*).

Moreover, in view of growing interests in the consumption of mandarin-like fruits, hybridization in citrus species was obtained by artificially cross-breeding various mandarin-like species (*185, 186*). Considered to be a natural hybrid of mandarin and oval kumquat (*Citrus reticulate* x *Citrus japonica*), calamansi (*Citrus microcarpa*), also known as calamondin, limau kastuari, kalamondin and kalamansi, has spread throughout Southeast Asia, India, Hawaii, West Indies, Central and North America. The fruit resembles a small and round lime with an average diameter of up to 4.5 cm. It has the orange color of a tangerine with a very thin green or orange colored peel (*182, 187, 188*).

In contrast to major citrus fruits such as orange, lemon and lime, there is very little systematic study on the volatile compounds of pomelo and calamansi (*34, 140, 176, 183, 184*). Research on flavor profiles of Asian indigenous citrus shed new light on novel citrus flavors (*143, 189*).

1.7.2. Arabica coffee in Asia

Coffee is one of the most widely consumed beverages as seen from the rising trend of cafés offering coffee beverages of different origins, roasts and brews. Among the diverse varieties of coffee beans (from the *Rubiaceae* family), Arabica coffee (*Coffea arabica*) is highly esteemed with its superior quality and delightful taste; and accounts for two thirds of world coffee production (*190, 191*). The perception of coffee aroma and taste is dependent on the volatile as well as the non-volatile compounds present in the roasted coffee beans (*192, 193*), unique to cultivars, geographical regions and roasting conditions (*194*).

Similar to the classic Arabica coffee like Jamaican Blue Mountain and Hawaiian Kona, Asian varieties are valued for their full body and smooth mouthfeel while offering alternative choices to coffee lovers. The coffee market has been gradually expanding in Asia owing to an increase in the number of urban dwellers in the region. As the third largest coffee producer, there are more than 20 varieties of Arabica coffee being grown commercially in Indonesia. Typica is one of the original cultivars, which has been rated with good cup quality. Unfortunately, much of the Typica was lost in the late 1880s, when Coffee Leaf Rust swept through Indonesia. Bergandal and Sidikalang varieties of Typica cultivar from North Sumatra are a few that survived (*195*). Other countries have also begun producing their own coffee beans (*196-198*), where Yunnan is one of the major coffee bean producers in China while Thailand has introduced Arabica varieties into the existing Robusta coffee cultivation, in hope to improve the quality of its coffee exports. Agricultural and geographical variations are crucial to the aroma

differences among Arabica coffees. The soaring interest in these exotic and new coffees of different origins and processing methods has triggered the curiosity of flavor scientists for their volatile and aromatic profiles.

The quality of a cup of coffee is also dependent on the non-volatile composition and quality of the green coffee beans used; the compounds within (the coffee beans) reacting during the roasting process to produce the flavor of the drink (*191, 194*). Some of the non-volatile components are also important as a quality control indicator. Chlorogenic acid, for example, confers undesired astringency to the final cup of coffee if it is poorer in quality (*199, 200*). Also, the organic acid content of roasted coffee beans can be used as an indicator of the degree of roasting. It was found that darker roasts would give rise to a lower organic acid content (*63*).

On the other hand, health effects of coffee have always been controversial. Coffee is considered as a functional beverage with the potential health benefits due to antioxidant capabilities contributed by a diverse array of phenolic components. Phenolic acids are not only contribute to the acidity, bitterness and astringency (*201*), more importantly, they contribute to the radical scavenging capabilities. In addition, phenolic acids possibly contribute to neuroprotective effects which could prevent diseases like Alzheimer's disease (*202*). Hence, several studies have been done to determine the antioxidant capacity of coffee (*95, 202-204*).

Underlying the unique aroma of coffee is a profound complexity that involves more than 800 different chemical compounds (*60-63*). Although the extensive studies have been carried out, the determination of volatiles in roasted coffee bean is still a challenging task as many of the important

odorants are present in trace amounts and/or are reactive and unstable (*62, 65, 93, 205*). Furthermore, systematic assessment of volatile and non-volatile components in coffee that directly affect the cup quality of coffee in terms of flavor and nutritional value is of utmost importance. This is to minimize any discrepancy arising from the geographic factors and processing conditions.

1.8. Objectives and research outline

To explore the flavor potential and to characterize the varieties of indigenous citrus and Arabica coffee from the Asian region, much effort and work is still required. It is believed that the varieties of Asian citrus and coffee have distinct characteristics but are underutilized. Furthermore, classical solvent extraction or HS-SPME might not be comprehensive in flavor analysis. Hence, this has led to the need to develop more effective and versatile analytical methods to extend the detection range and to overcome the interferences from the complex matrix.

As an attempt to diversify the range of citrus flavors and promote the utilization of new coffee varieties from Asia, the main objective of this research was to characterize the volatile components and aroma profiles in citrus and Arabica coffee from Asian countries. Four work themes were defined – each with its own specific aims, which are: -

- To perform a complete characterization of both the volatile compounds and aromatic profiles of pomelo blossoms, peels and juices using different analytical techniques.
- To characterize and correlate the volatile and aromatic profiles of calamansi from Southeast Asia through multivariate analysis.
- To develop an extraction technique for complex food matrices such as citrus juice or simultaneous quantification of volatile compounds.
- To assess the flavor profile of Asian coffee based on volatile and non-volatile components using different extraction techniques and multivariate analysis.

1.9. Thesis outline

Chemical compositions and aromatic profiles of selected citrus and coffee are presented in this thesis. The main body of this thesis consists of a general introduction on research background and literature reviews and overall conclusion as well as recommendations for future studies. Detailed methodologies and results are arranged according to the following chapters:-

Chapter 2-4 are a series of study of Malaysian pomelo including blossoms, peels and juices regarding their chemical compositions, sensory profiles and exploration on the correlation between these chemical compositions and sensory profiles; assisted by Loke Xiu Qing as part of her Hons project.

Chapter 5 and 6 report the characterization of calamansi peels and juices from Southeast Asian countries using multivariate analyses assisted by Chong Zhi Soon, Sng Jingting as part of their Hons projects and Zhu Danping as part of her MSc Chemistry by coursework project.

Chapter 7 describes the development of stir bar sorptive extraction on dealing with the target analytes with highly diverse physicochemical properties (i.e. volatility and polarity) and disparate concentrations, assisted by Justin Lee Yong Kiang as part of his MSc Chemistry by coursework project.

Chapter 8 and 9 study on characterization of Arabica coffee from Asia and development of PLE technique in order to enhance the extraction yield and

achieve desirable aromatic profiles, assisted by Tong Jia Xin, Jeremy Ong Jian Ming and Alena Tan Ann Ann as part of their Hons projects.

CHAPTER 2 CHARACTERIZATION OF VOLATILE COMPOUNDS AND AROMA PROFILES OF MALAYSIAN POMELO (*CITRUS GRANDIS* (L.) OSBECK) BLOSSOM AND PEEL

2.1. Introduction

Citrus peel oil has received much attention for a long time due to their highly versatile application (*206, 207*). In the context of citrus blossom oils (e.g. neroli oil), their chemical compositions have been traditionally studied using blossom oils extracted via steam distillation (*171, 208*). In spite of the high recovery ratio and low cost of this approach, the heat applied during the process distorted or even deteriorated the original aroma of citrus blossoms. Since citrus blossoms are known for their fresh, pleasant and highly desirable aroma, there is much academic and commercial interest to identify and replicate the authentic aroma from intact citrus blossoms. However, information on the volatile constituents in intact citrus blossoms is scarcely reported. Literature research indicates that, to date, there have been only three reports on volatile compositions of intact citrus blossom (*90, 209, 210*). Of

these, Jabalpurwala *et al.* (*90*) mentioned that intact pomelo blossom produced the highest level of volatiles among the 15 citrus cultivars, particularly 1-hexanol and linalool.

Pomelo has been identified as one of Malaysia's top exported fruit commodities. The best pomelo in Malaysia is reputed to be from Tambun, which is well known for their juiciness and tart taste. According to Department of Agriculture Malaysia (*211*), the popular cultivars of pomelo are PO 51 (white-fleshed pomelo) and PO 52 (pink-fleshed pomelo). Both cultivars are seedless and similar in appearance with the medium-thick rind, and are slightly pebbly, with PO 52 is slightly larger than PO 51. The knowledge of the chemical compositions and sensory profiles of their peels as well as blossoms would lead to better understanding of two cultivars of Malaysian pomelo.

The aim of this work was to determine the volatile compositions of Malaysian pomelo blossoms and peels through HS-SPME/GC-MS analysis as well as to identify their key aroma profiles by sensory evaluation.

2.2. Experimental procedures

2.2.1. Pomelo materials

The blossoms and fruits of PO 51 (white pomelo) and PO 52 (pink pomelo) were collected from Tambun pomelo farm near Ipoh in the state of Perak, Malaysia in August 2009. Pomelo blossoms were either yellowish white or plain white, fragrant, solitary and around encompassed 2.5 cm in diameter. Only those blossoms at an early stage of budding development (determined by their partially opened petals) were picked to ensure uniform

sampling. Intact blossoms (including petal, pistil, stamen and a short pedicel) with the branch were cut together from the major stalk split, and were stored in a sealed icebox (around 5 °C) until further experiments were carried out within 12 hours.

The mature fruits of each cultivar were harvested, and stored in a fridge (around 5 °C). After the pomelo fruits were washed with deionized water and dried under room temperature, the flavedo part was peeled off using a titanium fine grater (Fresco, ON, Canada), and then immediately used.

2.2.2. HS-SPME sampling procedure

One intact pomelo blossom (~2 g) and the grated peel (~2 g) were individually placed into a 20-mL glass vial with PTFE-coated silicone septum. In order to achieve better extraction efficiency, HS-SPME extraction conditions were optimized as described elsewhere (*212*) in terms of fiber type, sample volume, salt addition, extraction temperature and time (data not shown). A 85 μm Carboxen/PDMS StableFlex (Supelco, Bellefonte, PA, USA) was selected for this study due to being effective in the extraction of volatiles (*212*). Default extraction temperature was typically set at 40 °C, while 60 °C was also employed for the examination of high-boiling volatile compounds. After incubation for 30 min, the SPME fiber was inserted in a GC injector for 5 min, where the analytes were rapidly desorbed from the fiber under splitless mode and then were transferred to a GC column for subsequent separation and detection. The composition of each volatile was taken as the relative percentage of FID peak areas.

2.2.3. GC-MS analysis

GC-MS analysis was carried out in an Agilent 6890N GC coupled with a flame ionization detector (FID) and a 5975 inert mass spectrometer detector (MSD) (Agilent Technologies, Palo Alto, CA, USA). The GC was equipped with the fused silica capillary column (60 m x 0.25 mm x 0.25 μm DB-FFAP, Agilent, Woodbridge, VA, USA). Helium was used as carrier gas at a flow rate of 1.2 mL/min. The injector was set at 250 °C. The GC oven temperature was programmed from 50 °C for 5 min, then was raised to 230 °C at 5 °C/min and held at this temperature for 60 min. FID temperature was set at 250 ˚C, and MSD was operated in the electron impact (EI) mode at 70 eV. Identification of the eluted compounds was achieved by matching the mass spectra against NIST 8.0 MS library (National Institute of Standards and Technology, Gaithersburg, MD, USA), and confirmed with Linear Retention Indices (*LRI*). *LRI* values on FFAP column were determined using two series of alkanes (C5–C20 and C21–C40) (Fluka, St. Louis, MO, USA) run under identical conditions.

All standard compounds used in the identification of volatile constituents are obtained from Firmenich Asia Pte. Ltd..

2.2.4. Sensory evaluation

Intact pomelo blossoms were evaluated using quantitative descriptive analysis by a panel of six assessors (3 females and 3 males) from Firmenich Asia and National University of Singapore. Panelists were mainly comprised of trained flavorists and were familiar with the procedure used to evaluate. Preliminary sensory evaluations were performed and sensory descriptors were

collected from terminology provided by Firmenich. After consensus among the panelists, the appropriate descriptive sensory terms were established. There were eight descriptors for intact pomelo blossoms: aldehydic, animalic, citrus blossom, floral, fresh, fruity, hay, waxy. Each panelist was presented with a 20-mL headspace vial containing one fresh blossom and the intensity of the attributes was rated using a 9-point scale from 0 (uncharacterized) to 8 (very strong). The results were averaged for each attribute and plotted on a spider web diagram.

2.3. Results and discussion

2.3.1. Volatile composition of pomelo blossoms

Common extraction methods for analyzing blossoms include steam distillation (*171*), dynamic and vacuum headspace sampling (*213*). In the early stage of sample preparation, pomelo blossom extracts were prepared in the manner of solvent extraction. However, the result indicated that the process of solvent extraction, somewhat deteriorated the aroma profiles of pomelo blossom extracts. Consequently, HS-SPME was applied to extract the volatiles of pomelo blossom. HS-SPME is a relatively simple and solvent-free extraction technique. Because of its sensitivity and reliability, HS-SPME was subsequently extended to flavor analysis of citrus blossoms (*90, 209, 210*).

Table 2.1 lists the volatile compositions of pink and white pomelo blossoms. The identified compounds were categorized into hydrocarbons, acids, aldehydes, alcohols, esters, and others; and the relative amounts of volatile compounds were expressed as peak area percentages of the total area in FID.

Table 2.1. Identifications of the volatile compounds and their relative GC peak area of Malaysian pomelo (*Citrus grandis* (L.) Osbeck, pink and white type) blossoms through HS-SPME analysis

Compounds	*LRI*		Pink pomelo	White pomelo	Identification
	FFAP	Ref			
Hydrocarbons					
iso-Prene	510	-	*tr*	*tr*	MS
α-Pinene[I,II]	1008	1007	0.03	0.09	MS, *LRI*[b], STD
Camphene	1039	-	*tr*	*tr*	MS , STD
β-Pinene[I,II]	1102	1113	0.12	0.30	MS, *LRI*[a], STD
Sabinene[I,II]	1104	1093	0.12	0.21	MS, *LRI*[b]
δ-3-Carene[I,II]	1112	1180	0.21	0.58	MS, *LRI*[a], STD
β-Myrcene[I,II]	1161	1158	0.10	0.11	MS, *LRI*[b], STD
α-Terpinene[I,II]	1181	1178	2.51	2.75	MS, *LRI*[a], STD
Limonene[I,II]	1188	1185	15.46	48.16	MS, *LRI*[b], STD
β-Phellandrene[I]	1198	1194	0.21	0.26	MS, *LRI*[a]
trans-β-Ocimene[I,II]	1246	1242	0.14	0.18	MS, *LRI*[a], STD
cis-β-Ocimene[II]	1251	1252	3.99	11.99	MS, *LRI*[a], STD
γ-Terpinene[I,II]	1266	1274	0.05	0.04	MS, *LRI*[a], STD
ρ-Cymene[I,II]	1291	1267	0.32	0.27	MS, *LRI*[b], STD
allo-Ocimene[II]	1295	-	1.13	1.59	MS, STD
Terpinolene[I,II]	1298	-	0.13	0.22	MS, STD
dehydro-ρ-Cymene	1323	-	0.11	0.05	MS
ρ-1,3,8-Menthatriene	1339	1375	0.14	0.05	MS, *LRI*[b]
4,8-Dimethyl-1,3,7 Nonatriene	1349	-	2.00	0.47	MS
Sabinene hydrate[c]	1488	1465	0.04	0.03	MS, *LRI*[a]
δ-Elemene	1502	-	*tr*	*tr*	MS
α-Copaene	1513	1536	*tr*	*tr*	MS, *LRI*[a]
β-Elemene	1540	-	*tr*	*tr*	MS
γ-Elemene	1625	1625	*tr*	*tr*	MS, *LRI*[a]
β-Caryophyllene[I,II]	1638	1657	0.08	0.12	MS, *LRI*[a], STD
α-Humulene	1669	-	0.01	0.01	MS,
β -Farnesene	1695	1711	0.03	0.01	MS, *LRI*[a], STD
Germacrene D	1704	1722	0.03	0.02	MS, *LRI*[a], STD
β-Bisabolene	1762	1788	0.06	0.08	MS, *LRI*[a]
α -Farnesene[II]	1772	1801	0.17	0.06	MS, *LRI*[a], STD
Squalene	2985	-	*tr*[*]	*tr*[*]	MS
Acids					
Acetic acid	1449	1451	*tr*	*tr*	MS, *LRI*[b], STD
Neric acid	2331	-	*tr*[*]	*tr*[*]	MS
Geranic acid	2383	-	*tr*[*]	*tr*[*]	MS
Myristic acid	2527	-	*tr*[*]	*tr*[*]	MS, STD
Palmitic acid	3091	-	*tr*[*]	*tr*[*]	MS, STD
Linoleic acid	3106	-	*tr*[*]	*tr*[*]	MS, STD
Oleic acid	3135	-	*tr*[*]	*tr*[*]	MS, STD
Alcohols					
Ethanol[II]	958	936	8.89	13.78	MS, *LRI*[b], STD
Hexanol[II]	1346	1351	0.01	0.01	MS, *LRI*[b], STD
trans-2-Hexenol	1401	-	-	0.02	MS, STD
Linalool[I,II]	1533	1540	56.53	9.17	MS, *LRI*[b], STD
α-Terpineol[I]	1699	1711	*tr*	*tr*	MS, *LRI*[a], STD
Citronellol[I,II]	1790	1762	0.21	0.25	MS, *LRI*[b], STD
Nerol[I,II]	1836	1825	0.36	1.48	MS, *LRI*[b], STD
Geraniol[I,II]	1847	1840	0.36	1.28	MS, *LRI*[a], STD
Benzyl alcohol[I]	1884	-	0.06	0.20	MS, STD
Carveol[**]	1899	-	0.03	0.06	MS

Table 2.1. (Cont'd)

2-Phenyl ethanol	1958	1903	*tr*	*tr*	MS, *LRI*[b], STD
Spathulenol	2014	-	*tr*	*tr*	MS
trans-Nerolidol[II]	2079	-	0.14	0.10	MS, STD
cis-Farnesol[I,II]	2396	2371	1.75	0.39	MS, *LRI*[a], STD
Phytol	2583	2593	*tr*	*tr*	MS, *LRI*[a]
Aldehydes					
Hexanal[I]	1058	1079	0.01	0.03	MS, *LRI*[b], STD
Benzaldehyde[I,II]	1532	1525	0.01	0.01	MS, *LRI*[a], STD
Phenylacetaldehyde[I,II]	1688	1706	*tr*	*tr*	MS, *LRI*[a], STD
Neral[I]	1718	1724	0.02	0.15	MS, *LRI*[a], STD
Geranial[I,II]	1742	1744	0.03	0.29	MS, *LRI*[a], STD
Perillic aldehyde	1832	-	0.01	0.03	MS, STD
Esters					
Ethyl acetate	926	889	0.03	0.01	MS, *LRI*[b], STD
Ethyl butanoate	1016	1028	0.04	-	MS, *LRI*[b], STD
Ethyl 2-methyl butanoate	1455	-	0.04	-	MS, STD
Hexyl 2-methyl butanoate	1665	-	0.05	0.05	MS, STD
Citronellyl acetate	1679	-	0.01	0.01	MS, STD
Methyl geranate	1732	-	0.32	0.35	MS, STD
Neryl acetate	1752	1742	*tr*	0.01	MS, *LRI*[a], STD
Geranyl acetate	1790	1771	0.01	0.01	MS, *LRI*[a], STD
Methyl benzoate	1812	-	-	*tr*[*]	MS, STD
Ethyl benzoate	1819	-	-	*tr*[*]	MS, STD
Methyl salicylate[II]	1828	-	0.01	0.01	MS, STD
Methyl cinnamate	2137	2056	*tr*[*]	*tr*[*]	MS, *LRI*[b], STD
Ethyl cinnamate	2189	2123	*tr*[*]	*tr*[*]	MS, *LRI*[b], STD
Methyl anthranilate[I,II]	2307	-	0.65	1.23	MS, STD
Ethyl anthranilate	2336	-	0.01	0.12	MS, STD
Others					
Dimethyl sulfide	846		*tr*	*tr*	MS, *LRI*[b], STD
Acetone[II]	870	-	*tr*	*tr*	MS, STD
6-Methyl-5-hepten-2-one[I,II]	1333	-	0.27	0.58	MS, STD
trans-Linalool oxide[I]	1440	-	*tr*	*tr*	MS, STD
2-Methyl furan[II]	1441	-	*tr*	*tr*	MS, STD
cis-Linalool oxide[I]	1468	-	*tr*	0.01	MS, STD
γ-Butyrolactone	1680	-	0.01	0.02	MS, STD
Carvone	1749	1715	*tr*	*tr*	MS, *LRI*[a], STD
Caryophyllene oxide	1992	1999	*tr*[*]	*tr*[*]	MS, *LRI*[a]
Phenol	2058	-	*tr*	*tr*	MS, STD
2-methoxy-4-vinylphenol	2253	-	*tr*	*tr*	MS
2,6-dimethoxy phenol	2307	2307	*tr*	*tr*	MS, *LRI*[a]
4-vinyl phenol	2450	-	*tr*[*]	*tr*[*]	MS, STD
Indole[I,II]	2507	-	0.96	0.86	MS, STD
Caffeine	3122	-	*tr*	*tr*	MS, STD
Total identified (%)			97.96	98.11	
Total peak area (all volatiles)			1.50E+09	2.39E+09	

Unless otherwise specified, HS-SPME extraction condition is 40 °C for 30 min.
[I] Compounds reported in Ref. (*171*); [II] Compounds reported in Ref. (*90*).
Identification method: MS= mass spectrum; Linear Retention Indices (*LRI*) are compared with references from standards (STD) or literature values (*LRI*[a] referred to the values in Ref. (*214*), *LRI*[b] referred to the values in Ref. (*215*)).
"-", not detected at 40 °C and 60 °C; *tr* trace level, GC peak area below 0.01%; [*]compounds detected only at HS-SPME extraction temperature of 60 °C for 30 min; [**] unknown isomer.

A total of 88 compounds were identified in the headspace volatiles of pink and white pomelo blossoms, accounting for 97.96% and 98.11% of total peak areas respectively. Jabalpurwala *et al.* (*90*) detected 70 volatile compounds in intact grapefruit, sweet orange, sour orange, mandarin, lemon, lime and pomelo blossoms, of which 33 volatiles were for pomelo blossom. The likely explanation is due to the elevated HS-SPME extraction temperature in the present study. For pomelo blossoms, the group of hydrocarbons comprised predominantly of limonene, α-terpinene and ocimene. Further, white pomelo blossom contained much higher level of limonene (48.16%) than pink pomelo blossom (15.46%). Jabalpurwala *et al.* (*90*) found that pomelo blossoms differed from all other citrus cultivars in the highest level (13.6%) of ρ-cymene, which was detected at low concentrations (<0.32%) in Malaysian pomelo cultivars. Moreover, an unusual acyclic C11 homoterpene, 4,8-dimethyl-1,3,7-nonatriene (DMNT), rarely found in citrus blossoms, was detected here. DMNT was reported to be emitted by many species following herbivore damage and utilized in their chemical communication system. Biosynthesis of DMNT has also been shown to proceed via *trans*-nerolidol (*216*).

Oxygenated compounds are more important for organoleptic quality. Linalool, significant in floral scent, is a typical terpene alcohol in citrus blossoms, and also appears to be major component of fragrant flowers, such as lavender, jasmine, and rose. As shown in Table 2.1, linalool is the most abundant terpene alcohol in pomelo blossoms, and the significantly higher content in pink pomelo blossom further differentiated this cultivar from white pomelo. Also, it is worth of note that the concentration of linalool was

inversely proportional to that of limonene in pomelo blossom. As reported by Jabalpurwala *et al.* (*90*), linalool and limonene were the volatiles that accounted for the maximum variance in the data among citrus cultivars. In addition, the chemical variability was also observed among the flower oil (neroli), leaf oil (petitgrain) and peel oils (bitter orange oil) of four sour orange provenances, which were submitted to the same pedoclimatic and horticultural conditions. Marked variability was reported within the sour orange group, mainly due to the high proportion of mutants, as also occurred in the entire groups and its readiness to produce hybrids either naturally or artificially (*208*). However, further investigation on the molecular basis of genetic differences that could link to distinctive biochemical characteristic between the pink and white pomelo is required. *Trans*-nerolidol, a floral, woody, and fruity compound, was the main sesquiterpene alcohol in pomelo blossoms. It was also detected at higher levels in pink pomelo blossom (1.75%). However, some other terpene alcohols, such as nerol, geraniol and citronellol, were found to be more abundant in white pomelo blossom. In addition, it appeared unusual that high concentrations of ethanol (13.78% and 8.89%, respectively) were measured in pink and white pomelo blossoms. Ethanol may be formed as an artifact during the process of storage and HS-SPME extraction. The reason for relatively high concentration is partly due to the high selectivity of Carboxen/PDMS fiber for ethanol (*212*).

Aldehydes, such as hexanal, benzaldehyde, perillic aldehyde and citral, are commonly found in citrus blossoms (*90, 171, 209, 210*). Among them, citral normally occurs as a mixture of its stereoisomers (geranial and neral), which are the characteristic compounds of lemon and lime. Geranial and neral

were detected at low amounts in both pomelo cultivars, and relatively higher in pink pomelo blossom.

Methyl anthranilate is a nitrogen-containing ester that naturally occurs in Concord grapes and was commonly detected in citrus blossoms (*90, 171, 210*). It is also proven as an avian repellent derived from natural flavorants (*217*). Compared to pink pomelo blossom, white pomelo blossom contained relatively higher levels of methyl anthranilate. Even more interestingly, methyl geranate was found in both pink and white pomelo blossoms (0.32% and 0.35%, respectively). This compound, with waxy, green and fruity-notes, is commonly found in blossoms of *Araceae, Arecaceae, Berberidaceae, Ericaceae, Orchidaceae, Passifloraceae, Ranunculaceae, Rosaceae, Verbenaceae* and *Zamiaceae* (*214, 218*). To my knowledge, this is the first time that methyl geranate has been reported in citrus blossoms.

Indole is typically reported as one of the distinctive traits of citrus blossoms, and both pomelo cultivars also contained a tiny amount of indole. Essentially, indole should give a delicate floral character, but it is always misunderstood as a fecalic chemical. This is mainly because most of commercially available indole has traces of skatole that has an extremely strong fecalic note. In plants, it was reported to be formed by direct cleavage of the tryptophan precursor, indole-3-glycerol phosphate (*218*).

2.3.2. Volatile composition of pomelo peels

Table 2.2 shows that in pink and white pomelo peels, 85 and 88 volatile compounds, accounting for 97.82% and 98.10% of total peak areas respectively. In contrast with pomelo blossoms, the main constituents in

pomelo peels were hydrocarbons, amounting for 98% of the total volatiles detected. Limonene is the most prominent, followed by α-pinene, β-myrcene and β-pinene. It was also found that some interesting chemicals (e.g. methyl geranate) detected in pomelo blossoms were absent in pomelo peels.

Further, a wide range of aliphatic aldehydes (C8 to C14) were found in pink pomelo peel, particularly octanal and decanal known as character-impact constituents of orange (*176*). On the other hand, α- and β-sinensal, imparting ripe citrus notes, were detected at trace amounts in white pomelo peel, and virtually absent in pink pomelo peel. Nootkatone is the most important aromatic of pomelo and grapefruit peel oils.

In this study, no nootkatone was detected at 40 °C. After the temperature was increased to 60 °C, nootkatone was measured at trace-level in white pomelo peel but not in pink pomelo peel. Minh Tu *et al.* (*184*) reported that the content of nootkatone in the pomelo peel oil could probably be used as a measurement of the fruit maturity, and so lag time was required for its development in the pomelo peel oil. Hence, under the present condition, it is possible that the pink pomelo peel was too fresh to reach a measurable level of nootkatone.

Table 2.2. Identifications of the volatile compounds and their relative GC peak area of Malaysian pomelo (*Citrus grandis* (L.) Osbeck, pink and white type) peels through HS-SPME analysis

Compounds	*LRI*		Pink pomelo	White pomelo	Identification
	FFAP	Ref			
Hydrocarbons					
α-Pinene[III,IV,V,VI]	1008	1007	0.39	0.38	MS, *LRI*[b], STD
α-Thujene[VI]	1033	1031	*tr*	*tr*	MS, *LRI*[a]
Camphene[III,IV,VI]	1039	-	0.01	*tr*	MS, STD
β-Pinene[III,IV,V,VI]	1102	1113	0.09	0.01	MS, *LRI*[a], STD
Sabinene[III,IV,V,VI]	1104	1093	0.01	0.01	MS, *LRI*[b]
δ-3-Carene[iv]	1112	1180	0.03	0.01	MS, *LRI*[a], STD
β-Myrcene[III,IV,VI]	1161	1158	0.36	0.16	MS, *LRI*[b], STD
α-Terpinene[III,IV,V]	1181	1178	*tr*	*tr*	MS, *LRI*[a], STD
Limonene[III,IV,V,VI]	1188	1185	96.07	96.90	MS, *LRI*[b], STD
β-Phellandrene[IV,VI]	1198	1194	0.03	0.03	MS, *LRI*[b]
trans-β-Ocimene[IV,VI]	1246	1242	0.01	0.01	MS, *LRI*[a], STD
cis-β-Ocimene[IV,VI]	1251	1252	0.07	0.05	MS, *LRI*[a], STD
γ-Terpinene[III,V,VI]	1266	1274	0.01	*tr*	MS, *LRI*[a], STD
ρ-Cymene[III,VI]	1291	1267	*tr*	*tr*	MS, *LRI*[b], STD
Terpinolene[III,IV,V,VI]	1298	-	0.03	0.03	MS, STD
Sabinene hydrate[VI]	1488	1465	*tr*	*tr*	MS, *LRI*[a]
δ-Elemene	1502	-	0.01	*tr*	MS
α-Copaene[III]	1513	1536	*tr*	*tr*	MS, *LRI*[a]
β-Elemene[III]	1540	-	*tr*	*tr*	MS
γ-Elemene	1625	1625	*tr*	*tr*	MS, *LRI*[a]
β-Cubebene[III,VI]	1631	-	0.05	0.01	MS, STD
β-Caryophyllene[III,IV,VI]	1638	1657	0.04	0.03	MS, *LRI*[a], STD
α-Humulene[IV]	1669	-	*tr*	*tr*	MS
β -Farnesene	1695	1711	0.04	0.03	MS, *LRI*[a], STD
Germacrene D[IV,VI]	1704	1722	*tr*	*tr*	MS, *LRI*[a], STD
β-Bisabolene	1762	1788	*tr*	*tr*	MS, *LRI*[a],
α -Farnesene	1772	1801	0.01	0.01	MS, *LRI*[a], STD
Acids					
Acetic acid	1449	1451	0.01	*tr*	MS, *LRI*[b], STD
Alcohols					
Hexanol	1346	1351	0.01	0.01	MS, *LRI*[b], STD
cis-3-Hexenol	1354	1389	*tr*	*tr*	MS, *LRI*[b], STD
trans-2-Hexenol	1401	-	0.02	0.03	MS, STD
Octanol	1527	-	*tr*	*tr*	MS, STD
Linalool[III,IV,V,VI]	1533	1540	0.10	0.08	MS, *LRI*[b], STD
4-Terpinenol[VI]	1631	-	*tr*	*tr*	MS, STD
α-Terpineol[III,IV,V,VI]	1699	1711	0.04	*tr*	MS, *LRI*[b], STD
Decanol	1765	-	*tr*	*tr*	MS, STD
Citronellol[III,IV]	1789	1762	0.02	*tr*	MS, *LRI*[b], STD
Nerol[III]	1836	1825	0.05	0.05	MS, *LRI*[b], STD
Geraniol[III,IV]	1847	1840	0.07	0.07	MS, *LRI*[b], STD
Dodecanol	1850	-	*tr*	*tr*	MS, STD
Carveol[**IV,V]	1899	-	*tr*	*tr*	MS
2-Phenyl ethanol	1958	1903	*tr*	*tr*	MS, *LRI*[b], STD
Perillic alcohol[IV]	2046	-	*tr*	*tr*	MS, STD

Table 2.2. (Con'd)

trans-Nerolidol[III,IV,VI]	2079	-	0.01	*tr*	MS, STD
Elemol[**III,V]	2120	-	0.02	0.02	MS, STD
cis-Farnesol	2396	2371	*tr*	*tr*	MS, *LRI*[a], STD
Phytol	2583	2593	0.03	0.02	MS, *LRI*[a]
Aldehydes					
Hexanal	1058	1079	0.01	-	MS, *LRI*[b], STD
Octanal[III,IV,V,VI]	1280	1280	0.01	-	MS, *LRI*[b], STD
Nonanal[III,V,VI]	1390	1385	*tr*	-	MS, *LRI*[b], STD
Citronellal[III,IV,V,VI]	1479	1485	*tr*	*tr*	MS, *LRI*[a], STD
Decanal[III,IV,V,VI]	1516	1497	0.03	-	MS, *LRI*[b], STD
Neral[III,IV,V,VI]	1718	1724	0.02	*tr*	MS, *LRI*[a], STD
Geranial[III,IV,V,VI]	1742	1744	0.05	0.01	MS, *LRI*[a], STD
Dodecanal[III,IV,V,VI]	1744	1788	*tr*	-	MS, *LRI*[a], STD
Tridecanal	1759	-	*tr*	-	MS
trans,trans-2,4-Decadienal	1829	1804	*tr*	-	MS, *LRI*[a], STD
Perillic aldehyde[III,IV,V,VI]	1832	-	*tr*	*tr*	MS, STD
Tetradecanal[V]	1955	-	*tr*	-	MS, STD
β-Sinensal[V]	2233	2249	-	*tr*	MS, *LRI*[a], STD
α-Sinensal[IV,V]	2334	-	-	*tr*	MS, STD
Esters					
Methyl acetate	851	856	0.01	0.01	MS, *LRI*[a], STD
Methyl octanoate	1394	1378	*tr*	*tr*	MS, *LRI*[a], STD
Ethyl octanoate	1431	-	*tr*	*tr*	MS, STD
Methyl nonanoate	1450	-	*tr*	*tr*	MS, STD
Octyl acetate[III]	1475	-	*tr*	*tr*	MS, STD
Methyl decanoate	1548	-	*tr*	*tr*	MS, STD
iso-Bornyl acetate	1594	-	*tr*	*tr*	MS, STD
Carvyl acetate	1738	-	*tr*	*tr*	MS, STD
Neryl acetate[IV]	1752	1742	0.01	*tr*	MS, *LRI*[a], STD
Geranyl acetate[III]	1790	1771	0.01	*tr*	MS, *LRI*[a], STD
Methyl benzoate	1812	-	*tr*	*tr*	MS, STD
Methyl salicylate	1828	-	*tr*	*tr*	MS, STD
Methyl anthranilate	2307	-	*tr*	*tr*	MS, STD
Ethyl anthranilate	2336	-	*tr*	*tr*	MS, STD
Others					
Acetone	870	-	*tr*	*tr*	MS, STD
trans-Linalool oxide[IV]	1440	-	*tr*	*tr*	MS, STD
cis-Linalool oxide[IV]	1468	-	*tr*	*tr*	MS, STD
Carvone[V]	1749	1715	0.01	0.01	MS, *LRI*[a], STD
Caryophyllene oxide	1992	1999	*tr*	*tr*	MS, *LRI*[a], STD
Phenol	2058	-	*tr*	0.01	MS, STD
2-methoxy-4-vinylphenol	2253	-	*tr*	*tr*	MS
Osthole[VI]	2355	-	*tr*[*]	*tr*[*]	MS
3-ethyl phenol	2425	-	*tr*	*tr*	MS, STD
4-vinyl phenol	2450	-	*tr*	*tr*	MS, STD
Indole	2507	-	*tr*	*tr*	MS, STD
Nootkatone[III,IV,V,VI]	2568	-	-	*tr*[*]	MS, STD
Total identified (%)			97.82	98.10	
Total peak area (all volatiles)			3.90E+10	5.00E+10	

Unless otherwise specified, HS-SPME extraction condition is 40 °C for 30 min.

[III] Compounds reported in Ref. (*34*); [IV] Compounds reported in Ref. (*184*); [V] Compounds reported in Ref. (*122*); [VI] Compounds reported in Ref. (*219*).

Identification method: MS= mass spectrum; Linear Retention Indices (*LRI*) are compared with references from standards (STD) or literature values (*LRI*[a] referred to the values in Ref. (*214*), *LRI*[b] referred to the values in Ref. (*215*)).

"-", not detected at 40 °C and 60 °C; *tr* trace level, GC peak area below 0.01%; [*]compounds detected only at HS-SPME extraction temperature of 60 °C for 30 min; [**] unknown isomer.

2.3.3. Sensory evaluation

Compared to instrumental analysis, sensory evaluation provides insight into aroma profile of pomelo. The sensory profile of intact pomelo blossoms was described with eight attributes. The results are summarized in a spider diagram (Figure 2.1). Among the eight attributes, floral was evaluated as the most dominant note of both intact pomelo blossoms, followed by animalic and citrus blossom note. In additional, hay was the weakest note in both pomelo blossoms. Compared to white pomelo blossom, pink pomelo blossom exhibited higher scores of these two notes. However, white pomelo blossom was rated higher in fresh, fruity, aldehydic and waxy attributes than pink pomelo blossom. More significantly, white pomelo blossom had stronger citrus blossom attribute. This is in agreement with the analytical findings that pink pomelo blossoms had higher amount of linalool, *trans*-nerolidol and indole. On the other hand, volatiles namely limonene, citral, nerol and geraniol were found dominant in white pomelo blossoms represent the citrus attribute.

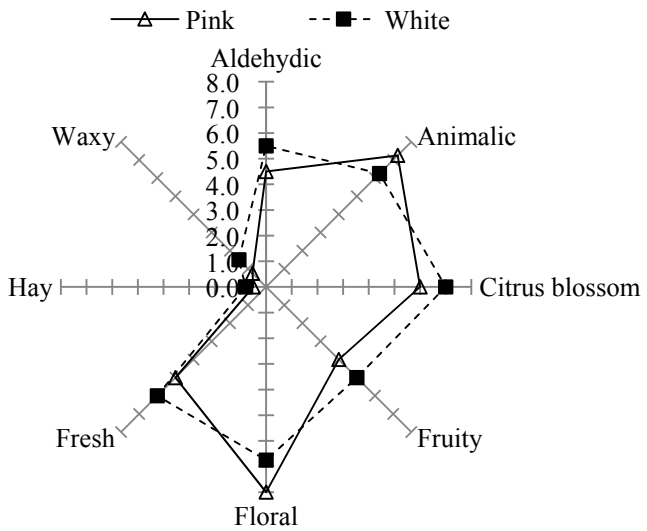


Figure 2.1. Sensory profile of intact Malaysian pomelo (*Citrus grandis* (L.) Osbeck, pink and white type) blossoms: Pink pomelo blossom; White pomelo blossom

2.4. Conclusion

The knowledge of the chemical compositions and sensory profiles of their blossoms and peels could lead to better understanding of two cultivars of Malaysian pomelo. This chapter was to determine the volatile compositions of Malaysian pomelo blossoms and peels through HS-SPME–GC/MS analysis and to identify the key aroma profiles of pomelo blossoms by sensory evaluation. Pink and white pomelo blossoms contained similar volatiles, which mainly consisted of limonene, *cis*-β-ocimene, α-terpinene, linalool, methyl anthranilate, and indole. The primary difference was in the concentration ratio between limonene and linalool. Major volatiles in pomelo peels were terpene hydrocarbons. Pink pomelo peel contained higher levels of aldehydes (e.g. octanal, decanal and citral), while some trace-level important compounds (e.g. b-sinensal, a-sinensal and nootkatone) were found only in white pomelo peel. Through sensory evaluation, both intact pomelo blossoms had similar aroma profiles, which comprised the dominant note of floral, followed by animalic and citrus blossom. The applicability of HS-SPME/GC-MS to measure volatile compounds in two cultivars of Malaysian pomelo blossom and peel was demonstrated. While the information on pomelo blossom would enable better comparison between two cultivars of Malaysian pomelo, the study of pomelo peel itself may serve the purpose of characterization and will be further explored in next chapter.

CHAPTER 3 IDENTIFICATION OF AROMA-ACTIVE COMPOUNDS IN MALAYSIAN POMELO (*CITRUS GRANDIS* (L.) OSBECK) PEEL BY GAS CHROMATOGRAPHY-OLFACTOMETRY

3.1. Introduction

Malaysian pomelo has a unique and strong aromatic peel oil, which could be utilized in both flavor and fragrance industries but has not been systemically studied. In the previous chapter, the volatile compounds and aroma profiles of Malaysian pink and white pomelo blossoms and peels were extracted using HS-SPME and analyzed through GC-MS/FID. The volatile compositions of both cultivars were quite similar but differed in intensity. In addition, it was observed that the pink pomelo peel possessed a stronger citrus, green and grapefruit-like note than the white pomelo peel. Nonetheless, it was difficult to reconstitute pomelo flavor due to the complexity of original pomelo aroma. Therefore, the aims of this chapter were to isolate the volatile compounds in Malaysian pomelo pink and white peels using solvent extraction, and determine their aroma-active compounds by aroma extract

dilution analysis (AEDA), and reconstruct the aroma models with these identified compounds.

So far, to my knowledge, only one study reported the utilization of GC-O to evaluate aroma-active compounds in Tosa-buntan (*Citrus grandis* Osbeck forma *Tosa*), the most popular pomelo cultivated in Japan (*140*). From the result, flavor dilution (FD) factor can be determined. Finally, the synthetic blends of odorants (aroma models) can be prepared based on the detected concentration of aroma-active compounds. Aroma reconstruction is made to compare the resemblance of the synthetic mixture to original flavor.

3.2. Experimental procedures

3.2.1. Preparation of pomelo peel extracts

Malaysian pink and white pomelos were collected as described in Chapter 2. Peels of white pomelo (3.8 kg) and pink pomelo (4.1 kg) were removed with a yield of 362 g and 370 g respectivley, and immediately covered with 1.0 L dichloromethane (Merck, Darmstadt, Germany). The mixtures were kept at 5 °C for four days, and stirred by vortex (TT3 digital, IKA, Staufen, Germany) for 30 min daily. After this, the mixtures were subsequently filtered through anhydrous sodium sulphate (Na_2SO_4, Sigma-aldrich). Finally, the filtrates were concentrated using a TurboVap II (Caliper Life Science, Hopkinton, MA, USA) to 0.5 mL. Both peel extracts thus prepared were dark green. The extracts were stored at 5 °C, and their quantity and quality were examined by GC-MS before sensory evaluation and sniffing test.

3.2.2. GC-MS/FID analysis

GC-MS/FID analysis was carried out as described in Chapter 2 (Section 2.2.3). For the quantification of pomelo peel extracts, 1 μL of pomelo peel extracts were spiked in 1 μL 5-methyl-2-hexanone (100 ppm in dichloromethane, Sigma-Aldrich) as internal standard (IS) and directly injected by an autosampler (Gerstel Multi Purpose Sampler, Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) under splitless mode. The percentages were obtained from the FID area corrected with the use of the response factors, previously measured with standard compounds under the same conditions. The response factor RRFs were calculated as: RRF = $(Mass_{compound} \times Area_{IS}) / (Mass_{IS} \times Area_{compound})$, where $Mass_{compound}$ and $Area_{compound}$ are the mass and corresponding GC peak area of the compounds, $Mass_{IS}$ and $Area_{IS}$ are the mass and GC peak area of the internal standard. For the commercially unavailable components, the RRFs were assumed to be 1.00 in the present study. Identification of the eluted compounds was achieved by matching the mass spectra against NIST 8.0 MS library.

All experiments were carried out at least in triplicate. Results were reported as mean values.

3.2.3. Sensory evaluation

A set of coded smelling strips of pomelo peel extracts was evaluated by a panel of six assessors (3 females and 3 males) as explained in Chapter 2 (Section 2.2.4). The selected attributes for pomelo peel extracts were: aldehydic, citrus, fresh, fruity, grapefruit, green, peely, woody.

3.2.4. Gas chromatography-olfactometry (GC-O)

GC-O instrument comprised a Shimadzu GC-MS QP 5000 with the olfactometer ODO II (SGE, Ringwood, VIC, Australia). The separation of volatile compounds was performed in the fused silica capillary column (60 m x 0.25 mm x 0.25 μm DB-FFAP) coated with 0.25 μm film thickness of polyethylene glycol modified with nitroterephthalic acid. Helium was used as carrier gas at a flow rate of 1.2 mL/min. The temperature of injector was 250 °C. Each sample was injected in 1 μL volumes in a split mode with ratio of 20: 1. The GC oven temperature was programmed from 50 °C for 5 min, then was raised to 240 °C at 5 °C/min and held at this temperature for 20 min. MS was operated in the electron impact (EI) mode at 70 eV. The outlet of the column was split into two ways using splitter and routed by deactivated fused silica capillaries to the mass detector and sniffing port, respectively. The sniffing port was always heated with a flexible heater to avoid condensation of the volatile materials in the glass tube. The carrier gas stream for sniffers was moistened at the outlet for sniffing to prevent the nose from drying out.

