Volatile compounds isolated and identified from Kohia (*Passiflora tetrandra*)

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A thesis submitted to
Auckland University of Technology
in partial fulfilment of the requirements for the degree
of Master of Applied Science (MAppSc)

2013

School of Applied Science

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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Abstract

Plants biosynthesise and release a large diversity of volatile compounds. The dominant types of these volatiles can be divided into three categories: carbohydrate-derived compounds, fatty acid derived compounds and amino acid derivatives.

In this research, the volatile organic compounds were investigated in three different parts of the New Zealand native plant – Kohia (*P. tetrandra*), which is also known as New Zealand passion fruit. The parts included leaves, flowers and fruit. Solid phase microextraction (SPME) was chosen as the analytical method. The identification of volatiles was by gas chromatography – mass spectrometry.

It was found that the compositions of different parts of Kohia were distinctly different. The volatiles in the leaves were mostly alcohols and carbonyl compounds, whereas the major components in flowers were terpenes, and esters were the principal components of the fruit.

In addition, the volatiles in female and male of Kohia leaves were also different. The main components in female leaves were cyclopentenone (31.49%), cyclopentanone (13.10%) and 2-hexenol (8.38%), while the predominant compounds in male leaves were cyclopentanone (17.97%), *cis*-hexenyl acetate (14.06%) and 2-hexenol (14.02%). Attached (still on the plant) and excised (cut off) flowers of female Kohia were mainly contained (*Z*)-ocimene and α-farnesene.

For the fruit part, two different passion fruit species were studied in this study: Kohia fruit and purple passion fruit (*P. edulis* Sims). The results showed that hexyl hexanoate (17.72%) and hexyl butanoate (16.99%) followed by 1-methylhexyl hexanoate (10.68%) and 1-methylhexyl butyrate (10.35%) composed the majority of purple passion fruit flavour. Methyl decanoate (16.97%), cyclopentenone (14.21%) and methyl dec-4-enoate (10.38%) were important compounds in Kohia fruit.

Acknowledgement

First and foremost, I would like to show my deepest gratitude to my primary supervisor Dr. Roger Whiting. He has provided me with valuable guidance in every stage of the writing of this thesis. Without his enlightening instruction, impressive kindness and patience, I could not have completed my thesis.

In addition, I shall extend my thanks to my second supervisor John Robertson for all his kindness and help. He helped me a lot on both experiments and writing of thesis. His keen and vigorous academic observation enlightens me not only in this thesis but also in my future study.

I would also like to thank all teachers and technicians who have helped me to develop the fundamental and essential academic competence and support me. Last but not least, I'd like to thank all my friends for their encouragement and support.

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Chapter 1 Introduction and Objectives

1.1 Introduction

Approximately 80 million years ago, the landmass that was to become New Zealand was separated from Gondwana. Then since around 55 million years ago, the gap between New Zealand and Australia has been getting wider until around 2000 km now and therefore the flora is a unique Gondwana assemblage (Brockie, 2013). Hence New Zealand native plants as a unique flora are worth investigated. In this research, Kohia – *Passiflora tetrandra* (Passifloraceae, Passiflora), which is a New Zealand native plant, was studied. According to the literature (Bortz, 2001), the majority of research on Passiflora genera plants is related to medical science. Some volatile compounds and extracts from plants can be used as fragrance ingredients in perfumery. The volatiles could be affected by some factors, such as physiological variation (type of plant material, organ development, and pollinator activity cycle), environmental conditions (climate, pollution, diseases and pests), geographic variation, genetic factors and evolution (Figueiredo, Barroso, Pedro, & Scheffer, 2008).

According to biosynthetic metabolism, plant volatiles can be classified into three groups: carbohydrate-derived flavour compounds, fatty acid-derived and other lipophilic flavour compounds together with amino acid-derived flavour compounds. Terpenoids, which are derived from carbohydrates, are the most important fragrance ingredients that are secondary metabolites. This kind of volatile compound occurred in the majority of plants, particularly in conifers. The volatiles derived from fatty acid are normally produced by three different oxidation metabolisms: oxidation via lipoxygenase, α-oxidation and β-oxidation. The products are straight-chain acids, alcohols, aldehydes, ketones and esters of these compounds. Also, amino acids are believed to be the precursor of volatile compounds in the plant kingdom. These compounds are often abundant in the flowers and fruits.

The techniques for extracting volatiles and essential oils have traditionally been processes like solvent extraction and distillation. However, SPME (Solid Phase Microextraction) has been developed to conveniently trap and inject low levels of volatile compounds into a gas chromatograph (GC). Although quantization can be difficulty, it is a relatively quick and sensitive method to address the need for fast and

solvent-free separation of compounds. When using the SPME method, some parameters must be optimized first to obtain reasonable reproducibility and sensitivity.

1.2 Objectives

This research was intended to investigate the volatile compounds in a New Zealand native plant – Kohia (*Passiflora tetrandra*). The volatiles were obtained from different parts of this plant using the SPME method. They are female and male leaves, female flowers and fruits. Also, the flavour compounds in purple passion fruit (*Passiflora edulis* Sims) were studied to compare with those of Kohia fruits. Analysis of the volatiles was by GC (Gas Chromatography) and GC-MS (Gas Chromatography – Mass Spectrometry).

Chapter 2 Literature Review

2. 1 New Zealand Flora

Between the late Cretaceous and early Tertiary, there was an ancient continent that became isolated progressively. This continental landmass is the place on which New Zealand biota grew. New Zealand was connected to the Gondwana supercontinent originally. For the last 55 million years, it has been isolated from Australia, its closest neighbour which is 2000 km away (Winkworth, Wagstaff, Glenny, & Lockhart, 2005). The present flora in New Zealand has been regarded as a good living case of 'Gondwana' vegetation, which was isolated by sea-floor spreading however the paleontological record shows that the majority of New Zealand flora arrived by 'post-drift'. That is New Zealand's entire forest-flora possibly got here by long-distance dispersal (Pole, 1994) after the Gondwana split.

New Zealand has a very lush and wide variety of plants because of abundant sunshine and rain. At least 80% are unique to New Zealand (Jack, 2003). While iconic plants like cabbage trees, flax and others are quite familiar to most native plants are not well known. There is a tendency to forget the place of New Zealand native flora in the world (Foster, 2006). Botanists describe the variety of characteristics of New Zealand native plants as showing 'divarication, juvenile forms, dioecious or separate gender on separate trees, a profusion of small white flowers and an immense variety of plants' (Foster, 2006).

2.1.1 Kohia – *Passiflora tetrandra* (Passifloraceae, Passiflora)

Kohia is an example of a New Zealand native plant, which is not known to most people. Little research has been carried out on this plant. Kohia belongs to Passiflora genera in the Passifloraceae family.

The Passifloraceae family is commonly called the passion flower family. It is in the Malpighiales order, which includes 16 genera and 705 different species (Berry, 2012b), containing herbaceous plants, shrubs, trees and woody vines often with unbranched tendrils formed between the stipules. The species of this family are distributed widely, mostly in the tropics and subtropics and especially in Neotropics and Africa. The flowers are usually beautiful, showy, and regular, like a saucer-shaped to tubular flora

cup. Concentrated on the petals there are usually rings of membranes and filaments formed inside. The flowers commonly have an androgynophore or gynophore, on which the stamens and ovary are held (Berry, 2012a). The fruits of Passifloraceae are often berries or capsules with seeds compressed and surrounded by pulpy aril or apical.

The largest genus in the Passifloraceae family is Passiflora, also known as passionflower genus. This genus is composed of 525 different species. Most of them are highly praised by people for their showy and unusual flowers (Berry, 2012b). Most species of Passiflora are found in China, Southern Asia, South America and New Guinea. Nine species are native to the USA, four or more have been discovered in Australia and only one endemic species in New Zealand, as the sole New Zealand representative (Wikipedia, 2013).

The New Zealand species is Kohia–*Passiflora tetrandra*. It is mostly called New Zealand passion fruit. It is quite different from its most common and well-known relative plant –*Passiflora edulis*, a commonly grown fruit. Kohia has potential to be more widely used in New Zealand. Although the fruit is inedible, it is an attractive climber with white flowers gradually turned to yellow (**Figure 1).** It can grow in the confined spaces and gives additional layers of vitality and interest to the garden.

Figure 1 *Passiflora tetrandra* flowers (Mitcalfe, 2013)

New Zealand native passion fruit is unique amongst *Passiflora*. It is usually found in some lowland forests and montane zone, throughout the whole North Island and also includes Banks Peninsula in the South Island. *Passiflora tetrandra* was discovered by Daniel Solander and Joseph Banks in 1769, who were the botanists on Cook's first voyage to New Zealand (Unknown, 2013). Kohia can grow to 10 meters high and many meters wide. This native plant is dioecious and both male and female produce tiny

flowers (**Figure 1**) from October to December. After the female is pollinated in summer it will produce bright orange and inedible fruit (**Figure 2**) in April or May. When the fruit ripen they are only the size of a berry or a small peach, always preferred by birds as food.

Figure 2 *Passiflora tetrandra* fruits

2.1.2 *Passiflora edulis*

Passiflora edulis is also one species of *Passiflora* genera in *Passifloraceae* family. It is commonly known as yellow passion fruit and spread widely throughout tropical countries in the world. There are two different types of *Passiflora edulis* grown for commerce. They are yellow form (*P. edulis* var. *flavicarpa* Degenerer) and purple form (*P. edulis* Sims) respectively (Patel, 2009).

Figure 3 Flower of *Passiflora edulis* Sims (Tony, 2008)

The leaves of this kind of passionfruit are evergreen and finely toothed when mature. On the upper side of leaves, the colour is deep green and glossy, while it is paler and dull underneath. The single flower (**Figure 3**) of passionfruit can be 5-7.5 cm wide. The bloom is composed of five greenish-white sepals and five petals, and also there are three green, leaf-like bracts inside. In the central structure, there are some white-tipped and purple based rays, five stamens with anthers and ovaries. The fruit (**Figure 4**) is nearly round and egg-sized (4-7.5 cm wide). The rind of fruit is tough and smooth, and the colour is dark purple when ripe. Inside the fruit, it is filled with aromatic double-walled membranous sacs, which contain pulpy juice and small dark brown seeds (Gray, Prestage, Linforth, & Taylor, 1999; Morton, 1987).

Figure 4 Fruit of Passiflora edulis Sims

2.1.3 Medicinal Use of Passiflora Extracts

So far, research on *Passiflora* species has been limited to medical uses, and the extracts could be classified into several kinds of medicinal activities: anxiolytic, spasmolytic, hypnotic, sedative, narcotic and anodyne (Bortz, 2001). For example these extracts are part of a treatment that has successfully given to patients with adjustment disorder and anxiety (Bourin, Bougerol, Guitton, & Broutin, 1997).

Several flavonoids were separated from some *Passiflora* species. One major function of flavonoids is to protect against a variety of diseases, often related to some oxidative process in the body. *C*-deoxyhexosyl flavones from *Passiflora edulis* leaves were investigated (Ferreres et al., 2007) and tested for antioxidant properties against DPPH radicals. Two flavonoids, apigenin and chrysin were found in *Matricaria chamomilla* and *Passiflora incarnate.* Both of these two flavonoids were able to have anxiolytic effect (Zanoli, Avallone, & Baraldi, 2000). In this research, the results also showed that chrysin had a clear anxiolytic effect, which was related to GABA-benzodiazepine receptor unit. The anxiolytic effect was blocked by the injection of benzodiazepine antagonist Flumazenil.

The extracts from *Passiflora edulis* Sims were used to evaluate the anxiolytic/sedative activity (Caccioni, Guizzardi, Biondi, Renda, & Ruberto, 1998). The results showed that the aqueous extract had an anxiolytic activity and no significant effect on the motor activity. However, the TFF (total flavoniods fraction) had an anxiolytic activity and a significant effect on the motor activity. The aqueous extracts of *Passiflora incarnate* L. including indole alkaloids maltol and flavonoids were studied to show that they had a sedative property in mice by the decrease of rears and steps climbed in the staircase test (Scora, Kumamoto, Esen, & Stone, 1976). Therefore, it can be shown that extracts from *Passiflora* species can be used as insomnia remedies.

2.2 Perfume Chemistry

Nowadays, fragrances are getting more and more popular. Meanwhile, they are accepted and expected to be used in a wide field, including haute couture perfumes and many consumer products, such as soaps, shampoos and some detergents. According to the different types of essential oils, plants can emit various aromatic and pungent fragrances, so that can be used for some condiments.

100 Years ago, perfumes were composed entirely of natural materials. Since then, fragrances and flavours were commercialized at the same time as single aromatic natural compounds were isolated. It was a technical breakthrough of great significance, mainly in the chemistry field. The first companies in flavours and fragrances were established by business people and scientists. This kind of industry is composed of four business sectors (see **Figure 5**). These sectors were, and still are closely related to each other and even overlapping (W. Schwab, Davidovich-Rikanati, & Lewinsohn, 2008). The situation of world trade is shown in **Figure 6**.

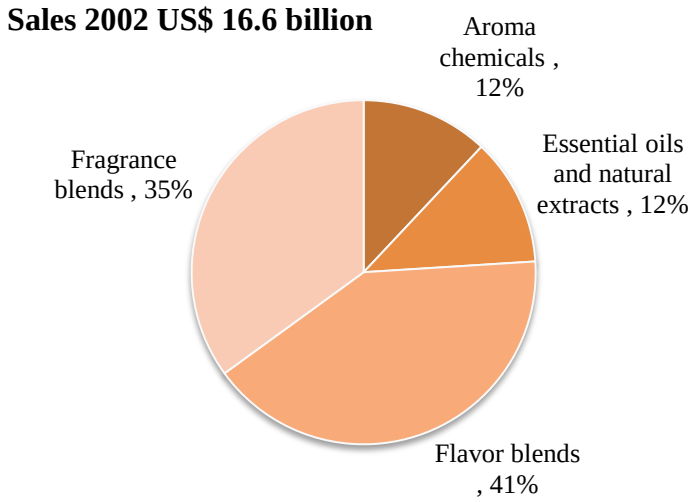


Figure 5 Sales in 2002 US$ 16.6 billion (W. Schwab et al., 2008)

In this figure, essential oils and natural extracts mean the aromatic materials from botanical and animal sources using various extraction methods. Essential oils are aromatic and mixtures of a number of chemical compounds. Aroma chemicals are produced through bio catalytic or organic synthesis or separated from natural sources. They are commonly used to compose fragrances and flavours. Fragrance and flavour blends are both very complex formulations because they are composed of essential oils, natural derivatives and up to 100 components of aroma chemicals. The flavours are formulated to be used in food, beverage and pharmaceutical industries. The formulated fragrances are used in some products, like cosmetics and detergents, because of their pleasant scents (W. Schwab et al., 2008).

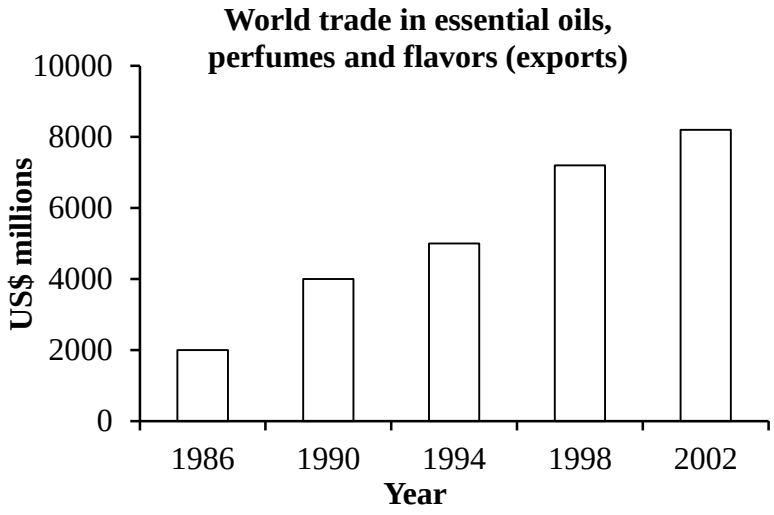


Figure 6 World trade in essential oils, perfumes and flavours (exports)

(W. Schwab et al., 2008)

There is an increasing tendency to formulate cosmetics using plant extracts today not only for skin care, but also for the pleasant odour. Formulated cosmetics and some hair care products, like dyes, colorants contain some active compounds from natural sources. In the future, more new plants will be identified to have the potential value as a source of essential oils (Aburjai & Natsheh, 2003).

However, the use of synthetic compounds in some modern perfumery industries is widespread because of the high cost and short supply of natural extracts. Today, there are around 3000 different fragrance ingredients available to be used, but the ingredients directly from natural sources represent no more than 5% of the total (Fortineau, 2004). Although the natural materials are often used less, the development and discovery of synthetic chemicals depends on the study and analysis on some natural sources, like plants, fruits and even animals.

There are three basic methods to produce the main fragrance components. These are expression, distillation, and solvent extraction. Because of these more complex techniques, natural materials are usually much more expensive than synthetic chemicals to isolate and produce.

As the improvement of techniques for extraction, characterization and chemical synthesis there is more potential to explore new fragrance materials. It is mostly of note that the perfume industries and pharmaceutical industries use the same methods and are also based on the analysis of natural materials. It is often necessary to identify the components existing in the nature first. To do this, “headspace” procedures to trap the volatiles from most flowers directly are often used. At next, an authentic sample should be prepared to compare with the products of synthesis so that determine the correct structure. At the end of sequence, a series of analogues need to be prepared. And these analogues should have similar structures, but different natural parent (Fortineau, 2004).

2.3 Plant-derived Volatiles

There is a variety of reasons for the plants producing odorous chemicals. The process by which all living organisms generate biological materials is known as biosynthesis. The materials can be divided into two main forms: primary and secondary metabolites. The common materials, like carbohydrates, lipids, proteins and nucleic acids are considered primary metabolites (Sell, 2006). Despite a few materials being degradation products of primary metabolites, the intermediates of primary metabolism also play an important

role in the biosynthetic pathways of plant volatiles. The compounds used for fragrance ingredients are mostly secondary metabolites (Sell, 2006).

Sell (2006) reported that the materials used as sources of fragrance ingredients, which contained in secondary metabolites, can be classified into four groups. They are in a decreasing sequence of significance as the perfume ingredients: terpenoids, shikimic acid derivatives, polyketides and alkaloids, and this is very few alkaloids or their derivatives are used as source of fragrance ingredients so that this is not discussed further (Sell, 2006).

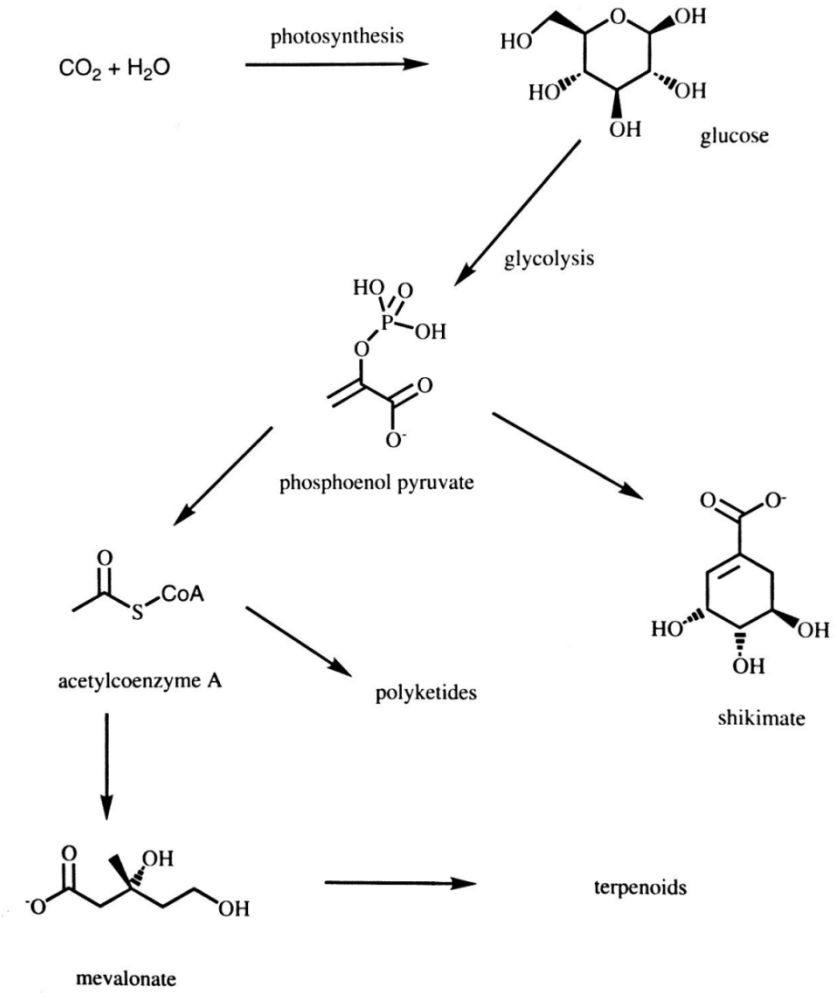


Figure 7 Basic biosynthesis routes (Sell, 2006)

Terpenoids, shikimic acid derivatives and polyketides are all originally derived from glucose (see **Figure 7**). This figure illustrates that green plants use carbon dioxide and water to produce glucose with the sunlight, which acts as an energy source to promote the photosynthesis process. The plant decomposes glucose to the enol form of pyruvic acid – phosphoenol pyruvate. This is then converted to either shikimic acid or acetyl coenzyme-A. The thiol function in coenzyme-A not only plays a role of an activating

group, but also acts as an efficient leaving group. An aldol type reaction produces long chain compounds. Then through self-condensation, polyketides are produced from these long chains. On the other hand, Acetyl coenzyme-A can also be modified to mevalonic acid to form terpenoids (Sell, 2006).

Schwab et al. (2008) also explained that there are three groups of the plant-derived volatiles are classified according to their biogenetic origin. These groups will be described in the following sections.

2.3.1 Carbohydrate-derived Volatile Compounds

Terpenoids Terpenoids are the basic components in the essential oils of many herbs. The process of terpenoid synthesis can be seen in **Figure 7**. Many terpenoids in the plant are non-volatiles and are involved in photosynthesis, growth regulation and membrane structure. Terpenoids of hemiterpenoids (C_5), monoterpenoids (C_{10}), sesquiterpenoids (C_{15}) and diterpenoids (C_{20}) are always volatiles. C_{10} and C_{15} terpenoids (**Figure 8**) were found to affect the scent of flora and flavour of fruits (Wilfried Schwab et al., 2008).

