Characterisation of volatile compounds from the leaves of six *Pittosporum* species; an instrumental and sensory evaluation

by

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Characterisation of volatile compounds from the leaves of six *Pittosporum* species; an instrumental and sensory evaluation

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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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Date: 20 August 2013

Abstract

Six *Pittosporum* species native to New Zealand, *P. cornifolium*, *P. crassifolium*, *P. eugenioides*, *P. kirkii*, *P. tenuifolium*, and *P. umbellatum*, were investigated in this study. The 88 volatile compounds released from their leaves were identified by head space SPME whose optimized conditions are fibre coating PDMS, extraction time 30mins and extraction temperature 70°C with GC-MS analysis. Most of these volatile compounds were identified as sesquiterpenes, 21 of which were shown to be major constitutes in the *Pittosporum* species. They are α-pinene, sabinene, β-pinene, β-myrcene, limonene, α-copaene, (-)-β-bourbonene, β-cubebene, α-gurjunene, β-caryophyllene, cis-thujopsene, humulene, alloaromadendrene, γ-muurolene, germacrene D, β-cadinene, bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-, naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, bicyclo[2.2.1]heptane, 2-methyl-3-methylene-2-(4-methyl-3-pentenyl)-, (1S-endo)-, 4,4-dimethyl-3-(3-methylbut-3-enylidene)-2-methylenebicyclo[4.1.0]heptane and cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-. The main components in *P. cornifolium*, *P. crassifolium*, *P. eugenioides*, *P. kirkii*, *P. tenuifolium*, and *P. umbellatum* are germacrene D, α-copaene, nonane, germacrene D, β-caryophyllene and cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-, respectively. The GCMS fingerprint spectra clearly showed the difference of these plants.

Six of the *Pittosporum* species were also subjected to projective mapping of their odour profiles. This showed "green" and "sweet" as general descriptors for all the *Pittosproum*. However, *P. kirkii* and *P. cornifolium* were also described as "fruity" and "floral". The aromas of *P. umbellatum*, *P.tenuifolium* and *P. crassifolium* as "bitter", "woody" and "grassy" with a sense of "spicy" and P. *eugenioides as having a "citrus"* odour.

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1 Introduction and purpose

1.1 Introduction

This study was conducted to determine the volatile compounds in six *Pittosporum* species native to New Zealand, viz. *Pittosporum cornifolium*, *Pittosporum crassifolium*, *Pittosporum eugenioides*, *Pittosporum kirkii*, *Pittosporum tenuifolium*, and *Pittosporum umbellatum*. The samples were extracted by headspace solid phase microextraction (HS-SPME) and analysed by gas chromatography-mass spectrometry (GC-MS). The odour descriptors of *Pittosporum* were investigated using a sensory analysis technique called projective mapping. The general profiling of *Pittosporum* are presented in this work.

1.2 Purpose of the study

No reports have been found to establish the expression of volatiles in leaves of *Pittosporum cornifolium*, *Pittosporum crassifolium*, *Pittosporum eugenioides*, *Pittosporum kirkii*, *Pittosporum tenuifolium*, *and Pittosporum umbellatum* plants by using HS-SPME. Therefore, the present study investigated the use of HS-SPME combined with GC-MS as a methodology for profiling the volatile signature of leaves from these six *Pittosporum*s. To optimize the extraction conditions, the SPME fibre types, extraction temperatures and extraction times were investigated as the most important parameters affecting the SPME extraction efficiency. Meanwhile, intuitionist sensory evaluation of projective mapping assisted the understanding of the odour similarities and differences among the six *Pittosporum species*.

1.3 Thesis overview

Chapter 2 reviews the botany of six selected *Pittosporum* species, their growth environment, biology and chemistry. It discusses four common extraction methods, which are hydrodistillation, steam distillation, supercritical fluid extraction and solid phase microextraction. It also covers the principle and procedure of sensory analysis called projective mapping for odour analysis.

Chapter 3 provides the materials and methods used in this study. It details the sample collection, conditions of SPME methods and GC-MS analysis. The sensory analysis is also described in this chapter.

Chapter 4 presents the optimization of SPME, including fibre coating selection, time and temperature. It covers the characterization of volatile compounds from six *Pittosporum* species by both chemical and sensory analysis. In this chapter, the classification of volatile chemicals, the major components and odour descriptors of *Pittosporum* are investigated.

Chapter 5 summarises the overall results of this study.

2 Literature Reviews

2.1 Background of *Pittosporum*

The *Pittosporaceae* family is represented by nine genera (Linnek, Mitaine-Offer, Paululat, & Lacaille-Dubois, 2012). *Pittosporum* is a genus of about 200 species in the family *Pittosporaceae* in the division of Angiosperms (Flowering plants) found all over the world (Cooper, 1956). They are evergreen shrubs or small trees. There are over 20 species native to New Zealand and they are found in many localities throughout most of the country (Wardle, 2011).

*Pittosporum*s grow well when planted in well-draining soil and exposed to full sunlight or partial shade. They grow taller in shaded spots, but retain their naturally rounded shape. Normally, *Pittosporum* plants can grow from two to thirty meters tall; depending on the species (Foster, 2008).

This study looks at six readily available *Pittosporum* species. They are described below.

2.1.1 *Pittosporum cornifolium*

Figure 1. Image of *Pittosporum cornifolium*

The Māori name for *Pittosporum cornifolium* is tāwhiri karo, which is also known as perching pittosporum (William, 1869). *P. cornifolium* grows as a shrub to 2m high, usually as an epiphyte on other trees, or growing on rocks. Slender, often drooping branches carry thin, leathery leaves, 4-7cm long and 3-5m wide, with young hairy branchlets. Small reddish flowers arise in umbels at ends of branchlets (Foster, 2008).

The capsule is 10mm long. *P. cornifolium* is found from North Cape to the Marlborough Sounds (Network, 2012a). It is referred to as being used to make the black dye by Maori (W., 1971).

2.1.2 *Pittosporum crassifolium*

Figure 2. Image of *Pittosporum crassifolium*

Pittosporum crassifolium is called Karo in Māori (Mary, 1990). *P. crassifolium* is an attractive species popular for its fast growth. It is a shrub or small tree up to 10m high, with ascending branches and grey to dark brown bark (Cooper, 1956). The tough, leathery leaves, 5-12 cm long by 2-2.5 cm wide, are robust grey-green above, and covered with thick white hair below. The plant produces showy, sweetly scented flowers and is resilient in coastal areas. A common adaptation of coastal plants is the hairs which act as a buffer from the harsh coastal winds and prevent excessive evaporation (Foster, 2008). *Pittosporum* crassifolium is found from Te Paki in the far North to Wellington (Network, 2011a).

2.1.3 *Pittosporum eugenioides*

Pittosporum eugenioides, commonly called lemon wood or tarata (Network, 2011a), is New Zealand's largest *Pittosporum* (Weston, 2004a).

Pittosporum eugenioides is an attractive shrub or small tree reaching 12-13m, with a trunk up to 60cm wide and pale bark. Glossy, light green leaves are 5-12 cm long by 2.5-4cm wide, with an undulating margin, a pale midrib, and citrus scent when crushed (hence the common name, lemonwood). The greenish-yellow flowers produced at the

end of the branchlets have a sweet, honey-like scent. The ripe seed capsules are black. *P. eugenioides* is distributed in both the North and South Islands and is commonly used as shelter in gardens.

Buchanan and Kirk both mentioned that the Maori used the leaves and flowers mixed with fat as a scent applied to their bodies due to their fragrance (H., 1883) (Fan, Liu, & Wang, 2011). Furthermore, the resin or gum obtained by chewing the leaves also was used as medicine to treat bad breath.

Figure 3. Image of *Pittosporum eugenioides*

2.1.4 *Pittosporum tenuifolium*

Figure 4. Image of *Pittosporum tenuifolium*

Pittosporum tenuifolium is called Kohuhu in Maori (Richard, 1870). It is a tree to 6-8 m tall, with a trunk to 40cm in diameter and dark brownish-black bark. The glossy leaves,

7 cm long by 2 cm wide, are usually pale green above, lighter below, with somewhat undulating margins. The flowers are fragrant, dark red to black, and 12 mm long, followed by three-valved capsule. *P. tenuifolium* is a very common tree, widespread throughout New Zealand (Network, 2011b; Wikipedia, 2012). Colenso referred to the fragrant gum being used to produce perfume oil obtained by bruising bark of tree (William, 1869).

2.1.5 *Pittosporum kirkii*

Pittosporum kirkii's common name is *kirk's kohuhu* or thick-leaved *kohukohu*, which is an openly branched shrub to 2-4m high, often epiphytic on trees or growing on rocks, with stout branches and purplish bark. The leaves are thick and leathery, 12cm long by 3cm wide.

The species is endemic to the northern half or the North Island, from Mangonui to Raetihi (Network, 2012b).

Figure 5. Image of *Pittosporum kirkii*

2.1.6 *Pittosporum umbellatum*

Pittosporum umbellatum is known as Heakaro by Maori. It is a tree reaching 6 m with leaves 5-10 cm long, alternate or almost in whorls, leathery and dark green. Pinkish-red flowers arise in many-flowered terminal umbels, followed by two-valved capsules 1cm wide, holding sticky seeds. *P. umbellatum* is found in the east coast scrublands from North Cape to Gisborne (Foster, 2008; Network, 2011c).

Figure 6. Image of *Pittosporum umbellatum*

2.2 Previous study on *Pittosporum*

Pittosporum is probably a Gondwana plant found in Australasia, Oceania, eastern Asia and some parts of Africa. When searching the key word "*Pittosporum*" in Scopus, there are around 96 articles related to the study of *Pittosporum*. 27 papers on *P. tobira*, 20 papers on *P. undulatum* and other papers relate to 35 species around the world.

2.2.1 The distribution of *Pittosporum* species

Investigations of *Pittosporum* in the environment tend to have two different aspects. The first is the discovery of new species. Recent examples are the discovery of *P. serpentinum de Lange* found in North Cape, New Zealand (De Lange, 2003). Also seven *Pittosporum, P. balansae, P. formosanum, P. glabratum, P. kweichowense, P. pentandrum, P. podocarpum* and *P. rehderianum* were found in China (Zhiyun & Turland, 2002) .The other aspect is the distribution, habitat and biomass of plants have been investigated to understand the effect of species invasion. The case of *P. undulatum* native to Australia growing in Azores archipelago, Portugal, has been greatly studied (Lourenço, Medeiros, Gil, & Silva, 2011; J. R. Medeiros, Campos, Mendonça, Davin, & Lewis, 2003; Rose, 1998).

2.2.2 The Chemistry of *Pittosporum*

Chemically, first of all, the chemical constituents have been studied. Hydrodistillation is the most common extraction method used for essential oil collection from leaves,

flowers, capsule, bark and stem (Ferreira et al., 2007; John, Karunakaran, George, Pradeep, & Sethuraman, 2007; Rodrigues et al., 2007).The method will be discussed later in 2.4. Some novel chemical compounds, neolignan glycosides (Zhao, Nie, Guo, Li, & Bai, 2012), triterpenoid saponins (Linnek et al., 2012) have been found. Meanwhile, the leaves of *P. tobira* are also referred to as indicators of airborne trace elements (Lorenzini et al., 2006) and atmospheric pollution (Matarese Palmieri, La Pera, Di Bella, & Dugo, 2005).

In previous studies, the leaves of twelve different species of *Pittosporum* from a wide range of countries have been investigated to elucidate the compounds present. Seven of these came from New Zealand; the others were from Australia, Portugal, India, South Korea, Brazil and Madagascar. That nearly all of these studies use hydrodistillation extraction, while the remainder use steam distillation (Table 1.). In view of the development of SPME combined with GC-MS techniques, it would seem timely to reinvestigate compounds present in the plants. The following section looks at some of the key compounds found by these researchers.

Table 1. Previous work on the composition of the essential oils of some *Pittosporum* species

Species	Main components	Extraction	Area	Reference
P. angustifolium	Bornyl acetate, tridecane, fenchyl acetate	Hydrodistillation	South-east Queensland, Australia	(Sadgrove & Jones, 2013)
P. anomalum	β-Elemene, dodecanal, bicyclogermacrene	Hydrodistillation	Wellington, New Zealand	(Weston, 2004b)
P. neelgherrense Wight et Arn.	α-Copaene, bicyclogermacrene	Hydrodistillation	Wellington, New Zealand	(Weston, 2004b)
P. tenuifolium	Phytyl acetate, heneicosane	Hydrodistillation	Wellington, New Zealand	(Weston, 2004b)
P. tobira (Thunb.) W. T. Aiton	Octyl acetate, decanol, (E)-3-hexenol	Hydrodistillation	Wellington, New Zealand	(Weston, 2004b)

Species	Main components	Extraction	Area	Reference
P. fairchildii	Germacrene D, bicyclogermacrene, α-copaene	Hydrodistillation	Wellington, New Zealand	(Weston, 2004b)
P. neelgherrense Wight et Arn.	Undecane, caryophyllene oxide, β–Caryophyllene	Hydrodistillation	Agasthyamala, Kerala, India	(John, George, Pradeep, & Sethuraman, 2008)
P. tenuifolium	β-Caryophyllene, bornyl acetate	Hydrodistillation	Wellington, New Zealand	(Weston, 2004b)
P. tobira (Thunb.) W. T. Aiton	Nonane, myrcene, undecane	Steam distillation	Lisboa, Portugal	(Rodrigues et al., 2007)
P. tobira against Aedes aegypti (L.)	Undecane, limonene, 4-methyl-1,3-pentadiene	Hydrodistillation	Jeiju Island, South Korea	(Chung, Seo, Kang, Park, & Moon, 2010)
P. umbellatum	Gicyclogermacrene, β-caryophyllene, γ-eudesmol	Hydrodistillation	Wellington, New Zealand	(Weston, 2004b)
P. undulatum	Limonene, bicyclogermacrene, eremophilene	Hydrodistillation	North-eastern New South Wales, Australia	(Sadgrove & Jones, 2013)
P. undulatum	Calamenene, farnesol, (8β,13β)-kaur-16-ene	Hydrodistillation	Azores island, Portugal	(J. R. Medeiros, Campos, Mendonça, Davin, & Lewis, 2003)
P. undulatum	Sabinene, limonene, terpinen-4-ol	Steam distillation	Azores island, Portugal	(Mendes et al., 2011)
P. undulatum	Limonene, sabinene, terpinene-4-ol	Steam distillation	Lisboa, Portugal	(Ferreira et al., 2007)
P. undulatum	Limonene, bicyclogermacrene,	Hydrodistillation	São Paulo, Brazil	(Logo, Fávero, & Romoff, 2006)

Species	Main components	Extraction	Area	Reference
	β-pinene			
P. viridiiflorum Culofondis var. viridiiflorum	α-Cadinol, δ-cadinene, α-amorphene	Hydrodistillation	Antananarivo, Madagascan	(Ramanandraibe et al., 2000)
P. viridulum	Spathulenol, caryophyllene oxide, α−cadinol	Hydrodistillation	Achenkovil Forest Division, Kerala, India	(John et al., 2007)

2.2.2.1 Limonene

Limonene (25.4-27.1%) is the major chemical compound in *P. undlatum* under steam distillation extraction (Ferreira et al., 2007; Mendes et al., 2011). By using hydro distillation extraction, limonene (28.4-80.8%) and biclyclogermacrene (5.3-26.1%) were major compounds (Ferreira et al., 2007; Sadgrove & Jones, 2013). It also occurs in some *Pittosporum* namely *P. undulatum, P. angustifolium and P. tobira*. Limonene is a typical monoterpene, but with a lemony smell. Jette T. Knudsen's review showed limonene was the most common compound in floral scents and occurs in more than 71% of the plant families (Knudsen, Eriksson, Gershenzon, & Ståhl, 2006).It has been frequently used in domestic and occupational products as a fragrance chemical (Rastogi, Heydorn, Johansen, & Basketter, 2001). Furthermore, anti-carcinogenic activity (Elson, Maltzman, Boston, Tanner, & Gould, 1988) and antimicrobial activity (Palá-Paúl, Velasco-Negueruela, José Pérez-Alonso, & Sanz, 2002) were found related to limonene from plant essential oils.

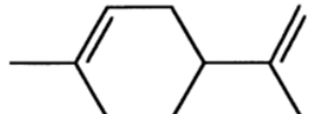


Figure 7. Chemical structure of limonene

2.2.2.2 Caryophyllene

Caryophyllene or (−)-β-caryophyllene, is a natural bicyclic sesquiterpene that is a constituent of many essential oils (Wikipedia). Caryophyllene is one of the chemical compounds that contribute to the spiciness of black pepper. Jirovetz studied (using SPME techniques) the black pepper (Piper nigrum) and black and white ''Ashanti pepper'' (Piper guineense) finding they contain 57.59% and 51.75% respectively (Jirovetz, Buchbauer, Ngassoum, & Geissler, 2002). It is found in many plants and Jette T. Knudsen's review showed that caryophyllene occurs in more than 52% of plants family (Knudsen et al., 2006). Caryophyllene is a sesquiterpene present in natural products such as the oil of cloves(Jirovetz et al., 2006), rosemary (Boutekedjiret, Bentahar, Belabbes, & Bessiere, 2003) and cinnamon leaves (Goñi et al., 2009). All of

them have been used as natural remedies and fragrances. Caryophyllene has been commonly used as a fragrance chemical since the 1930s (Opdyke, 1973).

Figure 8. Chemical structure of caryophyllene

It appears in all kinds of *Pittosporum* investigated before. It has been identified as the major compound in *P. neelgherrense* (9%), *P. viridulum* (17.6%) and *P. tenuifolium* (12.4%) (John et al., 2008; John et al., 2007; Weston, 2004b).