Sniffing test is used to describe the aroma character of each component. The sensory panel was composed of four well-trained flavorists (2 males and 2 females). The peel extracts were stepwise two-fold diluted with dichloromethane. All dilutions were sniffed twice by the panel until no odor was detected. The odor descriptors used here were based on Flavornet (*220*) and Leffingwell's Flavor-Base (*221*). AEDA method was performed to determine flavor dilution (FD) factor, which is the highest dilution at which an individual compound could be detected. Furthermore, to determine the relative contribution of each compound to the aroma of pomelo peel extracts, odor

activity value (OAV) and relative flavor activity (RFA) were calculated. The FD-factor was expressed by powers of two (2^n), and the RFA was calculated as log 2^n/(weight percentage of the compound %)$^{0.5}$. In order to obtain OAV, the concentration of the compound was calculated from its GC-FID peak with internal standard, while the corresponding odor threshold was referred to the published literature (*221-224*).

3.2.5. Aroma model

The blends of synthetic mixture (aroma models) were prepared with the aroma-active compounds identified in pink and white pomelo, respectively (as shown in Table 3.2 and 3.3) except *trans*-epoxy-ocimene, α-copaene and carveol. Triacetin was selected as a diluent for the aroma model. These models were presented to the panel to rate the following attribute intensity that used a 9-point scale from 0 (uncharacteristic) to 8 (very strong) for aldehydic, citrus, fresh, fruity, grapefruit, green, peely, and woody attributes. The results were then averaged for each attribute and plotted on a spider web diagram.

All standard compounds used in the identification and aroma model are obtained from Firmenich Asia Pte. Ltd..

3.3. Results and discussion

3.3.1. Volatile composition of pomelo peel extracts

Pomelo peel extracts were carefully prepared by solvent extraction and these extracts were examined by GC-MS/FID. A total of 96 compounds were identified as potential aroma-active compounds and listed in Table 3.1.

Table 3.1. Identifications of the volatile compounds and their relative GC peak area of Malaysian pomelo (*Citrus grandis* (L.) Osbeck, pink and white type) peel extracts

Compounds	*LRI*		Pink pomelo	White pomelo	Identification
	FFAP	Ref			
Hydrocarbons					
α-Pinene[III,IV,V,VI]	1008	1007	0.16	0.10	MS, *LRI*[b], STD
α-Thujene[VI]	1033	1031	*tr*	0.01	MS, *LRI*[a]
Camphene[III,IV,VI]	1039	-	*tr*	0.01	MS, STD
β-Pinene[III,IV,V,VI]	1102	1113	0.05	0.02	MS, *LRI*[b], STD
Sabinene[III,IV,V,VI]	1104	1093	*tr*	0.01	MS, *LRI*[b]
δ-3-Carene[IV]	1112	1180	*tr*	0.01	MS, *LRI*[a], STD
β-Myrcene[III,IV,VI]	1161	1158	0.06	0.03	MS, *LRI*[b], STD
α-Terpinene[III,IV,V]	1181	1178	0.01	0.04	MS, *LRI*[a], STD
Limonene[III,IV,V,VI]	1188	1185	93.11	95.40	MS, *LRI*[b], STD
β-Phellandrene[IV,VI]	1198	1194	0.03	0.07	MS, *LRI*[b]
trans-β-Ocimene[IV,VI]	1246	1242	0.01	0.01	MS, *LRI*[a], STD
cis-β-Ocimene[IV,VI]	1251	1252	0.01	0.03	MS, *LRI*[a], STD
γ-Terpinene[III,V,VI]	1266	1274	0.03	0.02	MS, *LRI*[a], STD
ρ-Cymene[I,VI]	1291	1267	0.02	0.04	MS, *LRI*[b], STD
allo-Ocimene	1295	-	*tr*	*tr*	MS, STD
Terpinolene[III,IV,V,VI]	1298	-	0.03	0.03	MS, STD
dehydro-ρ-Cymene	1323	-	*tr*	*tr*	MS
ρ-1,3,8-Menthatriene	1339	1375	*tr*	*tr*	MS, *LRI*[b]
Sabinene hydrate[VI]	1488	1465	*tr*	0.01	MS, *LRI*[a]
δ-Elemene	1502	-	0.10	0.10	MS
trans-epoxy-Ocimene	1508	-	-	0.01	MS, STD
α-Copaene[III]	1513	1536	*tr*	0.01	MS, *LRI*[a]
β-Elemene	1540	-	0.04	0.03	MS
γ-Elemene	1625	1625	0.05	0.03	MS, *LRI*[a]
β-Cubebene[III,VI]	1631	-	0.06	0.04	MS, STD
β-Caryophyllene[III,IV,VI]	1638	1657	0.16	0.07	MS, *LRI*[a], STD
α-Humulene[IV]	1669	-	0.03	0.01	MS
β-Farnesene	1695	1711	0.04	0.03	MS, *LRI*[a], STD
Germacrene D[IV,VI]	1704	1722	0.16	0.05	MS, *LRI*[a], STD
β-Bisabolene	1762	1788	*tr*	0.01	MS, *LRI*[a]
α-Farnesene	1772	1801	0.01	0.02	MS, *LRI*[a], STD
Squalene	2985	-	*tr*	*tr*	MS
Acids					
Acetic acid	1449	1451	0.01	0.02	MS, *LRI*[b], STD
Hexanoic acid	1867	-	0.01	*tr*	MS, STD
Octanoic acid	2091	-	0.01	*tr*	MS, STD
Decanoic acid	2309	-	0.05	0.01	MS, STD
Neric acid	2331	-	*tr*	*tr*	MS
Geranic acid	2383	-	*tr*	*tr*	MS
Myristic acid	2532	-	0.13	0.06	MS, STD
Lauric acid	2533	-	0.18	*tr*	MS, STD
Palmitic acid	3091	-	0.93	0.67	MS, STD
Linoleic acid	3106	-	0.37	0.27	MS, STD
Oleic acid	3135	-	0.14	0.19	MS, STD
Alcohols					
Ethanol	958	936	*tr*	*tr*	MS, *LRI*[b], STD
Hexanol	1346	1351	0.02	0.04	MS, *LRI*[b], STD
cis-3-Hexenol	1354	1389	0.05	0.08	MS, *LRI*[b], STD
trans-2-Hexenol	1401	-	*tr*	0.01	MS, STD
Octanol	1527	-	0.07	0.01	MS, STD
Camphor	1529	-	*tr*	0.01	MS, STD
Linalool[III,IV,V,VI]	1533	1540	0.25	0.17	MS, *LRI*[b], STD
4-Terpinenol[VI]	1631	-	0.01	0.01	MS, STD
α-Terpineol[III,IV,V,VI]	1699	1711	0.17	0.14	MS, *LRI*[b], STD

Table 3.1. (Cont'd)

Decanol	1765	-	0.02	*tr*	MS, STD
Citronellol[III,IV]	1789	1762	0.01	*tr*	MS, *LRI*[b], STD
Nerol[III]	1836	1825	0.22	0.24	MS, *LRI*[b], STD
Geraniol[III,IV]	1847	1840	0.28	0.27	MS, *LRI*[b], STD
Dodecanol	1850	-	0.02	0.01	MS, STD
Benzyl alcohol	1884		*tr*	*tr*	MS, STD
Carveol[**IV,V]	1899	-	0.08	0.06	MS
2-Phenyl ethanol	1958	1903	0.01	0.01	MS, *LRI*[b], STD
Perillic alcohol[IV]	2046	-	0.04	0.04	MS, STD
trans-Nerolidol[III,IV,VI]	2079	-	0.01	0.01	MS, STD
Elemol[**III,V]	2120	-	0.11	0.10	MS, STD
cis-Farnesol	2396	2371	0.02	0.01	MS, *LRI*[a], STD
Phytol	2583	2593	0.11	0.01	MS, *LRI*[a]
Aldehydes					
Hexanal	1058	1079	0.06	-	MS, *LRI*[b], STD
Octanal[III,IV,V,VI]	1280	1280	0.04	-	MS, *LRI*[b], STD
trans-2-Heptenal	1349	1305	*tr*	-	MS, *LRI*[b], STD
Nonanal[III,V,VI]	1390	1385	0.01	-	MS, *LRI*[b], STD
Citronellal[III,IV,V,VI]	1479	1485	0.01	*tr*	MS, *LRI*[a], STD
Decanal[III,IV,V,VI]	1516	1497	0.17	-	MS, *LRI*[b], STD
Neral[III,IV,V,VI]	1718	1724	0.06	0.03	MS, *LRI*[b], STD
Geranial[III,IV,V,VI]	1742	1744	0.12	0.06	MS, *LRI*[b], STD
Dodecanal[III,IV,V,VI]	1744	1788	0.02	-	MS, *LRI*[a], STD
Tridecanal	1759	-	0.01	-	MS
trans,trans-2,4-Decadienal	1829	1804	*tr*	-	MS, *LRI*[a], STD
Perillic aldehyde[III,IV,V,VI]	1832	-	0.02	0.01	MS, STD
trans-2-Dodecenal	1883	-	*tr*	-	MS, STD
Tetradecanal[v]	1955	-	*tr*	-	MS
Esters					
iso-Bornyl acetate	1594	-	0.01	0.01	MS, STD
Carvyl acetate	1738	-	*tr*	*tr*	MS, STD
Neryl acetate[IV]	1752	1742	0.03	0.02	MS, *LRI*[a], STD
Geranyl aceate[III]	1790	1771	0.03	0.03	MS, *LRI*[a], STD
Methyl palmitate	2289	-	0.14	0.13	MS, STD
Ethyl palmitate	2376	-	*tr*	*tr*	MS, STD
Others					
Dimethyl sulfide	846	-	*tr*	*tr*	MS, STD
Acetone	870	-	*tr*	*tr*	MS, STD
Acetoin	1293	-	*tr*	*tr*	MS, STD
6-Methyl-5-hepten-2-one	1333	-	*tr*	*tr*	MS, STD
trans-Linalool oxide[IV]	1440	-	0.03	0.03	MS, STD
2-Methyl furan	1441	-	*tr*	*tr*	MS, STD
cis-Linalool oxide[IV]	1468	-	0.03	0.03	MS, STD
Carvone[III]	1749	1715	0.01	0.01	MS, *LRI*[b], STD
Caryophyllene oxide	1992	1999	*tr*	0.01	MS, *LRI*[a]
Carvacrol	2223	-	*tr*	-	MS, STD
Osthole	2355	-	0.20	0.17	MS
Total identified (%)			98.37	99.17	
Total peak area (all volatiles)			4.90E+10	1.60E+11	

[III] Compounds reported in Ref. (*34*); [IV] Compounds reported in Ref. (*184*); [V] Compounds reported in Ref. (*122*); [VI] Compounds reported in Ref. (*219*).

Identification method: MS= mass spectrum; Linear Retention Indices (*LRI*) are compared with references from standards (STD) or literature values (*LRI*[a] referred to the values in Ref. (*214*), *LRI*[b] referred to the values in Ref. (*215*)).

"-", not detected; *tr* trace level, GC peak area below 0.01%; [**] unknown isomer.

The identified volatile compounds were classified according to their functional groups: hydrocarbons, alcohols, aldehydes, acids, esters and others, as described in previous chapter. Pomelo peel extracts were then injected into GC-O for sniffing test, and their aroma-active compounds were determined by AEDA method.

Following this, sensory evaluation was carried out for pomelo peel extracts. Figure 3.1 showed that pink pomelo peel extract was ranked highly for its grapefruit note at a score of 7.0, followed by green (6.0), citrus (6.0), aldehydic (5.0) and fruity (5.0) notes. The score for woody note was the weakest, being only 3. These attributes complied with the analytical results that pink pomelo peel have higher amount of citral, β-pinene, acetate ester, aliphatic aldehydes. On the other hand, white pomelo peel had a well-balanced aroma profile, mainly comprised of fresh (5.5), woody (5.0) and peely (5.0) notes.

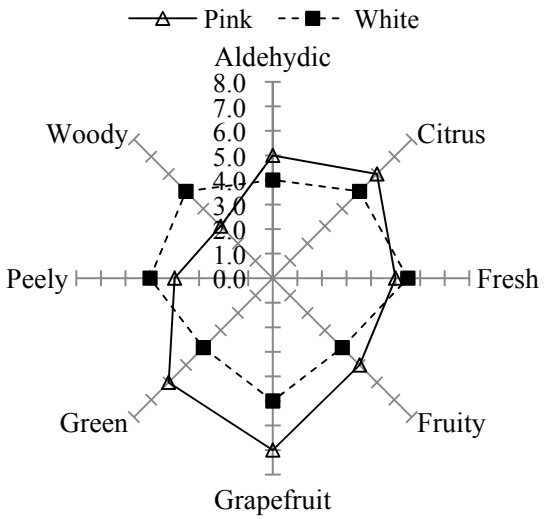


Figure 3.1. Sensory profile of Malaysian pomelo (*Citrus grandis* (L.) Osbeck, pink and white type) peel extracts: Pink pomelo peel extract; White pomelo peel extract

A GC-MS chromatogram together with an aromagram is shown in Figure 3.2. It clearly indicates that there is a significant difference between the responses of GC and sensory evaluation. Since volatile compounds exhibit various aroma activities, the potency of volatiles could be easily misinterpreted due to their absolute concentration (*142*). A trace-level potent aroma compound in GC chromatogram, cis,trans-2,6-nonadienal (peak 41), was detected as one of the most dominant peaks in the aromagram.

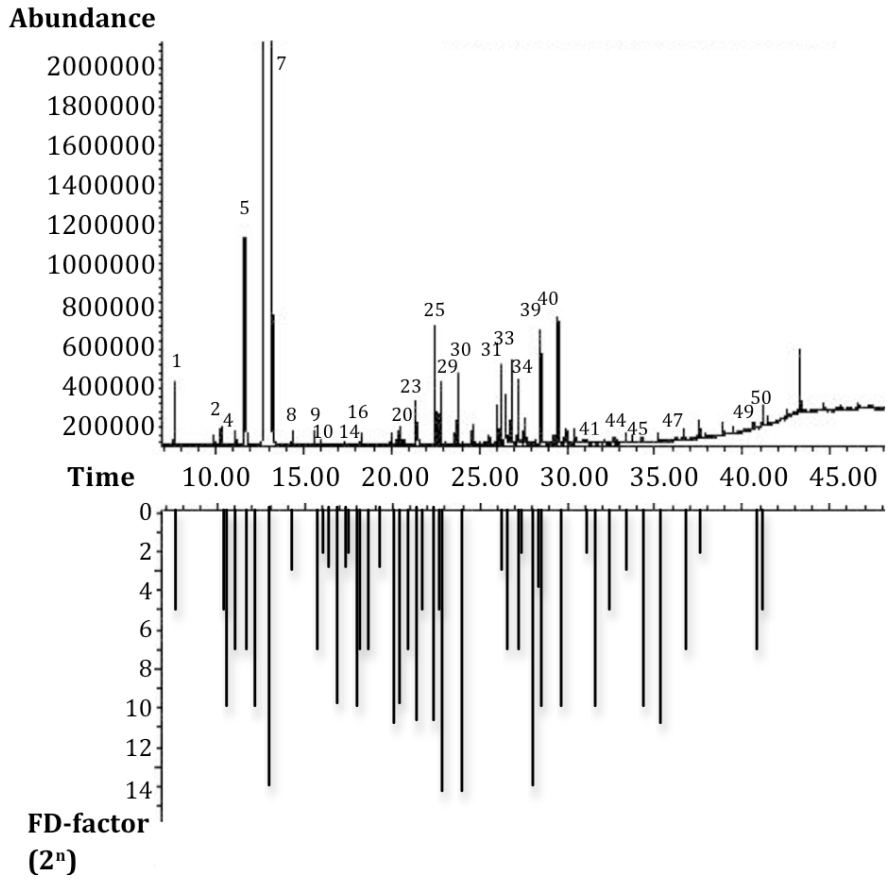


Figure 3.2. GC-MS chromatogram (top) and aromagram (bottom) attained by performing the AEDA on Malaysian pomelo peel extract

Labeled peaks: (1) α-Pinene; (2) β-Pinene; (4) Unknown; (5) β-Myrcene; (7) Limonene; (8) *trans*-β-Ocimene; (9) Octanal; (10) ρ-Cymene; (14) Hexanol; (16) *cis*-3-Hexen-1-ol; (20) *cis*-Linalool oxide; (23) Decanal; (25) Linalool; (29) β-Caryophellene; (30) α-Terpineol; (31) Germacrene D; (33) Carvyl acetate; (34) Geranial; (39) Nerol; (40) Geraniol; (41) *trans*-2-Dodecenal; (44) α-Farnesol; (45) *trans*-Nerolidol; (47) Carvacrol; (49) Indole; (50) Nootkatone

Tables 3.2 and 3.3 show that 50 and 47 aroma-active compounds were detected in pomelo pink and white peel extracts respectively. It is noted that in general, compounds that had high FD factor also had high RFA. On the other hand, since OAV often depends on the literature values for odor threshold, which are always very uncertain, the application of OAV may be discrepant due to variations of operation conditions. In this study, the concept of RFA could be used as an alternative to identify potent aroma-active compounds, and was created to compensate the limitations of OAV in the identification of the important aroma-active compounds.

In the pink pomelo peel extract, the compounds that exhibited strongest aroma activity (in decreasing order of RFA) were: *trans*-linalool oxide, *cis,trans*-2,6-nonadienal, octanal, citronellal, nonanal, *trans*-nerolidol, neryl acetate, indole, 6-methyl-5-hepten-2-one, terpinolene, *trans*-2-heptenal, perilla alcohol and nootkatone. The counterparts in the white pomelo peel extract were: terpinolene, α-copaene, 4-terpinenol, *trans*-nerolidol, *trans*-linalool oxide, nootkatone, *cis,trans*-2,6-nonadienal, citronellol, elemol, carvyl acetate, 6-methyl-5-hepten-2-one, perilla aldehyde and indole.

Hydrocarbon terpenes, such as limonene, α-pinene, β-pinene, sabinene, myrcene, *trans*-β-ocimene, were the predominant volatiles of Tosa-buntan pomelo peel extracts. However, most of them do not contribute significantly to aroma perception of pomelo peel extracts due to their high odor thresholds. Although limonene shows the highest FD-factor, it is not the key flavor compound in the both pomelo peel extracts due to a relatively low RFA value. Nevertheless, limonene is a necessary component of the following pomelo peel odor model.

Table 3.2. Aroma-active compounds with odor description identified in Malaysian pink pomelo peel extract achieved by means of GC-O

No.	*LRI* [a]	Compound[b]	Odor description[c]	Conc (ppm)	FD[d]	Odor threshold[e] (ppm)	OAV	RFA	Identification[f]
1	1010	α-Pinene	Terpenic, woody	837.5	32	0.19^{II}	4408	5.2	Odor, MS, STD, *LRI*[b]
2	1105	β-Pinene	Terpenic, woody	202.2	32	1.5^{II}	135	10.6	Odor, MS, STD, *LRI*[b]
3	1112	Unknown	Fermented, sulfurous	*tr*	1024	*u*	-	-	Odor
4	1116	Unknown	Tropical, sulfurous	*tr*	128	*u*	-	-	Odor, MS, STD
5	1164	β-Myrcene	Herbaceous, woody	6121.8	128	0.1^{II}	61218	2.7	Odor, MS, STD, *LRI*[b]
6	1169	Unknown	Nutty, pandan	*tr*	1024	*u*	-	-	Odor
7	1190	Limonene	Citrus, terpenic	299823.1	16384	0.2^{II}	1499115	0.8	Odor, MS, STD, *LRI*[b]
8	1249	*trans*-β-Ocimene	Green, terpenic	104.0	8	0.34^{II}	306	8.9	Odor, MS, STD, *LRI*[a]
9	1285	Octanal	Aldehydic, peely	20.8	128	0.082^{II}	253	46.2	Odor, MS, STD, *LRI*[b]
10	1295	ρ-Cymene	Oxidized lemon, woody	15.1	4	0.12^{II}	126	15.5	Odor, MS, STD, *LRI*[b]
11	1302	Terpinolene	Lime, terpenic	11.4	8	0.41^{II}	28	26.8	Odor, MS, STD, *LRI*[a]
12	1305	Unknown	Green, hazelnut-like	*tr*	1024	*u*	-	-	Odor
13	1336	6-Methyl-5-hepten-2-one	Green, lemony	10.1	8	1^{I}	10	28.5	Odor, MS, STD
14	1347	Hexanol	Fresh, green	35.6	4	3.125^{I}	11	10.1	Odor, MS, STD, *LRI*[b]
15	1350	*trans*-2-Heptenal	Fatty, intense green	17.3	1024	0.013^{III}	13315	22.9	Odor, MS, STD, *LRI*[b]
16	1359	*cis*-3-Hexen-1-ol	Fresh, leafy green	135.2	128	3.625^{I}	37	18.1	Odor, MS, STD, *LRI*[b]
17	1396	Nonanal	Aldehydic, citrus	23.8	128	0.1^{I}	238	43.2	Odor, MS, STD, *LRI*[b]
18	1405	*trans*-2-Hexen-1-ol	Green, leafy	20.2	8	4.25^{I}	5	20.1	Odor, MS, STD
19	1446	*trans*-Linalool oxide	Floral, tea-like	37.0	2048	0.32^{I}	1094	55.5	Odor, MS, STD
20	1472	*cis*-Linalool oxide	Floral, tea-like	350.0	1024	0.32^{I}	116	16.1	Odor, MS, STD
21	1479	Octyl acetate	Aldehydic, floral	34.9	2	0.45^{I}	78	5.1	Odor, MS, STD
22	1483	Citronellal	Green, lemongrass	22.2	128	0.046^{I}	482	44.7	Odor, MS, STD, *LRI*[a]
23	1519	Decanal	Aldehydic, citrus	678.0	2048	0.07^{I}	9686	12.7	Odor, MS, STD, *LRI*[b]
24	1532	Octanol	Orange, waxy	545.5	32	0.875^{I}	623	6.4	Odor, MS, STD, *LRI*[b]
25	1536	Linalool	Floral, woody	832.3	2048	0.028^{I}	29725	11.5	Odor, MS, STD, *LRI*[b]
26	1598	iso-Bornyl acetate	Camphoraceous, woody	232.2	2	1.38^{I}	168	2.0	Odor, MS, STD
27	1609	Fenchol	Camphoraceous, lime	496.0	32	1.2^{I}	413	6.8	Odor, MS, STD
28	1642	ρ-Menthene-8-thiol	Grapefruit, sulfurous	*tr*	32	*u*	-	-	Odor, STD
29	1645	β-Caryophellene	Spicy, woody	494.9	16384	0.15^{II}	3300	18.9	Odor, MS, STD, *LRI*[a]
30	1705	α-Terpineol	Citrus, lime	517.9	16384	15^{I}	35	18.5	Odor, MS, STD, *LRI*[b]

Table 3.2. (Cont'd)

31	1710	Germacrene D	Hay, woody	594.7	8	*u*	-	3.7	Odor, MS, STD, *LRI*[a]
32	1725	Neral	Fresh, lemon	271.7	128	0.1[I]	2717	12.8	Odor, MS, STD, *LRI*[b]
33	1745	Carvyl acetate	Green, herbaceous	626.2	2	0.0015[I]	417467	1.2	Odor, MS, STD
34	1749	Geranial	Fresh, lemon	587.1	128	0.1[I]	5871	8.7	Odor, MS, STD, *LRI*[b]
35	1754	Dodecanal	Aldehydic, waxy	1162.5	4	0.055[I]	21136	1.8	Odor, MS, STD, *LRI*[a]
36	1760	Carvone	Cooling, spearmint	118.9	2	0.067[I]	1774	2.8	Odor, MS, STD, *LRI*[b]
37	1762	Neryl acetate	Fresh, rosy	152.4	16384	2[I]	76	34.1	Odor, MS, STD, *LRI*[a]
38	1839	*trans,trans*-2,4-Decadienal	Fatty, vegetative	55.3	16	0.01[I]	55300	5.1	Odor, MS, STD, *LRI*[a]
39	1848	Nerol	Fruity, rosy	950.0	1024	0.68[I]	1397	9.8	Odor, MS, STD, *LRI*[b]
40	1858	Geraniol	Fruity, rosy	701.9	1024	0.01[I]	70190	11.4	Odor, MS, STD, *LRI*[b]
41	1896	*trans*-2-Dodecenal	Green, waxy	31.1	4	0.0014[I]	22214	10.8	Odor, MS, STD
42	1941	*cis,trans*-2,6-Nonadienal	Fatty ,vegetative	30.0	1024	0.00001[IV]	2997998	55.0	Odor, MS, STD
43	2048	Perilla alcohol	Green, spicy	46.6	32	7[I]	7	22.0	Odor, MS, STD
44	2065	α-Farnesol	Floral, tea-like	95.7	8	0.02[III]	4785	9.2	Odor, MS, STD
45	2070	*trans*-Nerolidol	Floral, woody	56.3	1024	2.25[I]	25	40.1	Odor, MS, STD
46	2119	Elemol	Floral, woody	1540.9	2048	0.1[I]	15409	8.4	Odor, MS, STD
47	2212	Carvacrol	Phenolic, spicy	210.4	128	2.29[I]	92	14.5	Odor, MS, STD
48	2223	β-Sinensal	Peely, sweet orange	9.1	4	0.082[I]	111	20.0	Odor, MS, STD, *LRI*[a]
49	2501	Indole	Animallic, floral	46.6	128	0.14[III]	333	30.9	Odor, MS, STD
50	2562	Nootkatone	Grapefruit, woody	49.1	32	0.28[I]	175	21.5	Odor, MS, STD

LRI on column (DB-FFAP) determined with two series of *n*-alkanes.

[b] Compounds are listed in their elution order on a DB-FFAP column.

[c] Odor description based on the description of panelist and literature references.

[d] Flavor dilution of the highest dilution at which an individual compound could be detected.

[e] Odor threshold in water reported in [I]Ref. (*142*); [II]Ref.(*143*); [III]Ref. (*144*); [IV]Ref. (*214*); "u" unidentified.

[f] Identification method: Odor= comparison of odor descriptors to literature GC-O database; MS= mass spectrum; Linear Retention Indices (*LRI*) are compared with references from standards ((STD) or literature values (*LRI*[a] referred to the values in Ref. (*215*), *LRI*[b] referred to the values in Ref. (*220*)

"Unknown" compounds tentatively identified without reference compound injection or no detection by MS

"*tr*" trace level, GC-FID peak area unable to record

Table 3.3. Aroma-active compounds with odor description identified in Malaysian white pomelo peel extract achieved by means of GC-O

No.	*LRI* [a]	Compound[b]	Odor description[c]	Conc (ppm)	FD[d]	Odor threshold (ppm)[e]	OAV	RFA	Identification[f]
1	1010	α-Pinene	Terpenic, woody	650.5	32	0.19^{II}	3424	5.9	Odor, MS, STD, *LRI*[b]
2	1090	Pentanethiol	Guava-like, tropical	*tr*	128	*u*	-	-	Odor, STD
3	1105	β-Pinene	Terpenic, woody	875.7	128	1.5^{II}	584	7.1	Odor, MS, STD, *LRI*[b]
4	1164	β-Myrcene	Herbaceous, woody	3710.3	4	0.1^{II}	37103	1.0	Odor, MS, STD, *LRI*[b]
5	1190	Limonene	Citrus, terpenic	167070.6	16384	0.2^{II}	835353	1.0	Odor, MS, STD, *LRI*[b]
6	1249	*trans*-β-Ocimene	Green, terpenic	96.4	8	0.34^{II}	284	9.2	Odor, MS, STD, *LRI*[a]
7	1295	ρ-Cymene	Oxidized lemon, woody	6.8	4	0.12^{II}	57	23.1	Odor, MS, STD, *LRI*[b]
8	1302	Terpinolene	Lime, terpenic	4.8	256	0.41^{II}	12	110.0	Odor, MS, STD, *LRI*[a]
9	1305	Unknown	Green, hazelnut-like	*tr*	128	*u*	-	-	Odor
10	1309	Unknown	Cooked milk, creamy	*tr*	2	*u*	-	-	Odor
11	1321	Unknown	Beany, woody	*tr*	8	*u*	-	-	Odor
12	1336	6-Methyl-5-hepten-2-one	Green, lemon	2.1	4	1^{I}	2	41.6	Odor, MS, STD
13	1347	Hexanol	Fresh, green	45.1	4	3.125^{I}	14	9.0	Odor, MS, STD, *LRI*[b]
14	1359	*cis*-3-Hexen-1-ol	Fresh, leafy green	138.4	32	3.625^{I}	38	12.8	Odor, MS, STD, *LRI*[b]
15	1405	*trans*-2-Hexen-1-ol	Green, leafy	15.1	8	4.25^{I}	4	23.2	Odor, MS, STD
16	1446	*trans*-Linalool oxide	Floral, tea-like	15.7	256	0.32^{I}	49	60.8	Odor, MS, STD
17	1472	*cis*-Linalool oxide	Floral, tea-like	53.2	32	0.32^{I}	166	20.6	Odor, MS, STD
18	1483	Citronellal	Green, lemongrass	8.5	8	0.046^{I}	185	31.0	Odor, MS, STD, *LRI*[a]
19	1512	*trans*-epoxy-Ocimene	Floral, woody	1127.4	32	*u*	-	4.5	Odor, MS, STD
20	1519	α-Copaene	Spicy, woody	1.8	16	*u*	-	89.8	Odor, MS
21	1531	Camphor	Camphoraceous, minty	7.6	4	4.6^{I}	2	21.8	Odor, MS, STD
22	1536	Linalool	Floral, woody	300.0	32	0.028^{I}	10714	8.7	Odor, MS, STD, *LRI*[b]
23	1598	iso-Bornyl acetate	Camphoraceous, woody	381.9	8	1.38^{I}	277	4.6	Odor, MS, STD
24	1638	4-Terpinenol	Lime, musty	7.2	128	6.4^{I}	1	78.5	Odor, MS, STD
25	1645	β-Caryophellene	Spicy, woody	110.6	16	0.15^{II}	737	11.5	Odor, MS, STD, *LRI*[a]
26	1687	Citronellyl acetate	Fruity, rosy	80.4	8	0.00468	17182	10.1	Odor, MS, STD
27	1705	α-Terpineol	Lime, woody	206.7	128	15^{I}	14	14.7	Odor, MS, STD, *LRI*[b]
28	1710	Germacrene D	Hay, woody	51.4	4	*u*	-	8.4	Odor, MS, STD, *LRI*[a]
29	1725	Neral	Fresh, lemon	67.5	128	0.1^{I}	675	25.7	Odor, MS, STD, *LRI*[b]
30	1745	Carvyl acetate	Herbaceous, spicy	11.3	32	0.0015^{I}	7533	44.8	Odor, MS, STD

Table 3.3. (Cont'd)

31	1749	Geranial	Fresh, lemon	125.3	32	0.1[I]	1253	13.5	Odor, MS, STD, *LRI*[b]
32	1760	Carvone	Cooling, spearmint	5.9	8	0.067[I]	89	37.0	Odor, MS, STD, *LRI*[b]
33	1762	Neryl acetate	Fresh, rosy	206.7	256	2[I]	103	16.8	Odor, MS, STD, *LRI*[a]
34	1798	Citronellol	Rosy, woody	25.5	256	0.062[I]	412	47.7	Odor, MS, STD, *LRI*[b]
35	1799	Geranyl acetate	Fresh, rosy	41.6	32	0.15[I]	278	14.0	Odor, MS, STD, *LRI*[a]
36	1822	Methyl benzoate	Jasmine, phenolic	28.8	2	0.000845[IV]	34107	5.6	Odor, MS, STD, *LRI*[b]
37	1839	Perilla aldehyde	Citrus, spicy	13.7	32	0.062[I]	221	40.7	Odor, MS, STD
38	1848	Nerol	Fruity, rosy	444.1	256	0.68[I]	653	11.4	Odor, MS, STD, *LRI*[b]
39	1858	Geraniol	Fruity, rosy	261.3	256	0.01[I]	26134	14.9	Odor, MS, STD, *LRI*[b]
40	1908	Carveol	Spearmint, spicy	131.1	8	4[I]	33	9.1	Odor, MS
41	1941	*cis,trans*-2,6-Nonadienal	Fatty, vegetative	15.6	128	0.00001[III]	1560000	53.4	Odor, MS, STD
42	2018	Benzothiazole	Rubbery, sulfury	24.4	32	0.08[III]	305	30.5	Odor, MS, STD
43	2070	*trans*-Nerolidol	Floral, woody	9.9	256	2.25[I]	4	76.5	Odor, MS, STD
44	2119	Elemol	Floral, woody	28.2	256	0.1[I]	282	45.4	Odor, MS, STD
45	2223	β-Sinensal	Peely, sweet orange	33.6	4	0.082[I]	410	10.4	Odor, MS, STD, *LRI*[a]
46	2501	Indole	Animallic, floral	28.2	128	0.14[III]	201	39.7	Odor, MS, STD
47	2562	Nootkatone	Grapefruit, woody	14.9	128	0.28[I]	53	54.6	Odor, MS, STD

[a] *LRI* on column (DB-FFAP) determined with two series of *n*-alkanes.
[b] Compounds are listed in their elution order on a DB-FFAP column.
[c] Odor description based on the description of panelist and literature references.
[d] Flavor dilution of the highest dilution at which an individual compound could be detected.
[e] Odor threshold in water reported in [I]Ref. (*142*); [II]Ref.(*143*); [III]Ref. (*144*); [IV]Ref. (*214*); "u" unidentified.
[f] Identification method: Odor= comparison of odor descriptors to literature GC-O database; MS= mass spectrum; Linear Retention Indices (*LRI*) are compared with references from standards ((STD) or literature values (*LRI*[a] referred to the values in Ref. (*215*), *LRI*[b] referred to the values in Ref. (*220*)
"Unknown" compounds tentatively identified without reference compound injection or no detection by MS
"tr" trace level, GC-FID peak area unable to record

In contrast, the relatively low oxygenated terpenes content produce the most aroma activity. Relatively high intensity FD-factors belonged to the group of oxygenated terpenes, considered being the most expressive class of terpenes used in perfumery (*225*). Aldehydes, identified as the characteristic aroma of citrus fruits, are secondary metabolites formed during the process of maturation. Some aldehydes imparted pleasant fresh and citrus notes to both pomelo peel extracts, while others contributed to a fatty note. Moreover, citral (neral and geranial), possessing a fresh lemony note, was also found to be key aroma-active volatiles. β-sinensal exhibits peely and sweet orange-like odor. In addition, other fatty-acid degradation aldehydes: octanal, nonanal, decanal (aldehydic); *trans*-2-heptenal, *trans*-2-dodecenal, *cis,trans*-2,6-nonadienal, and *trans,trans*-2,4-decadienal also exhibite intense aroma activity (fatty, intense green and oily notes). Sawamura *et. al.* (*140*) had identified 2-dodecenal to be a characteristic key compound of Tosa-buntan pomelo. Through careful sniffing, a solution of this compound below 2 ppm gave a pleasant and refreshing aroma. *Trans*-2-dodecenal was also detected in the pink pomelo peel extract, but it was not representative of the whole aroma profile even though it was approximately ten times higher at 31 ppm. This was probably due to the variation of volatile composition background among the different pomelo cultivars (*34*). Furthermore, other unsaturated aldehydes identified here were considered more impactful in aroma than *trans*-2-dodecenal. Relative high amount of unsaturated aldehydes might form during the plant tissue disruption.

The terpene alcohols (e.g. linalool, 4-terpineol, citronellol, farnesol, nerolidol, nerol and geraniol) are also key aroma compounds. As a group, they

impart floral, tea-like and woody flavor. Aliphatic alcohols that were present in small proportions also contributed to citrus perception. Hexanol, a fatty-degradation alcohol, exhibits fresh and green notes. In addition, *trans*-2-hexenol and *cis*-3-hexenol add a leafy-green note whereas higher aliphatic alcohols like octanol contribute to orange and waxy attributes. Sulfur compounds are always associated with key aroma chemicals in citrus and tropical fruits. They are commonly present at extremely low levels, but often have even lower odor thresholds (27-29).

Based on *LRI* and odor perception, ρ-menthene-8-thiol was tentatively detected in the pink pomelo peel extract, whereas pentanethiol was found in the white pomelo peel extract (see Tables 3.2 and 3.3). Pentanethiol was found to be the main component responsible for the characteristic tropical aroma of guava fruit (*Psidium guajava* L.) (*226, 227*). ρ-menthene-8-thiol has a prominent grapefruit-like sensory attribute, and was identified as one of the major grapefruit aroma-active components (*133, 228*). While there were a few unknown compounds contributed to atypical notes, e.g. beery, meaty, guava-like, tropical, and hazelnut-like. It is therefore suggested that some compounds with sulfur or nitrogen atom remain unidentified here. The present study has not been exhaustive with respect to these unknown. However, the source of these unknowns could be explained by the biosynthesis pathway that coincide in part with the route of the degradation of monoterpenes (e.g. limonene, β-pinene, and β-myrcene) or/and the reaction of sesquiterpenes (e.g. α-humulene and β-caryophyllene) with some sulfur chemicals (e.g. dimethyl sulfide) that were presented in the obtained extract due to aging or prolong storage together with exposure to light or oxidation.

In order to verify the analytical data obtained by quantification, an attempt was made to reconstruct the flavor of the pomelo peel from its identified compounds. In this work, triacetin was employed as a solvent during the reconstitution, which can dissolve both hydrophilic and hydrophobic compounds. It is odorless and inert. The amount of each compound used in the reconstituted model was based on the concentration determined in the both Malaysian pomelo peel extracts (Tables 3.2 and 3.3). As the concentration of ρ-menthene-8-thiol could not be determined due to weak MS and FID signals, several trials were made in order to find out their most suitable concentration. Results indicated that 0.0005% w/w of ρ-menthene-8-thiol produced a close resemblance to natural pink pomelo peel extract. Further increment of ρ-menthene-8-thiol had created overwhelmed sulfury and tropical notes. The aroma model was evaluated and found that the grapefruit note was significantly improved.

As shown in Figure 3.3, the profile of the pink pomelo aroma model was found to be more aldehydic and woody than that of the original pink peel extract. Furthermore, it lacked fresh and green characters. While several odorants with these descriptors were identified during sniffing, only some could be attributed to compounds identified in the pink pomelo extract, thus suggesting that other significant contributors to pomelo peel flavor may remain as yet unidentified.

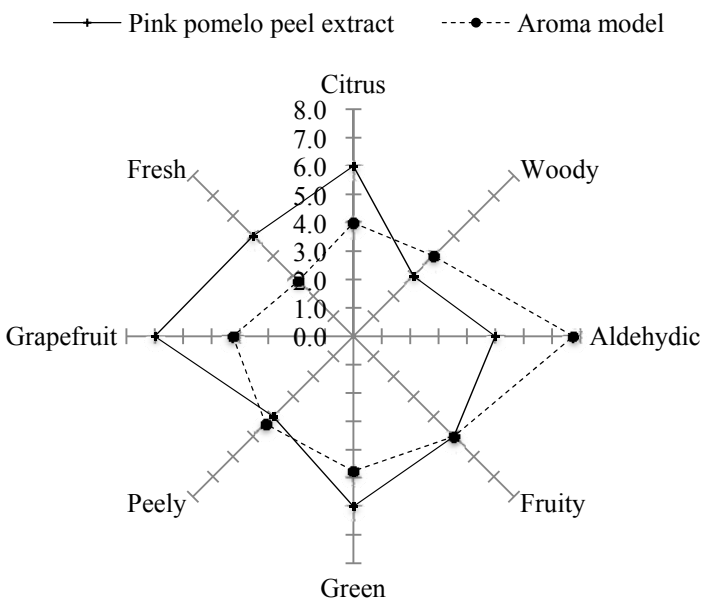


Figure 3.3. Flavor profile analysis of Malaysian pink pomelo peel extract and the reconstituted aroma model

For white pomelo peel extract, aroma model was prepared with the compounds with OAV ≥ 1. It showed good agreement compared to white pomelo peel extract, but it was rated lowest in grapefruit note, less green but more aldehydic (Figure 3.4). Overall, the sensory evaluation deduced that there were some unidentified molecules that had strong tropical and woody notes. In addition, *cis,trans*-2,6-nonadienal was considered characteristic in the white pomelo peel.

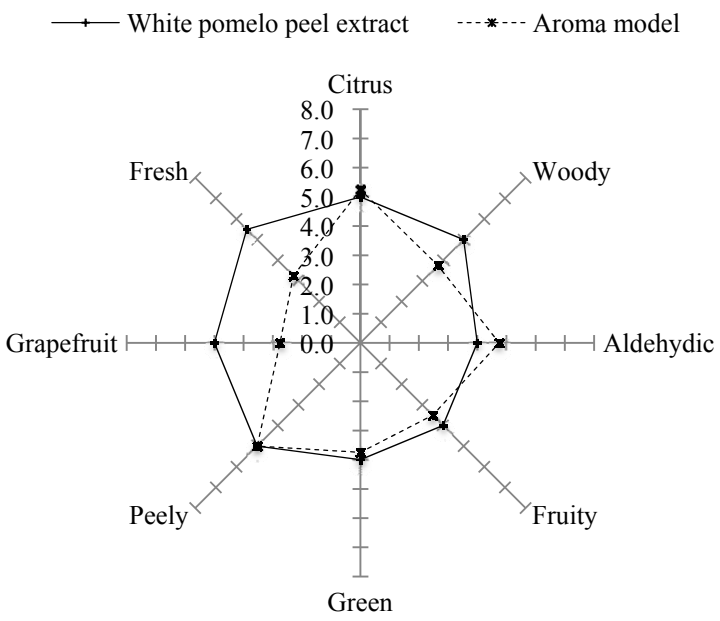


Figure 3.4. Flavor profile analysis of Malaysian white pomelo peel extract and the reconstituted aroma model

The above discussion was for the aroma model based on the analytical data. Similar sensory evaluation with various aroma models was also performed, which were prepared in the same manner but the proportion of each component was carefully adjusted. However, the effort on the reconstructing pomelo peel flavor by mixing the aroma-active compounds identified in the present study was not closely similar to the correspondent peel extracts, particularly for pink pomelo peel. So, under the present condition, some key aroma compounds to Malaysian pomelo peel flavors have yet to be chemically characterized.

3.4. Conclusion

Using GC-MS/FID and gas chromatography-olfactometry (GC-O), 50 and 47 aroma-active compounds were identified in pink and white pomelo peel extracts, respectively. On the basis of flavor dilution (FD) factor, odor activity value (OAV) and relative flavor activity (RFA), main odorants in pink pomelo peel extract: *trans*-linalool oxide, *cis,trans*-2,6-nonadienal, octanal, citronellal, nonanal, *trans*-nerolidol, neryl acetate, indole, 6-methyl-5-hepten-2-one, terpinolene, *trans*-2-heptenal, perilla alcohol and nootkatone were determined, while the most intense aromas in the white pomelo peel extract were terpinolene, α-copaene, 4-terpinenol, *trans*-nerolidol, *trans*-linalool oxide, nootkatone, *cis,trans*-2,6-nonadienal, citronellol, elemol, carvyl acetate, 6-methyl-5-hepten-2-one, perilla aldehyde and indole. Therefore, no single compound was determined as possessing a characteristic Malaysian pomelo like aroma.

CHAPTER 4 CHEMICAL COMPOSITION AND SENSORY PROFILE OF POMELO (*CITRUS GRANDIS (L.)* OSBECK) JUICE

4.1. Introduction

In the previous two chapters, the volatiles of pomelo blossoms and peels indicated that the pomelo possessed a unique aroma and taste. Among the 100 volatiles present in the pomelo peel, 50 aroma-active compounds (mainly unsaturated aliphatic aldehydes, terpene aldehydes, esters, terpene alcohols and nootkatone) were found characteristic for the complex pomelo flavor. Although some comparisons have been made on the volatile fractions of Nakon (*183*) and Chandler pummelo (*177*), the flavor profile of pomelo juice is still inexplicit. Therefore, the chemical composition and sensory profile of the pomelo juice were investigated in this chapter. Furthermore, the possibility of correlating instrumental data (volatile and non-volatile components) with the sensory profile through multivariate analyses (PCA and PLSR) was explored.

Citrus juices are complex mixtures of aromatic volatiles (e.g. esters, aldehydes, ketones and alcohols) and non-volatile components (e.g. organic

acids and sugars) (*132, 229*). Although aromatic volatiles are normally present at trace levels (*230, 231*), they are essential to characterize the aroma of citrus juices (*232*). Moreover, the composition of non-volatile components (i.e., °Brix/acid ratio) significantly contributes to their flavor perception (*233*). Therefore, the interaction between the volatile and non-volatile components of foods has been widely studied (*27, 234, 235*).

In order to better understand the relationship between the chemical composition and sensory profile, multivariate analyses (e.g. principal component analysis (PCA) and partial least square regression (PLSR)) have been widely applied to extract meaningful information from complex data sets. A study has been done to correlate the sensory profile of commercial orange juice with its aroma components during accelerated storage (*236*); however, there is still limited information on correlating chemical components with sensory perception of other citrus juices.