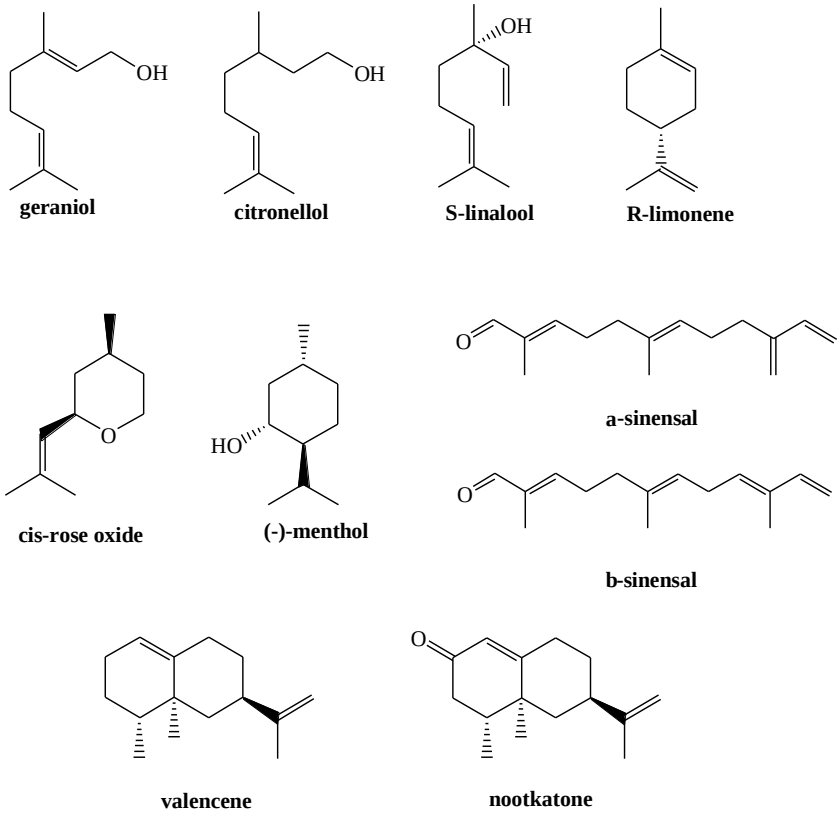


Figure 8 Some important plant-derived volatile terpenoids (Wilfried Schwab et al., 2008)

Caccioni et al. (1998) studied the volatile components in some different types of citrus fruits, and it was found that there was a large number of monoterpenes and sesquiterpenes in all the fruit. In the essential oil of oranges (*C.sinensiscv*), the monoterpene of limonene accounts for more than 90%. Although a small quantity of sesquiterpenes is present in the oil, both of monoterpenes and sequiterpenes are significant for the orange flavour. The characteristic aroma of the leaf essential oil of citrus species (Scora et al., 1976) is due to the combination of geraniol, citronellol and linalool. The lychee fruit (*Litchi chinesis Sonn*) was found to show the highest value of cis-rose oxide. *Cis*-rose oxide is an important component in the distinctive flavour of the lychee (Ong & Acree, 1999). The monoterpenes limonene, *α*-terpinene and *α*-pinene composed the main volatile compounds of redblush grapefruit peel oils and also nootkatone, *α*- and *β*-sinensal were important though they just account for a small amount of the oil (Njoroge, Koaze, Karanja, & Sawamura, 2005).

Furanones and Pyrones A limited number of natural volatile compounds are derived without degradation from the original carbohydrates, and these fall into two classes. These are furanones and pyrones and probably derived from fructose (Wilfried Schwab, 2013). They are present in fruit, leaves and bark of some trees (W Schwab & Roscher, 1997). Their structures are shown below in **Figure 9** (Bood & Zabetakis, 2002).

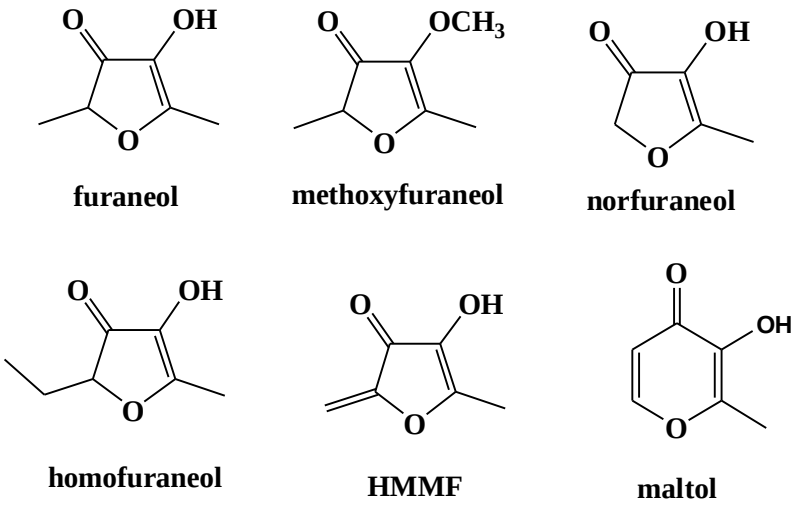


Figure 9 Furanones and pyrones (Wilfried Schwab et al., 2008)

It has been found that furaneols can be emitted only by the fruit of a few plant species. *2,5*-dimethyl-*4*-hydroxy-2H-furan-*3*-one (furaneol) is a very important volatile component producted by Maillard reaction in fresh strawberry (Zabetakis & Holden, 1997) and pineapple (Rodin, Himel, Silverstein, Leeper, & Gortner, 1965). Maltol was

also detected in the plants, such as leaves of *Cercidiphyllum japonicum* (Tiefel & Berger, 1993), fruit of *Tamarindus indica L.* as low quantities (Wong, Tan, Chow, & Chee, 1998), foliage of Fraser fir (*Abiesfraseri*) (Carlow et al., 2006) and four species of *Pinaceae* plants (Tiefel & Berger, 1993).

2.3.2 Fatty Acid-derived and Other Lipophylic Volatile Compounds

A large number of plant-derived volatile compounds are based on the saturated and unsaturated fatty acids. These groups of volatiles contain straight-chain acids, alcohols, aldehydes, ketones and esters of these compounds. These products are formed by three different metabolic pathways: *α*-oxidation, *β*-oxidation and the lipoxygenase pathway (Wilfried Schwab et al., 2008).

The lipoxygenase pathway is a process of in-chain oxidation. This is based on the polyunsaturated fatty acids, such as linolenic acid. The main products are saturated and unsaturated volatiles of C_6 and C_9 aldehydes and alcohols. In this pathway there are more than four enzymes involved (Wilfried Schwab et al., 2008). The first important one is lipoxygenase (LOX) (**Figure 10**).

C_6 and C_9 aldehydes and alcohols take significant responsibility for the "fresh green odour" of green leaves, the aroma of fruit and vegetables. Gray et al. (1999) investigated the composition of C_6 aldehydes (hexanal and hexenal) in cherry tomatoes and standard tomatoes. It was also found that *α*-linolenic acid in cherry tomatoes was about twice as much as that in standard tomatoes. Although both hexanals and hexenals contributed to the flavour of tomatoes, hexenals gave more flavour than hexanals in both types of tomatoes (Gray et al., 1999). Later, Wang et al. (2001) studied the volatile compounds in the tomato leaves. They found tomato leaves contained the highest amount of *2E*-hexenal compound. In cucumber, C_6 and C_9 aldehydes are formed by *9-*/*13*-hydroperoxides of polyunsaturated fatty acids and also formed after tissues disruption (Matsui et al., 2006). In addition, the authors also found C_9 aldehydes could prevent attack by fungal pathogens including *Botrytis cinerea* and *Fusarium oxysporum*.

α-and *β*-oxidation are also the major processes that form volatile compounds in plants. However the roles are not very certain. In *α*-oxidation, C_n fatty acids are degraded to C_{n-1} fatty aldehydes and C_n-hydroxy acids (Wilfried Schwab et al., 2008). These C_{n-1} fatty acids were studied in cucumber (*Cucumis sativus*) (Borge, Vogt, & Nilsson, 1999), potato (Laties & Hoelle, 1967) and green algae (*Ulva pertusa*) (Kajiwara et al., 1988).

The products of β-oxidation with an acyl CoA hydrolase are short and medium straight-chain carboxylic acids. These kinds of compounds have been found in many other plants as well (Wilfried Schwab et al., 2008).

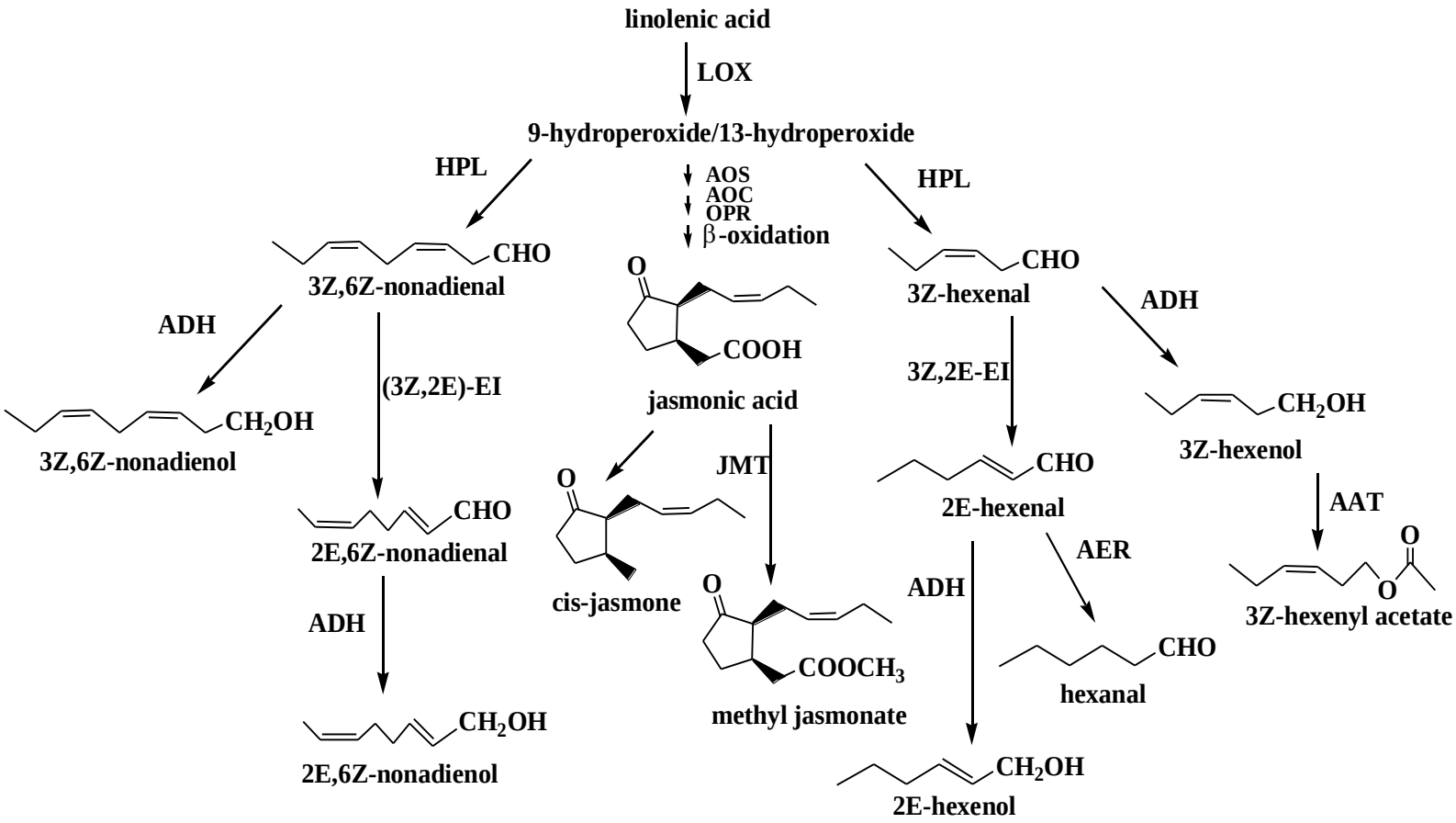


Figure 10 Linolenic acid-derived volatiles. (LOX-lipoxygenase, HPL-hydroperoxidelyase, AOS-allene oxide synthase, AOC-allene oxide cyclise, OPR-12-oxo-phytodienoic acid reductase, ADH-alcohol dehydrogenase, 3Z,2E-EI-3Z,2E-enal isomerase, JMT-jasmonate methyltransferase, AER-alkenal oxidoreductase, AAT-alcohol acyl CoA transferase) (Wilfried Schwab et al., 2008)

2.3.3 Amino Acid-derived Flavour Compounds

The especial important plant-derived volatiles come from branched-chain amino acids and the biosynthetic pathways of amino acid-derived volatiles are shown in **Figure 11**. Gonda et al. (2010) claimed that the first steps in the process of amino acids producing aroma molecules were not very certain. Despite these amino acids are believed to be the precursor of volatile compounds for the scent of flowers and the aroma of fruit. In their research, they found in the melon tissues that the initial steps of amino acid conversion to flavour compounds were based on an amino transfer process rather than decarboxylation or aldehyde synthesis (Gonda et al., 2010).

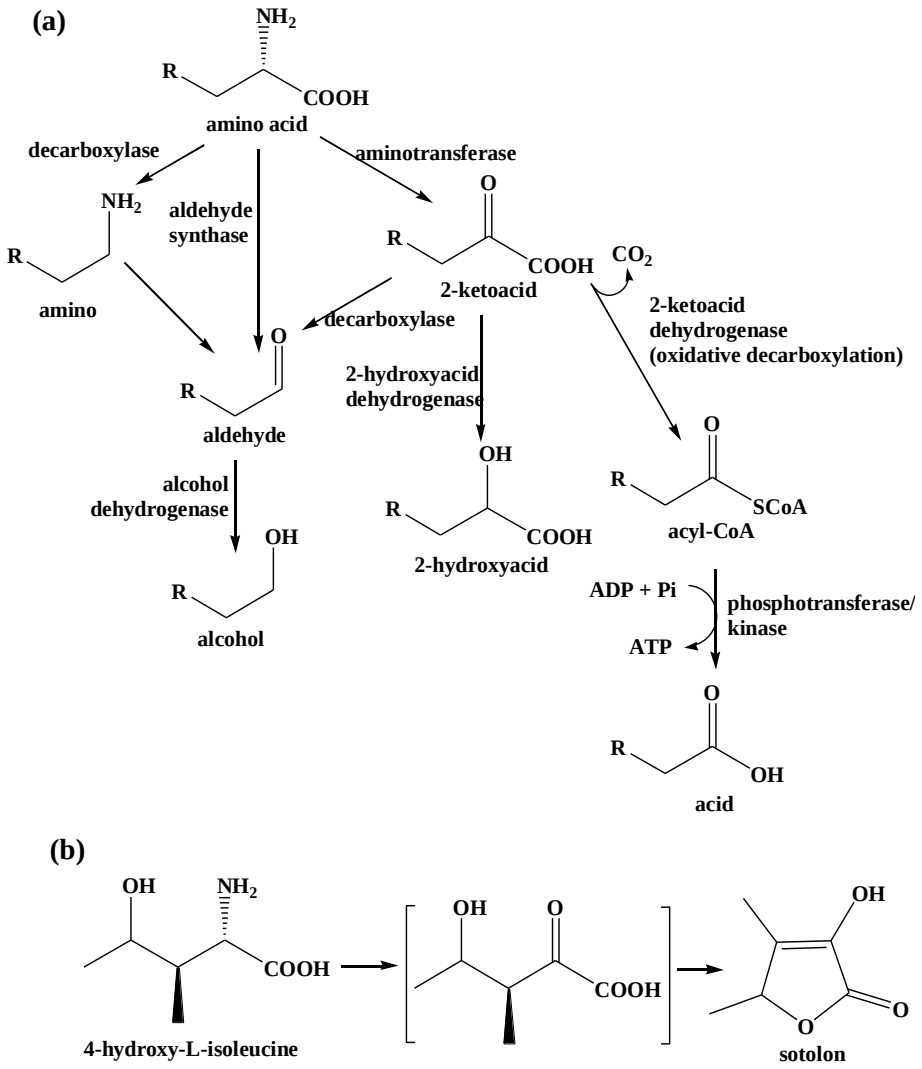


Figure 11 Amino acid-derived volatile compounds. (a) Biosynthesis of volatiles derived from branched-chain amino acid (b) Biosynthesis of sotolon (Wilfried Schwab et al., 2008).

In the fruits of grape, melon, strawberry, peach and tomato, there is a large number of leucine-derived and phenylalanine-derived volatile compounds (*3*-methylbutanol, *3*-methylbutanal, *3*-methylbutanoic acid, *2*-phenyllenthanol and phenylacetaldehyde) (Aubert, Baumann, & Arguel, 2005). Besides, *3*-methyl butyl butanoate ester formed by the amino acid-derived alcohol and acid was found to be the main constitute in banana cultivars and gives the important fruit odour to the banana (Nogueira, Fernandes, & Nascimento, 2003).

2.3.4 Factors Affecting the Difference of Volatiles and Essential Oils

When plants produce aromatic compounds, these can come from a number of different parts of the plant, such as leaves, flower, fruit and roots. Often the different parts have different aroma characteristics. Only few species give a similar composition of essential oil in these different organs. Likewise, entomophilous flowers also can produce a variety of volatiles, whose one function is to be used as orientation clues for the pollinators. Hence the volatiles from flowers or flower parts are typically different from those from other organs of the plants.

Research investigating *Achillea ptarmica* (Kuropka, Neugebauer, & Glombitza, 1991) found that there was no monoterpenes in the essential oils of the green parts and roots, but these were present in the flowers. This implies that the floral oils and odours were directly affected by the pollinator's attraction (Figueiredo et al., 2008). However there was no research addressing the chemistry of plants that included pollinator attraction until a study by Varassina et al. (2001) was carried out to compare nectar production, flower scents and interactions with the different pollinators in four Passiflora species: *P. galbana* and *P. mucmnata* were pollinated by bees, *P. alata* by bats, and *P. speciosa* by hummingbirds. The results showed that there were various classes of volatile compounds in the Passiflora species pollinated by bees and bats. Both of them have acute olfactory senses. Hummingbirds are not scent-orientated, so *P. speciosa* did not show the same patterns of scent production.

In addition to pollination, there are many other factors that affect the volatile compounds and essential oils, such as physical variations, environmental conditions and geographic variations. All these can lead to differences in the perfume of the plants.

2.4 Extraction Techniques

Essential oils are composed primarily of volatile and aroma constituents with low molecular weight. They are obtained from plant materials using different extraction methods, some of which have been used for many years.

2.4.1 Traditional Extraction Methods

In the perfume industry, the perfume materials can be obtained from different extraction methods (see **Figure 12**). The common methods used to extract the natural perfume ingredients and essential oils can be categorized three classes: expression, distillation and solvent extraction.

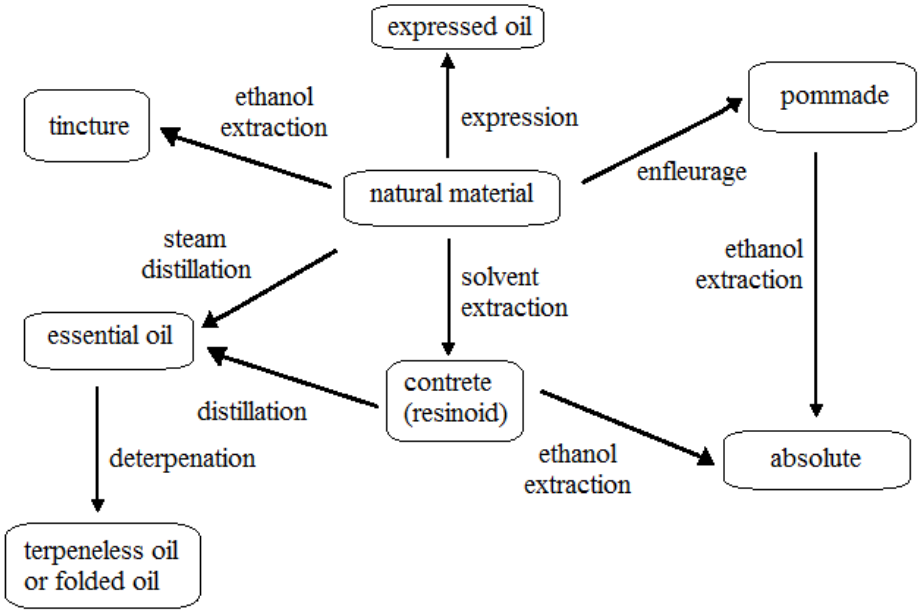


Figure 12 Types of extraction methods (Fortineau, 2004)

Expression is the simplest of the methods, but only used in the citrus, such as orange, mandarin and lemon. This method uses physical pressure to force the oils to be released from the natural sources, so that the product is called pressed oil.

The most commonly used distillation method is steam distillation. In this extraction method, the still pot is charged with water or steam and then plant materials are put into the still. The steam passes through the plant materials and both heat and moisture in the still will decompose the cell walls in the plant to release the aromatic compounds. The oils have low vapour pressures (otherwise you would not be able to smell them) and co-distill with the steam. Depending on the different densities, the oil and water will

separate from each other. In addition, distillation also includes dry distillation and hydro diffusion. In dry distillation, the plant materials are usually put in a vessel, whose surface has a high temperature caused by heat (most case is by direct flame). This technique is particularly employed for extracting the oils from wood, because this kind of oil usually has a high boiling point and needs high temperature to vaporise their chemical compounds. As a relatively new technique, hydro diffusion is similar to steam distillation, but the steam is produced at the top of the pot and the essential oil with water obtained at the bottom. The steam in hydro diffusion gives the same result as steam distillation. However, the difference is that in hydro diffusion the oil is not needed to vaporise with the heat of the steam because it is not distilled (Sell, 2006).

Solvent extraction is one of the most significant techniques of perfume ingredient extraction. The solvents typically used are acetone, ethyl acetate, hexane and dichloromethane, and the combination of these solvents is often used as well. Nowadays, supercritical fluids are also used as one of the extraction solvents. Carbon dioxide is the most common supercritical fluid because its critical point (7.4 MPa and 31 ^{o}C) is relatively easily reached and maintained. However, the liquefying of carbon dioxide needs quite a high pressure, which makes the equipment expensive. This method raises the expense of essential oil produced but there is an obvious advantage that CO_2 can be removed very easily hence there is need to worry about the levels of residual solvents (Sell, 2006).