2.2.2.3 Copaene

Copaene is a tricyclic sesquiterpene. It is found in a number of essential oil-producing plants, such as olive (Damascelli & Palmisano, 2012), cinnamon and clove oils (Goñi et al., 2009). Its aroma can be described as woody or spicy. Copaene was found in *P.crassifolium* (17.5%), *P. fairchildii* (5.9%), *P. viridulum* (2.3%), *P. undulatum* (2.8%) and *P. umbellatum* (2.1%) in Weston's work (Weston, 2004b).

Figure 9. Chemical structure of copaene

Other common terpenes including α-pinene, β-pinene were often found in *Pittosporum* species. Therefore, terpenes are a major class of chemicals in this study.

2.2.2.4 Alkanes

Alkanes are saturated hydrocarbons. Long chain alkanes are normally formed in a waxy coating on the plant surface (McDuffee et al., 2004). Short chain alkanes are not commonly found in plants. However, Thomas et al. studied the biosynthesis of short chain alkanes in Pinus jeffreyi, a kind of pine. They showed short-chain alkanes biosynthesis with high levels of heptane in its resin (T. J. Savage, Hamilton, & Croteau, 1996). Nonane is also another short chain alkanes, found in Rosa hybrida (Kim, Kim, Kim, & Lee, 2000) and Nuphar japonica (Azuma, 2013) as major volatile components.

Nonane (C_9H_{20}), undecane ($C_{11}H_{24}$) and tridecane ($C_{13}H_{28}$) have been found in *Pittosporum*. The distillate of steam distillation from *P. tobira* was found to contain 43.1% nonane. Undecane was identified as the first major compound in *P. tobira* (31.11%) (Chung et al., 2010) and *P. neelgherrense* (62.2%) (John et al., 2008) under hydrodistillation. Weston also noted that the essential oil from the leaves of *Pittosporum* included significant proportions of short chain n-alkanes (Weston, 2004b).

2.3 Volatile compounds in plants

2.3.1 Functional affect

Plants are sedentary organisms. Volatile compounds act as a language that plants use for their communication and interaction with the surrounding environment. A total of 1700 volatile compounds have been isolated from more than 90 plant families (Knudsen & Tollsten, 1993). The plants have to adjust to the surrounding environment during their life cycle. They release volatile compounds through their leaves, flowers and fruits to either defend against insects or attract them to pollinate their flowers. The volatile compounds can be antimicrobial compounds which also served to combat pests, thus providing plants with a type of immune system (Turlings & Tumlinson, 1992). The different volatile compounds also act as signals for communication between plants and insect. They are involved in protecting the plant (Dudareva & Pichersky, 2008). Plants have also developed various mechanisms for their interactions with the environment. The biosynthesis of volatiles gives the plant a defence mechanism and changes the scent and aroma properties of flowers and fruit. The study of the occurrence and distribution of plant volatiles has been significantly helped

in the last 20 years due to the simple, sensitive headspace sampling method and the availability of gas chromatography-mass spectrometry (Dudareva, Pichersky, & Gershenzon, 2004). There has been a lot of interest in the antimicrobial activity of *Pittosporum*. Such as the antifungal activity (R. T. S. Medeiros, Gonçalez, Felicio, & Felicio, 2011), larvicidal effects (Chung, Seo, Kang, Park, & Moon, 2012), antibacterial activity (Sadgrove & Jones, 2013) and the medicinal effects (Shyamal et al., 2006) of the bark, leaves and fruit of *Pittosporum*.

When plants release volatile compounds, these change depending on the environmental conditions. For instance, some species respond to lower light intensity or shorter day length. In addition, water stress also is a factor that affects volatile release. When less water is available to the plant, elevated levels of volatiles are released from infested individuals relative to non-water-stressed controls (Frans J. M. Harren, 2013).

2.3.2 Volatile Compounds

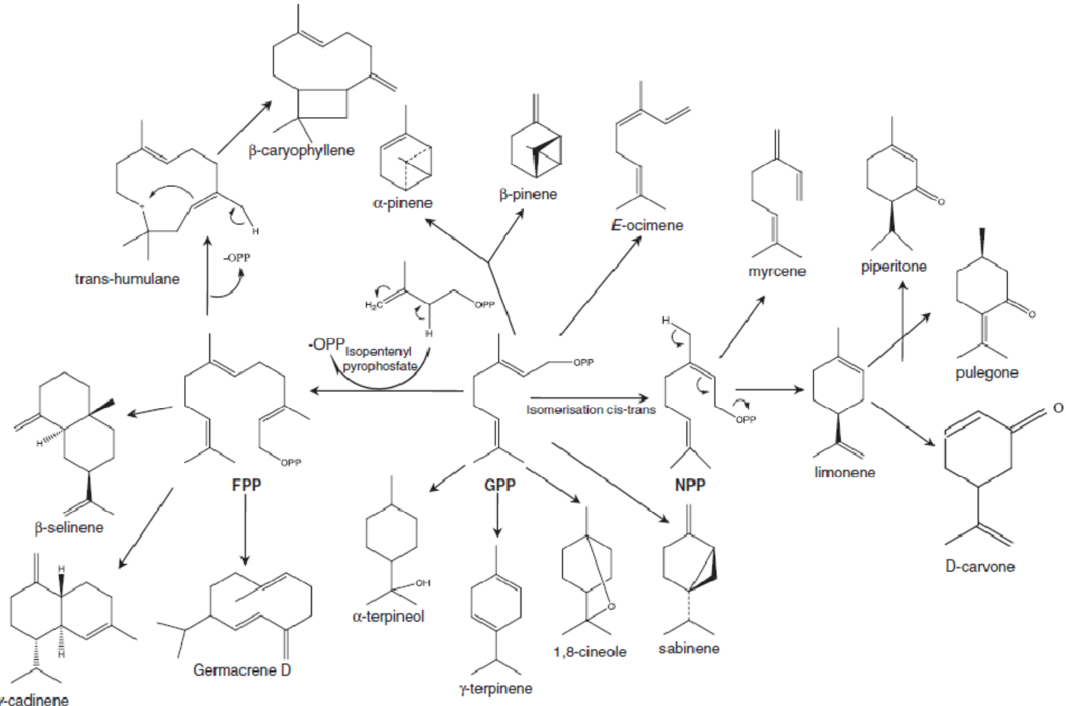
The volatile compounds are identified as largely lipophilic. Plant volatiles are normally low-molecular-weight compounds, which usually have a molecular weight below 300. The compounds emitted from vegetative parts including flowers, leaves, root and barks, are divided into fatty acid derivatives, terpenoids, benzenoids and phenylpropanoids. Fatty acid derivatives are subdivided into: alkanes, alkenes, acids, aldehydes, ketones, alcohols, esters and ethers. Terpenoids are subdivided into: monoterpenes, sesquiterpenes and diterpenes (Bouvier-Brown, Holzinger, Palitzsch, & Goldstein, 2007). Furthermore, the biosynthesis pathways of monoterpenes, sesquiterpenes, and diterpenes include the synthesis of the five-carbon precursor isopentenyl diphosphate and its allylic isomer dimethylallyl diphosphate, the synthesis of the intermediate diphosphate precursors, and the formation of the diverse terpenoids. Terpene syntheses play a key role in volatile compound synthesis (Cheng et al., 2007)

Many pathways lead to the formation of volatile compounds, which contribute to the volatile metabolomic character of plants. Maffei and Silva's (Maffei, Canova, Bertea, & Scannerini, 1999, Silva, 2013 #152) study gave a brief review of progress on some

volatile compounds, particularly in terpenes. These are germacrene D, β-caryophyllene, α-pinene, β-pinene, myrcene, limonene, humulane, selinene and sabinene as example. They are produced by biosynthesis, an enzyme-catalyzed process in cells of living organisms by which substrates are converted to more complex products (Alberts, 2008).

2.3.3 The biosynthesis of terpenes

The biosynthesis superficially appears to occur via the C-5 isoprene, 2-methyl-1,3-butadiene however the biosynthetic pathway is actually via phosphate activated forms, isopentenyl pyrophosphate (IPP, formed from acetyl-CoA via mevalonic acid in the HMG-CoA reductase pathway) and dimethlallyl pyrophosphate (DMAPP) (Wikipedia, 2013c). The process is shown below in Figure 10 (Maffei et al., 1999; Silva & Câmara, 2013).



FPP-farnesyl phosphate; GPP-geranyl phosphate; NPP-neryl phosphate.

Figure 10. Biosynthesis of several terpenes in plants
(Maffei et al., 1999; Silva & Câmara, 2013)

2.4 Common methods of extraction

Analysis of plants normally involves a sample preparation stage such as extraction or distillation followed by analysis with gas chromatography or liquid chromatography.

The methods commonly used currently for the isolation of essential oils from natural products are hydrodistillation and steam extraction (Ozel, Gogus, Hamilton, & Lewis, 2004). Losses of some volatile compounds, low extraction efficiency, degradation of unsaturated compounds through thermal or hydrolytic effects, may be encountered with these extraction methods. Recently, more efficient extraction methods such as solid phase microextraction (SPME) (Zhang & Pawliszyn, 1993), supercritical fluid extraction (SFE) (Simándi et al., 1998) and accelerated solvent extraction (ASE) (Schäfer, 1998), have been used for the isolation of organic compounds from various plants.

2.4.1 Cold pressing

Commonly, extra virgin olive oil is made by cold pressing technique, which is well known to produce a high quality cooking oil (Reboredo-Rodríguez, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2012). The term "cold pressed" is always on the label to express the technique of manufacturing, which means entirely by mechanical without the use of any solvents, and under temperatures, less than 86°F, 30°C, that will not degrade the oil.

The principle of cold pressed extraction is to simply squeeze the materials. Obviously, the advantage of cold press technology is that more natural flavour compounds and more antioxidant are protected under low temperature. Cold-pressing is able to extract bioactive compounds such as essential fatty acids, phenolics, flavonoids and tocopherol in the oils.

It was also found that, in the study of peel extracts of unripe Shiikuwasha (Citrus depressa Hayata) fruits that more phenolics where present that when the fruit were cold pressed that when they were steam distilled (Asikin et al., 2012). This gives stronger antioxidant activity. However cold pressing gave fewer volatile compounds in smaller amounts. Similar results showed in Peng's work, hot water heating increased the yields of essential oils from both peelings and the whole fruit of kumquat (Fortunella margarita Swingle), but the principal constituents of the oils were similar (Peng et al., 2013).

The cold pressing method is used to obtain oily extracts from any part of plant, including fruit, leaves, flower and seed. The cold pressed oil from the peel of citrus family has been studied widely. For example the volatile profile of 'Setoka', which is a new sweet citrus hybrid has been established (Song & Sawamura, 2010). The investigation of the influence of geographical region on the flavour compounds in various cold-pressed lemon oils is reported by Kostadinović (Kostadinović et al., 2010). Dugo also studied the aroma difference among Italian and Egyptian cold pressed and distilled oil from a bitter orange tree, they used fruits, leaves and flower as sources (Dugo et al., 2011). The aroma impact compounds were identified in cold-pressed grapefruit oil (Lin & Rouseff, 2001). Extra virgin cold-pressed avocado oil has a characteristic flavour of grassy and butter/mushroom-like, and is high in monounsaturated fatty acids (Wong, Requejo-Jackman, & Woolf, 2010).

2.4.2 Distillation

Distillation is a widely used method for separating mixtures based on differences in the conditions required to change the phase of components of the mixture (Fair, 2001). Essential oils are secondary metabolites produced in plant metabolism. They have low toxicity to humans and wildlife and are environmentally safe. Generally, distillation takes several hours to get the essential oil. The method is suitable for the production of large amounts and it is widely adopted by the flavour and fragrance industry to produce flavour additives.

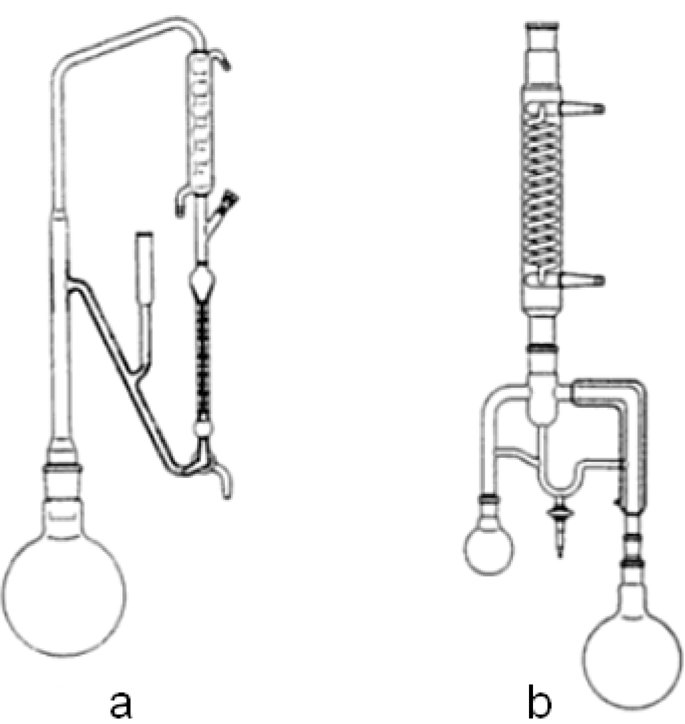
There are two types of distillation using water used to extract volatile compounds. These are hydrodistillation and steam distillation. Both of them operate using a carrier solvent such as water and ethanol or other organic solvent, or mixture of them. The volatile compounds are gently extracted into a solvent and the liquid can be easily stored in a container. It is believed that the rudiments of the methods existed over 4000 years ago. The world's first recorded chemist is considered to be a woman named Tapputi, a perfume maker who was mentioned in a cuneiform tablet from the 2nd millennium BC in Mesopotamia. She distilled flowers, oil, and calamus root with other aromatics then filtered and put them back in the still several times (Wikipedia). In Fragonard known as a museum located in France, a small perfume distillery is on

display (Figure 11.). It is clear from the photograph that essential oil will steam out with water from the main copper boiler to the collector.

Figure 11. Small copper perfume distillation device

In these latter days, some small and ingeniously designed glassware have been invented. Scientists operate glassware named a clevenger-type apparatus (See Figure 12a.) for the continuous hydrodistillation of essential oils. Oil which is lighter than water is collected in the conical bulb while water returns to the distillation flask (Nicholas, J.Walton, D.Diane, & Brown, 1999). Amvam studied thirteen essential oils of aromatic plants by the same glassware for 6 hours (Amvam Zollo et al., 1998). Chopa worked on rosemary essential oil by hydrodistillation for 4 hours using a cleavage apparatus (Sánchez Chopa & Descamps, 2012). Therefore, hydrodistillation is simple and easy setting method, but the disadvantage is time consuming. Furthermore, because the temperature during extraction can be high and the time is so long, the method is not suitable for extracting strong volatile compounds or molecules that are unstable.

Similarly to hydrodistillation, steam distillation also a frequently-used method to extract the volatile compounds from plants. Likens and Nickerson-type apparatus is one kind of continuous steam distillation extractions apparatus is shown in Figure 12b. Lee, Mitchell and Shibamoto carried out steam distillation using a modified Likens-Nickerson apparatus at 55° C and 95mmHg for 3 hours to extract the natural antioxidant, R-tocopherol (vitamin E), from fresh soybeans, mung beans, kidney beans, and azuki beans (Lee, Mitchell, & Shibamoto, 2000). Steam distillation is gentler than hydro distillation, because the volatile compounds are brought out with steam. It decreased the possibility of sample overheating or destroying. Meanwhile, it is found that the DPPH radical scavenging activities of steam distillation oils were higher than those of hydrodistillation oils from teucrium orientale L. var. orientale (Yildirim et al., 2004).



a: Clevenger-type apparatus; b: Likens and Nickerson-type apparatus

Figure 12. Glassware apparatus of hydrodistillation and steam distillation

(source: (Nicholas et al., 1999))

2.4.3 Supercritical Fluid Extraction (SFE)

Supercritical Fluid Extraction is a physical extraction technology. It is a solvent extraction method that was developed in the late 1970s as a new type of separation and purification technology. With public pressure to avoid the environmental effects of extraction technologies there has been much development of less contaminating methods of extraction. Supercritical extraction is one of these.

Supercritical fluids possess unique properties, intermediate between those of gases and liquids. Substances become supercritical when both their pressure and temperature exceed the supercritical values (Bowadt & Hawthorne, 1995). At lower temperatures and higher pressure, the gas will be converted into a liquid. Then with the temperature increasing, the volume of the liquid is increased. There is always a critical temperature (T_c) and critical pressure (P_c), for a particular substance. Once above the critical temperature and critical pressure, there is no phase change between liquid and gas. This is the critical point. The supercritical fluid is similar to a gas with strong penetration but with greater density and solubility of a liquid. With such good solvent properties this system can be used as the solvent for extraction, separation of monomers (Nicholas et al., 1999). Carbon dioxide, and water are the fluids used most frequently in reactions at supercritical conditions. CO_2, which has a critical temperature near ambient conditions, has often been used because of this experimental convenience (P. E. Savage, Gopalan, Mizan, Martino, & Brock, 1995). In recent years, SFE has gained use as a laboratory tool small-scale extraction of plant parts, mainly for sample preparation purposes.