4.2. Experimental procedures

4.2.1. Chemicals

Standards of 5-methyl-2-hexanone, organic acids (citric, malic, succinic and ascorbic acids) and sugars (glucose, fructose and sucrose) of HPLC grade from Sigma were used. Analytical grade dichloromethane, acetic acid, ethyl acetate, calcium chloride ($CaCl_2$, ≥99%) and anhydrous sodium sulfate were supplied by Merck Pte Ltd. (Darmstadt, Germany). HPLC-grade methanol, hexane and diethyl ether were obtained from Tedia (Fairfield, OH, USA). Analytical grade ascorbic and ethylenediaminetetraacetic acids

(EDTA) (Sigma-Aldrich), sodium hydroxide (NaOH) (Schedelco, Singapore) and hydrochloric acid (HCl) (VWR, Radnor, PA, USA) were used.

All standard compounds used in the identification of volatile constituents were obtained from Firmenich Asia Pte. Ltd. (Singapore).

4.2.2. Preparation of pomelo juice

Two batches of 50 kg mature pomelo fruits, *Citrus grandis* (L.) Osbeck PO 51 (white-fleshed pomelo) and PO 52 (pink-fleshed pomelo) were harvested in September and November 2010, respectively. The fruits were then transported and stored in a fridge (5 °C) before use. Pomelo juice was prepared by hand-squeezing the pulp after removing the peel to avoid contamination from the components in the flavedo and albedo. Freshly squeezed juice was immediately used for volatile extraction. Fifty millilitres of pomelo juice was subjected to centrifugation at 17,000 g for 15 min at 4 °C (Sigma 3-18K, Sartorius Mechatronics, Göttingen, Germany). The supernatant was directly used to determine its physicochemical properties. For HPLC analysis of sugars and organic acids, the supernatant was filtered through a 0.2 μm regenerated cellulose (RC) filter (Minisart RC15, Sartorius Stedim Biotech, Aubagne, France) and then kept at -30 °C before analysis.

4.2.3. Extraction of volatile compounds using HS-SPME

HS-SPME, as a qualitative method, was adopted to monitor the volatiles of pomelo juices. Five grams of freshly prepared pomelo juice were added into a 20-mL glass vial, followed by 3 g of saturated $CaCl_2$ solution. The glass vial was crimp-capped and subjected to headspace extraction. A 85

μm CarboxenTM/PDMS StableFlexTM (Supelco) SPME fiber was selected. The default extraction condition of HS-SPME was 40 °C for 30 min. The SPME fiber was then thermally desorbed into the GC injector (250 °C) for 5 min.

4.2.4. Extraction of volatile compounds using organic solvents

The solvent extraction method used was modified from that of Takeuchi *et. al.* (*237*). Several parameters were optimized prior to extraction, i.e. solvent type, solvent volume to juice weight ratio, extraction speed and extraction time. Firstly, several common organic solvents or their mixture such as dichloromethane (*86*), hexane (*188*), diethyl ether (DEE) (*189*) and a mixture of hexane with DEE (*89*) were examined. Then, dichloromethane was chosen due to its good solubility over a wide spectrum of volatiles (e.g. decanal, citronellal, citral and *trans*-2-dodecenal). Further, in order to retain the original profiles of calamansi juice, the extraction temperature was maintained at low temperature (~4 °C). In addition to increase distribution coefficient and extraction efficiency, the ratio of solvent volume to juice weight, extraction speed and time were also optimized (data not shown). Ten grams of pomelo juice, spiked with 100 μL of 10,000 ppm internal standard 5-methyl-2-hexanone, were extracted twice with 8 mL of dichloromethane. The mixture was vortex-mixed (Rotamax 120, Heidolph, Schwabach, Germany) at top speed for 30 min, and then centrifuged at 4,494 g for 5 min at 4 °C, where the aqueous phase was removed. The gel that formed at the interface was broken up and allowed to float to the surface. The collected organic phase was dried with anhydrous Na_2SO_4, and was filtered through a sintered filter and

concentrated to a final volume of 100 μL under a gentle stream of nitrogen. All extracts were stored at -30 °C before analysis.

4.2.5. GC-MS/FID analysis

Analyses of the volatile compounds of pomelo juices extracted using HS-SPME and organic solvents were carried out in the same manner as described in Chapter 2 (Section 2.2.3).

4.2.6. Physicochemical properties

Physicochemical properties of pomelo juices, i.e. pH, titratable acidity (TA) and total soluble solids (TSS), were determined. pH was measured using a pH meter (744 pH Meter, Metrohm AG, Herisau, Switzerland). TA was measured by titrating 1 g of the supernatant with 0.111 M NaOH and expressed as citric acid (g of citric acid/ kg of juice). The TSS content of the supernatant expressed as °Brix was determined with a refractometer (RX-5000α, Atago, Japan).

4.2.7. Ultra-fast liquid chromatography (UFLC) instrumentation

A Shimadzu Prominence UFLC system equipped with two pumps (LC-20AD), an autosampler (SIL-20AC), column oven (CTO-20AC) and an evaporative light scattering detector (ELSD-LT II) for sugar analysis or a photodiode array detector (SPD-M20A) for the analysis of organic acids was used (Shimadzu, Kyoto, Japan). Default column temperature was set at 40 °C and sample injection volume was 10 μL. Data were acquired and processed

using LC solution version 1.24 SP1 software (Shimadzu). Sugars and organic acids were identified by matching the retention times and spectral characteristics of standards.

4.2.7.1 HPLC analysis of sugars

For sugar analysis, Agilent ZORBAX carbohydrate analysis column (150 mm x 4.6 mm, 5 μm) attached with ZORBAX NH2 analytical guard column (4.6 mm x 12.5 mm, 5 μm) was used. The mobile phase used was acetonitrile/water (80:20, v/v) with the isocratic flow rate set at 1.40 mL/min. Detection was performed with an ELSD-LT II at 40 °C, gain 5 and pressure of 350 kPa. A series of standard solutions (ranging from 0.10 to 4.0 mg/mL of fructose, glucose and sucrose) were prepared. A standard curve with R^2 greater than 0.99 was plotted and the concentrations of the respective sugars in pomelo juices were quantified using the standard curves.

4.2.7.2 HPLC analysis of organic acids

Supelco Supelcogel C-610H ion exchange column (300 mm x 7.8 mm) with sulfonated polystyrene divinylbenzene packing was used for the separation of organic acids. Absorbance of the analytes was measured at 210 and 260 nm. Detection of ascorbic acid was performed at 260 nm to avoid interferences from other acids. The mobile phase used was 0.10% H_2SO_4 with the isocratic flow rate set at 0.40 mL/min. Standard solutions were prepared with a serial concentration of malic, ascorbic and succinic acids at 0.30 - 3.00 mg/mL and citric acid at 2.40 - 24.00 mg/mL. A standard curve with R^2

greater than 0.99 was plotted and the concentrations of the respective acids were quantified using the standard curves.

4.2.8. Sensory evaluation

The sensory profiles of fresh white and pink pomelo juices were evaluated by six experienced flavorists from Firmenich Asia Pte. Ltd., Singapore as explained in Chapter 2 (Section 2.2.4). After reaching a consensus, the appropriate descriptive sensory attributes were established, i.e. acidic, citrusy, fresh, green, peely and woody as odor (orthonasal) attributes while bitter, sour and sweet as taste (retronasal) attributes. Attribute rating was used with '0' indicating uncharacterized attribute intensity and '5' indicating very strong attribute intensity. A set of coded tasting cup was presented to the panelists, and the intensity of each attribute was rated after sniffing and sipping each of the pomelo juices. The average score for each descriptive attribute was expressed in a plotted web diagram.

All experiments were carried out in triplicate and the results were reported as the mean values.

4.2.9. Statistical analysis

Multivariate analysis was carried out using PCA based on the correlation matrix. Correlations between chemical compounds (X-variables) and sensory data (Y-variables) were investigated by partial least square regression (PLSR) analysis using Matlab version 7.6.0.324, R2008a software (The MathWorks Inc., MA, USA). PLSR was performed for each sensory variable. Not all the instrumental variables were measured on the same scale,

and they were standardized to have the same variance. This was not done for the sensory variables, since these were all measured using the same scoring scheme. The differences in physicochemical properties between the pink and white pomelo juices were analyzed by T-test (Microsoft Excel 2011, Microsoft Corporation, Washington D.C, USA).

4.3. Results and discussion

4.3.1. Volatile composition of pomelo juices

In spite of extensive work done on common citrus fruits, limited studies have been conducted on pomelo juice. In the present study, pink and white pomelo juices had different volatiles, generally categorized as hydrocarbons, esters, acids, alcohols, aldehydes and others. Table 4.1 shows the volatile compounds of dichloromethane extracts from freshly squeezed pomelo juices. According to the relative concentration (ppm) obtained from the dichloromethane extracts, the pink pomelo juice contained a higher amount of total volatiles relative to the white pomelo juice. Hydrocarbons (mono- and sesquiterpenes) that are associated with citrus-based flavors were found in both pomelo juices. Up to 90% of limonene, β-pinene, β-myrcene and α-terpinene constituted the major monoterpenes. The total amount of terpene hydrocarbons in the white pomelo juice was 1.5-fold higher than that of the pink pomelo juice. In contrast, the pink pomelo juice was characterized by higher amounts of alcohols and aldehydes.

Table 4.1. Identification of volatiles and their concentrations (ppm) in Malaysian pomelo (*Citrus grandis* (L.) Osbeck pink and white types) juice extracts

Compounds[a]	*LRI*		Odor description[c]	Pink	White	Identification[d]
	FFAP[b]	REF				
Hydrocarbons						
α-Pinene[I]	1010	1007	Terpenic, woody	248.41	341.26	MS, STD, LRI
β-Pinene[I]	1105	1113	Terpenic, woody	348.71	19.57	MS, STD, LRI
Sabinene	1107	1093	Terpenic, green	11.88	88.39	MS, LRI
β-Myrcene[I]	1164	1158	Herbaceous, woody	41.10	141.94	MS, STD, LRI
α-Terpinene	1183	1178	Terpenic, woody	-	15.92	MS, STD, LRI
Limonene[I]	1190	1185	Citrus, terpenic	86.49	542.66	MS, STD, LRI
β-Phellandrene	1198	1194	Citrus, spicy	-	99.59	MS, LRI
trans-β-Ocimene[I]	1249	1242	Green, terpenic	-	10.10	MS, STD, LRI
cis-β-Ocimene	1251	1252	Green, terpenic	-	7.72	MS, STD, LRI
ρ-Cymene[I]	1295	1267	Oxidized lemon, woody	-	2.25	MS, STD, LRI
Terpinolene[I]	1302	-	Lime, terpenic	-	4.57	MS, STD
4,8-dimethyl-1,3,7-nonatriene*	1349	-	Mild woody	20.50	4.47	MS
δ-Elemene	1502	-	Balsamic, mild woody	-	37.90	MS
β-Elemene	1540	-	Herbaceous, woody	-	8.07	MS
β-Caryophyllene[I]	1566	-	Spicy, woody	24.57	39.88	MS, STD, LRI
γ-Elemene	1608	1625	Woody	-	9.59	MS, LRI
α-Humulene	1669	-	Earthy, spicy	-	3.62	MS
Germacrene D[I]	1685	-	Hay, woody	-	3.01	MS, STD, LRI
α-Selinene	1696	-	Woody	27.41	-	MS
Esters						
Neryl acetate[I]	1762	1742	Fresh, rosy	-	38.69	MS, STD, LRI
Ethyl palmitate	2346	-	Faint, waxy, sweet	144.00	-	MS, LRI
Acids						
Acetic acid	1449	1451	Acidic	155.06	-	MS, STD, LRI
Palmitic acid	2889	-	Waxy	-	8.20	MS, LRI
Oleic acid	3129	-	Weak waxy	-	1.89	MS, LRI
Linoleic acid	3140	-	Fatty, oily	-	4.34	MS, LRI
Alcohols						
2-ethyl-1-hexanol	1390	-	Citrus, floral	162.51	-	MS
Linalool[I]	1507	1533	Floral, woody	92.52	69.30	MS, STD, LRI
Nerol[I]	1836	1825	Fruity, rosy	4.54	2.94	MS, STD, LRI
Geraniol[I]	1847	1840	Fruity, rosy	11.70	3.04	MS, STD, LRI
Carveol[I*]	1899	1899	Spearmint, spicy	-	9.40	MS
trans-Nerolidol[I]	1991	-	Floral, woody	8.42	2.90	MS, STD
Eugenol	2020	-	Clove-like, honey	76.61	-	MS, STD
Aldehydes						
Octanal[I]	1262	1280	Aldehydic, peely	20.50	70.22	MS, STD, LRI
Nonanal[I]	1365	1385	Aldehydic, citrus	155.06	3.25	MS, STD, LRI
Decanal[I]	1469	1497	Aldehydic, citrus	-	62.10	MS, STD, LRI
Citronellal[I]	1511	1485	Green, lemongrass	162.51	4.01	MS, STD, LRI
Neral[I]	1656	-	Fresh, lemon	-	102.69	MS, STD, LRI
Geranial[I]	1676	-	Fresh, lemon	26.91	123.86	MS, STD, LRI
trans,*cis*-2,4-Decadienal[I]	1737	-	Fatty, vegetative	195.92	2.59	MS, STD, LRI
Dodecanal[I]	1758	1788	Citrus, waxy	50.09	29.44	MS, STD, LRI

Table 4.1. (Cont'd)

Perilla aldehyde[I]	1768	-	Fatty, spicy	-	2.32	MS, STD, LRI
trans,trans-2,4-Decadienal[I]	1806	1804	Fatty, vegetative	-	1.20	MS, STD, LRI
trans-2-Dodecenal[I]	1849	-	Green, waxy	-	5.56	MS, STD
cis-trans-2,6-nonadienal[I]	1945	-	Fatty, vegetative	-	2.01	MS, STD
Octadecanal	2179	-	Fatty, tallow	189.10	-	MS, STD
Others						
Limonene oxide	1416	-	Citrus, faint spearmint	-	0.53	MS, STD
Dimethyl sulfone	1793	-	Sulfurous	5.55	-	MS, STD
1,10-Dihydronootkatone	2217	-	Strong grapefruit-like	3.39	-	MS
Indole[I]	2507	-	Animallic, floral	6.08	1.58	MS, STD
Nootkatone[I]	2601	-	Grapefruit, woody	58.20	1.79	MS, STD
Osthole	3145	-	Herbal, musty	27.72	54.14	MS
Total identified (ppm)				**2457.99**	**1988.48**	

[a] Compounds are listed in their elution order on a DB-FFAP column.
[b] Linear Retention Indices (LRI) on column (DB-FFAP) determined with two series of *n*-alkanes.
[c] Odor description based on the Leffingwell's Flavor-Base (*221*) and Flavornet (*220*)(*220*)(*220*)(*220*)(*220*)(*220*)(*220*)(*219*)(*218*)(*217*)(*216*)(*215*)(*215*)(*214*)(*213*)(*212*)(*211*)(*210*)(*210*)(*210*)(*210*)(*210*)(*209*)(*209*)(*210*)(*209*)(*209*)(*209*)(*209*)(*209*).
[d] Identification method: MS = mass spectrum; STD = comparison with standard compound; LRI is compared with references from standards or literature values. [I] Compounds were reported in pomelo peel extract; "-" not detected; * unknown isomer

The volatile compounds extracted by HS-SPME demonstrated some discrepancies between the pink and white pomelo juices (Table 4.2). This might be due to the different affinity of each molecule for the SPME fiber coating material and competition among the molecules to achieve thermodynamic equilibrium in the headspace and on the SPME fiber. Several volatiles were detected at trace levels that could contribute to the pomelo flavor profile. Although sesquiterpene hydrocarbons were the second most abundant group of volatiles detected by HS-SPME, only α-selinene was detected in the pink pomelo juice extracted with dichloromethane. Among the sesquiterpenes, valencene was perceived as an important aroma compound in distinguishing the citrus types (*177*).

Table 4.2. Identification of volatiles in Malaysian pomelo (*Citrus grandis* (L.) Osbeck pink and white types) juices through HS-SPME (relative percentages of FID peak area)

Compounds[a]	LRI[b]	Odor description[c]	Pink	White	Identification[d]
Hydrocarbons					
α-Pinene[I]	1010	Terpenic, woody	tr	3.24	MS, STD, LRI
β-Pinene[I]	1105	Terpenic, woody	0.14	2.33	MS, STD, LRI
Sabinene	1107	Terpenic, green	0.13	0.83	MS, LRI
β-Myrcene[I]	1164	Herbaceous, woody	3.84	0.85	MS, STD, LRI
α-Terpinene	1183	Terpenic, woody	2.03	0.27	MS, STD, LRI
Limonene[I]	1190	Citrus, terpenic	49.43	31.34	MS, STD, LRI
β-Phellandrene	1198	Citrus, spicy	0.33	tr	MS, LRI
trans-β-Ocimene[I]	1249	Green, terpenic	1.01	0.35	MS, STD, LRI
cis-β-Ocimene	1251	Green, terpenic	1.97	0.19	MS, STD, LRI
ρ-Cymene[I]	1295	Oxidized lemon, woody	1.30	1.50	MS, STD, LRI
Terpinolene[I]	1302	Lime, terpenic	0.92	0.33	MS, STD
dehydro-ρ-Cymene	1323	Citrus, herbaceous	6.79	0.49	MS
ρ-1,3,8-Menthatriene	1339	Green, herbaceous	1.05	0.37	MS, LRI
4,8-dimethyl-1,3,7-nonatriene*	1349	Mild woody	1.52	6.32	MS
δ-Elemene	1502	Balsamic, mild woody	0.16	3.57	MS
α-Copaene	1513	Woody, spicy	-	0.02	MS, LRI
β-Caryophyllene[I]	1566	Spicy, woody	1.75	0.61	MS, STD, LRI
γ-Elemene	1608	Woody	0.08	0.44	MS, LRI
α-Humulene	1669	Earthy, spicy	0.29	0.62	MS
Germacrene D[I]	1685	Hay, woody	0.35	0.47	MS, STD, LRI
Valencene	1689	Citrus, ripe orange	0.19	0.68	MS, STD, LRI
β-Selinene	1691	Herbaceous	0.04	0.58	MS
α-Selinene	1696	Woody	0.33	0.83	MS
allo-Aromadendrene	1709	Woody	0.16	0.58	MS
α-Farnesene	1715	Citrus, herbaceous	-	0.96	MS, STD, LRI
δ-Cadinene	1759	Spicy, woody	-	0.96	MS
Calamene	1809	Herbaceous, spicy	0.11	0.96	MS
Calacorene	1893	Woody	0.12	0.15	MS
γ-Gurjenene	2190	Balsamic, woody	0.04	0.61	MS
Esters					
Neryl acetate[I]	1762	Fresh, rosy	-	0.91	MS, STD, LRI
Ethyl palmitate	2346	Faint, waxy, sweet	0.50	0.11	MS, LRI
Acids					
Acetic acid	1449	Acidic	0.16	-	MS, STD, LRI
Octanoic acid	2017	Fatty, sweaty	-	0.21	MS, STD, LRI
Nonanoic acid	2116	Fatty, nutty	0.11	0.21	MS, STD, LRI
Decanoic acid	2230	Fatty, waxy	0.18	0.73	MS, STD, LRI
Alcohols					
Ethanol	967	Alcoholic	-	3.71	MS, STD, LRI
Prenol	1290	Fruity, green	2.83	-	MS
Hexanol[I]	1346	Fresh, green	4.02	4.17	MS, STD, LRI
cis-3-Hexenol[I]	1350	Fresh, leafy green	5.49	2.59	MS, STD
2-ethyl-1-hexanol	1390	Citrus, floral	0.50	0.99	MS
Linalool[I]	1507	Floral, woody	0.24	-	MS, STD, LRI
Octanol[I]	1532	Orange, waxy	0.52	1.66	MS, STD, LRI
Nerol[I]	1836	Fruity, rosy	0.13	0.94	MS, STD, LRI
Geraniol[I]	1847	Fruity, rosy	0.13	0.26	MS, STD, LRI
Carveol[I*]	1899	Spearmint, spicy	0.22	0.33	MS
trans-ρ-Mentha-2,8-dienol	1938	Green, cooling	0.09	-	MS, STD, LRI
trans-Nerolidol[I]	1991	Floral, woody	0.03	0.16	MS, STD
Aldehydes					
Hexanal	1024	Fatty-green, grassy	0.81	3.93	MS, STD, LRI
trans-2-Hexenal	1193	Fruity, leafy green	1.27	1.42	MS, STD
Decanal[I]	1469	Aldehydic, citrus	0.23	-	MS, STD, LRI
Neral[I]	1656	Fresh, lemon	0.83	1.75	MS, STD, LRI

Table 4.2. (Cont'd)

Geranial[I]	1676	Fresh, lemon	0.06	0.20	MS, STD, LRI
Perilla aldehyde[I]	1768	Fatty, spicy	-	0.57	MS, STD, LRI
trans-2-Dodecenal[I]	1849	Green, waxy	0.14	-	MS, STD
cis-trans-2,6-nonadienal[I]	1945	Fatty, vegetative	0.07	-	MS, STD
α-Sinensal[I]	2353	Peely, sweet orange	0.25	0.21	MS, STD, LRI
Others					
6-Methyl-5-hepten-2-one[I]	1333	Green, lemony	0.38	0.25	MS, STD
trans-Linalool oxide[I]	1440	Floral, tea-like	-	1.92	MS, STD
cis-Linalool oxide[I]	1468	Floral, tea-like	0.86	0.76	MS, STD
Isopiperitenone	1843	Fruity, herbal, sweet	-	3.24	MS, STD
Phenol,2,4-bis(1,1-dimethylethyl)	2058	Phenolic	0.12	0.23	MS, STD
Benzophenone	2212	Geranium-like, rose-like	0.17	0.26	MS, STD
1,10-Dihydronootkatone	2217	Strong grapefruit-like	0.27	1.48	MS
Nootkatone[I]	2601	Grapefruit, woody	0.70	2.71	MS, STD
Total identified (%)			95.38	96.47	

[a] Compounds are listed in their elution order on a DB-FFAP column.
[b] Linear Retention Indices (LRI) on column (DB-FFAP) determined with two series of *n*-alkanes.
[c] Odor description based on the Leffingwell's Flavor-Base (*221*) and Flavornet (*220*)
[d] Identification method: MS = mass spectrum; STD = comparison with standard compound; LRI is compared with references from standards or literature.
[I] Compounds were reported in pomelo peel extract; "-" not detected; * unknown isomer; "tr" trace

Aldehydes were present at comparatively high concentrations in both juices. It is clearly recognized that aldehydes play a major role in characterizing citrus flavor (*238*). The amount of aldehydes detected in the pink pomelo juice (c.a. 800 ppm) was 2-fold higher than that of the white pomelo juice (c.a. 409 ppm). Saturated aliphatic aldehydes (e.g. octanal, nonanal and decanal) are known to impart peely and citrus-like notes, and have been implicated as important contributors to citrus flavor, e.g. orange.

Typical isomeric monoterpenic aldehydes (e.g. neral and geranial) were also found. On the other hand, unsaturated aliphatic aldehydes (e.g. *cis,trans*-2,4-decadienal, *trans,trans*-2,4-decadienal and *cis,trans*-2,6-nonadienal) that typically contribute intense green, waxy odors have been identified as aroma-active compounds in characterising both white and pink pomelo peels. These compounds were also reported to be responsible for the separation between Chandler pummelo and other citrus varieties (Powell Navel orange,

Clemenules mandarine and Fortune mandarine) (*177*).

Esters are important aroma components in many fruits, including citrus. In contrast to Nakon pummelo (*183*), both pink and white pomelo juices had much less fruity aroma. Nevertheless, several prominent esters were found in both pink and white pomelo juices. Nootkatone, a sesquiterpene ketone, possesses a grapefruit-like aroma with woody and bitter tastes. Another compound, 1,10-dihydronootkatone, was found to possess approximately 3.5 times more intense grapefruit aroma than pure nootkatone at equimolar concentrations (*239*). Both compounds constitute an important trait for grapefruit and its parental fruit (*35, 240*). Apart from being one of the most important grapefruit aromatic compounds, nootkatone is also found to decrease the somatic fat ratio (*241*). Therefore, pomelo may be a viable and sustainable source of nootkatone production, which is highly demanded by the flavor, fragrance and cosmetic industries. Detailing the volatile composition of pomelo juices, it is noted that there were 36 volatiles that were aroma-active compounds contributing to the pomelo profile.

4.3.2. Physicochemical properties and non-volatile composition of pomelo juices

Physicochemical properties (i.e. TSS (°Brix), TA and pH) are crucial parameters in determining the maturity and quality of citrus fruits. Considered as a liquid-solid suspension system, citrus juices consist of a mixture of soluble and suspended solids. It has been reported that the physicochemical properties such as sugars and pulp in citrus juices alter headspace concentrations and aroma thresholds of many citrus volatiles (*132*). Table 4.3

summarizes the physicochemical properties, organic acids and sugars content of both pomelo juices.

Table 4.3. Physicochemical properties, sugars composition and organic acids content of Malaysian pomelo (*Citrus grandis* (L.) Osbeck pink and white types) juices

	Pink	**White**
Physicochemical properties		
pH***	3.67 ± 0.02	6.07 ± 0.06
Titratable acidity (g citric acid /kg)***	0.94 ± 0.04	0.04 ± 0.00
Total soluble solids (°Brix)***	10.90 ± 0.09	10.45 ± 0.21
Sugars (mg/mL)		
Fructose**	13.41 ± 1.50	11.02 ± 0.62
Glucose**	13.13 ± 1.42	10.43 ± 1.43
Sucrose	49.91 ± 8.91	56.51 ± 4.71
Total	*76.45*	*77.96*
Organic acids (mg/mL)		
Ascorbic	0.22 ± 0.13	0.39 ± 0.20
Citric*	14.15 ± 8.18	0.14 ± 0.08
Malic*	0.75 ± 0.42	1.94 ± 0.97
Succinic**	0.05 ± 0.02	0.39 ± 0.18
Total	*15.17*	*2.86*

T-test significantly different $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

Among these qualities, the difference between the TSS content and sugar content were insignificant between the two cultivars. The TSS contents (°Brix) of both pomelo juices were similar and comparable to other common citrus juices. On the other hand, the pH value and TA were the more distinguishing trait for the two cultivars. White pomelo was characterized by mild acidity and a higher pH value, while pink pomelo was found to be higher in the organic acid content, of which, citric acid was the main organic acid present. The total acid content and TA of pink pomelo juice were comparable to those of grapefruit juice, which was also pigmented (*242, 243*). However,

the white pomelo juice was exceptionally low in the organic acid content compared to other citrus fruits. As mentioned earlier, non-volatiles play an important role in determining the organoleptic profile and acceptability.

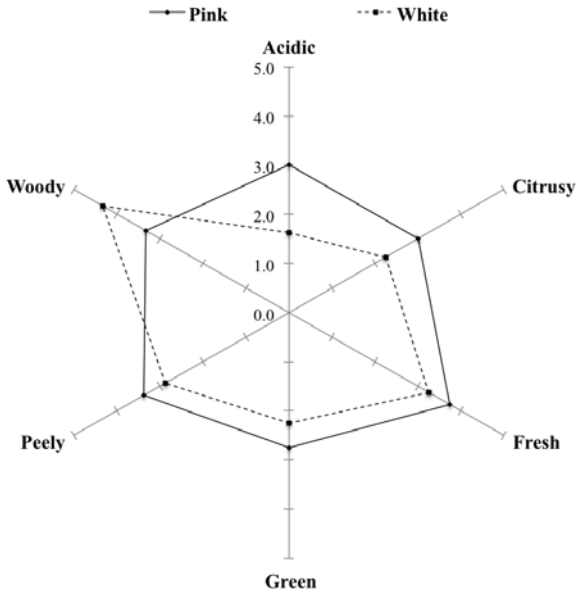
4.3.3. Sensory evaluation and correlation with instrumental data using multivariate analysis

As one basic principle in developing sensory techniques, multivariate analysis plays an elementary part in such sensory array. Pattern recognition and/or multivariate calibration methods are used to interpret complex signals and thus, producing qualitative and quantitative data (*156*). Specifically, PCA with the capability of distinguishing variations could visualise the degree of contribution of each interdependent chemical variable to the overall variability in a large data set. PLSR could assess the correlation between the sensory attributes and analytical data (*244*). Descriptive sensory analysis, also known as sensory profiling, is a technique of relating individual or a group of aroma volatiles to sensory perception (*245*).

Figure 4.1 (a) illustrates the distinctive orthonasal and retronasal attributes of freshly squeezed pomelo juices. For the orthonasal odor, seven attributes were selected, namely acidic, citrusy, fresh, green, peely and woody. The retronasal attributes selected for freshly pomelo juices were bitter, sour and sweet. The pink pomelo juice received higher scores for all of the orthonasal attributes, except for the woody note. The white pomelo juice with a higher amount of terpene hydrocarbons was rated 4.3 for the woody note compared to the pink pomelo juice (2.5).

Regarding the retronasal attributes, the white pomelo juice with higher sucrose content was rated higher at 4.0 for sweetness, while the pink pomelo juice was rated higher in bitter and sourness notes. It is agreed that both orthonasal and retronasal attributes were distinctive to aroma and taste profiles. Therefore, these sensory attributes have been directed towards relating the level of aroma volatiles to the sensory profile.

(a)



(b)

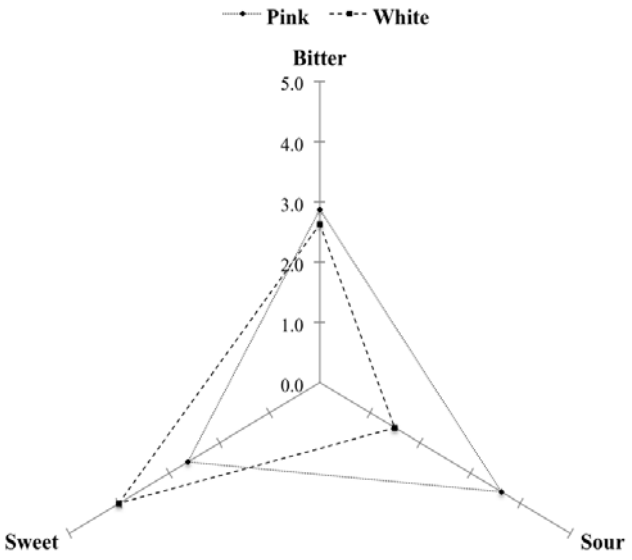


Figure 4.1. Sensory attributes of fresh pomelo juices: (a) orthonasal and (b) retronasal

Prior to the multivariate analysis, pre-screening was done by sniffing pomelo juice extracts (data not shown). It was noted that the aroma-active compounds that characterized pomelo juices closely resembled those of pomelo peel extracts. Hence, the 29 identified aroma-active compounds presented in pomelo juice were selected in order to understand the contributions of each variable to the overall variation. PCA was employed to understand the contributions of aroma-active compounds to the sensory attributes of pomelo juices. Two principal components (PCs) were obtained from the dichloromethane-extracted pomelo juice data sets, accounting for 98.4% of the cumulative percentage of total variations, where PC 1 and PC 2 accounted for 85.2% and 9.8% of the variance, respectively. Figure 4.2 illustrates the PCA biplots of pink and white pomelo juices with selected aromatic volatiles and non-volatile compounds. As the chemical profiles of both pink and white pomelo juices were significantly different, the score points were distant. It can be seen that the aromatic volatiles were the major differentiating factors reflected in PC 1, where the variations in the non-volatile compounds were shown in PC 2. Furthermore, more information could be retrieved through the biplot, which loadings implied that certain aromatic variables were closely clustered to each pomelo cultivar. As the score points were scaled to fit within the unit square, only their relative locations to the variables were determined from the plot. As indicated by the biplot, each loading contributed equal coefficient to the variance, none of the aroma-active compounds were eliminated.

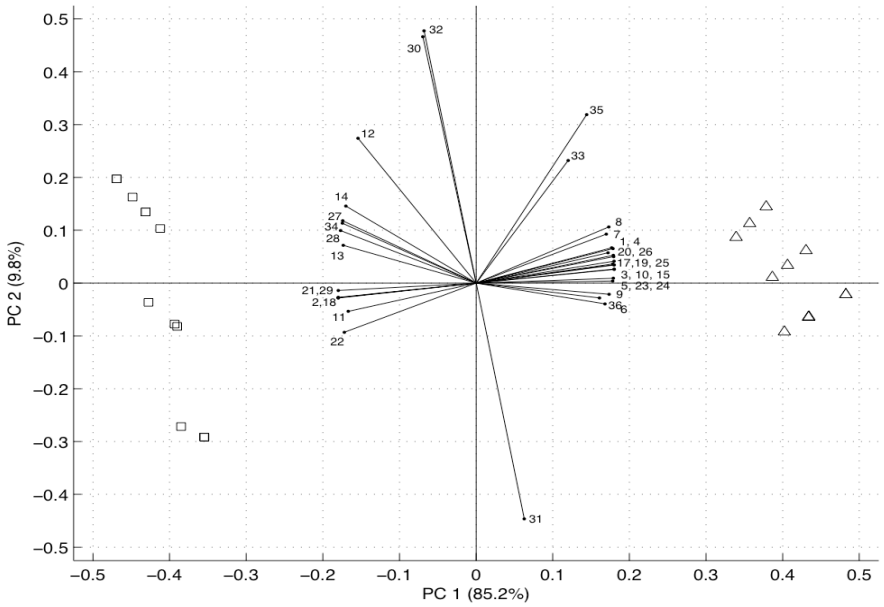


Figure 4.2. Biplot of volatile and non-volatile compounds of pink (□) and white (Δ) pomelo juice

Variables explained: (1) α-Pinene; (2) β-Pinene; (3) β-Myrcene; (4) Limonene; (5) *trans*-β-Ocimene; (6) ρ-Cymene; (7) Terpinolene; (8) β-Phellandrene; (9) Germacrene D; (10) Neryl acetate; (11) Linalool; (12) Nerol; (13) Geraniol; (14) *trans*-Nerolidol; (15) Octanal; (16); Nonanal; (17) Decanal; (18) Citronellal; (19) Neral; (20) Geranial; (21) *tran,cis*-2,4-Decadienal; (22) Dodecanal; (23) Perilla aldehyde; (24) *trans*-*trans*-2,4-Decadienal; (25) *trans*-2-Dodecenal; (26) *cis,trans*-2,6-Nonadienal; (27) 1,10-Dihydronootkatone; (28) Indole; (29) Nootkatone; (30) Fructose; (31) Glucose; (32) Sucrose; (33) Ascorbic acid; (34) Citric acid; (35) Malic acid; (36) Succinic acid

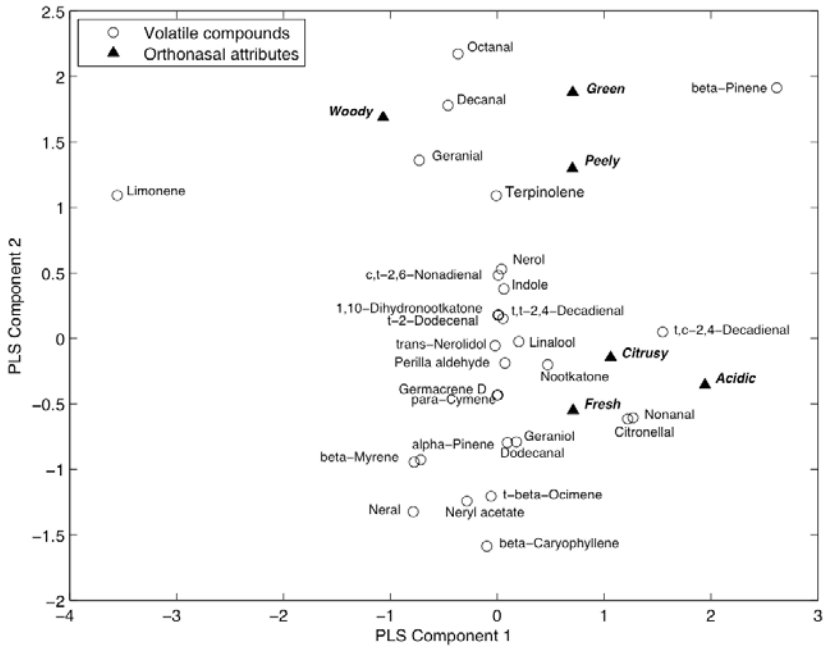
To further correlate the instrumental data with the sensory attributes, PLSR was employed. As a recent technique that can generalise and combine features from both PCA and multiple regression, PLSR can analyse a set of dependent variables from a set of independent variables (*166*). Table 4.4 presents the percentage of variations explained and the R-squared values for the PLSR analysis that included either 29 volatiles or 11 non-volatiles as dependent variables, in which the explanatory variables matrix comprised 10 sensory attributes (i.e. 7 orthonasal and 3 retronasal). Overall, the percentage explained of the orthonasal attributes was not as good as the retronasal attributes.

Table 4.4. Percentage of variation explained in the first two components of PLSR

Sensory variables	Percentage explained	R-squared
Orthonasal		
Acidity	68.38	0.6838
Citrusy	26.22	0.2622
Fresh	60.70	0.6070
Green	53.56	0.5356
Peely	72.70	0.7269
Woody	21.59	0.2122
Retronasal		
Bitter	25.86	0.2586
Sour	86.37	0.8637
Sweet	78.34	0.7834

In addition, not all sensory variables were well defined with the first two components, notably citrusy, woody and bitter. Therefore, it suggests that these attributes were not fully elucidated in the current PLSR model. As it can be seen from Figure 4.3(a), two correlated attributes were located closely to each other, namely citrusy and fresh notes; of which, potent aroma compounds such as terpene alcohols (linalool, *trans*-nerolidol, geraniol and nerol), aldehydes (citronellal, nonanal, *trans,cis*-2,4-decadienal) and nootkatone were situated around. The peely note was mainly correlated with octanal, decanal and geranial. On the other hand, sourness that was mainly influenced by the citric acid content was located far apart from other retronasal notes of sweetness and bitterness (Figure 4.3(b)). The PLSR analysis indicated that the present model is of little value in correlating bitterness. Finally, the effect of phenolic components on the bitterness of pomelo juice has yet to be investigated.

(a)



(b)

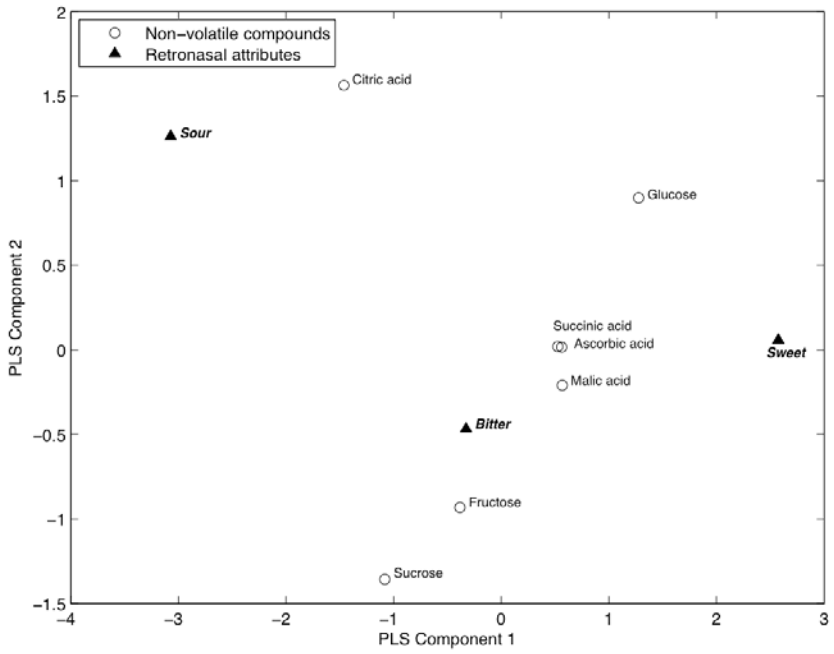


Figure 4.3. PLSR loading plots of volatile compounds correlated with orthonasal attributes (a) and non-volatile compounds correlated with retronasal attributes (b)

4.4. Conclusion

The juices of two cultivars of pomelo fruits from Malaysia were characterized through measuring their physicochemical properties, volatile and non-volatile components. As a pigmented variety, the pink pomelo juice shared a closer resemblance to grapefruit chemically in contrast to the white pomelo. Moreover, the more terpenic white pomelo juice with a milder acidity and lesser amounts of volatiles was suggested to be a kind of atypical citrus fruit. Both the chemical composition and sensory profiles were further correlated with the aid of multivariate analysis. Using PCA, the main variations of both cultivars were contributed by their aroma-active compounds. PLSR indicated that the most aroma-active compounds contributed to the sensory perception of acidic, fresh and peely notes while the non-volatile components were correlated with the sour and sweet tastes. Hence, this finding may benefit future work correlating sensory perception with instrumental data.

CHAPTER 5 CHARACTERIZATION OF CALAMANSI (*CITRUS MICROCARPA*): VOLATILES, AROMATIC PROFILE AND PHENOLIC ACIDS IN THE PEELS

5.1. Introduction

There have been limited studies on the volatile constituents of calamansi peel. Of these, the earliest study was performed by Nigam *et al.* (*246*) as a preliminary analysis of the calamansi peel from India. Further, Moshonas and Shaw (*188*) used hexane to extract the calamansi peel originated from United States, and detected 56 volatiles. Also using hexane, Takeuchi *et al.* (*237*) identified a trace amount of methyl-N-methyl anthranilate (characteristic mandarin-like aroma) in the calamansi peel obtained from the Philippines. More recent work compared the volatile composition of the calamansi peel with its leaf using hydro-distillation method (*247*). However, there is still a lack of systematic knowledge of calamansi peel, especially its sensory information.

Besides their characteristic flavor, the health-promoting properties of citrus fruits have been ascribed to their inherent phenolic compounds, including coumarins, flavanoids, lignins, phenolic acids and tannins (*248*).

Moreover, several studies of phenolic acids (e.g. caffeic, *p*-coumaric, ferulic and sinapic acids) in citrus fruits have been confined mainly to understand their distributions among the cultivars during maturation and exploration of their nutritional properties (*249-252*). To date, there is no report of phenolic acids content in calamansi peel. Therefore, the objective of this study was to characterize and compare calamansi peel through measuring its volatiles and aromatic profiles of calamansi from different geographical origins. The obtained data (volatiles and phenolic acids) was systematically studied using three statistical tools. In addition, the phenolic acids content could provide some information about the antioxidant capacity of calamansi peel.

5.2. Experimental procedures

5.2.1. Calamansi materials and chemicals

Based on the preliminary screening (i.e. examination on their physical qualities, e.g. fruit size, maturation date; comparative sensory analysis of their aromatic profiles; screening their volatiles using headspace solid-phase microextraction (HS-SPME)), calamansi fruits (*Citrus microcarpa*) from three locations (Johor Bahru, Malaysia; Quezon City, the Philippines; Hanoi, Vietnam) were chosen in the present study, and three batches of each location were collected on September 30 2010, November 26 2010 and February 20 2011, respectively. Only mature calamansi fruits were selected, and then cleaned with deionized water before use. Their thin peels (flavedo parts) were separated and cut into small pieces manually. For all calamansi fruits, each batch (*c.a*. 1300 g) was weighed to give *c.a*. 200 g of calamansi peel. Some were immediately used for extraction of volatiles, and others were vacuum-

dried (Shel Lab, Cornelius, OR, USA) at 40 °C until constant mass. The dried peel was ground into fine powder in a blender (Braun, Kronburg, Germany) for phenolic acid extraction.

All standard compounds used in the identification of volatile constituents were obtained from Firmenich Asia Pte. Ltd., Singapore.

5.2.2. Extraction of volatile compounds

A 100 g sample of calamansi peel was covered with 150 mL of dichloromethane. The mixture was then stirred using a vortex shaker at 300 rpm for 1 h. Solid residues were then separated from the extract by filtration. Excess anhydrous sodium sulfate was added into the filtrate to remove moisture. The extract was then filtered and concentrated to 0.5 mL using a TurboVap II rotary evaporator. The concentrate was dark green. Finally, the peel extract was diluted to 15 mL with dichloromethane, dispensed into amber sample vials and stored at -70 °C before being analyzed by GC-MS/FID and sensory evaluation. Another similar series of extraction experiments were performed using hexane (Tedia), and yellow-greenish extract was obtained.

5.2.3. GC-MS/FID analysis

The volatiles of calamansi peel extracts were detected using an Agilent 6890N GC coupled with FID and a 5975 inert MSD. For quantitative analysis of calamansi peel extracts, a similar procedure was carried out with spiking of 5-methyl-2-hexanone as described in Section 3.2.2 of Chapter 3, except at a split ratio of 1:2. The concentration of the compounds was expressed as parts per million (ppm) based on the relative FID peak area of each compound

against internal standard with the response factor, previously measured with standard compounds under the same conditions.