Unfortunately, distillation, solvent extraction, or combinations of these methods have the disadvantage that it takes several hours to extract. Although the crude extracts will be more similar to natural botanical material because there is little or no heating used in the process. When removing the solvents to concentrate the extract, these features may be lost.

2.4.2 Modern Methods

Modern extraction techniques include, as noted above, supercritical carbon dioxide extraction, subcritical water extraction and ultrasonic extraction.

Supercritical carbon dioxide extraction (SFE – CO_2) is currently the most widely used and advanced extraction technique to extract plant essential oils during the isolation of natural products. Supercritical fluids are compressible fluids with high density at a temperature and pressure above their critical points (P_C, T_C). They have properties

between those of liquids and gases. This makes them a good solvent. The diffusion coefficients and viscosities of supercritical fluids are close to those of gases, and the surface tension is near zero. Therefore, these fluids are very good at penetrating the pores in the solids and semi-solids. Through this process, the essential oil of plants can be extracted and isolated efficiently. Supercritical fluid extraction is able to use the strong solubility characteristics of the supercritical fluid to extract the components from the natural materials. When the pressure was reduced, the supercritical fluid vapors leave the extracted components behind (C. Chen, Li, & Li, 2011).

Supercritical CO_2 fluid extraction (SFE - CO_2) has some unique advantages. It is harmless, colorless and odorless, and therefore is very safe to use. The process of extraction is easily controlled and gives high yields of products. Also, supercritical CO_2 is cheap and can be reused. One especially useful aspect of SFE - CO_2 is that components that are thermally sensitive and easily oxidized are not affected. This is to say the original composition and quality are maintained. Nevertheless, SFE - CO_2 has some limitations. High pressure is required in the operation, so it needs higher equipment requirements than other techniques, which means a higher investment cost (C. Chen et al., 2011).

Nowadays, this method is used worldwide and has been applied successfully in the investigation of volatiles from different plants. For example, extraction of the essential oils of clove buds (Ong & Acree, 1999), valerian (*Valeriana officinalis* L.) roots (Njoroge et al., 2005), *Mentha pulegium* L. (Bood & Zabetakis, 2002) using SFE - CO_2 have been investigated.

Subcritical water extraction (SWE) is also a developed extraction technique. Under moderate pressure, water is heated between 100 °C and 374 °C (critical point). It still remains in the liquid state and is called subcritical water. The polarity of subcritical water is reduced with the increasing of temperature. Hence the water polarity can be controlled over a wide range through the control of the water temperature and pressure. As a result, the components can be extracted continuously and selectively according to their polarities (C. Chen et al., 2011).

SWE has the advantages of simple equipment, short extraction times, high yield and quality of the essential oils. As the extraction agent, pure water does no harm to the environment. This method is widely used in extracting essential oils from coriander seeds (*Coriandrum sativum* L.) (Zabetakis & Holden, 1997), leaves of *Thymbra spicata*

L. (Rodin et al., 1965), Fennel (*F. vulgare*) (Tiefel & Berger, 1993) and other plants.

2.4.3 Solid Phase Microextraction (SPME)

Solid phase microextraction (SPME) is an analytical laboratory scale sample preparation technique. A fused silica fibre is coated with a suitable stationary phase. When fibre is exposed to sample, the analyte in the sample will be extracted directly and establish an equilibrium with the analyte on the fibre. Then the fibre is transferred to a gas chromatograph injector with the help of syringe shaped device. The analyte is desorbed from the fibre at a high temperature (typically the GC injector temperature) and then analyzed by the GC or GC-MS (Wong et al., 1998).

SPME has some important advantages of solvent-free, small size of sample and fast sampling. It can be used with gaseous, liquid or some sample not clean. Two basic types of extraction modes can be used in SPME: direct extraction and headspace configuration. In the direct extraction mode, the coated fibre is directly inserted into the sample and the analytes are extracted directly from the sample matrix to the extracting phase. In the headspace configuration mode, the fibre is in the headspace and the analytes must be volatile from sample matrix to the headspace. Then the volatile analytes are indirectly extracted to the fibre coating (Wang et al., 2001).

Proper optimization of the solid phase microextraction conditions can reduce the number of experiments and achieve reproducibility and sensitivity. Some of the parameters include fibre coating, extraction time, sample temperature, salting and pH (Matsui et al., 2006).

2.4.3.1 Selection of Fibre Coating

There are several types of fibre coatings available currently. The general principle used for fibre selection is ‘like dissolves like’, so the selection of fibre depends on the polarity and volatility characteristics of target analytes. The four most commonly used fibre coatings are list in the following **Table 1** (Matsui et al., 2006).

Table 1 Common SPME Fibres (Borge et al., 1999)

Coating Material	Acrostic	d_F/μm[a]	Recommended Use
Polydimethylsiloxane	PDMS	100	Non-polar analytes
Polydimethylsiloxane / Divinylbenzene	PDMS/DVB	65[b]	Polar analytes (especially amines)
Carboxen / Polydimethylsiloxane	CAR/PDMS	75[b]	Volatile / low molar mass analytes
Polyacrylate	PA	85	Polar analytes (especially phenols)

a d_F=coating thickness.

b Highly cross-linked coating.

The most widely used fibre is polydimethylsiloxane (PDMS) (Borge et al., 1999). There are two main advantages of using this fibre. PDMS is very rugged and can withstand high temperatures, up to 300 °C, and also it has a greater stability than CAR/PDMS and PDMS/DVB. Furthermore, this fibre can evaluate the contribution constants of organic compounds according to retention times on PDMS-coated GC columns (Matsui et al., 2006).

2.4.3.2 Optimization of Extraction Conditions

In addition to fibre coating, extraction time, analyte concentration, addition of salt to suspensions of the sample, and changing temperature can also influence the extraction process. The extraction time is related to the partition coefficient of the analyte and agitation rate. Magnetic stirring is commonly used in SPME for agitation, which speeds up the transfer of analytes to fibre. The extraction efficiency is also improved by heating sample. Higher sample temperature always cause higher diffusion coefficients, but still low partition coefficients(Wong et al., 1998). Moreover, adding salts to sample is also an important method to improve efficiency due to the salting-out effect on the sample.

2.4.3.3 Limitations of Solid Phase Microextraction

However, solid phase microextraction (SPME) has some problems in the use. The fibre quality of different manufacturer produced is inconsistent, even though the same manufacturer and the quality of different batches are not the same. Thus each fiber must be optimized before use (Laties & Hoelle, 1967). When using a new fiber or a fibre that has not been used for a long time, it is necessary to perform the fibre conditioning again. The temperature and time for conditioning is provided by the manufacturer. Sometimes, the problem of residuals on the fibre is difficult to solve even with high temperature conditioning. Therefore, during the period of sampling, blank gas chromatography or liquid chromatography should be run for some time connected with fibre. If the sample contains a high percentage of suspended matter, then stirring in a sample will damage the fiber, and also macromolecules substances will be adsorbed into the fiber irreversibly so that changing the fibre coating properties. It is one reason for causing poor reproducibility and linearity when extracting components from contaminated water (Laties & Hoelle, 1967).

2.4.3.4 Applications in Botany Analysis

SPME is applied in a number of fields, such as food, environment, pharmaceuticals and physical chemistry. Most especially, it has been accepted in the flavor and fragrance area and also used for analysis of volatile compounds in the different parts of aromatic plants, such as leaves, flowers and fruits.

There is a lot of research reporting the volatiles extracted and isolated from plant leaves using SPME, like *L. alba* stems and leaves (Aubert et al., 2005), leaves of *Eucalyptus citriodora* (Zanoli et al., 2000) and rocket salad (Eruca sativa) of fresh leaves (Acree & Arn, 1984).

Volatiles of many flowers have the potential to be applied as perfume ingredients. Also SPME is currently used to isolate and identify these volatile components on different flowers: lavender (*Lavandula angustifolia* L.) flowers (Nogueira et al., 2003), fresh, frozen and withered flowers of *Michelia alba* (Jirovetz, Smith, & Buchbauer, 2002), *Chimonanthus praecox* flowers (Burdock, 1996), etc.

The flavors in the fruits directly influence the quality of products, so the volatiles are quite important for fruits. Nowadays, SPME technique has been developed to analyze the volatile compounds in several fruits, such as some tropical fruits including passion

fruit, sac hew and tamarind (Morton, 1987), tomato and strawberry (Palacios, Bertoni, Rossi, Santander, & Urzúa, 2009). Recently, SPME method is coupled with GC or GC-MS to analyze the various volatiles in different plant organs.

In this research, authentic compounds were not used and the equilibrium conditions were unknown so that peak areas were used instead of the absolute values. However, this is common in the literature where the authentic compounds and equilibrium conditions are not available.

Chapter 3 Materials and Methods

3.1 Chemicals

Deionised water was used in the experiment and sodium chloride (99.5%) to adjust the ionic strength of the fruit sample. The C_7-C_{20} n-alkanes series mixed standard was purchased from Sigma-Aldrich.

3.2 Materials

The SPME fibre (100 μm PDMS, fused silica) and holder were purchased from Supelco (Bellefonte, Pennsylvania, USA). 20 mL certified flat bottom headspace crimp top glass vials were obtained from Agilent Technologies and the aluminum headspace caps with grey butyl/PTFE were purchased from VWR Company.

3.3 Samples

There were two different plants used in this research: *Passiflora tetrandra* and *Passiflora edulis*. The different parts of *P. tetrandra* were investigated: leaves of both males and females, flowers of females and the fruits of females. *P. tetrandra* samples were obtained from Oratia Native Plant Nursery Ltd and the fruit of *P. edulis* was purchased from a local supermarket.

The male and female leaves were stored in the refrigerator with Ziploc bags. Before analysis, leaves were cut into small pieces and blended in rotating blade bench top coffee grinder for about 40 seconds at 2900 rpm. Both of two different kinds of passion fruits were opened and the pulp and seeds put into the blender to make the juice. Then the juice was transferred to glass bottles separately and stored at -18 ^{o}C until analysis.

3.4 Methods

3.4.1 Experiments on Leaves of *P. tetrandra*

3.4.1.1 SPME - Selection of Extraction Temperature and Time

In order to obtain high volatile compound recoveries and extraction efficiencies, extraction time and temperature were optimized. At first, the study was on the female leaves, and extractions were conducted at 20, 30, 40, 50 and 60 ^{o}C with a constant

extraction time of 10 min. When the most suitable extraction temperature (30 ^{o}C) was selected, then different extraction times at 30 ^{o}C of 10, 20, 40 and 60 min were tried. At last, the most suitable extraction time (10 min) was established. The male leaves were evaluated using the same extraction time and temperature. Each measurement was repeated three times.

3.4.1.2 SPME –Sampling

For each sampling (**Figure 13**), 0.55 ±0.03 g of blended leaves were weighed into a 20 mL headspace vial and tightly capped. After the sample had equilibrated for 30 min on a 30 ^{o}C hot plate, the syringe injector of SPME was inserted into the headspace of the sample and the fibre exposed for 10 min at 30 ^{o}C.

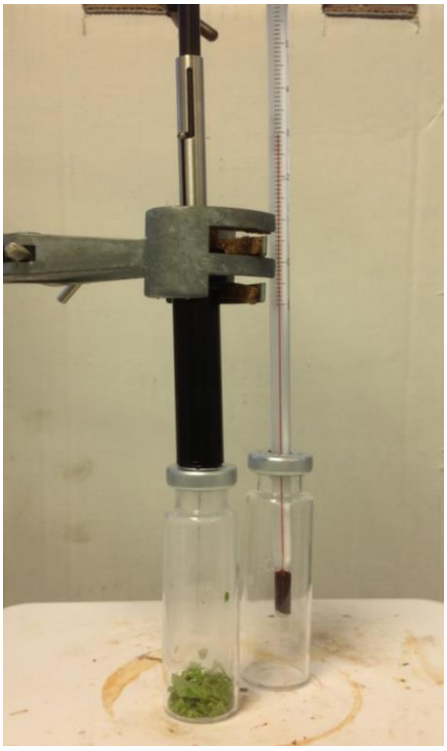


Figure 13 SPME sampling of *P. tetrandra* leaves

3.4.1.3 Gas Chromatography (GC) and Gas Chromatography – Mass Spectrometry (GC – MS) Analysis

After sampling, the SPME fibre was withdrawn into the needle and removed. Then the needle was inserted into the injector port at 200 ^{o}C and the fibre exposed to desorb the volatiles into the column of GC or GC-MS system. Although desorption is very fast a standard desorption time of 10 min was used for all samples.

GC conditions: The volatiles were analyzed by the Shimadzu GC-2010 gas

chromatography system equipped with a 30 m×0.25 mm i.d., 0.25 μm film thickness, ZB-5 fused-silica capillary column (5%-diphenyl-95%-Dimethylpolysiloxane Phase) (Phenomenex, Inc, Torrance, USA), coupled to a flame ionization detector (FID) (Japan). Nitrogen was used as the carrier gas, which was set at 74.3 kPa and the total flow rate was 34 mL/min. The mode of injections was splitless. The oven temperature was started from 40 °C with 1 min hold time, and it was heated to 230 °C at 5 °C/min, held 20 min at 230 °C. The temperature of injector and detector were held at 200 °C and 250 °C respectively.

GC – MS conditions: The Trace Ultra GC (Thermo Scientific, USA) was coupled with a 30 m×0.32 mm i.d., 0.50 μm film thickness, VF-5 ms low bleed/MS fused-silica capillary column (5%-Phenyl-95%-Dimethylpolysiloxane Phase) (Phenomenex, Inc, Torrance, USA). The mass spectrophotometer (Thermo Scientific, DSQ Series, USA) was performed for the detection. Helium was used as the carrier gas at a constant flow rate of 1.5 mL/min. The injector was splitless with a narrow bore splitless liner. The oven temperature was started from 40 °C with 1 min hold time, and it was heated to 230 °C at 5 °C/min, held 20 min at 230 °C. The temperature of injector and transfer line were held at 200 °C and 250 ^{o}C respectively. The mass spectrometer was operated with 70 eV electron ionisation mode, a source temperature of 200 °C. The mass scan range was from m/z 50 to m/z 650 at a rate of 500 amu/s.

3.4.2 Experiments on Flowers of *P. tetrandra*

When the flower buds on the branch come out, two different sampling ways were carried out. One was to extract the volatiles from fresh attached flowers (still on the branch) and the other one was done on the fresh excised flowers (cut the flowers off).

3.4.2.1 Attached Flowers Sampling

In this sampling, 3 fresh flowers on the branch were introduced into a glass chamber that fixed on the retort stand. Then both of top and bottom of the chamber were covered tightly with aluminium foil. After the flower sample in the chamber equilibrated for 2 hours at the room temperature, the syringe injector of SPME was inserted into the headspace of the sample and the fibre exposed for 3 different times: 10, 20, 30 min respectively (**Figure 14**).

Figure 14 SPME sampling of attached flowers of female *P. tetrandra*

3.4.2.2 Excised Flowers Sampling

For sampling, 3 fully developed fresh flowers were picked and immediately placed into a 20 mL headspace vial and tightly capped with cap containing grey butyl/PTFE septa.

The syringe injector of SPME was inserted into the headspace of the sample and the fibre exposed after an equilibration time of 2 hours at room temperature. Three different absorption times (10, 20, 30 min) were carried out and then the effect of the extraction time was determined (**Figure 15**).

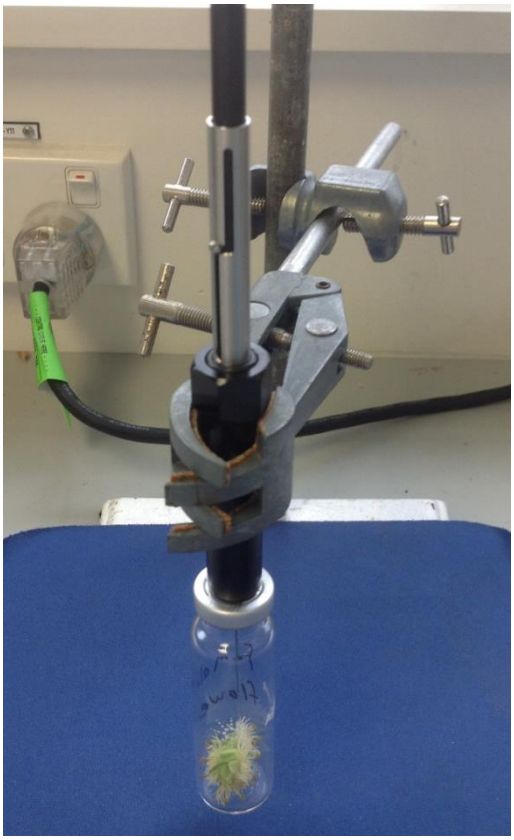


Figure 15 SPME sampling of excised flowers of female *P. tetrandra*

3.4.2.3 GC and GC – MS Analysis

When sampling was finished, the SPME carrier was taken out from the headspace of sample and analysed by GC or GC – MS. The procedures, conditions of GC and GC – MS and the method of volatile compounds identification were the same as that used in the analysis of *P. tetrandra* leaves using SPME. Each measurement was repeated three times.

3.4.3 Experiments on Fruits of *P. tetrandra* and *P. edulis* Sims

As a comparison and as a way of optimising the analytical method with a different, readily available passiflora species, *P. edulis* Sims (supermarket fruit) was analysed. Three extraction parameters were optimized in this part of the research: extraction time, extraction temperature and salt addition.

As noted above, the extraction time was conducted at 10, 20, 40 and 60 min with a constant extraction temperature of 30 ^{o}C. According to the results, the most suitable extraction time was 20 min. Then a series of extraction temperatures, 30, 40, 50 and 60 ^{o}C were tried and the best extraction time was found to be 20 min. After that, the preferred extraction temperature (30 ^{o}C) was used to investigate the effect of salt addition (0, 0.02, 0.05, 0.1 and 0.2 g of 99.5%w/w NaCl). In some other studies the addition of salt "salts out" organic volatiles and enhances recoveries. However, the total peak area decreased with the addition of salt (detail effects see next chapter). So no salt was used with passion fruits.

The *P. tetrandra* fruit juice was evaluated using the same most suitable extraction time and temperature (20 min, 30 ^{o}C） without salt addition. Each measurement was repeated three times.

3.4.3.1 Sampling

Approximately 2.6 g fruit juice was weighed in a 20 mL headspace vial with 2 mL deionised water and a magnetic stirring bar. Then the vial was tightly sealed with a septum. The sample was equilibrated for 30 min at 30 ^{o}C on the hot plate. Then the syringe injector of SPME was inserted into the headspace of the sample, and the fibre was exposed to the headspace of the sample for 20 min at 30 ^{o}C. All experiments (see **Figure 16**) were conducted under the constant stirring rate of 3000 rpm.

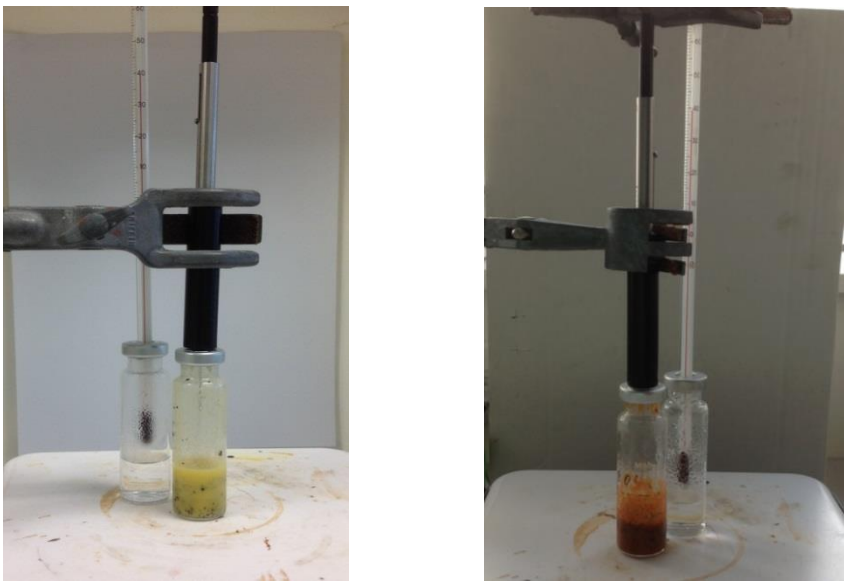


Figure 16 SPME sampling of fruits of *P. edulis* Sims (left) and *P. tetrandra* (right)

3.4.3.2 Gas Chromatography (GC) and Gas Chromatography – Mass Spectrometry (GC – MS) Analysis

When sampling was finished, the SPME device was taken out from the headspace of sample and then removed for GC or GC – MS analysis. The procedures and the method of volatile compounds identification were the same as that used in the analysis of *P. tetrandra* leaves using SPME, while the conditions of GC and GC – MS were a little different, which is shown in the following.

The conditions of GC and GC-MS applied in the analysis of passion fruit were nearly the same as that used in the analysis of leaves of passionfruit using SPME method. The only different one is the oven temperature programme. It was starting from 40 ^{o}C with a hold time of 1 min, then heated to 230 ^{o}C at a rate of 5 ^{o}C /min, held 10 min at 230 ^{o}C.

3.5 Volatile Compounds Identification

All the peaks were identified by comparing the MS spectrum of unknown peaks with those of the previously analysed authentic compounds stored in the NIST/EPA/NIH library. In order to confirm the peak identification, retention indices (RI) were also determined for each compound based on the retention time of compounds and C_7-C_{20} n-alkanes series. Then the RI of unknown peaks compared with that of previous analysed compounds, which were under similar conditions. The relative amounts of each compound were described as the percentage of peak area relative to total peak area and relative to the highest peak area (M Pontes, Marques, & Câmara, 2009).

Chapter 4 Results and Discussion

4.1 Leaves of *P. tetrandra*

4.1.1 Selection of Extraction Temperature and Extraction Time

During the process of optimizing extraction conditions, a number of variables were considered. In this work, the two main factors were studied including extraction temperature and extraction time.

The temperature of the sample can affect the diffusion of analytes into the fibre. Therefore, the selection of extraction temperature was worked out on female *P. tetrandra*, and the total peak area obtained from GC-MS for 10 min extraction at different temperature was shown in the followings (**Table 2** and **Figure 17**). The total peak area stands for the sum of peak area of all volatiles in three measurements.

Table 2 Total peak area of volatile compounds in female *P. tetrandra* leaves extracted for 10 min at different temperatures using SPME.