2.4.3.2 Applications of SFE

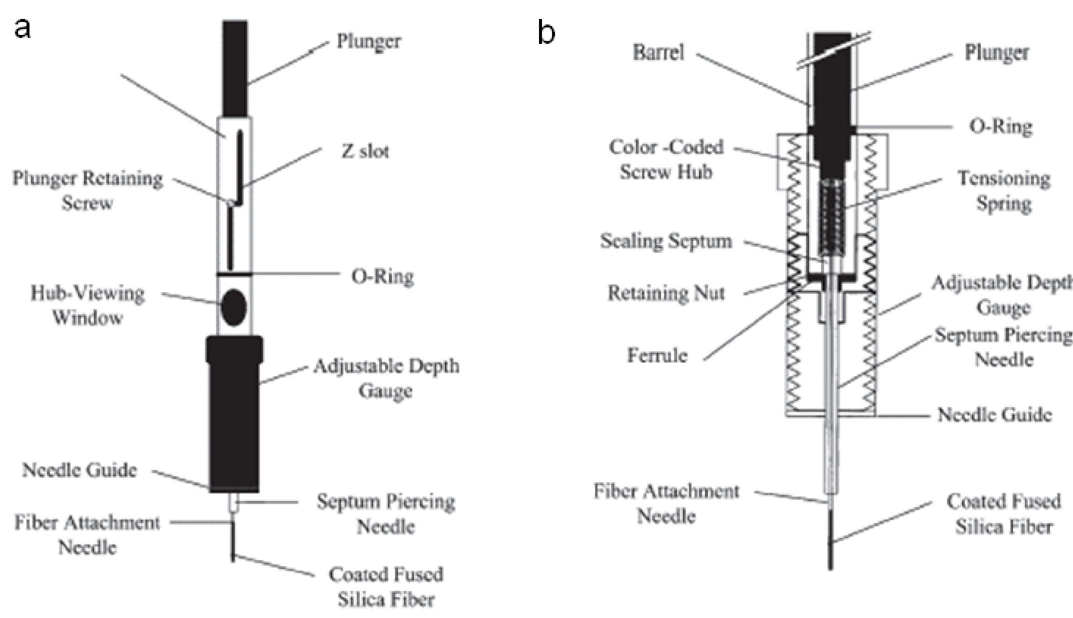
40 years age, SFE became recognize a clean, efficient new separation method with good selectivity. It has become widely used in laboratories to extract volatile compounds from plants. Stashenko and co-workers did a research about analysis of volatile secondary metabolites from Colombian xylopia aromatica (Lamarck) by different extraction methods, including hydrodistillation and supercritical fluid (CO_2) extraction. Results show the two methods are quite similar based on the contribution of compounds. Obviously, SFE is much faster-process technology (Stashenko, Jaramillo,

& Martínez, 2004). Furthermore, Homeira et al. found that SFE offers important advantages over steam distillation due to a higher recovery and reproducible results. This was for the analysis of essential oil of plant *Zataria multiflora* Boiss (Ebrahimzadeh, Yamini, Sefidkon, Chaloosi, & Pourmortazavi, 2003).

2.4.4 Solid Phase Microextraction (SPME)

SPME was developed as a solventless extraction by Janusz Pawliszyn and his team in 1990 (Janusz Pawliszyn, 1997). SPME can be thought of as a very short gas chromatography column turned inside out. The target analytes from an isolated complex mixture are cycling between the fibre coating and the sample mixture. The sample can be in liquid or gas phase. The concentration equilibrium established between the sample matrix and the extraction phase basically depends on time, temperature, the nature of the fibre and sample phases (Wikipedia, 2013d). The type of chemicals extracted depends on the type of fibre coating. When equilibrium conditions are reached, the fibre is removed, and then put in the instrument (gas chromatography or high performance liquid chromatography). Identification is done using the usual chromatography techniques.

An SPME device is illustrated on Figure 13 below, which uses a polymer-coated quartz fibre contained in a modified syringe holder.



a: SPME fibre holder   b: SPME fibre assembly

Figure 13. A commercial SPME device (Mester, Sturgeon, & Pawliszyn, 2001)

The advantages of this technique are speed, simplicity, sensitivity, time saving and selectivity for volatiles. The major disadvantages of this technique are the small sampling capacity of the fibre and low reproducibility of results due to ageing of the fibre and often difficult to reproduce equilibrium conditions which presents problems in quantitative measurements (J. Pawliszyn, 2000).

SPME is widely used for the analysis of volatiles related to aromatic, flavour and medicinal compounds from plants (Belliardo et al., 2006; Jeleń, Majcher, & Dziadas, 2012). It has the advantage of being fast and simple, normally the extraction time ranges from 10 mins to 1 hour. It has been used in the volatile characterisation with leaves of coriander (Carrubba, La Torre, Di Prima, Saiano, & Alonzo, 2002), flowers of rose (Bianchi, Nuzzi, Leva, & Rizzolo, 2007) , fruit of cherry (Piccirillo, Demiray, Silva Ferreira, Pintado, & Castro, 2013), wine (Carrillo, Salazar, Moreta, & Tena, 2007), tea (L. F. Wang et al., 2008), and lamb ham (Paleari, Moretti, Beretta, & Caprino, 2006).

The technology of SPME combined with GC-MS can provide the aroma fingerprint of target samples to identify the similarities and differences. Bowen (Bowen & Reynolds, 2012) compared table wine and ice wine from the same vineyard block. This technology helped researchers understand how the odour potency changes and what compounds may be affected. It is also very powerful for the identification of the differences between quite similar samples. Reboredo studied extra-virgin olive oils from the same and different olive varieties (Reboredo-Rodríguez et al., 2012).

2.5 Volatiles associated with aromas

In this study, the volatiles released from leaves of *Pittosporum* were not only investigated under chemical identification analysis, but also studied by aroma description using a sensory technique called projective mapping. Each of the *Pittosporum* species has its own distinctive aroma. For example, *P. eugenioides* has a lemon-like aroma (Weston, 2004b). *P. kirkii* has a floral smell. The aim of this research was to relate the perceived aromas to the volatile compounds found using GC-MS.

2.5.1 Sensory analysis

The field of sensory evaluation as a scientific discipline began with testing of food products in the late 1940's by the military (Stone & Sidel, 1993). These authors define sensory evaluation as a scientific method used to evoke, measure, analyze, and interpret responses to products as perceived through the senses of sight, smell, touch, taste, and hearing (Stone & Sidel, 2004). Therefore, researchers started to pursue the quantification of the flavour and aroma by modern analytical methods. For many reasons odour space is difficult to define. Taste has its traditional five qualities which are sweet, salty, sour, bitter, and possibly umami (Schiffman, 2000). However, smell seems to have a virtually limitless odour space (Deibler & Delwiche, 2003).

Sensory evaluation is a science of measurement, it is concerned with precision, accuracy, sensitivity and like other analytical procedures it is concerned in avoiding false positive results. Modern sensory evaluation replaced the use of the sensory acuity of a single expert (brew masters, cheese makers, dairy judges, chefs) with the use of trained panels of people that would give a more reliable, generalized and less biased result over time for a particular product (Lawless & Heymann).

2.5.2 Definition of odour

First of all, odour refers to biological, physical and psychological release caused by the interaction between chemical stimulant, aromas and fragrances, and the olfactory systems of living creatures. Odour descriptors are a biochemical link between sense and memory in the brain (S. Y. Wang, Wang, Tseng, Lin, & Liu, 2006). Human sensory data provides the best models for the way consumers are likely to perceive and react to aroma products in real life.

A fragrance classification chart, called the fragrance wheel, was developed in 1983 by Michael Edwards (Edwards, 2012). The first use of the chart was for retailers to suggest different fragrances in a similar category to ones that their customer's may prefer. Nowadays, the developed fragrance classification scheme has been applied to design, manufacture, identification and consumption of perfumes. Meanwhile, the sensory wheel can simplify fragrance classification as well as show the relationships among

each of the individual families. It can easily help a person clarify what the odour is. Since its creation, the wheel and the developed fragrance classification scheme has been modified several times through the addition of different groups to encompass different fragrance types (Zarzo & Stanton, 2009).

Figure 14. Micheal Edwards' fragrance wheel

2.5.3 Odour analysis with Projective mapping

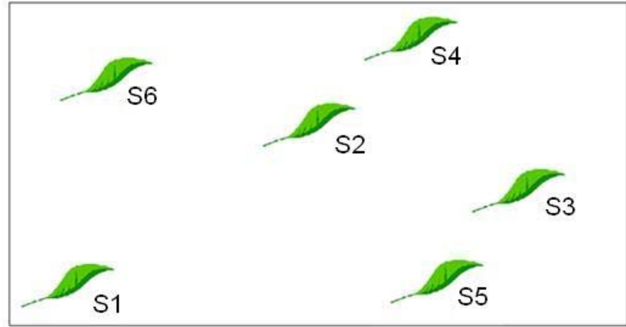
Projective mapping (PM), also known as napping is a tool for sensory analysis. It is a low cost and rapid method for assessing product similarities and producing the necessary descriptive terms. Projective mapping allow panellists to provide any words related to sense definitions, including odour, touch, taste from their own dictionary.

The principle of projective mapping is an alternative procedure to profiling and dissimilarity scaling with related attributes provided by panellists (Risvik, McEwan, Colwill, Rogers, & Lyon, 1994). Kennedy studied the different brands of milk and dark chocolates using PM, the results show the similarities among them with descriptive analysis (Kennedy & Heymann, 2009). Dehlholm et al. compared several rapid

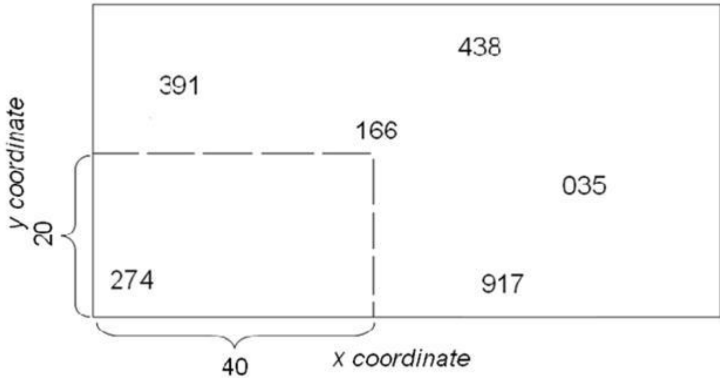
descriptive sensory methods, including PM to investigate the odour maps of liver pâté (Dehlholm, Brockhoff, Meinert, Aaslyng, & Bredie, 2012).

PM consists of a single session. All samples are randomly presented to each panellist in a different order. Panellists are instructed to sense (look, smell, taste or touch) all the samples and then to place the samples on an A3 paper (60×40cm) according to the similarities or dissimilarities between these samples (Valentin, Chollet, Lelièvre, & Abdi, 2012). Panellists can place the similar samples close to each other or the different samples far away (Figure 15.). Then, panellists are asked to describe each sample with a few descriptors (Figure 16.).

a



b

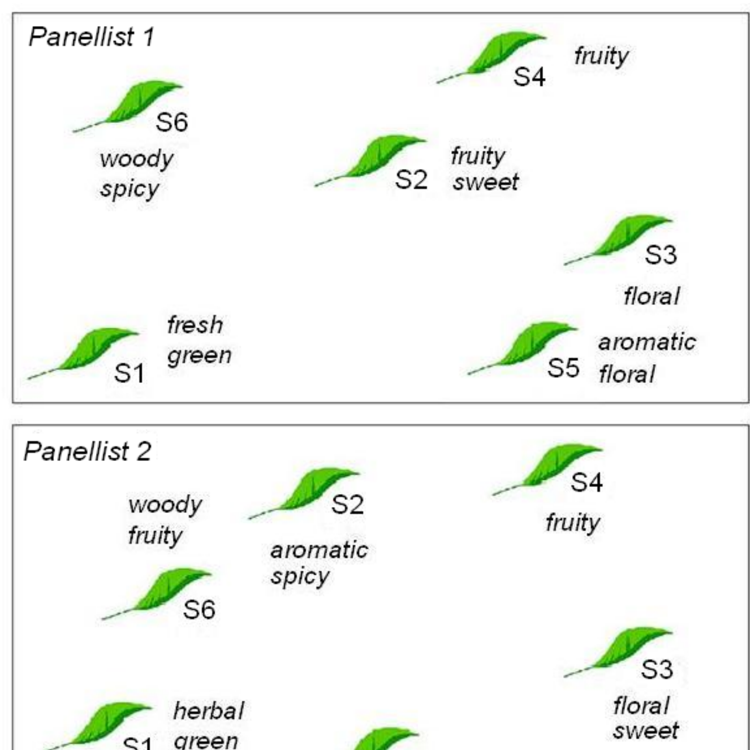


c

Sample	Code	*x*	*y*
S1	274	5	5
S2	166	40	20
S3	035	55	10
S4	438	45	35

S5	917	50	5
S6	391	10	30

Figure 15. An example of Projective mapping. Step a: Panellist place the position of samples on map; step b: x and y coordinates measure; step c: data collection



Sample	Panellist 1		Panellist 2		Descriptors								
	x1	*y 1*	*x2*	*y2*	*fresh*	*green*	*herbal*	*aromatic*	*spicy*	*fruity*	*sweet*	*floral*	*woody*
S1	5	5	7	5	1	2	1	0	0	0	0	0	0
S2	40	20	30	30	0	0	0	1	1	1	1	0	0
S3	55	10	70	20	0	0	0	0	0	0	1	2	0
S4	45	35	60	45	0	0	0	0	0	2	0	0	0
S5	50	5	45	5	0	0	0	0	0	0	1	2	0
S6	10	30	12	20	0	0	0	0	1	1	0	0	2

Figure 16. An example of data table obtained with PM (two samples' maps with descriptors)

2.6 Statistical analysis

Multivariate statistical analysis is widely used in science to assist our understanding of multifactor and complex data. With the increasing sophistication of computer based statistical packages, the use of multivariate statistical analysis has expanded (Lawless & Heymann). In this study, the technique of principal component analysis (PCA) was applied to the GC-MS data and sensory tests, which is in the class of methods commonly referred to as factor analysis.

PCA is a multivariate method to simplify the data; it can help researchers deal with the large amount of data produced in sensory techniques. PCA is often used to reduce the number of data dimensionality, while maintaining visualization retaining as much as possible of the information present in the original data (Lasekan, 2012). It is done by retaining the low-level main component and ignoring the high-level main component. Such low-level components are often able to retain the most important aspects of the data. It is a mathematical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components (Wikipedia, 2013b). The PCA finds these patterns of correlation and substitutes a new variable, called a factor, for the group of original attributes that were correlated. The analysis then seeks a second and third group of attributes and derives a factor for each, based on the variance left over (Lawless & Heymann).

This method permits extraction of data information, the relationship between samples and variables. The main features of PCA are the coordinates of the data in the new base (scores plot) and the contribution to each component of the sensors (loadings plot). The scores plot is usually used for studying the classification of the data clusters, while the loadings plot can provide information on the relative importance of the array sensors to each principal component and their mutual correlation. Principal component analysis (PCA) is able to differentiate between the similar samples. Mildner-Szkudlarz and his co-workers studied five oils—rapeseed, soybean, peanut, sunflower and olive oils—using multivariate analysis (MVA) principal component analysis (PCA) for the analysis of chromatographic and sensory data. They concluded

that PCA is a rapid tool for differentiation of these oils based on the comparison of volatile compound profiles (Mildner-Szkudlarz, Jeleń, Zawirska-Wojtasiak, & Wąsowicz, 2003).

3. Materials and methods

3.1 *Pittosporum* leaves collection

The leaves of six *Pittosporum*s were collected in October 2012 from the University of Auckland garden by the author. There are clear labels shown on plants showing the name of the *Pittosporum* plant. The locations are as follows

Table 2. GPS location of sample collection

Sample	GPS location
P. cornifolium	36.850926, 174.770899
P. crassifolium	36.848741, 174.769901
P. eugenioides	36.849449, 174.771365
P. kirkii	36.850668, 174.769219
P. tenuifolium	36.850874, 174.768822
P. umbellatum	36.850827, 174.769933

Fresh leaves were randomly picked from the trees. They were immediately placed in plastic bags and transported to the laboratory. The leaves were wiped with clean paper towel to remove the dust on the surface of leaves. Then they were stored at 4°C before analysis.

3.2 Volatiles compounds in *Pittosporum*

3.2.1 Solid-phase Microextraction

The coated fibres were polydimethylsiloxane (PDMS) (100 μm thickness), polydimethylsiloxand/divinylbenzene (PDMS/DVB) (65 μm thickness) and carboxen/polydimethylsiloxane (CAR/PDMS) (75 μm thickness) (Supleco, USA). The fibres were conditioned prior use according to the package instructions,*viz.* 0.5h at 250°C for PDMS and PDMS/DVB. The n-Alkanes (C7-C30) standard for retention indices was purchased from Sigma-Aldrich (USA).

3.2.2 Sample preparation

The leaf samples (0.5g) were milled in rotating blade coffee grinder (Breville CG2B Coffee ‘n’ Spice Grinder, Australia) and placed in 20 mL flat bottom headspace vials (Agilent, USA). The vial was immediately sealed with a PTFE/silicone septum and crimp cap (VWR, New Zealand). The head space vial was heated using a plate heater (IKA, USA). The SPME fibre was exposed to the head space 1 cm above the sample surface to adsorb the volatiles.

3.2.3 GC-MS analysis

After extraction, the fibre was inserted directly into the injection port equipped with a SPME-specific liner at 250°C and kept there for 5 min in splitless mode with septum purge (3 mL/min) on.

The GC analytical method was based on the method used by Roy Ma (Ma, 2011). Then optimised for sensitivity and resolution. Analyses were performed using the Trace GC Ultra (Thermo Scientific, USA) coupled with a DSQ series mass spectrometer detector (Thermo Scientific, USA). The column was a VF-5 ms low bleed/MS fused-silica capillary column (5%-phenyl–95%-dimethylpolysiloxane phase, 60 m×0.25 mm×0.25 µm) (Phenomenex, USA). Helium at a constant flow rate of 1.5 mL/min was used as carrier gas. Chromatographic conditions were as follows: the oven was held for 2 min at 40 °C, heated to 180 °C at 2 °C/min, and held 2 min at this temperature. The mass spectrometer was operated in the electron impact mode with a source temperature of 200 °C, an ionising voltage of 70 eV, and the transfer line temperature was 250 °C. The mass spectrometer scanned masses from 48 to 400 m/z at a rate of 3.41 scan/s. n-Alkanes were run under the same conditions as the samples to calculate the Kovats index (KI) of compounds. Peak identification was carried out by comparison of their mass spectra with spectra in the NIST/EPA/NIH Mass Spectral Database (National Institute of Standards and Technology, Gaithersburg, MD, Version 2.0a, 2002, USA), or NIST web book (http://webbook.nist.gov/chemistry/) with corroboration from the Kovats Index or authentic standards.