5.2.4. Extraction of phenolic acids

Phenolic acids were isolated from calamansi peel according to the method described by Bocco *et al*. (*253*). For free phenolic acids, 10 g of calamansi peel powder was extracted twice with 40 mL of 80% methanol through vortexing at a speed of 300 rpm for 30 min. The mixture was filtered, and then was evaporated to dryness under reduced pressure at 45 °C (Büchi Labortechnik AG, Flawil, Switzerland). Finally, the dried residue was reconstituted by dissolving in 10 mL of methanol and later filtered with a 0.20 μm PTFE filter (Agilent Technologies) for the determination of free phenolic acids.

For the isolation of bound phenolic acids, 10 g of the above dried was hydrolyzed with 80 mL of 2 M NaOH containing 10 mM EDTA and 1% ascorbic acid (w/v) under nitrogen for 4 h. By adding EDTA and ascorbic acid, degradation of phenolic acids during alkaline hydrolysis can be prevented (*254*). The aqueous phase was filtrated and then acidified with 6 M HCl to pH 2.0. The acidified water phase was extracted 3 times with 80 mL of ethyl acetate. Excess of anhydrous Na_2SO_4 was added to remove moisture. The extract was filtered prior to evaporation to dryness under reduced pressure at 45 °C. Finally, the dry residue was reconstituted by dissolving in 20 mL of methanol and later filtered with a 0.20 μm PTFE filter for further analysis.

5.2.5. UFLC/PDA analysis of phenolic acid content

The phenolic acid content of calamansi peel was measured using a Shimadzu Prominence UFLC system equipped with two LC-20AD pumps, a SIL-20A autosampler injector, CTO-20AC column oven and a SPD-M20A photodiode array detector. A C18 reverse-phase silica column coated with a dense monolayer of dimethyl-n-octadecylsilane stationary phase (150 mm x 4.6 mm x 5 μm ZORBAX Eclipse Plus C18) with a guard column (4.6 mm x 12.5 mm, 5 μm) was used for the separation of phenolic acids. The column oven temperature was set at 40 °C. Sample injection volume was 10 μL and flow rate was 1.0 mL/min. The mobile phase consisted of two solvents: solvent A, 0.1% acetic acid and solvent B, 100% methanol. The solvent gradient in volume ratio was as follows: 0 - 1 min, from 10% B to 16% B; 1 - 3 min, from 16% B to 42% B; 3 - 25 min, from 42% B to 65% B; 25 - 26 min, from 65% B to 20% B; 26 - 27 min, from 20% B to 10% B; 27 - 30 min, from 16 % B to 10 % B, followed by washing and reconditioning the column. Identification of phenolic acids was achieved by matching the retention time and UV-visible spectra of standards. External standards were used for phenolic acids quantification: chlorogenic and ferulic acids (Aldrich), caffeic and *p*-coumaric acids (Sigma), and sinapic acid (Fluka). The calibration curves were obtained within a concentration range of 1 to 400 mg/L.

5.2.6. Statistical analysis

One-way ANOVA (SPSS, Statistical Package for the Social Sciences Version 16.0, IBM Corporation, Armonk, NY, USA) was selected to determine the significant difference of the volatiles and phenolic acids data

sets obtained from calamansi peel extracts. Significant differences between the mean values were determined by Duncan's multiple range tests with the probability value of $p<0.05$. In additional, multivariate analysis of the same volatile data sets was carried out using PCA and CDA (Matlab, version 7.6.0.324, R2008a). A score plot of PCA was used to study the distribution of the volatiles relative to the overall variability of the data sets obtained from the three countries. Obtained from the volatile data, representations expressed a strong correlation in PCA and corresponded to significant F and p values. Furthermore, CDA was applied to verify the selected group of volatiles showing the maximum capacity of these discriminations. The detailed procedure was reported by Trujilo-Ortiz *et al*. (*255*).

All experiments were carried out in triplicate and the results were reported as the mean values.

5.2.7. Sensory evaluation

Sensory evaluation was carried out as described in Chapter 2 (Section 2.2.4). Selected descriptive sensory attributes include fatty, fruity, green, juicy, mandarin-like, peely, sweet and woody. A 9-point scale was used with '0' indicating unperceived attribute intensity and '9' indicating very strong attribute intensity.

5.3. Results and discussion

5.3.1. Volatile components of calamansi peel

Considering solvent efficiency in the extraction of volatiles from food matrices (*256*), both polar organic solvent (dichloromethane) and non-polar organic solvent (hexane) were selected. According to Table 5.1, it is noteworthy that the volatiles obtained in hexane peel extracts were relatively higher than those in dichloromethane peel extracts. This is likely due to solvent polarity, which affects extraction efficiency on different groups of volatile compounds and compositions. In comparison to hexane, dichloromethane has a relatively higher extraction affinity towards polar oxygenated volatile chemicals, resulting in a different aromatic profile (discussed below).

Table 5.1. Identification of volatile compounds and their concentrations (ppm) of calamansi (*Citrus microcarpa*) peel extracts from Malaysia, the Philippines and Vietnam through hexane and dichloromethane

Compounds	LRI	Hexane			F	*p*	Dichloromethane			F	*p*
		MAS	PHIL	VIET			MAS	PHIL	VIET		
Hydrocarbons											
α-Pinene[I,II,III]	902	34.93 ± 10.1	49.63 ± 20.1	69.52 ± 20.0	17.683	0.000	31.24 ± 10.1	47.39 ± 40.7	50.79 ± 13.3	10.515	0.001
Camphene	937	0.55 ± 0.1	0.62 ± 0.3	0.73 ± 0.1	4.407	0.028	0.73 ± 0.3	0.59 ± 0.6	0.70 ± 0.3	3.885	0.040
β-Pinene[I,II,III]	972	41.72 ± 18.9	50.47 ± 12.8	65.45 ± 6.3	15.968	0.000	35.56 ± 14.4	44.22 ± 15.4	46.29 ± 6.4	6.152	0.009
Sabinene[I,II,III]	983	18.04 ± 6.4	22.75 ± 7.3	30.88 ± 4.7	16.130	0.000	15.84 ± 5.9	21.28 ± 11.8	22.89 ± 1.4	6.669	0.007
δ-3-Carene[I]	1109	2.67 ± 1.2	2.63 ± 1.6	5.01 ± 2.3	19.071	0.000	2.83 ± 1.6	2.19 ± 1.2	4.24 ± 1.9	13.563	0.000
β-Myrcene[I,II,III]	1124	173.65 ± 63.9	226.75 ± 103.7	320.95 ± 109.8	15.874	0.000	158.16 ± 66.1	228.22 ± 170.0	257.04 ± 71.3	6.081	0.010
Limonene[I,II,III]	1176	8638.38 ± 3213.6	10980.04 ± 5070.0	15629.51 ± 5219.8	16.129	0.000	7651.41 ± 3247.3	10979.72 ± 8068.9	12375.75 ± 3234.7	6.097	0.010
β-Phellandrene[II]	1182	29.06 ± 13.8	35.23 ± 15.9	51.89 ± 22.0	16.851	0.000	26.09 ± 11.8	38.36 ± 36.4	41.03 ± 14.3	5.961	0.010
trans-β-Ocimene[II,III]	1204	0.41 ± 0.1	0.66 ± 0.8	0.73 ± 0.6	3.594	0.049	0.45 ± 0.1	0.45 ± 0.4	0.69 ± 0.6	7.623	0.004
γ-Terpinene[I,II,III]	1217	0.36 ± 0.1	0.51 ± 0.5	0.83 ± 0.7	5.080	0.018	0.54 ± 0.3	0.43 ± 0.5	0.60 ± 0.5	3.965	0.037
cis-β-Ocimene[II,III]	1221	3.82 ± 0.8	4.04 ± 2.0	5.16 ± 1.6	4.167	0.033	3.99 ± 1.4	3.94 ± 2.4	4.09 ± 0.4	1.668	0.216
para-Cymene[I,II]	1242	0.52 ± 0.2	0.40 ± 0.2	0.50 ± 0.4	2.354	0.124	0.61 ± 0.3	0.49 ± 0.4	0.55 ± 0.4	3.254	0.062
Terpinolene[I,II]	1255	0.81 ± 0.3	1.04 ± 0.7	1.34 ± 0.7	8.320	0.003	0.67 ± 0.15	0.91 ± 0.7	1.04 ± 0.5	6.713	0.007
allo-Ocimene	1382	tr	tr	tr	-	-	tr	tr	tr	-	-
α-Cubebene	1421	0.56 ± 0.4	0.35 ± 0.2	0.62 ± 0.3	2.037	0.159	0.94 ± 0.5	0.38 ± 0.3	1.10 ± 0.3	10.903	0.001
δ-Elemene[I,II]	1441	13.78 ± 4.5	16.76 ± 8.6	23.25 ± 6.4	1.436	0.264	16.10 ± 3.8	21.26 ± 11.9	23.61 ± 1.9	11.257	0.001
Bicycloelemene	1452	1.39 ± 0.2	1.48 ± 0.5	2.09 ± 0.3	1.067	0.365	2.02 ± 0.8	1.79 ± 0.4	2.45 ± 0.6	5.990	0.010
α-Copaene[II]	1461	0.33 ± 0.2	0.19 ± 0.1	0.26 ± 0.1	2.837	0.085	0.28 ± 0.1	0.22 ± 0.2	0.30 ± 0.2	2.812	0.087
β-Bourbonene	1493	0.48 ± 0.2	0.33 ± 0.2	0.49 ± 0.3	1.767	0.199	0.47 ± 0.1	0.39 ± 0.2	0.58 ± 0.2	6.451	0.008
β-Elemene[I,II,III]	1561	3.64 ± 1.4	4.31 ± 2.8	6.27 ± 2.3	10.671	0.001	5.21 ± 1.5	5.43 ± 2.8	6.87 ± 1.4	5.051	0.018
β-Caryophyllene[I,II]	1566	1.12 ± 0.3	1.39 ± 0.6	1.89 ± 0.4	0.886	0.429	2.13 ± 0.8	1.88 ± 0.9	2.24 ± 0.3	2.380	0.121
γ-Elemene[II]	1608	1.51 ± 0.2	1.93 ± 0.8	2.96 ± 0.6	2.329	0.126	1.93 ± 0.8	2.17 ± 0.7	2.95 ± 0.4	5.389	0.015
β-Cubebene	1612	0.58 ± 0.4	0.35 ± 0.4	0.44 ± 0.4	5.564	0.013	0.85 ± 1.0	0.41 ± 0.61	0.54 ± 0.84	2.519	0.109

Table 5.1. (Cont'd)

β-Farnesene[II]	1628	1.03 ± 0.4	0.65 ± 0.6	1.00 ± 0.8	1.377	0.278	1.18 ± 0.3	0.91 ± 1.2	1.00 ± 0.8	12.349	0.000
α-Humulene[II]	1642	1.79 ± 0.4	1.37 ± 0.6	2.83 ± 1.3	3.601	0.048	1.67 ± 0.4	2.46 ± 3.2	2.55 ± 0.4	17.842	0.000
Germacrene D[I,II]	1685	146.39 ± 38.5	137.45 ± 75.0	202.92 ± 75.9	0.710	0.505	140.64 ± 41.7	179.62 ± 140.9	169.14 ± 83.0	0.266	0.769
β-Selinene[I,II]	1689	6.18 ± 5.6	6.22 ± 7.2	10.96 ± 12.5	1.330	0.289	5.76 ± 4.57	9.64 ± 15.3	36.97 ± 69.9	1.524	0.245
α-Selinene[II]	1696	6.50 ± 2.9	6.38 ± 3.1	9.62 ± 4.3	11.579	0.001	7.23 ± 3.4	7.47 ± 3.31	10.32 ± 4.72	9.379	0.002
Bicyclogermacrene[I]	1609	14.52 ± 1.1	12.04 ± 5.2	17.08 ± 5.2	1.082	0.360	14.48 ± 2.5	15.84 ± 11.2	16.70 ± 2.6	4.392	0.028
α-Farnesene[II]	1715	3.08 ± 2.0	2.49 ± 2.2	2.54 ± 1.0	1.318	0.292	2.84 ± 2.1	3.40 ± 4.5	2.89 ± 1.2	2.531	0.107
Germacrene B	1823	2.89 ± 1.5	2.22 ± 2.0	2.34 ± 2.0	1.158	0.336	2.71 ± 0.8	2.84 ± 4.1	2.11 ± 1.8	1.933	0.174
Alcohols											
Hexanol[I]	1316	0.24 ± 0.1	0.17 ± 0.1	0.17 ± 0.1	1.993	0.165	0.90 ± 0.5	0.50 ± 0.3	0.32 ± 0.2	9.541	0.001
cis-3-Hexenol[I,II]	1350	6.12 ± 1.6	3.33 ± 2.0	4.34 ± 0.6	1.722	0.207	25.31 ± 7.3	13.43 ± 6.8	13.29 ± 0.5	21.257	0.000
Linalool[I,II,III]	1507	32.61 ± 9.6	25.27 ± 10.8	36.53 ± 7.2	2.003	0.164	37.64 ± 6.4	29.99 ± 18.3	39.02 ± 5.4	10.144	0.001
Sabinene hydrate[I]	1514	0.72 ± 0.5	0.30 ± 0.2	0.77 ± 0.7	2.392	0.120	0.91 ± 0.5	0.39 ± 0.3	1.09 ± 0.6	6.892	0.006
Octanol[I,II,III]	1532	12.68 ± 4.1	13.42 ± 8.5	27.95 ± 4.5	8.480	0.003	14.00 ± 2.8	17.13 ± 13.7	26.26 ± 1.0	12.841	0.000
Nonanol[I,II]	1617	3.81 ± 1.8	3.82 ± 2.2	8.40 ± 3.8	10.900	0.001	3.62 ± 1.9	4.42 ± 2.3	6.49 ± 2.9	10.370	0.001
α-Terpineol[I,II,III]	1664	13.96 ± 4.7	11.52 ± 5.3	16.62 ± 3.6	1.167	0.334	20.44 ± 4.1	17.79 ± 10.9	22.34 ± 4.1	7.480	0.004
Citronellol[I]	1723	1.96 ± 1.0	1.75 ± 0.8	2.44 ± 1.1	8.320	0.003	2.26 ± 1.3	1.74 ± 0.6	2.47 ± 1.3	5.461	0.014
trans-2-Decenol	1799	0.90 ± 0.6	0.49 ± 0.3	0.91 ± 0.5	1.895	0.179	1.22 ± 0.7	0.82 ± 0.4	1.63 ± 1.1	5.025	0.018
Carveol[* II]	1848	tr	tr	tr	-	-	tr	tr	tr	-	-
Perilla alcohol	1972	2.43 ± 2.1	0.97 ± 0.5	1.59 ± 0.8	3.434	0.055	1.68 ± 0.9	0.99 ± 0.4	2.00 ± 0.9	5.567	0.013
trans-Nerolidol[I]	1991	2.43 ± 1.5	1.04 ± 0.5	2.51 ± 0.8	4.866	0.020	2.35 ± 1.5	1.36 ± 0.9	2.18 ± 0.5	4.472	0.026
Elemol[*I,II,III]	2042	14.98 ± 3.7	15.95 ± 8.7	25.17 ± 5.9	2.373	0.122	17.32 ± 3.2	18.45 ± 8.5	24.93 ± 4.5	12.131	0.000
γ-Eudesmol[I]	2076	4.13 ± 1.6	2.26 ± 1.6	5.14 ± 2.3	5.644	0.013	3.62 ± 1.0	2.74 ± 2.7	5.12 ± 2.3	8.265	0.003
α-Eudesmol[I,II]	2190	6.97 ± 1.9	5.42 ± 8.9	9.22 ± 3.4	3.366	0.057	5.61 ± 1.5	6.16 ± 5.5	8.40 ± 2.4	9.383	0.002
β-Eudesmol[II]	2200	17.87 ± 4.6	15.57 ± 8.9	25.34 ± 8.1	10.442	0.001	15.54 ± 3.1	18.09 ± 15.5	21.90 ± 4.8	9.007	0.002
Phytol[I]	2572	11.44 ± 11.6	1.44 ± 0.6	5.28 ± 4.0	5.245	0.016	12.67 ± 7.8	2.62 ± 2.8	8.53 ± 9.9	6.005	0.010
Aldehydes											
Octanal[I,II,III]	1262	3.80 ± 2.7	4.43 ± 4.5	7.02 ± 2.1	1.579	0.233	5.12 ± 2.3	6.69 ± 8.5	11.35 ± 4.2	13.255	0.000
Nonanal[I,II,III]	1365	5.42 ± 5.3	5.83 ± 5.5	9.39 ± 4.2	15.543	0.000	6.36 ± 4.9	8.42 ± 11.8	12.36 ± 6.9	11.531	0.001
Decanal[I,II,III]	1469	10.14 ± 7.4	12.79 ± 11.4	21.65 ± 12.1	18.270	0.000	10.48 ± 5.8	17.19 ± 19.9	23.68 ± 10.2	11.738	0.001
Undecanal[I,III]	1573	4.15 ± 1.1	4.61 ± 2.3	7.72 ± 2.9	8.603	0.002	4.90 ± 1.4	6.22 ± 5.25	8.25 ± 2.6	8.431	0.003

Table 5.1. (Cont'd)

trans-2-Decenal[I]	1616	2.51 ± 0.6	3.10 ± 1.7	3.57 ± 0.7	1.820	0.191	3.12 ± 1.5	3.41 ± 2.3	4.53 ± 1.0	6.716	0.007
Neral[III]	1656	1.29 ± 0.5	1.04 ± 0.9	1.67 ± 0.6	1.559	0.237	1.23 ± 0.3	1.32 ± 1.4	1.93 ± 0.8	5.796	0.011
Geranial[III]	1676	1.94 ± 0.8	1.56 ± 1.3	2.50 ± 0.9	8.250	0.003	1.84 ± 0.5	1.98 ± 2.1	2.90 ± 1.2	5.796	0.011
trans,cis-2,4-Decadienal[I,II]	1737	1.87 ± 1.7	1.24 ± 1.8	2.13 ± 2.7	3.007	0.075	1.94 ± 1.0	0.73 ± 0.4	2.65 ± 3.0	7.266	0.005
Perilla aldeyhde[I,II,III]	1768	2.23 ± 0.9	1.98 ± 2.6	2.09 ± 0.9	4.609	0.024	2.00 ± 0.5	2.13 ± 3.1	2.87 ± 1.7	4.533	0.025
trans,trans-2,4-Decadienal[I,II]	1806	2.16 ± 0.9	2.11 ± 0.9	3.14 ± 1.6	4.558	0.025	1.72 ± 1.1	2.58 ± 3.3	3.01 ± 1.7	5.745	0.012
trans-2-Dodecenal	1849	1.18 ± 0.4	0.59 ± 0.3	2.11 ± 2.8	10.080	0.001	1.35 ± 0.3	1.30 ± 1.6	2.45 ± 2.1	2.790	0.088
trans,cis-2,6-dodecadienal	1860	1.30 ± 0.6	0.67 ± 0.4	1.24 ± 0.7	6.754	0.006	0.94 ± 0.7	1.25 ± 1.5	2.69 ± 2.9	2.169	0.143
Esters											
cis-3-Hexenyl acetate	1287	6.12 ± 0.5	3.33 ± 0.7	4.34 ± 0.4	7.409	0.004	25.31 ± 0.4	13.43 ± 1.0	13.29 ± 0.3	17.701	0.000
Heptyl acetate[I,III]	1342	0.55 ± 0.3	0.48 ± 0.2	0.68 ± 0.1	11.931	0.001	0.74 ± 0.3	0.50 ± 0.3	0.77 ± 0.2	9.048	0.002
Ethyl octanoate	1443	tr	tr	tr	-	-	tr	tr	tr	-	-
Octyl acetate	1446	0.73 ± 1.3	0.69 ± 1.7	0.93 ± 1.9	2.340	0.125	0.73 ± 1.2	0.99 ± 2.5	0.64 ± 1.1	1.755	0.201
Citronellyl acetate[II,III]	1625	1.03 ± 0.7	0.80 ± 0.6	1.33 ± 1.3	2.786	0.088	0.80 ± 0.2	1.12 ± 1.7	1.20 ± 1.0	3.045	0.073
Decyl acetate	1646	1.31 ± 0.8	1.14 ± 1.2	1.94 ± 2.0	1.843	0.187	1.54 ± 0.8	1.54 ± 2.2	1.85 ± 1.7	3.370	0.057
Geranyl acetate[III]	1719	34.39 ± 8.0	31.89 ± 11.0	52.27 ± 9.0	1.736	0.204	29.90 ± 10.5	38.27 ± 21.4	46.38 ± 3.7	7.835	0.004
Methyl salicylate[III]	1761	2.77 ± 0.9	1.34 ± 0.7	2.56 ± 0.7	14.952	0.000	3.18 ± 1.0	1.69 ± 1.0	3.09 ± 0.7	11.978	0.000
Geranyl propionate	1804	1.64 ± 1.0	1.19 ± 0.9	2.82 ± 1.3	8.056	0.003	1.67 ± 0.9	1.63 ± 1.2	2.57 ± 0.6	12.668	0.000
Dodecyl acetate	1854	0.99 ± 0.3	0.32 ± 0.2	0.79 ± 0.4	7.481	0.004	0.98 ± 0.5	0.86 ± 1.0	1.61 ± 1.6	1.713	0.209
Perillyl acetate	1935	2.07 ± 1.1	1.06 ± 0.6	2.29 ± 0.7	8.311	0.003	1.87 ± 0.8	1.45 ± 1.2	2.04 ± 0.7	4.423	0.027
Methyl-N-methyl anthranilate [I,II]	2061	3.27 ± 3.0	0.84 ± 0.8	2.37 ± 2.0	2.290	0.130	2.43 ± 1.9	0.94 ± 0.6	2.11 ± 1.7	4.358	0.029
Acids											
Acetic acid	1421	0.19 ± 0.1	0.02 ± 0.0	0.38 ± 0.2	8.108	0.003	0.30 ± 0.2	0.25 ± 0.2	0.54 ± 0.3	10.536	0.001
Octanoic acid	2017	4.09 ± 2.8	1.12 ± 0.8	3.49 ± 1.0	5.583	0.013	3.80 ± 2.1	2.27 ± 2.41	4.61 ± 2.3	3.984	0.037
Nonanoic acid[III]	2116	3.97 ± 2.4	1.44 ± 0.2	2.90 ± 1.4	2.759	0.090	3.45 ± 1.8	2.14 ± 0.3	4.01 ± 2.2	3.879	0.040
Decanoic acid	2230	2.04 ± 3.2	0.82 ± 0.2	2.11 ± 1.4	6.026	0.010	2.38 ± 2.8	1.14 ± 1.9	3.09 ± 3.6	2.389	0.120
Myristic acid[** II,III]	2659	0.39 ± 1.0	2.27 ± 2.0	6.96 ± 3.5	14.329	0.000	1.11 ± 2.9	2.72 ± 4.8	14.05 ± 13.0	9.392	0.002
Palmitic acid[** II,III]	2889	24.39 ± 20.6	5.05 ± 7.0	14.66 ± 9.7	6.527	0.007	36.10 ± 13.4	15.86 ± 23.5	30.17 ± 39.2	3.079	0.071
Stearic acid[**]	3109	21.09 ± 26.7	2.50 ± 3.0	17.81 ± 19.8	3.067	0.071	48.18 ± 31.6	16.84 ± 8.0	46.40 ± 60.8	2.543	0.107
Linoleic acid[**]	3140	0.98 ± 2.6	3.27 ± 4.5	14.99 ± 10.0	45.903	0.000	17.92 ± 11.2	7.40 ± 10.0	12.11 ± 10.2	4.578	0.025

Table 5.1. (Cont'd)

Others										
Limonene oxide[II]	1416	0.28 ± 0.3	0.40 ± 0.6	0.23 ± 0.3	1.101	0.354	0.37 ± 0.12	0.35 ± 0.7	0.31 ± 0.3	8.110	0.003
Camphor	1498	0.35 ± 0.1	0.29 ± 0.2	0.47 ± 0.3	1.979	0.167	0.47 ± 0.2	0.32 ± 0.3	0.48 ± 0.2	4.617	0.024
Isopiperitenone[I,II]	1843	1.12 ± 0.7	1.47 ± 3.3	1.05 ± 0.5	7.671	0.004	1.15 ± 0.7	1.73 ± 3.6	2.28 ± 2.3	2.124	0.149
Carvone[I,II,III]	1762	tr	tr	tr	-	-	tr	tr	tr	-	-
Total identified (ppm)		*9.43E03* ± 3.38E03	*1.18E04* ± 5.44E03	*1.68E04* ± 5.53E03			*8.53E03* ± 3.39E03	*1.19E04* ± 8.76E03	*1.36E04* ± 3.42E03		
Overall total identified (%)		*98.80*	*99.72*	*99.46*			*98.46*	*99.56*	*99.21*		

[I] Compounds reported in Ref. (*247*); [II] Compounds reported in Ref. (*237*); [III] Compounds reported in Ref. (*188*).
"-", not detected; "tr", trace; [*] unknown isomer; [**] semi-volatile compounds detected; MAS, Malaysia; PHIL, the Philippines; VIET, Vietnam.
LRI on column (DB-FFAP) determined with two series of n-alkanes (C5–C20 and C21-C40).
p-values in bold indicate compounds were significantly different ($p<0.05$) among countries of similar solvent extraction.

Moreover, in the hexane extracts, total volatile amounts ranged from 16844 ppm (Vietnam), 11,775 ppm (the Philippines) to 9,435 ppm (Malaysia), respectively. These compounds were categorized into the groups of hydrocarbons, alcohols, aldehydes, esters, acids and others. Hydrocarbons were dominant compounds in calamansi peel extracts, regardless of their geographical origins. The group of hydrocarbons comprised predominantly of monoterpenes (e.g. limonene, β-myrcene, β-pinene, α-pinene, β-phellandrene and sabinene) and sesquiterpenes (e.g. elemene, farnesene and germacrene isomers), which were also commonly reported in other citrus fruits (*184, 237, 247, 257*). Germacrene D and bicyclogermacrene were the important volatile components that characterize the unique flavor of Japanese yuzu (*Citrus junos*) and daidai (*Citrus aurantium*) (*257, 258*). A previous study reported that these two compounds could be used to indicate the quality and freshness of the peel extracts because they are susceptible to isomerization, oxidation and rearrangement processes during storage (*258, 259*).

Among 16 identified alcohols, linalool and elemol were major terpene alcohols. In addition to hexanol, *cis*-3-hexenol and *trans*-nerolidol are common in citrus fruits. As a group, they can impart floral, green and fresh notes. Some aldehydes exhibit intense aroma of citrus. For instance, both of citral stereoisomers (geranial and neral) are thought to be important for the aroma of kumquat, one parent hybrid of calamansi (*206*). Furthermore, a wide range of aliphatic aldehydes (C8 to C12) was found here. Hexanal, by-product formed from fatty acid degradation, was reported in the mandarin peel oil and the Philippines calamansi peel (*176, 237, 260*), but was not detected in any of the calamansi peel extracts. Other fatty acid degradation aldehydes, such as

trans,*cis*-2,4-dodecadienal and *trans*,*trans*-2,4-dodecadienal (intense green, fatty and oily notes), were identified in each of the peel extracts. Among all, Vietnam peel extract had the highest aldehydes. Moreover, eleven ester compounds were detected here, particularly methyl-N-methyl anthranilate (a key characteristic mandarin-like volatile). Also, methyl salicylate was reported as an important component, due to its green and minty properties (*188*). Besides straight-chain acetate esters (i.e. *cis*-3-hexenyl acetate and heptyl acetate), terpenes esters (i.e. geranyl acetate, geranyl propionate, citronellyl acetate and perillyl acetate) could attribute to fresh, fruity and green notes.

5.3.2. Statistical analysis

Being a classical statistic tool, ANOVA is often performed before chemometrics. Hibbert (*154*) recommended standard statistical tests for normality and outliers in order to find out the significant differences between variables. However, ANOVA is sensitive to non-normal distributions, which is a typical trend in aromatic volatiles or essential oil data. There was one study reported by Butcher (*261*) which concluded that ANOVA was not appropriate to be used on ratios of terpenes from 109 samples of tea tree oil (*Melaleuca alterifolia*).

In the present work, the variations of volatile compounds among different locations were evaluated via ANOVA. Nevertheless, it is noted that the variation between analytical analysis and sensory perception could not be expressively correlated. This might due to some potent aroma-active compounds that are present at trace levels, which might be quantitatively insignificant among different geographical origins but overwhelmed by the

synergistic effects among aromatic volatiles. Hence, the segregation and distribution of the volatile variables among the peel extracts were subjected to PCA without removing the insignificant variables via ANOVA.

Three principal components (PCs) were obtained from dichloromethane extract data sets, accounting for 80.52% of the cumulative percentage of total variations, whereby PC 1, PC 2 and PC 3 accounted for 56.54%, 15.66% and 8.32% of the variance, respectively. The first PC axis was not especially effective in separating the calamansi from Malaysia and the Philippines (Figure 5.1(a)). Although there was some information in the data sets related to the first PC, limited discrimination ability of PCA caused the correlation to the origin of calamani to be only visible in the latter PCs (*262*). In spite of this limitation, the peel extracts of different origins were easily distinguished via the score plot of PC 3 vs. PC 2 (Figure 5.1(b)). Each of volatile variables that differentiated the data sets in Figure 5.1(b) is represented in Figure 5.1(c) by a vector, in which the direction and length of vector indicate the contribution of variable to PC 2 and PC 3. Regardless of their origins, the peel extracts were characterized by oxygenated compounds, which had larger positive coefficients in PC 2, such as citronellol, nonanol, perillyl acetate, decyl acetate and *trans*-2-decenol.

Moreover, methyl-N-methyl anthranilate had the highest coefficient in PC 2, indicating its strong influence as a characteristic compound in calamansi peel. This is in agreement with the sensory evaluation that calamansi peel extract exhibited a characteristic mandarin-like note (see discussion below). Variables such as α-pinene, sabinene, β-myrcene, limonene, β-phellandrene, heptyl acetate and octyl acetate were negatively associated with PC 2.

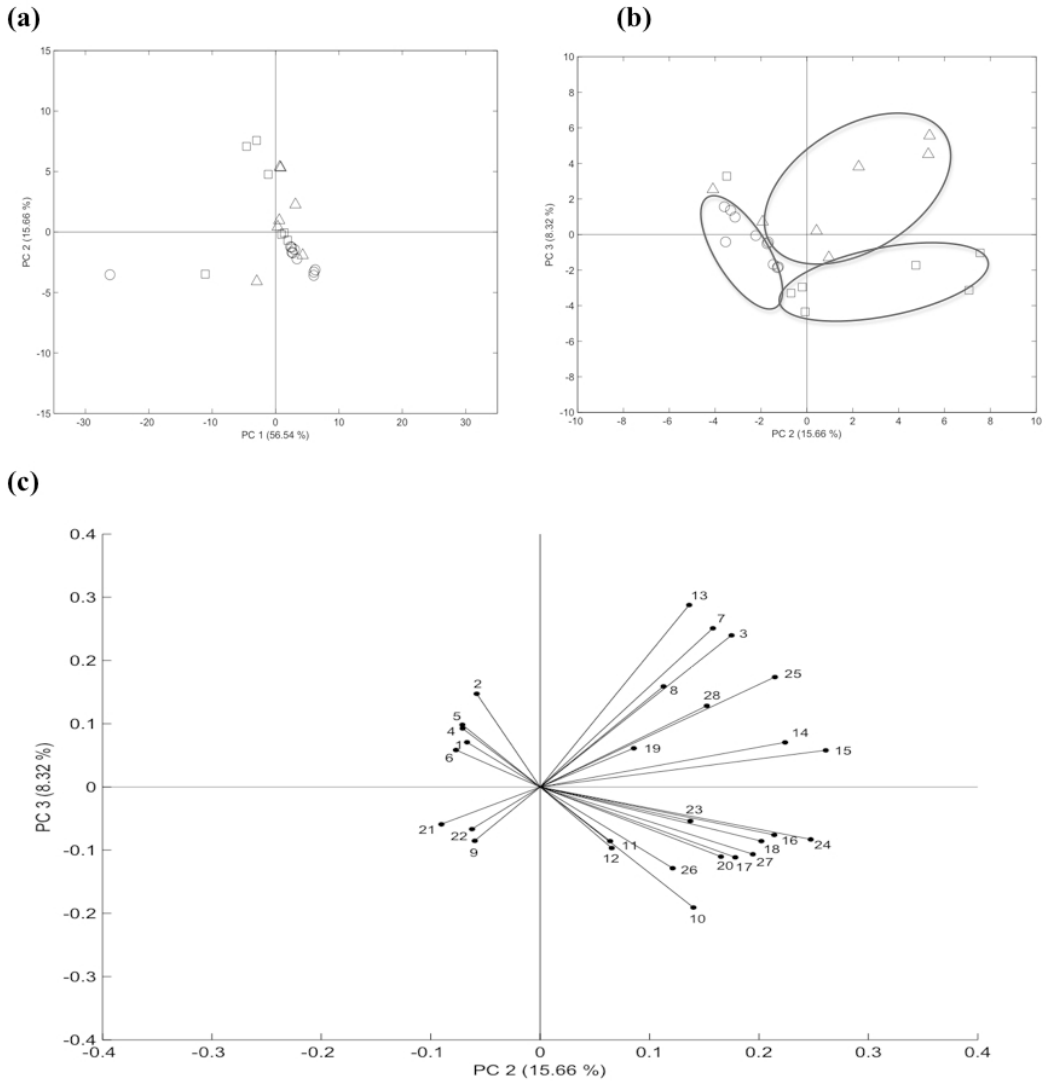


Figure 5.1. PCA of calamansi (*Citrus microcarpa*) peel extracts ((Δ) Malaysia; (○) the Philippines; (□) Vietnam)) using dichloromethane. (a) Score plot PC 2 against PC 1; (b) Score plot PC 3 against PC 2; (c) PCA plot on volatile variables of PC 3 against PC 2

Volatile variables explained: (1) α-Pinene; (2) Sabinene; (3) δ-3-Carene; (4) β-Myrcene; (5) Limonene; (6) β-Phellandrene (7) Bicycloelemene; (8) γ-Elemene (9) β-Cubebene; (10) α-Selinene; (11) Hexanol; (12) *cis*-3-Hexenol; (13) Sabinene hydrate; (14) Nonanol; (15) Citronellol; (16) *trans*-2-Decenol; (17) Perilla alcohol; (18) *trans*-Nerolidol; (19) Decanal; (20) *trans*,*cis*-2,6-Dodecadienal; (21) Heptyl acetate; (22) Octyl acetate; (23) Decyl acetate; (24) Perillyl acetate; (25) Methyl-N-methyl anthranilate; (26) Acetic acid; (27) Octanoic acid; (28) Decanoic acid. Other volatile variables listed in Table 1 are not shown due to their low loading factors in the linear combinations.

For hexane extracts, four PCs were obtained for PCA, accounting for 87.70% of cumulative variations of hexane extract data sets. The contributions for PC1, PC2, PC3 and PC4 were 44.36%, 21.75%, 13.05% and 8.54%, respectively. Similar to dichloromethane extract data sets, no separation for geographical differences was obtained in the first two PCs (Figure 5.1). However, the score plot of PC 4 against PC 3 clearly indicated the existence of three clusters within the peel extracts of Malaysia, the Philippines and Vietnam with respect to the volatile variables that segregate along PC 3 and PC 4 axes (Figure 5.2(b)). The contribution of each volatile variable to the score points in PC 3 and PC 4 is shown in Figure 5.2(c). By observing the loading factors of each variable in PC 3, the most important oxygenated compounds are methyl salicylate, nonanoic acid, octanoic acid, perillyl alcohol and phytol that expressed strong positive correlation to PC3, where terpene hydrocarbons (e.g. α-pinene, sabinene, β-myrcene and limonene), heptyl acetate and octyl acetate were found negatively correlated with PC 3.

As shown above, PCA is a useful tool in disclosing variances among volatiles or characteristic compounds. These compounds may be effective in accounting for the variances in the total data sets, but they may or may not be effective in discriminating between samples of different geographical origins. Thus, volatile compounds, of which had significant F values and p values (Table 5.1), were employed using CDA to verify the discrimination of the volatiles that showed strong correlations in PCs. In Figure 5.3, two canonical components (discriminant functions) contributed to class differentiation ($p<0.05$) for both dichloromethane and hexane extracts. So, the result

indicated that the volatile compounds with strong coefficients in PCA were confidently the discriminant among geographical origins.

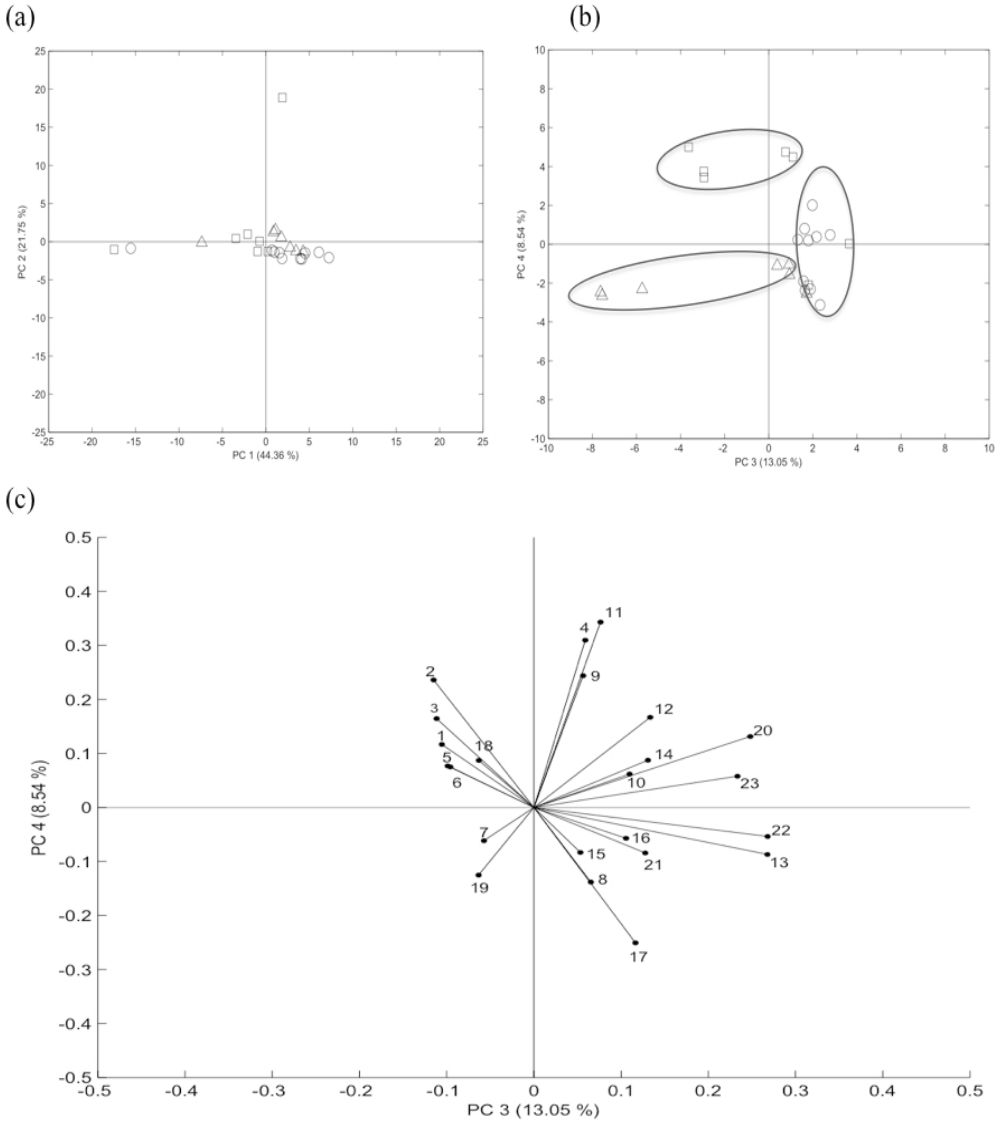


Figure 5.2. PCA of calamansi (*Citrus microcarpa*) peel extracts ((Δ) Malaysia; (○) the Philippines; (□) Vietnam)) using hexane. (a) Score plot of PC 2 against PC 1; (b) Score plot of PC 4 against PC 3; (c) PCA plot of volatile variables of PC 4 against PC 3

Volatile variables explained: (1) α-Pinene; (2) β-Pinene; (3) Sabinene; (4) δ-3-Carene; (5) β-Myrcene; (6) Limonene (7) *trans*-β-Ocimene; (8) ρara-Cymene; (9) Bicycloelemene; (10) Hexanol; (11) Nonanol; (12) Citronellol; (13) Perilla alcohol; (14) Geranial; (15) perillaldehyde; (16) trans,*cis*-2,6-Dodecadienal; (17) *cis*-3-Hexenyl acetate; (18) Heptyl acetate; (19) Octyl acetate; (20) Methyl salicylate; (21) Dodecyl acetate; (22) Octanoic acid; (23) Nonanoic acid. Other volatile variables listed in Table 1 are not shown due to their low loading factors in the linear combinations.

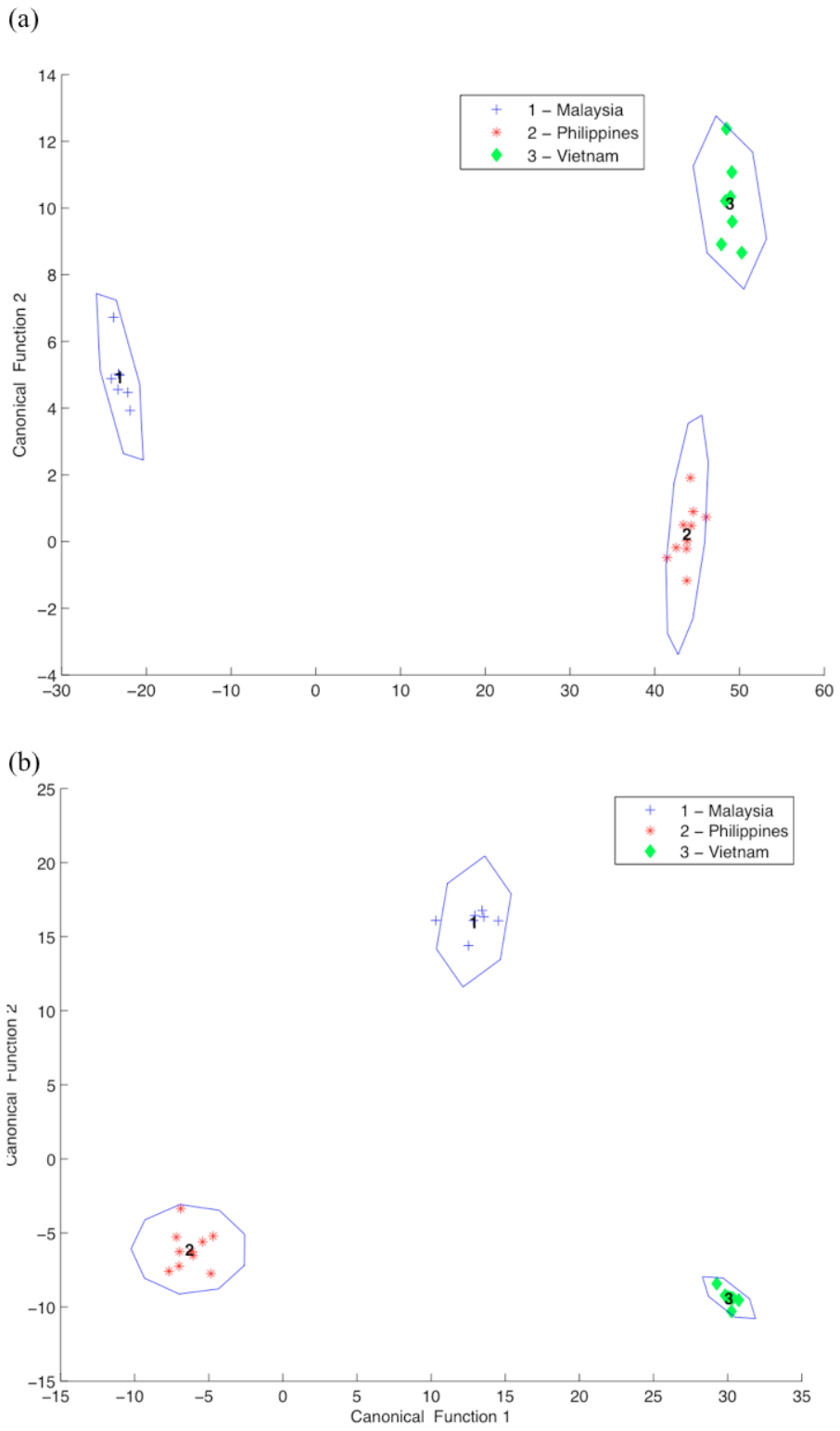


Figure 5.3. Canonical discriminant analysis employing country origin as grouping criterion. Projection of volatile variables on the discriminant space, selecting the two discriminant functions as axes: (a) Dichloromethane; (b) Hexane

5.3.3. Sensory evaluation

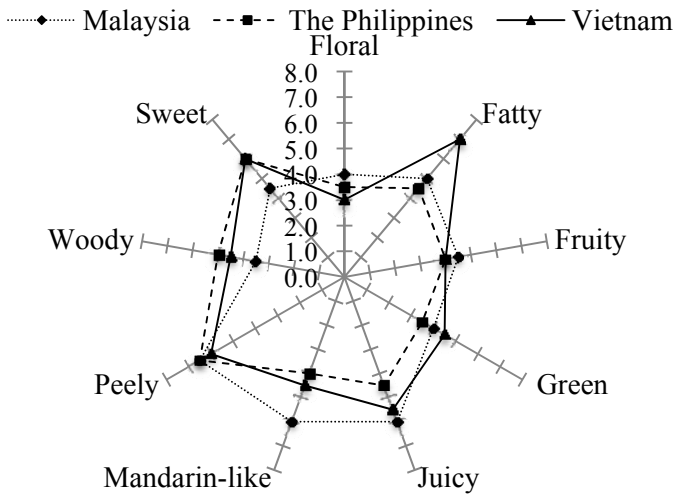
In addition to instrumental analysis, sensory evaluation is another useful technique to evaluate the human perception of flavor attributes (*132*). The aromatic profiles of calamansi peel extracts were presented in Figures 5.4(a) (dichloromethane peel extracts) and (b) (hexane peel extracts). Detailing the peel extracts obtained with the same solvent, volatile compounds having significant differences ($p<0.05$) among the three countries largely contributed to different aromatic profiles. For instance, Malaysia peel extract with the lowest amount of straight-chain aldehydes (octanal, nonanal, decanal and undecanal) that exhibited sweet and peely attributes was rated the lowest score in sweet attribute (4.5). However, this interpretation did not fully agree with other attributes. For example, Malaysia peel extract was rated high in peely (6.5) and waxy (7.0) attributes.

