GAS CHROMATOGRAPHY- MASS SPECTROMETRY AND GAS CHROMATOGRAPHY- OLFACTOMETRY ANALYSIS OF AROMA COMPOUNDS OF *VANILLA POMPONA* SCHIEDE

by

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ABSTRACT OF THE THESIS

Gas Chromatography –Mass Spectrometry and Gas Chromatography-Olfactometry Analysis of Aroma Compounds of *Vanilla Pompona* Schiede

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Vanilla is one of the most widely used flavor ingredients and the second most expensive spice in the world. Only three of the 110-130 species of vanilla are cultivated and have significant economic importance: *Vanilla planifolia* Andrews, *Vanilla tahitensis* Moore and *Vanilla pompona* Schiede. Among the three, *Vanilla pompona* is the only specie that has been highlighted as relatively resistant to climate change and diseases. These attributes have made this species a candidate for cross-breeding programs with *V. planifolia* to produce a more robust vanilla for commercial use.

The chemical and aroma composition of *V. planifolia* and *V. tahitensis* have been extensively analyzed. Surprisingly, studies on the chemical and odor characterization of *V. pompona* are scarce even though this species is frequently referred to in the literature as the third genus in order of economic importance. No study has been undertaken to identify which compounds are odor-active in this particular species of *Vanilla*. This study

provides a Gas Chromatography-Olfactometry (GC-O) analysis of Mexican *Vanilla pompona* Shiede for the first time.

A preliminary study was performed to select a representative aroma extract for Gas Chromatography-Spectrometry (GC-MS) and GC-O analysis. Three extracts were produced using different aroma extraction techniques. Based on sensory evaluation and preliminary chemical characterization of the extracts, the ethanol-dichloromethane solvent extraction method was selected to produce aroma extracts for in depth characterization by GC-MS and GC-O.

From the chemical characterization of the volatiles present in *V. pompona* extract, one hundred and twenty three volatiles were identified using GC-MS. Eighty compounds were identified in cured beans by means of Direct Thermal Desorption-Gas chromatography-Mass spectrometry (DTD-GC-MS). Twenty six of these constituents were identified in vanilla for the first time.

Forty five aroma impact compounds were identified by GC-O analysis of the extract of Vanilla pompona using a GC-NIF (Nasal Impact Frequency) modified method. Fifteen standard commercial samples were injected for confirmation and thirty five of the aroma impact compounds were characterized. The results of the GC-O analysis have indicated that the aroma profile consisted of thirteen primary aroma-impact compounds, eighteen identified as secondary aroma-impact compounds and thirteen odorants considered background. The aroma of *Vanilla pompona* extract is complex and rich with typical vanilla characteristics. It could be a valuable source for perfumery applications.

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1. Introduction

Vanilla is the most popular flavoring and the second most expensive spice in the world (Parthasarathy *et al.,* 2008). *Vanilla* comes from the fruit pods of a large climbing tropical vine that is a member of the Orchidaceae family. The genus, *Vanilla Plum*. ex *Mill*., includes the only orchid species of economic importance apart from the ornamental species. The current worldwide checklist of orchid vanilla species recognized 110-130 species of Vanilla (Bory *et al*., 2008). Most of the species are wild and only three species are cultivated: *Vanilla planifolia* G. Jackson, syn. *V. fragrans* (Salisbury) Ames, *Vanilla tahitensis* J.W. Moore and *Vanilla pompona* Schiede. The species *V. planifolia* and *V. tahitensis* cover most of the demand for food applications. *Vanilla pompona* has been utilized mostly for pharmaceutical and fragrance applications.

Vanilla occupies a prominent place in the food, beverage and fragrance global industry. This spice is used in all types of consumer products including nutraceuticals (Ranadive, 1994). The estimated world production is currently at least 9800 tons per year (FAOSTAT, 2012) with a continuously growing demand.

The use of natural vanilla is not limited to a flavor and fragrance additive. The main constituent of vanilla is vanillin. Researchers have been exploring numerous bioactive properties uses of this vanilla constituent. There is still work to do on alternative uses of vanilla and many growing opportunities such as the recent interest of the food industry in the use of natural compounds that exhibit antioxidant and antimicrobial activity. In this case, vanilla might be identified as a potential source of novel preservatives (Sinha *et al*., 2008).

The importance of vanilla to the global food and beverage industry has driven extensive scientific research directed to understand the chemical and aroma composition of the vanilla fruits, the biochemistry of the curing process, and to address concerns of adulteration of extracts and beans. Although the development and increase of sophistication of the analytical techniques reduced the constant threat of adulteration, the current challenges to the vanilla industry are related to environmental and social factors.

Most of the scientific research and efforts in the field have focused on the two commonly cultivated species of commerce: *V. planifolia* and *V. tahitensis*. However, events in the past 10 years highlighted the vulnerability of commercial vanilla to environmental factors such as climate change and diseases. In search of strategies to address these concerns, attention has been directed to the desirable properties of wild vanilla species, particularly to the *V. pompona* Schiede species, to endure climate changes and diseases compared to the commercial species. Although some efforts to incorporate the desirable attributes of wild vanilla species into commercial vanilla crops have been doing well, breeding wild vanilla species with commercial vanilla species do not always produce descendants with fragrant fruits. Success in obtaining resistant hybrids of *V. pompona* and *V. planifolia* without losing the characteristic fragrance of the fruits have been documented by Soto Arenas & Dressler (2010), Odoux and Grisoni (2011) and Havkin-Frenkel and Belanger (2011).

V. pompona, listed as the third vanilla species of commercial importance, is scarcely traded nowadays and absent from the United States market. This species deserves a second look by the vanilla industry due to its novel properties, aromatic

characteristics and agricultural potential in tune with the ongoing breeding efforts to incorporate its desirable properties to commercial vanilla crops (Cameron, 2011).

1.1. Geographical origin of cultivated vanilla

The bulk of vanilla cultivated for commerce is currently produced in Madagascar, Indonesia, China, Papua New Guinea, Mexico, Turkey, Tonga, Uganda and French Polynesia (FAOSTAT Database, 2012). Even though Mexico has lost its standing as the major exporter, this country continues to be the center of origin and genetic diversity for most of the species of the vanilla orchid (Hernández-Hernández, 2011).

China is considered the most recent producer of domesticated vanilla (*V. planifolia*). The production in this country started with the introduction of vines from Indonesia and Sri Lanka in the 1980's and has been progressively emerging as one of the most important vanilla producers since 1985 (Zhou *et al*., 2011).

Tahitian vanilla flavor is considered a "gourmet spice" in the global market and prized for luxury applications such as gastronomy and perfumery (Brunschwig *et al*., 2012). *V. tahitensis* is cultivated in the South Pacific, French Polynesia more commonly known as "Tahiti" and Papua New Guinea and primarily exported to France and Europe (Lubinsky *et al*., 2008).

Vanilla pompona is cultivated in the West Indies, Central and South America (Pascale et.al. 2004). This species of vanilla has been used as an extract for cooking in Mexico as well as in perfumery and pharmaceutical applications (Soto Arenas & Dressler, 2010). Although this species is easily cultivated and found extensively wild-

growing, it is only marketed locally and scarcely traded in international markets (Ehlers and Bartholomae, 1994).

1.2. Aspects of vanilla cultivation

Vanilla grows in a climatic belt, bordered by the Tropics of Cancer and Capricorn, north and south of the equator, respectively at temperatures ranging from 20-32°C (Dunphy and Bala, 2009). The vanilla plant is a climbing vine that requires a tree to provide physical support, shade, and organic material. The vines are propagated by means of stem cutting and take at least two years before the first flowering. The vines and supporting trees have to be regularly pruned to enable easy pollination and collection of the fruits as well as to provide the required 50% shade/sunlight.

1.2.1. Flowering and pollination of vanilla

The vanilla plants flower once a year over a period of about two months. The physiological cue that induces reproductively mature individuals of vanilla to flower is promoted by climatic or mechanical stress such as water, cool temperatures, and slight increases of sunlight intensity (Hernández-Hernández & Lubinsky, 2011).

Vanilla species, like other orchids, are characterized by the presence of a rostellum membrane separating the reproductive systems and limiting self-pollination. Therefore, the orchids require natural entities or human intervention to be pollinated and produce fruits (pods).

In the wild, vanilla flowers are pollinated by insects. However, natural pollinators are rare and only exist in South and Central America (Purseglove *et al*. 1981). Wild *V. planifolia* is pollinated by social bees of the Melipona genus and by hummingbirds.

Soto Arenas & Dressler (2010) reported the existence of three pollination systems among Mexican vanilla species. The first system involves carpenter bees of the Xylocopa genus and it is restricted to *V. inodora*. The second system, specific to *V. pompona, V. hameri* and *V. cribbiana*, involves bees from the Euglossa genus (Figure 1) and the reward of pollinators, usually males, is nectar. In Peru, two euglossini species, Eulaema meriana and Euglossa imperialis, are attracted to the fragrant flowers of *Vanilla pompona* that produces high quantities of limonene (Householder *et al.*, 2010). The third system, observed in Mexico wild species, concerns V. *planifolia, V. odorata* and *V. insignis* and it is a deceptive pollination system, meaning that the flowers do not reward the insects and the orchids are visited equally by males and females. Due to their pollination system, that includes offering of fragrant nectar as a reward, *V. pompona* is pollinated naturally more often than *V. planifolia*.

Hand pollination is performed with a small, thin stick similar to a toothpick, made from mambo, spines or other materials (Figure 2). Hand pollination is a daily task for a period of 3 months depending on the abundance of flowers, location, distance between plants, and efficacy of the pollinator. This is the most reliable method used for commercial cultivation of vanilla even in countries where natural pollinators are present (Hernández-Hernández & Lubinsky, 2011).

Figure 1. Natural pollinator Eulaema sp. (*jicote*) bees on *V. pompona* (Odoux and Grisoni, 2011).

Figure 2. Manual pollination of vanilla flower (http://www.vanillamexico.com)

1.2.2. Vanilla fruits and harvesting

The pod-like fruits or "vanilla beans" start developing after successful pollination. The morphology of the fruits depends on the species. Not all vanilla species produce aromatic fruits and the fragrant fruit spices are almost exclusively restricted to America (Soto Arenas & Cribb, 2010; Bory *et al*., 2011).

The vanilla beans present divergent morphological characteristics among the different species. The fruits of *Vanilla pompona* and *Vanilla planifolia* have the most noticeable difference in shape and size. The pods of *V. pompona* are significantly larger and thicker than those of *Vanilla planifolia* (Maruenda *et al*., 2013).

In addition, different physiological characteristics when the fruits ripen have been reported among different species. The beans of some species burst open while others do not. For instance, the fruits of *V. planifolia* begin to split and fall apart as soon as they ripen (dehiscence). *V. tahitensis* and *V. pompona* species do not easily develop split ends (indehiscence). Delayed splitting of beans upon ripening is a desirable attribute because they can then be harvested while fully ripe, with complete aroma potential (Odoux & Grisoni, 2011).

Another differentiating aspect among the different vanilla species is the rate aromatic compounds develop in the fruits. In *Vanilla planifolia,* the accumulation of glucosides starts in the fourth month of pod development and the production of glucovanillin increases progressively (Palama *et al*., 2009). According to Maruenda et al. (2013), the progress of the main glycosides in *Vanilla pompona* occurs at a very slow pace and increases sharply at the end of nine months. At that point, *V. pompona* beans reached concentrations of vanillin and aromatic aglycones at levels comparable to

commercially cultivated *V. planifolia* and *V. tahitensis*. Consequently, the harvesting time is an important factor to consider in the aroma quality of vanilla because this determines the level of glucosides in the fruits available for further convertion into aromatic aglycones during the process of curing.

Although it is well known that the vanilla beans harvested too early do not have the full aroma quality, commercial *Vanilla planifolia* fruits are frequently picked while still in developing stages to avoid the dehiscence of the fruits and the constant threat of thieves. In Mexico, early picking occurs due to the fixed date of harvesting on December 10th each year; a massive picking has been stipulated by agreement between growers and government. The growers harvest the entire crops in a single day, with the fruits at different stages of glucosides development as a consequence of flowering and pollination occurring over a period of three months (Hernández Hernández, 2011).

In Madagascar, early picking of vanilla beans is relatively common in many plantations to prevent the pods from being stolen from the vines, especially when prices are high. Using low quality vanilla beans require reformulation and process adjustment leading to increases in the cost of extracts and finished products.

Some minor growers in Uganda and French Polynesia harvest only when the fruits are mature, generally once a week, prolonging the harvest to two to three months (Odoux & Grisoni, 2011).

1.2.3. Curing process

Green vanilla beans are odorless and flavorless and require a curing process to develop the majority of the appreciated characteristic aroma and to reach microbiological

stability. The process consists of four basic stages: killing, sweating, drying and conditioning. This involves biological mechanisms (breakdown of tissue structures and plant response to mechanical stress), biochemistry (enzymatic and nonezymatic like oxidative reactions), enzymology and chemistry of flavor formation (Dunphy and Bala, 2009).

The curing method has been inherited from ancient Mexican cultures and modified by each region of cultivar. Despite the drawback of being long lasting and labor intensive, the traditional curing processes have prevailed over alternative more feasible methods that have been proposed (Havkin-Frenkel *et al*., 2011).

The three most popular traditional curing methods are Mexican (Beneficiado), Bourbon, and Tahitian. Even though the techniques differ from country to country, the basic stages are the same. Depending of the region, the entire curing process could last three to five months.

1.3. Challenges in vanilla commerce

Vanilla is a fragile crop with constant threat from plant diseases, volatile weather patterns typical of tropic climate, and economic hardship. All the efforts to increase knowledge about the vanilla species, both wild and domesticated, are potentially rewarding considering the continuous challenges the vanilla industry faces along with a continued growing demand. The most important of these challenges are outlined below.

1.3.1. The farming process

The cultivation of vanilla is difficult and labor-intensive. Prior to propagating the vines, the process requires the previous set up of support trees. During the subsequent two to three years before the first flowering, the support trees must be regularly pruned to provide the correct amount of sunlight for the beans. Each flower has to be pollinated by hand. The beans remain on the vines for 8-10 months until they reach maturity. When the beans are ready for harvest, they are picked individually by hand. Finally, the fruits require another hands-on process of curing during the next 3 to 5 months to develop their characteristic aromatic properties. During the process, it is also important to protect the fruits from thieves.

In many cases, farmers apply shortcuts such as deliberate “early picking” (unripe beans), plastic wrapping to retain moisture and increase the weight, propane heaters to speed up drying, and other improper practices that destroy the quality of beans. Good farming practices are essential to obtain vanilla beans of high quality and high aroma potential and any labor-saving adjustments to speed up the time to obtain a cash flow, result in detriment to the aroma quality of the vanilla beans.

1.3.2. The farmers and sustainability

Almost all vanilla throughout the world is grown as a cash crop by independent farmers in rural areas and remote villages in developing countries (Brownell, 2009). The farmers, who invest the most time and labor to grow the vanilla beans, usually have no direct access to the buyers and have to sell the beans to local intermediaries at unfairly

low prices. It is common that vanilla crops end up abandoned and substituted by other crops that offer more stability and consistency (Brownell, 2011).

After the "vanilla crisis" at the beginning of the 21st century, several companies that purchase large volumes of vanilla began independently making progress in implementing integral initiatives to promote the direct trade of beans from the farmers to the buyers, skipping futile intermediation. The most impacting initiatives to palliate the social issues involved in the cultivation of vanilla have been Fair Trade Certification and independent sustainability programs. The opinions concerning the positive impact of Fair trade certification in the price and availability of vanilla are divided (Pennestri, Hogan & Roques, 2009; Brownell, 2009, 2011).

Independent sustainability initiatives lead by several companies have been successful in ensuring their own supply and price stability of vanilla beans. Basically, the programs consist of providing training and education to small vanilla farmers on the curing process and good farming practices. All efforts directed to help small farmers to produce sustainable and high quality vanilla have been tangibly translated to a cost benefit and long term brand positioning for the companies that buy natural vanilla from the beans.

1.3.3. Vanilla diseases

Commercially cultivated vanilla is vulnerable to disease. Diseases of vanilla vines and beans damage and reduce the yield and productive period of plantations. Diseases have become a major concern over the past few decades and in huge part have

been attributed to the intensification of domesticated vanilla cultivation (Grisoni *et al.*, 2011).

This vulnerability to disease has been primarily linked to plant stress and lack of genetic diversity in vanilla plantations. Plant stress has been associated with adverse climate events (e.g. hurricanes, above average rainfall or drought), and poor farming practices such as excess shade and excessive pollination (Gleason, 2009).

Lack of genetic diversity in vanilla plantations has originated from the propagation method. Since plantations are established from stem cuttings, the plants come from the same vines over and over again. Growing the same crop species in the same soil year after year enriches the soil borne pathogens of the roots of that crop (Cook and Weller, 2004). Molecular studies have confirmed the very low level of genetic diversity in the vanilla plants cultivated throughout the world (*V. planifolia*) (Bory *et al*., 2011).

Vanilla diseases can be caused by pathogenic fungi or viruses. These can infect stems, roots, leaves, shoots, and the fruits causing rotting of the vines, spoilage of planted cuttings and shedding of fruits. Among all the vanilla diseases, the most lethal and widespread disease reported has been *Fusarium Oxysporum f. sp. Vanillae* (Hernández-Hernández, Grisoni *et al.*, Tombe and Liew, 2011).

1.3.3.1. Fusarium Oxysporum

The fungus *Fusarium oxysporum f. sp. vanillae* affects roots, stems, fruit, leaves and shoots of the vanilla vines and subsequently produces damage and death of the plants (Ranadive, 1994; Hernandez-Hernandez, 2011). Since the greatest damage is caused

when it affects the stem and root of the plant, this disease is often referred to as stem rot and/or foot rot.

The fungus is part of the normal vanilla environment, but this becomes pathogenic when the plant is subjected to stress factors. Once the fungus becomes pathogenic, it is difficult to eliminate because it propagates to all the vines within the plantation and spreads to the ground.

The financial impacts of the disease reported by leading growers were a real threat to the stability of commercial vanilla supply during the past 15 years. For instance, in Indonesia and Madagascar, the world's leading producers, Fusarium has caused significant economic losses over the last two decades. In Mexico, an outbreak killed 67% of plants in a 4-year-old plantation and infected 15% of the total fruit production. This fungus has been the main constraint to increase vanilla production in current plantations or establish new crops in smaller producers such as Puerto Rico and Costa Rica (Hernandez-Hernandez, 2011).

In this context, several vanilla connoisseurs in conjunction with Rutgers University convened to share concerns and discuss solutions in the 2009 Vanilla Conference held in New Jersey, and themed "Vanilla Diseases".

Among the strategies outlined to lessen the impact of this disease on vanilla plantations, it was proposed to expand the standard of identity to include *Vanilla pompona* and other species in the vanilla market spectrum (Brownell, 2009). In addition, the need to create a stronger breed of commercial *Vanilla planifolia* resistant to Fusarium and other diseases through crossing this species with American species such as *V. pompona* was emphasized (Purseglove *et al*., 1981). After these recommendations,

several initiatives have been reported. For instance, inter-specific hybridization has been conducted in Java using domestic *V. planifolia* and wild vanillas to develop lines resistant to stem rot caused by Fusarium oxysporum (Divakaran et al., 2011).

Some interspecific hybrids were successfully developed through breeding programs in Madagascar and Puerto Rico. Robust hybrids developed from *Vanilla planifolia* as the maternal parent and *Vanilla pompona* as the paternal parent, probably originated from the program in Madagascar have been successfully introduced and grow widely in Costa Rica (Varela Quirós, 2011).

1.4. *Vanilla pompona* Schiede

The name of the species *V. pompona* Schiede is based in a Mexican specimen collected by Schiede in Veracruz, near Papantla and Colipa in his work published in 1829 (Soto Arenas & Cribb, 2010). The documented history of *V. pompona* can be traced back to 1705 when it was identified as a *Suriname* species called "the greatest sort of Banille". In 1811, Humboldt published an account of a vanilla species growing wildly in Venezuela, Guiana and upper Amazon in Peru and described its fruits as of extraordinary size and very aromatic. In 1829, Dr. Schiede, who found this species during a trip to Mexico made in 1820 and published a description as a plant with bisulcate large fruits "abundant in ethereal oil and excellent scent", but difficult to dry at the extent required for trading. In 1872, M. Deltiel published an account of the cultivation of vanilla in Reunion, and reported that the "Great Vanilla" (*V. pompona* also briefly known as *V. surinamensis*) was introduced in the island from Cayenne by Commandant Philibert, but its cultivation never occurred at the extent of *V. planifolia* which had been established as

the standard of vanilla for commerce. However, “Baynilla Pompona” of Schiede was known at that time as an article of commerce and the fruits used in the same way as those of *V. planifolia* (Rolfe, 1895).