Repeat time	Different extraction temperature/°C				
	20	30	40	50	60
1	823498725	1339431296	780743647	1116674726	681452444
2	787535891	1270236954	813102828	1035323758	859865634
3	827799609	1369617014	928614640	1027693868	858392881
Total	2438834225	3979285264	2522461115	3179692352	2399710959
SD	22109538	50950026	77734512	49318334	102584399
Mean	812944742	1326428421	840820372	1059897451	799903653
RSD%	2.72	3.84	9.25	4.65	12.82

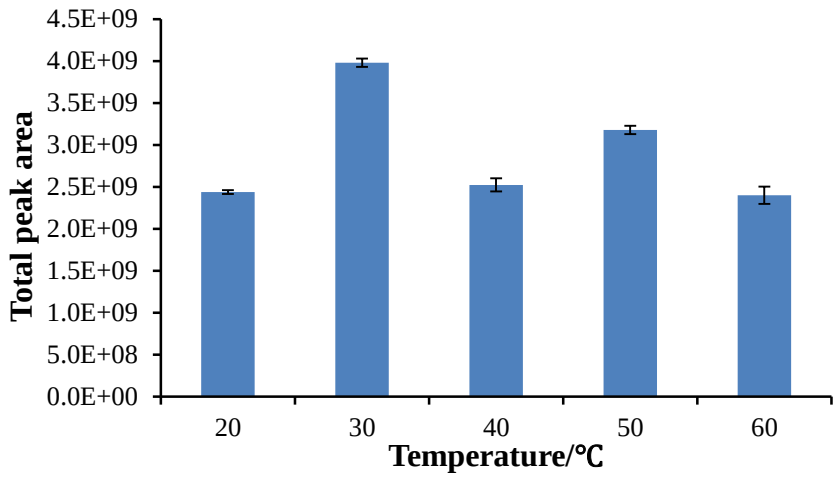


Figure 17 Influence of extraction temperature on SPME of volatile compounds in female *P. tetrandra* leaves with100 μm PDMS fibre (10 min extraction time). Error bars represent standard deviation (n=3 for each data point).

From the **Table 2** and **Figure 17**, it can be seen that the different extraction temperature for leaves influence the total area yield of three repeat times. As a result of the maximum area yield and a relative low standard deviation, the most suitable extraction temperature 30 ℃ was chosen for the next experiment.

The effect of extraction time has also been tested at 30 ℃ extraction temperature. The data of area yield is shown in **Table 3** and **Figure 18**.

Table 3 Total peak area of volatile compounds in female *P. tetrandra* leaves extracted for different times at 30 ℃ extraction temperature.

Repeat time	Different extraction time/min			
	10	20	40	60
1	1339431296	941559776	1719916909	958273666
2	1270236954	935985717	1757249031	1018003775
3	1369617014	1030883512	1622433036	1071751432
Total	3979285264	2908429005	5099598976	3048028873
SD	50950026	53253156	69608603	56765159
Mean	1326428421	969476335	1699866325	1016009624
RSD%	3.84	5.49	4.09	5.59

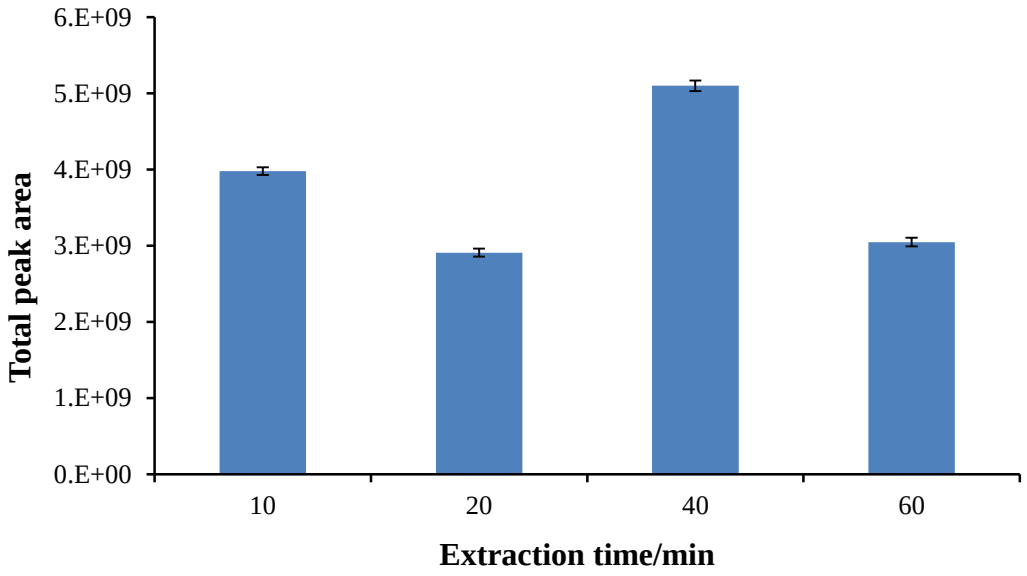


Figure 18 Effect of extraction time on SPME of volatile compounds in leaves of female *P. tetrandra* with100 μm PDMS fibre (30 ℃ extraction temperature). Error bars stand for standard deviation (n=3 for each data point).

Figure 18 illustrated that the maximum peak area yield was obtained at the extraction time of 40 min and the second highest yield was worked out at 10 min. Nevertheless, the standard deviation and relative standard deviation at 10 min were the lowest, and also the time of 10 min was a better choice to apply in the experiment operation. Therefore, the extraction time of 10 min and temperature of 30 °C were selected to be used in the sampling of male *P. tetrandra* leaves.

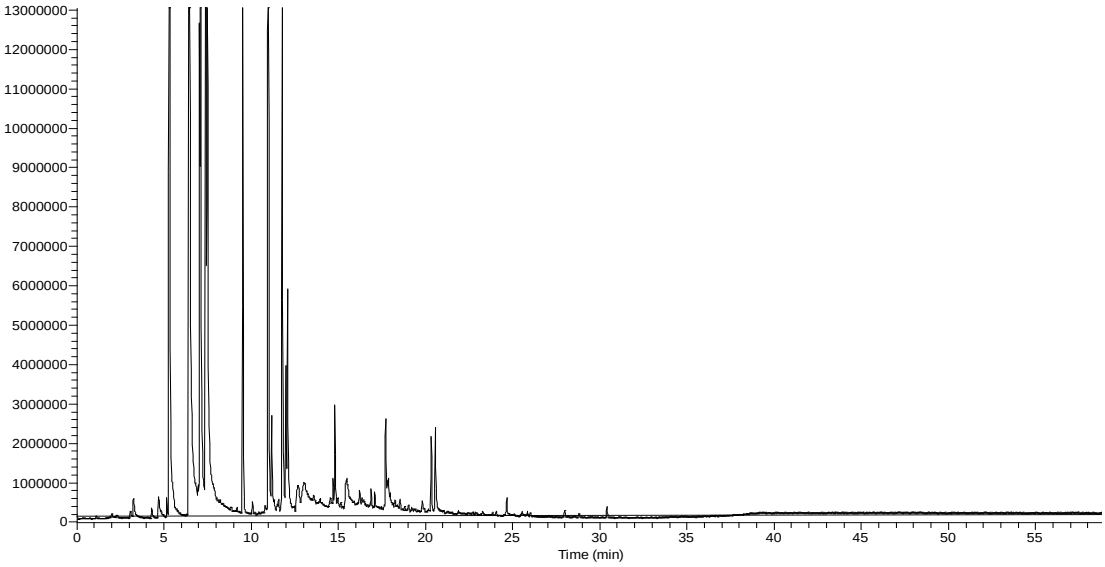
However, these results seem a little unreasonable, because the total peak area should increase with the increasing of temperature of sample and extraction time in principle. The possible explanation is that in each process of sample preparation and sampling new and fresh leaves of female Kohia were taken off, blended and used for the identification. That means the sample used was varied in each measurement. The preferred treatment is that blending a larger number, homogenising the mix and taking sub-samples.

4.1.2 Analysis of Volatile Compounds in Leaves

In the identification of volatile components, both male and female *P. tetrandra* leaves were analysed. With the optimised conditions, each sample was analysed three times and about twenty of volatile compounds were detected in the leaves. GC – MS chromatograms of male and female leaves obtained using PDMS fibre with the

experimental optimised conditions (extraction time: 10 min, extraction temperature: 30 °C) is shown in **Figure 19** and the identified compounds are described in **Table 4**.

(a)



(b)

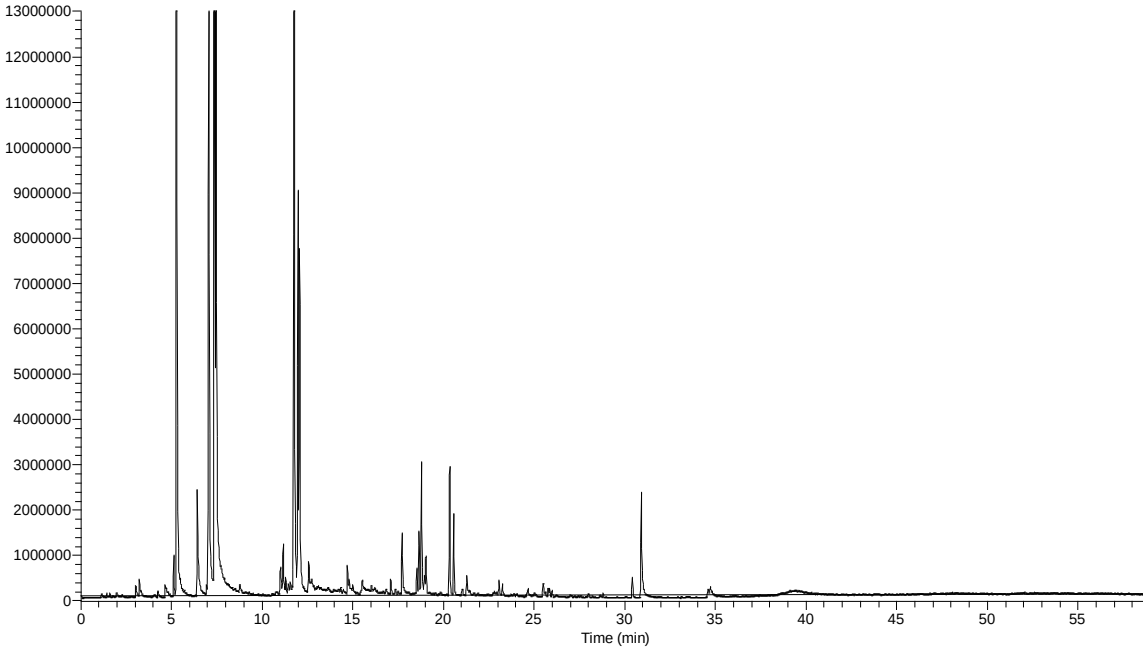


Figure 19 GC – MS chromatograms of volatiles in leaves of female Kohia (a) and male Kohia (b) using SPME technique with the experimental optimised conditions (extraction time: 10 min, extraction temperature: 30 ℃).

Table 4 The volatiles identified from the leaves of male and female Kohia (*P. tetrandra*) using SPME method.

RT (min)	CAS#	Formulas	Compound Names	RI_{ref} [a]	RI_{exp} [b]	Relative to total peak area (%)		Relative to highest peak area (%)		ID[c]	Odour (Nf = not found)
						Female leaves	Male leaves	Female leaves	Male leaves		
3.04	616-25-1	$C_5H_{10}O$	*1*-Penten-3-ol	672	<702	-	0.18	-	100	MS	bitter, mild green[f]
3.26	96-22-0	$C_5H_{10}O$	*3*-Pentanone	703	702	0.23	0.27	85	100	MS, RI	ether[d]
5.13	96-41-3	$C_5H_{10}O$	Cyclopentanol	781	788	0.13	0.40	33	100	MS, RI	Nf
5.28	120-92-3	C_5H_8O	Cyclopentanone	795	793	13.10	17.97	73	100	MS, RI	Nf
6.40	930-30-3	C_5H_6O	Cyclopentenone	802	835	31.49	2.01	100	6.4	MS, RI	Nf
7.03	6728-26-3	$C_6H_{10}O$	(*E*)-2-Hexenal	848	856	2.34	0.74	100	32	MS, RI	green, leaf[d]
7.05	928-96-1	$C_6H_{12}O$	(*Z*)-3-Hexen-1-ol	857	857	-	8.07	-	100	MS, RI	fresh, green grass-like[e]
7.11	544-12-7	$C_6H_{12}O$	(*E*)-3-Hexen-1-ol	856	859	5.43	0.12	100	2.2	MS, RI	fresh[d]
7.36	928-95-0	$C_6H_{12}O$	*2*-Hexenol	861	868	8.38	14.02	60	100	MS, RI	leaf, green, wine, fruit[d]
7.46	111-27-3	$C_6H_{14}O$	*1*-Hexanol	860	871	-	9.99	-	100	MS, RI	resin, flower, green[d]
7.50	4312-76-9	$C_6H_{14}O_2$	Hexyl hydroperoxide		872	5.08	-	100	-	MS	Nf
9.51	7785-70-8	$C_{10}H_{16}$	*1R*-α-Pinene		937	3.34	-	100	-	MS	pine, turpentine[d]
10.98	18172-67-3	$C_{10}H_{16}$	*L*-β-Pinene	990	983	8.04	-	100	-	MS, RI	pine, resin, turpentine[d]

11.02	3391-86-4	$C_8H_{16}O$	*3*-Octenol	983	984	-	0.41	-	100	MS, RI	champignon-like[e]
11.19	106-68-3	$C_8H_{16}O$	*3*-Octanone	990	989	0.80	0.70	100	88	MS, RI	herbal, buttery[e]
11.31	3777-69-3	$C_9H_{16}O$	*2*-Pentylfuran	994	993	-	0.26	-	100	MS, RI	green bean-like[e]
11.78	3681-71-8	$C_8H_{14}O_2$	*cis*-3-Hexenyl Acetate	1005	1008	5.26	14.06	37	100	MS, RI	powerful green[e]
12.00	142-92-7	$C_8H_{16}O_2$	Hexyl acetate	1010	1015	1.01	3.78	27	100	MS, RI	fruit, herb[d]
12.08	2497-18-9	$C_8H_{14}O_2$	*2*-Hexenyl acetate	1014	1018	2.19	4.26	51	100	MS, RI	Nf
12.57	104-76-7	$C_8H_{18}O$	*2*-Ethylhexanol	1024	1033	-	0.39	-	100	MS, RI	rose, green[d]
14.81	78-70-6	$C_{10}H_{18}O$	Linalol	1100	1104	0.74	-	100	-	MS, RI	flower, lavender[d]
16.24	N/A	$C_{10}H_{16}O$	Isopinocarveol		1152	0.13	-	100	-	MS	Nf
16.89	30460-92-5	$C_{10}H_{14}O$	Pinocarvone		1174	0.11	-	100	-	MS	Nf
17.72	112-40-3	$C_{12}H_{26}$	Dodecane	1200	1201	0.67	0.67	100	100	MS, RI	alkane[d]
18.54	432-25-7	$C_{10}H_{16}O$	*β*-Cyclocitral	1210	1230	-	0.31	-	100	MS, RI	mint[d]
18.80	35154-45-1	$C_{11}H_{20}O_2$	(*Z*)-3-Hexenyl 3-methylbutanoate		1239	-	1.72	-	100	MS	Nf
20.58	629-50-5	$C_{13}H_{28}$	Tridecane	1300	1301	0.71	0.91	78	100	MS, RI	alkane[d]
23.24	629-59-4	$C_{14}H_{30}$	Tetradecane	1400	1401	-	0.16	-	100	MS, RI	alkane[d]
Total compounds identified						19	22				
RSD (n=3)						0.04	0.03				

Notes:

-: not detected

a Retention indices from literature.

b Retention indices determined experimentally and relative to C_7-C_{20} hydrocarbons.

c Identification methods

d Odour description from Flavornet Database (Acree & Arn, 1984).

e Odour description from Jirovetz (Jirovetz et al., 2002).

f Odour description from handbook (Burdock, 1996).

Nf No reference found of the description of the odour

Through the optimized SPME method, there were 19 and 22 different volatile components identified from the female and male Kohia leaves respectively. These identified compounds were organized in different groups according to their chemical structures, such as alcohols, carbonyl compounds, monoterpenes and alkanes. It was concluded that alcohols and carbonyl compounds were the major volatile components and monoterpenes and alkanes were less. However, all of these compounds were the main part of the aroma in the studied female and male Kohia leaves and are potentially able to play an important role in their organoleptic profile.

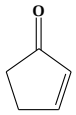
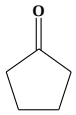
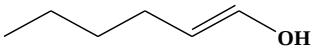
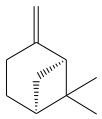
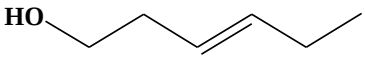
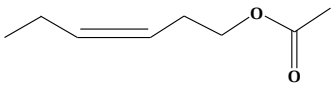
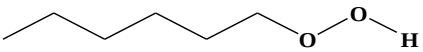
These volatiles were identified by matching the MS spectrum of unknown peaks with those of the previously analysed authentic compounds stored in the NIST/EPA/NIH library. In order to ensure the correct volatiles identification, retention indices (RI) were also determined for each compound based on the retention time of the peak and C7-C20 *n*-alkanes series. Then the RI of unknown peaks compared with that of previous analysed compounds, which were under similar conditions. **Table 4** gives the relative composition in the investigated leaves as a percentage relative to total peak area and the percentage relative to the compound with highest peak area in each Kohia leaves. This does not take account of the response factors of the individual components but because none were available as authentic standards it had to be assumed that all compounds had the same response factor and that the peak area was reasonably representative of the relative amounts in the head space.

Among the identified volatile compounds, there were 13 major volatiles in both of male and female Kohia leaves. They were *3*-pentanone, cyclopentanol, cyclopentanone, cyclopentenone, (*E*)-2-hexenal, (*E*)-3-hexen-1-ol, *2*-hexenol, *3*-octanone, *cis*-3-hexenyl acetate, hexyl acetate, *2*-hexenyl acetate, dodecane and tridecane with different relative contents. The other compounds in the different Kohia leaves such as *1R*-α-pinene, *L*-β-pinene, linalool and others also have been reported in the previous literature as the components in the plants (Majnooni, Gholivand, Nikbakht, & GR Bahrami, 2013; Palacios et al., 2009; Radulović, Đorđević, Zlatković, & Palić, 2009; Sajed, Sahebkar, & Iranshahi, 2012; Sheng-bi, 2011b).

Although female and male Kohia leaves shared 13 volatile compounds, the main compounds that composed the volatile composition were different. In the volatile composition of female Kohia leaves, 7 major compounds (details see **Table 5**) were assumed to constitute the aroma profile: cyclopentenone, cyclopentanone, *2*-hexenol, *L*-

β-pinene, (*E*)-3-hexen-1-ol, *cis*-3-hexenyl acetate and hexyl hydroperoxide, according to decreased percentage of relative content. The total percentage of these 7 major volatiles was about 75% of the total.

Table 5 Seven main volatiles in the female Kohia leaves

Compounds	Relative contents (%)	Structure	Odour (Nf = not found)
cyclopentenone	31.49		Nf
cyclopentanone	13.10		Nf
2-hexenol	8.38		leaf, green, wine, fruit
L-β-pinene	8.04		pine, resin, turpentine
(*E*)-3-hexen-1-ol	5.43		fresh
cis-3-hexenyl acetate	5.26		powerful green
hexyl hydroperoxide	5.08		Nf

In the female Kohia leaves, two volatile compounds cyclopentenone (31.49%) and cyclopentanone (13.10%) accounted for major components. Both of them are ketones. Stintzi et al. (2001) has investigated the role of cyclopentanones and cyclopentenones in the plant. They found both cyclopentanones and cyclopentenones can act alone or in concert with expression of defense genes. Jasmonic acid (JA) and 12-oxo-phytodienoic acid (OPDA) are both cyclopentenone precursors. Stintzi et al. (2001) analysed the levels of cyclopentanone JA and two cyclopentenone jasmonate family members OPDA and dnOPDA in wild-type leaves in response to mechanical wounding. Their study showed increased levels of JA, OPDA and dnOPDA responded to wounding.

2-Hexenol, (*E*)-3-hexen-1-ol and *cis*-3-hexenyl acetate, which are all derived from linolenic acid, accounted for 8.38%, 5.43% and 5.26% respectively in the volatiles of female Kohia leaves. Their biosynthesis in the plant is shown in **Figure 20**.

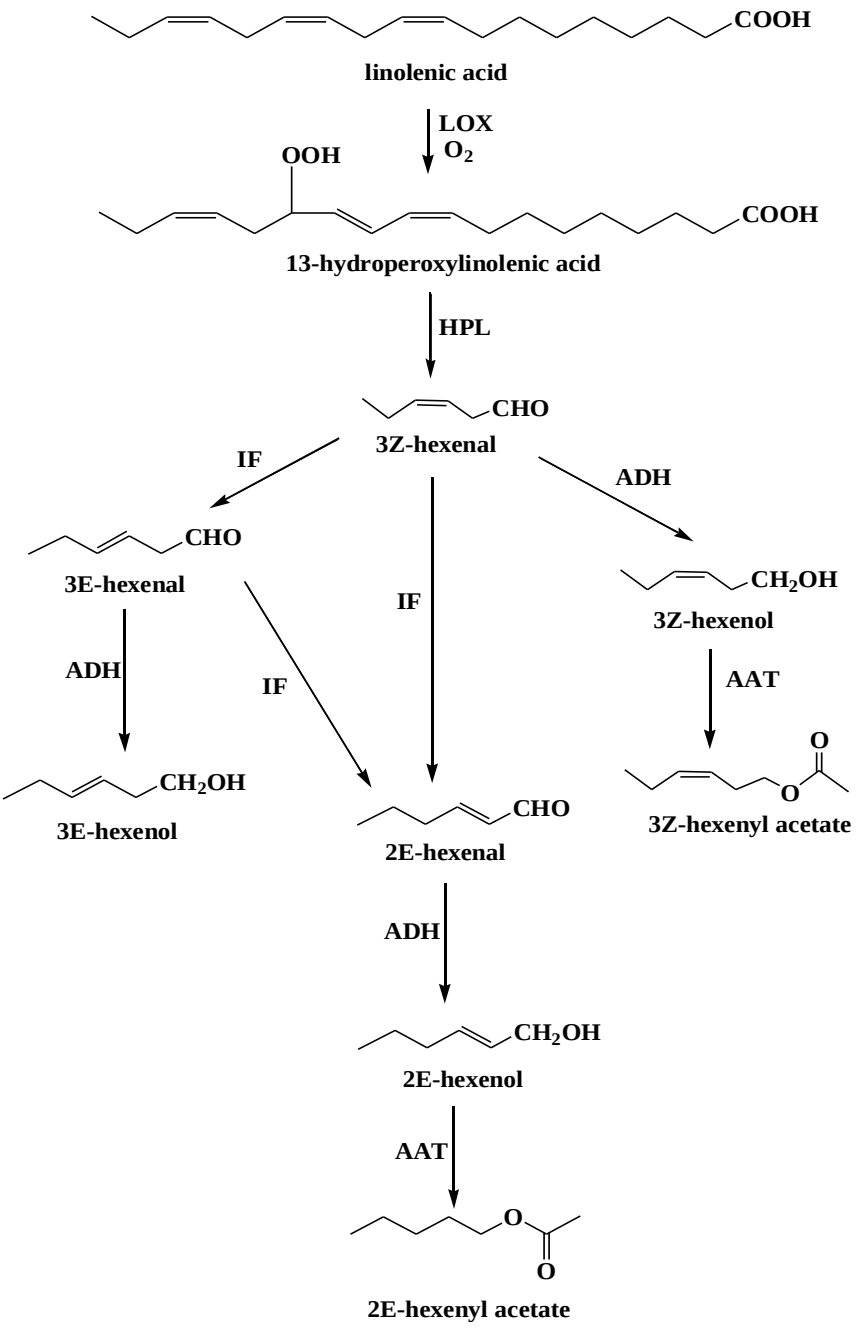


Figure 20 The biosynthesis of *2*-hexenol, *3E*-hexenol, *cis*-3-hexenyl acetate and *2*-hexenyl acetate from linolenic acid in plant. LOX-lipoxygenase, HPL-hydroperoxidelyase, IF-isomerization factor, ADH-alcohol dehydrogenase, AAT-alcohol acyl CoA transferase (Croft, Juttner, & Slusarenko, 1993).