3.2.4 Compound identification and quantitative analysis

To confirm the identity of volatile compounds, the KI was calculated for each volatile compound using the retention times of a homologous series of C7 to C30 n-alkanes and comparing the KI with compounds analysed under similar conditions previously. The peak area was used for quantitative analysis of volatiles in *Pittosporum*.

3.3 Sensory analysis

3.3.1 Materials

The milled leaf samples (0.2g) were placed in 21 mL glass scintillation vials, sealed with polypropylene caps with silver foil liner (MicroAnalytix, New Zealand).

3.3.2 Panellists

In this study, 18 consumers of perfume, 5 males and 13 females were recruited with the age ranging between 16-70 years old. They were invited to attend projective mapping sessions at the AUT Sensory Lab. The panellists were instructed to smell the leaves of *Pittosporum*, and describe the attribute for each sample of flavour. The panellists were asked to group the products and described the characteristic of the leaves on a computer screen.

3.3.3 Projective mapping

The projective mapping experiments were carried out three times during a two-hour period. A brief introduction was given to the panellists before they started smelling the samples to brief them about the project and explain the principle of projective mapping. Introduction was necessary as the panellists were naive. The evaluation was recorded by the software FIZZ 2.46b (Biosystemes, France). The test was done under red light to eliminate the effect of colour.

A simple questionnaire was given to all panellists prior to carrying out the projective mapping experiment. The questionnaire contained basic demographic questions, which included gender, sex, and frequency of perfume consumption.

Each panellist smelled the six *Pittosproum* species samples, *P. cornifolium, P. crassifolium, P. eugenioides, P. kirkii, P. tenuifolium* and *P. umbellatum*. They are randomised and coded with three-digital number in sensory booths, in the order presented from left to right. Ground, roasted coffee bean was used as a smell cleanser to remove any after-smell in the nose.

The panellists were asked to place the coded samples on two dimensional screens according to the similarities and differences of sample. The similar samples were placed close; the different samples were placed far away. While doing this, the panellists were asked to describe the samples in their own words, which were typed beside the samples on the screen.

3.3.4 Statistical Analysis

Consumer maps were analyzed by collecting the mapping data, x and y coordinates, that were recorded using FIZZ 2.46b (Biosystemes, France). All the data for seven different samples of leaves from each panellist were evaluated using XLSTAT to be further analyzed by GPA, and PCA (Kennedy, 2010).

Attribute descriptors were grouped and their frequency determined before further analysis. The attribute data was then used as supplementary variables when the analysis was carried out for PCA later. GPA showed the presence and degree of variability in the space used by each panellist. Additionally, it was used to determine the samples coordinate for PCA on two-dimensional biplots as well as the efficiency of each of the three individual GPA transformations by examining the F statistics and p-value. The data which are the supplementary data (described attributes) and the sample coordinates got from GPA were set into the PCA analysis. PCA was then carried out for each of the three replications to get a clear picture on the correlation between samples and the attributes.

4 Results and Discussion

4.1 Optimization of SPME

4.1.1 Fibre coating selection

The comparison of the performance of three fibres; polydimethylsiloxane (PDMS), polydimethylsiloxane/divinylbenzene (PDMS/DVB) and carboxen/polydimethylsiloxane (CAR/PDMS) was carried out.Based on total peak area, reproducibility and number of the extracted compounds, the PDMS coating was found to be the most effective SPME fibre to isolate the volatile compounds. The comparison of the efficiency of the fibres to extract volatile compounds from *P. eugenioides* is shown in Figure 17. The PDMS and PDMS/DVB coating extracted more volatile compounds than CAR/PDMS. This is probably due to the polarity of the three fibres which are in the order of PDMS<PDMS/DVB<CAR/PDMS (Silva & Câmara, 2013). Therefore, it was concluded that most of the volatile compounds from the leaves are of low polarity. The other reason for the PDMS coating being selected was its smaller standard deviation than PDMS/DVB (Fig. 17). Because of those factors, PDMS fibres were used for further optimization work.

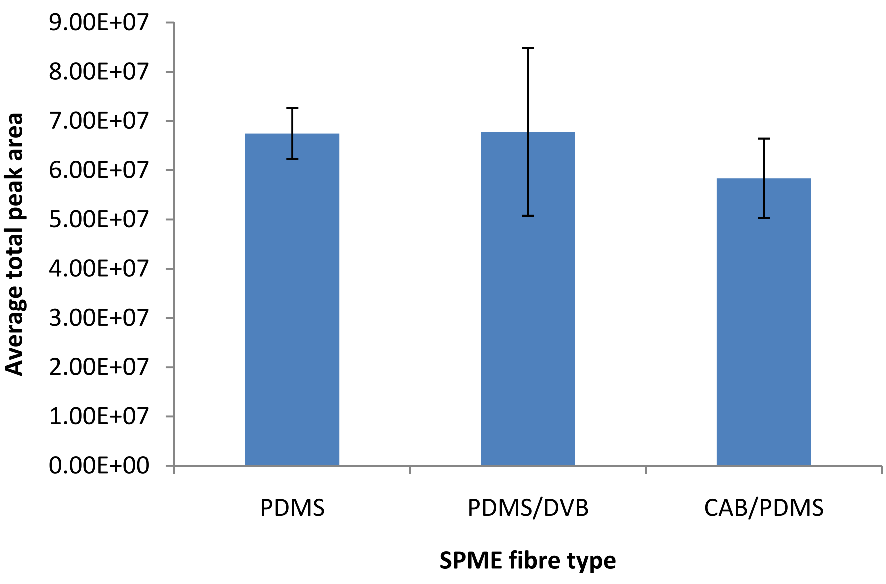


Figure 17. Optimization of the SPME: influence of fibre coatings on total peak area (extraction time: 10 mins; extraction temperature: 20°C)

4.1.2 Extraction time

The volatile compounds from leaves move among the three phases, leaves, headspace and SPME fibre. Over time, an equilibrium is achieved. The optimum time for extraction is the time required for equilibrium to be reached between the leaves and the fibre coating (Câmara, Alves, & Marques, 2006). The extraction time profile (10 to 40 mins), was based on the total peak area of volatile compounds in leaves of *P. eugenioides* plant (Figure 18.). It shows that in 30 mins the total peak area no longer increased suggesting saturation of the fibre. After 30mins, the adsorptive capacity actually appears to drop, but it was not considered significant. On the other hand, the slight decrease in the standard deviation between in 30 and 40mins suggests a more stable equilibrium has been achieved. As the number of compounds found did not increase after 30 mins, SPME exposure this was taken to be the optimum time. To give the greatest sensitivity an extraction time of 30 min was selected for further experiments.

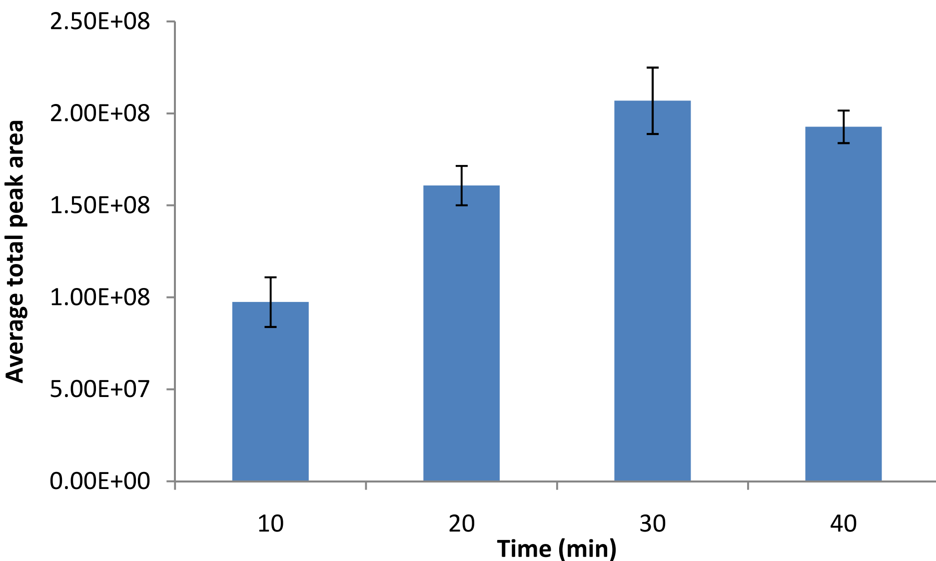


Figure 18. Optimization of the SPME: influence of the extraction time on total peak area (fibre: 100 µm PDMS coating; extraction temperature: 20°C)

4.1.3 Extraction temperature

The effect of the extraction temperature on the headspace SPME extraction of compounds in leaves of *P. eugenioides* was investigated. The temperature also is a key

factor to extraction. This is because it can influence the volatile compounds distribution coefficients between the sample and the headspace and between the headspace and the fibre coating. Especially for highly volatile compounds, increasing temperature increases the absorption of compounds by the fibre as a result of higher partial vapour pressure of the analyte in the headspace.

In the experiment, the extraction temperatures were 20, 40 and 70°C using PDMS coating fibre with 30 minutes extraction time. Figure 19 shows the effect of the extraction temperature based on the total peak area of volatile compounds fraction of *P. eugenioides* fresh leaves. This shows that total peak areas increased with the temperature increase. At 70 °C, the total peak area and the individual peak areas were both largest. So 70 °C was selected for the headspace SPME extraction of volatile compounds from fresh leaves of the selected plants (*P. cornifolium, P. crassifolium, P. eugenioides, P. kirkii, P. tenuifolium and P. umbellatum*).

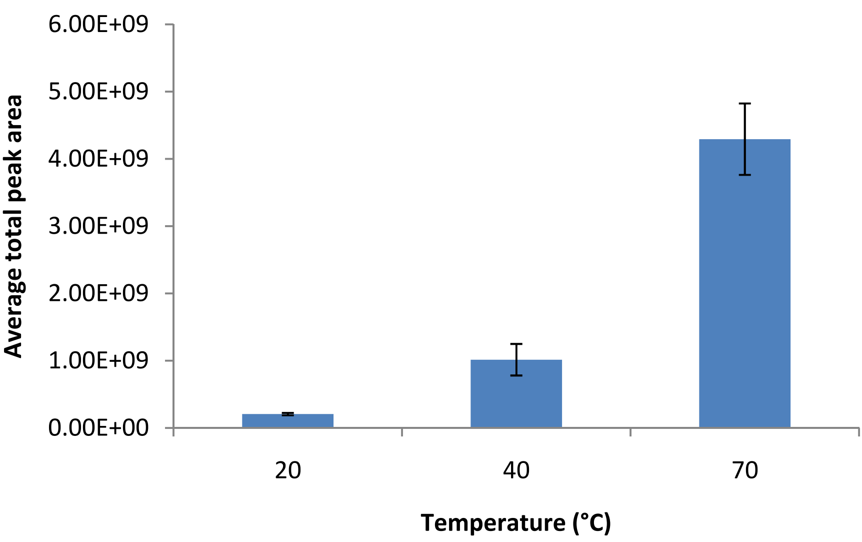


Figure 19. Optimization of the SPME: influence of extraction temperature on total peak area (fibre: 100µm PDMS coating; extraction time: 30mins)

4.2 Characterization of volatile compounds from fresh leaves of six *Pittosporum*

4.2.1 Chemical composition

The volatile compounds released from fresh leaves of selected plants were investigated using HS-SPME under optimized parameters with PDMS coating fibre, 30 mins of extraction time at an extraction temperature of 70°C. The HS-SPME coupled with GC-MS analysis of six *Pittosporum* family plants led to the identification of a total of 88 different volatiles. The total ion chromatograms of six selected plants are shown in Appendix A. classified by functional group: monoterpenes hydrocarbons (Mt; 11), sesquiterpene hydrocarbons (St; 38), alcohol (Alc; 11), aldehyde (Ald;3), alkane (Ala; 8), alkene (Ale; 10), Ester (Es;6). Oxygenated sesquiterpene (OSt; 1).

Identification of volatile compounds was carried out using their ion mass data and Kovtas index in Table 3. The peak number on chromatograms is given by “No” column in Table 3.

The analytical results showed that, the chemical composition of the *Pittosporum* plants differed in the volatile compounds present and in the relative percentage abundance of the volatile compounds. Table 3 shows the identified volatiles (in total, 88 compounds) in the *Pittosporum* plants studied. The RPA% is the mean percentage ratio of the respective peak areas relative to the total peak area after triplicate analysis of each species.

Table 3. Volatile metabolites identified in *P. cornifolium* (PCO), *P. crassifolium* (PCR), *P. eugenioides* (PEU), *P. kirkii* (PKI), *P. tenuifolium* (PTE) and *P. umbellatum* (PUM) fresh leaves using HS-SPME$_{PDMS}$/GC-MS

No	RT[a] (min)	RI[b]	RI$_{cal}$[c]	ID[d]	Metabolites	CAS No.	RPA[e](%)					
							PCO	PCR	PEU	PKI	PTE	PUM
1	3.33	-	691	MS+RI	Hexane	110-54-3	-	-	0.02	-	0.01	-
2	3.57	700	702	MS+RI	3-Pentanone	96-22-0	-	-	0.14	-	0.01	-
3	4.67	764	738	MS+RI	1-Pentanol	71-41-0	-	-	0.80	-	-	-
4	6.67	800	803	MS+RI	Octane	111-65-9	-	-	0.06	-	-	-
5	9.5	854	856	MS+RI	2-Hexenal	505-57-7	0.01	-	-	-	0.05	-
6	9.61	856	858	MS+RI	3-Hexen-1-ol, (Z)-	928-96-1	-	-	-	0.03	-	-
7	9.65	858	859	MS+RI	3-Hexen-1-ol	544-12-7	-	-	0.59	-	0.12	-
8	10.48	867	874	MS+RI	1-Hexanol	111-27-3	0.02	-	0.13	0.03	0.04	-
9	11.97	900	901	MS+RI	Nonane	111-84-2	-	0.07	32.73	0.03	-	9.08
10	13.59	-	926	MS+RI	Bicyclo[3.1.0]hexane, 4-methyl-1-(1-methylethyl)-, didehydro deriv.	58037-87-9	0.03	-	0.03	0.08	0.14	0.13
11	14.04	931	932	MS+RI	α-Pinene	80-56-8	0.05	6.95	0.24	0.13	7.51	13.70
12	15.15	947	949	MS+RI	Camphene	79-92-5	-	0.01	0.02	-	0.20	0.20
13	15.41	955	953	MS+RI	Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl)-	36262-09-6	-	-	-	0.01	-	-
14	16.79	969	973	MS+RI	Sabinene	3387-41-5	0.04	0.02	1.33	0.07	0.26	8.11
15	17.09	975	976	MS+RI	β-Pinene	127-91-3	0.04	0.21	0.10	0.08	0.17	1.15
16	18.07	992	991	MS+RI	β-Myrcene	123-35-3	0.07	0.08	0.20	0.20	0.18	1.25
17	18.77	1000	1002	MS+RI	Decane	124-18-5	-	-	0.12	-	-	0.01
18	19.16	1007	1007	MS+RI	α-Phellandrene	99-83-2	-	-	-	0.01	0.01	-
19	19.39	1009	1010	MS+RI	3-Hexen-1-ol, acetate, (Z)-	3681-71-8	0.04	-	0.15	0.17	0.04	-
20	19.98	1007	1018	MS+RI	Acetic acid, hexyl ester	142-92-7	0.04	-	0.05	0.12	0.05	0.03
21	20.52	1024	1025	MS+RI	p-Cymene	99-87-6	0.15	-	-	0.89	0.17	-
22	20.88	1027	1030	MS+RI	Limonene	138-86-3	0.02	0.03	0.18	0.05	0.22	0.14

No	RT[a] (min)	RI[b]	RI_{cal}[c]	ID[d]	Metabolites	CAS No.	RPA[e](%)					
							PCO	PCR	PEU	PKI	PTE	PUM
23	22.25	1041	1048	MS+RI	(Z)-Ocimene	3338-55-4	0.02	-	0.02	0.02	-	-
24	23.07	1064	1060	MS+RI	γ-Terpinene	99-85-4	0.22	-	-	0.83	0.34	0.06
25	25.04	1083	1086	MS+RI	δ-Terpinene	586-62-9	-	-	-	0.01	0.01	0.02
26	26.15	1100	1101	MS+RI	Undecane	1120-21-4	-	5.75	1.04	0.02	-	1.37
27	27.18	-	1115	MS+RI	Cyclohexane, 2-ethenyl-1,1-dimethyl-3-methylene-	95452-08-7	-	-	0.02	-	-	0.04
28	30.77	1164	1164	MS+RI	Undecane, 2-methyl-	7045-71-8	-	-	-	-	-	-
29	33.36	1199	1200	MS+RI	Dodecane	112-40-3	-	-	-	0.01	-	-
30	34.02	1203	1209	MS+RI	Decanal	112-31-2	-	-	0.03	0.01	-	-
31	34.25	-	1212	MS+RI	6,7-Dimethyl-1,2,3,5,8,8a-hexahydronaphthalene	107914-92-1	-	-	-	-	0.29	-
32	34.33	1215	1214	MS+RI	Acetic acid, octyl ester	112-14-1	-	-	0.28	-	-	-
33	36.51	-	1245	-	NI[f]	-	0.01	-	-	-	-	-
34	38.79	1272	1278	MS+RI	1-Decanol	112-30-1	-	-	0.06	-	-	-
35	39.07	-	1282	MS	Bicyclo[3.1.1]hept-2-en-4-ol, 2,6,6-trimethyl-, acetate	-	0.01	-	-	0.07	-	-
36	39.34	-	1285	MS	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	92618-89-8	-	-	-	-	0.02	0.07
37	40.09	-	1296	MS	(-)-trans-Pinocarvyl acetate	-	-	-	-	0.01	-	-
38	40.29	1300	1299	MS+RI	Tridecane	629-50-5	-	2.80	-	0.04	-	0.11
39	41.02	-	1310	-	NI	-	-	-	-	0.01	-	-
40	42.33	-	1331	-	NI	-	-	-	-	0.02	0.02	-
41	42.36	1340	1333	MS+RI	δ-Elemene	20307-84-0	-	0.03	-	-	-	0.10
42	42.55	-	1335	-	NI	-	-	-	-	-	0.34	-
43	43.35	1351	1346	MS+RI	α-Cubebene	17699-14-8	0.05	0.85	-	0.03	17.56	0.10
44	43.79	1433	1352	MS+RI	α-Guaiene	3691-12-1	0.08	0.06	-	0.05	0.17	0.02
45	44.01	-	1356	-	NI	-	-	0.01	0.03	-	-	-
46	44.37	-	1361	-	NI	-	-	-	0.02	-	-	-