The sensory profile of calamansi dichloromethane extracts revealed that Malaysia calamansi peel extract was ranked highly for peely note (6.5), followed by juicy note (6.0) and mandarin-like note (6.0) with woody note being the weakest (3.5). This could be partly attributed to relatively higher amount of acetate ester that was positively correlated in PCA, the highest amount of methyl-N-methyl anthranilate, and significantly less ($p<0.05$) amounts of terpene hydrocarbons.

On the other hand, the calamansi peel extract originated from the Philippines had a well-balanced aromatic profile with the attributes mainly comprised of peely (6.5), sweet (6.0) and woody (5.0) notes. Compared to Malaysia and the Philippines, Vietnam peel extract exhibited the highest score of green (4.5) and fatty (7.0) notes. Fatty note was mainly attributed to the

presence of unsaturated aldehydes and fatty acids, which was in agreement with the analytical findings that Vietnam calamansi peel had a significantly higher (p<0.05) content of *trans*,*trans*-2,4-decadienal, *trans*-2-dodecenal, perilla aldehyde, octanoic acid and nonanoic acid. Apart from the common sensory attributes (i.e. peely, green, woody and fatty) perceived from citrus peel, juicy attribute was selected here, which originated from a group of alcohols, aldehydes and esters (e.g., geranyl propionate, linalool, neral and geranial).

(a)



(b)

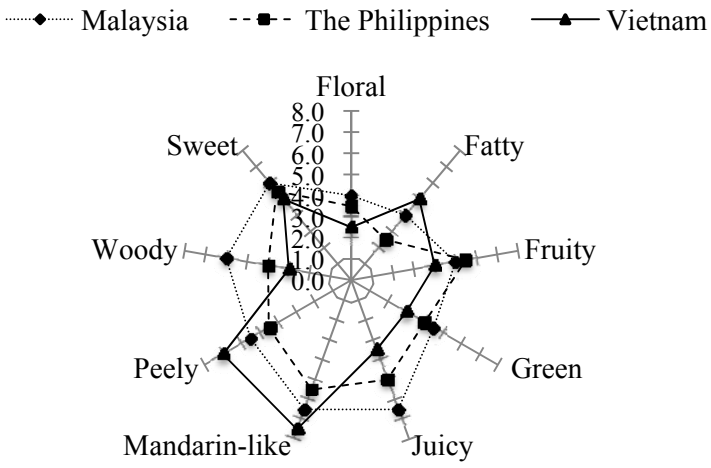


Figure 5.4. Sensory profiles of calamansi (*Citrus microcarpa*) peel extracts: (a) Dichloromethane; (b) Hexane

Similarly, another series of sensory evaluation was performed for calamansi hexane extracts. The aromatic profile of hexane extracts appeared to be less representative to its natural profile with higher hydrocarbon content but a significantly lower amount of carbonyls. By taking advantage of the differences in polarity, characterization of this complex citrus volatile profile could be complemented and more complete. Among nine aromatic attributes, woody, peely and mandarin-like notes were more expressive, of which mandarin-like note was the most dominant for all the hexane extracts of different origins with fatty note being the weakest, except for Vietnam peel extract in which floral (2.5) note had the lowest score (Figure 5.4(b)). In contrast to Malaysia and the Philippines aromatic profiles, asymmetrical attribute scores were observed in Vietnam peel extract, with scores ranging from mandarin-like (7.5), peely (7.0) and sweet (5.0) to floral (2.5) notes. On the other hand, a higher score of juicy (6.5) and green (4.5) notes was ranked for Malaysia peel extract. It suggested that oxygenated compounds such as methyl salicylate, perillyl alcohol, *cis*-3-hexenyl acetate and hexanol correlated positively with the flavor leading to the possibility of a synergistic effect in the Malaysia extract.

5.3.4. Phenolic acid content

Besides investigating the volatiles and aromatic profiles, four types of phenolic acids (caffeic, *p*-coumaric, ferulic and sinapic acids) in calamansi peel were listed in Table 5.2. A recent study done by Kashiwagi *et al. (263)* reported that the addition of antioxidants to *Citrus* peel oils can prevent the deterioration of flavor quality by inhibiting the formation of oxidation

artefacts. This finding indicated that phenolic acids, as antioxidants, might be important in maintaining the complex mixture of the volatile chemical substances. Under the present condition, calamansi has lower concentrations of free phenolic acids than bound phenolic acids. This implied with literatures that most of phenolic acids in citrus are bound to the fruit cell walls through the formation of ester and glycosidic linkages (*249*). It is observed that the phenolic acids contents of calamansi peel were statistically different among the three countries ($p<0.05$), except for the bound ferulic acid content of Malaysia and Vietnam calamansi peel. Moreover, *p*-coumaric acid was the most dominant free phenolic acid, but ferulic acid was one in the bound one. Ferulic acid is known to be the precursor of *p*-vinyl guaicol, an off-flavor product in citrus fruits (*264*). It can be derived from *p*-coumaric and caffeic acids as well as be converted to sinapic acid via enzymatic biotransformation.

Table 5.2. Free and bound phenolic acids content (mg/kg) of the calamansi (*Citrus microcarpa*) peel from Malaysia, the Philippines and Vietnam

Compounds	Retention time (min)	Malaysia	Philippines	Vietnam
Free phenolic acids				
Caffeic acid	10.88	$4.20^{a} \pm 0.65$	$5.55^{b} \pm 0.45$	$1.89^{c} \pm 0.16$
p-Coumaric acid	15.38	$194.83^{a} \pm 7.53$	$162.33^{b} \pm 12.41$	$106.31^{c} \pm 7.59$
Ferulic acid	17.42	$45.29^{a} \pm 0.92$	$48.99^{ab} \pm 3.07$	$30.07^{c} \pm 1.82$
Sinapic acid	18.17	$118.89^{a} \pm 0.47$	$28.64^{b} \pm 1.30$	$32.63^{c} \pm 4.67$
Bound phenolic acids				
Caffeic acid	10.88	$4.80^{a} \pm 1.35$	$25.75^{b} \pm 0.82$	$11.75^{c} \pm 0.48$
p-Coumaric acid	15.35	$131.49^{a} \pm 20.00$	$263.25^{b} \pm 4.93$	$173.69^{c} \pm 15.12$
Ferulic acid	17.39	$275.35^{a} \pm 31.59$	$410.92^{b} \pm 19.17$	$272.52^{a} \pm 10.71$
Sinapic acid	18.15	$109.79^{a} \pm 13.20$	$88.66^{b} \pm 7.70$	$76.75^{c} \pm 4.58$

Different superscripts within the same row indicate the statistical differences ($p<0.05$).

5.4. Conclusion

In this study, the volatiles and phenolic acids in calamansi peel were extracted and measured. With the aid of ANOVA, variations among these volatiles and phenolic acids were found to be significant (p<0.05) among three geographical origins (Malaysia, the Philippines and Vietnam). Furthermore, PCA and CDA approaches were employed to understand the correlation and segregation among these volatile compounds. Through sensory evaluation, the aromatic profiles of the calamansi peel extracts were identified and expressed in nine attributes, which could lead to a better insight. Moreover, the knowledge of phenolic acids provided the information of nutrition on calamansi fruit. This approach may also prove to be effective in studying the discrimination of citrus from different origins.

CHAPTER 6 CHARACTERIZATION OF CALAMANSI (*CITRUS MICROCARPA*): VOLATILES, PHYSICOCHEMICAL PROPERTIES AND NON-VOLATILES IN THE JUICE

6.1. Introduction

Calamansi juice has the combination of a sweet mandarin-like aroma with a zesty taste of lime, a slightly peely note of orange and a hint of acidic astringency. It has been used as a seasoning in food, and is also often used as a flavoring or as a food additive to enhance iron absorption. Literature search indicated that, to date, there have only been four reports on calamansi juice (*237, 265-267*).

Measurement of volatiles in citrus juice still remains a challenge. For example, to preserve the authentic aromatic profile of citrus juice during extraction, it is virtually impossible to obtain a high aroma compound recovery (*268*). This is partly due to the complex matrix and a scarce amount of volatile compounds present in citrus juice. In addition to various bacterial and enzyme activities, desirable yet unstable aroma compounds are easily

transformed while some off-flavor compounds are also quickly formed during extraction (*238*). In previous reports, citrus juice was commonly prepared through pressing citrus fruit with the peel together. Due to the incorporation of peel oil, this resulted in the intensified flavor of citrus juice, but interfered its original aroma profile (*238, 269*).

Besides the volatiles, the aroma and taste of citrus juice also depend on the balance between sugars and organic acids, which are among the major non-volatiles. Their nature and concentrations significantly influence the organoleptic quality of citrus juice (*270*). Hence, the detection and measurement of sugars and organic acids in calamansi juice warrant detailed exploration.

Phenolic acids are also major components that widely distributed in the different parts of citrus fruit, such as flavedo, albedo, endocarp and juice sacs (*253, 267, 271*). Peleg et al. (*249*) reported various phenolic acids in each part of orange and grapefruit and found that a concentration gradient of phenolic acids appeared to exist from the peel, and especially flavedo towards the juice. Furthermore, Gattuso et al. (*272*) reviewed a collection of phenolic compounds in citrus juice using different extraction methods, from which most of the juice samples were prepared by hand-squeezing in a domestic juicer or a commercial extraction process along with the peel. Mechanical pressure of the whole citrus fruit definitely caused the unintentional penetration of phenolic acids from its peel into the juice, increased their concentrations in the juice, and potentially caused the objectionable flavor during storage (*264*).

Therefore, the objective of this study was to characterize calamansi juice from selected geographical origins, focusing not only on the volatile compounds, but also on the non-volatiles (sugars, organic acids and phenolic acids) and other physicochemical properties. Through multivariate analysis, it is believed that information about calamansi juice would be more comprehensive and insightful.

6.2. Experimental procedures

6.2.1. Calamansi materials and chemicals

Prior to squeezing, calamansi fruits were washed with deionized water and wiped dry with paper serviettes; the peel and visible albedo of calamansi fruits were carefully peeled off. Calamansi juice was then obtained by squeezing manually with the use of a stainless steel sieve. Some of the freshly squeezed juice was immediately used for extracting volatiles, and the rest was subjected to centrifugation at 17,000 *g* for 15 min at 4 °C (Sigma 3-18K). Some of the supernatant was directly used to determine the physicochemical properties, and the remaining was filtered through a 0.2 μm regenerated cellulose (RC) filter (Minisart RC15), and then kept at -30 °C until further analysis of sugars and organic acids.

All standard compounds used in the identification of volatile constituents were obtained from Firmenich Asia Pte. Ltd., Singapore. Chemicals and organic solvents were stated in Chapter 5 (Section 5.2.1.).

6.2.2. Solvent extraction of volatiles

To avoid contaminating freshly squeezed juice with peel oil, calamansi peel was carefully removed prior to juicing. Moreover, based on the total concentration of volatiles and sensory profile, hand squeezing was selected here instead of mechanical squeezing. A solvent extraction method modified from that of Takeuchi et al. (*237*) was detailed in previous chapter (Section 4.2.2.).

6.2.3. Headspace-solid phase microextraction (HS-SPME)

Five grams of freshly prepared calamansi juice were added into a 20-mL glass vial, followed by three grams of saturated $CaCl_2$ solution. The glass vial was crimp-capped and subjected to headspace extraction. The optimized extraction condition of HS-SPME analysis was finally set at 40 °C for 30 min, and the SPME fiber was inserted into the GC injector for 5 min for thermal desorption.

6.2.4. GC-MS/FID analysis

Analysis of the volatile compounds of calamansi juice was carried out using an Agilent 6890N GC coupled with a FID and a 5975 inert MSD as described in Section 4.2.4. of Chapter 4 .

6.2.5. Physicochemical properties

Physicochemical properties of calamansi juice, i.e. pH, titratable acidity and total soluble solids (TSS) were determined as described in Section 4.2.6. of Chapter 4.

6.2.6. Extraction of phenolic acids

The extraction procedure was modified from that described by Peleg et al. (*249*). In the present study, calamansi juice was first centrifuged at 5,000 *g* for 20 min at 4 °C (Harrier 18/80, MSE, London, UK). For the extraction of free phenolic acids, five grams of the supernatant were extracted with 10 mL of methanol by vortexing for 5 min. The methanol-juice mixture was then centrifuged at 5,000 *g* for 10 min at 4 °C and the top layer of extract was removed. The extraction process was then repeated. The top layer of extract in each mixture was pooled and dried with five grams of anhydrous Na_2SO_4. Subsequently, the combined extract was filtered and concentrated to about 1 mL under reduced pressure at 35 °C. The concentrate was then diluted with methanol to 5 mL. The extract was filtered with a 0.20 μm PTFE filter prior to analysis.

For the determination of bound phenolic acids, hydrolytic procedures were modified from those described in the literature (*249, 251, 254*). Five grams of the centrifuged juice were hydrolysed using 5 mL of 2 M NaOH (containing 1% ascorbic acid and 10 mM EDTA) under nitrogen for 4 h at room temperature. After which, the mixture was acidified to pH 2.0 using 6 M HCl and centrifuged at 14,000 *g* for 15 min at 4 °C. The supernatant obtained was extracted using the same procedures as those for the extraction of free

phenolic acids, but 15 mL of methanol was used here. Bound phenolic acids were determined by comparing the differences before and after alkaline hydrolysis.

6.2.7. Ultra-fast liquid chromatography (UFLC) analysis

Analysis of non-volatiles was performed using a Shimadzu Prominence UFLC system equipped with two pumps (LC-20AD), an autosampler (SIL-20AC), column oven (CTO-20AC), an evaporative light scattering detector (ELSD-LT II) for sugar analysis, and a photodiode array detector (SPD-M20A) for the analysis of organic acids. The default column temperature was set at 40 °C and the sample injection volume was 10 µL. Data were acquired and processed using LC solution version 1.24 SP1 software. Sugars and organic acids were identified by matching the retention times and spectral characteristics against standards as detailed in Section 4.2.7. of Chapter 4 .

6.2.8. Statistical analysis

Comparison of physicochemical properties, organic acids, sugars and phenolic acids in calamansi juices (Malaysia, the Philippines and Vietnam) was performed using ANOVA and Duncan's multiple range tests with the probability value of $p<0.05$. Further, multivariate analysis was carried out using PCA based on the correlation matrix as explained in Chapter 5 (Section 5.2.6.).

All experiments were carried out in triplicate and the results were reported as the mean values.

6.3. Results and discussion

6.3.1. Volatile components of calamansi juice

A total of 60 volatile compounds in calamansi juice were detected, identified and are listed in Table 6.1. Apart from the components reported in previous studies ((*229, 237, 256, 265*), 17 compounds were reported here for the first time (e.g. geranyl propionate, *trans*-2-decenol, neral and *trans*-2-dodecenal). The Vietnam calamansi juice gave the highest amount of total volatiles, up to three-folds higher than others. However, the volatiles in the Vietnam juice mainly consisted of hydrocarbons that contributed relatively little to aroma due to their high odor threshold values. In contrast, the Philippines calamansi juice with the lowest amount of total volatiles consisted of the highest acids, alcohols and aldehydes.

Terpene hydrocarbons are known to be the major components of citrus essential oils, which contribute to characteristic citrusy and woody notes. It is noted that the calamansi juice from the three countries shared the same monoterpene and sesquiterpene profiles, which were also detected in the calamansi peel (Table 5.1.).

For all the calamansi juices, the group of hydrocarbons comprised predominantly of limonene, germacrene D and β-myrcene. Previous studies suggested that β-selinene and limonene, together with small amounts of oxygenated terpenes, were responsible for the aroma of calamansi fruit (*265, 273*).

Table 6.1. Identification of volatiles and their concentrations (ppm) in calamansi (*Citrus microcarpa*) juices from Malaysia, the Philippines and Vietnam

No.[a]	Compounds	LRI		Malaysia	Philippines	Vietnam	Identification[c]
		FFAP[b]	REF				
	Hydrocarbons						
1	α-Pinene[I,III,IV]	902	-	6.76[a] ± 0.23	4.92[b] ± 0.02	12.84[c] ± 0.03	MS, STD
2	β-Pinene[II,III]	972	-	8.18[a] ± 0.18	10.11[b] ± 1.11	16.84[c] ± 0.35	MS, STD
3	Sabinene	983	-	2.76[a] ± 0.08	2.04[b] ± 0.08	6.23[c] ± 0.16	MS
	δ-3-Carene[d, I,II,III,IV]	1070	-	*tr*	*tr*	*tr*	MS, STD
4	β-Myrcene[I,II,III,IV]	1124	1158	28.67[a] ± 0.85	18.98[b] ± 0.29	68.59[c] ± 0.49	MS, STD, LRI
	α-Terpinene[d]	1168	1178	*tr*	*tr*	*tr*	MS, STD, LRI
5	Limonene[I,II,III,IV]	1176	1185	1353.65[a] ± 45.30	922.00[b] ± 12.64	3323.62[c] ± 1.25	MS, STD, LRI
6	β-Phellandrene[II,III]	1182	1194	4.41[a] ± 0.16	3.18[b] ± 0.13	10.36[c] ± 0.05	MS, LRI
7	*trans*-β-Ocimene[III]	1204	1242	1.61[a] ± 0.14	2.05[a] ± 2.07	2.25[a] ± 0.02	MS, STD, LRI
	cis-β-Ocimene[d]	1210	1252	*tr*	*tr*	*tr*	MS, STD, LRI
8	ρara-Cymene[III]	1242	1267	0.02[a] ± 0.00	0.03[a] ± 0.01	0.05[a] ± 0.01	MS, STD, LRI
9	Terpinolene[I,II,III]	1255	-	0.23[a] ± 0.02	16.66[b] ± 18.87	0.41[a] ± 0.01	MS, STD
	dehydro-p-Cymene[d]	1289	-	*tr*	*tr*	*tr*	MS
	4,8-dimethyl-1,3,7-nonatriene[d]	1347	-	*tr*	*tr*	*tr*	MS
10	δ-Elemene[II,IV]	1441	-	18.82[a] ± 1.51	25.59[b] ± 1.50	17.53[a] ± 0.02	MS
11	Bicycloelemene	1452	-	1.88[a] ± 0.09	0.24[b] ± 0.09	0.73[b] ± 0.02	MS
12	β-Bourbonene	1493	-	0.09[a] ± 0.02	0.10[a] ± 0.03	0.03[a] ± 0.00	MS
	α-Copaene[d,III]	1528	1536	*tr*	*tr*	*tr*	MS, STD, LRI
13	β-Elemene[II]	1561	-	2.32[a] ± 0.22	2.94[b] ± 0.06	1.81[c] ± 0.02	MS
14	β-Caryophyllene[II,III]	1566	-	1.87[a] ± 0.14	1.99[a] ± 0.09	3.20[b] ± 0.02	MS, STD
15	γ-Elemene	1608	1625	3.57[a] ± 0.33	3.13[a] ± 1.72	4.48[b] ± 0.07	MS, LRI
16	β-Cubebene[II]	1612	-	1.03[a] ± 0.06	2.95[a] ± 2.61	2.97[a] ± 0.03	MS, STD
17	β-Farnesene[II]	1628	1711	0.35[a] ± 0.30	1.93[b] ± 0.20	0.65[a] ± 0.01	MS, STD, LRI
18	γ-Cadinene	1631	-	0.41[a] ± 0.02	0.18[b] ± 0.06	0.08[c] ± 0.01	MS
19	α-Humulene	1642	-	0.82[a] ± 0.41	1.08[a] ± 0.59	1.71[b] ± 0.02	MS
20	Germacrene D[II,IV]	1685	1722	47.04[a] ± 3.10	51.49[a] ± 2.03	71.53[b] ± 0.06	MS, STD, LRI
21	β-Selinene[IV]	1689	-	1.43[a] ± 0.09	1.03[b] ± 0.05	3.40[c] ± 0.07	MS

Table 6.1. (Cont'd)

22	α-Selinene	1696	-	0.18[a] ± 0.02	0.14[a] ± 0.01	0.42[b] ± 0.04	MS
23	Bicyclogermacrene	1609	-	3.74[a] ± 0.29	4.39[b] ± 0.17	0.38[c] ± 0.01	MS
24	α-Farnesene[III]	1715	1772	0.14[a] ± 0.06	1.81[b] ± 0.74	0.36[a] ± 0.04	MS, STD, LRI
25	δ-Cadinene[II,III,IV]	1759	-	0.44[a] ± 0.05	0.70[b] ± 0.08	0.40[a] ± 0.01	MS
26	Germacrene B[II]	1823	-	0.69[a] ± 0.07	1.02[b] ± 0.52	0.76[a] ± 0.04	MS
	Esters						
27	Heptyl acetate[IV]	1342	-	0.09 ± 0.01	-	0.13 ± 0.04	MS, STD
28	Octyl acetate[II,III,IV]	1446	-	1.74[a] ± 0.28	1.00[a] ± 1.05	2.10[b] ± 0.08	MS, STD
29	Citronellyl acetate[III]	1625	-	0.07[a] ± 0.02	0.75[b] ± 0.78	0.28[b] ± 0.01	MS, STD
30	Decyl acetate[II,IV]	1646	-	1.26[a] ± 0.67	1.46[a] ± 0.65	1.48[a] ± 0.01	MS, STD
31	Geranyl acetate[I,III,IV]	1719	-	5.03[a] ± 0.26	2.10[b] ± 0.26	13.17[c] ± 0.09	MS, STD
32	Geranyl propionate	1804	-	0.21[a] ± 0.01	0.95[a] ± 0.98	0.81[a] ± 0.02	MS, STD
33	Perillyl acetate	1935	-	-	-	0.16 ± 0.01	MS, STD
	Acids						
34	Decanoic acid[IV]	2230	-	-	-	0.69 ± 0.06	MS, STD
35	Palmitic acid	2889	-	19.28[a] ± 0.92	27.36[b] ± 0.01	18.24[a] ± 0.01	MS
36	Stearic acid	3109	-	16.97[a] ± 0.19	16.19[a] ± 1.53	16.77[a] ± 0.09	MS
37	Linoleic acid	3140	-	5.04[a] ± 0.04	5.28[a] ± 0.37	4.62[b] ± 0.10	MS
	Alcohols						
	Ethanol[d,I]	967	936	*tr*	*tr*	*tr*	MS, STD, LRI
38	*cis*-3-Hexenol[IV]	1350	1389	0.43[a] ± 0.08	3.02[b] ± 0.03	0.41[a] ± 0.04	MS, STD, LRI
39	Linalool[I,III,IV]	1507	1540	7.41[a] ± 1.36	11.16[b] ± 0.11	8.01[a] ± 0.02	MS, STD, LRI
40	Octanol[II,IV]	1532	-	1.19[a] ± 0.04	3.50[b] ± 0.31	1.60[a] ± 0.02	MS, STD
	4-Terpineol[a,I,III,IV]	1608	-	*tr*	*tr*	*tr*	MS, STD
41	Nonanol[II,IV]	1617	-	0.10 ± 0.01	-	0.14 ± 0.02	MS, STD
42	α-Terpineol[I,III,IV]	1664	-	2.47[a] ± 0.20	12.41[b] ± 0.10	3.37[a] ± 0.09	MS, STD
43	*trans*-2-Decenol	1799	-	-	0.96 ± 0.18	0.29 ± 0.02	MS, STD
44	Perilla alcohol[IV]	1972	-	-	-	0.42 ± 0.00	MS, STD
45	β-Elemol[II]	2042	-	2.12[a] ± 0.12	1.83[a] ± 0.01	5.92[b] ± 0.05	MS, STD
46	γ-Eudesmol[IV]	2076	-	0.24[a] ± 0.06	0.22[a] ± 0.08	0.59[b] ± 0.05	MS
47	α-Eudesmol[IV]	2190	-	0.60[a] ± 0.06	0.52[a] ± 0.43	1.65[b] ± 0.01	MS
48	β-Eudesmol[II,IV]	2200	-	2.09[a] ± 0.10	0.61[b] ± 0.08	3.34[b] ± 0.13	MS
	Carvacrol[d]	2218	-	*tr*	*tr*	*tr*	MS, STD
49	Phytol	2572	-	0.34 ± 0.04	1.03 ± 0.07	-	MS

Table 6.1. (Cont'd)

	Aldehydes						
50	Octanal[I,II,IV]	1262	1280	0.63 ± 0.63	-	2.41 ± 0.04	MS, STD, LRI
	trans-2-Heptenal[d]	1345	1305	*tr*	*tr*	*tr*	MS, STD, LRI
51	Nonanal[I,II,III,IV]	1365	1385	0.03 ± 0.00	0.08 ± 0.06	-	MS, STD, LRI
52	Decanal[I,II,III,IV]	1469	1497	0.52[a] ± 0.02	0.37[a] ± 0.08	6.30[b] ± 0.01	MS, STD, LRI
53	Undecanal[II]	1573	-	1.33[a] ± 0.07	0.24[b] ± 0.10	0.08[c] ± 0.02	MS
54	*trans*-2-Decenal[II]	1616	-	0.31[a] ± 0.02	0.97[b] ± 0.19	0.35[a] ± 0.02	MS, STD
55	Neral	1656	-	-	-	0.61 ± 0.06	MS, STD
56	*trans,cis*-2,4-Decadienal[IV]	1737	-	0.19[a] ± 0.01	0.38[a] ± 0.19	0.47[a] ± 0.01	MS, STD
57	Perilla aldeyhde[I,IV]	1768	-	0.22[a] ± 0.02	5.30[b] ± 0.06	0.15[a] ± 0.03	MS, STD
58	*trans,trans*-2,4-Decadienal[IV]	1806	1804	0.22[a] ± 0.01	0.10[a] ± 0.02	1.66[b] ± 0.01	MS, STD, LRI
59	*trans*-2-Dodecenal	1849	-	-	-	0.54 ± 0.02	MS, STD
	Others						
60	Isopiperitenone[IV]	1843	-	0.71[a] ± 0.02	5.71[b] ± 0.07	0.28[a] ± 0.01	MS
	Total identified			**1610.78**	**1166.92**	**3649.05**	

[I]Compounds reported in Ref. (*265*); [II]Compounds reported in Ref. (*266*); [III]Compounds reported in Ref. (*229*); [IV]Compounds reported in Ref. (*237*).

[a] Compounds listed as in principal component analysis (PCA)

[b] LRI on column (DB-FFAP) determined with two series of n-alkanes (C5–C20 and C21-C40).

[c] Identification method: MS = mass spectrum; STD = comparison with standard compound; LRI is compared with references from standards or literature values.

[d] Compounds only detected at HS-SPME; "*tr*" , trace; "-", not detected. Different superscripts within the same row indicate the statistical differences ($p<0.05$).

As shown in Table 6.1, linalool and α-terpineol were the major oxygenated compounds identified with varied amounts according to the geographical origins of calamansi fruits. The amounts of both compounds present in citrus juice determine the organoleptic quality of citrus juice (*274*). Linalool is a typical terpene alcohol found in citrus fruits that imparts a characteristic floral attribute of citrus blossom, while α-terpineol is transformed from linalool through oxidation and cyclisation, indicating the poor flavor quality of citrus juice. Among the three countries, linalool, *cis*-3-hexenol and octanol were found at higher levels in the Philippines calamansi juice. On the other hand, β-elemol and β-eudesmol were detected at higher levels in the Vietnam calamansi juice. As a group together with other alcohol compounds such as octanol, *cis*-3-hexenol, β-elemol, and β-eudesmol, they impart a well-balanced juice profile with floral, fresh, fruity, green and woody perceptions.

Aldehydes (e.g. decanal, nonanal, octanal, undecanal, and perilla aldehyde, etc), contributing to aldehydic, fatty, green and peely notes, were detected with a similar profile to that of some previous reports (*229, 237, 265, 266*). Moreover, it was intriguing to find a trace amount of neral in the Vietnam calamansi juice (0.61 ppm). As neral is the characteristic compound of lemon and lime, the Vietnam calamansi juice was described as having a slight lemon-like note.

Ester compounds with low odor thresholds are also important in contributing to floral, waxy and aldehydic notes with green and fruity nuances. For the total ester compounds, the Vietnam calamansi juice had a higher amount (18.92 ppm) than those of the Malaysia (9.47 ppm) and the

Philippines (3.04 ppm) juices. However, the highest proportion of esters in the Malaysia calamansi juice (0.6%) could make esters more perceptible, as compared to those from other countries.

Besides solvent extraction, HS-SPME was adopted to extract compounds at trace levels and to study the original volatile profile with minimum changes to that of the fresh juice. To this end, extraction temperature was set at 40 °C to give an original volatile profile of the fresh calamansi juice close to that at room temperature while achieving satisfactory extraction efficiency. Although a salting-out effect had not profoundly increased the yield of volatile release, a saturated calcium chloride solution was added into the juice to inhibit the enzymes and bacterial activity. There were 10 trace compounds that were detected only by HS-SPME, namely, δ-3-carene, α-terpinene, *cis*-β-ocimene, dehydro-p-cymene, *trans*-4,8-dimethyl-1,3,7-nonatriene, α-copaene, ethanol, 4-terpineol, carvacrol and *trans*-2-heptenal.

6.3.2. Physicochemical properties of calamansi juice

Physicochemical properties including pH, titratable acidity and total soluble solids play a significant role in taste, color and microbial stability of juice. For the pH of calamansi juice, it was found that the Vietnam calamansi juice had a significantly lower pH value (Table 6.2). The pH value of a solution may not be directly related to its titratable acidity as pH is only a measurement of free hydrogen-ion activity while titratable acidity measures the total acid concentration (*275*). ^{o}Brix value is a measurement of total soluble solids, which includes organic acids and sugars in the juices. It was

found that the Malaysia calamansi juice had the lowest °Brix value, followed by the Philippines and Vietnam juices, respectively.

Table 6.2. Physicochemical properties, sugars, organic acids and phenolic acids of calamansi juices from Malaysia, the Philippines and Vietnam

	Malaysia	**Philippines**	**Vietnam**
Physicochemical properties			
pH	$2.57^{a} \pm 0.02$	$2.54^{ab} \pm 0.03$	$2.50^{b} \pm 0.03$
Titratable acidity (% citric acid)	$5.72^{a} \pm 0.19$	$5.66^{a} \pm 0.07$	$6.14^{b} \pm 0.15$
Total soluble solids (°Brix)	$7.56^{a} \pm 0.11$	$8.04^{b} \pm 0.31$	$8.09^{b} \pm 0.08$
Sugars (%)			
Fructose	$0.22^{a} \pm 0.04$	$0.302^{b} \pm 0.04$	$0.27^{c} \pm 0.01$
Glucose	$0.20^{a} \pm 0.03$	$0.243^{b} \pm 0.04$	$0.28^{c} \pm 0.01$
Sucrose	$0.29^{a} \pm 0.03$	$0.306^{a} \pm 0.07$	$0.27^{a} \pm 0.02$
Total	$0.71^{a} \pm 0.04$	$0.85^{b} \pm 0.05$	$0.81^{b} \pm 0.02$
Organic acids (%)			
Ascorbic	$0.030^{a} \pm 0.00$	$0.036^{ab} \pm 0.01$	$0.052^{a} \pm 0.01$
Citric	$5.287^{a} \pm 0.26$	$4.997^{b} \pm 0.23$	$5.518^{a} \pm 0.27$
Malic	$0.228^{a} \pm 0.04$	$0.213^{a} \pm 0.01$	$0.211^{a} \pm 0.06$
Succinic	$0.070^{a} \pm 0.02$	$0.148^{b} \pm 0.03$	$0.077^{a} \pm 0.02$
Total	$5.62^{a} \pm 2.59$	$5.40^{b} \pm 1.62$	$5.86^{a} \pm 2.71$
Free phenolic acids (mg/kg)			
Caffeic acid	$1.60^{a} \pm 0.13$	$2.54^{b} \pm 0.44$	$1.59^{a} \pm 0.073$
p-Coumaric acid	$18.03^{a} \pm 1.14$	$22.52^{b} \pm 2.37$	$23.74^{b} \pm 1.05$
Ferulic acid	$1.27^{a} \pm 0.15$	$1.79^{b} \pm 0.26$	$1.29^{a} \pm 0.22$
Sinapic acid	-	-	-
Bound phenolic acids (mg/kg)			
Caffeic acid	-	-	2.72 ± 0.64
p-Coumaric acid	$21.84^{a} \pm 2.16$	$26.44^{b} \pm 2.25$	$27.05^{b} \pm 2.07$
Ferulic acid	$22.65^{ab} \pm 3.74$	$25.40^{b} \pm 2.04$	$21.77^{a} \pm 2.51$
Sinapic acid	$5.24^{a} \pm 0.28$	$6.03^{b} \pm 0.31$	$6.55^{b} \pm 1.22$

Different superscripts within the same row indicate significant differences ($p<0.05$).

6.3.3. Sugar content of calamansi juice

The main components of carbohydrates in citrus fruits are three simple sugars: sucrose, glucose and fructose (*233*). As shown in Table 6.2, glucose and fructose contents of calamansi juices were statistically different ($p<0.05$).

For the fructose and glucose contents, the Philippines calamansi juice had the highest concentration, followed by the Vietnam and Malaysia juices. In contrast, the sucrose contents of calamansi juices were not statistically significant (p>0.05). Fructose, glucose and sucrose in calamansi juices were found to be in similar proportions with lower contents in my study, compared to a previous study (*265*). The differences in the sugars composition could be due to the maturity and origin of calamansi fruits.

6.3.4. Organic acid content of calamansi juice

Organic acids are widely distributed in citrus fruits, and contribute to the tartness of citrus fruits (*242, 250, 270, 276*). Similar to other citrus fruits, citric acid was determined to be the major organic acid in calamansi juices while ascorbic and succinic acids were at much lower levels (Table 6.2). Although dehydroascorbic acid was detected in calamansi juices, the level was too low to be quantified. In general, the Philippines calamansi juice had the lowest amount of organic acids, notably citric acid, while the Vietnam calamansi juice had the highest amount. L-Malic acid was the second most abundant organic acid, and was not statistically different among the three countries (p>0.05). Compared to a previous study where citric and malic acids in calamansi juices were determined (*265*), my results showed a higher citric acid content while malic acid content was similar. Additionally, the Philippines calamansi juice had the highest amount of succinic acid (0.15%), and its concentration was about twice as high as those of the Malaysia and Vietnam calamansi juices.

6.3.5. Phenolic acid content of calamansi juice

Although the phenolic acids composition of calamansi juices has not been investigated, studies on the phenolic composition of other citrus juices such as grapefruit, orange and mandarin have been conducted (*249, 250, 252, 277-280*). Table 6.2 shows the free phenolic acids extracted from calamansi juices. The Malaysia calamansi juice had the lowest amount of free phenolic acids content. Similar to calamansi peel, *p*-coumaric acid was found to be the major free phenolic acid. The free phenolic acids composition obtained in this study differed from that of a mandarin juice (*280*). The total concentrations of free phenolic acids extracted from the calamansi juices were notably higher than those of mandarin juice except for ferulic acid (see Table 6.2). On the other hand, free sinapic acid was not detected in all of the calamansi juices. While free sinapic acid was not detected in the calamansi juices, 0.11 mg/kg was found in orange juice sacs (*249*). This could be due to the difference in the extraction solvent used. Although many studies used ethyl acetate for the extraction of phenolic acids (*249, 254, 281*), some other reports showed that extraction with methanol improved yields and prevented the formation of troublesome emulsions (*282*). Furthermore, my initial trials also indicated that methanol was a better solvent as it gave a higher extraction yield. Therefore, methanol was used in this study.

After alkaline hydrolysis, the total levels of phenolic acids increased significantly by 138%, 115% and 108% in the Malaysia, the Philippines and Vietnam calamansi juices, respectively. In all calamansi juices, the largest increase was observed in the level of ferulic acid, indicating that it was one of the two major phenolic acids in calamansi juices after hydrolysis, the other

being *p*-coumaric acid. Calamansi is a different citrus hybrid, whose phenolic acids composition would be different from that of other citrus fruits. Unlike mandarin (*280*), orange juice (*249, 251, 252*) and grapefruit juice (*250*), the most abundant free phenolic acid in the calamansi juices was found to be *p*-coumaric acid. As hydrolysis also released bound sinapic acid, the sinapic acid content in the Malaysia, Philippines and Vietnam calamansi juices was determined to be 5.24 mg/kg, 6.03 mg/kg and 6.55 mg/kg, respectively (Table 6.2). In general, the Philippines calamansi juice had the highest content of phenolic acids. Moreover, the Malaysia calamansi juice had the lowest concentrations of *p*-coumaric and sinapic acids, while the Philippines calamansi juice had the highest concentration of ferulic acid.

Caffeic acid was not detected in the Malaysia and Philippines calamansi juices after alkaline hydrolysis, although bound caffeic acid in the Vietnam calamansi juice was found to be 2.71 mg/kg. This could be due to the loss of caffeic acid during alkaline hydrolysis as they contained the reactive o-dihydroxy phenols (*249*). Although the addition of EDTA and ascorbic acid could reduce the loss of caffeic acid (*254*), some loss of caffeic acid could still have occurred during 4-hour alkaline hydrolysis. Hydrolysis was also performed under nitrogen as phenolic acids were unstable in alkaline hydrolysis in the presence of oxygen (*281, 283*). One study on orange (*Citrus sinensis* (L) Osbeck var Shamuti) juice determined the phenolic acids after hydrolysis, and sinapic, ferulic, coumaric and caffeic acids in orange juice sacs were 8.57 mg/kg, 27.95 mg/kg, 5.30 mg/kg and 3.47 mg/kg, respectively (*249*). In comparison, present findings indicated that the level of *p*-coumaric

acid was significantly higher in calamansi juices, while sinapic, ferulic and caffeic acids were slightly lower after hydrolysis (Table 6.2).

In grapefruit and orange juices, most of phenolic acids were present in the bound forms (*249*). For the calamansi juice, this did not apply to all types of phenolic acids (Table 6.2). For *p*-coumaric acid, it almost exclusively existed in the free form as the level of *p*-coumaric acid only increased to a small extent after hydrolysis. On the other hand, most of ferulic acid existed in the bound form as hydrolysis greatly increased the level of ferulic acid in calamansi juices. Different extraction methods, maturity and varieties may be responsible for these divergences (*248*).

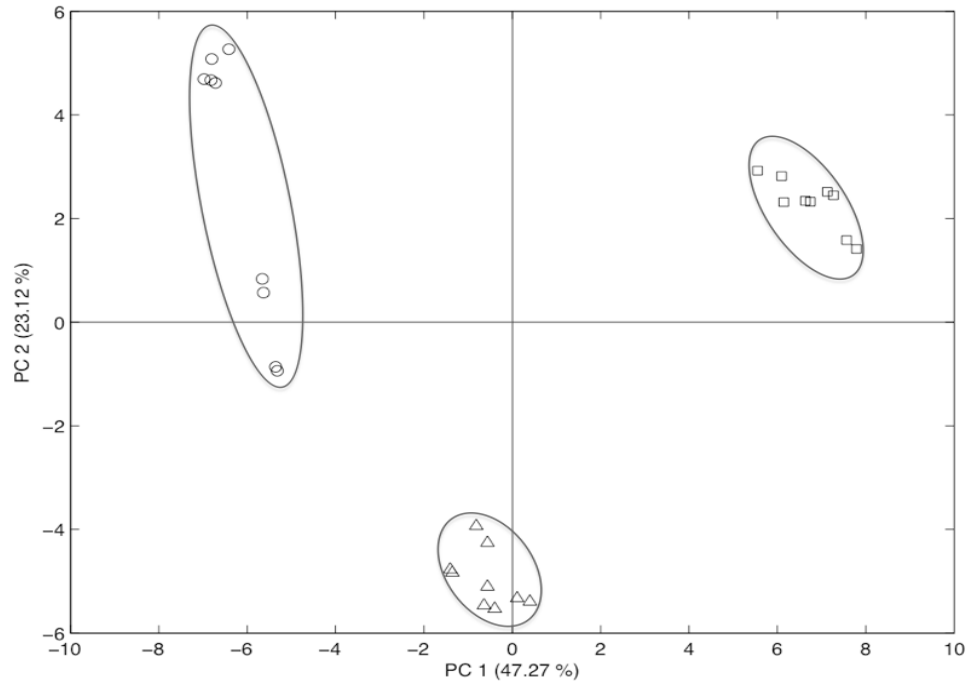
6.3.6. Principal component analysis (PCA)

PCA was conducted to understand the correlation and segregation among the volatile compounds obtained from the calamansi juices. Through PCA, four principal components (PC) were obtained accounting for 89.65% of the total variance. Figure 6.1 illustrates the correlations between chemical variables and the first two dimensions using PCA built on the normalized variables. Observing the score plot of PC 1 and PC 2, three clusters were observed in the segregation of volatile compounds according to their geographical origins (Figure 6.1(a)). The Vietnam calamansi juice was grouped at the upper right quadrant suggesting that it was strongly and positively correlated to PC1, whereas the Malaysia calamansi juice was negatively correlated with PC 2. The Philippines calamansi juice exhibited a positive correlation with PC 2 and a negative correlation with PC 1. Upon further analysis of variable loadings, it was found that the dense loading of

variables was located at the upper left and right quadrants of PCA plot (Figure 6.1(b)), indicating their positive correlations with PC 1 and PC 2. Several major terpene hydrocarbons (e.g. limonene, β-myrcene, α-pinene, β-pinene and germacrene D) and oxygenated compounds (e.g. decanal, octanal, neral and geranyl acetate) had positive correlations to PC 1, which might be attributable to the higher volatile contents in the Vietnam calamansi juice.

Similar observation was found in the Philippines calamansi juice that was positively correlated to PC 2; of which, several sesquiterpenes (e.g. elemene isomers and β-farnesene), *cis*-3-hexenol, linalool, octanol, nonanal and other compounds were detected in higher amounts in the Philippines juice.

(a)



(b)

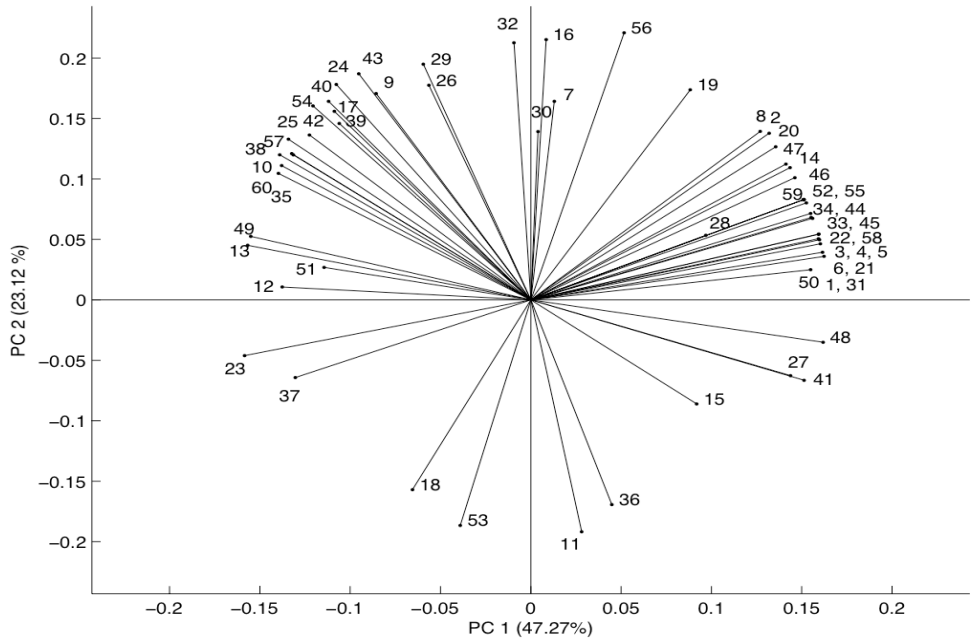


Figure 6.1. PCA analysis of calamansi (*Citrus microcarpa*) juice dichloromethane extracts [(Δ) Malaysia; (○) the Philippines; (□) Vietnam]: (a) Score plot of PC 2 against PC 1; (b) Variables plot of PC 2 against PC 1

Volatile variables explained according to the identified compounds in Table 6.1.