1.4.1. Botany

Vanilla pompona Schiede has been described as a plant with large, deep yellow and strongly fragrant flowers that are very colorful and ostentatious. It is a plant more vigorous and xerophytic than other vanilla species. Unlike other species, it can grow in zones granitic, volcanic and calcareous and is the only species of fragrant fruits in western Mexico (Householder *et al.*, 2010). A detailed description of plants and flowers of *Vanilla pompona* was provided by Soto Arenas & Dressler (2010) and illustrated in Figure 3.

The flowers are characterized by a bright yellow color and trumpet-shaped, tubular labellum as shown in Figure 4. The pollination of the orchids results in large, fat fruits, having a length about 12-15 cm (6 inches) long and a width of about 2.5 cm (1 inch) (Ehlers and Pfister, 1997; Cameron, 2011). The short, thick, trigonous and banana-like shape aroma fruits of *Vanilla pompona* are different from the more familiar pendulous and narrowly cylindrical fruits of *V. planifolia*. A remarkable quality of this vine is the capability to produce more count than other vanilla species. Also, it is apparently able to maintain the high fruit set without negative impact on the survival of the plant and future reproductive event (Soto Arenas & Dressler, 2010).

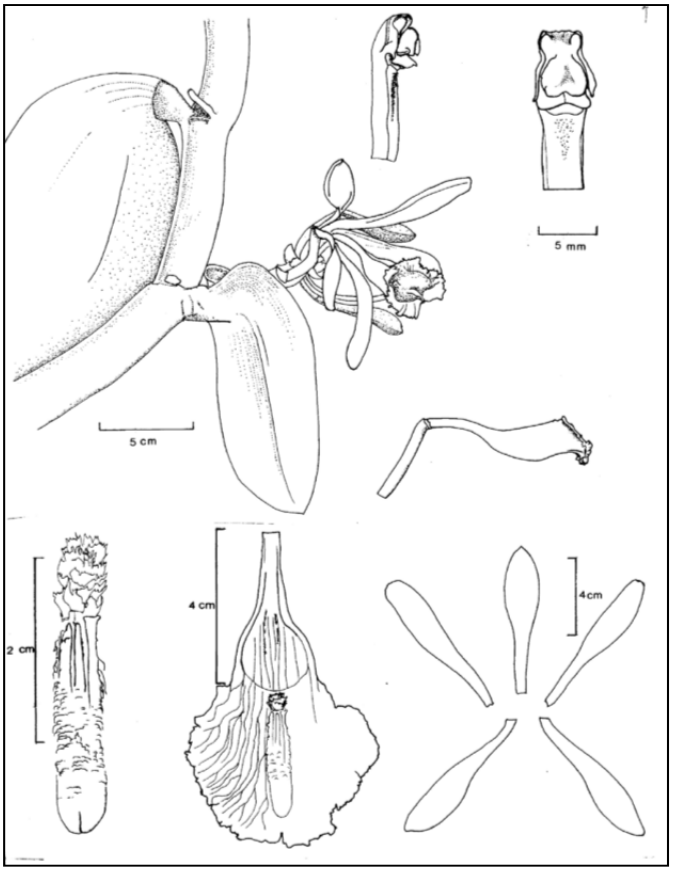


Figure 3. Drawing of *Vanilla pompona* Schiede subsp. pompona (Soto Arenas & Cribb, 2010).

1.4.1.1. Subspecies

Vanilla pompona is the most variable species in the genus and it is considered a species complex. Soto Arenas & Dressler (2010) described samples from Mexico, Central and South America and this study recognized three sub-species:

- ***Vanilla pompona* subsp. *Pompona*** is native to Mexico (Figure 3).
- ***Vanilla pompona subsp. Grandiflora (Lindl.)*** is located in South America.

- ***Vanilla pompona subsp. Pittieri (Schltr.)*** is distributed in Central America particularly in Honduras, Nicaragua, West Costa Rica and the Pacific side of Panama.

Figure 4. Picture of *Vanilla pompona* Schiede referenced in Householder, 2007 (http://atrium.andesamazon.org).

1.4.2. Cultivation, uses and trading of *V. pompona*

Vanilla pompona is widely and disjunctively distributed in Mexico, Central and Latin America. This species is native of Mexico, Nicaragua, Panama and Costa Rica and naturalized in the West Indies, Guadalupe islands and other sparse areas.

Recent studies reported that this species is one of the six most abundant vanilla species present in wetland ecosystems within the southern Peruvian Amazon (Householder *et al*., 2010; Maruenda et.al, 2013).

In Mexico, the cultivation of *V. pompona* is currently more localized to the regions of Nayarit and Oaxaca. This species is known in these regions by several common names such as vainilla platanillo, platanillo, vainilla pompona, plátano, vainilla cimarrona, bania (oaxaca), nuguyu or nejuyu (Oaxaca, Zoque), vainilla, litsmoya (southern Veracruz; Popoluca); "vainilla gruesa" (Guerrero and S Oaxaca) (Soto Arenas & Cribb, 2010).

Beans and extracts of this vanilla species are reported to be commercialized primarily for use in cooking. In some areas in Mexico, this species can be found cultivated in *V. planifolia* and coffee plantations, as a curiosity or charm, rarely offered for commerce, and protected to be used only for local consumption. Similarly, some plantations in Ecuador and Guatemala grow a mixture of wild vanillas including *V. pompona*, besides the true *V. planifolia* (Soto Arenas & Dressler, 2010).

Across the Atlantic and Pacific Ocean, this vanilla species is specifically cultivated in Guadeloupe, Martinique, and Dominica, with the products being sold under the name "***Vanillons"*** (Ehlers and Pfister, 1997; Reineccius, 2006). Genetic studies of specimens of *V. pompona* in these regions were compared and found to be associated to specimens that grow in Veracruz, Mexico, confirming that this was the origin from where the vines were introduced to the West Antilles (Soto Arenas & Dressler, 2010).

According to Ehlers and Pfister (1997) Vanillons pods or its extracts are rarely used for the production of food, and are applied primarily in perfumery and

pharmaceutical preparations in the European market because of their deviating flavor. Likewise, the cultivation of this species in Guadeloupe has been extensively referred to uses for perfumery and pharmaceutical applications by Correll (1944), Purseglove *et al.* (1981) and Lepers *et al*. (2011) and Odoux & Grisoni (2011), however, no further information or details can be found on those specific applications.

Despite *Vanilla pompona* being considered the third vanilla species in economic importance, it is scarcely traded nowadays. The scant attention by the vanilla industry might be in part due to the following:

1) This species was not included in the FDA standard of identity of vanilla for use in foods and beverages in the U.S., presumably due to a safety concern related to natural occurrence of coumarin as further explained in Section 1.4.3.
2) The inaccessibility to cured fruits of *V. pompona* (Ehlers and Pfister, 1997) and the current lack of supply and demand in web sites or any other means used for trading vanilla.
3) This species has been referred in literature as "poor" or "lower" quality compared with the other two commercial species (Reineccius, 2006). This statement has been called into question in recent studies (Section 1.8.1).

Introduction of this species to international markets for use in fragrance, perfumery or pharmaceutical applications is a desirable goal of local growers to maintain its cultivation and to preserve the genetic heritage of this novel vanilla.

1.4.3. *Vanilla pompona* and adulteration

Given the cost of cultivating and producing vanilla products, the practice of adulteration has been constantly an issue in vanilla trading. During the 20th century, *Vanilla pompona* species has been mentioned in several discussions related to misleading practices. For instance, Gnadinger (1925) developed a chemical method to differentiate "expensive" Bourbon extracts (Madagascar *V. planifolia)* from "less expensive" vanilla extracts produced using *V. tahitensis* and *Vanillons (Vanilla pompona)*, species that were considered of lower quality then.

Concerns of adulteration of vanilla extracts with Tonka beans were published on the web page of the Food Drug Administration (FDA) Consumer's updates in 2009. The beans of Tonka trees smell and taste like vanilla extract due to the presence of natural occurring coumarin. A high level of coumarin is toxic to the liver and kidneys. For a long time, *Vanilla pompona* was deemed as the only species to naturally contain coumarin. This theory remained for years, and the discussion was documented by Ehlers and Pfister (1997). The authors published a study that confirmed that this compound is not naturally occurring in this species. The initial affirmations and the scarce scientific studies on *Vanilla pompona* species could produce a safety concern in the use of this vanilla in food, and may be the reason this cultivated species was excluded from the standard of identity of vanilla extracts for use in foods and beverages in the U.S.

Another topic of discussion related to *Vanilla pompona* was the presumable presence of the compound piperonal, a recognizable vanilla adulterant that has heliotrope, flowery sweet, powdery and vanillic aroma. This compound either as naturally occurring or as adulterant was regarded as being responsible for the floral fingerprint of both *V.*

tahitensis and *V. pompona*. The presence of floral notes in vanilla extracts, deviant from the most popular *V. planifolia* (Bourbon) aroma, was not considered a positive attribute. The reports of the natural occurrence of piperonal in these species were confirmed to be incorrect, and the results attributed to either the use of unsuitable analytical methods or adulteration (Ehlers and Pfister, 1997). Most recently, Adedeji *et al*. 1993 and Lee, 2006 characterized samples of *V. pompona* beans using Direct Thermal Desorption-Gas Chromatography-Mass Spectrometry (DTD-GC-MS) and did not report coumarin or piperonal in these beans.

1.5. Volatile composition of vanilla

The typical aroma of the cured vanilla beans results from a complex and varied mixture of chemical compounds. The volatiles composition of vanilla depend upon the vanilla species, the geographical origin (climate, soil composition), the curing process (Mexican, Bourbon, Tahitian), the farming practices (growth conditions, planting and harvesting), and also the analytical technique used to extract the aromatic components (Hartman, 1992; Nakasawa *et al*., 1981).

Up to now, more than 300 volatile compounds have been identified from cured vanilla fruits and commercial extracts (Toth, 2012; Lee, 2006; Havkin-Frenkel & Belanger, 2011; Hartman, 1992). The main vanilla flavor backbone of commercial vanilla species is constituted by the so called marker compounds: vanillin, *p*-hydroxybenzaldehyde, vanillic acid, and *p*-hydroxybenzoic acid. The structures are shown in Figure 5 (Ranadive, 1994). The ratios of these compounds have been used as an indicator of quality of commercial vanilla to detect adulteration of beans and extracts.

Although these ratios are now part of the standard of identity of vanilla extracts in the European regulations, such generalizations are not completely accurate for all vanilla beans (Gassenmeier *et al*., 2008).

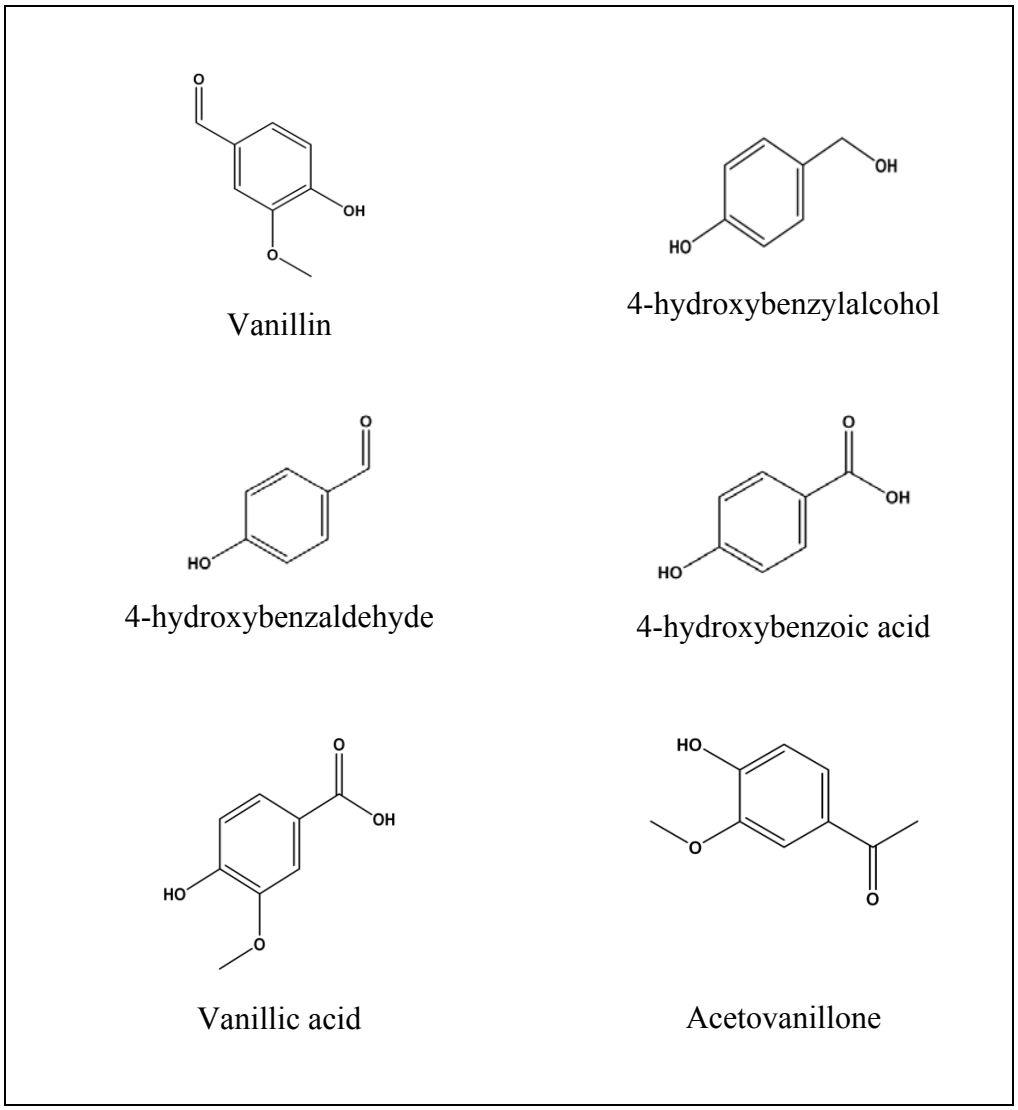


Figure 5. Main volatile constituents and aroma impact compounds in *Vanilla planifolia* (Perez Silva et al., 2006).

The aroma precursors of vanilla are present in green beans as glycosides which are released upon curing. The biochemical pathways forming aromatic aglycones during

the curing process have been thoroughly studied and discussed (Adedeji *et al*., 1993; Ranadive, 1994).

The quality of cured vanilla beans was formerly determined by the concentration of vanillin, which has been reported to occur at levels of 0.3-3.4 percent (Morison-Smith, D., 1964; Brodelius, 1994). Although the presence of vanillin enhances the quality of vanilla, this is only a contributor and cannot replace alone the characteristic natural vanilla flavor because vanilla beans also contain hundreds of constituents, some of them at trace concentrations that play significant roles in the richness, complexity and strength of the overall aroma (Perez Silva *et al*., 2006).

The aroma characteristics among species differ not only in the concentration of compounds, but also in chemical composition; for instance, compounds such as anisaldehyde, anisyl acetate and methyl anisate are present in Tahitian vanilla flavor, but these are not present in *V. planifolia*. On the other hand, classical "vanilla" notes identified in *V. planifolia* and attributed to vanillyl or *p*-hydroxybenzyl compounds were not identified in the species *Vanilla tahitensis* (Brunschwig *et al*., 2012).

The volatile composition of *Vanilla planifolia* Jackson and *Vanilla tahitensis* Moore has been extensively analyzed using different analytical techniques in more than 100 publications. In contrast, only two studies have been published on chemical characterization of the species *Vanilla pompona* (Ehlers and Pfister, 1997; Maruenda *et al*., 2013).

A comprehensive review of the volatile composition of selected vanilla beans can be found in the Handbook of Vanilla Science and Technology published by Havkin-

Frenkel & Belanger (2011), which covers information published on vanilla flavor analysis up to that time.

1.6. Characteristic vanilla aroma and flavor

The characteristic aroma qualities of the three commercially important species of *Vanilla* were described by Ranadive (1994) and Toth (2012) and are summarized in Table 1.

Table 1. List of vanilla flavor terminology (Ranadive, 2006).

Acidic	Fruity	Smokey	Creamy	Chocolate
Anisic	Hay-Like	Sour	Earthy	Pungent
Aromatic	Moldy	Spicy	Floral	Vanillin
Balsamic	Musty	Sweet	Raisin	Vinegar
Barnyard	Phenolic	Tea-Like	Resinous	Rummy
Caramelized	Prune	Tobacco-Like	Woody	Heliotrope

1.7. Aroma compounds in vanilla

Among the hundreds of volatiles that have been identified in vanilla beans and extracts using advanced analytical techniques, just a fraction of these chemical compounds have been recognized as aroma-odor active. Many of the volatile compounds present in vanilla are known to have a certain odor quality to some extent. However, a measure of the extent that each of these chemicals impact on the overall odor quality is a question that can be determined only after performing Gas Chromatography-Olfactometry (GC-O) analysis.

Aroma compounds that are only characteristic of certain vanilla species are considered fingerprints. For instance, *Vanilla tahitensis* contains unique aroma components such as anisyl alcohol, anisic acid and para-anisaldehyde (Figure 6) that are responsible for the characteristic perfume and floral aroma of this species (Brunschwig *et al*., 2012).

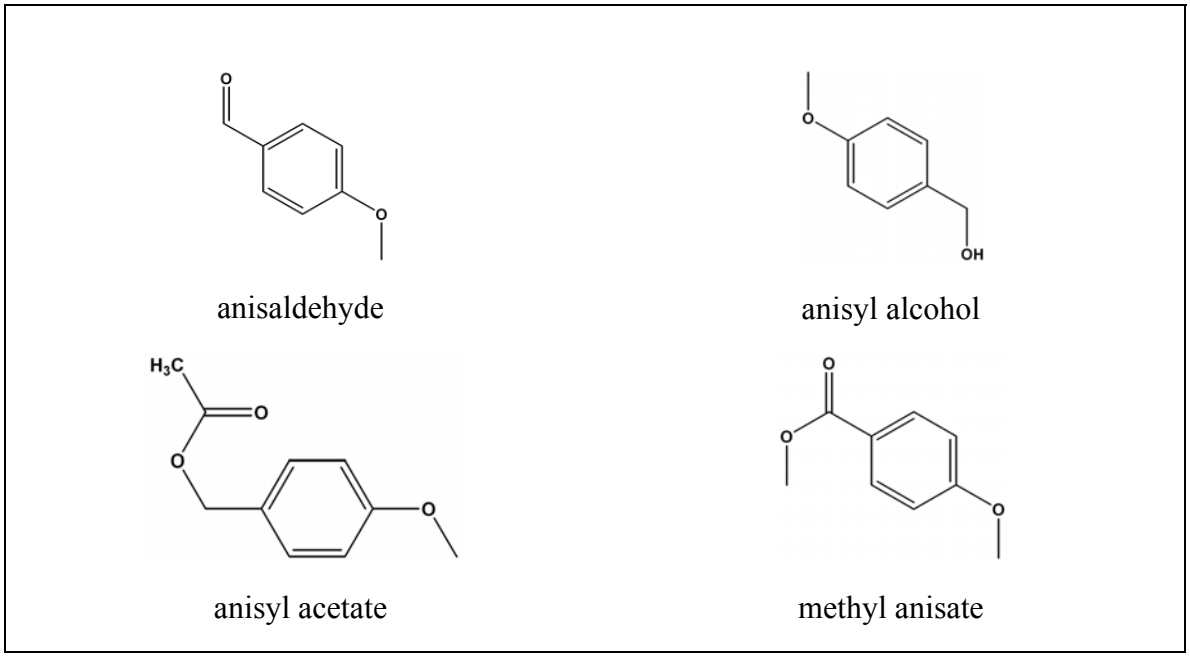


Figure 6. Aroma impact compounds considered fingerprint of *Vanilla Tahitensis* (Brunschwig et al., 2012).