No	RT[a] (min)	RI[b]	RI_{cal}[c]	ID[d]	Metabolites	CAS No.	RPA[e](%)					
							PCO	PCR	PEU	PKI	PTE	PUM
47	44.77	1374	1367	MS+RI	Ylangene	14912-44-8	-	0.10	0.02	-	0.85	0.02
48	45.29	1376	1375	MS+RI	α-Copaene	3856-25-5	1.34	38.03	0.08	0.96	14.33	1.74
49	45.71	1385	1382	MS+RI	(-)-β-Bourbonene	5208-59-3	0.62	0.19	0.19	0.31	0.25	0.13
50	46.02	1388	1386	MS+RI	β-Cubebene	13744-15-5	1.02	8.01	0.14	1.09	6.72	1.38
51	46.13	1391	1388	MS+RI	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1α,2β,4β)]-	515-13-9	0.57	-	0.15	-	-	-
52	46.31	1393	1391	MS+RI	2H-2,4a-Methanonaphthalene, 1,3,4,5,6,7-hexahydro-1,1,5,5-tetramethyl-, (2S)-	1135-66-6	-	0.25	0.79	-	-	-
53	46.38	-	1392	-	NI	-	0.01	-	-	-	0.07	-
54	46.58	1396	1395	MS+RI	1,4-Methano-1H-indene, octahydro-4-methyl-8-methylene-7-(1-methylethyl)-, [1S-(1α,3aβ,4α,7α,7aβ)]-	3650-28-0	-	0.03	-	-	-	-
55	46.73	-	1397	MS+RI	7-Tetracyclo[6.2.1.0(3.8)0(3.9)]undecanol, 4,4,11,11-tetramethyl-	74842-43-6	-	-	-	-	0.01	-
56	46.89	1408	1400	MS+RI	1H-3a,7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-, [3R-(3α,3aβ,7β,8aα)]-	469-61-4	-	0.13	0.26	-	-	-
57	47.33	1411	1407	MS+RI	α-Gurjunene	489-40-7	20.71	0.28	0.60	35.43	2.99	0.10
58	47.66	1413	1413	MS+RI	4,4-Dimethyl-3-(3-methylbut-3-enylidene)-2-methylenebicyclo[4.1.0]heptane	79718-83-5	0.44	0.36	1.49	0.35	0.03	0.02
59	48.04	1415	1418	MS+RI	β-Caryophyllene	87-44-5	1.47	3.53	24.52	0.93	19.77	20.23
60	48.24	1468	1421	MS	β-Chamigrene	18431-82-8	0.25	1.91	8.28	0.17	-	-
61	48.42	-	1424	-	NI	-	-	0.06	0.08	-	-	-
62	48.67	1431	1428	MS+RI	cis-Thujopsene	470-40-6	0.54	0.07	0.03	0.15	0.16	0.02
63	48.96	1433	1433	MS+RI	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	17699-05-7	0.21	1.48	4.20	0.15	0.63	2.33
64	49.18	1439	1437	MS+RI	Aromadendrene	109119-91-7	0.02	-	-	0.01	0.02	0.05
65	49.39	-	1440	MS+RI	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	483-75-0	0.08	0.02	0.27	0.01	0.69	0.13
66	49.84	1460	1447	MS+RI	Bicyclo[2.2.1]heptane, 2-methyl-3-methylene-	25532-78-9	0.03	0.03	0.07	0.04	1.11	0.12

No	RT[a] (min)	RI[b]	RI_{cal}[c]	ID[d]	Metabolites	CAS No.	RPA[e](%)					
							PCO	PCR	PEU	PKI	PTE	PUM
					2-(4-methyl-3-pentenyl)-, (1S-endo)-							
67	49.98	1486	1449	MS+RI	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-Dimethyl-7-(1-methylethenyl)-, [1S-(1α,7α,8aα)]-	10219-75-7	-	0.21	0.78	-	-	-
68	50.25	1451	1452	MS+RI	Humulene	6753-98-6	3.14	1.69	3.02	2.30	3.16	2.98
69	50.52	1455	1460	MS+RI	Alloaromadendrene	25246-27-9	21.26	4.11	3.62	2.95	2.24	0.30
70	50.74	1458	1462	MS+RI	Seychellene	20085-93-2	-	0.30	0.48	-	-	-
71	50.94	1462	1465	MS+RI	Spiro[4.5]dec-7-ene, 1,8-dimethyl-4-(1-methylethenyl)-, [1S-(1α,4β,5α)]-	24048-44-0	-	0.08	0.70	0.01	-	-
72	51.07	1440	1468	MS+RI	Aromandendrene	489-39-4	0.06	0.08	0.42	0.01	0.03	-
73	51.34	-	1472	-	NI	-	0.10	-	-	0.04	0.05	-
74	51.59	1474	1474	MS+RI	γ-Muurolene	30021-74-0	0.06	0.72	0.14	0.04	0.34	0.07
75	51.87	1480	1480	MS+RI	Germacrene D	23986-74-5	36.87	2.77	2.08	44.31	8.26	7.70
76	52.35	1479	1488	MS+RI	Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(1α,3aβ,4α,7β)]-	22567-17-5	0.20	0.18	0.15	0.10	0.17	-
77	52.76	1492	1495	MS+RI	Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-	3242-08-8	5.99	12.11	2.29	5.02	0.50	23.93
78	52.99	1497	1498	MS+RI	α-Muurolene	31983-22-9	-	-	0.04	-	0.17	-
79	53.12	1508	1500	MS+RI	cis-α-Bisabolene	29837-07-8	-	0.08	0.56	-	-	-
80	53.24	1505	1502	MS+RI	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2α,4aα,8aβ)]-	473-13-2	0.02	-	-	-	-	-
81	53.48	1509	1504	MS+RI	α-Farnesene	502-61-4	0.51	-	0.37	0.52	2.22	-
82	53.54	1511	1507	MS+RI	β-Bisabolene	495-61-4	-	0.44	2.43	-	-	0.80
83	53.73	-	1511	MS+RI	1H-Benzocycloheptene, 2,4a,5,6,7,8,9,9a-octahydro-3,5,5-trimethyl-9-methylene-, (4aS-cis)-	3853-83-6	-	0.03	0.43	-	-	-
84	53.84	1511	1512	MS+RI	γ-Cadinene	39029-41-9	0.10	0.03	0.02	0.03	0.03	-
85	54.15	1518	1518	MS+RI	β-Cadinene	523-47-7	0.95	5.20	0.03	0.11	3.34	1.01

No	RT[a] (min)	RI[b]	RI_{cal}[c]	ID[d]	Metabolites	CAS No.	RPA[e](%)					
							PCO	PCR	PEU	PKI	PTE	PUM
86	54.36	1521	1521	MS+RI	Calamenene	483-77-2	-	0.14	0.61	-	0.42	-
87	54.49	1523	1523	MS+RI	β-Sesquiphellandrene	20307-83-9	-	-	1.15	-	-	1.04
88	54.63	-	1526	MS+RI	ç-Elemene	30824-67-0	-	0.02	-	-	0.14	-
89	54.99	1524	1532	MS+RI	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	16728-99-7	0.02	0.08	-	-	0.77	-
90	55.10	-	1534	MS	Tricyclo[5.4.0.0(2,8)]undec-9-ene, 2,6,6,9-tetramethyl-, (1R,2S,7R,8R)-	5989-08-2	-	0.07	0.15	-	-	-
91	55.18	-	1536	-	NI	-	0.03	-	-	0.02	0.25	-
92	55.86	-	1547	-	NI	-	-	0.16	0.85	-	-	-
93	56.52	-	1558	MS+RI	1-Methyl-2,4-bis(1-methylethylidene)-1-vinylcyclohexane	339154-91-5	0.05	-	-	-	1.94	-
94	56.71	1515	1561	MS+RI	Isolongifolan-8-ol	1139-08-8	0.08	-	0.05	-	0.16	0.22
95	56.86	-	1564	-	NI	-	0.04	-	-	0.02	-	-
96	57.24	1567	1570	MS+RI	Palustrol	5986-49-2	0.88	-	-	0.71	0.08	-
97	57.65	1571	1578	MS+RI	Spathulenol	6750-60-3	-	-	-	0.01	-	-
98	57.95	1578	1582	MS+RI	Caryophyllene oxide	1139-30-6	-	-	0.08	-	0.11	-
99	58.69	1593	1595	MS+RI	Viridiflorol	552-02-3	0.17	0.12	-	0.07	-	-
100	59.28	1584	1605	MS+RI	Globulol	51371-47-2	1.26	0.01	0.02	1.11	0.06	-

[a] RT—Retention time (min).

[b] RI—NIST Standard Reference Database Number 69

[c] RI $_{cal}$=retention index relative n-alkanes (C6 to C18) on a VF-5ms (5% phenyl-methyl) capillary column.

[d] The reliability of the identification or structural proposal is indicated by the following: MS—NIST and Wiley libraries spectra and the literature;

[e] RPA (%)—percentage ratio of the respective peak area relative to the total peak area

[f] NI—Not Identified

4.2.2 Identification of volatiles from the Six *Pittosporum* species

Eighty-eight volatile compounds with peak areas > 1% of the total ion count (TIC) seen in the GC-MS chromatograms were found from the leaves of all six selected *Pittosporum.* Of these peaks most were identified but some remain unidentified. The number of identified substances is as follows: 51 in *P. cornifolium* (99.02-99.50%), 53 in *P. crassifolium* (99.77-99.92%), 62 in *P. eugenioides* (96.41-97.98%), 57 in *P. kirkii* (99.56-99.65%), 59 in *P. tenuifolium* and 40 in *P. umbellatum* (99.33-99.88%), respectively (The percentages shown above indicate the percentage of the total peak area that was identified. The range of values was that over three injections).

5 monoterpenes, 2 alkanes and 14 sesquiterpenes are found in all six selected *Pittosporum* plants. They are:

1	α-pinene	2	sabinene	3	β-pinene
4	β-myrcene	5	limonene	6	α-copaene
7	(-)-β-bourbonene	8	β-cubebene	9	α-gurjunene
10	β- caryophyllene	11	cis-thujopsene	12	humulene
13	alloaromadendrene	14	γ-muurolene	15	germacrene D
16	β-cadinene				

17 bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-
18 naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-
19 bicyclo[2.2.1]heptane, 2-methyl-3-methylene-2-(4-methyl-3-pentenyl)-, (1S-endo)-
20 4,4-dimethyl-3-(3-methylbut-3-enylidene)-2-methylenebicyclo[4.1.0]heptane
21 cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-

Most of these volatile compounds were also found in previous work of other *Pittosporum* species, such as in *P. neelgherrense* investigated by John(John et al., 2008), in *P. tobira* investigated by Rodrigues (Rodrigues et al., 2007) , in *P. balfourii* investigated by Gurib-Fakim (Gurib-Fakim & Demarne, 1994). So it would appear that these volatile compounds are commonly found in *Pittosorum* species.

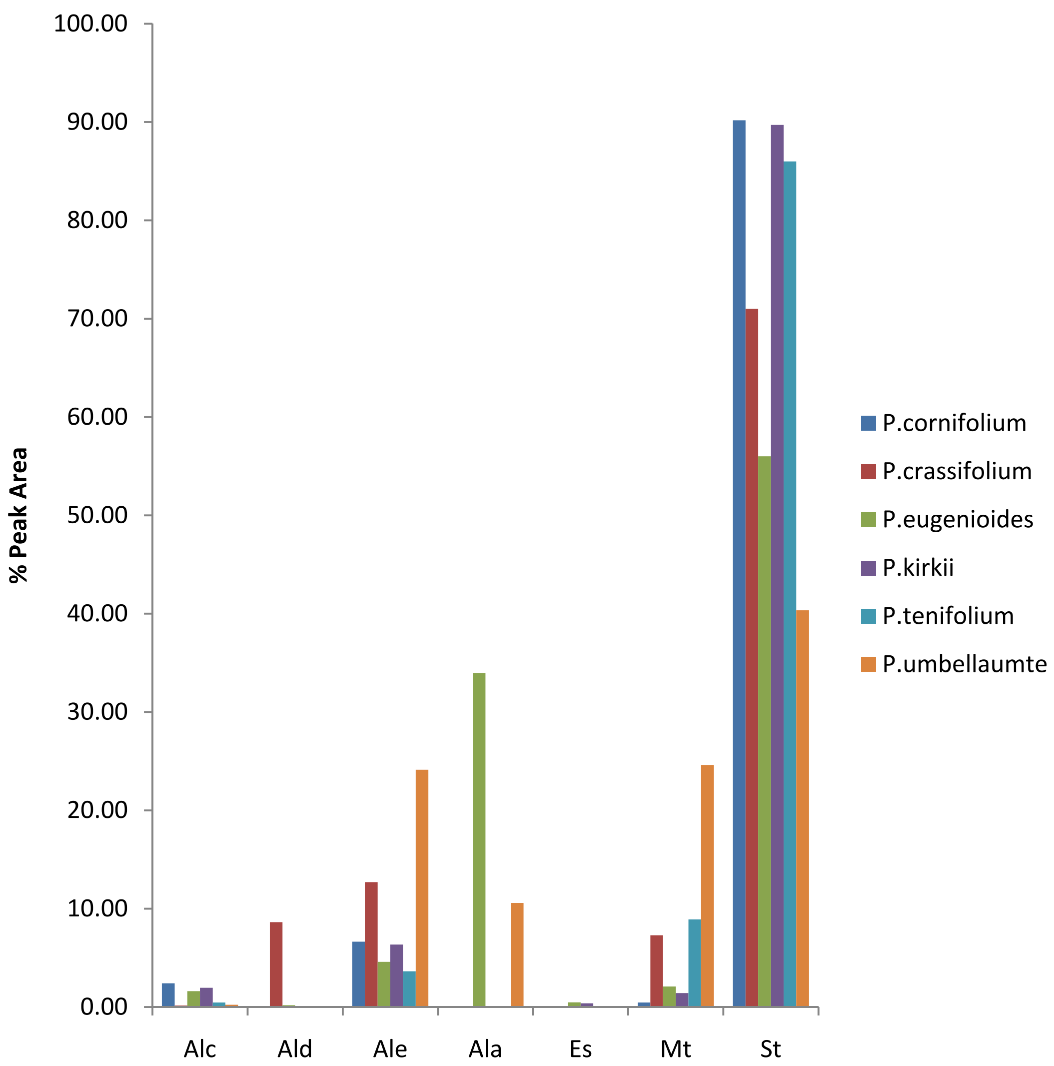


Figure 20. Distribution of volatile compound by chemical classes, found in fresh leaves of six selected *Pittosporum* plants; (Alc-Alcohol; Ald-Aldehyde; Ale-Alkene; Ala-Alkane; Es-Ester; Mt-Monoterpene, St- Sesquiterpenes)

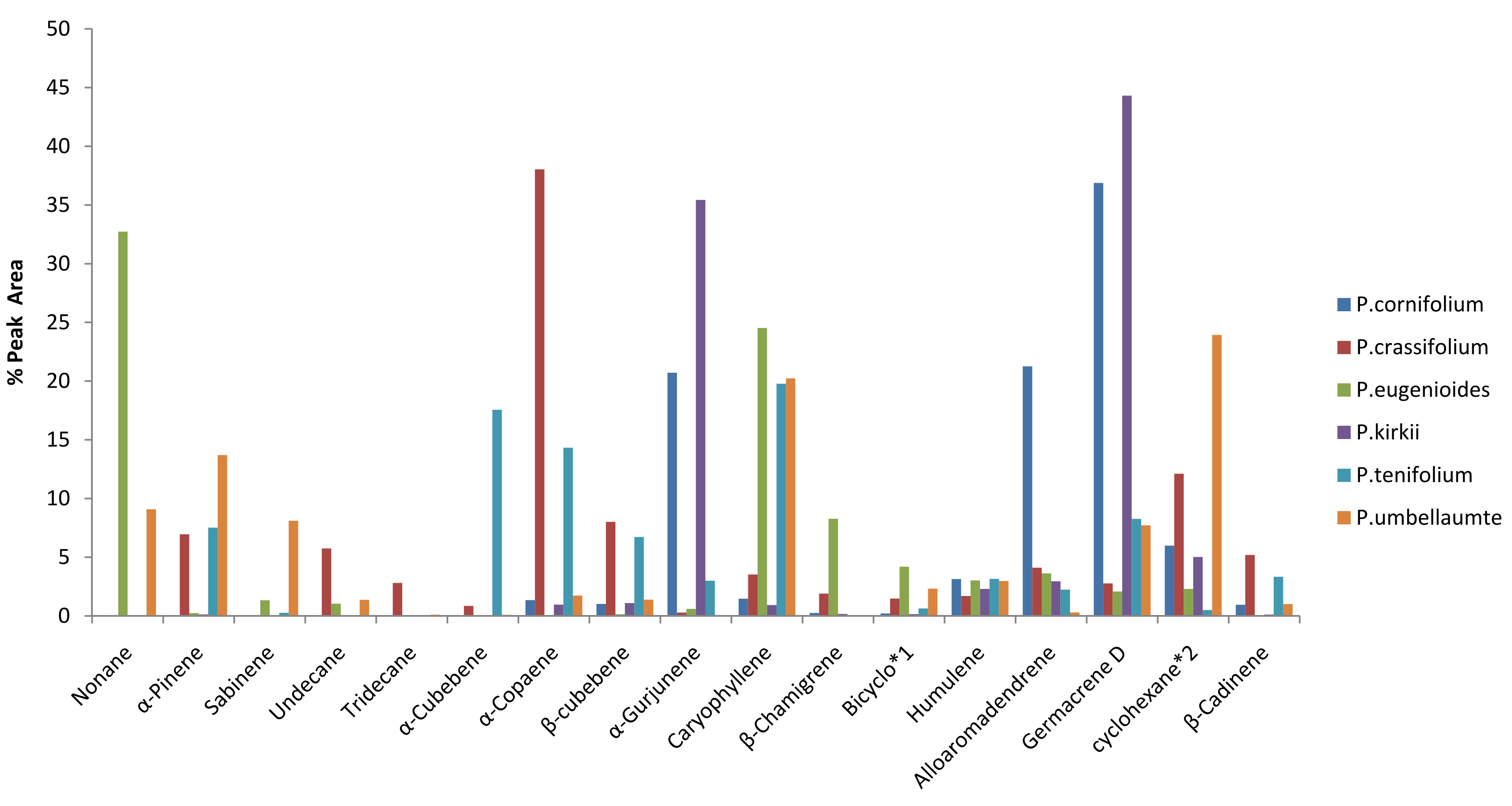
While, it can be found that the major chemical class in fresh leaves of the six selected *Pittosporum* were sesquiterpenes in Table 4. The percentages of the chemical classes are shown in Table 3. The alkanes are the second most common chemical class in *P. cornifolium* (6.65%), *P. crassifolium* (12.70%), *P. eugenioides* (33.98%), and *P. kirkii* (6.35%). However, in *P. tenuifolium* and *P. umbellatum* the monoterpenes exceed the alkanes. In *P. tenuifolium*, it is 3.63% of alkene compared with 8.91% of monoterpenes. In *P. umbellatum* it is 24.12% of alkene compared with 24.63% of monoterpenes. The percentages of each chemical class in total peak area of each species are showed in Table 4.