Studies on the fresh and macerated tea leaves (Hatanaka & Harada, 1973) found *2*-hexenol in the macerated tea leaves, but not in fresh tea leaves. It can be supported by

the combination of this result and as shown in **Figure 20**, *3Z*-hexenal is isomerized to *2E*-hexenal, and then reduced to *2E*-hexenol by the enzyme of alcohol dehydrogenase (ADH). This result was confirmed by them using labelling experiments with incubation of linolenic acid-[U-^{14}C] with chloroplasts isolated from tea (Hatanaka, Kajiwara, & Sekiya, 1976). In 1968, reduction of *2E*-hexenal to *2E*-hexenol by another alcohol enzyme of NAD oxidoreductase (E. C. 1.1.1.1.) has also been reported by Eriksson (1968) in peas. Besides, *2*-hexenol was first identified in tomatoes by Nelson and Hoff (1969) and Granny Smith and Red Delicious apples (Rowan, Allen, Fielder, & Hunt, 1999).

3E-hexenal also can be modified to *3E*-hexenol directly by alcohol dehydrogenase (ADH). *3E*-hexenol, which is a leaf alcohol and has green and fresh notes, was found in Indian Walnut (*Juglans regia*) (Verma, Padalia, Chauhan, & Thul, 2013) and banana leaf (Kuo et al., 2006). *Cis*-3-hexenyl acetate was found to be the main volatile ester emitted by the flowers of the scented rose var. "Fragrant Cloud" (Shalit et al., 2003).

2-Hexenol and *3E*-hexenol have defense functions when the plant injured (Kokubo & Yamamoto, 2008), have done research that supports this function. The results show that the oxidation of ascorbic acid and biosynthesis of green odour including *2*-hexenol and *3E*-hexenol were two origins when the cucumber was cut and exposed to the air. In addition, *cis*-3-hexenyl acetate has also a defense function when the plant damaged. Turlings et al. (1998) have studied on the Maize (*Zea mays* L.) and found it released specific volatiles including *cis*-3-hexenyl acetate in response to herbivory by caterpillars.

β-pinene has two forms: *L*-β-pinene and *R*-β-pinene. Both of them are monoterpenes. The biosynthesis of monoterpenes is occurred in the chloroplastids or leucoplasts and all from the same fundamental precursor - isopentenyl diphosphate (IPP). There are two different pathways to form IPP: classical mevalonate pathway and non-mevalonate pathway (also called rohmer pathway) (Lindmark-Henriksson, 2003). Moreover, these two pathways have been confirmed by site-specific deuterium distribution (H/D-isotope portraits) (Gerdov et al., 2005). Biosynthesis of *β*-pinene can be divided into three steps: IPP synthesis (**Figure 21**), GPP synthesis (**Figure 22**) and formation of *β*-pinene and *α*-pinene (**Figure 23**) are shown in the following:

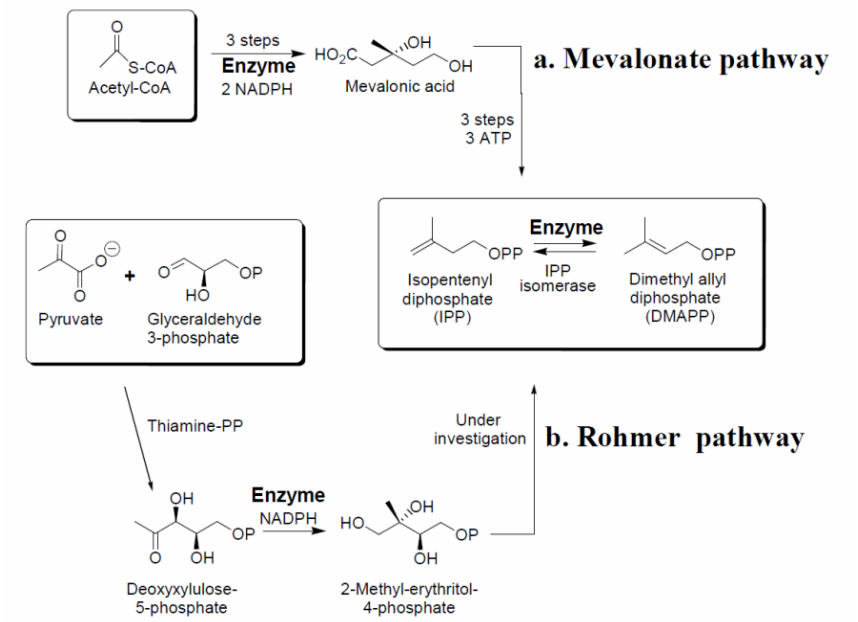


Figure 21 Biosynthesis of IPP. The diphosphate group is abbreviated as –OPP (Lindmark-Henriksson, 2003).

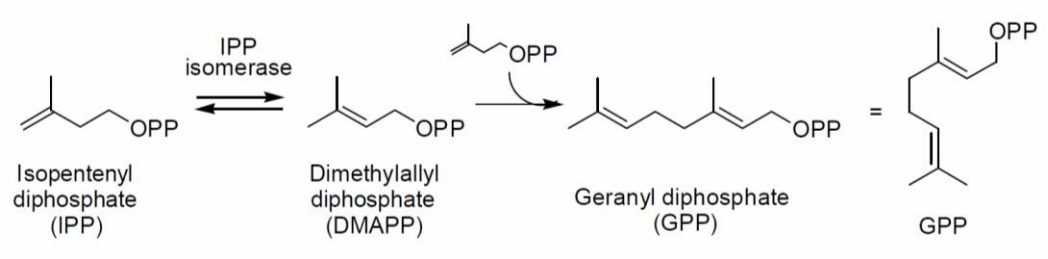


Figure 22 Biosynthesis of GPP (geranyl diphosphate) (Lindmark-Henriksson, 2003).

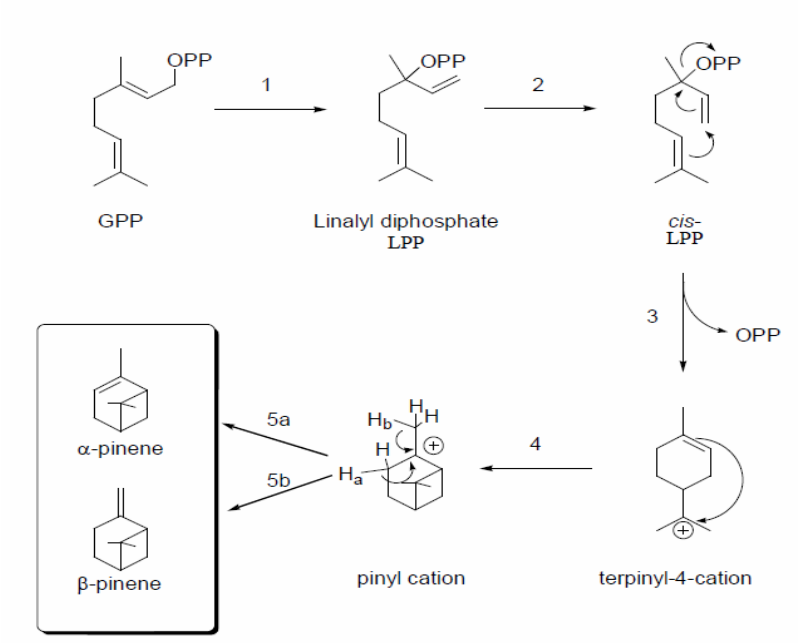


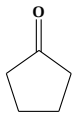
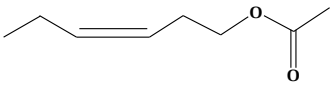
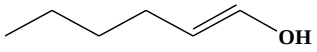
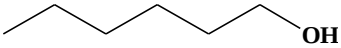
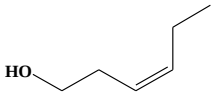
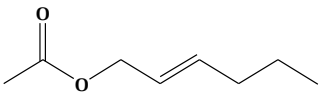
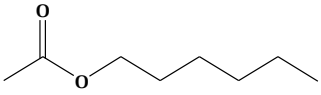
Figure 23 Biosynthesis of pinenes (Lindmark-Henriksson, 2003).

L-β-pinene, like other monoterpenes, is an important aroma and flavour in leaves, flowers and fruits. It was found in the neddles of *P. tabulaeformis* (H. Chen, Tang, Gao, Chen, & Li, 2006), avocado criollo fruit (*Persea americana* var. *drymifolia*) (Torres-Gurrola, Delgado-Lamas, & Espinosa-García, 2011) and also in the cortical tissues of five different Norway spruce clones (Silvestrini, Michelozzi, Skroppa, Brancaleoni, & Ciccioli, 2004). *L*-β-pinene has also been evaluated the toxicity against the mosquito larvae of *Culex pipiens* (Diptera: Culicidae) and house fly, *Musca Domestica* L. (Palacios et al., 2009).

The rather surprising hexyl hydroperoxide accounted for 5.08% in the volatile composition of female Kohia leaves. In the previous literature, there was a limited amount of research that reported the hexyl hydroperoxide. The only one could be found was the research done by Sheng-bi et al. (2011a). They found this volatile compound was contained in the essential oils of *Trifolium repens* L. obtained by a combination method of steam distillation and solvent extraction.

Unlike the female Kohia leaves, male Kohia leaves were characterised by 22 volatile compounds. The 7 dominant components (details see **Table 6**) found, which of total content exceed 60%, were cyclopentanone, *cis*-3-hexenyl acetate, *2*-hexenol, *1*-hexanol, (*Z*)-3-hexen-1-ol, *2*-hexenyl acetate and hexyl acetate in the decreased order of relative content.

Table 6 Seven main volatiles in the male Kohia leaves

Compounds	Relative contents (%)	Structure	Odour (Nf = not found)
cyclopentanone	17.97		Nf
cis-3-hexenyl acetate	14.06		powerful green
2-hexenol	14.02		leaf, green, wine, fruit
1-hexanol	9.99		resin, flower, green
(*Z*)-3-hexen-1-ol	8.07		fresh, green grass-like
2-hexenyl acetate	4.26		Nf
hexyl acetate	3.78		fruit, herb

Cyclopentanone, *cis*-3-hexenyl acetate, *2*-hexenol and (*Z*)-3-hexen-1-ol were also found in the female Kohia leaves but with different relative contents, and have been discussed in the above section. Therefore, these four compounds will not be discussed in the following.

1-Hexanol and *2*-hexenyl acetate are derived from linolenic acid, as well as other green leaf alcohols and aldehydes. In the process, alcohol dehydrogenase converts hexanal to *1*-hexanol and *2E*-hexenol is transformed to *2*-hexenyl acetate by alcohol acyl transferase (AAT). The biosynthesis of hexanal and *2*-hexenyl acetate was shown in **Figure 20** Hexyl acetate is derived from linoleic acid after leaf wounding (**Figure 24)** and supported by Fall et al. (1999).

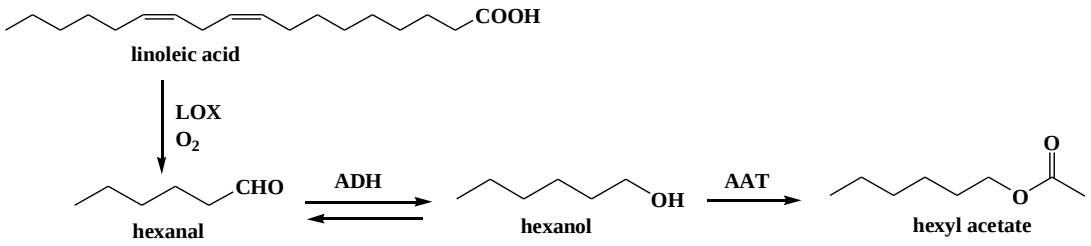


Figure 24 Biosynthesis of hexyl acetate from linoleic acid (Fall et al., 1999).

1-Hexanol can be found in many plant species. It has been reported as a volatile compound of potato foliage (*Solanum tuberosum* L.) (Visser, Van Straten, & Maarse, 1979), *Rosa* (EM Dobson, Bergström, Bergström, & Groth, 1987) and also one of the mixture volatiles that emitted by cabbage plants in response to caterpillar predation. These volatiles are thought to attract parasitic wasps that attack the herbivores (Mattiacci, Dicke, & Posthumus, 1995).

2-Hexenyl acetate has been reported in the charentais melon (*Cucumis melo* var. *cantalupensis*) (El-Sharkawy et al., 2005). Both of *2*-hexenyl acetate and hexyl acetate were found in the Cv. Anna Apples (Lurie, Pre-Aymard, Ravid, Larkov, & Fallik, 2002) and roses (Shalit et al., 2003).

4.2 Flowers of *P. tetrandra*

4.2.1 Attached female flowers of *P. tetrandra*

In the analysis of volatile compounds in attached *P. tetrandra* female flowers, only one parameter (extraction time) was optimised. It was carried out three times for each sample at room temperature. The results are shown in **Table 7** and **Figure 25**.

Table 7 Total peak area of volatile compounds in attached female *P. tetrandra* flowers extracted for different time at room temperature.

Repeat time	Different extraction time/min		
	10	20	30
1	2145041374	3585896896	1841943001
2	3216172639	3280007774	1871820292
3	3911442839	2969212066	1896950849
Total	9272656852	9835116736	5610714142
SD	889840526	308345668	27538037
Mean	3090885617	3278372245	1870238047
RSD%	28.79	9.41	1.47

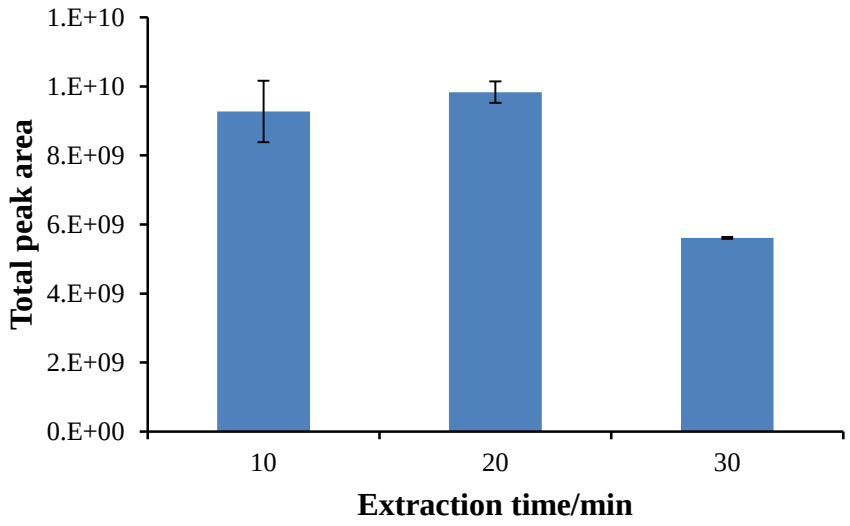


Figure 25 Effect of extraction time on SPME of volatile compounds in attached flowers of female *P. tetrandra* with 100 μm PDMS fibre at room temperature. Error bars stand for standard deviation (n=3 for each data point).

The data indicated the peak area reached the maximum value with a good standard deviation at the extraction time of 20 min. Thus 20 min was selected for extraction and analysis.

4.2.2 Excised flowers of female *P. tetrandra*

In this section, the extraction parameter optimised was extraction time as well. It was conducted with three different extraction time (10, 20, 30 min) at room temperature. The peak area yield was show in **Table 8** and **Figure 26**.

Table 8 Total peak area of volatile compounds in excised female *P. tetrandra* flowers extracted for different time at room temperature.

Repeat time	Different extraction time/min		
	10	20	30
1	8004937824	10243478475	13650765785
2	9690152482	11876257574	10519756185
3	11443667245	13455104568	12256740040
Total	29138757551	35574840617	36427262010
SD	1719477755	1605888517	1568632196
Mean	9712919184	11858280206	12142420670
RSD%	17.70	13.54	12.92

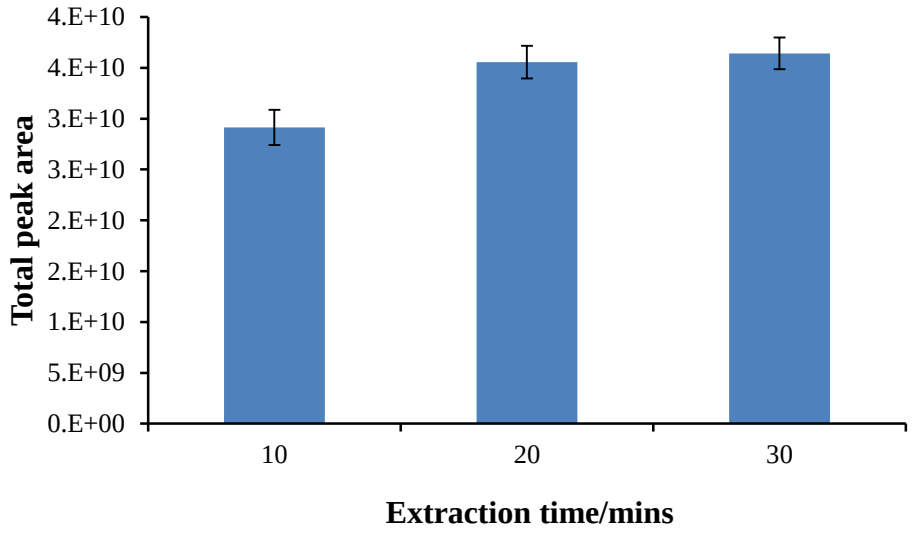


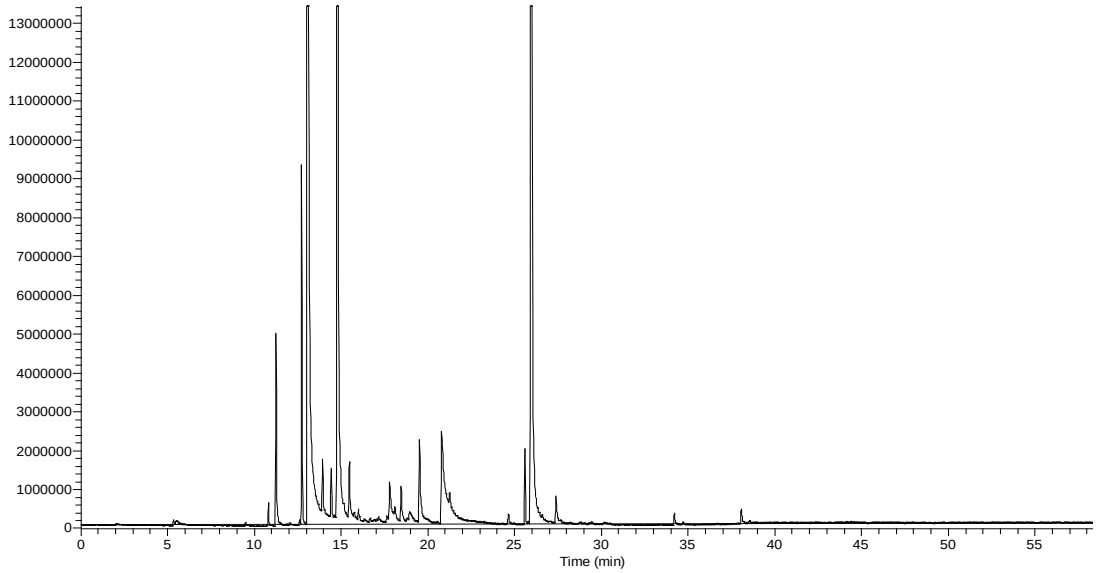
Figure 26 Effect of extraction time on SPME of volatile compounds in excised flowers of female *P. tetrandra* with 100 μm PDMS fibre at room temperature. Error bars stand for standard deviation (n=3 for each data point).

The results show that the total peak area increased with increasing extraction time between 10 and 30 min. When the time was at 20 and 30 min, almost maintained peak area was observed from the response. For the shorter sampling and analysis time, 20 min of extraction time was selected.

4.2.3 Analysis of Volatile Compounds in Flowers

The volatiles emitted from both attached and excised flowers of female *P. tetrandra* were investigated. Then the PDMS fibre with the optimised extraction time of 20 min was also used in the investigation and each sample was analysed three times. The chromatograms (see **Figure 27**) from GC – MS were obtained with the conditions described in chapter 2. The volatiles identified were shown in **Table 9**.