These compounds are considered fingerprints because they are absent or present at trace levels in *V. planifolia*, which lacks the floral notes (Toth, 2012; Havkin-Frenkel & Belanger, 2011; Nakazawa *et al*., 1981). Strong floral notes have been similarly perceived in *Vanilla pompona* (Nakasawa *et al*., 1981; Ehlers and Pfister, 1997).

From the GC-O/MS analysis of *Vanilla planifolia* and *Vanilla tahitensis* (Perez Silva *et al*. 2006, Zhang and Muller, 2012; Brunschwig *et al*., 2012), thirty eight aroma

impact compounds present in commercially cultivated vanilla have been identified. Additionally, sixty eight tentative compounds were proposed by Zhang and Mueller (2012). Table 2 compiles these aroma impacting compounds, including the selected tentative odorants proposed by Zhang and Mueller (2012).

1.8. Aroma and volatile composition of *Vanilla pompona*

The aroma of Guadeloupe vanilla (*V. pompona*) was reported by Ranadive *et al*. (1994) as perfumy, floral and sweet. A complete characterization and identification of aroma compounds in *Vanilla pompona* itself has yet to be undertaken. However, observations in published studies have indicated that *Vanilla tahitensis* and *Vanilla pompona* share a strong floral/perfumery/heliotropin-like flavor which is an attribute not found in *V. planifolia* (Ehlers and Pfister, 1997). Nakazawa *et al.* (1981) reported similarity of the aroma quality in these two species, and also perceived unique anise-like notes in *V. pompona*. More recently, Lee (2006) referring to cured beans of *Vanilla pompona* from Madagascar, described its overall aroma quality as strong vanillin-like, sweet, creamy, flowery, perfumery, resinous, and slightly woody with intermediate aroma characteristics between that of *Vanilla planifolia* and *Vanilla tahitensis*.

Concerning the chemical composition of *V. pompona*, Gnadinger (1925) reported the presence of anisyl alcohol in vanillons. The compound *p*-hydroxybenzyl alcohol was identified, using thin layer chromatography, as a characteristic component of pods of *V. pompona* (Prat and Stoll, 1968; Ehlers and Pfister, 1997).

Table 2. Odor active compounds identified in cured beans and extracts of species *Vanilla planifolia* and *Vanilla tahitensis* by GC-O/MS analysis (Perez Silva et al., 2006; Brunschwig et al., 2012; Zhang and Mueller, 2012).

VOLATILE COMPOUND (MULTIPLE REFERENCES)	DESCRIPTORS (REFERENCE)
2,3 butanedione (diacetyl)[1,3]	Sweet, buttery, creamy, milky[1], butter[3]
acetic acid[1,2]	Sour, vinegar-like[1,2]
acetol (hydroxyacetone)	Aromatic, caramellic[1]
3-methylbutanal (isovaleraldehyde)[1,3]	Acrid, fruity, peach, cocoa-like[1], chocolate[3]
isoamyl alcohol	Fresh, ethereal, fusel-like, fermented and yeasty[1]
2,3 butanediol (Isomer I)	Soft ethereal[1]
2,3 butanediol (isomer II)[1,2]	Soft ethereal[1], floral, oily[2]
butanoic acid (butyric acid)[1,2]	Penetrating, reminiscent of rancid butter[1], buttery, oily[2]
hexanal[1,3]	Green, fruity, aldehydic[1], somewhat green apple-like, green, grassy, fruity[3]
2-furaldehyde (2-furfural)	Sweet caramel-like, nutty, baked bread, almond[1]
2-furfurol	Burnt, sweet, caramel, brown[1]
isovaleric acid[1,2,3]	Acidic, cheese-like [1],buttery, oily[2], cheese, unpleasant[3]
2-methylbutyric acid[1,3]	Acidic, sweaty [1],cheese, fruity, animal[3]
1-hexanol	Roasty, nutty; pleasant cheesy[1]
pentanoic acid (valeric acid)[1,2]	Strongly acidic, caprylic[1], cheese[2]
2-hydroxyisobutyric acid[1]	Suffocating odor[1]
2-acetylfuran	Balsamic[1]
benzaldehyde	Sweet aromatic, spicy, bitter almond-and dark cherry like[1]
phenol [1]	Strongly phenolic, medicinal odour[1]
guaiacol[1,2,3]	Smoky, vanilla bean-like[3]
4-methylguaiacol (creosol)[2,3]	Sweet, woody[2] Smoky[3]

Table 2. Continued. Odor active compounds identified in cured beans and extracts of species *Vanilla planifolia* and *Vanilla tahitensis* by GC-O/MS analysis (Perez Silva *et al*., 2006; Brunschwig *et al*., 2012; Zhang and Mueller, 2012).

VOLATILE COMPOUND (MULTIPLE REFERENCES)	**DESCRIPTORS** (REFERENCE)
methyl salicylate	medicinal, phenolic, sweet, characteristic wintergreen[1]
1,2-dimethoxy-4-methylbenzene (methyl creosol)	candy sweet[1]
methyl nonanoate (methyl pelargonate)	oily, fatty; slightly fruity[1]
γ-octalactone	sweet creamy with coconut character[1]
p-anisaldehyde (4-methoxybenzadehyde)[1,3]	sweet, herbaceous-spicy; creamy, powdery, vanilla[1] , anise-like, almond[3]
4-(2 -propenyl)-phenol (chavicol, 4-allylphenol)	aromatic spicy, medicinal, phenolic[1]
phenelthyl acetate	sweet, floral, fruity, green, rose, dried fruit[1]
trans-cinnamaldehyde	sweet aromatic spicy, Cinnamic, cassia-like, balsamic[1]
nonanoic acid(pelargonic acid)	oily, fatty, caprylic cheesy[1]
anisyl alcohol4-methoxybenzylalcohol) [1,2,3]	sweet aromatic, balsamic, caramel, nutty [1], herbal[2] Anise-like[3]
methyl cis-cinnamate[1,2]	fruity, balsamic, strawberry-like[1], sweet[2]
3-phenol-2-propen-1-ol (cinnamyl alcohol)	sweet-warm balsamic, slightly cinnamon[1]
5-isopropyl-2-methyl phenol (carvacrol)	spicy, herbal phenolic[1]
3-methyl-5-propyl-2-cyclohexen-1-one (celery ketone, livescone)	slightly sweet, warm, celery like[1]
2-methoxy-4-vinylphenol (varamol 106)	aromatic, spicy, phenolic[1]
dihydroedulan II	sweet, rose like[1]
heliotropine (piperonal)/ (3,4-Methylenedioxybenzaldehyde)	cherry, powdery, vanilla and sweet anisic[1]
methyl decanoate (methyl caprate)	winey, slightly sweet, honey like[1]
γ -nonalactone	creamy-fatty, coconut-and apricot-like[1]
4-allyl-2-methoxyphenol (eugenol, 4-allylguaiacol)	strongly warm spicy, clove-like[1]
trans methyl cinnamate[1,2,3]	fruity[1,3], balsamic, strawberry like[1], sweet[2]
phenylethanol	floral, rose[3]

Table 2. Continued. Odor active compounds identified in cured beans and extracts of species *Vanilla planifolia* and *Vanilla tahitensis* by GC-O/MS analysis (Perez Silva *et al*., 2006; Brunschwig *et al*., 2012; Zhang and Mueller, 2012).

VOLATILE COMPOUND (MULTIPLE REFERENCES)	**DESCRIPTORS** (REFERENCE)
vanillin[1,2]	intensive sweet, tenacious creamy, characteristic vanilla aroma[1], vanilla, sweet[2]
β-damascenone	woody, floral, herbal, green and fruity[1]
vanillyl methyl ether	sweetish, fruity[1]
2,5-dihydroxybenzaldehyde	mild aromatic, spicy, medicinal[1]
trans-cinnamic acid	sweet aromatic, balsamic, cinnamic-like[1]
methyl 4-hydroxybenzoate (methylparaben)	sweet aromatic, phenolic, fruity[1]
ethyl trans-cinnamate	cinnamon[1]
acetovanillone (apocynin) (4-acetylguaiacol;4-hydroxy-3-methoxy-acetophenone)[1,2]	sweet aromatic, vanilla-like[1], vanilla, sweet, honey[2]
germacrene D	minty, woody, herbal, sweet, hay-and tea-like with tobacco nuances[1]
methyl vanillate	sweet aromatic, spicy, slightly vanilla[1]
methyl vanillyl ketone (guaiacylacetone)	sweet powdery, vanilla creamy balsamic[1]
4-hydroxy-3-methoxybenzoic acid (vanillic acid)	sweet aromatic, vanilla, creamy, milky[1]
dodecanoic acid (lauric acid)	mild fatty[1]
3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde, 5-methoxyvanillin) [1,2]	sweet aromatic, slightly floral[1], vanilla like, biscuit[2]
4-isopropyl-1,6-dimethylnaphthalene (cadalene)	herbal, savory[1]
4-vinylguaiacol [2,3]	chemical phenolic[2], smoky, phenolic[3]
4-vinylphenol	sweet, woody[2]
vanillyl alcohol	vanilla-like[2]
p-hydroxybenzaldehyde (4-hydroxybenzaldehyde)	vanilla-like,biscuit[2]
p-hydroxybenzyl alcohol	vanilla-like, sweet[2]
isobutyric acid	buttery[2]
1-octen-3ol; 1-octen-3-one	mushroom[2,3]
p-menthenal	fat, floral[3]

Table 2. Continued. Odor active compounds identified in cured beans and extracts of species *Vanilla planifolia* and *Vanilla tahitensis* by GC-O/MS analysis (Perez Silva *et al*., 2006; Brunschwig *et al*., 2012; Zhang and Mueller, 2012).

VOLATILE COMPOUND (MULTIPLE REFERENCE)	**DESCRIPTORS** (REFERENCE)
2-heptenal	green, oily[3]
(E)-2-decenal[2,3]	herb-like, floral[2], aldehyde, olive[3]
(E,Z)-2,4-decadienal[2,3]	herb-like, fresh[2], Fat, wax[3]
(E,E)-2,4-decadienal[2,3]	fatty, wood [2], cooking fat[3]
methyl salicylate	chalk[2]
ethyl linolenate	sweet[2]
1H-pyrrole-2,5-dione,ethyl-4-methyl	bread, nut[2]
3-hydroxy-2-butanone	buttery[2]
isobutanal	chocolate[3]
2,3-pentanedione	butter[3]
valeraldehyde	chocolate[3]
3-methyl-2-buten-1-ol	glue[3]
3-methyl-2-butene-1-thiol	meat, burnt, sulfur[3]
2-methylfuran-3-thiol	meat, bacon[3]
methional	cooked potato[3]
2-acetylpyrroline	grilled hazelnut[3]
dimethyltrisulfide	cabbage-like, sulfur[3]
(Z)-1,5-octadien-3-ol	mushroom, methalic,earthy[3]
2,4-heptadienal	oily, green, aldehyde, fatty[3]
octanal	fat, green orange, fruity[3]
phenylacetaldehyde	honey, floral[3]
(Z) 6-nonenal	melon[3]
(E,Z)-2,6-nonadienal	melon[3]
(Z) 2-nonenal	aldehyde, leather, fatty[3]
methyl anisate	anise-like[3]
nonanal	fat, green, orange[3]
p-cresol	animal, leather[3]
anisyl acetate	fresh, anise-like[3]

(1) Zhang and Mueller Firmenich (2013) GC-O/MS of *Vanilla planifolia* from Madagascar and Uganda.
(2) Perez Silva *et al*. (2006) GC-O/MS of *Vanilla planifolia* Jackson from Mexico.
(3) Brunschwig *et al*. (2012) GC-O/MS of *Vanilla tahitensis* from French Polynesia.

Nakasawa *et al.* (1981) characterized *Vanilla pompona* from the West Indies as part of a GC-MS analysis of several vanilla species. The concentrations of anisaldehyde, cinnamic acid, trans-cinnam-aldehyde and anise alcohol in *Vanilla pompona* were the highest among the vanilla species in this study. The strength of relevant aromatic compounds such as vanillin and vanillic acid was comparable to those found in *V. planifolia* and *V. tahitensis.*

Ehlers and Pfister (1997) studied a sample of *V. pompona* by means of HPLC and confirmed the presence of *p*-hydroxybenzoic acid, vanillic acid, *p*-hydroxybenzaldehyde, *p*-anisyl alcohol, *p*-anisic acid and *p*-anisaldehyde and vanillin (Table 3). The most comprehensive chemical characterization of *Vanilla pompona* was made by Lee (2006) using beans from Madagascar and identifying 79 volatile and semi-volatile compounds using DTD-GC-MS.

Table 3. Main compounds in *Vanilla pompona, V. planifolia* and *V. tahitensis* from studies made by Ehlers and Pfister (1997) and Ehlers and Bartholomae (1993, 1994) using HPLC.

Compound	Percentage			Method of identification
	V. pompona	*V. tahitensis*[a]	*V. planifolia*[b]	
p-hydroxybenzoic acid	0.05	0.48	<0.05	1
vanillic acid	0.07	0.06	0.10	1
p-hydroxybenzaldehyde	0.02	0.09	0.11	1
vanillin	0.32	0.50	1.80	1
p-anisyl alcohol	0.14	0.60	0	1
"ethyl vanillin"[c]	<0.001	<0.001	0	1
piperonal	<0.001	<0.001	0	1, 2
coumarin	<0.001	<0.001	0	1
p-anisic acid	0.04	0.56	0	1
p-anisaldehyde	0.03	0.02	0	1
m-anisaldehyde	<0.001	trace?	0	1
water	3.2	5.5	-	3

1 = HPLC; 2 = Color Test by Gnadinger; 3 = Karl Fischer; [a] = data from (26), sample 2; [b] = data from (27), sample 25; [c] = 3-ethoxy-4-hydroxybenzaldehyde; 0 = negative; - = no data retained

The Handbook of Vanilla Science edited by Havkin-Frenkel and Belanger (2011) compiled volatiles and semivolatiles found in *V. pompona* from the studies published by Gnadinger (1925), Simony (1953), Klimes and Lamparsky (1976), Shiota and Itoga (1975) and Lee (2006). A summary of this information, organized by chemical class, is presented in Table 4.

The most recent publication on *Vanilla pompona* reported the concentration of glucosides and aglycones of the eight main compounds in uncured beans of *Vanilla pompona* from a Peruvian forest. The author reported high concentrations of vanillin, 4-hydroxybenzyl alcohol and anisyl alcohol. Interestingly, the author reported the absence of anisaldehyde and vanillic acid in uncured beans of *V. pompona* in nine month-old fruits (Maruenda et al., 2013).

Table 4.Volatile compounds identified in cured beans of *Vanilla pompona* from samples sourced from unknown origin and from Madagascar (Havkin-Frenkel & Belanger, 2011).

COMPOUNDS	**CAS#**
Alcohols	
4-methoxyphenyl) methanol (anisyl alcohol)	105-13-5
1,2-dihydroxybenzene (catechol, pyrocatechol)	120-80-9
2,2-dimethylpentan-1-ol (neoheptanol)	2370-12-9
2-ethylcyclobutanol	35301-43-0
2-methoxy-*p*-cresol (2-hydroxy-5-methyl anisole)	93-51-6
2-methoxyphenol (guaiacol, methylcatechol)	9009-62-5
4-(hydroxymethyl)-2-methoxyphenol (vanillic alcohol)	498-00-0
4-(hydroxymethyl) phenol (*p*-hydroxy benzyl alcohol)	623-05-2
4-ethenyl-2-methoxyphenol (4-hydroxy-3-methoxystyrene)	7786-61-0
heptacosan-1-ol	2004-39-9

Table 4. Continued. Volatile compounds identified in cured beans of *Vanilla pompona* from samples sourced from unknown origin and from Madagascar (Havkin-Frenkel & Belanger, 2011).

COMPOUNDS	**CAS#**
hexacosan-1-ol	506-52-5
phenol (phenyl alcohol, benzenol)	108-95-2
Aldehydes	
3,3-dimethylhexanal	139-85-5
4-hydroxy-3 methoxybenzaldehyde (vanillin)	121-33-5
4-hydroxy-3,5-dimethoxybenzaldehyde (syringic aldehyde)	134-96-3
4-hydroxybenzaldehyde	123-08-0
decanal	112-31-2
hexacosanal	26627-85-0
nonanal (nonaldehyde)	124-19-6
Esters	
(4-methoxyphenyl) methyl hexadecanoate (anisyl stearate)	
4-methoxyphenyl) methyl pentadecanoate (anisyl palmitate)	
2,3-dihydroxypropyl acetate (glycerolmonoacetate)	93713-40-7
bis (6-methylheptyl) benzene-1,2-dicarboxylate (isooctylphthalate)	27554-26-3
ethyl acetate	141-78-6
ethyl hexadecanoate (ethyl palmitate)	628-97-7
methyl-4-hydroxy-3-methoxybenzoate (methyl vanillate)	3943-74-6
methyl acrylate (methyl prop-2-enoate)	96-33-3
methyl-3-phenylprop-2-enoate (methyl-trans- cinnamate)	1754-62-7
propan-2-yl acetate (isopropyl acetate)	108-21-4
propyl 4-hydroxybenzoate (propyl paraben)	94-13-3
Ethers	
1-methoxyhexane (methyl hexyl ether)	4747-07-3

Table 4. **Continued.** Volatile compounds identified in cured beans of *Vanilla pompona* from samples sourced from unknown origin and from Madagascar (Havkin-Frenkel & Belanger, 2011).

COMPOUNDS	**CAS#**
Ketones	
1-hydroxypropan-2-one (hydroxyl acetone, pyruvic alcohol)	116-09-6
ciclohexanone	108-94-1
cyclopent-4-ene-1,3-dione	930-60-9
heptacosene -2,4 –dione	
nonacosene -2,4 –dione	
Acids	
(9Z)-Octadec-9-enoic acid (oleic acid)	112-80-1
3-methylbutanoic acid (isovaleric acid)	503-74-2
4-hydroxy-3-methoxybenzoic acid (vanillic acid)	121-34-6
4-hydroxybenzoic acid (*p*-hydroxybenzoic acid)	99-96-7
4-oxopentanoic acid (levulinic acid)	123-76-2
9,12-octodecanoic acid (linoleic acid)	60-33-3
9-hexadecanoic acid (palmitoleic acid)	373-49-9
acetic acid (ethanoic acid)	64-19-7
formic acid (methanoic acid)	64-18-6
heptadecanoic acid (margaric acid)	506-12-7
hexadecanoic acid (palmitic acid)	57-10-3
octadecanoic acid (stearic acid)	57-11-4
pentadecanoic acid (pentadecylic acid)	1002-84-2
tetradecanoic acid (myristic acid)	544-63-8
Alkanes	
1,3,5 trimethylcyclohexane	1839-63-0
docosane	629-97-0
heptacosane	593-49-7
hexacosane	630-01-3

Table 4. **Continued.** Volatile compounds identified in cured beans of *Vanilla pompona* from samples sourced from unknown origin and from Madagascar (Havkin-Frenkel & Belanger, 2011).

COMPOUNDS	**CAS#**
nonacosane	630-03-5
pentacosane	629-99-2
tetracosane	646-31-1
tricosane	638-67-5
Alkenes	
1-tricosene	18835-32-0
squalene	111-02-4
nonacos-1-ene	18835-35-3
pentacos-1-ene	16980-85-1
Heterocyclics	
1,3-benzodioxole-5-carbaldehyde (heliotropine, piperonal)	120-57-0
1-furan-2-ylethanone (acetyl furan,2-furl methyl ketone)	80145-44-4
2,3 –dihydro-1-benzofuran (coumarin)	496-16-2
2,3-dihydro-2,5-dimethylfuran	
3,4 –dimethylfuran-2,5-dione (dimethylmaleic)	766-39-2
3H-pyran-2,6-dione (glutanoic anhydride)	
3-hydroxy-2-methylpyran-4-one (maltol)	118-71-8
4-hydroxy-2,5-dimethylfuran-3-one (furaneol, strawberry furanone)	3658-77-3
5-(hydroxymethyl) furan-2 –carbaldehyde (hydromethylfurfural)	76330-16-0
5-ethylfuran-2-carbaldehyde (5-ethylfurfural)	23074-10-4
5-methylfuran-2-carbaldehyde (5-methyl-2-furfural)	620-02-0
furan-2-carbaldehyde (furfural)	98-01-1
furan-2-ylmethanol (furfuryl alcohol, 2-furancarbinol)	98-00-0

Table 4. **Continued.** Volatile compounds identified in cured beans of *Vanilla pompona* from samples sourced from unknown origin and from Madagascar (Havkin-Frenkel & Belanger, 2011).