Table 4. Percentage ratio of the respective peak area relative to the total peak area in volatile compound by chemical classes

	PCO	PCR	PEU	PKI	PTE	PUM
Alcohol	2.42	0.14	1.62	1.96	0.47	0.22
Aldehyde	0.01	8.63	0.17	0.01	0.06	-
Alkane	-	-	33.98	0.09	0.01	10.58
Alkene	6.65	12.70	4.59	6.35	3.63	24.12
Ester	0.09	-	0.47	0.37	0.10	0.10
Monoterpene	0.45	7.30	2.09	1.41	8.91	24.63
Sesquiterpene	90.19	71.00	56.01	89.71	85.99	40.35

4.2.3 Major volatile chemicals

According to the individual chemical compounds showed in Table 3, there are 21 major volatile compounds frequently present in all six *Pittosporum*. (See in Table. 5). The histogram of these major compounds in six *Pittosporum* species (Figure 21.).



*1: Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-
*2: Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-

Figure 21. Major volatile compounds in six selected *Pittosporum* plants

Table 5. Molecular, chemical structure and aroma descriptors of the major volatile compounds identified in fresh leaves of six selected *Pittosporum*

Compound	Molecular	Chemical structure	Aroma descriptor
Nonane	C_9H_{20}		
α-Pinene	$C_{10}H_{16}$		pine, turpentine
Sabinene	$C_{10}H_{16}$		pepper, turpentine, wood
Undecane	$C_{11}H_{24}$		
Tridecane	$C_{13}H_{28}$		
α-Cubebene [a]	$C_{15}H_{24}$		herb, wax
α-Copaene [a]	$C_{15}H_{24}$		wood, spice
β-Cubebene [b]	$C_{15}H_{24}$		citrus, fruit
α-Gurjunene [a]	$C_{15}H_{24}$		wood, balsamic
β -Caryophyllene [a]	$C_{15}H_{24}$		wood, spice
β-Chamigrene [a]	$C_{15}H_{24}$		
Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)- [c]	$C_{15}H_{24}$		
Humulene	$C_{15}H_{24}$		wood
Alloaromadendrene [a]	$C_{15}H_{24}$		wood
Germacrene D	$C_{15}H_{24}$		wood, spice

Compound	Molecular	Chemical structure	Aroma descriptor
Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)- [a]	$C_{15}H_{24}$		
β-Cadinene [b]	$C_{15}H_{24}$		

(The structure of chemicals from a: sigma aldrich, b: chemspider and c: chemfrog, the rest of these were drawn by MDL ISIS Draw 2.5. The aroma descriptor from www.flavornet.org)

Since this study was aimed to characterise the volatile compounds and understand the difference and similarity between the *Pittosporum* species. The volatile fingerprints were classified and grouped by the percentage of components as shown in Table 5.

These compounds with the high percentages vary between the different species, although most compounds do appear in most species to some extent. For example, β-caryophyllene appears to be a major component in *P. eugenioides, P. tenuifolium* and *P. umbellatum*, but it is a minor compound in *P. cornifolium, P. crassifolium* and *P. kirkii*. Similarly, cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene) appears in all species, but it is a major compound in *P. cornifolium, P. crassifolium, P. kirkii* and *P. umbellatum*.

The largest component (RPA%) of *P. cornifolium* and *P. kirkii* was germacrene D, 36.87% and 32.73% respectively. Germacrenes are a class of volatile organic hydrocarbons. They are typically produced in a number of plant species for their antimicrobial and insecticidal properties, though they also play a role as insect pheromones (Wikipedia, 2013a). Germacrene D is present to various plants. Laouer et al. (2009) analysed Marrubium deserti essential oil from the aerial parts of the plant with regard to its composition and antioxidant activity. Germacrene D (45.7%) is found as major component in total thirty-seven compounds by GC-MS (Laouer et al., 2009).It is considered as a precursor of many sesquiterpene hydrocarbons. In the past, researchers discovered that all the rearrangement products obtainable from germacrene D were eventually also found as natural products (Bülow & König, 2000). Furthermore, the derivatives of germacrene D are also found as insect pheromones

emitted from plants (Müller & Buchbauer, 2011), either to protect the plant or attack the insect.

The largest component (RPA%) of *P. crassifolium* was α-copaene (38.03%), followed by cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene) (12.11%).

The largest component (RPA%) of *P. eugenioides* was nonane (32.73%), followed by β-caryophyllene (24.52%). Caryophyllene was detected in 33% of 300 analyzed cosmetic products on the Dutch market in the beginning of the 1990s (de Groot et al., 1994). In another study (Rastogi et al., 1998), chemical analyses of 71 deodorants on the European market 1998, revealed that 45% of the analyzed products contained caryophyllene.

In Weston's paper (Weston, 2004a), he suggested there are two or more chemotypes of *P. eugenioieds.* Because a comparison of his study with the results obtained by Carter and Heazlewood research, there are different major components. Weston identified the octyl acetate (33%) and the three other significant components were terpinen-4-ol (13%), decanol (6%) and (Z)-hex-3-enol (5%). But Carter and Heazlewood found a a high level (60%) of nonane. This phenomenon was recently observed in Leptospermum *scoparium J.R et G. Forst.*(Myrtaceae), a common leaf oil bearing plant in New Zealand. The chemotypes of *L.scoparium* were differentiated geographically and such a suggestion for *P.eugenioides* is rational since the plant material examined by Carter and Heazlewood was very probably collected from the environs of Dunedin, whereas that obtained for the present work was collected in Wellington, and these locations are geographically and climatically, quite different. Further research is needed to clarify this.

The largest component (RPA%) of *P. tenuifolium* was β-caryophyllene (19.77%), followed by α-cubebene (17.56%).

The largest component (RPA%) of *P. umbellatum* was cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)- (23.93%), followed by β-caryophyllene (20.23%).

From the data, it found that the major compounds in *P. cornifolium* and *P. kirkii* are almost same, including germacrene D, alloaromadendrene, α-gurjunene, cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-, humulene, β-caryophyllene, α-copaene, globulol and β-cubebene. In addition to, the order of RPA% from large to small are almost same. But according to Table 3, P.*cornifolium* is the only plant with an alloaromadendrene content significantly greater than 5%.

The GCMS fingerprint spectra clearly showed the differences between the six *Pittosporum* Species. This method can be applied to identify species of plant species.

4.2.4 Statistical analysis of compounds in *Pittosporum*

According to multivariate statistics analysis, biplot of the PCA performed on the chemical components of six *Pittosporum* showed on Figure 22.

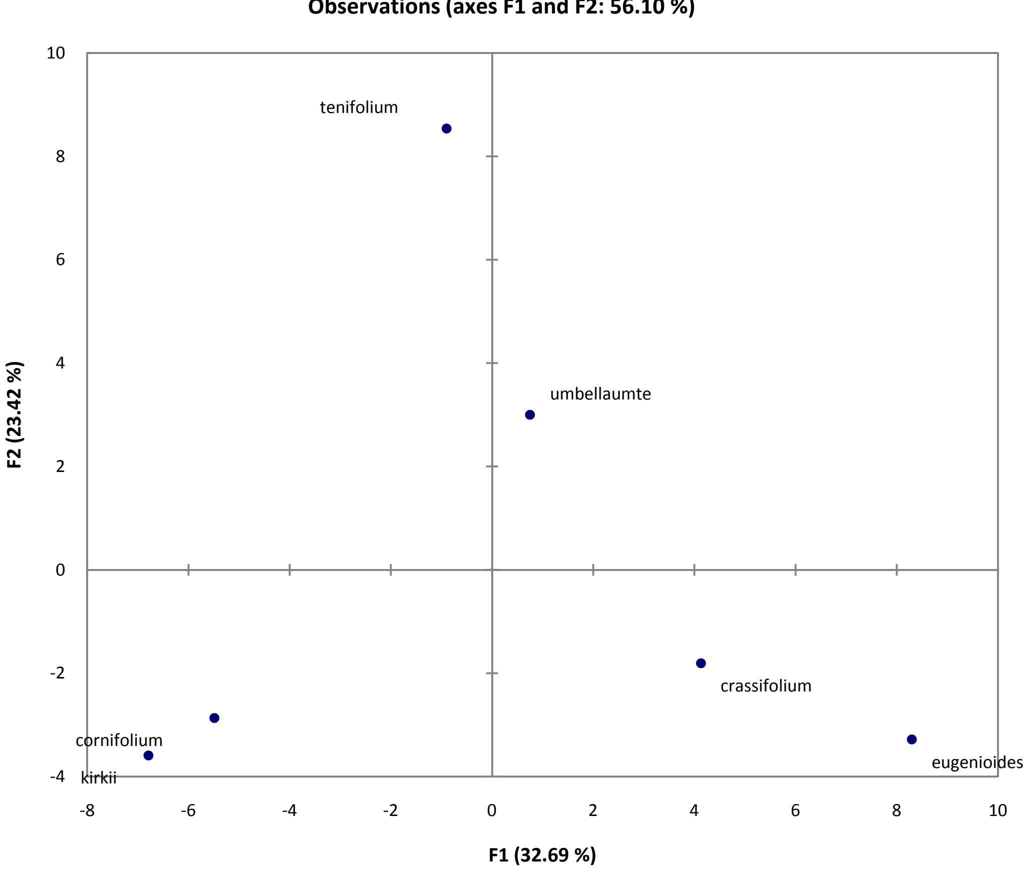


Figure 22. Biplot of PCA performed on chemical components of six *Pittosporum*

In this chart, principal component 1 (PC 1) and PC2 represented 56.10% of the total variance. PC1 accounted for 32.69% of the total variance while PC2 accounted for 23.42%. *P. kirkii* and *P. cornifolium* are positioned on both negative area of F1 and F2. They are very close to each other. Therefore, the volatile chemical components of them released from leaves are very similar to each other. The other four are a dispersed distribution on the plot chart. However, *P. eugenioides* and *P. crassifolium* are positioned on the same area (positive area of F1 and negative area of F2).

4.2.5 Statistics analysis of *Pittosporum* with sensory data

Panellists produced individual vocabularies ranging in size from one to three terms for each *Pittosporum* leaf. In total 294 descriptions were generated by the panellists. The complete list of the vocabulary developed by the jury included 37 different terms. The assessors frequently used the same words. In detail, descriptors terms of *"lemon grass", "mint", "aromatic"* and *"cinnamon"* are only for *P. umbellatum*; *"vegetative", "acidic", "ginger"* and *"musty"* are only for *P. tenuifolium*.

Table 6. Descriptors developed by the panellists from the six leaves of *Pittosporum* species, content of the six vocabulary classes and number of descriptors developed by the panellists for each term (in parentheses)

floral (16)	**fruity (79)**	**green (85)**	**citrus (43)**	**aromatic (42)**	**woody (29)**
floral (14)	fruity (17)	grassy (53)	lemon (21)	vanilla (9)	toasted (4)
honey (2)	sweet (55)	green (12)	orange (13)	pepper (4)	pine (3)
	mango (3)	fresh (10)	lime (8)	oily (22)	forest (3)
	melon (2)	parsley (4)	citrus (1)	raw (1)	roasted (1)
	cherry (2)	herbal (3)		acidic (1)	tobacco (2)
		mint (1)		alcohol (1)	woody (2)
		lemon grass (1)		aromatic (1)	bitter (12)
		vegetative (1)		cinnamon (1)	rancidity (1)
				ginger (1)	musty (1)

To describe the odour (orthonasal perception) of *Pittosporum* leaves, the panellists did not use exclusively accurate terminology. Three accuracy levels were observed: generic terms (for example, flora or woody), precise terms (lemon or orange), and

intermediate level terms (citrus or lime). To exploit the complete list of terms, including the more general ones, six according to overall classes were proposed from the fragrance wheel which developed by Michael Edwards (Edwards): floral, fruity, green, citrus, aromatic and woody. Thus, all 37 terms generated by the panellists were distributed into the six classes. The more precise content of each class is detailed in Table 6. Despite the inexperience of the judges, the results shows that the generic terms were frequently used, especially for floral (floral: 87.5%), fruity (sweet: 69.6%), green (grassy: 62.4%), citrus (lemon: 48.8%), and woody (bitter: 41.4%) classes. This fact justified the use of overall classes. The scores were obtained by numbering the descriptors belonging to each class.

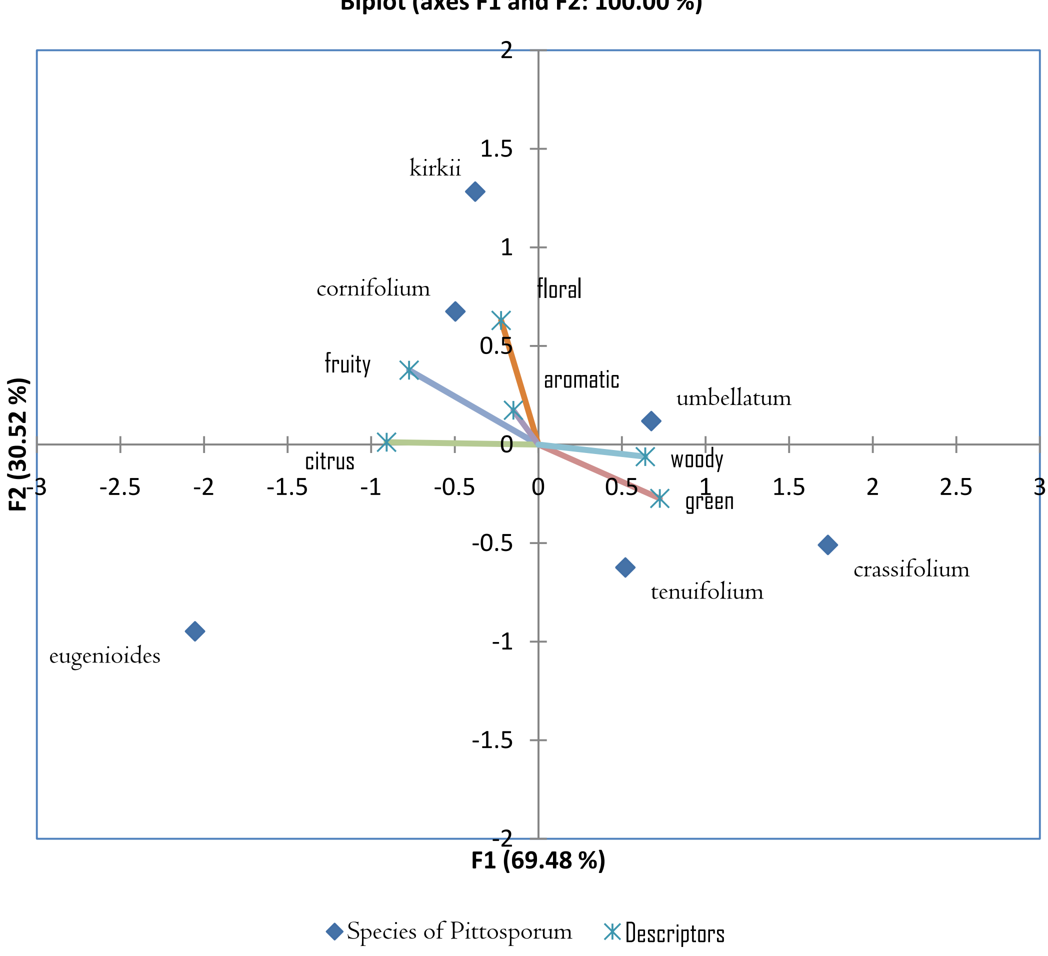


Figure 23. PCA related to sensory data: relative positions of six *Pittosporum* species and six overall classes of descriptors in the plane formed by the first two dimensions

The consecutive contingency table was submitted to PCA. The results of the PCA for each *Pittosporum* and each vocabulary class are presented in Figure 23. The two dimensions accounted for 100.0% of total variance. The horizontal axis F1 (69.48% of the total information) and the vertical axis F2 (30.52% of the total information) distinguished the descriptors in two groups. Thus they were opposed to each other. Two vocabulary classes were located on the positive part of axis F1 while the negative part of axis F2: woody and green. On the opposite, the second group was composed of citrus, fruity and floral. No conclusion can be established from the aromatic attribute because they were not well explained in the space confined within the two axes. The possible reason is the descriptor terms contributing to aromatic classes occur to all six *Pittosporum*. According to these descriptors “vanilla”, “pepper”, “oily”, “aromatic”, “cinnamon” and “ginger”, it also can be understand that the overall aroma of *Pittosporum* leaves are linked to spicy flavours.