(a)



(b)

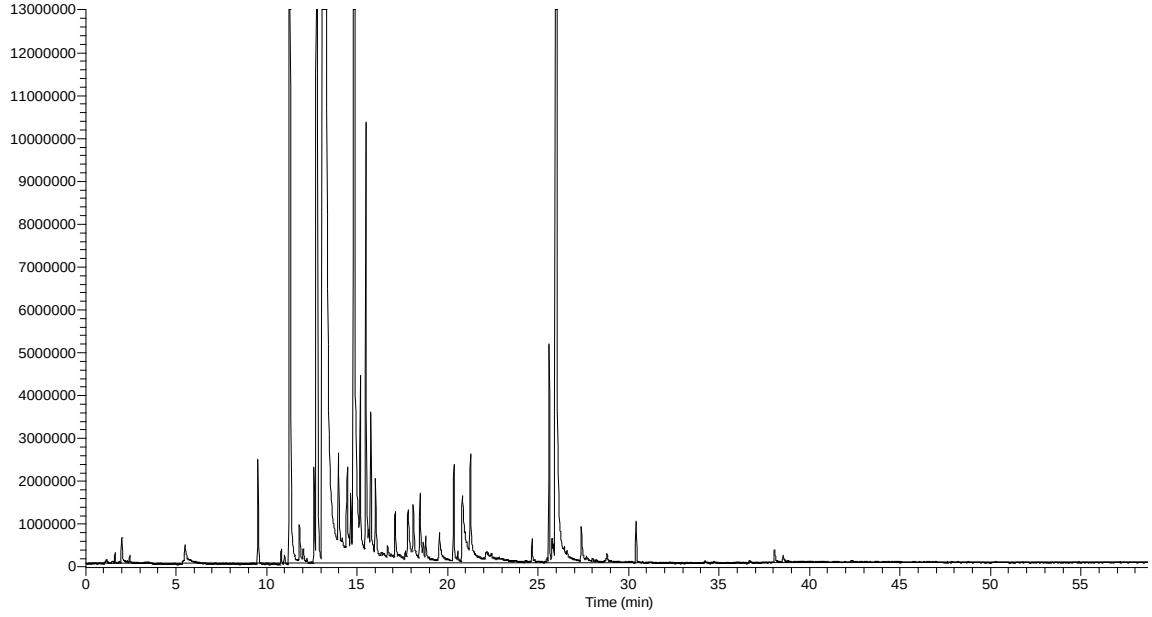


Figure 27 GC – MS chromatograms of volatiles in attached flowers (a) and excised flowers (b) of female Kohia extracted by SPME technique with 20 min extraction time at room temperature.

Table 9 The volatiles identified from the attached and excised female Kohia flowers (*P. tetrandra*) using SPME method.

RT (min)	CAS#	Formulas	Compound Names	RI_{ref} [a]	RI_{exp} [b]	Relative to total peak area (%)		Relative to highest peak area (%)		ID[c]	Odour (Nf = not found)
						Attached flowers	Excised flowers	Attached flowers	Excised flowers		
5.48	141-79-7	$C_6H_{10}O$	Mesityl oxide	800	803	-	0.02	-	100	MS, RI	sweet, chemical[d]
9.53	7785-70-8	$C_{10}H_{16}$	*1R*-α-Pinene		961	-	0.07	-	100	MS	pine, turpentine[d]
11.27	18172-67-3	$C_{10}H_{16}$	*L*-β-Pinene	990	992	0.56	2.39	23	100	MS, RI	pine, resin, turpentine[d]
11.82	3681-71-8	$C_8H_{14}O_2$	*cis*-3-Hexenyl Acetate	1005	1009	-	0.05	-	100	MS, RI	Powerful green[e]
12.63	10198-23-9	$C_{12}H_{20}O_2$	*β*-Terpinyl acetate		1035	-	0.06	-	100	MS	Nf
12.73	3779-61-1	$C_{10}H_{16}$	(*E*)-Ocimene	1049	1038	0.87	2.65	33	100	MS, RI	sweet, herb[d]
13.10	3338-55-4	$C_{10}H_{17}$	(*Z*)-Ocimene	1040	1050	51.64	76.99	67	100	MS, RI	citrus, herb, flower[d]
13.96	5989-33-3	$C_{10}H_{18}O_2$	*cis*-Linalool Oxide	1075	1077	0.19	0.07	100	37	MS, RI	flower[d]
14.83	78-70-6	$C_{10}H_{18}O$	Linalool	1100	1105	10.68	5.15	100	48	MS, RI	flower, lavender[d]
15.18	95452-08-7	$C_{11}H_{18}$	*1,1*-Dimethyl-3-methylene-2-vinylcyclohexane		1116	-	0.13	-	100	MS	Nf
15.48	111-11-5	$C_9H_{18}O_2$	Methyl octanoate	1132	1126	0.22	0.31	71	100	MS, RI	orange[d]
15.77	460-01-5	$C_{10}H_{14}$	(*3E,5E*)-2,6-Dimethyl-1,3,5,7-octatetraene	1134	1136	-	0.11	-	100	MS, RI	Nf

18.11	N/A	$C_{10}H_{16}O$	*E,E*-2,6-Dimethyl-3,5,7-octatriene-2-ol		1215	-	0.06	-	100	MS	Nf
20.78	274-40-8	C_8H_7N	Indolizine		1309	0.90	0.17	100	19	MS	Nf
21.27	110-42-9	$C_{11}H_{22}O_2$	Methyl decanoate	1324	1327	-	0.11	-	100	MS, RI	wine[d]
25.59	17699-05-7	$C_{15}H_{24}$	*α*-Bergamotene	1430	1494	0.18	-	100	-	MS, RI	Nf
25.61	26560-14-5	$C_{15}H_{24}$	(*Z,E*)-α-Farnesene	1496	1494	-	0.12	-	100	MS, RI	Nf
26.00	502-61-4	$C_{15}H_{24}$	*α*-Farnesene	1508	1510	32.20	10.67	100	33	MS, RI	wood, sweet[d]
27.38	40716-66-3	$C_{15}H_{26}O$	±-trans-Nerolidol	1569	1568	0.13	-	100	-	MS, RI	wood, flower, wax[d]
27.40	142-50-7	$C_{15}H_{26}O$	Nerolidol	1533	1569	-	0.03	-	100	MS, RI	Nf
Total compounds identified						10	18				
RSD (n=3)						0.09	0.13				

Notes:

-: not detected

a Retention indices from literature.

b Retention indices determined experimentally and relative to C_7-C_{20} hydrocarbons.

c Identification methods

d Odour description from Flavornet Database (Acree & Arn, 1984).

e Odour description from literature (Jirovetz et al., 2002).

Nf No reference found of the description of the odour

According to the optimized SPME method and GC-MS analysis, there were 10 and 18 different volatile components isolated and identified from the attached and excised female Kohia flowers respectively. Their compositions have similar principal components but vary in the concentrations of the lesser components. The identified compounds can be organized in different groups according to their chemical structures, such as monoterpenes, sesquiterpenes, esters, alcohols and alkaloids. It was found that monoterpenes and sesquiterpenes were the major volatile compounds but esters, alcohols and alkaloids being relatively less. However, all of these compounds were the main part of the aroma in the studied attached and excised female Kohia flowers and also they were able to take a significant responsibility for their organoleptic profile.

These volatiles were identified by the NIST/EPA/NIH library and retention indices (RI). **Table 9** described the composition in the investigated flowers and the relative composition is shown as the percentage relative to total peak area and the percentage relative to the compound with highest peak area in each flower with different sampling (as with the leaf results).

Among the identified volatile compounds, there were 8 volatile components found in both attached and excised female Kohia flowers. They were (*Z*)-ocimene, *L*-β-pinene, (*E*)-ocimene, *cis*-linalool oxide, linalool, methyl octanoate, indolizine and *α*-farnesene with different relative contents. The other compounds in the flowers such as *1R*-α-pinene and *α*-bergamotene also have been reported in many other plants (Bestmann, Winkler, & Helversen, 1997; Jayaprakasha, Rao, & Sakariah, 2002; Michaelakis et al., 2009; Zheng et al., 1990).

In the volatile composition of attached female Kohia flowers, 3 major compounds (details see **Table 10**) composed the aroma profile: (*Z*)-ocimene, *α*-farnesene and linalool. The total percentage of these 3 major volatiles was above 90%. The relative contents of other compounds were all less than 1% each.

In the composition of volatile compounds in excised flowers of female Kohia, 5 main components were composed the majority of aroma characteristics. They were (*Z*)-ocimene, *α*-farnesene, linalool, (*E*)-ocimene and *L*-β-pinene in the order of decreasing relative contents and the structures are shown below (**Table 11**). The total percentage of these five compounds in the volatile composition is reached above 95%. The relative amount of other each component was all less than 1%. Therefore, just these 5 main constituents will be discussed in the following.

Table 10 Three main volatile compounds in attached flower of female Kohia

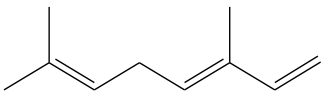
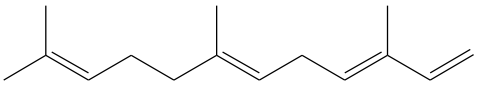
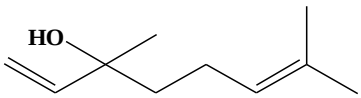
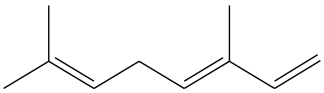
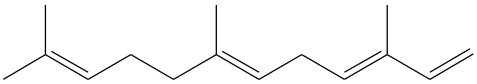
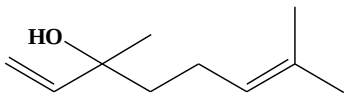
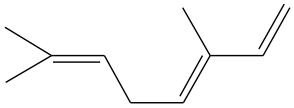
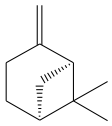
Compounds	Relative contents (%)	Structures	Odour (Nf = not found)
(*Z*)-ocimene	51.64		citrus, herb, flower
α-farnesene	32.20		wood, sweet
linalool	10.68		flower, lavender

Table 11 Five main volatiles in the excised flowers of female Kohia

Compounds	Relative contents (%)	Structures	Odour (Nf = not found)
(*Z*)-ocimene	76.99		citrus, herb, flower
α-farnesene	10.67		wood, sweet
linalool	5.15		flower, lavender
(*E*)-ocimene	2.65		sweet, herb
L-β-pinene	2.39		pine, resin, turpentine

(*Z*)-ocimene, (*E*)-ocimene and *L*-β-pinene are all monoterpenes. *L*-β-pinene was reported in the volatile composition of female Kohia leaves and also has been discussed before. (*Z*)-ocimene and (*E*)-ocimene are two of most common volatile chemicals released from plants. The formation mechanisms (**Figure 28**) of these two compounds were studied by Fäldt et al. in *Arabidopsis thaliana* (L.) (Fäldt, Arimura, Gershenzon, Takabayashi, & Bohlmann, 2003).

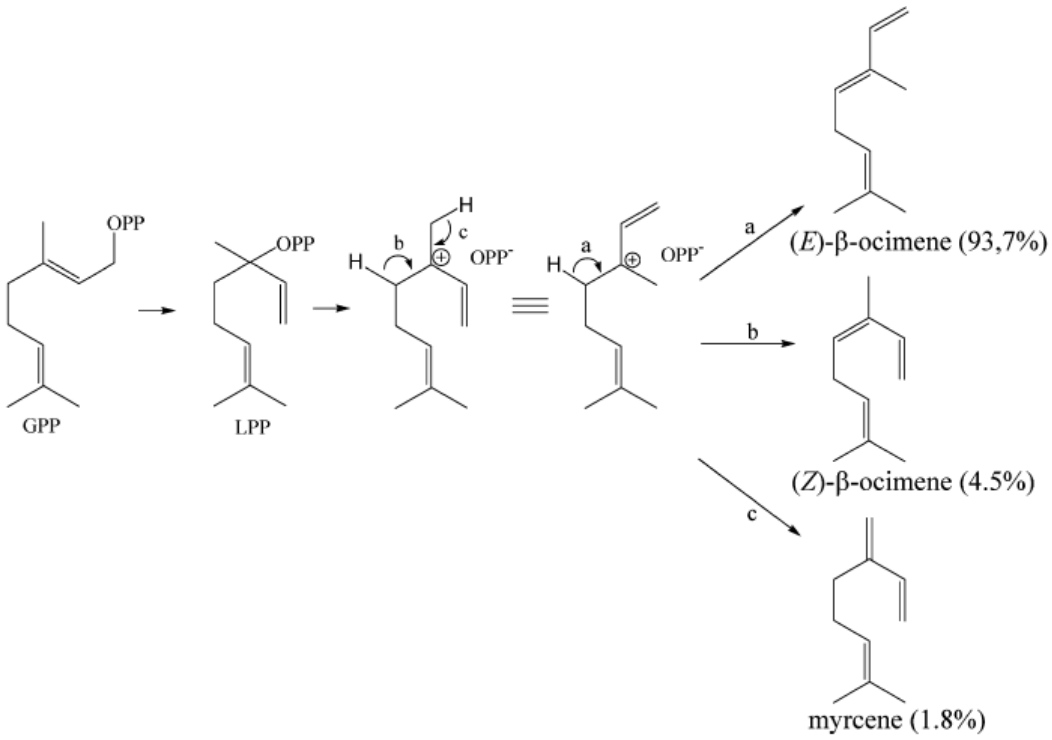


Figure 28 Biosynthesis mechanism for the formation of (*E*)-β-ocimene and (*Z*)-β-ocimene, from geranyl diphosphate (GPP) by AtTPS03 in *Arabidopsis thaliana* (L.) (Fäldt et al., 2003).

In this process, OPP denotes the diphosphate moiety. Formation of monoterpenoids is initiated by the ionization of GPP and subsequent isomerization to form linalyl diphosphate (LPP). Then the resulting linalyl carbocation can undergo deprotonation at the C4 methylene group (a, b) to form (*E*)-β-ocimene or (*Z*)-β-ocimene (Fäldt et al., 2003). Both of these two monoterpenes have been identified in the Arabidopsis flowers (F. Chen et al., 2003), lima bean leaves (Arimura et al., 2000) and *Haplophyllum tuberculatum* (Al-Burtamani, Fatope, Marwah, Onifade, & Al-Saidi, 2005).

(*Z*)-β-ocimene (highest amount in flower volatile composition) and (*E*)-β-ocimene were found as volatile chemicals emitted by plants in response to herbivory (Paré & Tumlinson, 1999). For instance, when lima bean plant was infested with two-spotted spider mites, it released (*E*)-β-ocimene and (*Z*)-β-ocimene to attract predatory mites, which is a natural enemy of two-spotted spider mites (Dicke, Sabelis, Takabayashi, Bruin, & Posthumus, 1990). Besides, (*E*)-β-ocimene and (*Z*)-β-ocimene were also found to be plant-to-plant signals that can up-regulate the pathways of ethylene and jasmonic acid in the uninfested lima bean leaves (Arimura et al., 2000). In addition to the lima bean plant, (*Z*)-β-ocimene was also found in peonies and contributes a lot to the floral fragrance (Li, Chen, Xu, Wang, & Wang, 2012).

The volatile with second highest amount in the female Kohia flowers was *α*-farnesene. It is an acyclic sesquiterpene hydrocarbon. Rupasinghe et al. (1998) found that *α*-farnesene was derived from trans,trans-farnesyl pyrophosphate (FPP) in 'Delicious' apple skin using vivo labeling studies. The study also showed *α*-farnesene was converted from FPP by trans,trans-α-farnesene synthase (a sesquiterpene synthase enzyme), not via farnesol (an intermediate) (Rupasinghe et al., 1998). The biosynthetic pathway of *α*-farnesene in apple skin is given in **Figure 29**.

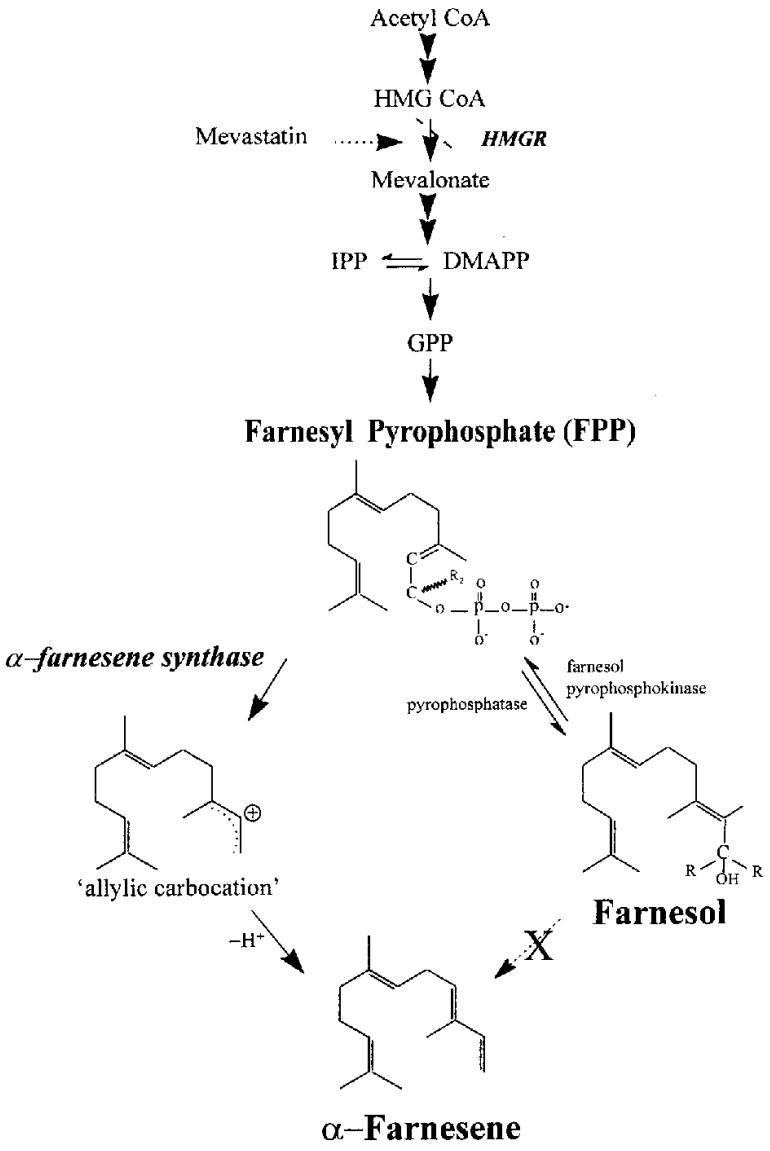


Figure 29 The biosynthesis of α-farnesene in apple skin tissue. HMG CoA-3-hydroxy-3-methylglutaryl-CoA, HMGR-HMG CoA reductase (Rupasinghe et al., 1998).

In addition to apple skin, *α*-farnesene also can be found in the petals of many peony cultivars (Li et al., 2012) and *Michelia alba* flowers (Shang, Hu, Deng, & Hu, 2002) with low relative content. The role of *α*-farnesene in the host plant on codling moth

(*Cydia pomonella* L.) has been tested by Bradley and Suckling (1995). The results showed that α-farnesene was a very important mediator for behavioural actions of neonate larvae guided to the source of α-farnesene. The α-farnesene dosages in response of female and male codling moth were studied, and the results showed that females were attracted to low dosages and repelled by high dosages. Males had no responses to low dosages or high dosages (Hern & Dorn, 1999). However, the content of α-farnesene in plant is often not very high.

Linalool is also an important volatile component in the studied female Kohia flowers. It is an acyclic monoterpene alcohol and can be found in many flowers and spice plants. The formation of linalool is initially by ionization of GPP and catalyzed by linalool synthase (a monoterpene synthase). This linalool synthase was first reported to be separated and characterized in the flower of *Clarkia breweri* (Pichersky, Lewinsohn, & Croteau, 1995). The formation of linalool was shown below (**Figure 30**).

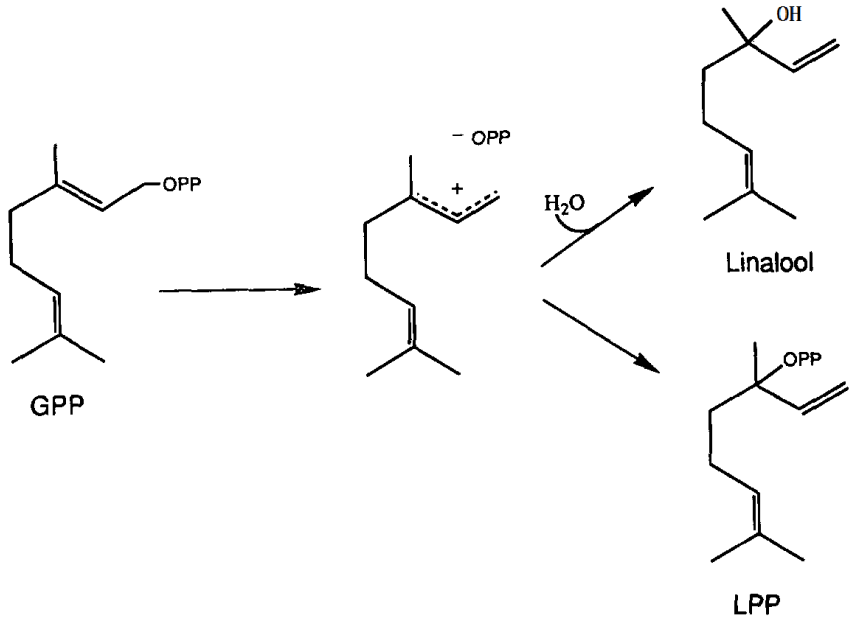


Figure 30 Formation of linalool (Pichersky et al., 1995)

Linalool occurs in a wide number of flowering plant families, examples are the flower buds of *Jasminum sambac* (Moon et al., 1994), flower of *Myrtus communis* L. (Jerkovic, Radonic, & Borcic, 2002), fresh elder flower of *Sambucus nigra* L. (Jørgensen, Hansen, Christensen, Jensen, & Kaack, 2000), etc. The fungistatic effect of linalool was found in rough lemon (*Citrus jambhiri* Lush) (Yamasaki, Kunoh, Yamamoto, & Akimitsu, 2007). In 2012, the antifungal activity of linalool was also investigated and the results showed that linalool can inhibit the formation of *C. albicans* biofilms and reduce existing *C.*

albicans biofilms (Hsu, Lai, Chuang, Lee, & Tsai, 2013). Besides, Yamasaki et al. found that linalool had insect-repellant effects on wild-type *Drosophila melanogaster* (Yamasaki et al., 2007).

4.3 Fruits of *P. tetrandra* and *P. edulis* Sims

4.3.1 Optimising of extraction conditions

The SPME extraction conditions (extraction time, extraction temperature and salt addition) were investigated. This was first investigated using the readily available fruit of *P. edulis* Sims.

These results for extraction time (see **Table 12** and **Figure 31**), temperature (see **Table 13** and **Figure 32**) and salt addition (see **Table 14** and **Figure 33**) on the peak area of volatile compounds were analysed and shown in the following tables.