COMPOUNDS	CAS#
hydroxydihydromaltol	
oxalan-2-one (gamma-butyrolactone)	96-48-0

1.8.1. Vanillin content in *Vanilla pompona*

The species *V. pompona* has been reported in literature as "lower quality" vanilla (Ranadive, 1994; Reineccius, 2006) mainly due to reports that this species contains the lowest concentration of vanillin compared to the other two main species as presented by Ehlers and Pfister (1997) in Table 3.

However, several studies have shown different results. For instance, Nakazawa (1981) performed GC analysis on several vanilla species from different geographical origins, including *Vanilla pompona* (Guadalupe vanilla) from West Indies and compared its composition with *V. planifolia* and *V. tahitensis*. The vanillin content of cured beans of *V. pompona* was comparable to the commercially cultivated vanilla species, and even higher than the sample of Tahitian vanilla.

Similarly, Lee (2006) evaluated the characteristic compounds of several vanilla species using DTD-GC-MS, and determined that the vanillin content in *Vanilla pompona* from Madagascar was higher (2.33%) than in *V. tahitensis* (1.04%) and *V. planifolia* (1.92%).

Maruenda *et al*., (2013) determined by GC-MS that the concentrations of vanillin and other main aromatic compounds in Peruvian *Vanilla pompona* at 9 months of fruit development was comparable to the levels found in commercial *V. tahitensis* and *V.*

planifolia. Giving the different physiology of fruit development of *V. pompona*, whereby the levels of glucovanillin spike after 8 months instead of gradually developing as in *V. planifolia*, the different concentrations of vanillin in the different studies could be explained by the unknown origin of the fruits used. Therefore, it is reasonable to include harvesting time as a parameter to control and document for a consistent and accurate comparison of aroma quality of this species, particularly when the intention is to compare its quality with the other two species that are traditionally cultivated.

1.9. Gas Chromatography – Olfactometry (GC-O) analysis of vanilla

Gas chromatography-olfactometry (GC-O) is an analytical technique that combines the separation capability of gas chromatography with the sensitivity of the human nose to identify key aromas and off odors in food samples. Incorporating a panel of subjects that sniff the effluents of the gas chromatograph through a hyphenated sniffing port has made it possible to evaluate the contribution of the compounds to the aroma quality of a sample simultaneously with the GC run. Trained panelists can describe not only the sensory contribution of a single odor-active compound, but also the intensity of the individual compound. The most common and well characterized scientific approaches that have been developed to perform and interpret the GC- sniffing data are described below.

1.9.1. Dilution to threshold methods

These GC-O methods use the number of times a sample needs to be diluted until it is not longer detected as a measure of odor impact. The most common techniques of

this kind are Combined Hedonic of Aromatic Response Measurement (CHARM) and Aroma Extract Dilution Analysis (AEDA). The techniques are based on injecting to the GC successive dilutions of the original aroma extract (twofold, threefold, fivefold or tenfold dilution levels), until the panelist no longer detects the odor at the sniffing port. For each odor, a peak of the olfactogram reflects the highest dilution level at which it is perceivable. This quantitative value is either the Flavor Dilution (FD) value in AEDA or the "Charm" in CHARM analysis.

Dilution methods have proven to be efficient for screening impact odor contributors of an aroma. However, they have notorious drawbacks such as the long time required for GC-O data acquisition, the impossibility to measure the intensity of the odor stimuli and more importantly, the assumption that the odor intensity of all components in a sample increase linearly with the concentration. It is well known that threshold dilutions do not give a true measure of the perceived odor intensity of a volatile compound because the perceived intensity (aroma potency) of an odorous sample is not a linear function of the sample odorant concentration, nor of the threshold dilution ratio. Therefore, threshold concentration does not necessarily correlate with aroma potency (Pollien et al., 1997; Blank, 2002).

1.9.2. Direct intensity methods

These methods require the use of trained panelists to provide odor descriptions and intensity ratings of undiluted GC effluents to assess their odor impact. The most used of this type of method is called OSME (smell in Greek) and it was proposed to incorporate direct measurement of the odor intensities (Blank, 2002). According to Da

Silva *et al*., 1994, this method correlates well with Steven's law for GC-sniffing experiments and provides good reproducibility of peak intensities. The olfactogram is similar to chromatograms obtained with the use of a conventional GC detector and represents odour intensity as a function of retention time. The height of the peak corresponds to the maximum odour intensity of a given analyte, while the width corresponds to odour duration.

The intensity methods are the least time consuming, easiest to conduct and highly reproducible; another advantage is that their results reflect fluctuations related to the "day to day" normal variations within a given population (Plutowska and Wardencki, 2008).

The limitation of Intensity methods is that the result obtained is only related to the given concentration of the analyte in the sample, and it might be similarly reported by the panelists at any concentration above the detection threshold of the compound. Therefore, this type of method is not suitable for distinguishing between two flavor mixtures which differ only by their ingredient concentrations (Pollien *et al*., 1997). Another drawback of the OSME method is the significant amount of time required to initially train the panelists before obtaining satisfactory reproducibility of odor intensities.

1.9.3. Frequency detection methods (GC-SNIF/NIF)

The Gas Chromatography-Olfactometry Surface Nasal Impact Frequency (GC-SNIF/NIF) method was originally proposed by Pollien *et al*. (1997) and developed for quantitative purposes. The method is based on the number of coinciding detection responses to a stimulus as an indicator of its odor impact (Plutowska and Wardencki,

2008). The premise is that compounds which are sensed more frequently than others are acknowledged as having the most important influence on the odour of a given sample.

During the data acquisition, several panelists continuously smell odors eluting from the chromatopraphic column and press a button for the whole duration of the perception of a given odorant, this operation generates an olfactogram comprised of square signals as shown in Figure 7. The peaks of the aromagram indicate the odour impacting regions. Each region is quantified by olfactometric indices such as NIF (Nasal Impact Frequency), that correspond to the peak height or the peak area called SNIF (Surface of Nasal Impact Frequency).

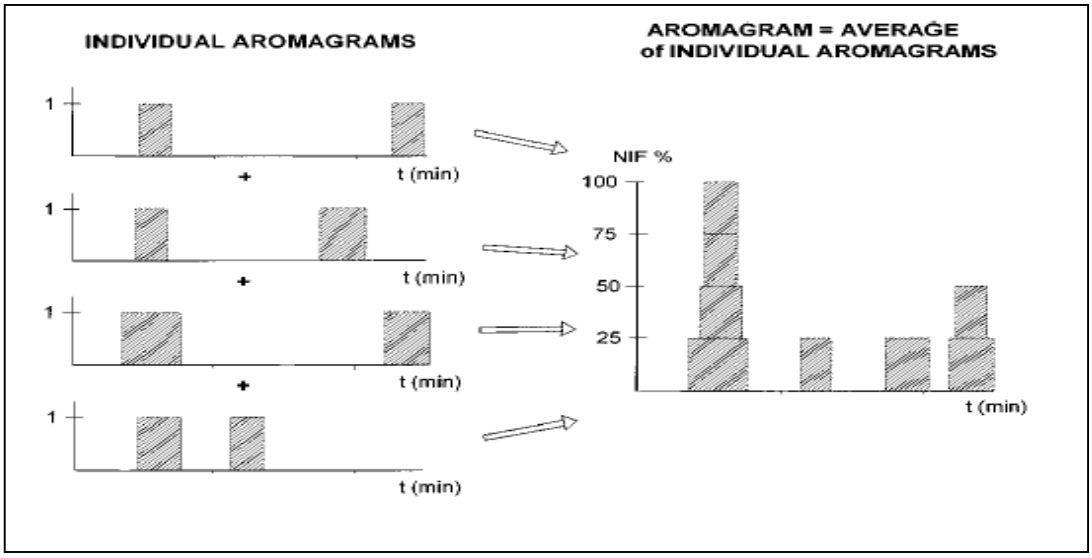


Figure 7. Principle of the GC-"SNIF" data treatment (Pollien et al., 1997)

A modified version of the method, called GC-NIF (Gas Chromatography-Nasal Impact Frequency), has been applied successfully to determine aroma compounds in food samples (Wijaya *et al*., 2005; Rochat *et al*., 2009; Tietel *et al*., 2011; Wang *et al*., 2011).

In this GC-NIF method, frequency and intensity values are the key parameters to select and categorize the relevance of the aroma impact compounds in a food sample. This criteria allows the identification of the most relevant odorants in the food sample and separate these from the noise. Noise is a term referring the odors perceived randomly due either to interferences of odorants or contributors below the detection threshold (Pollien *et al*., 1997).

A high frequency value (NIF value of 100%) indicates that the odor is being detected by panelists in most of the sessions, which means that the odorant concentration is above the odor threshold in the sample. A smaller peak corresponds to an aroma compound present below the detection threshold for more than one panelist. Intensity is an independent variable to measure the strength of the perception or potency of an odorant. In the GC-NIF modified method, instead of using odor duration as proposed in the original GC-SNIF method, the intensity value is assigned by the panelist at the instant an odor is perceived at a given detection time, similar to the concept used in the OSME method. The intensity measurement can only be carried out with well-trained panelists (Marsili, 2002).

2. Significance and aims of this study

2.1. Significance

Among the 110-130 species of Vanilla that produce aromatic fruits, *Vanilla pompona* (also called Vanillon and Guadaloupe vanilla) is considered the third genus in order of economic importance; however, studies on chemical and odor characterization of this genus of vanilla are scarce.

According to field observations, this species exhibits strong aromatic and flavorful properties and has been identified as a potential source of vanilla aromatic compounds including vanillin (Maruenda *et al*., 2013; Soto Arenas & Cribb, 2010). Another important property of this vanilla is its resistance to climate change and diseases such as Fusarium or root rot disease (Purseglove *et al*., 1981). This robustness is the reason this species has been preferred for hybridization programs with *V. planifolia* to produce a more resistant hybrid against those factors that continue to cause economic loss to vanilla growers (Varela Quirós, 2011). Despite its novel attributes, *Vanilla pompona* is scarcely traded nowadays, and particularly absent in the US market.

Characterization of the odor-active compounds of *Vanilla pompona* Schiede by Gas Chromatography Olfactometric analysis (GC-O/MS) would reveal information about the chemical and aroma composition of this species providing key parameters indicative of its quality. Knowledge of the chemical and aroma composition would be an important step to raise interest in this vanilla as a potential raw material for more widespread commercialization into multiple applications.

2.2. Aims of this study

The main objective of this study is to generate detailed information about the aroma quality of *Vanilla pompona* Schiede. The specific aims are:

1) To evaluate different extraction techniques and select the sample preparation method for further Gas Chromatography- Olfactometric (GC-O) and Gas Chromatography-Mass spectrometric (GC-MS) analysis.

2) To characterize volatile compounds present in *Vanilla pompona* by means of GC-MS analysis and Direct Thermal Desorption-Gas Chromatography-Mass spectrometric (DTD-GC-MS) analysis.
3) To determine the odor active compounds in *Vanilla pompona* by GC-O analysis.

3. Materials and methods

3.1. Sample of *Vanilla pompona*

The cured beans of *Vanilla pompona* Schiede used in this study came from the company Desarrollo Agroindustrial Gaya, S.A. de C.V. located in the region of Veracruz, Mexico. The sample was cured using the traditional Mexican process (oven/sun drying) in which the green beans are scalded using a typical oven at temperature of 60 °C for 48 hours. A diagram of the curing process or "beneficiado" applied to the sample is shown in Appendix 6. The cured pods were characteristic dark, and had a mellow odor described as nutty, roasted, earthy, sweet and cherry. The pods were curved, thicker and shorter than the usual spike-like *Vanilla planifolia* pods. The validity of the species used in this study was certified by the company that supplied the sample of *Vanilla pompona* Schiede.

The vanilla pods were transported in a vacuum sealed package at room temperature from Veracruz, Mexico and kept stored at -80°C during the analysis. For each experiment, the beans were frozen in nitrogen which aided managing the powder during the grinding and weighing process and more importantly, to minimize the loss of volatiles and formation of artifacts during the extraction process.

3.2. GC-MS method with polar column

For identification of compounds in *Vanilla pompona*, an Agilent 6890 gas chromatograph was used with an Agilent model 5973N mass spectrometer. The GC column used was a Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 μm DF, Restek). Samples (1 μL) for GC-O analysis were directly introduced to the column via splitless injection. Helium was used as carrier gas at a constant flow rate of 2.6 ml/min for the working column. The temperature of GC injectors was 250°C. Oven temperature program: 40°C (1min. hold) to 100°C at 5°C/min, to 200°C at 8°C/min, then to 240°C (21.83 min. hold) at 15°C/min, giving a total run time of 50 min. The mass spectrometer was operated in EI mode with an ionization voltage of 70 eV. The temperatures of the quadrupole and ion source were 150°C and 230°C respectively. The MSD transfer line temperature was 280°C. The mass scan range was m/z 13 to m/z 450. The olfactory port transfer line temperature was 240°C.

3.3. GC-MS method with non polar column

Vanilla pompona extract (1μL) was injected in an Agilent 6890 gas chromatograph equipped with a mass spectrometer MSDS 5973 and FID. A Restex Rxi®-1ms GC column (0.32 mm x 60 m x 1 μm df) was used. Helium was used as carrier gas at a constant flow rate of 2.6 ml/min for the working column. The temperature of GC injectors was 250°C. Oven temperature program: 40°C (5min. hold) to 300°C at 4°C/min, then 300°C (20 min. hold), giving a total run time of 80 min. The split ratio was 10:1. The mass spectrometer was operated in EI mode and 70 eV. The temperatures of quadrupole and ion source were 150°C and 230°C respectively. The MSD transfer line

temperature was 280°C. The mass scan range was m/z 15 to m/z 450. The olfactory port transfer line temperature was 240°C.

3.4. Extraction of volatiles in *Vanilla pompona*

Three commercialized approaches for extraction of volatiles from natural products were used to obtain a representative extract of *Vanilla pompona*: hydrodistillation, solvent extraction followed by GPC, and solvent extractions using separatory/centrifugation. The chemical characterizations of the volatiles and semi volatiles present in the extracts were made by GC-MS using two GC-columns of different polarities. In addition, the chemical characterization of the cured beans of *Vanilla pompona* was done using the DTD-GC-MS technique.

3.4.1. Preparation of the extract

The beans of *Vanilla pompona* were chopped into 0.5-1 cm pieces and frozen in liquid nitrogen, then ground to a fine powder before each experiment by means of an analytical mill (IKA® ALL basic). Solvents for flavor extractions used in the experiments were HPLC quality dichloromethane and methanol (Sigma-Aldrich's high-purity CHROMASOLV®), high-purity water (18 MΩ) and a food grade ethanol-water solution (95% w/w). The concentration of the vanilla extract for the experiment outlined in 3.4.4.2 was at 3X (3Fold), equivalent to 30g cured beans per 100mL of food grade alcohol.

3.4.2. Hydrodistillation followed by Solid Phase Extraction (SPE)

Vanilla pompona powder (50 g) was transferred to the vacuum distillation system (Figure 8). The distillate was collected and passed through two pre-conditioned Oasis® HLB cartridges (20cc/200 mg, 30 µm). Dichloromethane and methanol, high-purity water (18 MΩ), and flavor grade ethanol were used as solvents for elution.

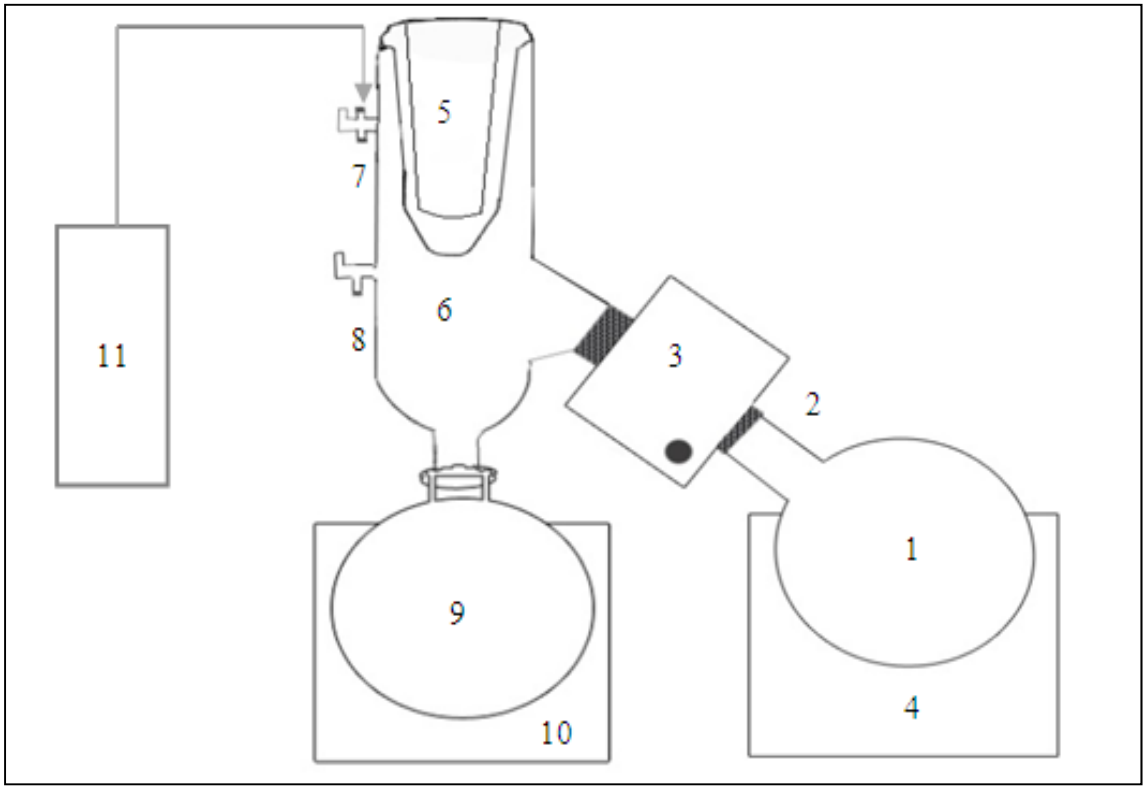


Figure 8. Vacuum distillation apparatus. (1) sample bottom flask, (2) clamp connection, (3) rotor, (4) controlled temperature bath; (5) inner cooling vessel, (6) condenser, (7) inlet vacuum snoozer; (8) vacuum control valve; (9) condensate bottom flask; (10) ice bath, (11) vacuum pump.

The extract collected from the elution was dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated to an approximate volume of 5 mL and

the aroma was evaluated. The extract was further concentrated to approximately 0.5 mL and analyzed by GC-O.

3.4.3. Gel Permeation Chromatography (GPC)

Extract of *Vanilla pompona* beans (50 g) made using dichloromethane was injected to a GPC system (Figure 9). The system consisted of a chromatographic column connected to a "Biotage SP1™ Purification System equipped with a pump, a UV detector and an automatic fraction collector. The column (25 mm i.d. x 500 mm l.) was previously packed with 50 g of Biobeads® S-X3 using 150 mL of DCM according to supplier directions and the bed length of the column was 400 mm.

The mobile phase moved the sample at an average rate of 5 mL/min and the eluent was progressively collected into fractions (6 mL each). The fractions were evaluated one by one and selected based on the presence of aroma. The selected fractions were combined and concentrated to 2.5 mL. Next, the selected fractions were further concentrated to 0.5 mL with a gentle stream of nitrogen and injected into the GC-MS.