Representation of vocabulary classes on axis F1 and F2 clearly separated terms denoting aroma profile of six selected *Pittosporum*. Representation of leaves of *Pittsoproum*s allowed *P. eugenioides* to be associated with a strong citrus odour which was referred to “lemon”, “orange” and “lime” odours. The position of *P. eugenioides* is far away from the other *Pittosporum* species. The odour of *P. eugenioides* leaf is very different from the other *Pittosporum* species. *P. crassifolium* and *P. tenuifolium* are easily associated with woody and green odours. More precisely, they were described by woody attributes such as “bitter”, “pine” and “forest”, and by green attributes such as “grassy”, “green” and “fresh”. The distance between *P. crassifolium* (1.7, -0.5) and zero point is two times longer than that between *P. tenuifolium* (0.5, -0.6), which proved *P. crassifolium* presents a stronger woody and green odour. *P. umbellatum* positioned in positive area of axis F1 and F2, which is close to woody and green attributes. However, due to the positive area of F2 is linked to fruity and floral attributes, *P. umbellatum* can be described as having grassy and woody odours with a sense of sweet floral. The last two *Pittosporum*s, *P. kirkii* and *P. cornifolium* were very close to each other and both set in negative part of axis F1 and positive of axis F2. They are likely the most similar *Pittosporum* in leaf odour with fruity and floral attributes.

The relationship between chemical components and sensory of six Pittosporums are not significant. The chemical analysis

5 Conclusions

The characterisation of volatile compounds from the leaves of six *Pittosporum* species was investigated by instrumental analysis and sensory evaluation. The six *Pittosproum*s are native to New Zealand and were collected from Auckland on October 2012.

A total 100 volatile chemical compound were identified by SPME combined with GC-MS. Eighty eight volatile compounds were confirmed by comparing Kovats Indices. The remaining 12 volatiles were not clearly identified. The number of identified compounds and the total fractions in each plant were 51 in *P. cornifolium*, 53 in *P. crassifolium*, 62 in *P. eugenioides*, 57 in *P. kirkii,* 59 in *P. tenuifolium* and 40 in *P.umbellaumte*, respectively. The main volatile compound of each *Pittosporum* species was germacrene D (36.87%) in *P. cornifolium*, α-Copaene in *P. crassifolium*, nonane in *P. eugenioides*, germacrene D in *P. kirkii*, caryophyllene in *P. tenuifolium* and γ-elemene in *P. umbellatum*. Sesquiterpenes as the major chemical classifications are identified in all six *Pittosporum*s, range from 90.19% to 40.35%. A PCA plot of the chemical composition data showed principal component 1 (PC 1) and PC2 represented 56.10% of the total variance. PC1 accounted for 32.69% of the total variance while PC2 accounted for 23.42%. *P. kirkii* and *P. cornifolium* were positioned on both negative area of F1 and F2. The biplot clearly shows *P. kirkii* and *P.cornifolium* are most similar species among all six *Pittosporum*s.

The sensory analysis profiles showed that the general odour of *Pittosporum* leaves described as "green" and "sweet". More specifically, *P. kirkii* and *P. cornifolium* are described more like "fruity" and "floral". The aromas of *P. umbellatum*, *P. tenuifolium* and *P. crassifolium* are "bitter", "woody" and "grassy" with a sense of "spicy". *P. eugenioides* as its common name lemonwood suggests, has a citrus odour. Projective mapping of the sensory analysis results suggested that *P. kirkiii* and *P cornifolium* are very similar while *P umbellatum* and *P tenuifolium* are less similar and a *P eugenoides* is quite different from the others.

6 Further directions

1. Examine the gene sequence of the *Pittosporum* species to check the closeness of the different species

2. Compare the odours of mixtures of authentic compounds identified in this study to the odour of the plant, to evaluate how similar synthetic mixtures are to the smell of the plant. Discrepancies may highlight the presence of minor compounds with intense odours or other anomalies which would be worth investigating.

3. Explore other descriptive sensory techniques to detail the descriptor of odour released from leaves.

4. To investigate the compounds in the flowers of *Pittosporum* species so that this can be added to further study to complete the profiling of *Pittosporum*.

5. The instrument that was used to identify the compounds in the plant material has a limited capacity to identify compounds in peaks that overlap. The opportunity to inspect the data using more powerful techniques could enable the identification of more compounds.

6. To investigate the differences between different plants of the same species from different locations and at different time of the year to evaluate the magnitude of any individual variations.