6.4. Conclusion

Using the improved extraction method, 17 volatile compounds that had not been reported in calamansi juice were identified in the present study. Two principal components of calamansi juice volatiles that accounted for variances of 47.27% and 23.12% were selected as two axes, enabling major differentiation between the calamansi juices from Malaysia, the Philippines and Vietnam, although the physicochemical properties and non-volatiles (sugars, organic acids and phenolic acids) from different geographical origins were insignificantly different. Thus, this approach may be useful in understanding the characteristic properties of citrus juices.

CHAPTER 7 SIMULTANEOUS QUANTITATION OF VOLATILE COMPOUNDS IN CITRUS BEVERAGE THROUGH STIR BAR SORPTIVE EXTRACTION COUPLED WITH THERMAL DESORPTION-PROGRAMMED TEMPERATURE VAPORIZATION

7.1. Introduction

Quantitation of flavor compounds in food samples still remains a challenging task due to their highly diverse physicochemical properties (i.e. volatility and polarity) and disparate concentrations. As discussed in previous chapters, distinctive flavors of citrus juices are contributed by aroma-active volatile compounds ranging from ppm to ppb levels while some potent polar oxygenated compounds are present at low ppt levels (*132, 284*). Indeed, there is still limitation on extracting volatiles from citrus juices with current extraction techniques, of which a method that enables the correlation between instrumental data and sensory evaluation could be better. Moreover, the various soluble solids (e.g. acids, sugars, and pectins) that are usually found in citrus beverages give rise to matrix effects that would further complicate the

extraction process (*285*). Hence, this has led to the need to develop a more effective and versatile SBSE method for flavor analysis.

SBSE could be generally viewed as a two-step process – the first step involves partitioning of analytes from aqueous phase into sorbent materials; the second step is to desorb the extracted analytes through thermal desorption or solvent dissolution, with the former being more commonly employed. The thermally desorbed analytes can be transferred into a gas chromatograph through a programmed-temperature-vaporization (PTV) inlet, which could focus the compounds in a cryofocusing trap before transferring them into the column (*286*). The combination of SBSE and TD-PTV injection is a sensitive yet complicated technique. To improve the performance of SBSE-TD-PTV method, different approaches were attempted in previous studies with one-variable-at-a-time univariate approach (*108, 113, 115, 117*). However, response surface methodology would be more appropriate in optimising multiple experimental factors whether extraction conditions only were optimized (*117*) or both important extraction and GC conditions were treated together in a set of sequential experimental designs (*120, 287*). In preliminary experiments, it was found that the effects of SBSE extraction conditions and TD-PTV injection parameters were basically unrelated (data not shown). In fact, the process of experimental design, all variables should be interrelated/correlated among themselves. Otherwise, the interpretation on the responses could disregard certain unfavourable conditions/ discrimination towards certain group of analytes (*120, 287*). Thus, these factors should be separately optimized by examining the response of each compound and took advantage of multi-responses optimisation approach to maximize these

responses. Through understanding the influence of TD-PTV factors (i.e. thermal desorption time, flow and cryofocusing temperature) and extraction parameters (i.e. extraction time, temperature stirring speed, electrolyte concentration and pH) on the performance of each compound, analyte discrimination could be alleviated. The targeted compounds are significantly different in physiochemical properties (e.g. boiling point, solubility, etc.).

Therefore, the objective of this chapter was to develop a SBSE-TD-PTV method for simultaneous determination of a wide range of volatile compounds using model citrus beverage. RSM was applied to understand the interactive parameters in the TD-PTV process, while partial factorial was used to prescreen extraction condition. Furthermore, the optimized method was evaluated and validated through various performance parameters (i.e. linearity, repeatability, precision and limit of detection).

7.2. Experimental procedures

7.2.1. Materials and sample preparation

Milli-Q water was generated from a Millipore water system (Milford, MA, USA). Analtyical grade ethanol 96% was obtained from Gadot-Lab, Hezlia, Israel and methanol from VWR International Ltd., Poole, UK; HPLC grade dichloromethane was purchased from Tedia.

A group of 36 common food flavorings was obtained from Firmenich Asia, Singapore (Table 7.1). Then, these compounds were diluted with ethanol (10 mg mL^{-1}) as flavoring for further analysis. For each SBSE extraction, 10 µL of this flavoring was spiked into 10.00 mL of Milli-Q water.

Table 7.1. RSM model and method validation for all volatile compounds

Compound	Default extraction concentration (µg/ L)	MP[a] (°C)	BP[b] (°C)	log $K_{o/w}$ [c]	RSM model	Precision (%RSD)		Linear Range (µg/L)	R^2	LOD (µg/ L)	LOQ (µg/ L)
						Repeatability (Intra-day; *n*=6)	Intermediate (Inter-day; *n*=5)				
Hydrocarbons											
Limonene	100	–74	176	4.38	Quadratic with positive interaction	4.96	13.20	1.00 - 10.00	0.996	0.80	2.67
Ocimene	40	50	100	4.80 (est.)	Quadratic	4.00	16.32	0.50 - 4.00	0.991	0.50	1.65
β-Myrcene	20	<-10	166-168	4.17	Quadratic with positive interaction	4.22	15.80	0.20 - 2.00	0.997	0.15	0.50
ρ-Cymene	20	–68	177	4.1	Quadratic with positive interaction	4.01	15.45	1.00 - 10.00	0.998	0.61	2.02
α-Pinene	10	–64	155	4.44	Quadratic with positive interaction	5.10	17.06	0.10 - 1.00	0.999	0.03	0.09
Terpinolene	10	n.a.	183-185	4.47	Quadratic with positive interaction	4.57	20.67	1.00 - 10.00	0.996	0.81	2.71
β-Caryophyllene	10	n.a.	262-264	6.30 (est.)	Constant	4.23	13.23	0.20 - 2.00	0.998	0.12	0.40
Valencene	10	n.a.	271	6.3 (est.)	Constant	5.48	12.87	0.25 - 5.00	0.999	0.19	0.23
Aldehydes											
Octanal	40	12-15	171	2.78 (est.)	Quadratic with positive interaction	1.98	13.60	2.00 - 20.00	0.998	1.16	3.87
Citral	40	n.a.	229	3.45	Constant	2.40	6.42	0.50 - 5.00	0.997	0.37	1.24
Nonanal	20	–18	195	3.27 (est.)	Quadratic with positive interaction	2.37	4.56	0.50 - 10.00	0.999	0.36	1.19
Decanal	20	n.a.	207-209	3.76 (est.)	Constant	2.67	4.86	0.20 - 2.00	0.995	0.18	0.60
Dodecanal	20	12	184-186	4.75 (est.)	Constant	4.09	7.08	1.00 - 10.00	0.998	0.57	1.91
Perillic aldehyde	10	n.a.	237	3.13	Constant	2.24	5.25	0.10 - 2.50	0.999	0.07	0.25
Decatrienal	10	n.a.	252	3.12 (est.)	Quadratic with negative interaction	2.44	3.35	0.19 - 1.00	0.979	0.19	0.62

Table 7.1. (Cont'd)

Alcohols											
Ethanol	259	−114	78	–0.31	Linear	13.90	20.53	2.59 – 25.90	0.997	1.76	5.88
Borneol	40	208	213	2.69	Quadratic with positive interaction	7.58	21.09	3.00 - 20.00	0.999	1.07	3.58
α-Terpineol	40	18	219	3.28	Constant	3.17	3.63	0.40 – 4.00	0.996	0.31	1.03
1,4-Cineole	20	n.a.	172-174	2.97	Quadratic with positive interaction	1.89	5.11	0.20 - 2.00	0.994	0.20	0.66
Eucalyptol	20	1.5	176-177	2.74	Quadratic with positive interaction	2.60	12.30	0.20- 2.00	0.999	0.08	0.27
Linalool	20	<-20	198-199	2.97	Constant	3.25	4.59	0.50 - 5.00	0.998	0.26	0.85
Citronellol	20	n.a.	225	3.91	Quadratic with negative interaction	3.80	2.93	0.20 - 4.00	0.996	0.31	1.03
Geraniol	20	15	229	3.47	Quadratic with negative interaction	2.87	4.58	0.20 - 2.00	0.998	0.12	0.41
Nerol	10	n.a.	224-245	3.47	Quadratic with negative interaction	3.56	4.39	0.25 – 2.50	0.999	0.09	0.29
Nerolidol	10	n.a.	121 (at 3mm Hg)	5.68 (est.)	Linear	4.43	3.35	0.25 - 5.00	0.999	0.18	0.60
Esters											
Ethyl butyrate	40	–93	120-121	1.85 (est.)	Quadratic with positive interaction	9.16	10.56	0.42 - 10.00	0.999	0.42	1.38
Citronellyl acetate	20	n.a.	240	4.56 (est.)	Quadratic with negative interaction	2.70	3.27	0.20 - 2.00	0.996	0.16	0.52
Linalyl acetate	10	85	220	3.93	Quadratic with negative interaction	4.80	21.35	0.10 - 1.00	0.999	0.02	0.07
Decyl acetate	10	n.a.	244	4.79 (est.)	Quadratic with negative interaction	4.96	6.45	0.25 - 2.50	0.999	0.12	0.39
Styrallyl acetate	10	n.a.	357	2.50 (est.)	Quadratic with negative interaction	2.78	5.66	0.10 - 0.75	0.993	0.09	0.29
Geranyl acetate	10	<25	240-245	3.98	Quadratic with negative interaction	1.48	3.76	0.25 - 2.50	0.996	0.20	0.66
Methyl jasmonate	10	<25	88-90 (at 0.1 mmHg)	2.76 (est.)	Linear	2.44	4.70	0.25 - 2.50	0.998	0.15	0.51

Table 7.1. (Cont'd)

Methyl-N-methyl anthranilate	10	17-19	255-256	2.81(est.)	Quadratic with negative interaction	2.28	5.46	0.25 - 5.00	0.999	0.13	0.45
Others											
Nootkatone	20	36	170	4.88 (est.)	Quadratic with negative interaction	2.99	3.57	1.83 - 20.00	0.998	1.83	6.09
β-Ionone	10	–49	126-128	4.42 (est.)	Quadratic with negative interaction	1.62	6.42	0.25 - 5.00	0.999	0.19	0.63
Methyl-N-methyl anthranilate	10	17-19	255-256	2.81(est.)	Quadratic with negative interaction	2.28	5.46	0.25 - 5.00	0.999	0.13	0.45
Indole	1	52-54	253-254	2.14	Quadratic with positive interaction	6.65	9.89	3.89 - 10.00	0.971	3.89	12.98

[a] Melting point at 760 mm Hg.
[b] Boiling point at 760 mm Hg.
[c] Expressed by the estimated logarithm of the n-octanol/water partition coefficient (from KOWIN v.1.67).

7.2.2. SBSE procedure

Stir bars coated with 24 µL of polydimethylsiloxane (PDMS) (10 mm length × 0.5 mm thickness) were purchased from Gerstel GmbH & Co. KG. Prior to use, stir bars were conditioned for 1 h at 300 °C in a flow of helium at 80 mL min^{-1}. Reconditioning of stir bars was done after use by soaking in Milli-Q water and a mixture of dichloromethane-methanol (1:1) for 2 h, as described elsewhere (*117*) SBSE was performed using a multiple position magnetic stirrer (Variomag Poly15, Thermo Fisher Scientific, MA, USA). Prior to optimization, the extraction time profile was examined by stirring solutions spiked with the flavoring (10 mg mL^{-1}) at room temperature at 800 rpm for durations between 10 min and 24 h. After extraction, the stir bars were dried with a lint-free tissue and placed in a glass thermal desorption tube.

7.2.3. Analytical procedure

TD–PTV–GC–MS/FID analysis was performed using a thermal desorption unit (TDU) coupled with an Agilent 7890C gas chromatograph with a 5975C mass-selective detector and a flame ionization detector with two-way splitter kit (Agilent Technologies). Thermal desorption unit (TDU) was mounted on top of a cooled injection system (CIS-4), a programmed-temperature-vaporization (PTV) type universal GC inlet (Gerstel). The entire system was operated under Maestro (Gerstel) integrated with Chemstation (Agilent Technologies).

Initially, the default condition for TD-PTV was set based on the recommendation by Gerstel, where stir bar was thermally desorbed from 40 °C (held for 1 min) to 250 °C (held for 5 min) at 720 °C min^{-1} with the

desorption flow of 60 mL min^{-1}. Using a glass wool liner (ID 2.0 mm), the desorbed compounds were cryofocused inside the CIS-4 at −100 °C. After desorption, CIS-4 was programed from −100 to 250 °C (held for 5 min) at 12 °C s^{-1} to transfer the trapped compounds into the analytical column. Splitless transfer of analytes was performed through solvent vent mode, and the effect of splitless time on the peak areas obtained was predetermined by varying opening time of split valve between 1 min to 7 min.

The separations were carried out on a DB-FFAP fused-silica capillary column of dimensions 60 m × 320 µm and 0.25 µm film thickness (Agilent Technologies). The oven temperature was programmed from 40 °C (held for 5 min) to 145 °C at 5 °C min^{-1}, then to 178 °C at 3 °C min^{-1}, and finally to 230 °C (held for 23 min) at 5 °C min^{-1}. Helium was used as the carrier gas at a flow rate of 1.3 mL min^{-1}. The mass spectrometer was operated in the scan mode with electron ionization of 70 eV.

7.2.4. Optimization of TD-PTV injection process

As shown in Table 7.2, three interactive parameters were desorption flow (40 – 80 mL min^{-1}), thermal desorption time (5 – 15 min) and cryofocusing temperature in the PTV injection system (–120 – –40 °C). Central composite design (CCD) was applied in this work, where a total of 20 experimental runs were constructed with 6 central points, 8 cubic points and 6 axial points at α value = 1.68 using Design Expert Version 6.0.10 software (Stat-Ease, MN, USA) (*152, 153*).

Table 7.2. Central composite design for three factors

Factor		Experimental levels				
		−α	−1	0	1	α
A	Desorption flow (mL/ min)	26.36	40.00	60.00	80.00	93.64
B	Thermal desorption time (min)	1.59	5.00	10.00	15.00	18.41
C	Cryofocusing temperature (°C)	−147.27	−120.00	−80.00	−40.00	−12.73

The experimental data were fitted by multiple regression equation including up to the second-order polynomial terms and interaction terms (*153*). The generalized response surface model to describe the variations in response variables is given as follows (*153*):

$$y = \beta_0 + \sum_{j=1}^{q} \beta_j x_j + \sum_{i=1}^{q} \beta_{jj} x_j^2 + \sum \sum_{i<j} \beta_{ij} x_i x_j$$

where y is the predicted response; β_o is a constant; β_j is the linear regression coefficient; β_{jj} is the quadratic coefficient, β_{ij} is the interaction coefficient; and x_i and x_j are independent variables. The adequacy of the model was determined by evaluating the coefficient of determination (R^2) and lack-of-fit tests obtained from the analysis of variance (ANOVA), while statistical significance of the model and model terms were determined at 95% confidence level. The terms found to be non-significant ($p > 0.05$) were dropped from the initial model and refitted with the significant ($p < 0.05$) independent variables in order to obtain the final reduced model. However, some insignificant linear terms were retained in the model if a quadratic or interaction term containing these variables was significant. Three dimensional response surface plots were used to visualize the modeled region and to determine the optimal experimental conditions.

Simultaneous optimisation was carried out through an objective function in the Design Expert software. With the objective function, individual desirability of each response variable was combined (*288*), as follow:

$$d_i = \begin{cases} 0 & y_i \le y_{i\,min} \\ \left[\dfrac{y_i - y_{i\,\min}}{y_{i\,\max} - y_{i\,\min}}\right] & y_{i\,min} < y_i < y_{i\,max}, \text{ for } i = 1, 2, \ldots, 14 \\ 1 & y_i \ge y_i \end{cases}$$

$$D = \left(\prod_{i=1}^{n} d_i^{w_i}\right)^{\frac{1}{\sum w_i}} = \left(\prod_{i=1}^{n} d_i\right)^{\frac{1}{n}}$$

where d_i is the individual desirability value of i^{th} response, the value of $y_{i\,min}$ and $y_{i\,max}$ are the minimum and maximum acceptable value of y_i, overall desirability (D) with n is the total number of responses and w_i, is the individual response importance, in our case w_i=1 as desirability function was set as linear.

7.2.5. Partial factorial design for SBSE extraction

A partial factorial experimental design (2^{5-1}) was used to evaluate the significance of the extraction conditions, as well as the interactions between them. The factors investigated were ionic strength (sodium chloride concentration), stirring speed (rpm), extraction time (h), temperature (^{o}C) and pH. Extraction was carried out in a temperature controlled water bath. All variables were evaluated at two levels, low (denoted as −1) and high (denoted as +1). The significant factors were indicated by the Pareto chart, which was obtained after multiple linear regression and analysis of variance (see Table 7.3).

Table 7.3. Experimental domain for screening significant factors affecting extraction of SBSE

Factor	Low (–)	High (+)
A: Ionic strength (% w/v NaCl)	0	30
B: Stirring speed (rpm)	300	900
C: Extraction time (h)	2	6
D: Temperature (^{o}C)	24	60
E: pH	2	7

7.2.6. Model evaluation and validation on model citrus beverage

Linearity was determined over an eleven-point calibration with citrus flavoring spiked Milli-Q water ranging from 10.3 to 515 μg L^{-1}. The calibration curves were prepared by calculating FID absolute peak areas against concentrations was obtained for individual compounds, with a correlation coefficient R^2 of at least 0.99. Based on the calibration curves obtained, figures of merits such as linearity, limit of detection (LOD) and limit of quantification (LOQ) were also determined for each compound. The LOD and LOQ for each compound were established by using the equations: LOD = $3s_y/b$ and LOQ = $10s_y/b$, where s_y is the SD of the peak areas obtained from at least five different concentrations within the linear range and b is the slope of the calibration curve.

Model citrus beverage was prepared by spiking 0.10 g of the citrus flavoring (10 mg mL^{-1}) into a 100.00 g synthetic juice matrix, which was made of Milli-Q water (88.82 g), sucrose (10.80 g, SAFC, St. Louis, MO, USA), anhydrous citric acid (0.26 g, SAFC), pectin (0.05 g, Grindsted® AMD783, Danisco, Lakeland, FL, USA), sodium citrate dihydrate (0.04 g,

SAFC), ascorbic acid (0.03 g, SAFC). Ten milliliter of model citrus beverage was used for SBSE extraction.

All experiments were carried out in triplicate and the results were reported as the mean values together with standard deviations.

7.3. Results and discussion

7.3.1. Optimisation of TD-PTV injection process

Through allowing compounds to be injected under temperature controlled conditions, PTV technique alleviates the problems of compound discrimination and decomposition, which always occur when analytes are flash vaporized in a hot split/splitless injector (*289*). In this work, PTV solvent vent mode was chosen to allow a suitable high desorption flow rate for desorption of the analytes into PTV and consecutively maximize the transfer of desorbed analytes into the GC column through splitless injection. Moreover, the split valve of CIS was closed during desorption, and remained close after desorption until the transfer of analytes was complete. The time period was defined before the split vent valve opens as splitless time. The duration of splitless time should be carefully set to ensure the complete transfer of analytes into the column. An insufficient splitless time could lead to an incomplete transfer of analytes and resulted in poor reproducibility of peak areas, as well as the discrimination for high boiling compounds.

Figure 7.1 shows the effect of splitless time on the peak areas of the various classes of volatile compounds. Most classes of compounds were maximally transferred into the column after opening the split vent time at 3 min, except for aldehydes and esters, which required a splitless time of 5 min.

On the other hand, a progressive loss of the compounds with lower boiling points was observed after a longer splitless time of 5 min, suggesting that prolonged vent time could be a disadvantage or discrimination against these compounds. Thus, a vent time of 3 min was set as the default to ensure the quantitative transfer of a good majority of the compounds, while minimizing the loss of more volatile compounds.

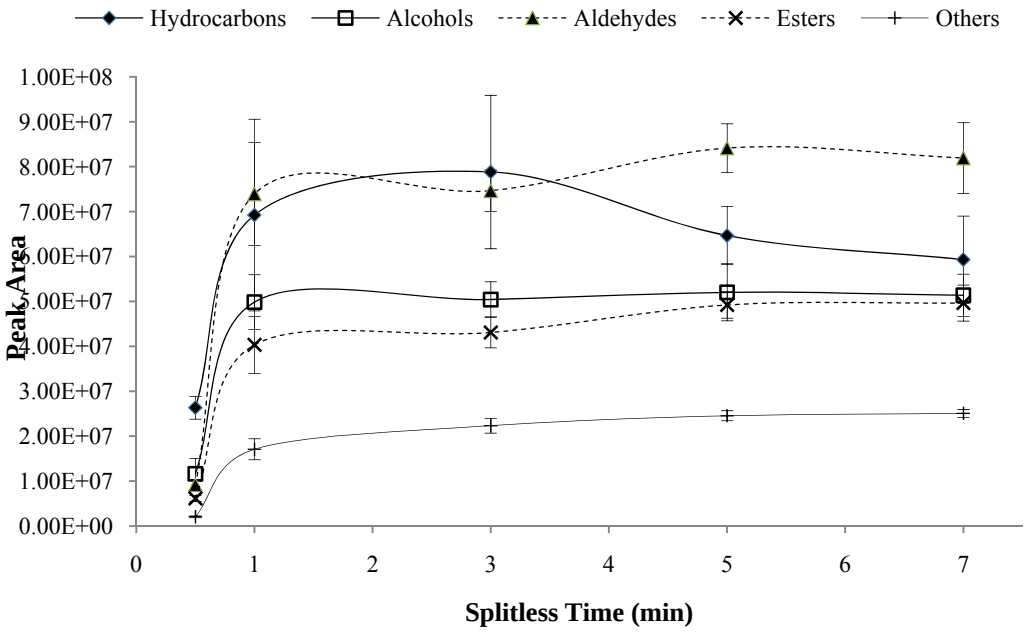


Figure 7.1. Effect of splitless time on the quantitation of each class of volatile compounds

The mode of the injection could affect the quantification of volatiles where thermal desorption process could be the most critical part in enhancing quantification proficiency. Previous studies that focused on the optimisation of PTV operating parameters have identified that injection temperature, desorption flow, vaporization temperature, vaporization time and cryofocusing temperature could significantly affect the efficiency of thermal desorption (*290-292*). A preliminary screening resulted in three interrelated factors (i.e. desorption flow, thermal desorption time and cryofocusing temperature) being

selected for further optimization using RSM (data not shown). The common practice to optimize an extraction process using RSM is to observe total responses or total peak areas (*152, 153, 289, 290*). However, such an approach does not account for differences among peak areas in contributing towards extraction efficiency of different compounds. In contrast, these compounds were studied by establishing the relationship between the response of each compound in terms of peak area and three operating parameters.

Figure 7.2 illustrates the response surface plots of three representative responses – constant, linear and quadratic, where the nature of the response surface system depends on the signs and magnitudes of the coefficients in the model terms. The estimated responses for all compounds are also listed in Table 7.1. In order to increase the model's predictive accuracy, a stepwise approach was applied to fit the full response surface and eliminate the terms not significant at the $p = 0.05$ level.

Although no clear-cut relationship between the models obtained for each compound and its physicochemical properties could be identified, it was observed that those compounds with constant models generally had lower $K_{o/w}$ values and higher solubility in water (e.g. citral, *alpha*-terpineol and linalool), suggesting that they were poorly extracted by SBSE. Therefore, a constant response reflected that the corresponding compound was non-responsive towards the parameters, while a linear response was obtained when the detected peak area was in proportional to the main parameters. The significant first-order terms of ethanol, nerolidol and methyl jasmonate reflected the fact that the lower cryofocusing temperature was due to their low boiling points. However, extreme low cryofocusing temperature (i.e. -150 °C) could possibly

crystallize other higher boiling compounds and the glasswool in the TDU inlet liner. Thus, those less volatile compounds would be trapped before they reached the column.

In addition, as suggested by Tredoux et. al (*112*) a trapping temperature of -100 °C instead of -150 °C greatly improved the peak shapes for early eluting compounds. It is believed that faster heating of the liner to the injection temperature leads to reduced injection times and therefore less band broadening. On the other hand, quadratic models with significant second-order coefficients (pure quadratic and interaction terms) played a vital role in estimating the responses and could shed some light on the thermal desorption behavior of these analytes. For instance, decyl acetate with a high boiling point decreased in its peak area as cryofocusing temperature decreased; while peak area of ocimene with a low boiling point showed an increase in response to a decrease in cryofocusing temperature.

Among the compounds with quadratic models, most of them did not have strong interaction effects, but did have strong quadratic terms on main effects. Among these three main effects, cryofocusing temperature had the greatest influence on the analytical responses. Nevertheless, a sufficient thermal desorption time and a high purge flow rate were important to maximize the transfer of analytes (e.g. terpinolene).

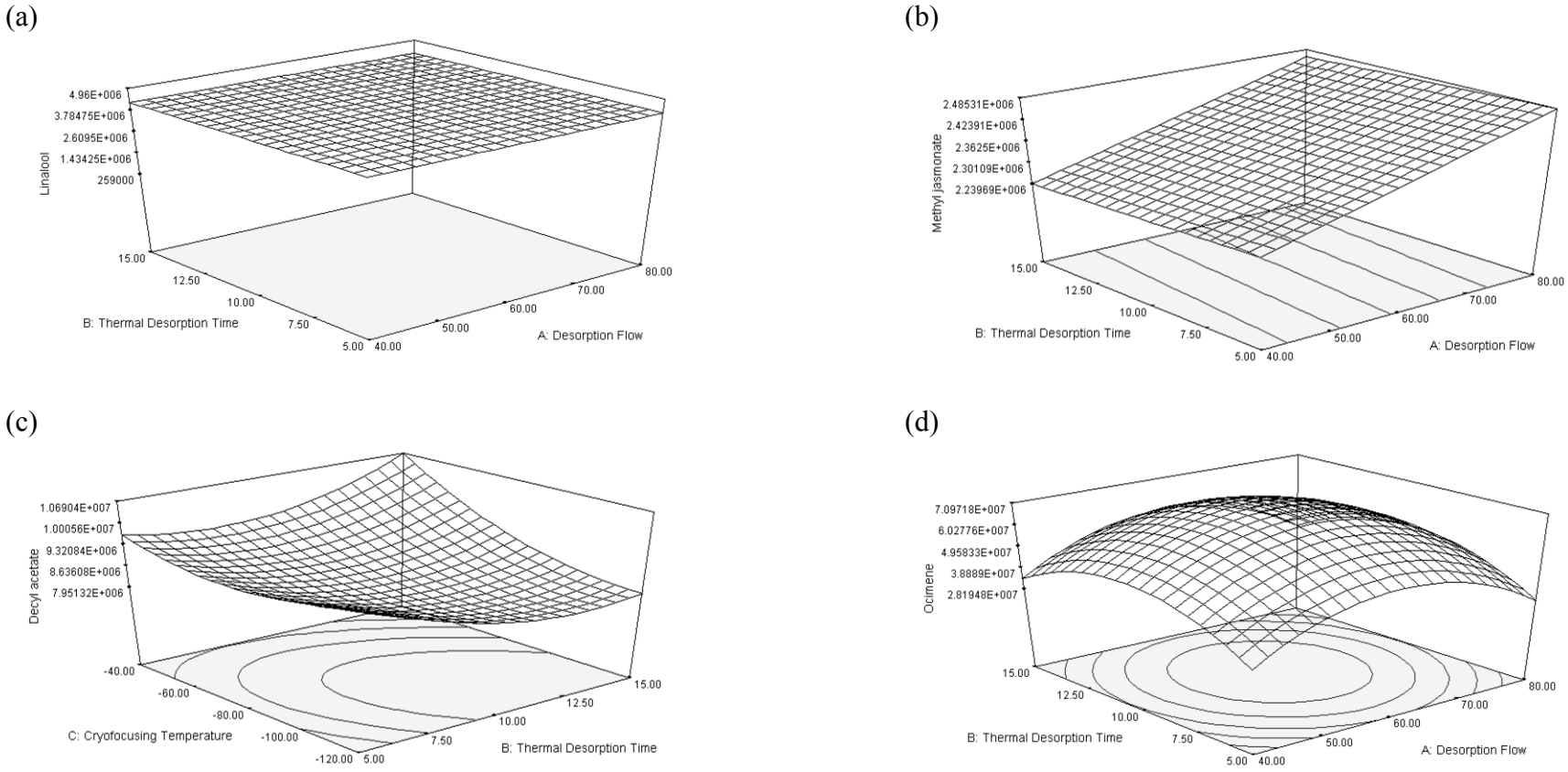


Figure 7.2. Typical profiles of surface response generated from a quadratic model in the optimization of three variables (thermal desorption time, desorption flow and cryofocusing temperature): (a) Constant – exemplified by linalool; (b) Linear – exemplified by methyl jasmonate; (c) Quadratic with minimum response– exemplified by decyl acetate; (d) Quadratic with maximum response – exemplified by ocimene

The response models also revealed that the dissimilarity of responses was mainly due to the physicochemical properties. Compounds with low boiling point (e.g. limonene, ocimene, *beta*-myrcene, octanal and ethyl butyrate) resulted in a maximum response, so they favored a moderate desorption temperature and a sufficiently low cryofocusing temperature. In contrast, a minimum value indicated the thermal desorption was operated under a condition remote from the optimum (e.g. decatrienal, citronellol, nootkatone). Hence, due to their low volatilities, these analytes required a longer desorption time.

Several studies have been done on wine whether focused on volatile phenols (*113*) or major wine volatiles (*112, 114, 115, 293*), however, to the best of my knowledge, there was none of the reported study optimized the operating condition based on all target analytes. In addition, it is often necessary to use constrained optimization to attain the best operating condition. This is particularly true in the present work to avoid the optimum point to fall outside the operating parameters.

The optimized factors were determined based on maximizing the desirability of the responses for the flavoring as a whole, which combined the individual desirability of each response into the objective function. The desirability of the response for each compound ranged from 0.00 to 1.00, corresponding to the increase from lowest to the highest in the response values obtained by the experiments. The optimized values of the three factors were A = 74.00 mL min^{-1}, B = 5.00 min and C = –120.00 °C, with an overall desirability of 0.54. Compared to the default thermal desorption method prior to the optimisation with overall desirability of 0.32, some of the peak

responses were suppressed while some were enhanced, thereby the peak responses of each target analytes varied from –35% – 100%, with an average enhancement of 32%. This reflected that under the optimized set parameters the variability of peak responses among the analytes was less discriminated.

7.3.2. Understanding of SBSE extraction

From the extensive reviews on SBSE, the recovery of an analyte could be approximated by its partition coefficient between PDMS and water ($K_{PDMS/W}$) and the phase ratio of PDMS phase/sample volumes (*84, 117, 294*). Moreover, previous studies revealed that some other experimental factors (e.g. extraction temperature, rate of agitation and salt content) could also affect the efficiency of SBSE (*108, 113, 120*). From a preliminary study on extraction-time profile, most of the compounds were recovered substantially after first 2 h of extraction and then gradually increased up to about 4 h. The increase in the amount extracted became less pronounced beyond 4 h and equilibrium was reached after about 6 h, except for hydrocarbons (data not shown).

The differences in equilibration time for different classes of analytes were thus suggested that kinetic aspect is also important to achieve equilibrium though the uptake rate is mainly determined by diffusion constants, stirring conditions, sample volume etc. (*101*). Even though an extraction time of 12 h could ensure the maximum recovery of all the compounds, such a long extraction time would simply be unrealistic for routine extraction. In addition, equilibration during the extraction process was not necessary in practice since calibration could be carried out for any

consistent extraction time (*117*). Hence, extraction time of 2 h was chosen as the default extraction time in this study.

Subsequently, a partial factorial experimental design was chosen to investigate the effects of the extraction factors on the efficiency of SBSE. The level of extraction time, temperature and stirring speed took into account the consideration on the sample throughput and minimal evaporation of the analytes for a combination of the extremes of these variables (at 900 rpm, 60 °C for 6 h). The sample pH was studies in the between range of 2 to 7 referring to the pH range of different food beverages. The sodium chloride concentration range studied involved no addition to saturation.

Under the present condition, according to the Pareto charts shown in Figure 7.3, extraction time, temperature and stirring speed had no significant effects on enhancing the extraction of SBSE. Nonetheless, pH had a positive effect, especially alcohol group and the other compounds like nootkatone and indole, which was in contrast to the insignificant enhancement on the extraction of volatile phenols from wine (*293*). On the other hand, highly positive effect of ionic strength was observed for alcohol group, since the addition of sodium chloride reduces the water solubility of polar analytes.

Thus, this resulted in the increased partitioning coefficient between the PDMS and analytes (*101*). Finally, esters and hydrocarbons were negatively affected by ionic strength. This could be due to the higher solute concentration increased viscosity of the solution and hinder the diffusion efficiency of analytes. During extraction, the solutes should migrate from the sample into the PDMS coating (*101*). In such case, sufficient convection or stirring speed is important, so that diffusion efficiency of analytes will not be affected.

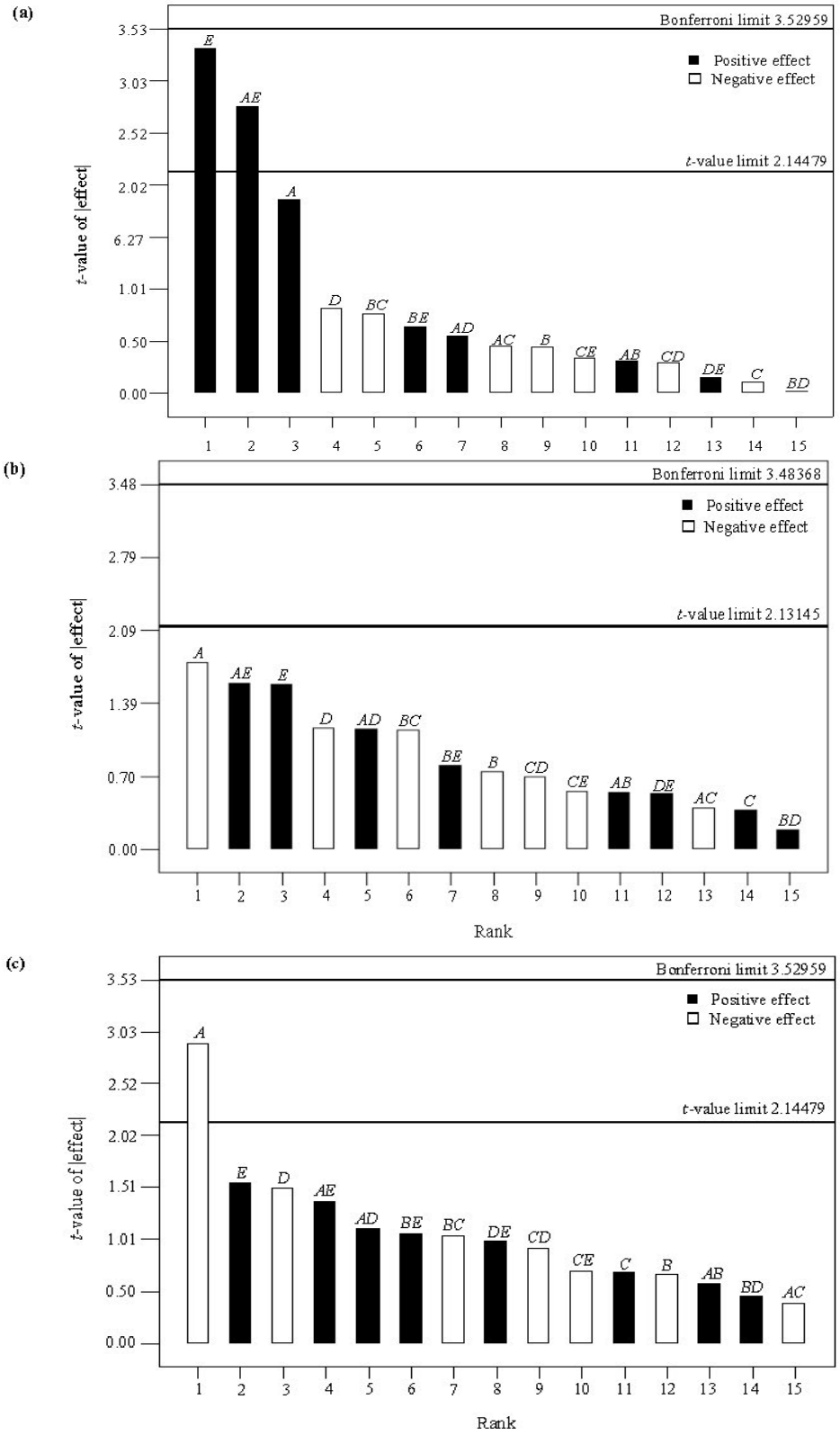


Figure 7.3. Pareto chart of the statistical analysis of the screening of factors for the extraction step of (a) alcohols; (b) aldehydes; (c) esters. The vertical line indicates the threshold value for proclaiming the statistical significant terms on the effect of (A) ionic strength; (B) stirring speed; (C) extraction time; (D) temperature; (E) pH

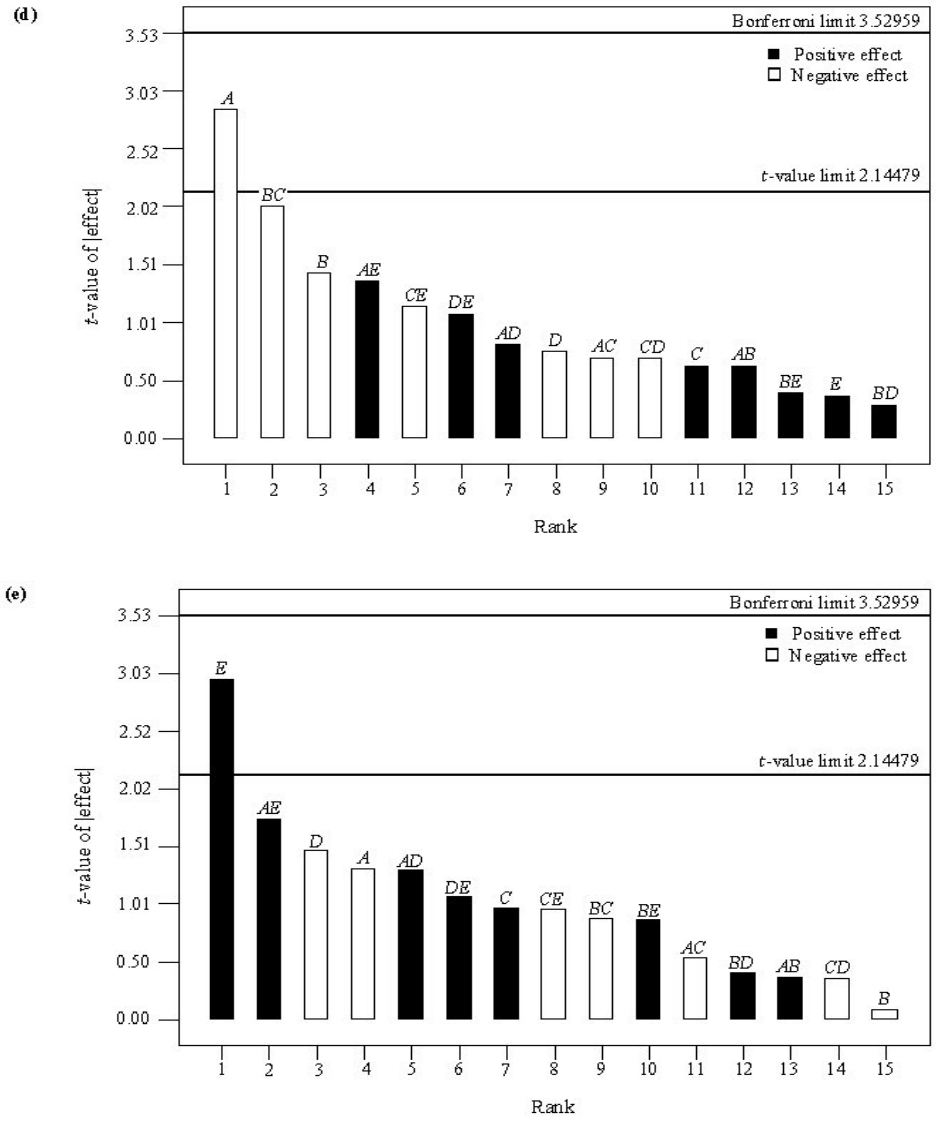


Figure 7.3. (Cont'd) Pareto chart of the statistical analysis of the screening of factors for the extraction step of (d) hydrocarbons; and (e) others. The vertical line indicates the threshold value for proclaiming the statistical significant terms on the effect of (A) ionic strength; (B) stirring speed; (C) extraction time; (D) temperature; (E) pH

7.3.3. Method evaluation and validation

With the aid of RSM, solute discrimination during thermal desorption and cryofocusing was reduced. Furthermore, SBSE extraction was found to be more favourable in neutral pH, while temperature and stirring speed were not the major factors as long as there was sufficient extraction time. In addition, extraction efficiency of polar organic analytes can be improved by increasing ionic strength. In the following experiment, sample matrix was not altered in order to maintain the true ratio in the flavoring.

In order to validate the multiple regression equations obtained, FID peak responses of each compound was examined under two combinations within the experimental range: (1) A = 74.00 mL min^{-1}, B = 15.00 min and C = –120.00 °C; (2) at A = 48.00 mL min^{-1}, B = 5.00 min and C = –54.00 °C. Results indicated that the values of the peak areas obtained from the actual experiments fall within 95% confidence interval of the predicted range of the regression model.

The precision of each compound was evaluated in terms of its repeatability and reproducibility. Repeatability was reported as the RSD of the peak area obtained from six consecutive analyses within the same day, whereas intermediate precision was reported as the RSD of the peak areas obtained from five consecutive analyses on different days. Table 7.3 shows the repeatability and intermediate precision for each compound. RSD of 1.7% and 6.3% were obtained for the total peak areas from intra-day and inter-day analyses, respectively.

A linear relationship of absolute peak areas against concentrations was obtained for individual compounds, with a correlation coefficient R^2 of at least

0.99. Based on the calibration curves obtained, the linearity, limit of detection (LOD) and limit of quantification (LOQ) were also determined for each compound (see Table 7.3). All the compounds had low LOD values from 0.03 to 3.89 μg L^{-1}. This data indicated that the analytical method developed could facilitate the simultaneous quantitation of nearly all of the compounds present when the flavoring was spiked in water.

In contrast to previous studies that semi-quantified the extracted aroma compounds using internal standard (*118*) or estimated the relative levels of target compounds in wine samples from the ratios of their MS areas to that of the relevant internal standards using scan mode (*116*), all the above discussions were based on the absolute FID peak area. In the present study, two internal standards (i.e. *n*-undecane and 6-methyl-5-hepten-2-one) were selected in an attempt to determine the concentration of each compound. However, owing to the diversity of compounds in citrus flavoring, the peak responses obtained for the two selected internal standards were not proportional to those obtained for all compounds (data not shown). Hence, this would have given rise to an over-estimation or bias when the concentrations of all the compounds were calculated based on normalizing their responses against the internal standards.

7.3.4. Matrix effect of model citrus beverage on SBSE extraction

The results presented thus far were obtained from experiments carried out by extracting citrus flavoring from a blank matrix (aqueous solution only) to develop the analytical method with minimum interferences from the matrix. A recent study determined the presence of nine synthetic musks using SBSE

in four kinds of aqueous matrices (i.e. effluent and influent of wastewater treatment plant, effluent of a reverse osmosis treatment plant and river water) which no matrix effect was observed (*295*).

On the other hand, the presence of ethanol in a model wine sample matrix resulted in decreased sensitivity of the SBSE method toward most of the volatile compounds (*118*). Beverage products usually contain different types of soluble solids (e.g. acids, sugars, and pectins) give rise to matrix effects that would further complicate the extraction process (*285*). The presence of flavor-hydrocolloid interaction does have an impact on the amount of volatile compounds released from the food matrix (*64, 285, 296*). As there is limited information of the flavor-hydrocolloid interaction on SBSE efficiency, therefore, a model citrus beverage was prepared to evaluate the matrix effect on SBSE extraction. To verify the applicability of the developed analytical method in real citrus beverage, the method was applied to the model citrus beverage.