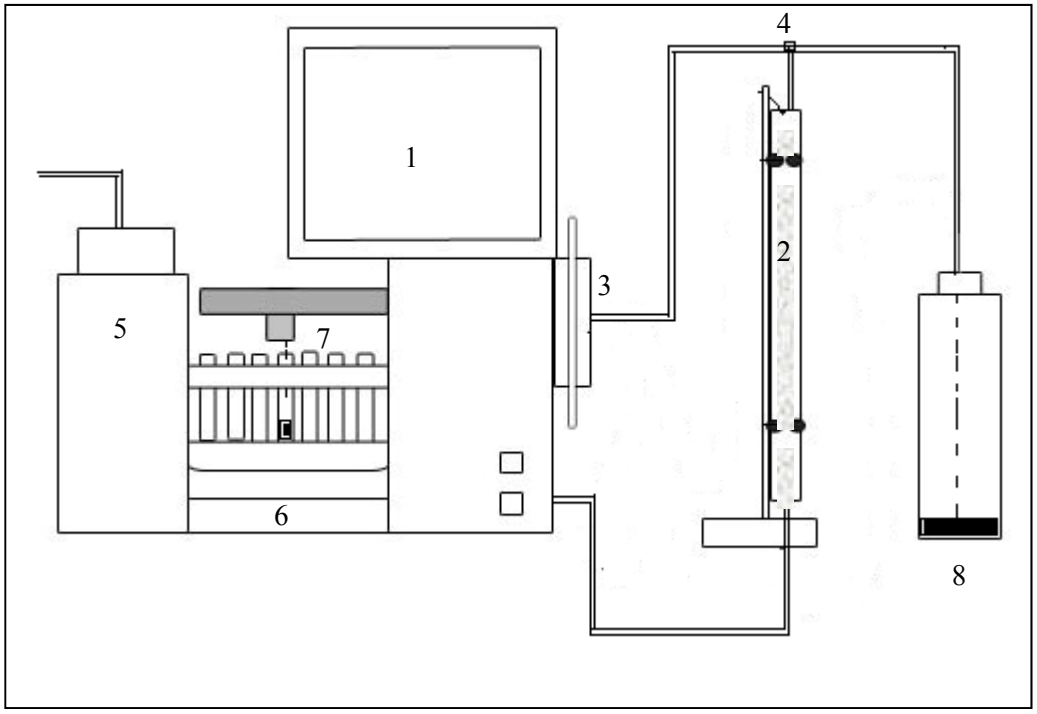


Figure 9. Gel Permeation Chromatography (GPC) system. (1) Monitor and data input, (2) GCP column, (3) Loop injector, (4) Three way valve; (5) Solvent input system, (6) Collector, (7) Sample tubes tray; (8) Waste container.

3.4.4. Liquid-liquid extractions

Flavor extraction was achieved using separatory funnel and centrifugation as illustrated in Figure 10. Dichloromethane (DCM), methanol, high-purity water (18 MΩ) and food grade ethanol solution (95%w/w) were the solvents used for flavor extraction. Both methods required an Evaporator (TurboVap® II) and stream of nitrogen for sample concentration.

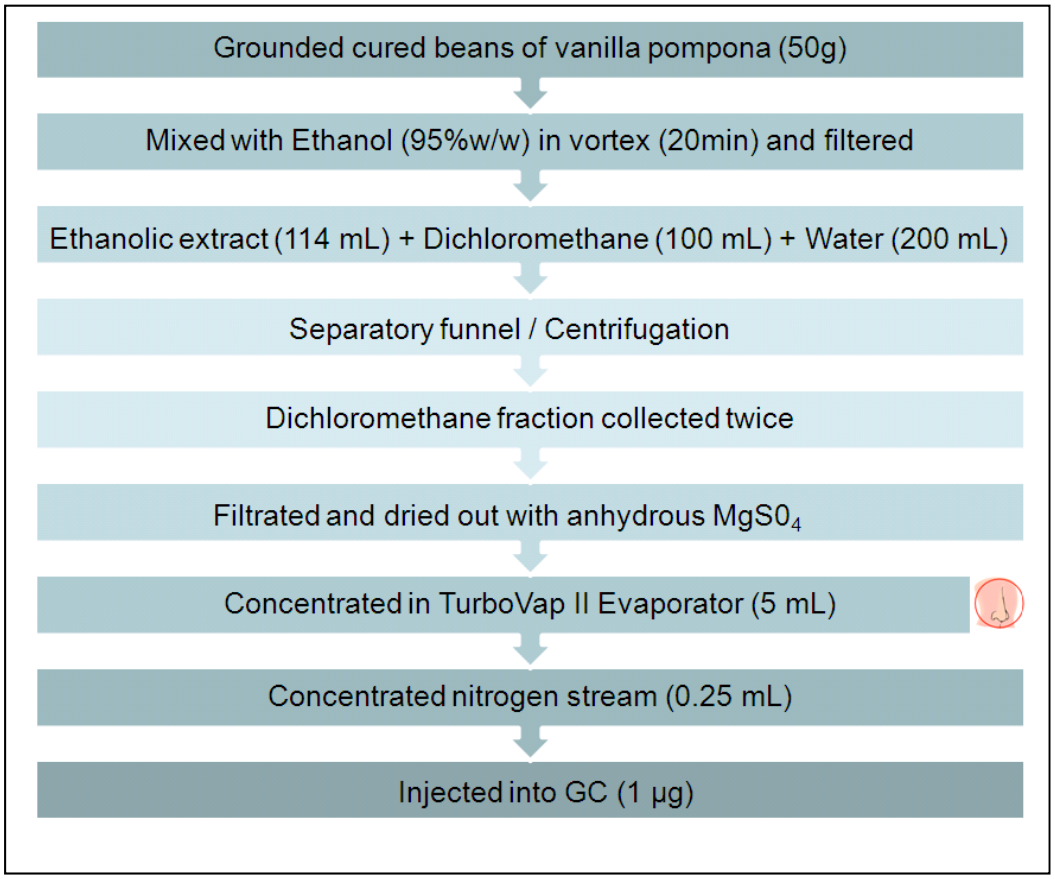


Figure 10. Procedure for the aroma extraction of volatiles and semi-volatiles of *V. pompona* by EtOH with CH2Cl2 back extraction.

3.4.4.1. Separatory funnel

Cured beans of *Vanilla pompona* powder (30 g) and 100 mL of flavor grade ethanol were used to produce an extract of approximately 3-fold concentration as described in Section 3.4.1. Food grade ethanol solution (14 mL) was added during the filtration process to wash any remaining volatiles. Finally, the collected sample (approx. 114 mL) was a dark and watery liquid. Next, dichloromethane (100 mL) and water (200 mL) were added to the *Vanilla pompona* extract in a separatory funnel and agitated, by shaking, several times to produce a substantial physical mixing. After agitation, the

phases were allowed to separate and the analytes were collected from the bottom phase (dichloromethane fraction) twice. The extract collected was filtered, concentrated and dried over anhydrous $MgS0_4$ to remove any remaining water. The extract was concentrated to 5 mL using an evaporator (TurboVap II Concentration Evaporator Workstation) and finally, concentrated to 0.5 mL under nitrogen stream. Finally, the sample was injected and analyzed by GC-MS.

3.4.4.2. Centrifugation

Vanilla pompona extract (3-fold) prepared as described in Section 3.4.1. was weighed (5 g) in a centrifuge tube using an analytical scale (Mettler Toledo). Water (20 mL) and DCM (20 mL) were added, then 100 µL of chlorocyclohexane (CCH) measured with an analytical pipette was added as an internal standard (solution 0.5 % CCH in methanol). The sample was vortexed for 5 min and centrifuged for 5 min at 1800 rpm (Beckman Coulter, model Allegra^{TM} 25R). The upper layer phase was discarded using suction equipment (VacuSafe aspirator) and the DCM phase layer (bottom layer) was kept. The sample was filtered and dried by pouring on top of a paper filter filled with anhydrous magnesium sulfate (Mg_2SO_4) previously prepared. Next, DCM (20 ml) was poured gently on top of the paper filter. The filtrate was concentrated down to a final volume of 0.5 mL by means of a TurboVap II concentrator. Finally, the sample was injected and analyzed by GC-O/MS.

3.5. Direct Thermal Desorption-Gas Chromatography-Mass Spectrometry (DTD-GC-MS) analysis

A short path thermal desorption unit (TD-2), located on the top of the GC injection port, is a device that allows thermally desorbing volatiles from a food sample to be directly injected into the GC column. Previously conditioned desorption tubes (Silanized glass lined stainless steel 3.0 mm idx 10 cm. length) from Scientific Instrument Services Inc. were used for this purpose. The thermal desorption conditions were 10 seconds helium purge, 30 seconds injection and desorption at 220°C for 5 minutes.

A sample of vanilla beans was weighed (9 mg) into a desorption tube and plugged with silanized glass wool. The sample loaded desorption tube was spiked with 10µg of 2,6-dimethoxyphenol (Aldrich Chemical with 99% purity) internal standard by injecting 1.0 µL of a methanol stock solution (10 mg/ml) using a solvent flush technique to ensure quantitative delivery. Next, the spiked desorption tube was connected into a Short Path Thermal Desorber, located at the injection port, of the GC and directly injected into the GC-MS. Semi-quantification of compounds was made by relative GC-MS area counts of analyte compared to internal standard using a response factor.

A Varian 3400 GC was used with an HP-5 (30m. x 0.32mm I.D. x 0.25µm - 60/325 (350) °C) capillary column. The end of the capillary column was inserted into the mass spectrometer. The heat transfer line was maintained at 280^{o}C from the GC to the mass spectrometer. Temperature was programmed starting at -20^{o}C (held for 5 min during the thermal desorption interval) to 40^{o}C at the rate of 10^{o}C/min, then to 320^{o}C at 4º C/min, held for 20 minutes. Injector temperature was 250^{o}C and detector temperature

was 325^{o}C. Helium was used as a carrier gas with a flow rate of 1.0 ml/min with a split ratio of 100: 1.

A Finnigan Mat 8230 high-resolution double focusing magnetic sector mass spectrometer was used for the analysis. The mass spectrometer was operated in electron ionization (EI) mode, scan masses were 35-350, scan time was 0.6 s and interscan time was 0.8 s. The resulting DTD-GC-MS chromatograms were processed using AMDIS deconvolution program in simple mode for identification of volatiles.

3.6. Automatic Mass Spectral Deconvolution and Identification System (AMDIS).

AMDIS is an acronym for Automated Mass spectral Deconvolution and Identification System, a software routine developed by NIST (National Institute of Standards and Technology). The extraction of chemical components by AMDIS software is called deconvolution. AMDIS analyzed data acquired by GC/MS and presented mass chromatograms for each integer m/z value in the data acquisition range. The algorithm automatically compares the mass chromatographic peak shapes with one another to allow for an assignment of ions to a spectrum representing a single compound; in addition, AMDIS makes visible the presence of coeluting substances by showing ions in common and assign the appropriate amount of the ion current to the spectrum of each substance. The system automatically removed peaks from the mass spectrum attributed to background and found the separate components by means of comparing the spectra against a library of target compounds. After the deconvolution or data analysis, the program presented a list of possible components with results of factors such as "net number" and "weight number" that reflected the quality of the match. A minimum

similarity of 60% in Retention Index values was set for identification of compounds by comparing the extracted component spectra and the target library spectra.

In this study, AMDIS was used in "Simple mode" for preliminary extractions and DTD-GC-MS analysis. The mode "Retention Index" was used for the solvent extraction directed to be used in GC-O analysis. AMDIS in Retention Index mode calculated automatically the RI of all the constituents based on a calibration library created by eluting n-alkanes. These Retention Indices were compared to those stored in RI databases.

Despite the capabilities of AMDIS, limitations were found in cases where the materials were present at trace amounts and isomers and/or materials with similar MS fragmentation coeluted. To overcome these limitations, additional confirmation procedures were applied, such as manual reviews (peak by peak) and injection of standard compounds.

3.7. Gas Chromatography-Olfactometry (GC-O) analysis

This was performed on a custom-designed AromaTrax™ GC-O System that integrates an Agilent 6890 gas chromatograph with an Agilent model 5973N mass spectrometer and an olfactory port with humidifier. The eluent of the column was split between the MSD and the olfactory detectors at a ratio of about 1:1 via an open split interface.

The approach used to determine the aroma impact compounds in *Vanilla pompona* extract is considered a modified version of the GC-SNIF or GC-NIF method explained in Section 1.9.3.

3.7.1. Gas Chromatography sniff sessions

Four trained GC-O analysts with proficient olfactory skills and experience with sniff sessions were recruited. They were asked to smell the GC effluents using the sniff port, shown in Figure 11, and to provide their odor description using either the AromaTraxTM panel (Figure 12) or free vocabulary. The panelists recorded the detection times and intensity values of each odor event simultaneously by pressing a button on the AromaTrax^{TM} panel shown in Figure 12. The steps were repeated until completing the fifty minute sessions.

Each assesor performed the GC-sniffing session twice, and each session was considered independent towards the characterization of aroma impact compounds. The number of sessions was selected according to the adequate range (6 to 12) recommended by Pollien *et al.* (1997). The GC-MS/O data was acquired simultaneously using two hyphenated systems: Aroma Characterization and Identification software AromaTRAX® and the GC-MS Chemstation. The hyphenated system provided the GC-O/MS information in overlay diagrams with TIC (Total Ion Chromatogram) results at the top and the aromagram results at the bottom. Four selected pairs from each run are shown in Figure 37 (Appendix 3).

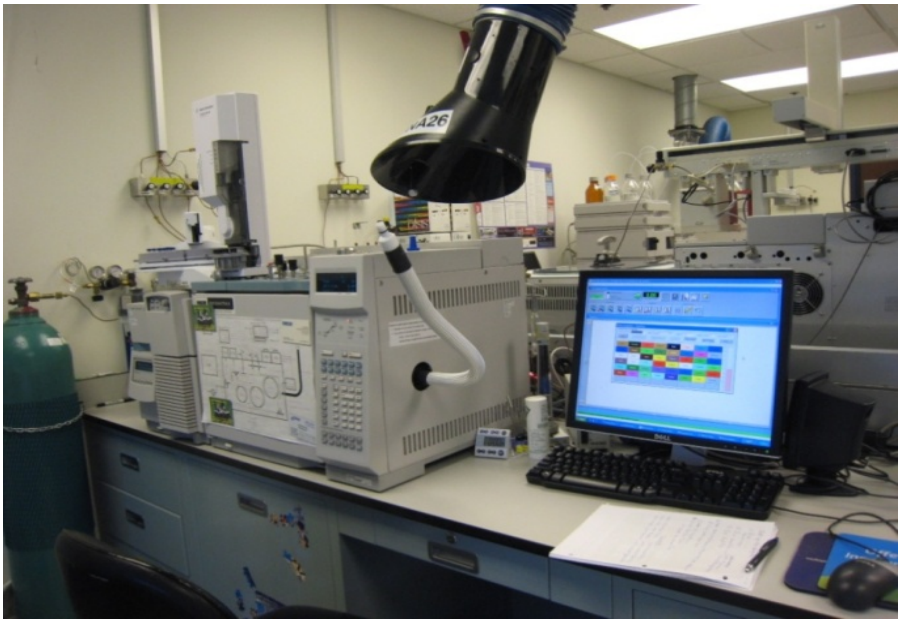


Figure 11. Gas Chromatograph-Mass Spectrometer equipped with sniff port for GC-O analysis.

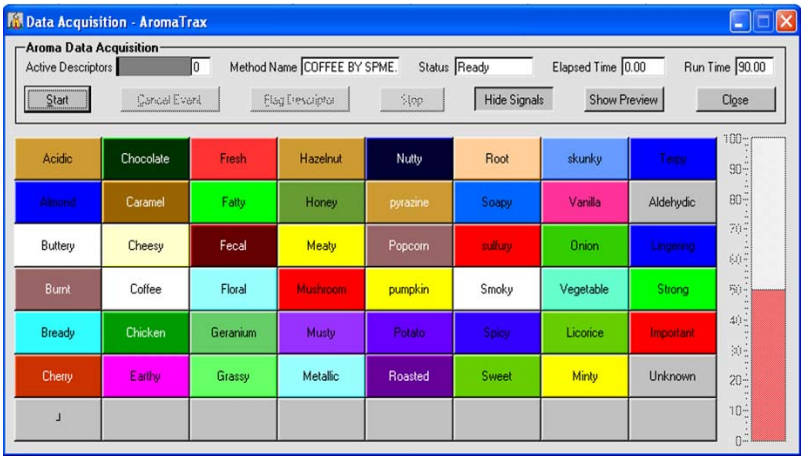


Figure 12. AromaTraxTM panel used for recording the olfactory data for GC-O analysis.

3.7.2. Selection of the aroma impacting compounds

The approach used for the selection and identification of aroma impact compounds is considered a modified GC-SNIF/NIF method. In this method, the total aroma intensity (I) and detection frequency values (DF) were used to rank the relative

contributions of the aroma-active compounds perceived by a panel. These frequency values were obtained by summarizing the number of recognitions and the intensity values of each combined event from the eight single aromagrams (Appendix 1).

The olfactory data (detection times, intensities values and odor qualities) collected during the GC-sniff sessions was exported to Excel format. Eight GC-O runs were performed by four panelists. The bulk of the data were aligned based on the detection times and similarity of the odor descriptions as shown in Table 14 in Appendix 1. The coincident odor events at a given detection time were combined. A composite aromagram based on the detection frequency and total intensity values for each combined event was thus obtained. The NIF (Nasal Impact Frequency) value, also called "frequency", corresponded to the percentage or number of panelists that recognizes an odor event at a given detection time with a maxium value of eight (8) equivalent to NIF value of 100%. The intensities of the single odor events were recorded using a scale from 1 to 100 on the Aromatrax^{TM} screen (Figure 12). The sum of all intensity values assigned by the panelists to an odor active compound was called Total Intensity, with a maximum value of 800.

The odor events with a frequency less than 4 recognitions and intensity values less than 200 were disregarded. Hence, the olfactory data was significantly reduced to those events considered aroma impact compounds (example in Table 15, Appendix 2).

3.7.3. Identification of aroma impacting compounds

3.7.3.1. Odor Retention Indices and criteria for identification

The parameter called "Odor Retention Index" ($RI_{(O)}$) was calculated from the curve of area abundance versus retention times of the n-alkanes under the same

conditions of analysis. The average of the detection times of each combined odor events registered during the Sniff session were used in these linear equations. The Retention Indices of the odorants allowed to narrow down the search of odorants in Retention Index databases (Rochat and Chaintreau, 2009). The linear equations used are shown in Figures 39 and 40 in Appendix 4.

The approach towards identification of each aroma impact compound consisted of the following criteria:

- The aromagrams were superimposed upon their corresponding MS Total Ion Chromatograms (TIC). Compounds were selected based on similarity of odor descritptions and the odor Retention Index (**$RI_{(O)}$**) versus the Retention Index of the GC-MS data (**$RI_{(MS)}$**). The match was considered acceptable when AMDIS results of matching factors: Net, Reverse and Weighted numbers had values close to 100. The ID criterion was identified as "MS" on Table 8.
- Compounds were proposed based on similarity of odor quality and odor Retention Indices ($RI_{(O)}$) versus $RI_{(MS)}$ or $RI_{(lib)}$ (Retention index from libraries or publications). In this study, the compounds were considered acceptable when the difference was less than 20. This ID criterion was indicated as "RI" on Table 8.
- Compounds were proposed based on similarity of Olfactory Retention Indices (**$RI_{(O)}$**) and odor qualities with those authentic standards found in Firmenich internal library and other databases, such as goodscents.company.com, vcf-online, flavor-base2010 and IOFI.org. The compounds that comply with this ID criterion is indicated as "O" on Table 8.

- Injection of selected standard compounds to obtain the elution pattern of the compound under the instrumental conditions used in the study served as confirmation in absence of Mass Spectra data or tentative comounds. This ID criterion was indicated as “I” on Table 8.

While comparing the $RI_{(O)}$ with $RI_{(l)}$, for some of the stock odorants, the standard process had to be repeated several times until a suitable compound was identified. Any chemical compound that complied at least with three of the criteria was considered “Confirmed”; otherwise the identification was considered “Tentative” (T).

4. Results: Comparison of isolation techniques for volatiles in *Vanilla pompona*

4.1. Isolation techniques for *Vanilla pompona*

It was not clear which method of isolating volatile aroma compounds would be more suitable to use for GC-MS and GC-O analysis of *Vanilla pompona*. A preliminary study was required to select an extraction technique that provided an extract representative of the aroma of *Vanilla pompona*. The following three aroma isolation techniques were selected for this purpose:

- Hydrodistillation under vacuum followed by solid phase extraction (SPE).

-Solvent extraction with dichloromethane $(CH_2Cl)_2$ followed by Gel Permeation Chromatography (GPC) cleanup.