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Appendix

Appendix A. Chromatograms of *Pittosporum Species*

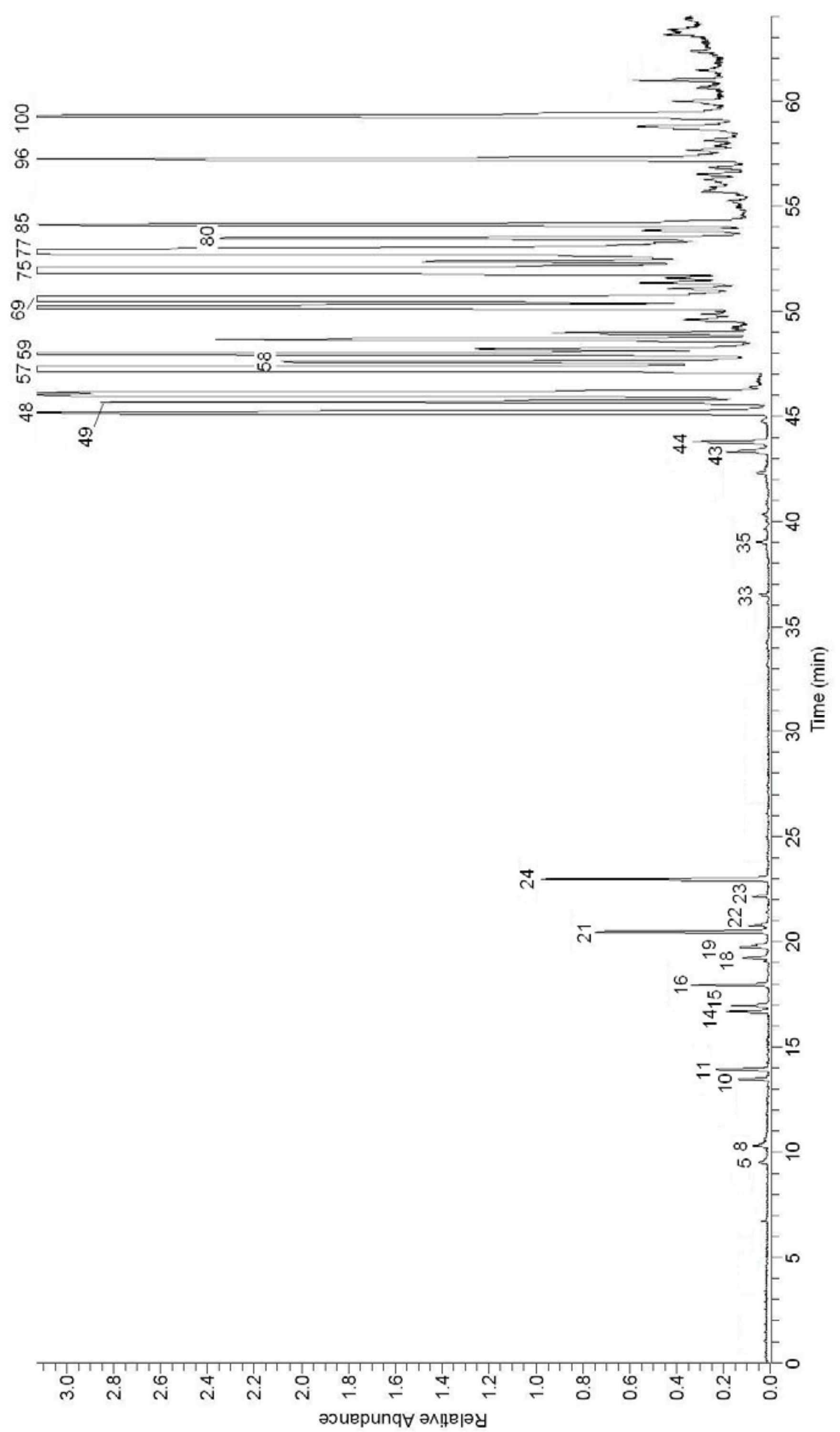

Figure 24. Chromatogram of *P. cornifolium*

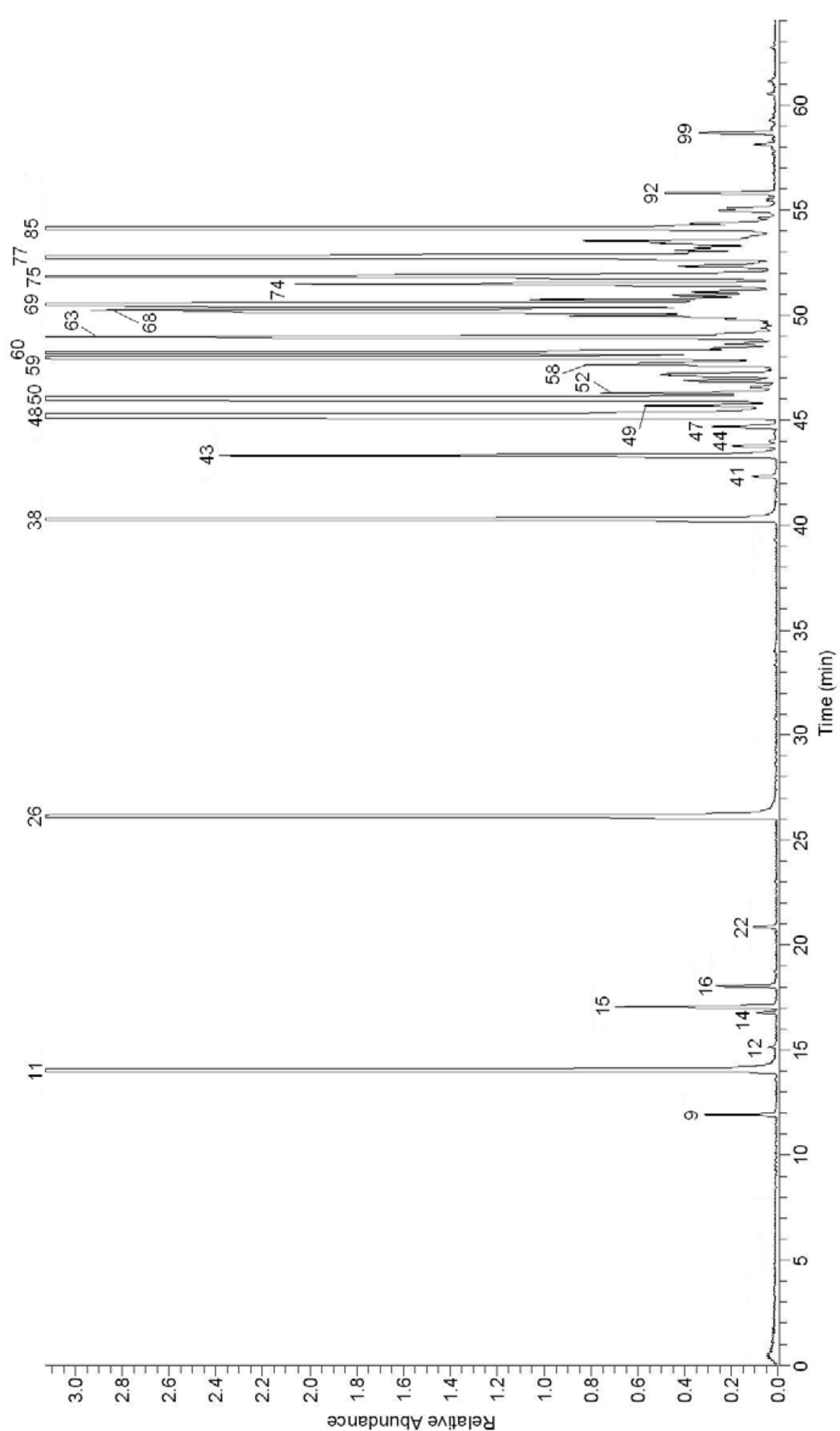

Figure 25. Chromatogram of *P. crassifolium*

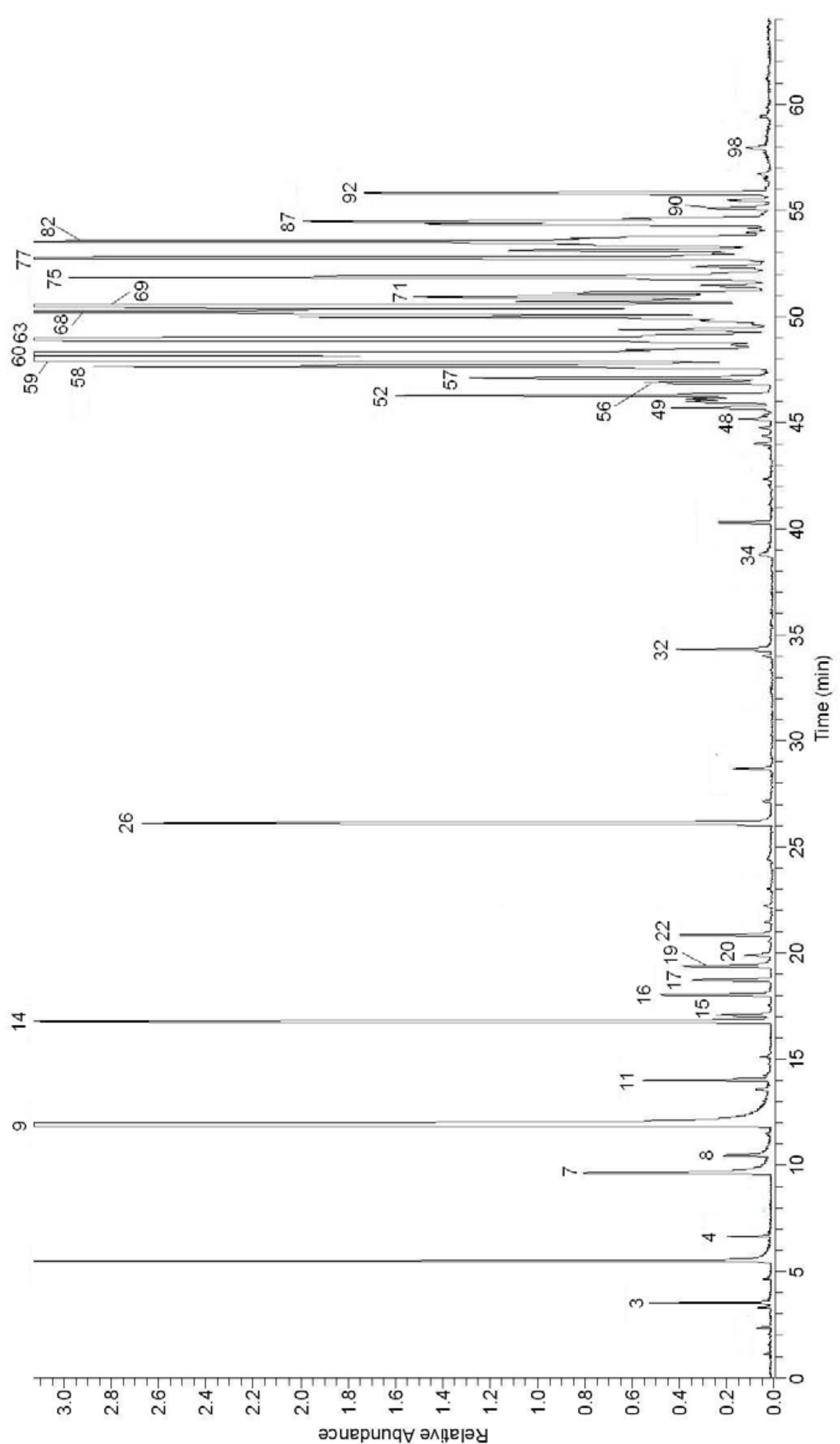

Figure 26. Chromatogram of *P. eugenioides*

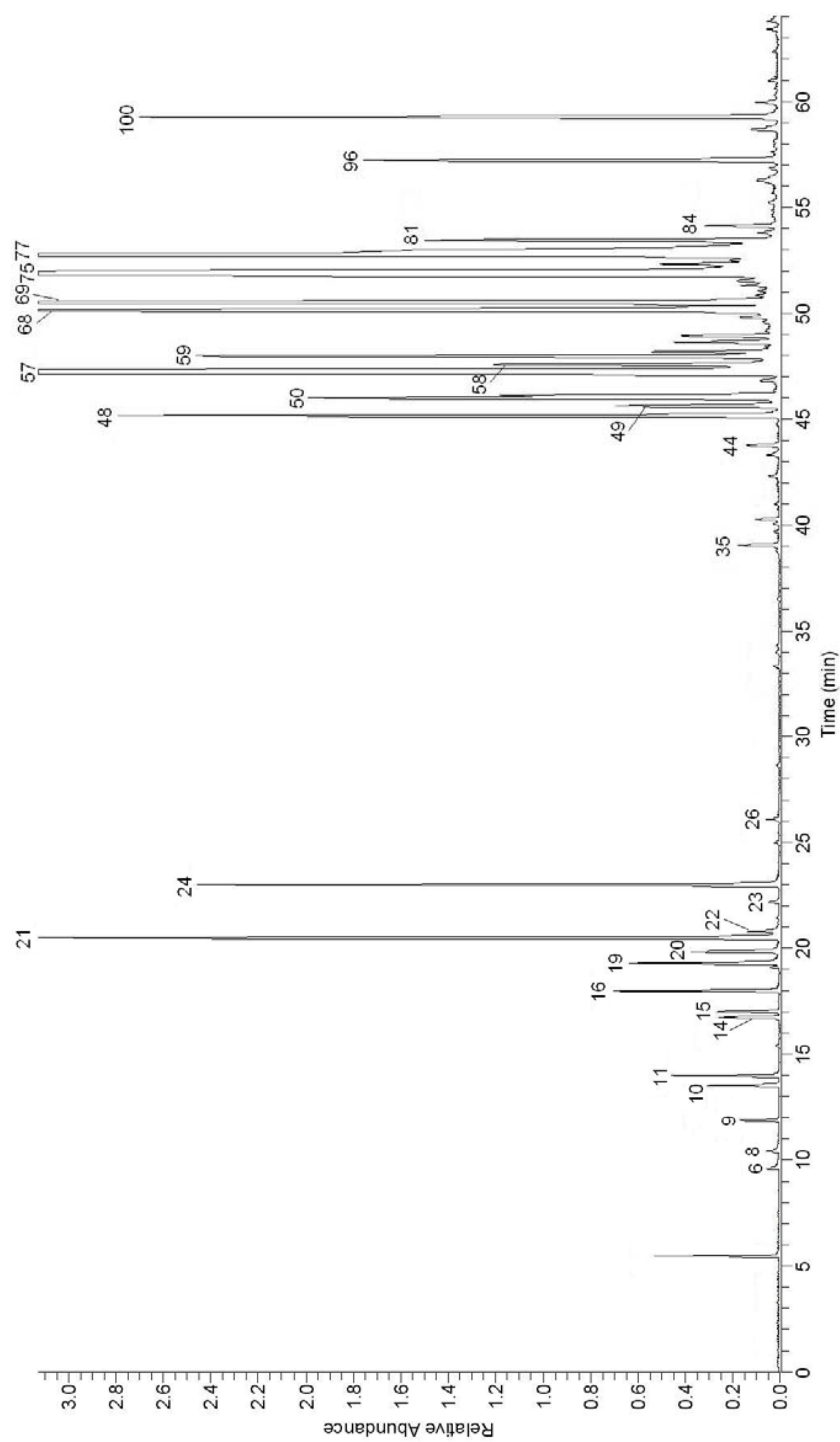

Figure 27. Chromatogram of *P. kirkii*

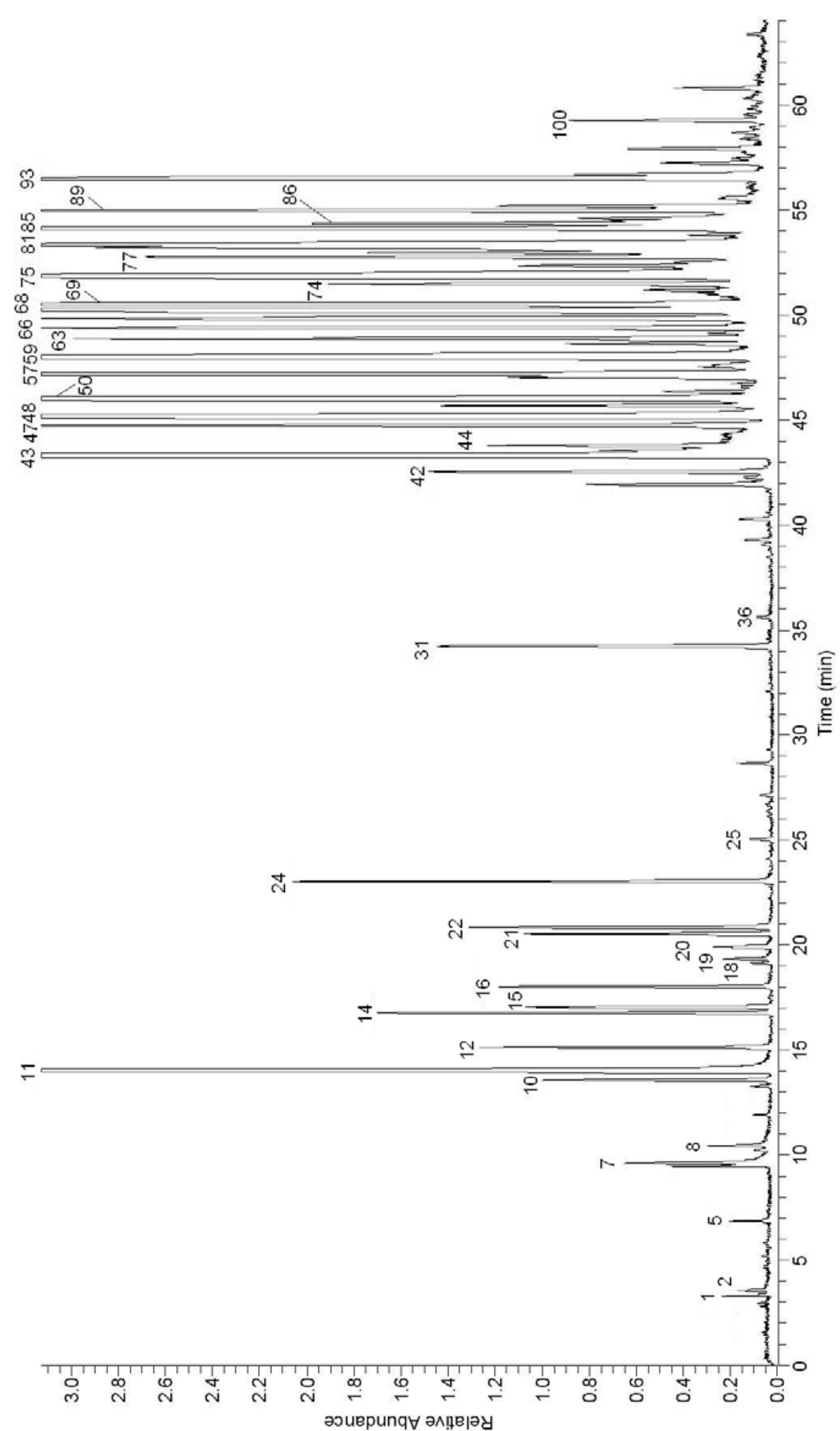

Figure 28. Chromatogram of *P. tenuifolium*

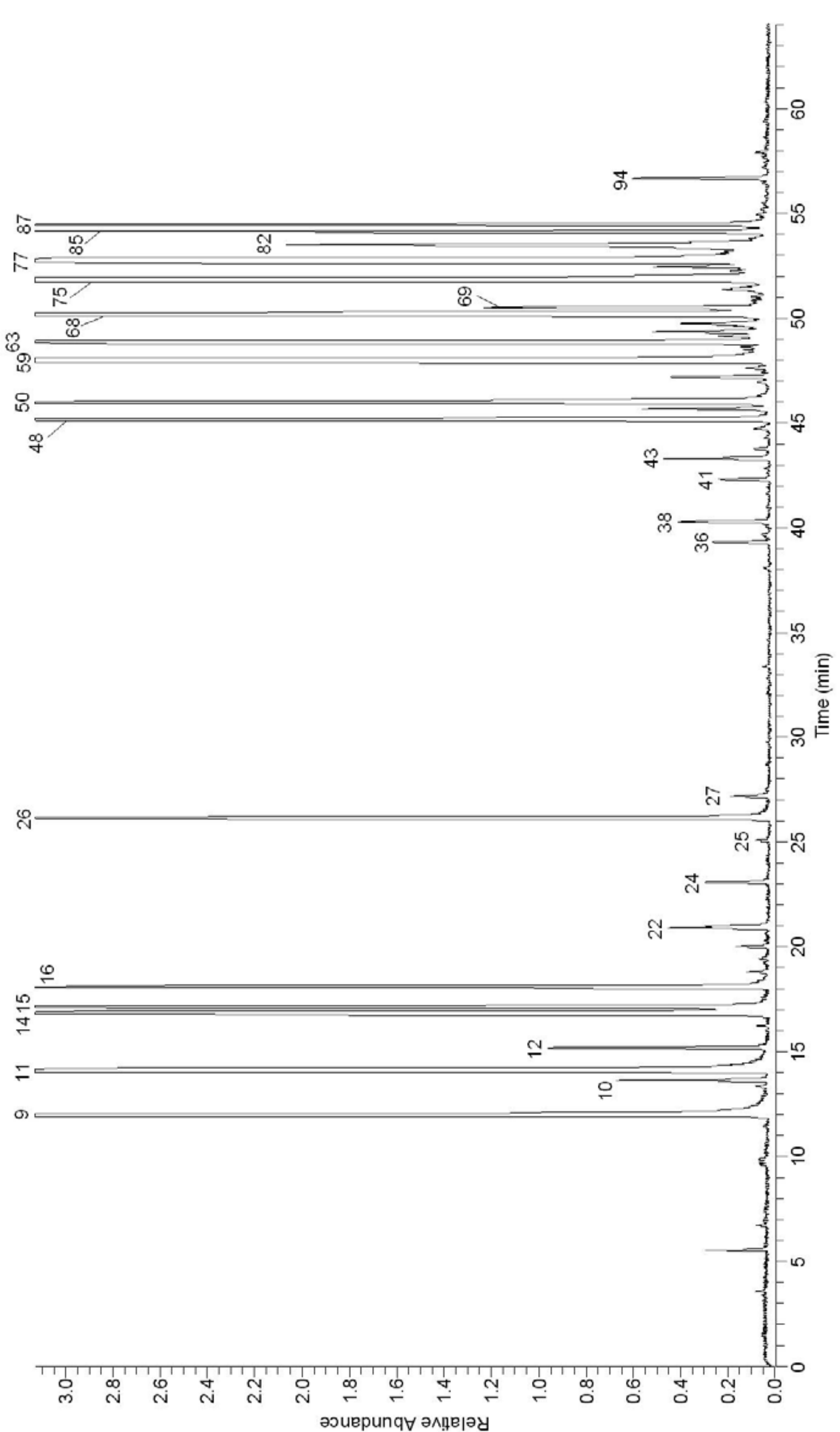

Figure 29. Chromatogram of *P. umbellatum*

Appendix B. x and y coordinate of projective mapping (output from Fizzlab sensory software)

Sample	PCR	PCO	PKI	PEU	PUM	PTE
J1_X	24	79.5	126	100.5	24	58.5
J1_Y	75	49	70	57	89	64
J2_X	69	60	133.5	64.5	88.5	63
J2_Y	64	59	22	60	73	59
J3_X	132	58.5	58.5	61.5	76.5	130.5
J3_Y	91	23	41	34	56	91
J4_X	82.5	114	42	24	76.5	121.5
J4_Y	63	82	22	34	78	63
J5_X	133.5	64.5	81	27	120	15
J5_Y	13	90	91	93	12	94
J6_X	79.5	124.5	51	31.5	42	130.5
J6_Y	58	77	26	28	36	79
J7_X	141	10.5	12	9	141	138
J7_Y	79	78	86	70	70	63
J8_X	79.5	30	27	67.5	52.5	66
J8_Y	85	63	71	38	32	82
J9_X	81	79.5	75	96	76.5	94.5
J9_Y	78	56	57	57	57	78
J10_X	129	114	136.5	36	55.5	30
J10_Y	82	64	72	38	47	29
J11_X	52.5	87	88.5	15	51	15
J11_Y	90	39	29	75	79	84
J12_X	16.5	114	142.5	127.5	28.5	63
J12_Y	10	71	96	92	16	38
J13_X	19.5	19.5	105	21	39	30
J13_Y	98	12	31	59	51	52
J14_X	55.5	66	55.5	64.5	28.5	42
J14_Y	87	32	30	88	88	86
J15_X	66	70.5	100.5	82.5	88.5	103.5
J15_Y	26	74	81	81	74	64
J16_X	61.5	93	82.5	135	61.5	135
J16_Y	91	33	34	27	83	37
J17_X	120	124.5	42	43.5	121.5	46.5
J17_Y	81	66	59	34	74	52
J18_X	123	24	51	105	94.5	81
J18_Y	76	28	34	86	58	70
J19_X	43.5	70.5	121.5	111	27	39
J19_Y	66	54	72	76	82	73

Sample	PCR	PCO	PKI	PEU	PUM	PTE
J20_X	52.5	133.5	31.5	99	61.5	58.5
J20_Y	58	90	76	27	50	60
J21_X	118.5	31.5	46.5	52.5	66	105
J21_Y	85	29	30	22	45	70
J22_X	127.5	100.5	97.5	27	42	100.5
J22_Y	77	57	69	41	21	93
J23_X	24	93	124.5	37.5	105	136.5
J23_Y	97	94	26	96	94	24
J24_X	81	124.5	45	33	117	40.5
J24_Y	55	77	33	34	82	40
J25_X	9	135	133.5	136.5	12	9
J25_Y	75	80	67	74	70	82
J26_X	112.5	31.5	34.5	73.5	37.5	111
J26_Y	71	74	60	24	67	78
J27_X	94.5	63	64.5	51	51	94.5
J27_Y	58	59	59	58	59	59
J28_X	129	78	130.5	34.5	57	19.5
J28_Y	79	55	86	30	41	31
J29_X	76.5	84	13.5	82.5	13.5	79.5
J29_Y	62	83	22	90	35	74
J30_X	18	109.5	121.5	124.5	135	45
J30_Y	9	63	88	78	93	23
J31_X	84	40.5	24	57	55.5	60
J31_Y	39	53	94	87	44	48
J32_X	46.5	102	85.5	31.5	28.5	30
J32_Y	37	23	33	94	54	46
J33_X	39	63	84	103.5	88.5	75
J33_Y	50	73	75	82	82	83
J34_X	37.5	106.5	54	99	100.5	37.5
J34_Y	55	81	59	26	19	66
J35_X	129	39	75	108	129	70.5
J35_Y	82	74	75	81	88	68
J36_X	69	133.5	132	130.5	34.5	67.5
J36_Y	65	73	81	88	74	71
J37_X	63	60	70.5	48	54	108
J37_Y	43	44	94	48	51	17
J38_X	109.5	88.5	28.5	75	15	126
J38_Y	33	79	94	79	94	33
J39_X	81	19.5	124.5	34.5	36	115.5
J39_Y	63	35	22	30	40	18
J40_X	133.5	9	10.5	10.5	132	133.5
J40_Y	81	84	76	89	90	86
J41_X	100.5	28.5	111	61.5	27	97.5
J41_Y	86	74	80	41	81	78

Sample	PCR	PCO	PKI	PEU	PUM	PTE
J42_X	78	58.5	79.5	58.5	76.5	78
J42_Y	61	62	61	62	62	61
J43_X	126	76.5	54	18	120	16.5
J43_Y	74	45	35	20	63	29
J44_X	18	108	57	106.5	21	57
J44_Y	43	73	62	86	28	72
J45_X	25.5	91.5	79.5	36	16.5	10.5
J45_Y	92	26	30	28	73	95
J46_X	127.5	81	72	28.5	49.5	36
J46_Y	23	42	45	93	60	63
J47_X	61.5	75	69	52.5	90	61.5
J47_Y	33	66	74	75	66	66
J48_X	75	73.5	108	105	34.5	120
J48_Y	36	27	80	70	72	73
J49_X	120	111	25.5	36	130.5	99
J49_Y	88	67	83	83	88	68

Appendix C. Attribute descriptors of projective mapping (output from Fizzlab sensory software)

	Attribute					
	PCR	PCO	PKI	PEU	PUM	PTN
raw	1	0	0	0	0	0
green	2	2	2	1	1	2
grassy	16	4	12	7	15	11
oily	4	3	4	4	4	4
herb	2	0	0	0	0	1
lighty	1	0	1	0	0	0
toast	4	0	0	0	0	0
tobacco	2	0	0	0	0	0
lemon	3	3	4	3	3	4
bitter	4	3	3	0	1	0
sweet	0	10	10	15	12	7
ice cream	0	3	1	1	1	1
fruity	0	4	4	2	3	2
mild	0	1	0	0	0	0
fresh	0	2	0	4	3	2
rancidity	0	1	0	0	0	0
orange	0	3	2	5	2	1
flora	0	0	4	3	3	2
alcohol	0	0	1	0	0	0
vanilla	0	1	0	1	1	0
pine	1	0	0	0	1	0
parsley	0	0	1	1	2	0
mint	0	0	0	0	1	0
musty	0	0	0	0	0	1
acidic	0	0	0	0	0	1
ginger	0	0	0	0	0	1
roast wood	1	0	0	0	0	0
medicine	1	0	0	0	0	0
pepper	1	1	0	0	0	1
melon	0	1	0	1	0	1
forest	0	0	1	1	1	0
cherry	0	0	1	0	0	0
refreshing	0	0	2	0	0	0
lime	0	0	1	2	0	1
mango	0	0	0	0	3	0
honey	0	0	0	0	0	2
essential oil	0	0	0	0	0	1
wood	1	0	0	0	0	0
citrus	0	1	0	0	0	0

	Attribute					
	PCR	PCO	PKI	PEU	PUM	PTN
vegetation	0	0	0	0	0	1
lemon grass	0	0	0	0	1	0
cinnamon	0	0	0	0	1	0
aromatic	0	0	0	0	1	0