Among food hydrocolloids, high-ester pectin is one the most commonly used. Hydrocolloids can be used as emulsifying and stabilizing agents in various applications such as acidified beverages (*285*). Sucrose and high-ester pectin were two main ingredients that could alter the rheological properties of the matrix and influence flavor partitioning in an emulsion system. The presence of sucrose and high-ester pectin generally resulted in changes of liquid partition coefficients. The salting-out effect was likely to be the reason for this phenomenon, whereby the sugar interacted with water, enhancing the concentration of flavor compounds. On the other hand, pectin was added in model citrus beverage with a pH value of 3.25, a weak gel network was

formed with hydrogen bonds and hydrophobic interactions. This macromolecular network could form flavor-matrix interactions with the volatile compounds and thereby enhancing or suppressing the release of the compounds to be extracted by SBSE (*285*).

From observations in this study, the variance of volatile extractions between the model citrus beverage and blank matrix was insignificant, except for hydrocarbons and alcohols (Figure 7.4). This could be explained by the hydrophobic interaction between non-polar terpenes and pectin network, thereby reducing their availability for extraction by SBSE. A previous study has demonstrated that both orthonasal and retronasal odor thresholds were much higher in reconstituted orange juice than in water (*284*). On the other hand, the noticeable enhancement of alcohol extraction could be due to the reduced water activity with the presence of soluble solids, thus, alcohol could be more easily absorbed by PDMS.

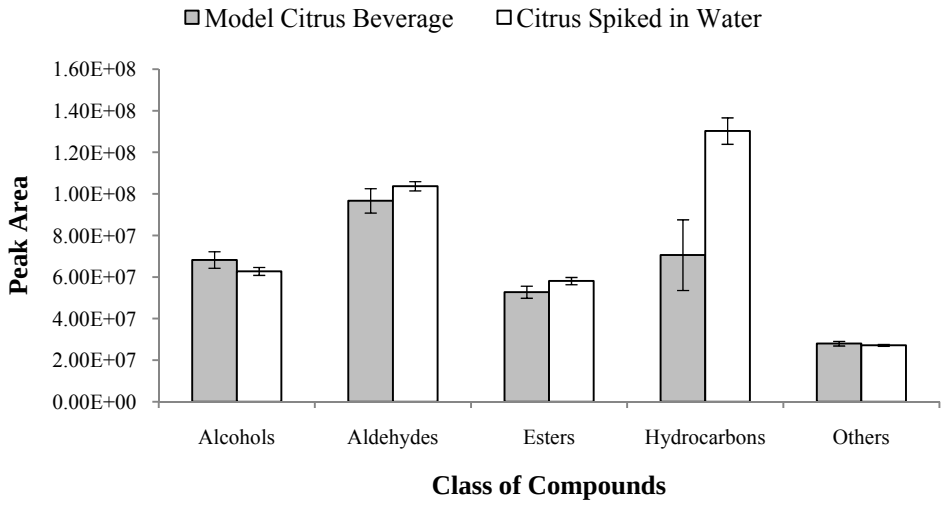


Figure 7.4. FID peak areas of SBSE extraction on different matrices

7.4. Conclusion

A systematic approach was applied to understanding the factors that would affect SBSE-TD-PTV analysis of a complex mixture. Detailing the responses of different flavor compounds, cryofocusing temperature was found to be the most influential factor among three TD-PTV parameters. Consequently, variability of GC peak responses among the analytes was alleviated. Furthermore, through partial factorial design, the results of SBSE extraction indicated the positive influence of ionic strength and neutral pH on the extraction of alcohol compounds. Finally, the optimized method was evaluated and validated through measuring linearity, the detection limits and repeatability values. Therefore, this methodology may be effective in improving the performance of SBSE-TD-PTV analysis.

CHAPTER 8 VOLATILE COMPOSITION AND ANTIOXIDANT CAPACITY OF ARABICA COFFEE

8.1. Introduction

Because of the scarcity and unique aroma, Sidikalang coffee is highly sought after by exporters and coffee drinkers today (*195*). Civet-treated Sidikalang coffee or Kopi Luwak is one of the most valued and most expensive types of coffee beans to be produced (*297*). In addition, highland arabica coffee, which grows on the foothills of the Himalaya in Yunnan and Doi Chang in Northern Thailand have been highly rated for their unique and distinctive flavors. However, there is limited information on these exotic and new coffee varieties. In view of the agricultural and geographical considerations relative to aroma differences, it is worth to explore the volatile and aromatic profiles of Asian coffee.

Green coffee beans are increasingly used in beverages due to a growing trend to create low-calorie refreshments with green coffee extracts. Hence, measuring the phenolic acid content and antioxidant activity of these beans would help assess their potential as a source of natural antioxidants (*298*). Since there is no standardized method for such tests, more than one test is

usually employed for antioxidant activity determination to take into account the different mechanisms of the antioxidants (*299*). Common *in vitro* antioxidant assays used include the Folin-Ciocalteu method, diphenyl-1-picryl hydrazyl assay (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-suslfonic acid) (ABTS) assay, and ferric reducing antioxidant potential (FRAP) assay (*298, 300-302*). Their mechanisms are generally based on a hydrogen atom transfer, a single electron transfer or both (*303, 304*).

The objective of this study was to evaluate and compare the chemical compositions and antioxidant capacities of four varieties of Asian Arabica coffee as potential Asian-grown specialty coffees.

8.2. Experimental procedures

8.2.1. Coffee beans and chemicals

Four varieties of green coffee beans were collected, i.e. Doi Chang (*Coffea arabica* L. cv. Catimor) from Doi Chang village located in Chiang Rai province of Northern Thailand, Sidikalang (*Coffea arabica* L. cv. Typica) and civet-treated Sidikalang (Kopi Luwak) from Dairi district, North Sumatra Indonesia and Yunnan (*Coffea arabica* L. cv. Catimor) from Pu'er district in Yunnan province of China.

Among these varieties, the Typica coffee is known for elongated oval shaped of bean compared to the Catimor coffee, while the Yunnan coffee beans had smaller bean sizes compared to the Sidikalang coffee beans. They were stored away from light at 25 °C. A sample of 150 g of green beans with initial moisture content of 7.2 – 9.6% was roasted with a home coffee roaster (Imex, Seoul, Korea) for 12 min, and then cooled down by blowing in the

roaster for 4 min. Each batch of roasted beans was cooled on a tray prior to grinding using a coffee grinder (Braun KMM30, Braun, Melsungen Germany). The ground coffee was sifted into the size range of 1.77 – 2.36 mm (Coffee Grind Sizer, Coffee Chemistry, CA, USA) and then was sealed in aluminum pouches and refrigerated at -30 °C until use.

Analytical grade acetone, dichloromethane, hexane, and methanol were from Tedia, and petroleum ether was from ACS Chemical Inc. (NJ, USA). Anhydrous Na_2SO_4 was used as a drying agent (Sigma-Aldrich) and acetic acid were purchased from Merck.

An internal standard for coffee extract, 5-methyl-2-hexanone was from Sigma-Aldrich and a series of alkane standards (C8 – C40) was purchased from Fluka. External standards were used for phenolic acids quantification: chlorogenic and ferulic acids, caffeic and *p*-coumaric acids (Sigma-Aldrich), and sinapic acid (Fluka). All standard compounds used in the identification of the volatile compounds were obtained from Firmenich Asia Pte. Ltd., Singapore.

8.2.2. Preparation of coffee extracts

8.2.2.1 Extraction of volatile compounds

Coffee extract was prepared from 10 g of ground coffee beans with a volume of 40 mL of solvent (i.e. methanol, hexane or dichloromethane). The suspension was stirred using a vortex mixer at 200 rpm and 25 °C for 1 h. Subsequently, the extract was filtered from the ground coffee beans and was dried with Na_2SO_4. The solvent was removed under a purified nitrogen stream using TurboVap II until the volume was reduced to approximately 1 mL. The

concentrated extract was dark brown. The sample was stored at -80 °C before use.

8.2.2.2 Extraction of phenolic acids

Ground green and roasted coffee beans were defatted with petroleum ether by soxhlet extraction. The defatted beans were collected and left to dry overnight in a fume hood and subsequently, freeze-dried in a freeze dryer (VirTis AdVantage, Genevac, SP Scientific, Ipswich, UK). The freeze-dried beans were stored in a desiccator. The extraction of phenolic acids was carried out as previously described by Krygier, et al. (*305*) with modifications. Two grams of defatted ground coffee beans were extracted with six portions of 40 mL of an acetone-methanol-water (7:7:6) mixture. The extract was evaporated to dryness under reduced pressure using a Rota-Vap at 40 °C. The residue was then reconstituted with 50 mL of 80% aqueous methanol and stored at -80 °C prior to analysis.

8.2.3. Instrumental analysis

8.2.3.1 GC-MS/FID analysis

GC-MS/FID analysis was carried out using Agilent 6890N GC coupled with FID and a 5975 inert MS. Identification and quantification of the eluted compounds were as described in previous chapter 3 (Section 3.2.2).

8.2.3.2 UFLC/PDA analysis

Phenolic acid analysis was carried out using a Shimadzu Prominence UFLC system equipped with two LC-20AD pumps, a SIL - 20A autosampler injector, CTO-20AC column oven and a SPD-M20A PDA detector for phenolic acid analysis (Shimadzu). The HPLC system was controlled using the software, LabSolutions (Shimadzu). Phenolic acid extracts were filtered with a 0.2 μm PTFE filter (Agilent Technologies) prior to analysis, and were analyzed as described in Section 5.2.5. of Chapter 5.

8.2.4. Determination of total polyphenol content

The determination of total polyphenol content by the Folin-Ciocalteu method was based on the ISO method for tea (*306*). A sample of 0.1 mL of the phenolic extract was diluted to 5 mL with deionised water. One mL of the diluted extract was then mixed with 5 mL of 10% diluted Folin-Ciocalteu phenol reagent (Merck). The mixture was allowed to stand for at least 3 min before 4 ml of 7.5% sodium carbonate was added. The reaction mixture was then mixed and allowed to stand at room temperature. After 60 min, the absorbance of the solution was measured at 765 nm, using a spectrophotometer (Pharmaspec UV-1700, Shimadzu, Kyoto, Japan). The blank was prepared by replacing the sample solution with water. Standard solutions of gallic acid (Acros Organics, New Jersey, USA) with concentrations of 10 - 50 mg/L were used for calibration.

8.2.5. Determination of antioxidant activity

8.2.5.1 DPPH assay

The radical scavenging activity of the phenolic extract was tested according to the method described by Nebesny & Budryn (*301*) and Kelebek & Selli (*307*) with modifications. A sample of 3.9 mL of DPPH solution (7.5 mg DPPH per 100 mL of methanol) was mixed with 0.1 mL of each phenolic extract and the mixture was left to stand in the dark at room temperature. A control was prepared by mixing 3.9 mL of DPPH solution with 0.1 mL of methanol. After 10 min, the absorbance of each mixture was measured at 517 nm. Methanol was used as a blank. The radical scavenging activity for each solution was measured using the following formula:

$$Radical\ scavenging\ activity = \left(\frac{A_{Control} - A_{Sample}}{A_{Control}}\right) \times 100$$

8.2.5.2 FRAP assay

The FRAP assay was conducted according to the procedure described by Vignoli et al. (*300*). The FRAP reagent was first prepared by mixing 2.5 mL of 10 mM 2,4,6-tris-2-pyridyl-1,2,5-triazine (TPTZ) (Sigma) solution in 40 mM HCl, 2.5 mL of 20 mM ferric chloride hexahydrate (GCE Laboratory Chemicals, Singapore) in deionised water, and 25 mL of 0.3 mM acetate buffer of pH 3.6. The mixture was incubated at 37 °C for 30 min. A 50 µL aliquot of the phenolic extract was diluted to 1 mL with deionised water and standard solutions of Trolox in ethanol with a concentration range of 100 – 800 mg/L were prepared for calibration. The reducing power of the phenolic extracts was then measured by mixing 900 µL of freshly prepared FRAP

reagent with 90 μL of deionised water and 10 μL of the phenolic extract or the standard solutions. The mixture was incubated at 37 °C and its absorbance was measured at 595 nm. The reducing power of coffee beans was expressed in mg Trolox per g coffee beans.

8.2.6. Statistical analysis

One-way analysis of variance (ANOVA) was carried out to determine the significant difference of the non-volatile composition and antioxidant activity between the green and roasted coffee bean samples. Distribution of the volatiles relative to the overall variability of the data sets was attained using principal component analysis (PCA) as explained in Section 5.2.6 of Chapter 5.

8.2.7. Sensory evaluation

The aroma profiles of the four different coffee extracts were evaluated as described in Section 2.2.4 of Chapter 2. The corresponding coffee descriptors were berry-like, buttery, caramelic, nutty, roasted, smoky, spicy, sulfury, sweet and roasted. A 6-point scale was used with ‘0’ indicating the uncharacterized attribute intensity and ‘5’ indicating the very strong attributes intensity.

8.3. Results and discussion

8.3.1. Volatile composition

Three organic solvents were screened for extracting coffee volatiles (data not shown). Compared to hexane and methanol, dichloromethane with its polar aprotic property was the most suitable solvent by allowing the potent polar compounds to be dissolved and giving a more comprehensive profile of coffee volatiles (data not shown). Most of the potent odorants in roasted ground Arabica coffee are known to be polar (*44*). Therefore, dichloromethane was chosen for further study.

The aroma of coffee is characterized by the diverse combinations and disparate concentrations of volatile compounds from different classes. The chromatograms of different coffee extracts showed similar volatile profiles but different in quantity. Among the hundreds of compounds identified, 62 aroma-active compounds were categorized into different classes and are listed in Table 8.1.

Sulfur-containing volatiles are known to be largely responsible for the roasty coffee aroma (*190*). Due to their low flash points and susceptibility to oxidative degradation reactions, sulfur compounds are usually present in trace amounts (less than 0.01% of the total amount of volatiles) but play a crucial role in the freshness of roasted coffee (*44, 308*); in which, 2-furfurylthiol is an important odorant with a low odor threshold of 0.05 ppb in air (*93*). The extracts from Yunnan and Doi Chang coffee beans were found higher in the amount of 2-furfurylthiol. Although the amounts of 2-furfurylthiol present in the different coffee extracts were relatively low, it displayed the greatest impact on aroma as revealed by subsequent sensory evaluation.

Table 8.1. Volatiles and their concentrations (ppm) of dichloromethane extracts of coffee varieties from different geographic origins.

	Compounds	*LRI*		Concentration (ppm)				Identification
		FFAP	Literature	Doi Chang *(Thailand)*	Sidikalang *(Indonesia)*	Yunnan *(China)*	Kopi Luwak *(Indonesia)*	
	Acids							
1	Acetic acid [I, III]	1430	1468	33.80 ± 8.38	40.47 ± 3.40	17.21 ± 3.88	35.00 ± 4.43	MS, $LRI^{b,c}$, STD
2	Propanoic acid	1524	-	11.84 ± 0.75	12.77 ± 0.52	8.14 ± 0.95	9.97 ± 1.52	MS, STD
3	Butanoic acid [I]	1609	-	2.53 ± 0.10	3.39 ± 0.08	2.94 ± 0.21	2.10 ± 0.25	MS, STD
4	3-methylbutanoic acid [I, II, III]	1653	1687	18.06 ± 1.80	32.18 ± 0.76	24.23 ± 3.29	23.56 ± 1.92	MS, LRI^{b}, STD
	Furans							
5	Fufuryl methyl ester [I]	1233	1260	2.87 ± 0.25	2.21 ± 0.62	2.58 ± 0.06	1.95 ± 0.32	*MS,* LRI^{c}
6	Furfural [I]	1467	1473	13.86 ± 1.10	17.46 ± 1.09	5.88 ± 0.27	19.37 ± 1.47	MS, $LRI^{a,c}$, STD
7	Furfuryl formate [I]	1497	1507	6.42 ± 0.33	5.09 ± 0.14	5.52 ± 0.69	4.06 ± 0.36	MS, $LRI^{a,b,c}$
8	2-acetylfuran [I]	1515	1513	13.27 ± 1.69	15.84 ± 0.99	11.87 ± 1.26	13.52 ± 1.05	MS, $LRI^{a,b}$
9	Furfuryl acetate [I]	1538	1547	32.92 ± 2.15	32.14 ± 1.75	40.04 ± 6.55	24.52 ± 1.43	MS, $LRI^{a,b,c}$, STD
10	5-methylfufural	1581	1582	24.76 ± 1.60	32.87 ± 1.59	8.88 ± 0.76	31.79 ± 1.81	MS, $LRI^{a,b,c}$
11	Furfural propionate	1599	1626	4.52 ± 0.26	4.63 ± 0.61	5.51 ± 0.31	3.72 ± 0.18	MS, LRI^{c}
12	2,2'-methylenebis furan [I]	1615	1637	5.22 ± 0.59	6.38 ± 1.38	9.23 ± 0.89	4.56 ± 1.48	MS, LRI^{c}
13	5-methyl-2-acetylfuran	1634	1653	3.74 ± 0.30	5.70 ± 0.26	4.64 ± 0.33	3.42 ± 0.21	MS, LRI^{c}
14	Furfuryl alcohol	1644	1671	237.95 ± 20.38	186.95 ± 4.72	207.31 ± 47.20	152.81 ± 7.86	MS, $LRI^{a,b}$, STD
15	Furfuryl ether	1991	1996	9.87 ± 0.40	9.51 ± 0.71	14.52 ± 1.20	7.51 ± 0.79	MS, $LRI^{a,c}$
16	5-(hydroxymethyl)-2-furfural [I]	2493	2516	12.09 ± 9.35	14.64 ± 0.56	16.23 ± 5.67	22.60 ± 4.50	MS, LRI^{b}
	Furanones							
17	Dihydro-2-methyl-3(2H)-furanone[I]	1255	1282	12.70 ± 1.20	15.36 ± 0.90	7.58 ± 1.27	13.60 ± 0.40	MS, $LRI^{b,c}$
18	2,5-dimethyl-3(2H)-furanone [I]	1513	-	4.06 ± 0.15	3.06 ± 0.87	4.54 ± 1.32	2.57 ± 0.25	MS, STD
19	γ-butyrolactone [V]	1638	1637	47.65 ± 3.18	30.53 ± 3.41	65.82 ± 10.90	32.29 ± 2.31	MS, $LRI^{a,b}$, STD
20	3,4-dimethyl-2,5-furandione	1749	-	10.89 ± 0.50	10.50 ± 0.32	13.54 ± 2.26	8.49 ± 0.71	MS
21	Furaneol [I, II, III, IV]	2016	2062	11.93 ± 1.17	13.62 ± 0.232	11.59 ± 0.69	10.93 ± 1.60	MS, $LRI^{b,c}$, STD
22	5-hydromethyldihydrofuranone	2481	2516	19.03 ± 3.03	20.81 ± 2.13	28.64 ± 8.11	26.40 ± 6.90	MS, LRI^{b}
	Ketones & Lactones							
23	Acetoin [I, V]	1267	1291	7.16 ± 7.89	7.85 ± 0.76	29.0 ± 6.51	7.01 ± 0.71	MS, $LRI^{a,b,c}$, STD
24	1-hydroxy-2-propanone	1278	-	15.31 ± 1.25	17.50 ± 0.98	8.40 ± 1.38	21.37 ± 0.91	MS
25	1-hydroxy-2-butanone [I]	1363	1361	6.57 ± 0.15	7.96 ± 0.27	6.15 ± 1.01	4.73 ± 0.22	MS, $LRI^{a,b,c}$
26	3-methyl-2-cyclopentenlone	1378	-	1.99 ± 0.35	1.80 ± 0.37	2.37 ± 0.29	1.29 ± 0.14	MS

Table 8.1. (Cont'd)

27	1-(acetyloxy)-2-propanone	1461	1477	24.66 ± 1.82	24.59 ± 1.61	17.33 ± 2.06	21.37 ± 0.91	MS, *LRI*[b]
28	1-(acetyloxy)-2-butanone	1534	-	5.05 ± 0.35	5.62 ± 0.33	3.66 ± 0.19	4.73 ± 0.22	MS
29	2,3-dimethyl-2-cyclopentenlone	1553	1573	2.71 ± 0.17	3.59 ± 0.33	5.00 ± 0.07	2.47 ± 0.27	MS, *LRI*[b]
30	1-(6-methyl-2-pyrazinyl)-1-ethanone[I]	1716	-	4.86 ± 0.28	4.50 ± 1.16	6.12 ± 0.26	3.01 ± 0.35	MS
31	3,5-dimethyl cyclopentadione	1790	-	8.87 ± 0.54	9.58 ± 0.64	12.38 ± 0.28	8.01 ± 0.94	MS, STD
32	Maple lactone [I, IV]	1834	1857	16.83 ± 1.33	17.01 ± 0.23	19.87 ± 2.23	13.72 ± 1.81	MS, *LRI*[b]
33	Ethyl maple lactone [I]	1894	-	10.01 ± 0.32	12.64 ± 0.79	14.72 ± 0.39	10.00 ± 0.31	MS
34	γ -dodecalactone	2071	-	13.44 ± 1.95	11.40 ± 1.40	16.18 ± 4.49	9.70 ± 1.04	MS
	Phenols							
35	Guaiacol [I, II, III, IV]	1861	1871	12.78 ± 1.42	12.08 ± 0.39	17.97 ± 1.06	9.20 ± 0.82	MS, *LRI*[a,b,c], STD
36	Phenol [I]	1995	2030	11.74 ± 1.31	11.43 ± 0.70	20.50 ± 0.57	8.33 ± 0.34	MS, *LRI*[b], STD
37	4-ethylguaiacol [I, II, III, IV]	2041	2065	14.88± 1.98	19.82 ± 1.50	24.80 ± 1.79	13.29 ± 2.33	MS, *LRI*[c], STD
38	p-vinylguaiacol [I, II, III, VI]	2206	2225	41.65 ± 3.75	46.54 ± 2.43	55.48 ± 3.76	31.64 ± 3.97	MS, *LRI*[b], STD
	Pyrazines							
39	Pyrazine [I]	1208	1215	3.73 ± 10.56	2.31 ± 0.37	4.49 ± 0.89	1.95 ± 0.15	MS, *LRI*[a,b,c]
40	2-methylpyrazine [I]	1261	1267	28.26 ± 2.85	18.78 ± 0.66	29.0 ± 6.51	13.25 ± 0.20	MS, *LRI*[a,b,c], STD
41	2,5-dimethylpyrazine [I]	1324	1324	11.39 ± 0.87	7.27 ± 0.40	11.73 ± 2.34	4.55 ± 0.13	MS, *LRI*[a,b,c], STD
42	2,6-dimethylpyrazine [I]	1329	1330	19.40 ± 2.64	16.12 ± 1.19	19.68 ± 4.56	11.94 ± 1.35	MS, *LRI*[a,b,c], STD
43	2-ethylpyrazine [I]	1333	1336	9.15 ± 0.58	6.07 ± 0.55	9.81 ± 0.54	5.53 ± 0.38	MS, *LRI*[a,b,c]
44	2,3-dimethylpyrazine	1354	1348	2.73 ± 0.67	3.36 ± 0.18	6.10 ± 0.76	2.58 ± 0.25	MS, *LRI*[a,b,c]
45	2-ethyl-6-methylpyrazine [I]	1405	1388	8.42 ± 0.42	6.92 ± 0.45	9.70 ± 1.40	5.51 ± 0.47	MS, *LRI*[a,b,c]
46	2-ethyl-5-methylpyrazine [I]	1414	1394	6.26 ± 0.39	4.38 ± 0.20	6.94 ± 0.87	3.22 ± 0.36	MS, *LRI*[a,b,c]
47	2,3,5-trimethylpyrazine [I, II, III, IV]	1428	1429	3.83 ± 1.08	-	6.29 ± 0.65	-	MS, *LRI*[b], STD
48	Ethenylpyrazine [I]	1453	1447	-	-	2.53 ± 0.07	-	MS, *LRI*[c]
49	2-ethyl-3,6-dimethylpyrazine [I, IV]	1478	-	5.18 ± 0.53	3.34 ± 0.53	5.98 ± 0.15	2.57 ± 0.78	MS
	Pyridines							
50	Pyridine [I]	1179	1182	36.86 ± 4.08	27.4 ± 1.56	65.52 ± 14.64	21.28 ± 0.95	MS, *LRI*[a,b,c], STD
	Pyrroles							
51	2-acetyl-1-methylpyrrole [I]	1671	1663	7.28 ± 0.36	6.90 ± 1.01	11.96 ± 1.01	5.72 ± 0.79	MS, *LRI*[a,b,c]
52	1-furfuryl pyrrole	1838	1839	11.20 ± 1.04	10.20 ± 0.64	16.92 ± 0.98	8.12± 1.38	MS, *LRI*[a,c]
53	2-acetylpyrrole [I]	1970	1983	23.22 ± 3.63	21.73 ± 0.94	34.48 ± 1.47	16.26 ± 1.48	MS, *LRI*[a,b]
54	1H-pyrrole-2-carboxyaldehyde [I]	2022	2038	16.52 ± 1.77	15.57 ± 0.77	16.87 ± 1.06	13.29 ± 1.45	MS, *LRI*[a,b]
55	1-methyl-1H-pyrrole-2-carboxyaldehyde [I]	2108	-	18.44 ± 8.31	13.42 ± 1.96	24.44 ± 6.81	11.64 ± 4.17	MS

Table 8.1. (Cont'd)

	Sulfur containing compounds							
56	2-Furfurylthiol [I, II, III, IV]	1439	-	3.29 ± 0.82	3.06 ± 0.17	5.08 ± 0.24	2.80 ± 0.50	MS, STD
57	Furfuryl methyl sulfide [I, IV]	1503	1506	5.03 ± 0.26	4.44 ± 0.64	7.55 ± 0.73	4.19 ± 0.47	MS, *LRI*[a,c]
	Miscellaneous							
58	2,3-pentanedione [I, II, III, IV]	1070	1067	7.32±12.06	7.90 ± 1.28	3.54 ± 0.77	7.99 ± 0.23	MS, *LRI*[a]
59	Methyl levulinate [I]	1566	-	2.85 ± 0.23	3.59 ± 1.18	2.53 ± 0.68	1.71 ± 0.15	MS
60	Maltol [I, IV]	1967	2006	29.21 ± 2.51	30.04 ± 0.89	36.34 ± 2.49	20.25 ± 3.00	MS, *LRI*[b,c], STD
61	Acetanisole [I]	2028	-	9.07 ± 1.33	8.87 ± 0.86	12.18 ± 1.61	6.48 ± 1.60	MS, STD
62	3,4-dimethoxystyrene	2052	-	16.23 ± 2.29	19.54 ± 0.23	20.51 ± 3.71	16.50 ± 2.74	MS
	Total concentration			1084.18	1016.17	1239.04	845.53	

[I]Compounds reported in Ref. (*63*); [II] Compounds reported in Ref. (*205*); [III] Compounds reported in Ref. (*93*); [IV] Compounds reported in Ref. (*190*).
Identification method: MS= mass spectrum; STD = standards and LRI=Linear Retention Indices obtained from references or literature values (*LRI*[a] referred to the values in Ref. (*309*), *LRI*[b] referred to the values in Ref. (*44*) and *LRI*[c] referred to the values in Ref. (*51*); "-", not detected

Other major compounds like furans, pyridines and pyrroles are not considered as potent odorants owing to their high threshold values in air. As a group, furans impart burnt and caramel base notes while pyridines and pyrroles express smoky and burnt coffee aroma, respectively (*310*). The presence of carboxylic acids such as acetic, propanoic, butanoic and 3-methylbutanoic acids accounts for the sourness of coffee (*192*). In contrast, pyrazines, furanones and phenolic volatiles are often known for their strong association with coffee aroma.

Pyrazines are the second dominant volatiles found in the coffee extracts, which impart roasty and earthy aroma in roasted ground coffee (*192*). The most abundant pyrazine was 2-methylpyrazine characterized with a nutty odor, yet the most aromatic pyrazines were 2-ethylpyrazine, 2-ethyl-6-methylpyrazine and 2-ethyl-3,6-dimethylpyrazine, which were present in relatively smaller amounts.

Likewise, furanones also belong to an important group of volatiles in coffee aroma; of which, furaneol or 2,5-dimethyl-4-hydroxy-3(2H)-furanone is one of the key aroma compounds most frequently reported (*205*). The expression of this chemical was highest in the Sidikalang coffee extract, followed by Doi Chang, Yunnan and Sidikalang Kopi Luwak.

Another member of the potent odorant group comes from volatile phenolic derivatives; among them, guaiacol, 4-ethylguaiacol and *p*-vinylguaiacol were identified. The highest concentration of phenolics was detected in the Yunnan coffee extract with *p*-vinylguaiacol as the major compound. This suggests a larger amount of phenolic acids could be present in the green Yunnan coffee beans compared to other coffee types and they were

first degraded to phenol and later to guaiacol during roasting (*192*). In addition, γ-butyrolactone, having an oily note with fatty nuance, is known to be generated from a chlorogenic acid lactone (*49*) and an elevated amount of 6582 ppm was detected in the coffee extract from Yunnan. Strecker aldehyde, 2,3-pentadione is a key odorant responsible for the buttery aroma in coffee (*310*) whereas ketones are characterized as less sharp compared to aldehydes (*311*) such as maple lactone which is known to possess a sweet caramellic-spicy scent.

The volatile compositions were segregated by principal component analysis (PCA), which would further advance the knowledge of the coffee extracts from different geographic origins. The relationships between the amounts of volatile chemicals and their aroma profiles were also explored with sensory analysis subsequently.

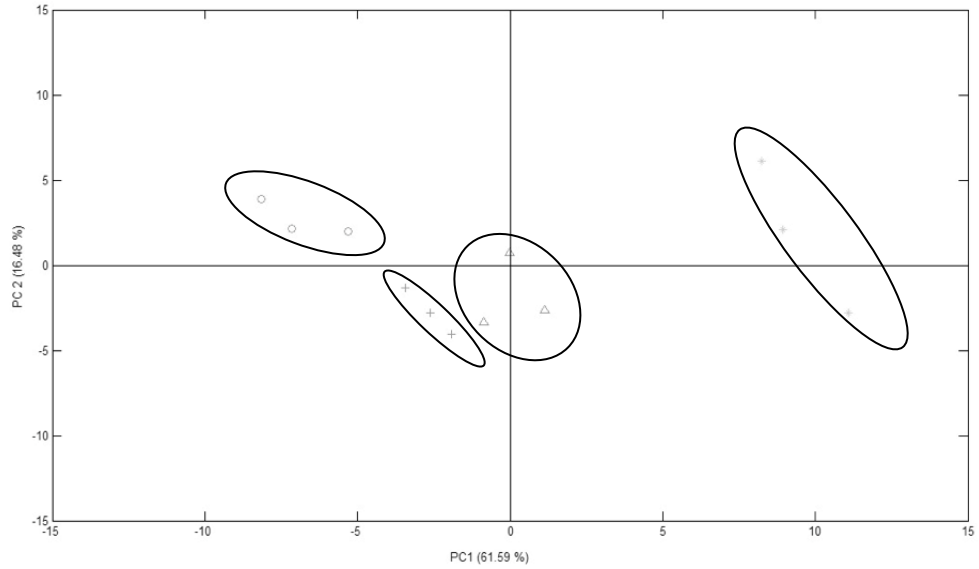
8.3.2. Principal component analysis (PCA)

The application of PCA plot can ease the interpretation of data via visualization. The first two principal components (PC) accounted for 78.08% of the cumulative percentage of total variation and represented the dominant information underlying the volatile profiles. The segregation of coffee extracts at each observation (Figure 8.1) was clearly distinguished by plotting PC 2 against PC 1. A distinct categorisation according to the total concentration of volatiles expressed in the extracts was observed along PC 1. The distance of clusters between each other corresponds to the proximity in the expression of volatile constituents.

As seen from Figure 8.1(a), the close proximity and distribution trend between Sidikalang coffees suggests a certain degree of similarity in aroma profiles, given that the coffees were of the same species and origin. Nevertheless, both were further discriminated by PC 2, suggesting a significant difference in the aroma profiles between each other. This is probably due to the fact that the proteolytic reactions that had occurred in the digestive tract of civet cats altered the amino acid content of green coffee beans, resulting in the differences of Maillard reaction products.

To further understand the contribution of each volatile variable in distinguishing the overall chemical profile, a biplot is displayed in Figure 8.1(b) where the direction and length of each variable (vector) indicated the contribution. Regardless of their origins and types, the coffee extracts were generally characterized by sulfur-containing compounds and pyrazines, which expressed larger positive coefficients in PC 1, such as 2-furfurylthiol, furfuryl methyl sulfide and 2-ethyl-6-methylpyrazine. In this work, 2,3,5-trimethylpyrazine had the highest coefficient in PC 1, indicating its strong influence as a characteristic chemical in coffee aroma. This was in agreement with the finding that the aroma sensory data of coffee extracts from different geographical origins exhibited distinguishable levels of a nutty note. However, it was not possible to differentiate between the nutty note in non-treated and civet-treated Sidikalang coffee extracts due to the similar amount of pyrazines expressed. In addition, compounds 57-62 were furfuryl methyl sulfide, 2,3-pentanedione, methyl levulinate, maltol, acetanisole, 4-dimethoxystyrene, respectively.

(a)



(b)

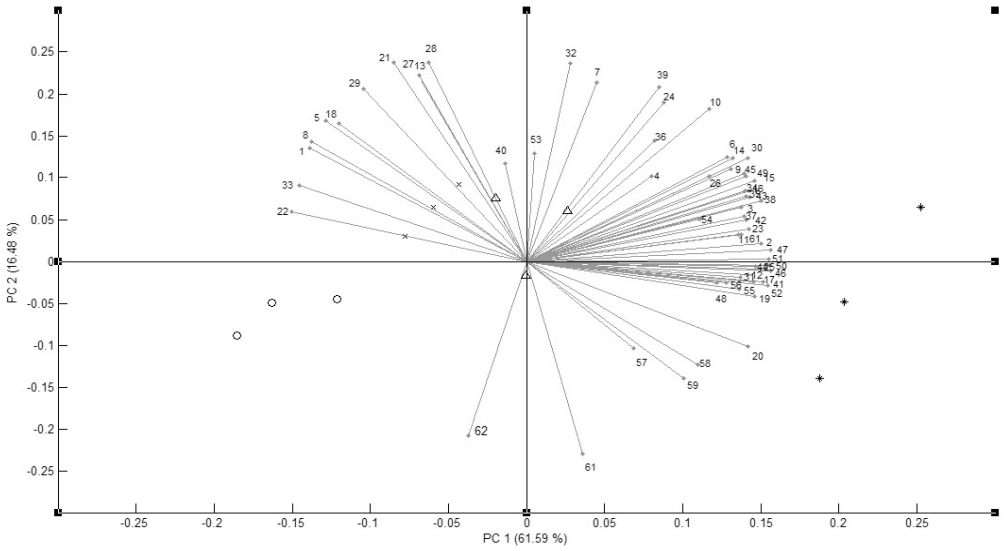


Figure 8.1. PCA score plot (PC 2 against PC 1) of coffee (*Coffea arabica*) extracts of dichloromethane (a); PCA biplot (PC 2 against PC 1) of coffee (*Coffea arabica*) extracts (b): (O) Sidikalang Kopi Luwak; (+) Sidikalang; (Δ) Doi Chang and (*) Yunnan

Volatile variables explained: Numbering identical to Table 8.1.

It is observed that 2,3-pentanedione, methyl levulinate and maltol with high coefficients in PC 1 show closer correlations towards the Yunnan origin. However, compounds such as acetanisole and 4-dimethoxystyrene with low coefficients in PC 1 were distant from all sources of origins. It is thus suggested that these two compounds were less significant in distinguishing among the origins.

8.3.3. Phenolic acid components

Phenolic compounds are responsible for the astringency, flavor and antioxidative activity of food (*312*). The predominant type of phenolic acids in plants is chlorogenic acid and it is of importance to study its content in coffee beans (*313*). A previous study (*200*) found that the chlorogenic acid content in defective green coffee beans is more than that present in graded coffee beans. This may affect the quality of the coffee beverage (*199*). As observed in Table 8.2, the phenolic acid content of both green and roasted coffee samples are statistically different except for the sinapic acid content in roasted coffee beans (*201, 314*). The major phenolic acid in all coffee samples was chlorogenic acid. However, while the quantity of this acid was similar for the green coffee samples, that the amount in roasted coffee samples was statistically different, with roasted Doi Chang and Kopi Luwak having the higher amounts of chlorogenic acid at 18.70 and 18.60 mg/g, respectively, while roasted Yunnan having the lowest content at 6.05 mg/g. Sinapic, caffeic and *p*-coumaric acids were present in small amounts in the roasted coffee samples.

Table 8.2. Phenolic acid components and their respective concentrations (mg/g dry wt.) of coffee beans from different geographic origins

Compounds	Green coffee beans			
	Kopi Luwak (Indonesia)	Sidikalang (Indonesia)	Doi Chang (Thailand)	Yunnan (China)
Chlorogenic acid	30.19 ± 0.36^{a}	30.76 ± 1.27^{ab}	29.32 ± 1.10^{a}	32.18 ± 0.97^{b}
p-Coumaric acid	-	-	-	-
Ferulic acid	0.09 ± 0.00^{a}	0.08 ± 0.01^{b}	0.05 ± 0.00^{c}	0.05 ± 0.00^{c}
Caffeic acid	0.57 ± 0.01^{a}	0.38 ± 0.03^{b}	0.18 ± 0.00^{c}	0.17 ± 0.00^{c}
Sinapic acid	-	-	-	-
Total	30.87	31.22	29.55	32.40
	Roasted coffee beans			
Chlorogenic acid	18.60 ± 0.29^{a}	13.69 ± 1.33^{b}	18.70 ± 0.66^{a}	6.05 ± 0.51^{c}
p-Coumaric acid	2.16 ± 0.19^{a}	1.52 ± 0.17^{b}	2.05 ± 0.17^{a}	0.72 ± 0.08^{c}
Ferulic acid	0.03 ± 0.00^{a}	0.08 ± 0.01^{b}	0.03 ± 0.01^{a}	0.10 ± 0.01^{b}
Caffeic acid	0.04 ± 0.02^{a}	0.40 ± 0.03^{a}	0.37 ± 0.05^{a}	0.29 ± 0.02^{b}
Sinapic acid	0.07 ± 0.02^{a}	0.06 ± 0.01^{a}	0.08 ± 0.02^{a}	0.05 ± 0.01^{a}
Total	20.90	15.75	21.23	7.21

Mean values with different letters (a-c) in the same row within the green or roasted coffee bean samples indicate statistical differences at the 0.05 ($p < 0.05$)

8.3.4. Antioxidant activity

8.3.4.1 Determination of total polyphenol content

As the measurement of the polyphenol content is based on a redox reaction, the Folin-Ciocalteu method can be considered as a general method of antioxidant activity determination (*300*). The total polyphenol content of the coffee beans from each origin is tabulated in Table 8.3. The lower polyphenol content found in roasted coffee beans (33.67 - 43.13 mg GA per g coffee) relative to green coffee beans (43.07 - 53.76 mg GA per g coffee) was observed. This can be attributed to their polymerization, auto-oxidation or degradation during roasting, leading to the decreased polyphenol level present (*203*). This corresponds to the higher amounts of chlorogenic acid in green coffee beans (Table 8.2). A statistical analysis of variance found that the polyphenol content in the samples from each country was statistically different for both green and roasted coffee beans. Among the green coffee beans,

Yunnan had the highest amount of polyphenols; while Sidikalang had the highest amount among the roasted coffee beans.

8.3.4.2 Radical scavenging activity by DPPH assay

The DPPH radical scavenging activity of different concentrations of the phenolic extract of each coffee sample was measured and the EC_{50} of each coffee sample was determined (Table 8.3). EC_{50} is known as the half maximal effective concentration and in this case, it is defined as the concentration of the coffee sample that causes a decrease in 50% of the initial DPPH concentration (*303, 304*). A lower EC_{50} value indicates a better antioxidant activity. The EC_{50} values obtained for roasted and green coffee beans, in general, had a range of 8.23 – 9.96 mg GA g^{-1} and 9.53 – 11.17 mg GA g^{-1} respectively. This can be attributed to the higher total polyphenolic content of green coffee beans. A good correlation between the total polyphenol content and DPPH scavenging radical activity was expected (*299*). However, a low R^2 value of 0.33 was calculated (Figure 8.2) as the EC_{50} values for the samples were determined to be similar. This discrepancy could be due to a larger natural variation in the polyphenolic content such as melanoidins with apolar properties that were less soluble and extracted by the acetone-methanol-water mixture used in the current study. Furthermore, DPPH radical scavenging activity depends on the hydrogen donating ability of the extracted antioxidants (*303*).

Table 8.3. Antioxidant activity of coffee beans from different geographic origins

	Kopi Luwak (Indonesia)	Sidikalang (Indonesia)	Doi Chang (Thailand)	Yunnan (China)
Green coffee beans				
Folin-Ciocalteu (mg GA/g)	49.88 ± 3.35^{a}	48.51 ± 0.82^{a}	43.07 ± 1.37^{b}	53.76 ± 0.71^{c}
FRAP (mg Trolox/g)	123.40 ± 7.85^{a}	147.46 ± 5.20^{c}	128.29 ± 5.68^{ab}	142.98 ± 9.88^{bc}
DPPH, EC_{50} (mg GA/g)	11.08 ± 1.57^{a}	11.17 ± 1.03^{a}	9.53 ± 0.74^{a}	10.21 ± 0.42^{a}
Roasted coffee beans				
Folin-Ciocalteu (mg GA/g)	40.42 ± 1.24^{a}	43.13 ± 2.99^{a}	33.67 ± 0.69^{b}	36.17 ± 1.60^{b}
FRAP (mg Trolox/g)	94.44 ± 2.67^{a}	109.02 ± 5.20^{b}	78.92 ± 3.75^{c}	81.42 ± 5.47^{c}
DPPH, EC_{50} (mg GA/g)	8.23 ± 0.81^{a}	9.96 ± 2.00^{a}	9.19 ± 0.53^{a}	8.80 ± 0.50^{a}

Mean values with different letters (a-c) in the same row within the green or roasted coffee bean samples indicate statistical differences at the 0.05 ($p < 0.05$)
GA: gallic acid; DPPH: diphenyl-1-picrylhydrazyl assay; FRAP: ferric reducing antioxidant power assay

8.3.4.3 Ferric reducing antioxidant power by FRAP assay

The ferric reducing capacity of each coffee sample was expressed in terms of Trolox equivalents and is tabulated in Table 8.3. A range of 123.40 – 147.46 mg Trolox/g and 78.92 – 109.02 mg Trolox/g for green and roasted coffee samples were obtained, respectively, where Sidikalang showed the highest ferric reducing capacity among both the green and roasted coffee samples. A positive correlation was observed between the total polyphenol content and ferric reducing capacity of the samples (Figure 8.2) as expected. Even though both the FRAP and DPPH assays were used to determine the antioxidant activity of the coffee samples, differing results were obtained due to the nature of each antioxidant assay as each assay is based on a different reaction mechanism. The FRAP assay measures only antioxidants that transfers a single electron while DPPH measures antioxidants that transfers both a single electron and a hydrogen atom. Hence, it is difficult to obtain

good agreement between the results obtained from both assays (*303*). However, no one standardized test can be used to determine the antioxidant capabilities of a substance and more than one test should be carried out so as to take into account the different modes of actions of the antioxidants present in a food matrix (*299, 304, 315*).

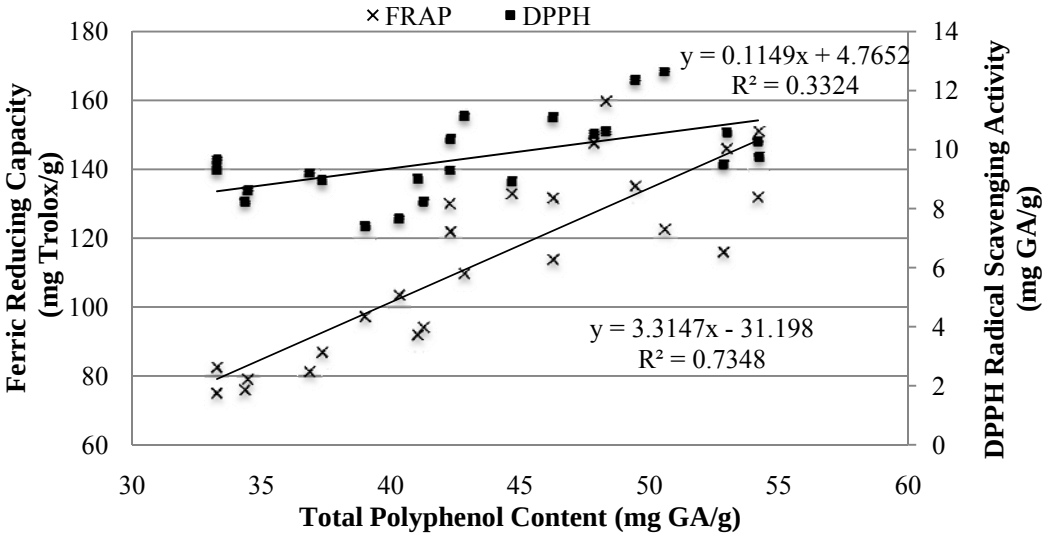


Figure 8.2. Correlation between FRAP and DPPH assays with the total polyphenol content of coffee

8.3.5. Sensory evaluation

The aromatic profiles (Figure 8.3) of the dichloromethane extracts of coffee from different origins (i.e. Doi Chang, Sidikalang, Yunnan and Sidikalang Kopi Luwak) revealed distinct sensory profiles, with descriptions such as roasted (3.5), caramelic (3.0), smoky (3.0) and sulfury (3.0) being highly ranked. The Sidikalang Kopi Luwak profile with a less smoky and well–balanced aromatic profile was more favourable relative to the untreated Sidikalang though the variations on the ratings of sensory attributes such as

nutty, spicy, sulfury and sweet notes were small. The aroma profiles of Sidikalang and Sidikalang Kopi Luwak were similar, which may be due to their close expression of volatile chemicals. Doi Chang and Yunnan coffee extracts exhibited the highest score for sulfury and nutty notes, respectively. The highly rated sulfury note could be mainly ascribed to the potent sulfur-containing compounds such as 2-furfurylthiol.