-Ethanol extraction with back CH_2Cl_2 extraction.

4.1.2. Evaluation of aroma of *Vanilla pompona* extracts

The aroma qualities of the extracts of *Vanilla pompona* produced by each sampling method were evaluated by two flavorists on a perfumer's blotter. The overall descriptions are presented in Table 5. The most frequent odor descriptors in all three samples of *Vanilla pompona* were sweet, floral or heliotropin, fruity, phenolic, anisic, smoky and barnyard.

Table 5. Sensory description of *Vanilla pompona* extracts produced using three different analytical methods.

Extraction method	**Vacuum distillation followed by SP (A)**	**Gel Permeation Chromatography (B)**	**Liquid-liquid extraction (C)**
Aroma description	*Sweet, creamy, vanillic, anisic-aldehyde like, heliotropin, phenolic, smoky, horse-banyard, horseyard, benzaldehyde, cherry-like, fruity, something savory as cumin-like.*	*Sweet, very creamy, helitropin, barnyard-horse radish, grapefruit, rich-creamy, pruny, raisin, dates, cinnamon, floral note, acetate type and cereal notes.*	*Heliotropin like, caramel brown sugar, fruit, sweet cherry like, heavy sweet-vanillic, anysil aldehyde, banyard note, tobacco notes, prune, dry fruits (fig, raisin, etc).*

In terms of aroma differentiation among the *Vanilla pompona* extracts, it was noticed that typical vanilla odors such as resinous, balsamic, chocolate, tobacco-like and caramel brown sugar, were not perceived from the extract produced by vacuum distillation (Section A in Table 5). However, savory and cumin-like notes were detected in this vacuum distillation extract, presumably due to the presence of artifacts derived from thermal degradation during the extraction process at 50°C. Similarly, Perez Silva et

al. (2006) reported non-characteristic aromas using vacuum distillation and disregarded this method for GC-O analysis. Non-characteristic vanilla odors such as cereal-like and acetate were perceived only in the extract using GPC cleanup (Section B in Table 5).

4.1.3. Volatile composition of the extracts

The number of volatiles extracted and identified by GC-MS from all *Vanilla pompona* extracts ranged from 100 to 150. The chromatograms are shown in Figure 13. Noticeable similarities exist accross the volatile profiles. For instance, most of the main compounds (peaks 1,2,3) eluted at similar retention times. Also, the most numerous concentrations of peaks occurred at retention times between 25-35 minutes.

The volatile profiles of the vanilla extract using three different sampling methods also presented dissimilarities. For instance, two of the chromatograms in Figure 13 (B, C) showed a compound eluting after vanillin, approximately at 45 min, identified as acetovanillone, a common compound in vanilla beans. However, this component was absent in the results from vacuum distillation method (Figure 13A).

In addition, a number of peaks corresponding to compounds eluting in the range of 35-40 minutes were present only in the resulting chromatogram from the organic solvent extraction followed by GPC cleanup (Fig. 13B). The absence of such volatiles from the separatory funnel extract (Figure 13C) was most likely due to these compounds being trapped in the emulsion.

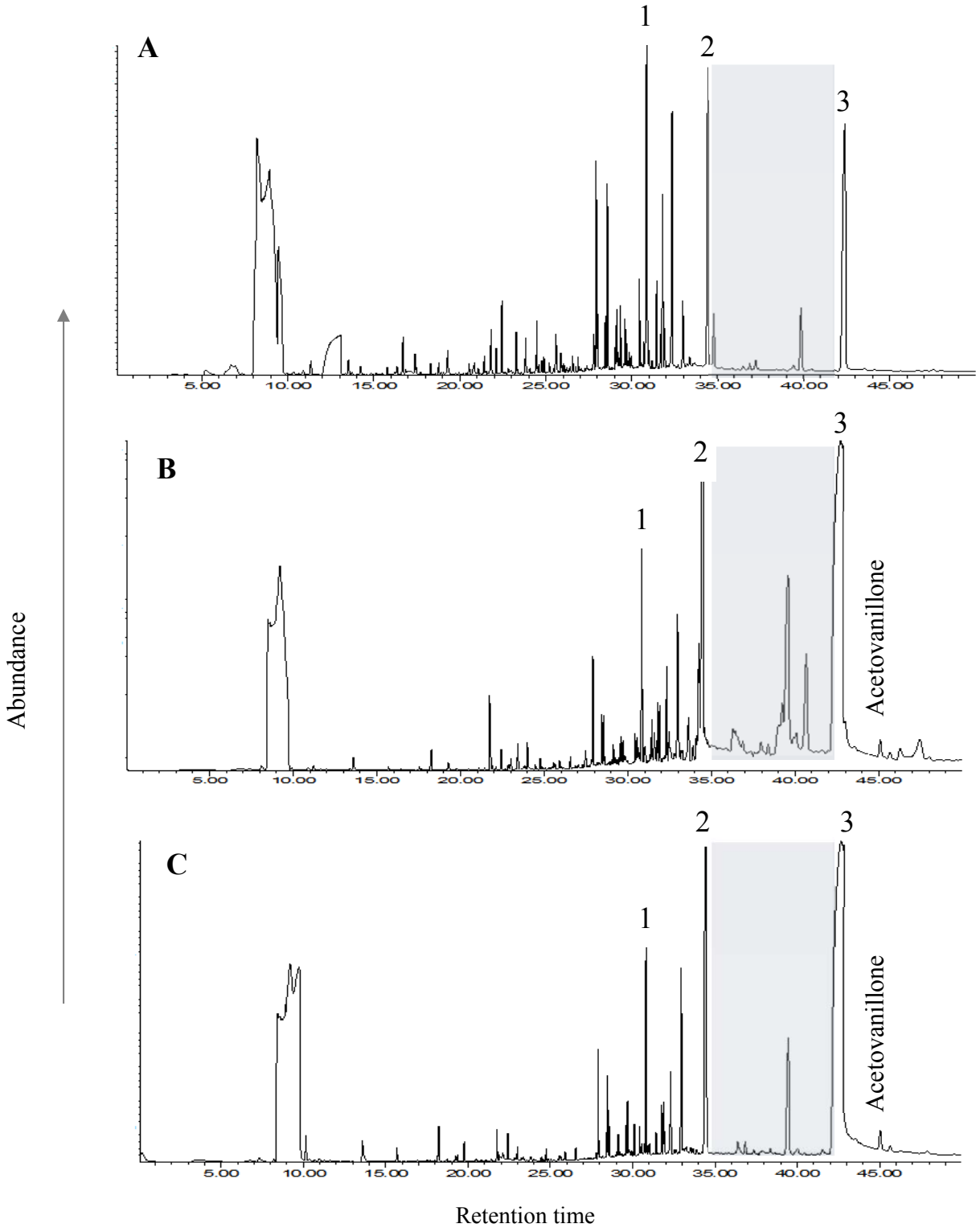


Figure 13. Chromatograms of *Vanilla pompona* extracts using three analytical methods: (A) Hydrodistillation and SPE, (B) Organic solvent extraction and GCP cleanup, (C) Liquid-liquid extraction.

The compound 5-(hydroxymethyl)-2-furfural (HMF), a product of Maillard reactions, was abundantly present in extracts produced by solvent extractions. However, this was present at trace amounts in the extract produced with vacuum distillation. In addition, the volatile profile of vacuum disillation extract lacked important peaks in the range of 35-40 minutes. Therefore, the vacuum distillation method proved to be the most selective for eliminating semi-volatiles and most of the materials that served as substrates for the chemical reactions producing HMF.

Vanillin was the most abundant compound in most of the extracts, except in the one produced by solvent extraction followed by GPC cleanup. In this extract, anisyl alcohol was the most abundant, and vanillin was the fifth in order of abundance; this was probably due to a loss of vanillin in the fractions that were excluded from the final combined extract.

4.1.4. Odor quality of the extracts

An experienced GC-O analyst performed a "Sniff" session on each of the extracts and characterized the odor experiences by descriptions and detention times. Intensity values were not used in this preliminary study. A range between 50 and 62 aroma impact zones were perceived and recorded from the *Vanilla pompona* extracts during the sniffing sessions.

All the odor events were annotated and grouped into odor families. The selection of odors belonging to each group was based on the similarity of odor qualities. Similarities in odor qualities can be associated to the presence of functional groups and characteristic chemical structures as described by Brunschwig *et al*. (2012) who applied a

similar approach for a GC-O study of *Vanilla tahitensis*. A representative name was assigned to each odor family using common aroma descriptors (Table 6). Once each odor event was placed in the most suitable odor family the events belonging to each category were numerated and the result called "relative frequency".

Table 6. Chemical compounds and correspondent odor characteristics identified during preliminary study of *Vanilla pompona* extracts prior to GC-O/MS.

ODOR DESCRIPTORS	**CATEGORIES**
Sweet, caramel, sugary, candy, honey, brown aldehyde, benzaldehyde	SWEET
Geranium, floral, perfumy, heliotropin, aldehyde	FLORAL
Green, grassy, fresh, veggie, vegetable, cool, herbaceous, hay	GREEN
Bready, baked, bread crust, doughy, cracker like, rice, popcorn/pyrazine, pretzel, Brown, hazelnut, nutty, roast, chocolate	BAKED-NUT
Smoky, phenolic, burnt, sandalwood, woody, hay, warm, bamboo	SMOKY-WOODY
Anisic, spicy, cinnamic, clove, peppery, eugenol	ANISIC-SPICY
Earthy, musty, mushroom	EARTHY
Vanillic, vanilla, powdery, coumarinic, familiar, lactonic, creamy	VANILLIC
Fruity, cherry, berry, redfruit, citrusy, tropical	FRUITY
Sour, acetic, rancid, skunky, cheesy, acidic, artichoke, savory, meaty	CHEESY-SOUR
Fatty, oily, waxy, fried, butyric, soapy	OILY-BUTTERY
Plastic, vial, metallic, play dough, weird, odd, solventy, ethereal, terpenic, unknown	OTHER

The values of relative frequency were converted to percentage based on the total of events for each extract (Table 7). Next, all the frequency results were plotted by odor

family and presented in Figure 14. The results were placed in one graph for comparison analysis of the odor profiles and selectivity of the extraction methods. Selectivity was not a desirable attribute in this study as the goal of the extraction methods was to obtain the broadest number of volatiles and semi-volatiles from the sample.

Table 7. Relative frequency of odor characteristics in *Vanilla pompona* extracts tabulated by odor family.

Extration / Odors	FLORAL	GREEN	SWEET-VANILLIC	FRUITY	OILY-BUTTERY	BAKED-NUT	ANISIC-SPICY	PHENOLIC-SMOKY	EARTHY	CHEESY-SOUR	OTHER
Hydrodistillation & SPE	9	9	20	7	7	4	4	14	6	9	11
Liq-liq & GPC	16	10	23	3	6	3	5	13	5	11	5
Liq-liq extraction 1	9	6	26	6	9	2	2	18	2	12	8

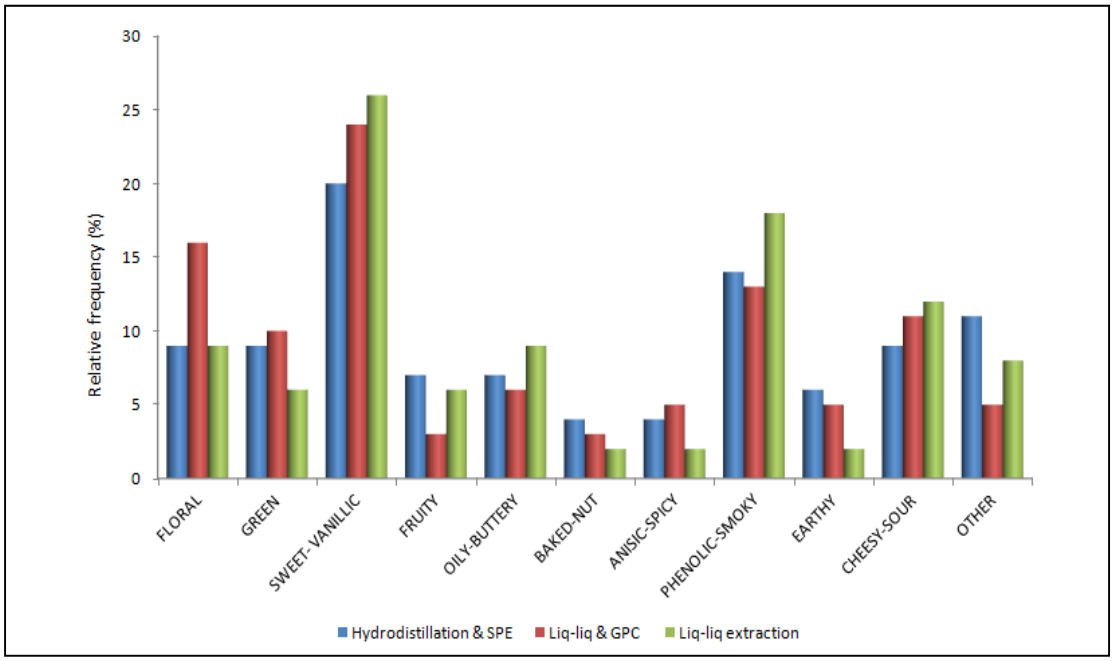


Figure 14. Relative frequency of odor families in extracts using three different analytical approaches for extraction and isolation of volatiles and semi-volatiles in *Vanilla pompona* Schiede.

Figure 14 demonstrates that all the odor categories were present in all extracts. Additionally, the concordance among the frequency values in each odor family indicates the aroma profiles obtained using the three analytical approaches were very similar. Therefore, there was no sign of selectivity of these analytical techniques for any particular odor and consequently for any corresponding chemical group or compound. Additional observations from the comparative graph (Figure 14) are:

1) Organic solvent extraction techniques produced the highest recovery of vanillin and phenolic-smoky odors, which are characteristic descriptors of vanilla (Table 1).
2) Floral aromas were most abundantly perceived from the extract using dichloromethane extraction followed by GPC.
3) The highest relative frequency of "Other" category was found in extracts produced by the hydrodistillation method. Non-characteristic odors were probably due to the presence of artifacts from thermal degradation during the extraction at 50° C.

4.1.5. Selection of the method for GC-MS and GC-O analysis

From the similarity of the results in both parameters, yield of volatile compounds by GC-MS and number of odor active zones found by GC sniffing from all the extracts, it is reasonable to conclude that the three analytical approaches used in this study produce extracts that are representative of the aroma of *Vanilla pompona*, and therefore suitable for use in GC-O/MS analysis. However, the presence of non-characteristic odors in the hydrodistillation method suggests some degree of thermal degradation and artifact formation. Consequently, this analytical method was disregarded.

Gel Permeation Chromatography successfully served as a cleanup method for extraction of volatiles and semi-volatiles in the vanilla extract. However, manual selection of fractions based on odor, would produce bias toward small size molecules to the detriment of important larger molecules, as evidenced with vanillin. Validation of this method requires the correlation of molecular size with volatility of the compounds as well as odor impact. This would be an interesting objective in another study.

The procedure of extracting vanilla compounds using ethanol-water-dichloromethane solvents with a separatory funnel was modified as described in Section 3.4.4.2. The modification consisted of using centrifugation instead of separatory funnel with the purpose of increasing the recovery of characterizing volatile compounds.

Based on convenience, simplicity of the extraction process, low requirement of solvents, shortest time, feasibility for standardization and reproducibility, the organic solvents (ethanol-water-dichloromethane) extraction technique was arbitrarily selected for the GC-O/MS analysis of *Vanilla pompona.*

5. Results: volatile composition of *Vanilla pompona* extract by GC-MS

5.1. Results

One hundred and twenty three volatile and semi-volatile compounds were identified by GC-MS in *Vanilla pompona* extract. The highest peaks in the Total Ion chromatogram (Figure 15) reflect the most abundant compounds. These are vanillin, anisyl alcohol, 5-(hydroxymethyl)-2-furfural, *p*-anisaldehyde, *p*-vinyl guaiacol, anisyl acetate, methyl linoleate, methyl linolelaidate and tricosane. The remaining volatiles were

present at levels less than 1% of the total abundance, which represents concentrations less than 10 ppm in the 3-fold extract.

The volatile profile of the *Vanilla pompona* extract is comprised of nine chemical classes: hydrocarbons (24), alcohols (20), esters (18), ketones (13), aliphatic acids (13), phenols (11), heterocyclic compounds (11), aldehydes (12), and ether (1). The percentages of these classes are shown in Figure 16.

The specifics of the chemical composition of *V. pompona* extract are presented in Table 8. The compounds are listed by chemical groups in descending order based on area abundance. The table includes the calculated Retention Indices (RI) for both, polar and non polar GC columns. The "literature" Retention Indices (RI_{lib}) from several databases for each type of column are shown in parenthesis on the table if available.

The compounds grouped in the phenolic class in *V. pompona* extract were not the most numerous (Figure 16), but it was the most abundant representing at least 48.4% of the total area despite of some phenolic compounds with particular functional groups not being counted in this section, but counted in the corresponding sections as alcohols, aldehydes, acids, and esters; for instance, *p*-anisaldehyde and anisyl alcohol. Vanillin was by far the most abundant phenol representing near 45% of the total volatiles.

Anisyl alcohol was the second most abundant compound in *Vanilla pompona*, with 18.85% of the total area, which corresponds to the 91.46% of the abundance of the alcohols. The vanillin and anisyl alcohol peaks were so high that they saturated the MS detector (Figure 15).

Among other anisic compounds, *p*-anisaldehyde was present at level of 3.26% of the total area in this *Vanilla pompona* extract. In addition, anisyl acetate and anisyl formate were present at relevant levels of 1.22% and 0.69% respectively.

A total of 13 fatty acids (FA) were found in *Vanilla pompona* samples. There were 10 saturated fatty acids (SFA). Short chain fatty acids (SCFA, C<8) eluted first in the polar GC column and medium chain FA and long chain FA eluted during the range of 25 to 39 min (Figure 15).

Hydrocarbons contained the most numerous compounds (24), but only represented the 3.30% of the total area in this extract (Figure 16). Among them, seven terpenoids and three sesquiterpenes were present in the *V. pompona* extract at trace concentrations (≤0.1% area).

In spite of the long history of research in vanilla, new compounds are continuously discovered, this time facilitated by the use of a powerful tool: AMDIS deconvolution program. Sixteen compounds present in the extract of *Vanilla pompona* had not been previously identified in vanilla; in all cases, they can be found in natural materials. The list of these compounds and the correspondent natural sources are presented in Table 9. Selected chemical structures are shown in Figure 18.

The principal biochemical and/or chemical routes of formation of the variety of chemical compounds occurred during the curing process and include phenolics originating from the phenylpropanoid secondary metabolic pathway from phenylalanine, C6-C10 aldehydes and alcohols from the polyunsaturated fatty acid/lypoxygenase pathway, aliphatic acids from intermediary metabolism and C4 dioxy compounds from the Glycolysis/pyruvate/acetolactate pathway (Dunphy & Bala, 2009).