Table 12 Total peak area of volatile compounds in *P. edulis* Sims fruit extracted for different time (10, 20, 40 and 60 min) at 30 ℃ of extraction temperature without salt addition.

Repeat time	Different extraction time/min			
	10	20	40	60
1	1407841342	2511681703	3467007390	3452857084
2	1417940913	2625914667	3238116360	3352800893
3	1456133411	2721558951	3146516630	3188355768
Total	4281915666	7859155321	9851640380	9994013745
SD	25471527	105075733	165073691	133550482
Mean	1427305222	2619718440	3283880127	3331337915
RSD%	1.78	4.01	5.03	4.01

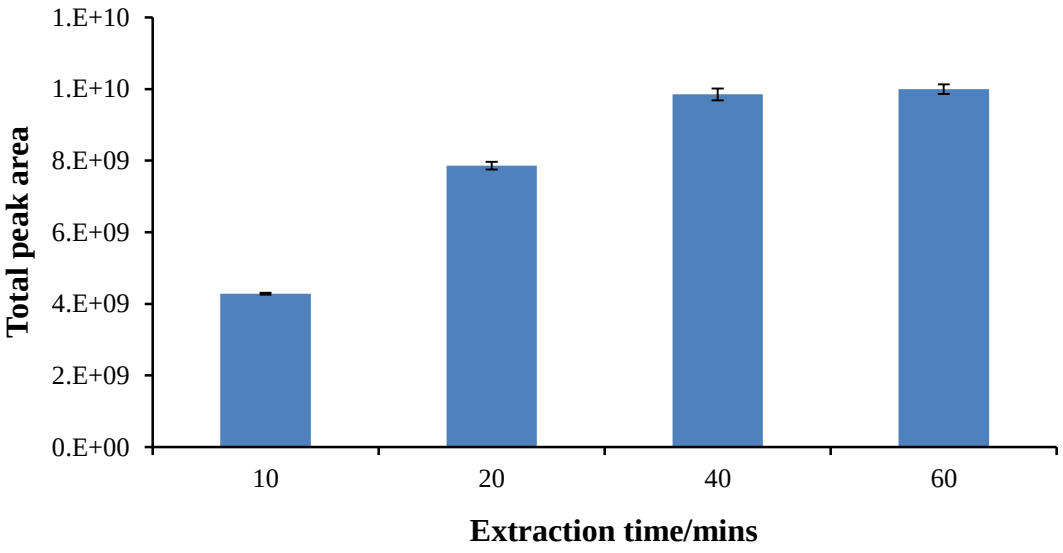


Figure 31 Effect of extraction time on SPME of volatile compounds in *P. edulis* Sims fruits with 100 µm PDMS fibre at 30 ℃ extraction temperature without salt addition. Error bars stand for standard deviation (n=3 for each data point).

The total peak area increased with increasing extraction time up to about 40 min however for greater consistency with previous results 20 min extraction time was selected.

Table 13 Total peak area of volatile compounds in *P. edulis* Sims fruit extracted for 20 min at different extraction temperature (30, 40, 50 and 60 ℃) without salt addition.

Repeat time	Different extraction temperature/°C			
	30	40	50	60
1	2511681703	5286281326	5879798760	5512027137
2	2625914667	4741087516	5196259172	6137631681
3	2721558951	5128710636	6364339572	6353580796
Total	7859155321	15156079478	17440397504	18003239614
SD	105075733	280569806	586858584	437078869
Mean	2619718440	5052026493	5813465835	6001079871
RSD%	4.01	5.55	10.09	7.28

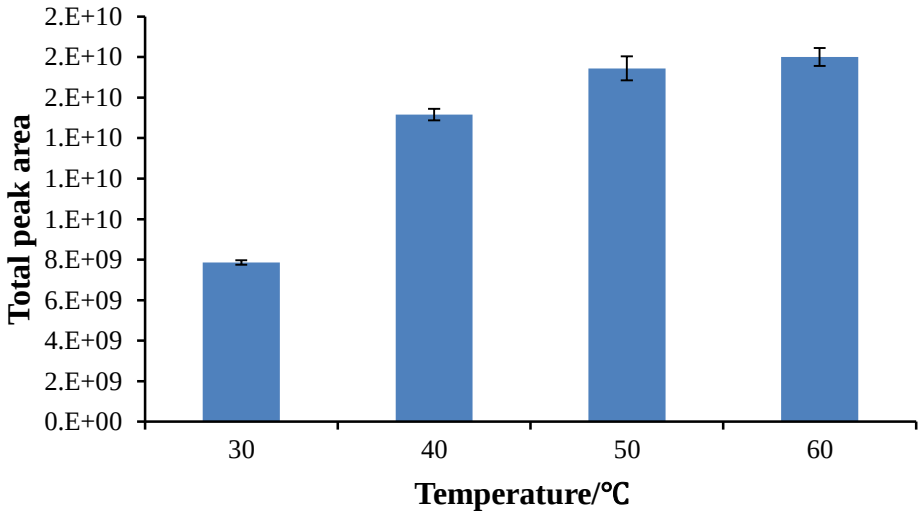


Figure 32 Effect of extraction temperature on SPME of volatile compounds in *P. edulis* Sims fruits with 100 μm PDMS fibre for 20 min of extraction time without salt addition. Error bars stand for standard deviation (n=3 for each data point).

These results show that the amount of extracts increased with the extraction temperature from 30 to 60 ℃ (30, 40, 50 and 60 ℃). The higher temperature contributes to the higher mobility of volatiles through between liquid and gas phases, so that leads to a higher concentration. However, because extraction temperature of 50 and 60 ℃ showed little improvement, 40 ℃ was selected for the next steps.

Table 14 Total peak area of volatile compounds in *P. edulis* Sims fruit extracted for 20 min at 40 ℃ extraction temperature with different amount of salt addition (0, 0.02, 0.05, 0.1 and 0.2 g).

Repeat Time	Different Amount of Salt Addition/g				
	0	0.02	0.05	0.1	0.2
1	5286281326	4064087948	3941179248	3895862177	4219037381
2	4741087516	4511674536	4257807047	3828510540	4132183355
3	5128710636	4139629534	4301931812	3992050723	4387498121
Total	15156079478	12715392018	12500918107	11716423440	12738718857
SD	280569805.9	239603063.6	196783540.3	82192731.68	129812860.5
Mean	5052026493	4238464006	4166972702	3905474480	4246239619
RSD%	5.55	5.65	4.72	2.10	3.06

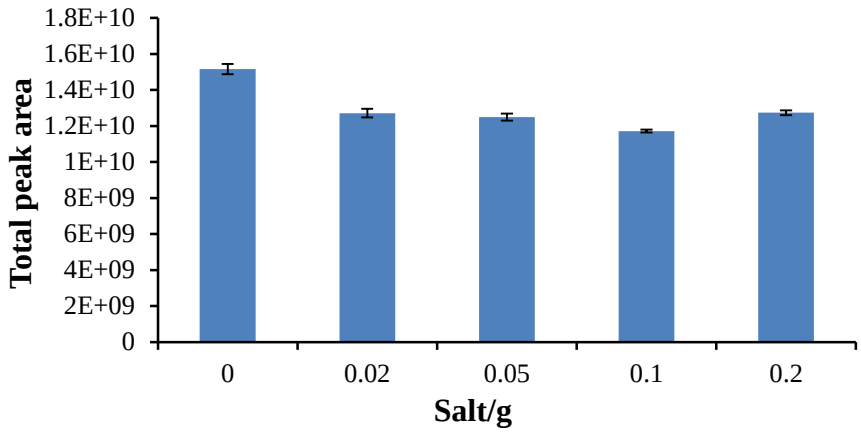


Figure 33 Effect of salt addition on SPME of volatile compounds in *P. edulis* Sims fruits with 100 μm PDMS fibre for 20 min at 40 ℃. Error bars stand for standard deviation (n=3 for each data point).

These results show that, contrary to what was expected, the addition of salt to the sample caused decreased yields of volatiles opposite to the principle. This was surprising because typically the "salting out" process will decrease the solubility of organic substances in water. It may be the effects of the polarity of extracted volatiles and extraction medium and their interplays on the yields of volatiles (my thanks to the thesis examiner for this suggestion). The influences of salt addition on the main volatiles in the both fruits of *P. edulis* Sims and *P. tetrandra* could be found in the **Figure 34** and **Figure 35**.

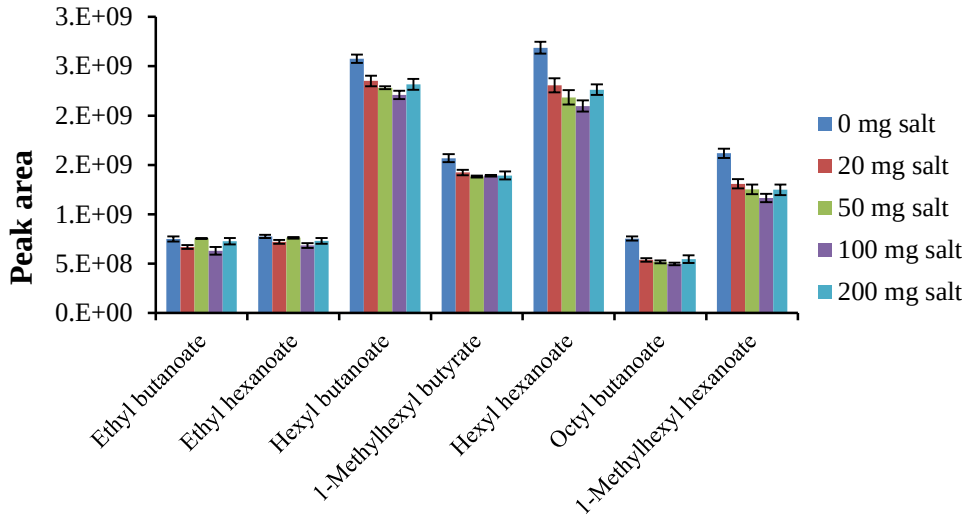


Figure 34 The influence of different amount of salt addition on seven key volatile compounds in sample of *P. edulis* Sims (extraction time: 20 min, extraction temperature: 40 °C).

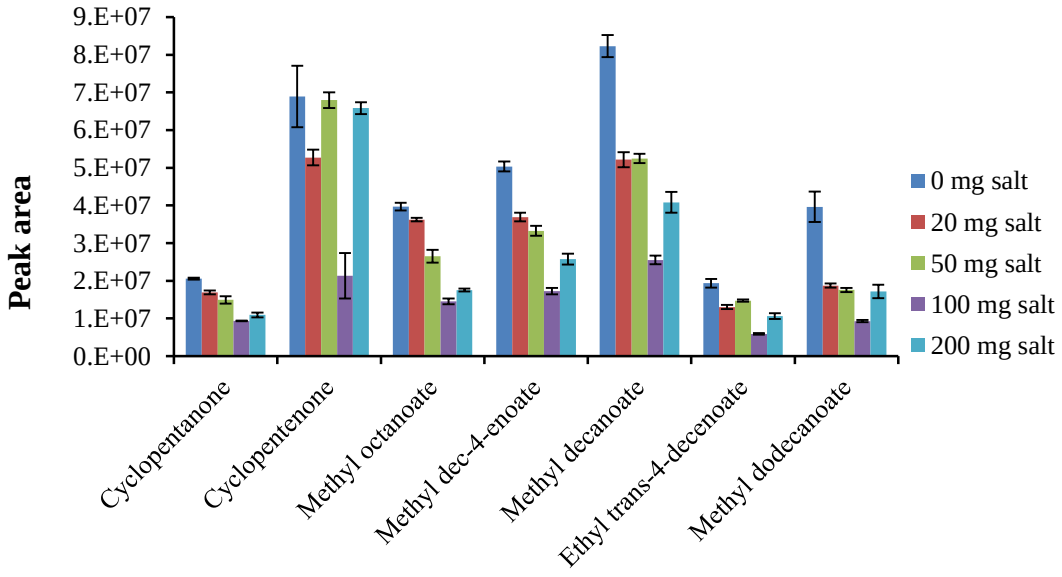


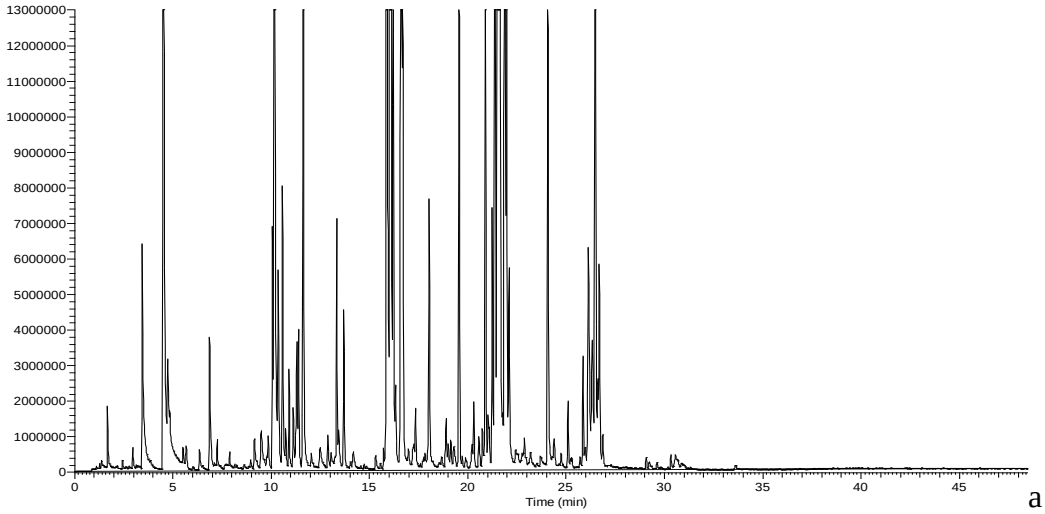
Figure 35 The influence of different amount of salt addition on seven key volatile compounds in sample of *P. tetrandra* fruit (extraction time: 20 min, extraction temperature: 40 °C).

4.3.2 Analysis of Volatile Compounds in Fruits

Using the above PDMS fibre SPME conditions, 40 ^{o}C, 20 min and no added salt, the volatile components in two different species of passion fruits (*P. edulis* Sims and *P. tetrandra*) were measured. Each measurement was determined three times under the best optimised conditions (extraction time: 20 min, extraction temperature: 40 ℃).

There were approximately fifty volatile compounds detected. The GC-MS profiles of two kinds of passion fruit were shown in **Figure 36** and the identified volatile compounds were expressed in **Table 15**.

(a)



a

(b)

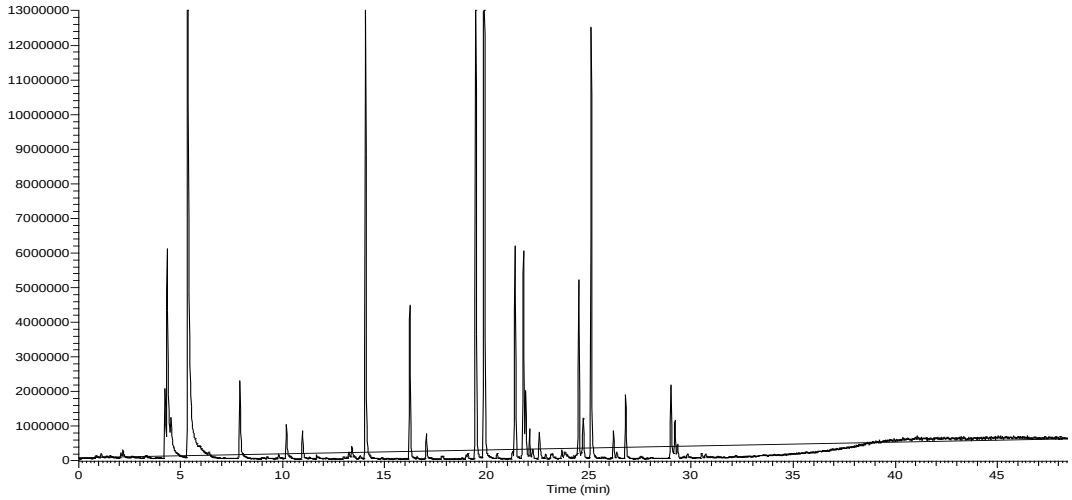


Figure 36 GC – MS chromatograms of volatiles in *P. edulis* Sims fruit (a) and Kohia fruit (b) extracted by SPME technique with best optimised conditions (extraction time: 20 min, extraction temperature: 40 ℃) using PDMS fibre without salt addition.

Table 15 The volatile compounds identified from fruits of *P. edulis* Sims and *P. tetrandra* (Kohia) using SPME method.

RT (min)	CAS#	Formulas	Compound Names	RI_{ref} [a]	RI_{exp} [b]	Relative to total peak area (%)		Relative to highest peak area (%)		ID[c]	Odour (Nf = no reference)
						P. edulis Sims	Kohia fruit	*P. edulis* Sims	Kohia fruit		
1.67	141-78-6	$C_4H_8O_2$	Ethyl Acetate	612	<747	0.13	-	100	-	MS	pineapple[d]
2.95	623-42-7	$C_5H_{10}O_2$	Methyl butanoate	723	<747	0.05	-	100	-	MS	ether, fruit, sweet[d]
4.24	96-41-3	$C_5H_{10}O$	Cyclopentanol	781	747	-	1.27	-	100	MS, RI	Nf
4.36	120-92-3	C_5H_8O	Cyclopentanone	795	753	-	4.24	-	100	MS, RI	Nf
4.5	105-54-4	$C_6H_{12}O_2$	Ethyl butanoate	799	759	4.94	-	100	-	MS, RI	apple[d]
5.36	930-30-3	C_5H_6O	Cyclopentenone	802	799	-	14.21	-	100	MS, RI	Nf
6.36	111-27-3	$C_6H_{14}O$	*1*-Hexanol	860	833	0.05	-	100	-	MS, RI	resin, flower, green[d]
6.87	110-43-0	$C_7H_{14}O$	*2*-Heptanone	891	851	0.34	-	100	-	MS, RI	soap[d]
7.23	543-49-7	$C_7H_{16}O$	*2*-Heptanol	904	863	0.08	-	100	-	MS, RI	mushroom[d]
7.88	106-70-7	$C_7H_{14}O_2$	Methyl hexanoate	934	885	-	1.45	-	100	MS, RI	fruit, fresh, sweet[d]
9.17	100-52-7	C_7H_6O	Benzaldehyde	962	927	0.06	-	100	-	MS, RI	almond, burnt sugar[d]
9.86	18172-67-3	$C_{10}H_{16}$	*L*-β-pinene	990	948	0.06	-	100	-	MS, RI	pine, resin, turpentine[d]
10.06	109-21-7	$C_8H_{16}O_2$	Butyl butanoate	994	954	0.43	-	100	-	MS, RI	fruity[g]
10.19	123-66-0	$C_8H_{16}O_2$	Ethyl hexanoate	996	958	5.12	0.59	100	12	MS, RI	apple peel, fruit[d]

10.37	3681-71-8	$C_8H_{14}O_2$	*cis*-3-Hexenyl Acetate	1005	964	0.52	-	100	-	MS, RI	powerful green[e]
10.57	142-92-7	$C_8H_{16}O_2$	Hexyl acetate	1010	970	0.59	-	100	-	MS, RI	fruit, herb[d]
10.90	60415-61-4	$C_9H_{18}O_2$	*1*-methylbutyl butanoate		980	0.19	-	100	-	MS	Nf
10.96	106-73-0	$C_8H_{16}O_2$	Methyl heptanoate	1026	982	-	0.51	-	100	MS, RI	fruity, orris-like odor, currant-like[f]
11.12	10198-23-9	$C_{12}H_{20}O_2$	*β*-Terpinyl acetate		987	0.09	-	100	-	MS	Nf
11.39	5921-82-4	$C_9H_{18}O_2$	*1*-Methylhexyl acetate	1034	996	0.28	-	100	-	MS, RI	Nf
11.63	3338-55-4	$C_{10}H_{16}$	(*Z*)-Ocimene	1040	1003	1.30	-	100	-	MS, RI	citrus, herb, flower[d]
12.90	586-62-9	$C_{10}H_{16}$	Terpinolen	1088	1077	0.05	-	100	-	MS, RI	sweet, citrus[f]
13.34	78-70-6	$C_{10}H_{18}O$	Linalool	1100	1057	0.41	0.24	100	59	MS, RI	flower, lavender[d]
13.69	95452-08-7	$C_{11}H_{18}$	*1,1*-Dimethyl-3-methylene-2-vinylcyclohexane		1068	0.30	-	100	-	MS	Nf
14.06	111-11-5	$C_9H_{18}O_2$	Methyl octanoate	1132	1080	-	8.19	-	100	MS, RI	orange[d]
15.32	140-11-4	$C_9H_{10}O_2$	Benzyl acetate	1177	1121	0.04	-	100	-	MS, RI	fresh, boiled vegetable[d]
15.79	53398-84-8	$C_{10}H_{18}O_2$	(*3E*)-3-Hexenyl butyrate		1137	0.03	-	100	-	MS	Nf
15.89	16491-36-4	$C_{10}H_{18}O_2$	(*3Z*)-3-Hexenyl butyrate	1183	1140	3.69	-	100	-	MS, RI	wine, green[d]
16.07	2639-63-6	$C_{10}H_{20}O_2$	Hexyl butanoate	1190	1146	16.99	-	100	-	MS, RI	apple peel[d]
16.20	106-32-1	$C_{10}H_{20}O_2$	Ethyl octanoate	1196	1151	1.32	2.47	53	100	MS, RI	fruit, fat[d]