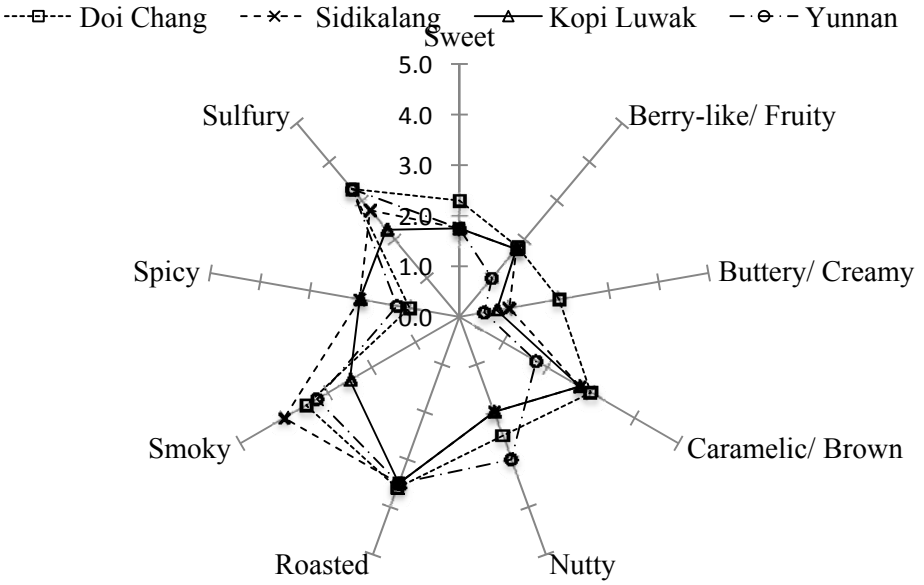


Figure 8.3. Aroma sensory profile of coffee (*Coffea arabica*) extracts using dichloromethane

A distinctive roasted note may be mainly contributed by the high concentrations of pyrazines, however, no distinctive difference was perceived among the extracts, though Kopi Luwak contained only half of the amount relative to that in Yunnan. The buttery note in the Yunnan coffee extract was perceived as the lowest (0.5) among all. This may be due to its lowest concentration of 2,3-pentanedione in the Yunnan coffee extract. Similarly, the

Yunnan coffee extract exhibited the strongest nutty note, corresponding to its highest level of 2–methylpyrazine and 2-acetylpyrrole. The relationship between the volatile components and sensory evaluation facilitates understanding the aromatic profile of Arabica coffees from different geographical origins in Asia.

8.4. Conclusion

Based on the total volatile compounds identified, Yunnan coffee had most of its volatile chemicals abundantly expressed, given its largest total peak area. Sensory evaluation revealed a stronger sulfury note in the Doi Chang and Yunnan coffee extracts but a lower buttery note in Yunnan coffee. Furthermore, successful characterisation of coffee extracts was represented by the distinct segregation of volatile clusters in PCA plots. Chlorogenic acid as the major phenolic acid component was determined significantly different, indicating a difference for coffee beans from different geographical locations. The total polyphenol contents of the green coffee beans were similar while those of the roasted coffee samples were marginally different. The DPPH radical scavenging activity was insignificantly different among the green and roasted coffee samples. However, the ferric reducing capacity of the samples was statistically different and both green and roasted Sidikalang beans gave the best antioxidant activity.

CHAPTER 9 PRESSURIZED LIQUID EXTRACTION ON COFFEE BEAN

9.1. Introduction

Recent advancement in developing rapid and sensitive extraction techniques has become increasingly important (*65, 76, 78-80, 316*). PLE could be significantly increased the extraction yield with the decrease of extraction time. Extraction procedures become versatile by tuning different operating parameters (e.g. temperature, pressure, time, extraction cycles and solvent) (*317*). Nevertheless, even minor adjustments of PLE parameters could affect the composition of extracted compounds. Thus, a detailed optimization demonstrated the posibility of manipulating the composition of the extract by adjusting the extraction parameters (*318*). In contrast to other common PLE applications in environmental aspects (*75, 79, 319-324*), PLE has been found very limited reports in flavor isolation (*317, 318*). For example, PLE was applied to studying volatile compounds in tumeric leaves, where simultaneous optimization of several response variables was carried out based on the desirability function and evaluated the flavor intensity of tumeric leave extracts (*318*). To the best of my knowledge, there is only one study reported

on the PLE extraction of polycyclic aromatic hydrocarbon from coffee (*75*), and has yet to be applied on any other coffee volatiles.

In this work, the objective was to extract volatiles in the coffee bean using PLE. Initially, the feasibility of PLE on extracting coffee volatiles was evaluated through comparison to solvent extraction. Furthermore, the factors affecting PLE extraction of volatiles in coffee beans were systematically optmized through response surface methodology (RSM). With the linear or square polynomial functions obtained from RSM, the significant effects of main factors as well as their interactive effects could be identified and predicted. Multiple responses of targeted compounds were transformed with different scalings into a desirability function in order to comphrehend the interactions between PLE parameters (*153, 288*). Under elevated temperature and pressure during PLE process, the composition of delicate aroma can be easily distorted majorly due to thermal degradation of labile compounds and some side-reactions. Hence, besides the optimization of operating parameters, thorough sniffing is required to identify and monitor the desirable flavor profiles of different extracted products that are concomitant to the analytical work (*190, 325*).

9.2. Experimental procedures

9.2.1. Coffee beans and chemicals

In this study, Boncafé International Pte. Ltd., Singapore provided the roasted Sumatra Mandheling coffee (*Coffea arabica* L. cv. Catimor), which underwent a roasting process for 14 min with an initial temperature of 160 °C and discharged at 223 °C. Coffee beans were grounded and sieved into the

size range of 1.77–2.36 mm. The ground coffee was sealed in the aluminium pouch and stored at -20 °C until use.

Acetone, dichloromethane, hexane and methanol from Tedia were of analytical grade. Anhydrous Na_2SO_4 purchased from Merck was used as a drying agent while hydro super gel diatomaceous earth (hydromatrix) obtained from Sigma-Aldrich was applied as drying agent and dispersing agent during extraction.

All standard compounds used in the identification of the volatiles were obtained from the Firmenich Asia Pte. Ltd., Singapore.

9.2.2. PLE procedure

Ten grams of ground coffee were evenly mixed with 5 g of diatomaceous earth, and then packed into a 40-mL stainless steel cell secured with a neoprene filtration end cap. Extraction was performed with a Power-Prep PLE (Fluid Management Systems, Watertown, MA, USA). The automated extraction cycle was operated using DMS6000 software as follows: the cell containing ground coffee was prefilled with extraction solvent (i.e. methanol, hexane, and dichloromethane), pressurized and heated for a static period (see Table 9.1).

Table 9.1. Face-centered central composite design (CCD)

Factor	Low (-)	High (+)	Centre
Temperature, x_1	50	100	75
Pressure, x_2	1000	2000	1500
Static extraction time, x_3	5	15	10

The design was a two-level full factorial design with 8 cube points, 6 centre points in cube, 6 axial points and alpha value 1.

The cell was then flushed with fresh extraction solvent and purged with a flow of nitrogen gas and the extracts were eluted out of the extraction cell into the collection bottle placed in an ice bath. The extract was cooled for 30 min and dried by 10 g of anhydrous Na_2SO_4 before being concentrated to 1.0 ml using TurboVap II. Finally, the extract was transferred to a 2-ml vial and stored at -30 °C until further analysis by GC-MS/FID and sensory evaluation.

9.2.3. Solvent extraction

Coffee extract was prepared from 10 g ground coffee with a volume of 40 mL dichloromethane. The suspension was stirred by vortex shaker at 200 rpm for 1 h. The extract was then filtered from the ground coffee beans and dried over anhydrous Na_2SO_4. The solvent was removed under purified nitrogen stream using TurboVap II until the volume of sample was reduced to 1 mL. The experiment was performed in triplicate and stored at -30 °C until used for analysis.

9.2.4. GC-MS/FID analysis

GC-MS/FID analysis was carried out using Agilent 6890N GC coupled with FID and a 5975 inert MS. Identification and quantification of the eluted compounds were as described in previous chapter 3 (Section 3.2.2).

9.2.5. RSM and statistical analysis

A face-centered central composite design (CCD) was constructed and analyzed using the Design Expert Version 6.0.10 software (Stat-Ease,

Minneapolis, MN, USA), where the effects of three independent variables on the selected key odorants (*62, 63, 93, 190, 205*) were taken as the responses. Three main factors were selected as reported in the literature (*321*), i.e. temperature (x_1, 50 – 100 °C), pressure (x_2, 1000 – 2000 psi) and static extraction time (x_3, 5- 15 min) (Table 9.1). The 20 runs were in triplicate in order to calculate the averages and standard deviations. Detailed responsed surface model was described in Section 7.2.4 of Chapter 7.

9.2.6. Optimization and validation procedures

Selective optimization based on individual desirability to response variables was obtained through an objective function. With the objective function, individual desirability of each response variable was combined (*288*), as follow:

$$d_i = \begin{cases} 0 & y_i \le y_{i\,min} \\ \left[\dfrac{y_i - y_{i\,\min}}{y_{i\,\max} - y_{i\,\min}}\right] & y_{i\,min} < y_i < y_{i\,max}, \text{ for } i = 1, 2, \ldots, 14 \\ 1 & y_i \ge y_i \end{cases}$$

$$D = \left(\prod_{i=1}^{n} d_i^{w_i}\right)^{\frac{1}{\sum w_i}} = \left(\prod_{i=1}^{n} d_i\right)^{\frac{1}{n}}$$

where d_i is the individual desirability value of i^{th} response, the value of $y_{i\,min}$ and $y_{i\,max}$ are the minimum and maximum acceptable value of y_i, overall desirability (D) with n is the total number of responses and w_i, is the individual response importance, in this case w_i=1 as desirability function was set as linear.

9.2.7. Sensory evaluation

Sensory profiles of the coffee extracts were evaluated as described in Section 8.2.7 of Chapter 8. The corresponding descriptive attributes were ashy, beany, berry-like, burnt, caramellic, earthy, nutty, roasted, smoky/phenolic and sulfury. A 6-point scale was used with '0' indicating the uncharacterized attribute intensity and '5' indicating the strongest attribute intensity.

9.3. Results and discussion

PLE is conducted under elevated temperature and pressure within short time period (*78*). Several factors, which may be interrelated, significantly influence PLE, mainly extractant, temperature, pressure and static extraction time (*80, 321, 326*). Generally, pressure helps to force liquid into the pores and maintain the solvent in liquid or condensed state at operating temperatures. Temperature can enhance the solvent wetting of the sample, permit the analytes to dissolve faster into the solvent, and then achieve an improved extraction process (*80*). With an improved diffusion rate and mass transfer process, it allows shorter extraction time and reduces the risk of degradation in the process of PLE. On the other hand, the selectivity of extraction could decrease at higher temperature due to the co-extraction of interfering matrix components such as fatty acids (*327*). In addition, degradation or evaporation of volatile components might occur at elevated temperatures. Therefore, a systematic assessment of the interrelated factors mentioned above is crucial.

9.3.1. Selection of extraction solvent, ratio of hydromatrix to sample and extraction cycle

Optimization of an extraction process commonly begins with an appropriate choice of the extraction solvent, which is able to solubilize the analytes of interest and minimize the co-extraction of other matrix components (*321*). The main classes of volatile compounds identified in the coffee bean were acids, furans, phenols, pyrazines, pyridines and sulfur-containing compounds. Among three selected solvents (i.e. hexane, dichloromethane and methanol), it is observed that methanol was more effective to extract polar compounds such as pyridine, acetic acid (Table 9.2).

Regarding to the total extracted amount, dichloromethane provided the highest. In addition, some key volatile compounds (i.e. furfury mercaptan and furaneol) were only detected in dichloromethane extract. Therefore, for better extraction, dichloromethane is the preferable among three solvents. Subsequently, PLE extraction was compared with conventional solvent extraction, of which both extracts shared similar volatile profiles but PLE extracts consisted of nearly 3 times higher of total extracted amount.

The suitability of PLE technique for the volatile analysis in ground coffee was also justified with the efficiency in the extraction time. Other experimental parameters were further optimized. Hydromatrix, serving not only as a dispersing agent but also as a dehydrating agent might alter the extraction efficiency (*326*).

Table 9.2. Identification of volatiles and their concentrations (ppm) in coffee beans extracted using hexane, dichloromethane and methanol

Compounds	*LRI*		Solvent extraction	PLE			Identification
	FFAP	Ref	Dichloromethane	Hexane	Dichloromethane	Methanol	
Acids							
Acetic acid [I]	1444	1468	22.73 ± 2.51	10.20 ± 5.05	34.17 ± 7.81	152.93 ± 47.82	MS, *LRI*[b], STD
3-methylbutanoic acid [I]	1657	1687	13.18 ± 0.46	20.81 ± 8.21	41.31 ± 13.22	-	MS, *LRI*[b], STD
trans-2-butenoic acid	1764	-	-	-	4.17 ± 1.64	-	MS, STD
Furans							
Furfural [I]	1473	1473	4.99 ± 0.48	5.00 ± 2.34	11.19 ± 3.74	5.93 ± 1.96	MS, *LRI*[a], STD
Furfuryl acetate [I]	1538	1507	27.50 ± 3.82	40.35 ± 17.10	62.97 ± 18.42	14.01 ± 9.29	MS, *LRI*[a], STD
Furfuryl alcohol	1653	1671	141.47 ± 20.05	106.26 ± 14.36	415.80 ± 99.83	244.75 ± 119.30	MS, *LRI*[a], STD
2-acetyl-5-methylfuran [I]	1623	1653	6.33 ± 3.77	4.16 ± 2.41	3.52 ± 1.42	0.78 ± 0.24	MS, *LRI*[c]
Furfuryl ether	1987	1996	9.12 ± 1.03	10.91 ± 5.95	19.18 ± 7.68	2.40 ± 1.03	MS, *LRI*[a]
Phenols							
Guaiacol I, II, III, IV	1865	1886	9.28 ± 2.04	13.12 ± 6.19	25.49 ± 12.50	6.14 ± 4.67	MS, LRIb, STD
Phenol I	2004	2030	12.33 ± 1.08	9.53 ± 5.05	26.98 ± 12.67	9.66 ± 6.37	MS. LRIb, STD
4-ethylguaiacol [I, II, III, IV]	2035	2065	17.00 ± 3.85	14.36 ± 7.43	28.23 ± 12.55	2.79 ± 1.44	MS. *LRI*[c], STD
p-cresol I	2090	-	-	2.03 ± 1.45	8.80 ± 7.93	0.57 ± 0.21	MS, STD
p-vinylguaiacol I, III, VI	2206	2225	34.02 ± 5.20	46.03 ± 27.72	86.73 ± 29.30	12.02 ± 7.33	MS, LRIb, STD
Pyrazines							
2-methylpyrazine [I]	1256	1267	21.35 ± 2.34	9.72 ± 2.54	32.24 ± 8.55	14.03 ± 5.74	MS, *LRI*[a]. STD
2,5-dimethylpyrazine [I]	1308	1324	7.55 ± 1.23	12.09 ± 4.52	21.76 ± 8.51	5.07 ± 1.08	MS. *LRI*[a], STD
2,6-dimethylpyrazine [I]	1314	1330	9.60 ± 1.51	-	18.46 ± 5.23	5.99 ± 2.85	MS. *LRI*[a], STD
2,3-dimethylpyrazine	1339	1348	2.45 ± 0.42	5.00 ± 2.96	4.60 ± 1.63	1.46 ± 0.96	MS, *LRI*[a]
2-ethyl-6-methylpyrazine [I]	1383	1388	5.91 ± 0.99	10.87 ± 5.27	10.03 ± 5.57	-	MS, *LRI*[a]
2-ethyl-5-methylpyrazine [I]	1392	1394	2.81 ± 0.49	5.55 ± 2.82	4.01 ± 3.70	2.10 ± 2.85	MS, *LRI*[a]
2,3,5-trimethylpyrazine [I, II, III, IV]	1407	1429	5.41 ± 0.41	9.27 ± 4.83	9.27 ± 3.11	2.22 ± 1.43	MS. *LRI*[b], STD

Table 9.2. (Cont'd)

Pyridines							
Pyridine [I]	1176	1182	34.75 ± 3.90	1.66 ± 0.38	63.42 ± 13.59	63.50 ± 15.76	MS, *LRI*[a], STD
2-acetylpyridine [I]	1613	-	-	-	-	0.28 ± 0.49	MS, STD
Sulphur-containing compounds							
Furfuryl mercaptan [I, II, III, IV]	1438	-	0.58 ± 0.35	-	2.31 ± 2.43	-	MS, STD
Furfuryl methyl sulfide [V]	1496	1506	3.05 ± 0.60	2.90 ± 1.42	2.55 ± 2.01	-	MS, *LRI*[b], STD
Miscellaneous							
2,3-pentanedione [I, II, III, IV]	1070	1067	2.6 ± 0.56	-	3.14 ± 0.52	-	MS, *LRI*[a]
Acetoin [V]	1270	1291	4.74 ± 0.44	-	14.64 ± 5.89	6.73 ± 2.42	MS, *LRI*[a], STD
γ-butyrolactone [V]	1645	1637	43.45 ± 7.95	19.11 ± 7.04	115.65 ± 32.42	59.58 ± 28.73	MS, *LRI*[a], STD
Maple lactone [I]	1830	1857	14.61 ± 3.32	-	25.37 ± 5.89	7.74 ± 3.03	MS, *LRI*[b]
Maltol [I,IV]	1975	2004	16.52 ± 4.45	12.50 ± 3.50	37.57 ± 23.45	13.36 ± 10.89	MS, *LRI*[b], STD
2-acetylpyrrole [I]	1977	1983	21.70 ± 3.14	24.16 ± 9.57	27.66 ± 15.91	15.15 ± 8.48	MS, *LRI*[a], STD
Furaneol [I, II, III, IV]	2028	2062	4.94 ± 1.46	-	9.59 ± 2.50	-	MS, *LRI*[b], STD
2-pyrrolidinone	2055	-	9.59 ± 1.20	5.05 ± 3.28	45.19 ± 35.84	8.61 ± 6.91	MS
Methyl palmitate [I]	2228	-	31.65 ± 1.95	39.52 ± 23.66	77.58 ± 33.76	74.16 ± 44.90	MS
3-pyridinol [I]	2426	-	33.96 ± 1.77	10.74 ± 7.87	161.31 ± 66.88	72.28 ± 25.43	MS
Total concentration			571.42	465.81	1473.80	806.93	

[I] Compounds reported in (*63*); [II] Compounds reported in Ref. (*205*); [III] Compounds reported in Ref. (*93*); [IV] Compounds reported in Ref. (*190*).
Identification method: MS= mass spectrum; LRI=Linear Retention Indices obtained from references or literature values and STD = standards. *LRI*[a] refers to the values in Ref. (*309*), *LRI*[b] refers to the values in Ref. (*44*) and *LRI*[c] refers to the values in Ref. (*51*); "-", not detected

Different ratios of sample to hydromatrix (i.e. 1:2, 2:1 and 1:1) were compared in order to understand its effect on the extraction efficiency. There was a slightly higher yield of volatiles extracted at a sample to hydromatrix ratio of 2:1 compared to the ratio of 1:2 and 1:1. It implied that a good approximation of phase ratio was required in order to aid the extraction process (data not shown here).

All the above discussion was based on one extraction cycle. For comparison, the number of extraction cycle was also studied as an affecting factor. In general, an increment in the number of extraction cycle allows the exposure of the matrix to fresh solvents and favours the solvent-to-sample equilibrium, thereby improving compound partition into the solvent phase (*320, 328*). The percentage yield was obtained by dividing the concentration obtained in a particular cycle by the total concentration obtained in all successive cycles.

Under the present condition, the first extraction cycle was able to extract more than 60% of the compounds, and the percentage yield of the compounds decreased as the number of extraction cycle increased. It was also noted that the aromatic profiles of extracts in subsequent cycles significantly changed and became undesirable (data not shown here). In the following experiments, the number of extraction cycle was set as one.

9.3.2. Face-centered central composite design

Considerations of the interrelated factors are crucial to the efficiency of PLE on volatile extraction. Fourteen compounds, reported as key volatiles in coffee bean (*62, 63, 93, 190, 205*), were selected for further optimization

(Table 9.3). These response variables were assessed as a function of main, quadratic and interactive effects of temperature (x_1), pressure (x_2) and static extraction time (x_3). For regression coefficients, positive values indicate that the yield of the compounds is favored toward the increasing values of the respective variables within the range studied, while negative coefficients indicate the decrease of yield toward the increasing value of studied range (*153*).

9.3.2.1 Effect of PLE operating parameters

Table 9.3 summarizes the predicted functions, coefficient of determination (R^2), along with the corresponding lack-of-fit test (*F*-and *p*-values) and individual probability of the independent variables in the final reduced models. The results suggested that the final reduced models were significantly ($p<0.05$) fitted for 14 response variables studied with relatively high R^2, ranging from 0.646 to 0.929. Moreover, extraction temperature was the most critical factor on PLE where most of the compounds were significantly affected ($p<0.05$). This further emphasized the volatile nature of these compounds as a slight change in extraction temperature affected their amount, which was especially significant ($p<0.0001$) for furfuryl mercaptan, furfural, furfuryl alcohol and maltol. Detailing the coefficient of the linear temperature factor (x_1) with a negative sign indicated that the amount of furfuryl mercaptan and furaneol decreased as extraction temperature increased. This implied that temperature must be restricted for thermally labile volatile components.

Table 9.3. Odour description, polynomial equation, R^2, probability values, lack-of-fit and significance probability of regression coefficients in the final reduced models

Compound	Odor description	Polynomial equation	R^2	Regression	Lack-of-Fit		Factors	
				p-value	*F*-value	*p*-value		*p* value
Furfural	Bread-like, caramellic, sweet	$Y= -22.38+0.52x_1 +0.25x_3$	0.871	0.0021	1.80	0.2677	x_1 x_3	**< 0.0001** **0.0046**
Furfuryl alcohol	Caramellic, fruity, sweet	$Y= 1077.22+20.37x_1+28.02x_3$	0.898	0.0007	2.21	0.2026	x_1 x_3	**< 0.0001** **0.0022**
Guaicol	Phenolic, spicy, vamilla	$Y= -16.53+0.31x_1$	0.646	0.1433	5.52	0.0421	x_1	**0.0117**
Phenol	Phenolic, plastic, rubber	$Y= -14.08+0.088x_1-0.26x_3-+0.02x_{13}$	0.830	0.0071	4.50	0.0623	x_1 x_3 x_{13}	**0.0003** **0.0091** **0.0406**
2-methylpyrazine	Cocoa, nutty, roasted	$Y= -72.29+1.56x_1+1.28x_3$	0.784	0.0203	1.21	0.4184	x_1 x_3	**0.0014** **0.0192**
2,5-dimethylpyrazine	Cocoa, roasted nut-like	$Y= -17.15+0.49x_1+0.50x_3$	0.674	0.1060	1.73	0.2802	x_1 x_3	**0.0260** **0.0469**
2,6-dimethylpyrazine	Cocoa, nutty, roasted	$Y= -22.32+0.70x_1+0.42x_3$	0.709	0.0690	1.06	0.4741	x_1 x_3	**0.0148** **0.0385**
Pyridine	Amine-like, fishy	$Y= -175.49+3.47x_1+5.90x_3$	0.863	0.0027	1.42	0.3540	x_1 x_3	**0.0001** **0.0037**

Table 9.3. (Cont'd)

Furfuryl mercaptan	Coffee-like, roasted, sulfury	$Y= 0.79-0.02x_1 +0.0006x_2-0.04x_3+0.0002x_1^2-0.000004x_{12}-0.00001x_{23}$	0.863	<0.0001	-	-	x_1 x_2 x_3 x_1^2 x_{12} x_{23}	**<0.0001** **0.0230** **0.0346** **0.0041** **0.0134** **0.0314**
Acetoin	Caramellic, toasted grain-like	$Y= -19.68+0.33x_1+0.006x_2+1.07x_3-0.0008x_{23}$	0.794	0.0167	4.38	0.0654	x_1 x_2 x_3 x_{23}	**0.0002** 0.3223 0.3452 **0.0472**
Maltol	Caramellic, fruity, sweet	$Y= -60.61+0.61x_1+0.01x_3+0.06x_{13}$	0.929	0.0001	5.81	0.0381	x_1 x_3 x_{13}	**< 0.0001** **0.0006** **0.0175**
2-acetylpyrrole	Musty, sweet, walnut-like	$Y= -17.18+0.53x_1+0.20x_3$	0.783	0.0204	2.92	0.1325	x_1 x_3	**0.0007** **0.0303**
Maple lactone	Fruity, maple, sweet caramel	$Y= -38.37+0.96x_1-1.18x_3$	0.840	0.0054	3.44	0.1005	x_1 x_3	**0.0002** **0.0070**
Furaneol	Caramellic, fruity, strawberry	$Y= 19.51-0.25x_1 -0.26x_3+0.02x_{13}$	0.789	0.0184	4.31	0.0675	x_1 x_3 x_{13}	**0.0006** 0.0796 **0.0277**

x_1, x_2 and x_3: the main effects of temperature, pressure and static extraction time, respectively. x_1^2, x_2^2, x_3^2: the quadratic effects of temperature, pressure and static extraction time, respectively. x_{12}: the interaction effect of temperature × pressure, x_{13}: the interaction effect of temperature × static extraction time, x_{23}: the interaction effect of pressure × static extraction time. Model terms with statistical significance (p<0.05) are shown in bold.

The application of high pressure in PLE allows the use of extraction temperature above the boiling point of the solvent while maintaining the solvent in its liquid state. High pressure could also improve the solvent accessibility to the analytes that are bound within the matrix pores (*79*). However, single-factor pressure term was insignificant for most compounds except for furfuryl mercaptan (p>0.05). Under the present condition, pressure seemed to play a minimum role, as long as the solvent was maintained in a condensed phase. This result was in good agreement with previous work on essential oil extracted from herbal plants where the effect of pressure on the amount of most substances was almost negligible (*329, 330*).

In PLE, static extraction time refers to the duration of the heat and maintenance of pressure step in the extraction cycle. It determines the time for the solute to equilibrate and partition between sample matrix and extraction solvent. Table 9.3 shows that the effect of static extraction time was significant for some compounds such as pyridine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine and furfuryl alcohol ($p<0.05$). Thus, static extraction time to a certain extent affected the extraction of target compounds, but was less pronounced than temperature.

9.3.2.2 Interaction between PLE operating variables

The interaction of independent variables is especially important to promote PLE extraction. For instance, temperature helps to enhance solvent penetration inside sample matrix, pressure can improve the solvent accessibility to the analytes, and static extraction time facilitates equilibration between solvent and matrix. As shown in Table 9.3, most of the significant

interactions existed between temperature and static extraction time. Figure 9.1 depicts the response surface plots of interaction effects between factors on the variation of selected responses (amounts extracted). For example, the amount of maltol was found to be a quadratic function of the temperature and time where the amount tended to increase with increasing temperature and time. These results could be associated with the increased ability of the solvent to solubilise maltol in the coffee bean matrix and the reduction of the viscosity of the extractant which allowed more effective penetration into the matrix at higher temperatures and prolonged heating. In contrast, furfuryl mercaptan was negatively correlated with the increase of temperature and time. By referring to the model terms, the amount of furfuryl mercaptan was significantly influenced by all three linear factors, quadratic effect of temperature and also interaction between *temperature* × *pressure* and *pressure* × *static extraction time*. In a particular study, it was reported that this compound was not stable as 40 day-storage at room temperature lowered its concentration by 81%, even though the coffee sample was vacuum-packed (*192*).

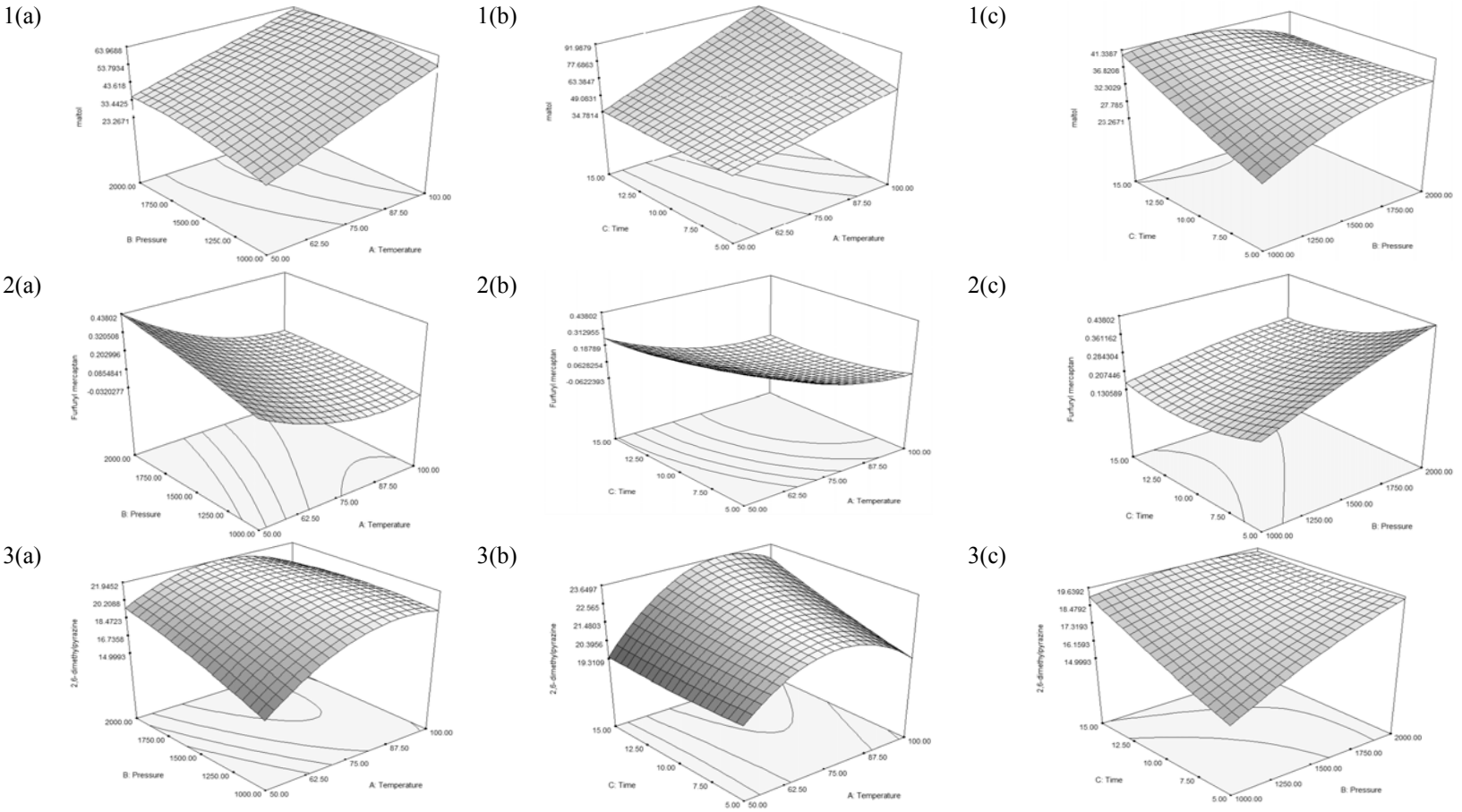


Figure 9.1. Response surface plots showing the effects of temperature, pressure and static extraction time of selected compounds: 1. maltol; 2. furfuryl mercaptan; 3. 2,6-dimethylpyrazine. (a) interaction between temperature and pressure; (b) interaction between temperature and time; (c) interaction between pressure and time

9.3.2.3 Optimization of PLE operating variables

Under elevated temperature and pressure, interfering substances may be extracted along with desired compounds during PLE extraction process. Moreover, it is worthy to note that no single experimental condition can be found under which the extraction of all volatile compounds is maximized due to the difference in physicochemical properties of the compounds (*288*). In order to assess the feasibility of PLE in flavor analysis, it was attempted to selectively maximise the target compounds while minimising the interferences, and thus several combinations were obtained through multi-response optimisation to manipulate the compositions of coffee extracts.

With the aim of maximising the total amount extracted, the optimal conditions obtained were 100 °C, 1000 psi and 15-min static extraction time with a desirability function of 0.731. In the second optimisation, the emphasis was placed on compounds that responded significantly in the response surface models. The results gave an optimal point of 75 °C, 1300 psi and 15-min static extraction time with a median desirability factor of 0.40. The optimal conditions and relatively low desirability were different from those obtained previously due to the differences in pre-selection of response goals.

Some thermal labile compounds (e.g. furfuryl mercaptan and furaneol) are key odorants of coffee aroma. The importance of their contribution strengthens the necessity to maximise their concentration. Thus, the third optimisation was performed only for furfuryl mercaptan and furaneol. The optimal conditions derived were 50 °C, 2000 psi and 5 min with a desirability factor of 0.911.

9.3.2.4 Validation of response surface model

In order to assess the long-term variability of the response surface model, intermediate precision was validated by selecting two experimental points within the experimental range. Each experimental point was performed in triplicate and the averaged concentrations of the compounds, together with the standard deviations are tabulated in Table 9.4. The experimental points chosen were 75 °C, 1500 psi and 10 min (centre point) and 50 °C, 2000 psi and 15 min. Due to high variability, furfuryl mercaptan was not included for the validation. The average concentration obtained fell well within the predicted response range at 95% confidence level.

Table 9.4. Validation of response surface model

Compounds	Concentration (ppm)			
	Prediction	**95% CI* low**	**95% CI* high**	**Average**
75 °C, 1500 psi and 10 min				
Pyridine	85.83	76.77	94.89	82.44±5.67
Acetoin	12.14	10.08	14.19	11.93±0.98
2,5-dimethylpyrazine	16.32	14.95	17.68	15.28±1.33
2,6-dimethylpyrazine	22.83	21.08	24.58	23.57±1.79
2-methylpyrazine	40.36	36.28	44.43	37.95±2.81
Furfural	14.26	12.83	15.68	13.96±1.05
Furfuryl alcohol	552.43	497.71	607.15	533.30±36.33
Maple lactone	23.43	20.43	26.43	21.27±3.48
Guaiacol	16.47	14.67	18.28	16.26±2.19
Maltol	59.47	53.74	65.19	57.63±10.65
Phenol	20.95	18.80	23.10	20.98±0.70
Furaneol	10.82	9.04	12.61	10.36±2.86
2-acetylpyrrole	44.24	39.91	48.58	39.56±2.61
50 °C, 2000 psi and 15 min				
Pyridine	55.88	32.41	79.35	63.86±20.02
Acetoin	6.94	1.63	12.26	9.46±3.16
2,5-dimethylpyrazine	14.24	10.70	17.78	14.73±4.56
2,6-dimethylpyrazine	19.42	14.88	23.95	21.01±4.70
2-methylpyrazine	30.24	19.69	40.80	33.27±9.95
Furfural	8.92	5.21	12.62	10.40±2.66
Furfuryl alcohol	337.48	195.72	479.24	383.98±113.65
Maple lactone	17.06	9.28	24.84	17.79±5.29
Guaiacol	13.63	8.96	18.30	14.23±3.51
Maltol	35.07	20.24	49.89	37.77±10.70
Phenol	14.39	8.82	19.96	14.81±4.52
Furaneol	5.90	1.28	10.52	6.26±2.02
2-acetylpyrrole	33.98	22.75	45.20	33.68±11.49

*Confidence interval with 95% confidence level

9.3.3. Sensory evaluation

The varying concentrations of the key odorants in the coffee bean extract give rise to its overall aroma and odor. During the three types of PLE optimization, different goals were set in order to obtain the optimal extraction condition required to yield coffee extracts with desirable aromatic profiles. Figure 9.2 reveals the notable differences in the aromatic profiles of the coffee extracts obtained under different extraction conditions. As 14 selected compounds possess distinctive attributes, odor descriptions are listed in Table 9.3. Under the operating condition of 100 °C, 1000 psi and 15 min, all responses were given equal emphasis. The coffee extract obtained exhibited weaker perception, except for the attribute of caramellic (3.0). Thus, this suggested that although most of the target compounds were extracted maximally, the coffee extract lost its genuine aromatic profile especially nutty (1) and sulfury (0) notes. The high score of the caramellic note was possibly attributed to furfuryl alcohol, furfural, maple lactone, maltol and furaneol.

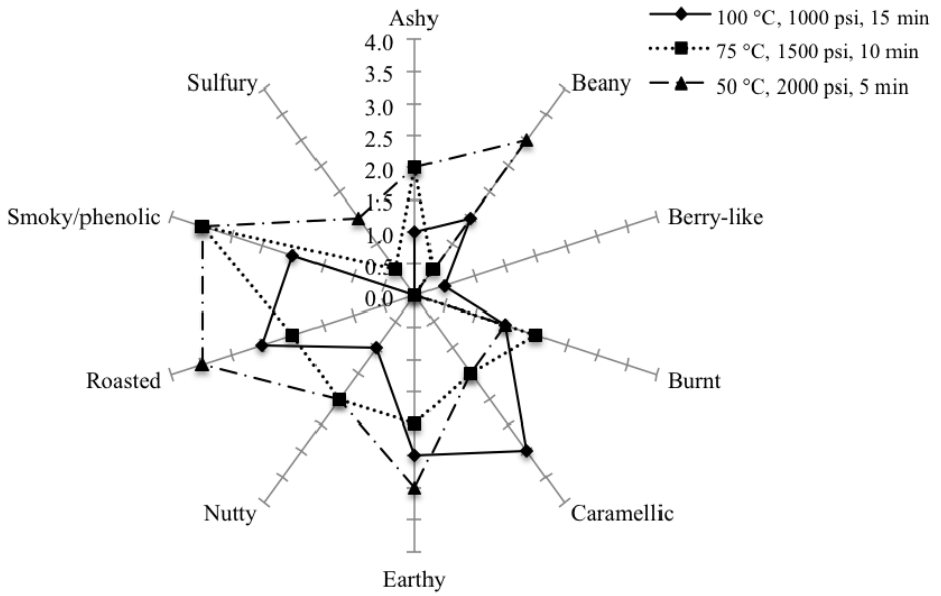


Figure 9.2. Sensory profile of coffee extracts under three optimized extraction conditions

On the other hand, the sensory profile of the coffee extract obtained at 75 °C, 1500 psi and 10 min revealed an average score of 2.0 for ashy, burnt, earthy, nutty, roasted notes. However, a strong smoky/phenolic attribute (3.5) was perceived, which overwhelmed the other attributes. This could be due to the extraction condition being favourable to the extracted compounds that responded significantly to the RSM model, particularly phenol, which are known to be responsible for smoky and phenolic odors in coffee aroma.

Due to the pre-set goal of maximising furfuryl mercaptan and furaneol, the sensory profile of the extract obtained at 50 °C, 2000 psi and 5 min revealed a higher sulfury note (1.5) compared to other sensory profiles. This was in accordance with the aim to develop an extraction condition suitable for furfuryl mercaptan exhibiting coffee-like, roasted and sulfury notes.

9.4. Conclusion

A novel approach to extraction of volatile compounds in coffee bean by PLE was demonstrated. RSM was applied to optimizing the PLE operating conditions (i.e. temperature, pressure and static extraction time). Temperature was clearly found to be the most important factor followed by pressure. Moreover, significant interactions existed between temperature and static extraction time. Through descriptive sensory analysis, the aromatic profiles of the coffee extracts under three different optimum conditions were expressed. Therefore, PLE is a convenient, reliable and flexible technique, which may be useful for flavor isolation.

CHAPTER 10 CONCLUSIONS, RECOMMENDATION AND FUTURE WORK

In this research, characterization of the volatile and aromatic profiles in Asian citrus – pomelo and calamansi, and Arabica coffee varieties from Asia were carried out using different analytical approaches. In the initial study, the blossoms and peels of two Malaysia pomelo varieties (i.e. pink and white-fleshed) were extracted and characterized. Some interesting chemicals were first detected in pomelo blossoms and calamansi juices. Through careful sniffing using GC-O and AEDA techniques, aroma-active compounds of pomelo peel extracts were first reported; however, no single compound was determined as possessing a characteristic Malaysian pomelo-like aroma. In addition, there were a few unknown compounds that contributed to atypical notes, e.g. beery, meaty, guava-like, tropical and hazelnut-like. It is therefore suggested that some compounds with sulfur or nitrogen atom remain unidentified. Further research in discovering novel compounds may require high throughput and higher sensitivity instruments such as high-resolution gas chromatographic, real time mass spectrometry, quadrupole time-of-flight to volatile compounds at a the level as low as picograms. This is especially

critical for volatile sulfur compounds, which can be easily transformed or arise from thermal breakdown or reaction in the injection port of a normal gas chromatograph.

In order to understand the relationship between chemical composition and sensory profile of food, pomelo juices were used as a model to correlate between their chemical components and ten sensory attributes (i.e. 7 orthonasal and 3 retronasal). Overall, the percentage explained of the orthonasal attributes was not as good as the retronasal attributes, notably citrusy, woody and bitter. Therefore, it is suggested that these attributes were not fully elucidated in the current PLSR model due to those unidentified compounds in pomelo. The effect of phenolic components on the bitterness of pomelo juice is yet to be investigated, thus, the present model is of little value in correlating bitterness. Correlation between instrumental data and sensory attribute is of extreme importance in deciphering aroma-active compounds. In view of the limitations present in current data analysis, improvements on extraction techniques and multivariate analyses, which can support flavor analysis, are needed.

Subsequently, the chemical components (volatiles and non-volatiles) of calamansi peels and juices were characterized. Variations among the volatile compounds of calamansi peels and juices were found to be significant (p<0.05) among three geographical origins (i.e. Malaysia, the Philippines and Vietnam). However, differences in non-volatile components in juices (sugars, organic acids and phenolic acids) from different geographical origins were insignificant. Advance analytical insturments such as ESI LC/MS may be

applied for lower detection level and structural confirmation of non-volatile compounds, especially phenolic acid isomers.

Due to disparate concentrations and physiochemical properties of volatile compounds present in food samples, some difficulties in terms of sensitivity and reproducibility were encountered. Extraction technique of SBSE-TD-PTV was developed. After optimization, this method can be applied as a very simple and fast technique with better sensitivity and reliability. This methodology could be extended to develop the analytical method for similar highly complex systems. However, the fundamental understanding of the diffusion coefficients of analytes with different physicochemical properties has yet to be fully resolved. Indeed, the interaction between flavor compounds and food components is very complex. Quantification of flavor being released is another important topic to precisely gauge the flavors perceived from food. Therefore, much research is required in order to understand the flavor release in the presence of fats, proteins, hydrocolloids and etc. It is believed that better insight can be gained through the combination of analytical techniques and mathematical modeling.

Knowledge of the chemical compositions and antioxidant capacities would facilitate a better understanding of Asian coffee. Therefore, volatile and non-volatile constituents of four Asian coffee varieties were also studied. It was found that the Sidikalang Kopi Luwak coffee was most favourable with a well-balanced aromatic profile, though its overall profile was similar to that of Sidikalang. On the other hand, the total polyphenol content of the Sidikalang beans was the highest. Between the green and roasted coffee beans, the radical scavenging activity was similar, whereas the Arabica Sidikalang variety

registered the highest ferric reducing capacity ($p<0.05$). Furthermore, the extraction yield of Sumatra Mandheling coffee bean using PLE was nearly three times higher. PLE demonstrated the feasibility of producing a series of coffee extracts under controllable extraction conditions in correlation with desirable sensory attributes. This approach has not previously reported to characterize the aroma of coffee bean.

In conclusion, the current study has demonstrated several approaches of flavor analytical techniques. With the aids of multivariate analysis, analytical data can be correlated to sensory data or classified by intrinsic (cultivars) and extrinsic factors (geographical origins). It is believed that chemical profiles obtained through the study could provide new insights of these indigenous citrus or new varieties of Arabica coffee. The knowledge could enrich the flavor spectrums of citrus fruits and Arabica coffee and could be utilized as flavoring for various food products.

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