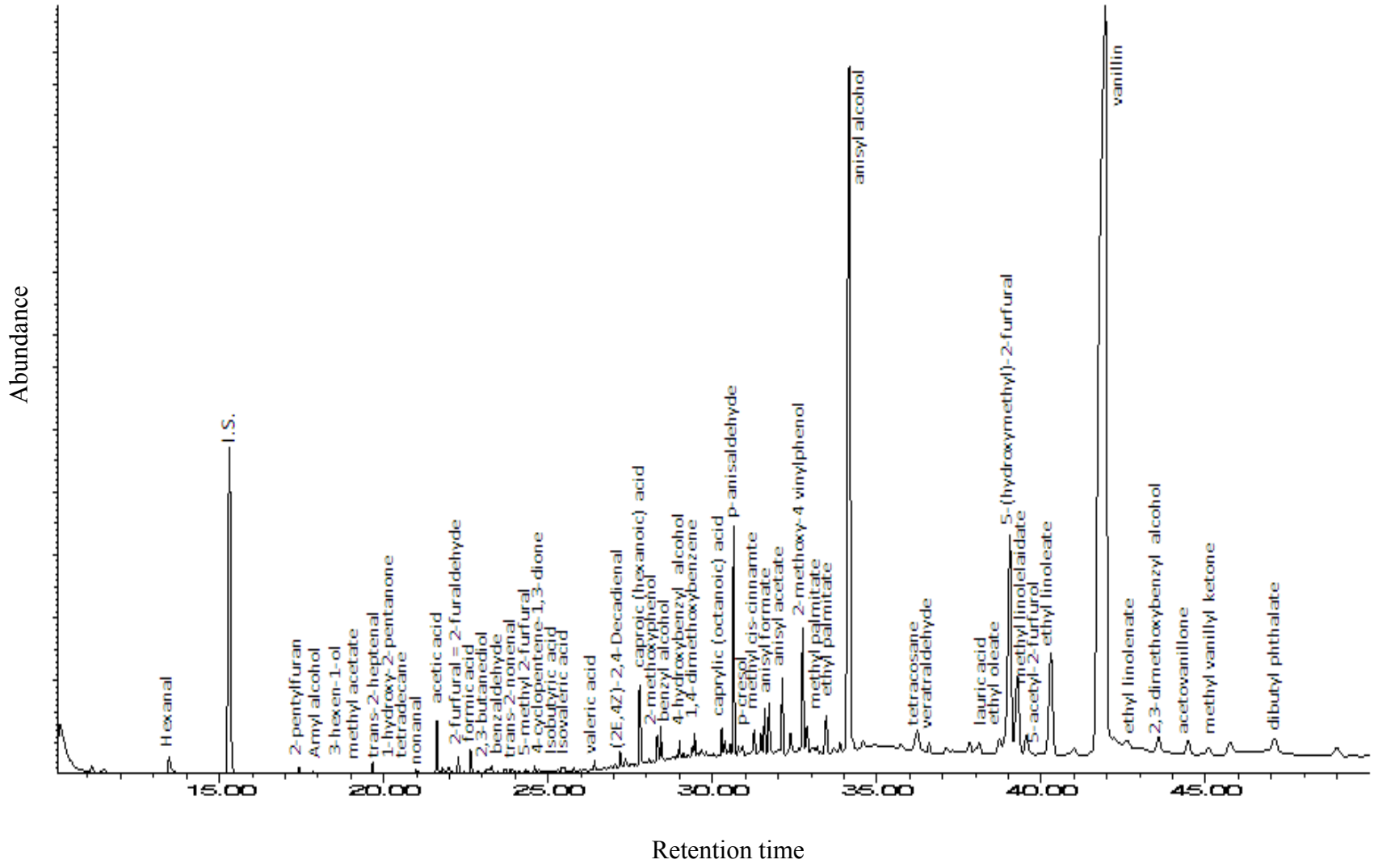


Figure 15. Volatile and Semivolatile composition of *Vanilla pompona* extract 3Fold.

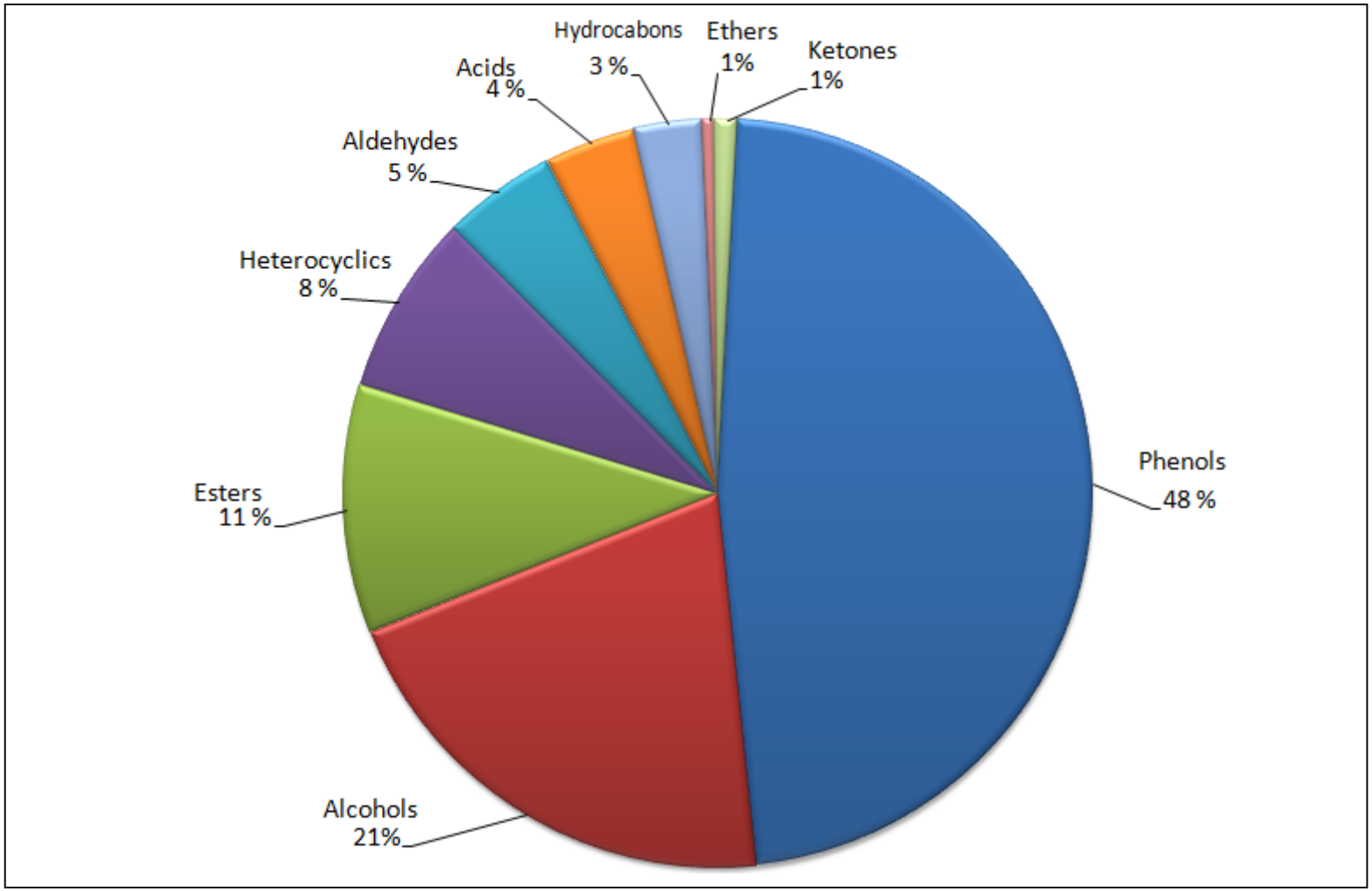


Figure 16. Percentages of area distribution of volatile compounds in ethanolic-water-dichloromethane extract 3X from cured *Vanilla pompona* beans using polar column: Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek).

Table 8. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No.	RT (min)	Compound Name	CAS #	Abundance (Area %)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
			Phenols -11 (48.47%)				
1	41.96	vanillin	121-33-5	44.84	2592 (2586)	1363(1357)	MS,RI
2	32.75	2- methoxy-4-vinylphenol=4-vinyl guaiacol		2.22	2219 (2206)	1288 (1287)	MS,RI
3	44.48	apocynin = acetovanillone	498-02-2	0.52	2653 (2640)	1448 (1446)	MS,RI*
4	29.46	2-methoxy-4-methyl-phenol (4-methylguaiacol=creosol)	93-51-6	0.28	1981(1988)	1169 (1173)	MS,RI
5	28.32	2-methoxyphenol=guaiacol	90-05-1	0.20	1883 (1893)	1063 (1062)	MS ,RI
6	36.15	*p*-vinylphenol	2628-17-3	0.15	2395 (2390)	1184 (1185)	MS,RI
7	30.79	*p*-cresol = 4-methylphenol	106-44-5	0.12	2087 (2084)	1046 (1047)	MS,RI
8	36.27	4-methoxyphenol (*p*-hydroxyanisole)	150-76-5	0.06	2400 (1866)	1179 (1182)	MS,RI*
9	29.81	phenol	108-95-2	0.05	2010 (1974)	954 (958)	MS,RI
10	27.56	1,2-dimethoxy-4-methylbenzene = methyl creosol	494-99-5	0.02	1818 (1807)	1206 (1212)	MS,RI
11	29.12	4-hydroxybenzyl alcohol	623-05-2	0.02	1952	(1337)	MS^T

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
			Alcohols -20 (20.61%)				
12	34.16	anisyl alcohol	105-13-5	18.85	2301 (2313)	1251 (1249)	MS,RI*
13	43.58	3,4-Dimethoxybenzyl alcohol= veratryl alcohol	93-03-8	0.52	2632	**	MS^T
14	28.43	benzenemethanol=benzyl alcohol	100-51-6	0.34	1893 (1904)	1005 (1008)	MS,RI*
15	39.54	5-acetyl-2-furfurol	55087-82-6	0.28	2519	**	MS,RI
16	11.12	*tert*-amyl alcohol	75-85-4	0.15	1009 (1007)	623 (625)	MS,RI
17	23.28	2,3-butanediol I	513-85-9	0.06	1535 (1529)	761 (761)	MS,RI
18	33.70	T-muurolol = epi-alpha-muurolol	19912-62-0	0.06	2274 (2190)	1654 (1656)	MS,RI*
19	35.73	1-hexadecanol = cetyl alcohol	36653-82-4	0.05	2375 (2386)	(1872)	MS,RI
20	32.89	alpha-cadinol	481-34-5	0.05	2228 (2221)	1654 (1644)	MS,RI
21	30.90	viridiflorol = himbaccol	552-02-3	0.04	2095 (2091)	1613 (1604)	MS,RI
22	23.84	2,3-butanediol II		0.04	1567 (1563)	751 (752)	MS,RI

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
23	28.91	phenethanol	60-12-8	0.04	1934 (1915)	1086 (1088)	MS,RI*
24	32.62	T-cadinol = epi-alpha-cadinol	5937-11-1	0.03	2212 (2167)	1641 (1640)	MS,RI
25	15.56	trans-3-penten-2-ol	3899-34-1	0.02	1169 (1171)	**	MS,RI
26	21.70	1-octen-3-ol	3391-86-4	0.02	1447 (1445)	963 (966)	MS,RI
27	17.56	1-pentanol = amyl alcohol	71-41-0	0.02	1250 (1254)	750 (755)	MS,RI
28	29.26	1-dodecanol	112-53-8	0.02	1964 (1965)	(1472)	MS,RI
29	11.89	2-methyl-3-buten-2-ol = dimethyl vinyl carbinol	115-18-4	0.01	1036 (1034)	**	MS,RI
30	25.33	2-furfurol	98-00-0	0.01	1658 (1663)	**	MS,RI
31	19.83	1-hexanol	111-27-3	0.01	1353 (1355)	**	MS,RI

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
Heterocyclics (Furans, pyrans, pyrazines,lactones) – 11 (7.62%)							
32	39.06	5-(hydroxymethyl)-2-furfural	67-47-0	6.63	2505 (2505)	1176 (1174)	MS,RI
33	22.27	2-furfural = 2-furaldehyde	98-01-1	0.22	1478 (1467)	799 (803)	MS,RI
34	17.42	2-pentylfuran	3777-69-3	0.13	1244 (1238)	980 (984)	MS,RI
35	37.09	dihydroactinidiolide = 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	15356-74-8	0.10	2431 (2351)	1506 (1509)	MS,RI
36	34.27	3,5-dihydroxy-2-methylpyran-4-one=5-hydroxymaltol	1073-96-7	0.10	2306 (2238)	1155 (1155)	MS,RI
37	29.66	maltol = corps praline=3- hydroxy-2-methyl-4-pyranone	118-71-8	0.07	1999 (1995)	1076 (1076)	MS,RI
38	24.31	5-methyl-2-furfural	620-02-0	0.04	1595 (1565)	929 (929)	MS,RI
39	29.62	2-acetylpyrrole = methyl 2-pyrrolyl ketone	1072-83-9	0.04	1994 (1970)	**	MS,RI
40	15.41	2-ethylpyrazine	13925-00-3	0.03	1163 (1334)	**	MS,RI

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
41	27.25	2(5H)-furanone	497-23-4	0.02	1793 (1795)	**	MS,RI
42	23.12	2-acetylfuran	1192-62-7	0.01	1525 (1513)	880 (883)	MS,RI
		Carbonyls, aldehydes - 12 (4.54%)					
43	30.65	*p*-anisaldehyde = 4-methoxybenzaldehyde	123-11-5	3.26	2076 (2064)	1220 (1220)	MS,RI
44	13.47	hexanal	66-25-1	0.52	1092 (1090)	776 (778)	MS,RI
45	27.83	E2,E4-decadienal	25152-84-5	0.27	1841 (1818)	1292 (1296)	MS, RI
46	36.59	veratraldehyde = 3,4-dimethoxybenzaldehyde	120-14-9	0.22	2413 (2393)	1430 (1435)	MS,RI
47	19.66	trans-2-heptenal	18829-55-5	0.19	1344 (1325)	930 (930)	MS,RI
48	27.19	E2,Z4-decadienal	25152-83-4	0.18	1789 (1793)	1270 (1274)	MS,RI
49	21.78	trans-2-octenal	2548-87-0	0.08	(1419)	1033 (1031)	MS,RI
50	21.79	*cis*-3-nonenal	31823-43-5	0.08	1345 (1452)	**	MS
51	23.73	*trans*-2-nonenal	18829-56-6	0.05	1561 (1560)	1124 (1137)	MS

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
52	23.67	benzaldehyde	100-52-7	0.02	1557 (1527)	931 (931)	MS,RI
53	16.30	heptanal	111-71-7	0.01	1197 (1189)	879 (877)	MS,RI
54	12.24	crotonaldehyde = 2-butenal	123-73-9	0.003	1048 (1047)	**	MS,RI
			Esters -18 (10.85%)				
55	40.30	ethyl linoleate	544-35-4	3.79	2542 (2524)	2143 (2142)	MS,RI
56	39.29	methyl linoleate	112-63-0	2.69	2512 (2478)	2074 (2073)	MS,RI
57	32.13	anisyl acetate	104-21-2	1.22	2181 (2149)	1382 (1388)	MS,RI
58	31.59	anisyl formate = *p*-methoxybenzyl formate	122-91-8	0.69	2144 (2108)	1298 (1317)	MS,RI
59	33.47	ethyl palmitate	628-97-7	0.67	2261 (2254)	1975 (1977)	MS,RI
60	47.09	dibutyl phthalate	84-74-2	0.44	2715	1923 (1922)	MS

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
61	32.87	methyl palmitate = methyl hexadecanoate	112-39-0	0.43	2227 (2206)	1906 (1909)	MS,RI
62	38.72	ethyl oleate	111-62-6	0.33	2493 (2477)	2150 (2149)	MS,RI
63	31.27	methyl *trans*-cinnamate	19713-73-6	0.20	2122 (2113)	**	MS
64	37.82	methyl cis-12-octadecenoate	2733-86-0	0.18	2459 (2462)	**	MS,RI
65	42.63	ethyl linolenate	1191-41-9	0.17	2610 (2566)	**	MS
66	43.00	methyl vanillate	3943-74-6	0.02	2618 (2598)	1480 (1482)	MS,RI
67	19.04	methyl acetate	79-20-9	0.01	1315	**	MS^T
68	21.66	ethyl caprylate	106-32-1	0.01	1445 (1439)	**	MS, RI
69	25.18	ethyl caprate	110-38-3	0.01	1649 (1645)	**	MS,RI
70	30.18	isopropyl myristate	110-27-0	0.004	2040 (2048)	**	MS,RI
71	26.20	benzyl formate	104-57-4	0.003	1715 (1688)	1049 (1049)	MS,RI

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
72	14.53	*n*-amyl formate	638-49-3	0.001	1130 (1123)		MS,RI
			Hydrocarbons -24 (3.30%)				
73	34.10	tricosane	638-67-5	1.10	2298 (2300)	2298 (2300)	MS,RI
74	36.24	tetracosane	646-31-1	0.50	2399 (2400)	2397 (2400)	MS,RI
75	38.91	pentacosane	629-99-2	0.37	2500 (2500)	2497 (2500)	MS,RI
76	32.38	docosane	629-97-0	0.32	2198 (2200)	2198 (2200)	MS,RI
77	33.88	cadalene = 4-isopropyl-1,6-dimethylnaphthalene	483-78-3	0.20	2285 (2262)	1670 (1672)	MS
78	28.34	1,6-dimethyl-4-propan-2-yl-1,2,3,4-tetrahydronaphthalene =calamenene	6617-49-8	0.17	1885 (1845)	1523 (1528)	MS,RI
79	29.40	4,7-dimethyl-1-propan-2-yl-1,2-dihydronaphthalene=alpha-calacorene	021391-99-1	0.11	1976 (1941)	1543 (1550)	MS,RI
80	33.08	alpha-d-curcumene	000644-30-4	0.10	2239	1477 (1480)	MS,RI
81	27.36	delta-cadinene	000483-76-1	0.09	1801 (1768)	**	MS,RI

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
82	34.59	*cis*-9-tricosene	027519-02-4	0.08	2321	2272 (2276)	MS,RI
83	30.93	heneicosane	000629-94-7	0.06	2098 (2100)	2098 (2100)	MS,RI
84	46.84	heptacosane	000593-49-7	0.03	2708 (2700)	**	MS
85	27.33	octadecane	000593-45-3	0.03	1799 (1800)	**	MS,RI
86	24.38	hexadecane	000544-76-3	0.03	1599 (1600)	**	MS,RI
87	25.97	heptadecane	000629-78-7	0.02	1699 (1700)	**	MS,RI
88	20.79	tetradecane	000629-59-4	0.02	1399 (1400)	**	MS,RI
89	16.36	dodecane	000112-40-3	0.02	1199 (1200)	**	MS,RI
90	18.71	tridecane	000629-50-5	0.01	1299 (1300)	**	MS,RI
91	13.67	undecane	001120-21-4	0.01	1099 (1100)	**	MS
92	26.50	gamma-muurolene	030021-74-0/317819-80-0	0.01	1737 (1690)	**	MS^T

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
93	22.67	pentadecane	000629-62-9	0.01	1499 (1500)	**	MS,RI
94	18.43	bromocyclohexane	000108-85-0	0.01	1287	957 (958)	MS,RI
95	16.85	limonene	000138-86-3	0.005	1220 (1200)	**	MS
96	18.18	vinylbenzene=styrene	000100-42-5	0.004	1276 (1255)		MS
Aliphatic acids -13 (3.65%)							
97	27.79	caproic (hexanoic) acid	142-62-1	0.83	1838 (1847)	955 (957)	MS,RI
98	31.72	pelargonic (nonanoic) acid	112-05-0	0.79	2153 (2156)	1241 (1244)	MS,RI
99	21.61	acetic acid	64-19-7	0.77	1443 (1434)	570 (579)	MS,RI
100	22.64	formic acid	64-18-6	0.33	1498 (1484)	**	MS,RI
101	30.28	caprylic (octanoic) acid	124-07-2	0.31	2047 (2058)	1144 (1157)	MS, RI

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
102	38.12	lauric (dodecanoic) acid	143-07-7	0.21	2470 (2477)	1536 (1541)	MS,RI
103	29.00	oenanthic (heptanoic) acid	111-14-8	0.18	1942 (1956)	1048 (1061)	MS,RI
104	26.41	valeric (pentanoic) acid	109-52-4	0.11	1731 (1737)	859 (860)	MS,RI
105	25.42	isovaleric acid	503-74-2	0.04	1664 (1672)	**	MS,RI
106	23.21	propionic acid	79-09-4	0.02	1531 (1534)	**	MS,RI
107	25.45	2-methylbutyric acid = N293	116-53-0	0.02	1666 (1668)	**	MS,RI
108	24.73	butyric (butanoic) acid	107-92-6	0.02	1621 (1618)	**	MS,RI
109	23.74	isobutyric acid	79-31-2	0.02	1561 (1557)	735 (738)	MS,RI
Carbonyls, Ketones -13 (0.55%)							
110	31.48	6,10,14-trimethyl-2-pentadecanone (Hexahydrofarnesyl acetone)	000502-69-2	0.25	2136 (2118)	**	MS
111	24.60	4-cyclopentene-1,3-dione	000930-60-9	0.09	1612 (1625)	**	MS,RI

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib}) Δ Non-Polar column	ID Criteria
112	20.99	nonanal	000124-19-6	0.06	1409 (1399)	**	MS,RI
113	45.13	methyl vanillyl ketone = guaiacylacetone = 4-hydroxy-3-methoxyphenylacetone	002503-46-0	0.04	2668 (2702)	1490 (1492)	MS,RI
114	20.33	1-hydroxy-2-pentanone	064502-89-2	0.03	1377	814 (824)	MS,RI
115	22.85	3-hydroxy-2-pentanone	003142-66-3	0.02	1510		MS^T
116	40.12	benzophenone	000119-61-9	0.02	2536 (2484)	1603 (1606)	MS,RI
117	21.31	3-octen-2-one	001669-44-9	0.02	1427 (1420)	**	MS, RI
118	19.33	2,3-octanedione = acetyl caproyl	000585-25-1	0.01	1329 (1331)	**	MS,RI
119	16.22	2-heptanone = methyl amyl ketone	000110-43-0	0.01	1194 (1184)	**	MS,RI
120	26.85	propiophenone = 1-phenyl-1-propanone = phenyl ethyl ketone	000093-55-0	0.004	1763	1139 (1143)	MS,RI

Table 8. Continuation. Volatile compounds in ethanolic-water-dichloromethane extract from cured *Vanilla pompona* beans using polar column Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek) and non polar column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No	RT (min)	Compound Name	CAS #	Area (%)	RI (RI_{lib}) Δ Polar column	RI (RI_{lib})Δ Non-Polar column	ID Criteria
121	20.88	2-nonanone = methyl heptyl ketone	000821-55-6	0.002	1404 (1392)	**	MS,RI
122	17.98	3-octanone	000106-68-3	0.001	1268 (1260)	**	MS,RI
		Ethers - 1 (0.03%)					
123	22.00	*p*-methylanisole = *p*-cresyl methyl ether	000104-93-8	0.03	1463	1001 (1000)	MS,RI

**Spaces in blank indicated that the Retention indices values were not available in library (for reference) or/and they were not detected by GC column.