16.62	39026-94-3	$C_{11}H_{22}O_2$	*1*-Methylhexyl butyrate		1165	10.35	-	100	-	MS	Nf
16.69	2198-61-0	$C_{11}H_{22}O_2$	Isopentyl hexanoate	1254	1167	0.88	-	100	-	MS, RI	Nf
17.03	1731-84-6	$C_{10}H_{20}O_2$	Methyl nonanoate	1224	1178	-	0.42	-	100	MS, RI	coconut[d]
17.34	10032-15-2	$C_{11}H_{22}O_2$	Hexyl 2-methylbutanoate	1247	1189	0.12	-	100	-	MS, RI	Nf
18.91	5870-93-9	$C_{11}H_{22}O_2$	Heptyl butanoate		1243	0.11	-	100	-	MS	Nf
19.43	1191-02-2	$C_{11}H_{20}O_2$	Methyl dec-4-enoate		1261	-	10.38	-	100	MS	Nf
19.56	41678-29-9	$C_{13}H_{20}O$	trans-Edulan	1309	1266	1.20	-	100	-	MS, RI	Nf
19.86	110-42-9	$C_{11}H_{22}O_2$	Methyl decanoate	1324	1276	-	16.97	-	100	MS, RI	wine[d]
20.90	51468-86-1	$C_{13}H_{20}$	Megastigma-4,6(E),8(E)-triene		1313	0.96	-	100	-	MS	Nf
21.25	16491-54-6	$C_{12}H_{22}O_2$	*1*-Octen-3-yl butyrate		1326	0.54	-	100	-	MS	fruity, buttery, strawberry, mushroom[f]
21.37	76649-16-6	$C_{12}H_{22}O_2$	Ethyl trans-4-decenoate	1378	1331	-	3.99	-	100	MS, RI	Nf
21.39	31501-11-8	$C_{12}H_{22}O_2$	*cis*-3-Hexenyl Hexanoate	1386	1332	4.27	-	100	-	MS, RI	fruit, prune[d]
21.53	6378-65-0	$C_{12}H_{24}O_2$	Hexyl hexanoate	1387	1337	17.72	-	100	-	MS, RI	apple peel, peach[d]
21.61	110-39-4	$C_{12}H_{24}O_2$	Octyl butanoate		1340	4.98	-	100	-	MS	green, herbaceous, sweet, melon-like[f]
21.80	110-38-3	$C_{12}H_{24}O_2$	Ethyl decanoate	1391	1347	-	3.49	-	100	MS, RI	grape[d]
21.88	6624-58-4	$C_{13}H_{26}O_2$	*1*-Methylhexyl hexanoate		1350	10.68	-	100	-	MS	Nf

21.90	4493-42-9	$C_{11}H_{18}O_2$	Ethyl (2E,4Z)-2,4-nonadienoate		1351	-	1.24	-	100	MS	Nf
21.98	69727-42-0	$C_{13}H_{26}O_2$	Butanoic acid, 1-methyloctyl ester		1354	0.90	-	100		MS	Nf
22.10	111-81-9	$C_{12}H_{22}O_2$	Methyl undecenoate		1358	-	0.57	-	100	MS	Nf
22.55	1731-86-8	$C_{12}H_{24}O_2$	Methyl undecanoate	1430	1375	-	0.58	-	100	MS, RI	Nf
22.88	17283-81-7	$C_{13}H_{22}O$	Dihydro-β-ionone	1433	1387	0.06	-	100	-	MS, RI	Nf
23.67	109119-91-7	$C_{15}H_{24}$	Aromadendrene	1434	1418	-	0.15	-	100	MS, RI	wood[d]
24.06	79-77-6	$C_{13}H_{20}O$	(*E*)-β-Ionone	1482	1433	1.08	-	100	-	MS, RI	seaweed, violet, flower, raspberry[d]
24.49	10152-60-0	$C_{13}H_{24}O_2$	Methyl 9-cyclopropylnonanoate		1450	-	3.37	-	100	MS	Nf
24.71	483-76-1	$C_{15}H_{24}$	*δ*-cadinene	1520	1459	-	1.02	-	100	MS, RI	thyme, medicine, wood[d]
25.10	111-82-0	$C_{13}H_{26}O_2$	Methyl dodecanoate	1527	1474	-	8.17	-	100	MS, RI	fatty, floral, wine[f]
25.10	141-16-2	$C_{14}H_{26}O_2$	Citronellyl butyrate	1531	1474	0.11	-	100	-	MS, RI	fruit, sweet, rose[d]
25.85	2345-26-8	$C_{14}H_{24}O_2$	Geranyl isobutyrate	1516	1504	0.17	-	100	-	MS, RI	Nf
26.49	2306-88-9	$C_{16}H_{32}O_2$	Octyl octanoate		1531	1.49	-	100	-	MS	faint, fatty odor, green tea, oily, fruity, sweet[f]
26.79	106-33-2	$C_{14}H_{28}O_2$	Ethyl dodecanoate	1598	1543	-	1.23	-	100	MS, RI	leaf[d]
Total compounds identified						41	22				
RSD (n=3)						0.06	0.12				

Notes:

-: not detected

a Retention indices from literature.

b Retention indices determined experimentally and relative to C_7-C_{20} hydrocarbons.

c Identification methods

d Odour description from Flavornet Database (Acree & Arn, 1984).

e Odour description from literature (Jirovetz et al., 2002).

f Odour description from handbook (Burdock, 1996).

g Odour description from handbook (Burdock, 2009)

Nf No reference found of the description of the odour

These volatiles were analysed and identified by optimized SPME method and GC-MS with NIST/EPA/NIH library. In order to ensure the correct volatiles identification, retention indices (RI) were also determined. **Table 15** described the composition in the investigated fruits and the relative composition is shown as the percentage relative to total peak area and the percentage relative to the compound with highest peak area in each fruit sample.

According to **Table 15**, there were 41 and 22 different volatile components isolated and identified from the fruits of *P. edulis* Sims and *P. tetrandra* (Kohia) respectively. The identified compounds can be organized in different groups according to their chemical structures, such as terpenes, esters, alcohols, ketones and aldehydes. It can be seen that esters were the major volatile compounds with terpenes, alcohols and ketones were relatively less.

Among the identified volatile compounds, there were only 3 volatile components found in both of these two fruits. They were ethyl hexanoate, linalool and ethyl octanoate with different relative contents. The other compounds in the fruits also have been reported in many other plants.

In the volatile composition of Kohia fruit, 7 major compounds (details see **Table 16**) were composed the majority of aroma profile: methyl decanoate, cyclopentenone, methyl dec-4-enoate, methyl octanoate, methyl dodecanoate, cyclopentanone and ethyl trans-4-decenoate according to decreased percentage of relative content. The total percentage of these 7 major volatiles was above 65%.

In the composition of volatile compounds in fruit of *P. edulis* Sims, 7 main components were composed the majority of aroma characteristics. They were hexyl hexanoate, hexyl butanoate, *1*-methylhexyl hexanoate, *1*-methylhexyl butyrate, ethyl hexanoate, octyl butanoate and ethyl butanoate in the order of decreasing relative contents and the structures are shown below (**Table 17**). The total percentage of these seven compounds in the volatiles reached 70%.

Table 16 Seven main volatile compounds in fruit of Kohia fruit

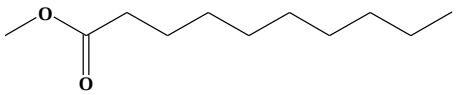
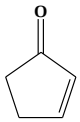
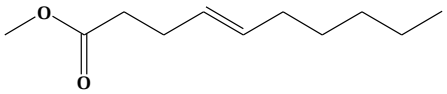
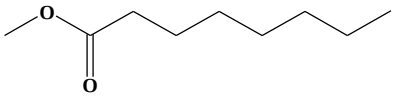
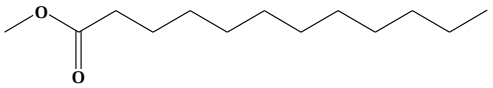
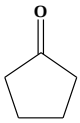
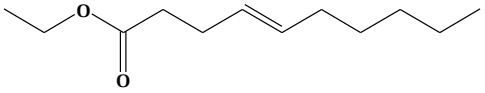
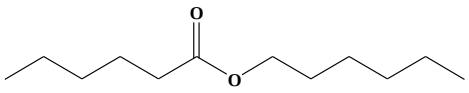
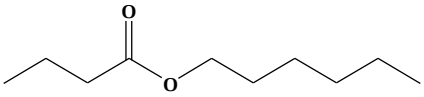
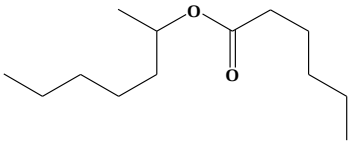
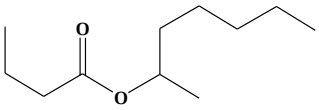
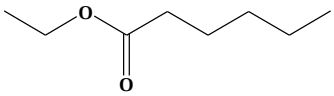
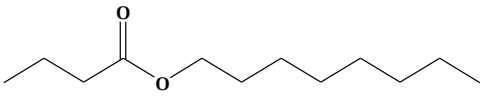
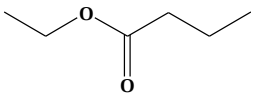
Compounds	Relative contents (%)	Structures	Odour (Nf = not found)
methyl decanoate	16.97		wine
cyclopentenone	14.21		Nf
methyl dec-4-enoate	10.38		Nf
methyl octanoate	8.19		orange
methyl dodecanoate	8.17		fatty, floral, wine
cyclopentanone	4.24		Nf
ethyl trans-4-decenoate	3.99		Nf

Table 17 Seven main volatile compounds in fruit of *P. edulis* Sims

Compounds	Relative contents (%)	Structures	Odour (Nf = not found)
hexyl hexanoate	17.72		apple peel, peach
hexyl butanoate	16.99		apple peel
1-methylhexyl hexanoate	10.68		Nf
1-methylhexyl butyrate	10.35		Nf
ethyl hexanoate	5.12		apple peel, fruit
octyl butanoate	4.98		green, herbaceous, sweet, melon-like
ethyl butanoate	4.94		apple

Cyclopentenone and cyclopantenone have been discussed in the section 4.1.2 of chapter 4, so they will not be explained in the following. The other five main volatile compounds in Kohia fruit and all of 7 main volatiles in *P. edulis* Sims fruit are all esters. These esters play the major role in fruity aroma of the fruit. The biosynthesis of esters (**Figure 37**) has been studied in the strawberry (Zabetakis & Holden, 1997).

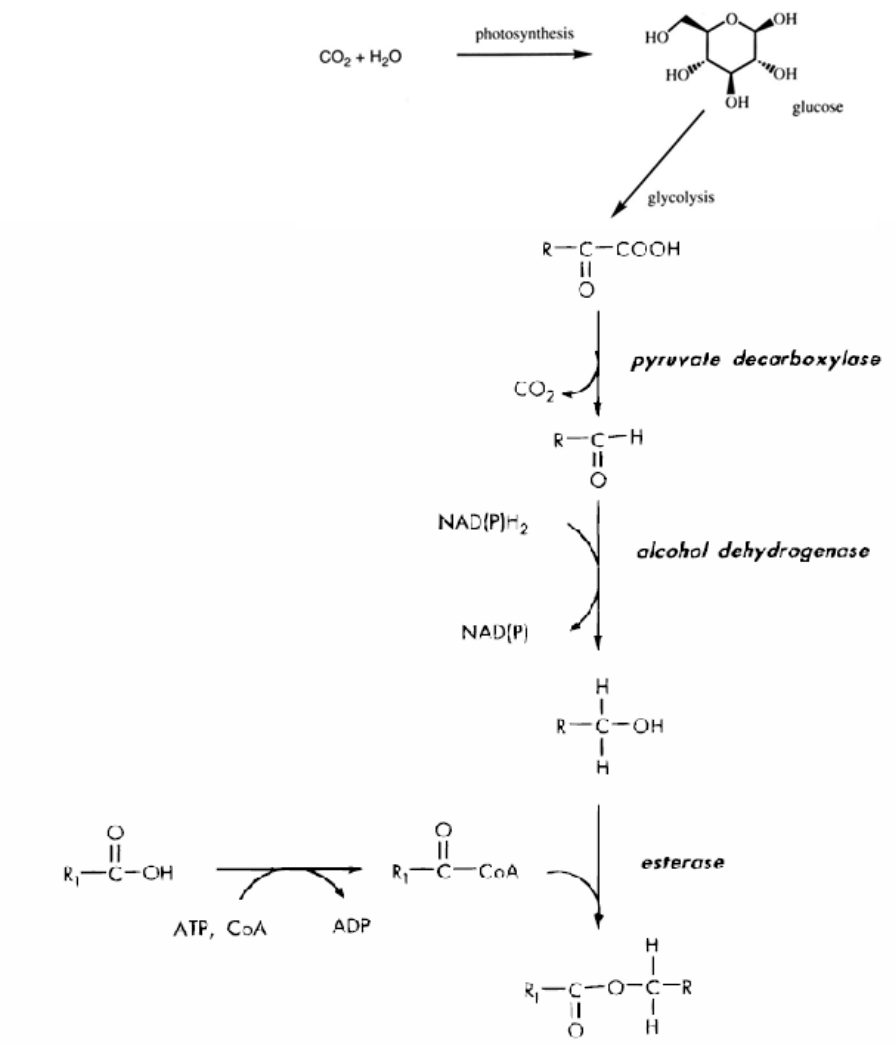


Figure 37 Biosynthesis of esters. NAD(P)H2- Nicotinamide Adenine Dinucleotide Phosphate Hydrogen, NAD(P)- Nicotinamide Adenine Dinucleotide Phosphate, ATP- Adenosine-5'-triphosphate, ADP- Adenosine Diphosphate, CoA- Coenzyme A (Zabetakis & Holden, 1997).

Methyl decanoate, methyl octanoate and ethyl hexanoate were also found in the ripe fruits of *Morinda citrifolia* (Farine, Legal, Moreteau, & Le Quere, 1996) and are an important part of the fruit aroma. The other esters list in **Table 16** and **Table 17** can be identified in the fruits of peach (Yang, Balandrán-Quintana, Ruiz, Toledo, & Kays, 2009), marula (*Sclerocarya birrea* subsp. *caffra*) (Viljoen, Kamatou, & Başer, 2008), papaya (*Carica papaya* L.) (Pino, Almora, & Marbot, 2003), banana cultivars (Marisela Pontes, Pereira, & Câmara, 2012), and strawberries (Zabetakis & Holden, 1997).

Pontes et al. (2009) have investigated volatile compounds in another two species of passion fruit: yellow passion fruit (*P. edulis* Sims *f. flavicarpa*) and banana passion fruits (*P. mollissima*). The volatiles and the percentages of volatile peak area relative to total peak area in these four passion fruit species are shown in **Table 18**.

Table 18 The volatile compounds composition in four different four passion fruit species (Kohia, purple passion fruit, yellow passion fruit and banana passion fruit).

No.	Compounds	Respective peak area relative to total peak area%			
		Kohia	Purple passionfruit	Yellow passionfruit	Bnana passionfruit
1	Ethyl Acetate	0.00	0.13	0.00	0.00
2	Methyl butanoate	0.00	0.05	0.41	0.00
3	Cyclopentanol	1.27	0.00	0.00	0.00
4	Cyclopentanone	4.24	0.00	0.00	0.00
5	Ethyl butanoate	0.00	4.94	0.46	0.00
6	Cyclopentenone	14.21	0.00	0.00	0.00
7	*1*-Hexanol	0.00	0.05	0.00	3.05
8	*2*-Heptanone	0.00	0.34	0.00	0.00
9	*2*-Heptanol	0.00	0.08	0.00	0.00
10	Methyl hexanoate	1.45	0.00	32.87	0.00
11	Benzaldehyde	0.00	0.06	0.00	0.00
12	*L*-β-pinene	0.00	0.06	0.00	0.00
13	Butyl butanoate	0.00	0.43	0.00	0.78
14	Ethyl hexanoate	0.59	5.12	3.19	0.00
15	*cis*-3-Hexenyl Acetate	0.00	0.52	0.00	0.00
16	Hexyl acetate	0.00	0.59	0.00	0.56
17	*1*-methylbutyl butanoate	0.00	0.19	0.00	0.13
18	Methyl heptanoate	0.51	0.00	0.00	0.00
19	*β*-Terpinyl acetate	0.00	0.09	0.00	0.00
20	*1*-Methylhexyl acetate	0.00	0.28	0.00	0.00
21	(*Z*)-Ocimene	0.00	1.30	1.90	56.57
22	Terpinolen	0.00	0.05	0.00	0.00
23	Linalool	0.24	0.41	0.00	0.00
24	*1,1*-Dimethyl-3-methylene-2-vinylcyclohexane	0.00	0.30	0.00	0.00
25	Methyl octanoate	8.19	0.00	0.00	0.00
26	Benzyl acetate	0.00	0.04	0.00	0.00
27	(*3E*)-3-Hexenyl butyrate	0.00	0.03	0.00	0.00

28	(*3Z*)-3-Hexenyl butyrate	0.00	3.69	0.00	0.00
29	Hexyl butanoate	0.00	16.99	2.49	16.83
30	Ethyl octanoate	2.47	1.32	0.00	0.00
31	*1*-Methylhexyl butyrate	0.00	10.35	0.00	0.00
32	Isopentyl hexanoate	0.00	0.88	0.00	0.00
33	Methyl nonanoate	0.42	0.00	0.00	0.00
34	Hexyl 2-methylbutanoate	0.00	0.12	0.00	0.00
35	Heptyl butanoate	0.00	0.11	0.00	0.00
36	Methyl dec-4-enoate	10.38	0.00	0.00	0.00
37	trans-Edulan	0.00	1.20	0.00	0.00
38	Methyl decanoate	16.97	0.00	0.00	0.00
39	Megastigma-4,6(E),8(E)-triene	0.00	0.96	0.00	0.00
40	*1*-Octen-3-yl butyrate	0.00	0.54	0.00	0.00
41	Ethyl trans-4-decenoate	3.99	0.00	0.00	0.00
42	*cis*-3-Hexenyl Hexanoate	0.00	4.27	0.00	0.00
43	Hexyl hexanoate	0.00	17.72	0.62	13.95
44	Octyl butanoate	0.00	4.98	0.00	0.00
45	Ethyl decanoate	3.49	0.00	0.00	0.00
46	*1*-Methylhexyl hexanoate	0.00	10.68	0.00	0.00
47	Ethyl (2E,4Z)-2,4-nonadienoate	1.24	0.00	0.00	0.00
48	Butanoic acid, 1-methyloctyl ester	0.00	0.90	0.00	0.00
49	Methyl undecenoate	0.57	0.00	0.00	0.00
50	Methyl undecanoate	0.58	0.00	0.00	0.00
51	Dihydro-β-ionone	0.00	0.06	4.89	0.00
52	Aromadendrene	0.15	0.00	0.00	0.00
53	(*E*)-β-Ionone	0.00	1.08	0.00	0.00
54	Methyl 9-cyclopropylnonanoate	3.37	0.00	0.00	0.00
55	*δ*-cadinene	1.02	0.00	0.00	0.00
56	Methyl dodecanoate	8.17	0.00	1.50	0.00
57	Citronellyl butyrate	0.00	0.11	0.00	0.00
58	Geranyl isobutyrate	0.00	0.17	0.00	0.00
59	Octyl octanoate	0.00	1.49	0.00	0.38
60	Ethyl dodecanoate	1.23	0.00	0.00	0.00
61	Pentan-2-one	0.00	0.00	0.38	0.32
62	*2*-methyl methylbutanoate	0.00	0.00	0.40	0.05
63	Butyl acetate	0.00	0.00	0.00	0.93
64	Butan-1-ol	0.00	0.00	0.00	0.12

65	*β*-myrcene	0.00	0.00	0.00	0.09
66	Eucalyptol	0.00	0.00	0.00	0.83
67	(*E*)-Ocimene	0.00	0.00	0.00	1.40
68	*3*-carene	0.00	0.00	0.00	0.09
69	(*E*)-methyl-2-hexenoate	0.00	0.00	11.66	0.00
70	Butyl hexanoate	0.00	0.00	0.11	1.00
71	*3*-methyl hexylbutanoate	0.00	0.00	0.00	0.11
72	Hexyl-2-bytenoate	0.00	0.00	0.00	0.89
73	Octan-1-ol	0.00	0.00	0.00	0.33
74	Methyl benzoate	0.00	0.00	11.25	0.00
75	*3*-hydroxymethyl hexanoate	0.00	0.00	4.04	0.00
76	P-menth-1-en-8-ol	0.00	0.00	0.00	0.39
77	*2*-(2-butoxyethoxy)-ethanol	0.00	0.00	1.59	0.00
78	*α*-Ionone	0.00	0.00	4.47	0.00
79	Hexanoic acid	0.00	0.00	3.12	0.00
80	*2*-methyl-3-hydroxy-2,4,4-trimethylpentyl propanoate	0.00	0.00	2.71	0.00
81	Isopropyl myristate	0.00	0.00	2.41	0.00
82	Nonanoic acid	0.00	0.00	1.06	0.00
83	Methyl hexadecanoate	0.00	0.00	0.64	0.00
84	*2*,*3*-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one	0.00	0.00	1.96	0.00
85	Methyl dihydrojasmonate	0.00	0.00	6.17	0.00

This table shows that the compositions of yellow passion fruit and banana passion fruit volatiles are also mainly esters. The main components in yellow passion fruit were methyl hexanoate (32.87%), (*E*)-methyl-2-hexenoate (11.66%), methyl benzoate (11.25%) and methyl dihydrojasmonate (6.17%). In banana passion fruit, the compounds of (*Z*)-ocimene (56.57%), hexyl butanoate (16.83%) and hexyl hexanoate (13.95%) took the main responsibility for the aroma. This shows that cyclopentenone was present at very high concentration (14.21%) in the New Zealand species, but was not present at all in the other three species. The possible explanation is that the cyclopentenone may be the result of mechanical wounding (Stintzi et al., 2001) but this was not investigated.

Chapter 5 Conclusion

Conclusion

The goal of this work was to find and identify volatile organic compounds in three different parts of the New Zealand passion fruit (Kohia) using solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry. After optimization of the SPME method, there were 19 and 22 volatiles found in female and male Kohia leaves, 10 volatiles in attached flowers and 18 in excised female Kohia flowers. There were 41 different volatiles in the commercial passion fruit and 22 in the Kohia fruit.

In the volatile composition of Kohia leaves, alcohols and carbonyl compounds were the major components of the aroma. The largest components in female leaves were cyclopentenone (31.49%), cyclopentanone (13.10%), *2*-hexenol (8.38%) and *L*-β-pinene (8.04%). In male leaves the predominant compounds were cyclopentanone (17.97%), *cis*-hexenyl acetate (14.06%) and *2*-hexenol (14.02%).

The two terpenes (*Z*)-ocimene and *α*-farnesene were the principal volatiles in both attached and excised female flowers Kohia flowers.

Two different passion fruit species were studied in this research and the main volatile compounds in both of fruits were esters. Hexyl hexanoate (17.72%) and hexyl butanoate (16.99%) followed by *1*-methylhexyl hexanoate (10.68%) and *1*-methylhexyl butyrate (10.35%) composed the majority of purple passion fruit flavour. The major volatiles in Kohia fruit aroma were methyl decanoate (16.97%), cyclopentenone (14.21%) and methyl dec-4-enoate (10.38%).

This work provides a better understanding of how these native flowers can be used in developing unique New Zealand perfumes.

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