Δ Retention Indices (RI_{lib}) in this table are sourced from olfactory databases (vcf-online and Firmenich internal database) and/or published papers (Perez Silva *et al*., 2006; Brunschwig *et al*., 2012; Zhang and Mueller, 2013).

[T]Compound is considered tentative.

5.2. Discussion

The volatile profile of *Vanilla pompona* extract is typical of vanilla species in the aspect of having more than 90% of the volatiles compounds present at trace levels (<0.1% area in this case). Klimes and Lamparsky (1976) and Perez Silva *et al*., (2006) stressed the importance of the hundred volatiles that are present at trace concentrations in the aroma identity of the vanilla beans and what makes vanilla from beans difficult to substitute by vanillin and other synthetic substitutes.

5.2.1. Phenolic composition

The phenolic composition of *Vanilla pompona* extract consists of 11 compounds. The high levels of vanillin, anisyl alcohol and *p*-anisaldehyde in this species is in good agreement with the proportions reported by Ehlers and Pfister (1997) and Nakazawa *et al.* (1981). Vanillin was by far the most abundant phenol representing near 45% of the total volatiles. This percent is close to the range reported in commercial species, which is between 50 and 85% (Perez Silva *et al*., 2006). Other phenol compounds such as *p*-vinyl guaiacol (2,22%), acetovanillone (0.52%), 4-methyl guaiacol (0.28%) and guaiacol (0.20%) are present at far less quantities than vanillin, which is consistent with the proportions found by Lee (2006) in *Vanilla pompona* from Madagascar.

All phenol compounds found in this species have been reported previously in vanilla, with the exception of 4-methoxyphenol (8). This isomer of guaiacol has been previously found in honey, licorice, sesame seed, and alpinia species.

Important phenolic compounds such as *p*-hydroxybenzoic acid and vanillic acid (Figure 5), known to be used as indicators of quality and possibly the origin of

commercial vanilla species, were not detected in this *Vanilla pompona* extract using the GC polar column under these analytical conditions. However, trace levels of these constituents were detected using the non-polar GC column. This is in agreement with previous GC-MS studies. Perez Silva *et al*. (2006) reported the absence of *p*-hydroxybenzoic acid in *Vanilla planifolia* Jackson, and attributed this absence to the polarity of the column used (DB-Wax). Also, Brunschwig *et al*. (2012) pointed out the absence of these compounds in *Vanilla tahitensis* and explained that this was due to either low concentrations of these vanillyl compounds or their co-elution with other compounds. On the other hand, Ehlers and Pfister (1997) characterized *Vanilla pompona* using High Performance Liquid Chromatography (HPLC) and reported the presence of vanillic acid and *p*-hydroxybenzoic acid (Table 4). HPLC seems to be a more suitable analytical technique than Gas Chromatography (GC) to identify and quantify the presence of these semi-volatiles (*p*-hydroxybenzoic acid and vanillic acid) in vanilla.

5.2.2. Alcohols

Twenty alcohols were identified in this *Vanilla pompona* extract. The high level of anisyl alcohol present in this extract coincides with levels reported in all previous studies independent of the analytical method used (Gnadinger, 1925; Ehlers and Pfister, 1997; Lee, 2006; Maruenda *et al*., 2013). Similarly, high amounts of this compound have been considered typical in *V. tahitensis* (Ehlers and Pfister, 1994; Brunschwig *et al*., 2012).

The compound *tert*-amyl alcohol present in this *Vanilla pompona* extract at a level of 0.15% of total area, have been identified by Zhang and Mueller (2013) in

Bourbon and Ugandan vanilla (*V. planifolia)* at very low levels utilizing AMDIS deconvolution program.

Another constituent, 3, 4 dimethoxybenzyl alcohol (veratryl alcohol), present at a salient level of 0.52% of total area in this extract, was found previously in cured beans of an unknown wild vanilla species from Peru using DTD-GC-MS (Lee, 2006; Havkin-Frenkel & Belanger, 2011).

The compound 2-methyl-3-buten-2-ol (0.01% area) was newly identified in vanilla in this study. This compound can be found naturally occurring in bilberry, cardamom, cherimoya, coffee, cranberry, mango, lemon, lavender oil, black currant and hops. Likewise, the compound trans-3-penten-2-ol (0.01% area) was not reported previously in vanilla, but its ketone counterpart, trans-3-penten-2-one, possibly produced by oxidation of this alcohol, was reported by Klimes and Lamparsky (1976) and Zhang and Mueller (2013) in Bourbon Vanilla species. The structures of these above mentioned compounds are shown in Figure 18.

5.2.3. Esters

Eighteen esters were identified in this *Vanilla pompona* extract. Esters of long chain fatty acids were most likely produced as a result of the presence of long chain fatty acids in plant material and alcohols in extracts.

The presence of the esters anisyl acetate (1.22% of total area) and anisyl formate (0.69% of total area) in *Vanilla pompona* can be considered a fingerprint (Figure 17) as it is in the case of *Vanilla tahitensis* because these are absent or present only at trace concentrations in *Vanilla planifolia (*Brunschwig *et al*., 2012). This is one of the

confirming elements that the volatile profile of *Vanilla pompona* resembles *Vanilla tahitensis* rather than *Vanilla planifolia* in agreement with previous observations. Amyl formate is being reported in vanilla for the first time in this study at the trace level of 0.001% area. This is naturally occurring in strawberry, honey, strawberry, prickly pear and tomato.

5.2.4. Heterocyclic compounds

Eleven heterocyclic compounds were identified in this study. Furanoids and pyrans in this extract may have formed from the thermal degradation of sugars as result of the desorption temperature (250°C) used during GC analysis of the vanilla sample (Adedeji et al., 1993).

The compounds 2-acetylpyrrole (Figure 18) and lactone dihydroactinidiolide were found in this *V. pompona* extract, and had been previously reported by Klimes and Lamparsky (1976) in *V. fragrans* of unknown origin. Also identified in this extract was the compound 2-furfurol, which was previously reported in Madagascar *Vanilla pompona* (Lee, 2006) and also in *Vanilla planifolia* (Zhang and Mueller, 2013).

In addition, ethyl pyrazine (Figure 18) was identified in this vanilla extract at level of 0.03% of total area. This is the first time this material has been reported to be in vanilla.

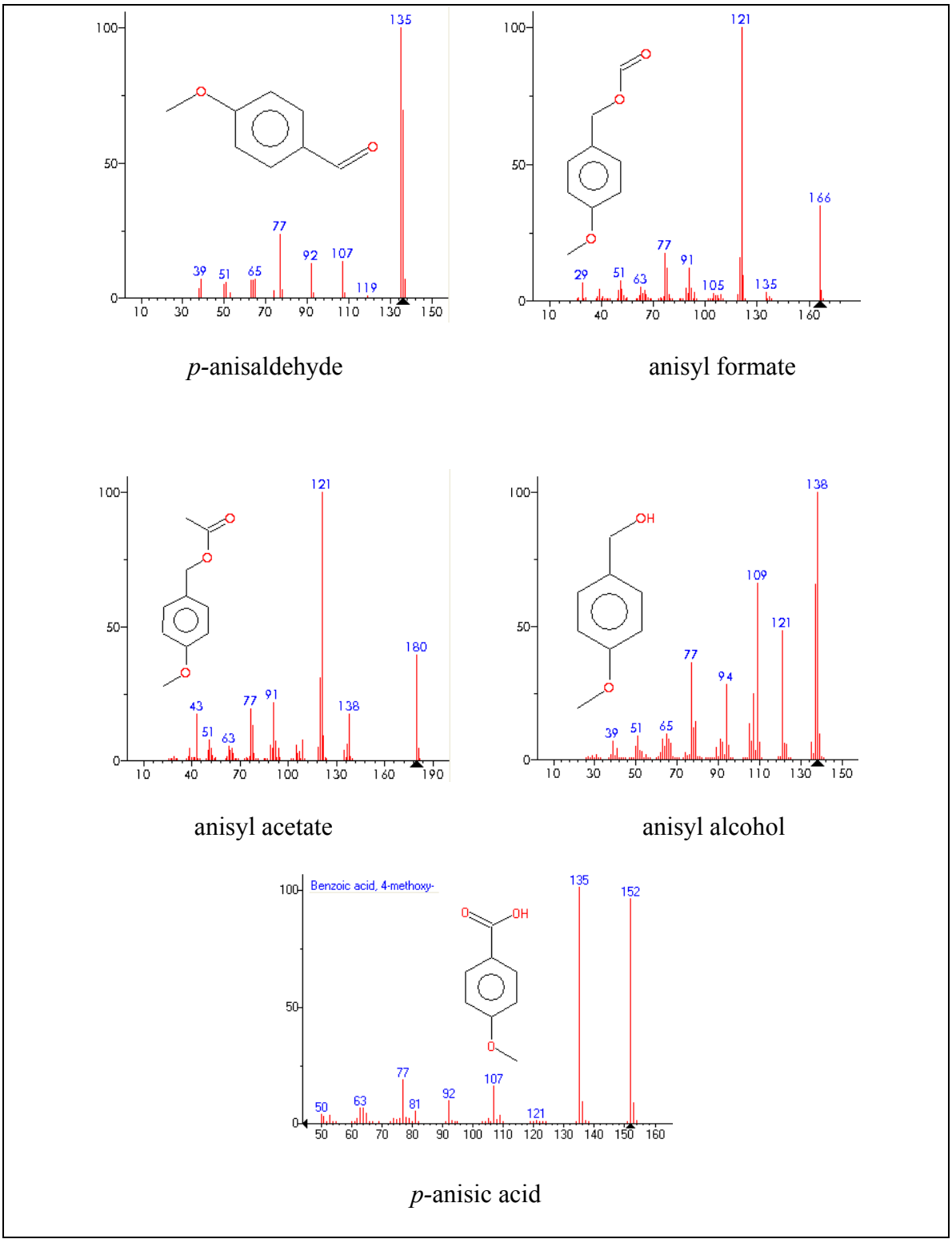


Figure 17. Anisyl compounds found in extract of *Vanilla pompona* Schiede.

5.2.5. Carbonyls, aldehydes and ketones

Twelve carbonyls, aldehydes and 13 carbonyls, ketones were identified in this *Vanilla pompona* extract. Among them, the compound 2-heptenal was found at level of 0.19% of area in this study. This compound was tentatively identified by Perez Silva *et al*. (2006) and the presence was confirmed by Toth (2012) in *Vanilla planifolia* species.

The compound benzophenone (Figure 18) that was present in this extract at a level of 0.02% was previously reported at same level in cured beans of Bourbon vanilla (Toth, 2012). Interestingly, benzophenones are considered active molecules known to be used for treatment of various pathological conditions (Prabhakar et al., 2006; Ranganatha et al., 2013; Vijay et al., 2014).

The compounds *trans* 2-nonenal and *cis* 3-nonenal had not been previously identified in vanilla pods; but they are naturally occurring in cucumber. Likewise, the compounds *trans* 2-octenal and 2-butenal, newly identified in this study, are natural occurring in cherry, apple, honey, guava, tomato, carrot, citrus fruits and other natural products.

The compounds piperonal (CAS# 120-57-0, CAS Name: 1, 3-benzodioxole-5-carboxaldehyde) coumarin (2H-1-benzopyran-2-one) were not found in the chemical characterization of this *Vanilla pompona* extract under the conditions of this study, confirming the results of Ehlers and Pfister (1993) from analysis of Vanillons.

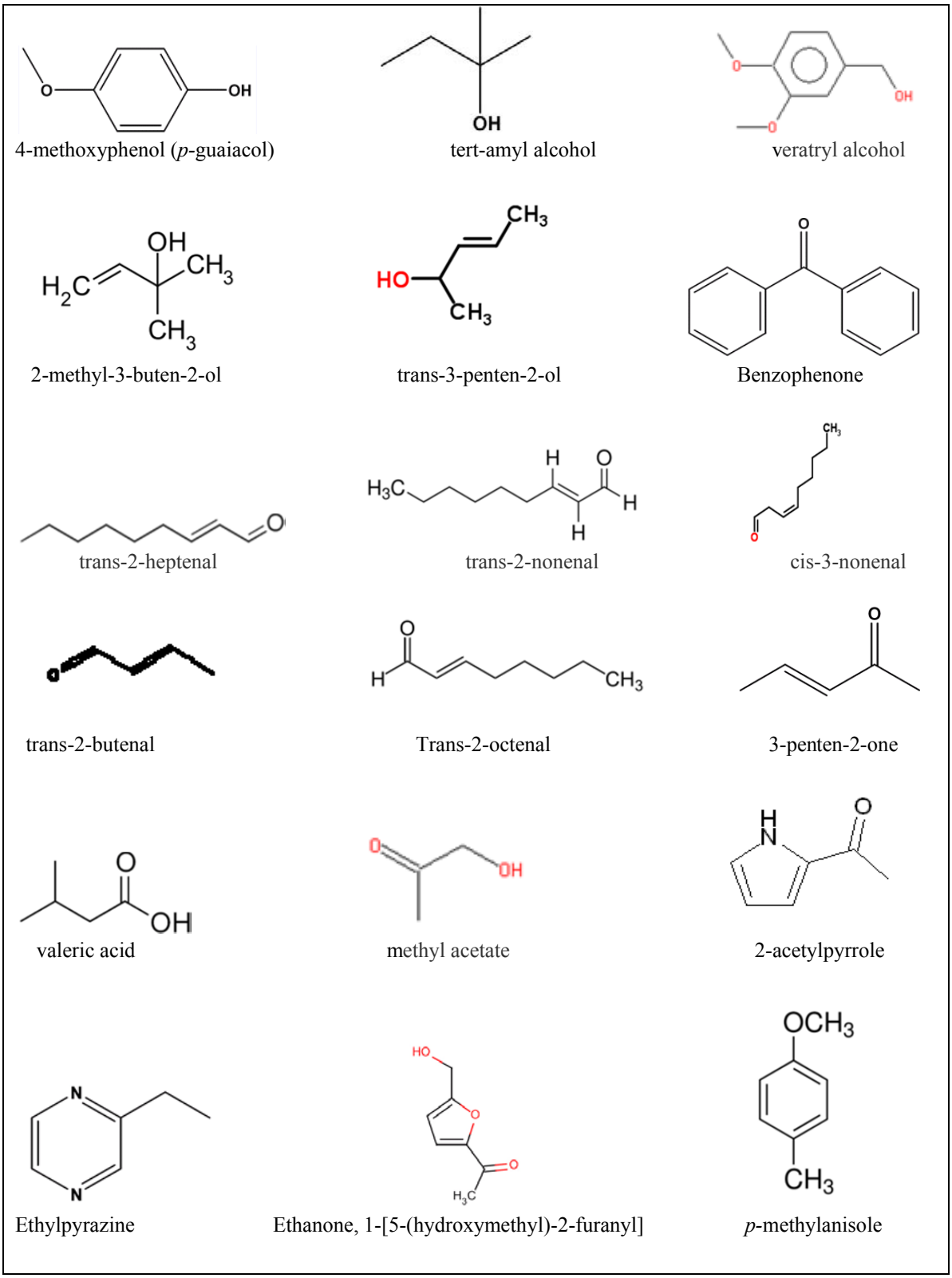


Figure 18. Volatile compounds found in *Vanilla pompona* extract.

5.2.6. Ethers

Benzyl ethers, produced from phenyl aldehydes and alcohols, such as vanillyl methyl ether, vanillyl ethyl ether, *p*-hydroxybenzyl methyl ether and *p*-hydroxybenzyl ethyl ether have been reported in cured beans of *Vanilla planifolia* (Galletto & Hoffman, 1978; Klimes & Lamparsky, 1976). Compounds *p*-cresyl isopropyl ether, anisyl ethyl ether and anisyl methyl ether have been reported in *Vanilla tahitensis* (Frenkle and Belanger, 2012; Da Costa and Panini, 2006). However, Lee (2006) reported only one ether (Methyl hexyl ether) present in *Vanilla pompona* from Madagascar. Likewise, in this extract of *Vanilla pompona*, only one ether was identified *p*-methylanisole (*p*-cresyl methyl ether) which has not been reported previously in vanilla.

5.2.7. Aliphatic acids

A total of 13 fatty acids were found in this *Vanilla pompona* extract. Among them, acetic acid is a typical constituent of cured beans and commonly the most abundant acid in this chemical class. However, in this study the fatty acid fraction (caproic acid and pelargonic acid) was the most abundant. The fatty acid composition in cured beans of vanilla has been proposed as key to discriminate among vanilla species and the curing method used (Brunschwig et al., 2009).

5.2.8. Hydrocarbons

Hydrocarbons have been previously identified in cured beans of *Vanilla planifolia* and *Vanilla tahitensis* (Adedeji et al., 1993; Lee, 2006) using DTD-GC-MS, and a copious presence of these compounds are now confirmed in this species including

terpenoids and sesquiterpenes found in vanilla for the first time (Table 9). The chemical structures of selected hydrocarbons are presented in Figure 19.

The numerous hydrocarbons (24) found in this extract of *Vanilla pompona* contrasts with the reported by Perez Silva *et al.* (2006) who only detected two hydrocarbons (tricosane and pentacosane) using GC-MS in an extract of Pentane/Ether (P/E) of *Vanilla planifolia* Jackson.

The compound *cis* 9-tricosene, also called muscalure was also identified. Interestingly it is also known as a pheromone of the female common housefly and bees. Cadalene was confirmed by identification in both polar and nonpolar GC columns. These two compounds were recently reported by Zhang and Muhler (2012) in *Vanilla planifolia* using GC-MS analysis with AMDIS deconvolution program.

Traces of styrene (No. 95 in Table 8) can occur naturally in some plants and foods, such as cinnamon, coffee beans, and peanuts. Also, it was reported in *Vanilla fragans* by Klimes and Lamparsky (1976). However, it is very likely that the presence of trace levels of styrene as well as bromocyclohexane (No. 93 in Table 8) in *Vanilla pompona* extract came from plastic packaging.

The compounds gamma curcumene (91 in Table 8) and germacrene (Figure 19) were tentatively identified because of their similar spectra. The compound gamma curcumene coeluted with germacrene at retention time of 26.5 min. The similarity of both spectra at trace concentrations in a highly crowed background limited the clear identification despite the use of the powerful AMDIS deconvolution program.

The formation of terpenes arises from carbohydrate and lipid metabolism of the plant. The presence of these hydrocarbons may not be considered significant in terms of

aroma contribution and potency of odors, but terpenes (mono- and sesquiterpenes) are considered important to the aroma of certain fruits. The presence of volatile terpenoids can be also associated to woody background odors.

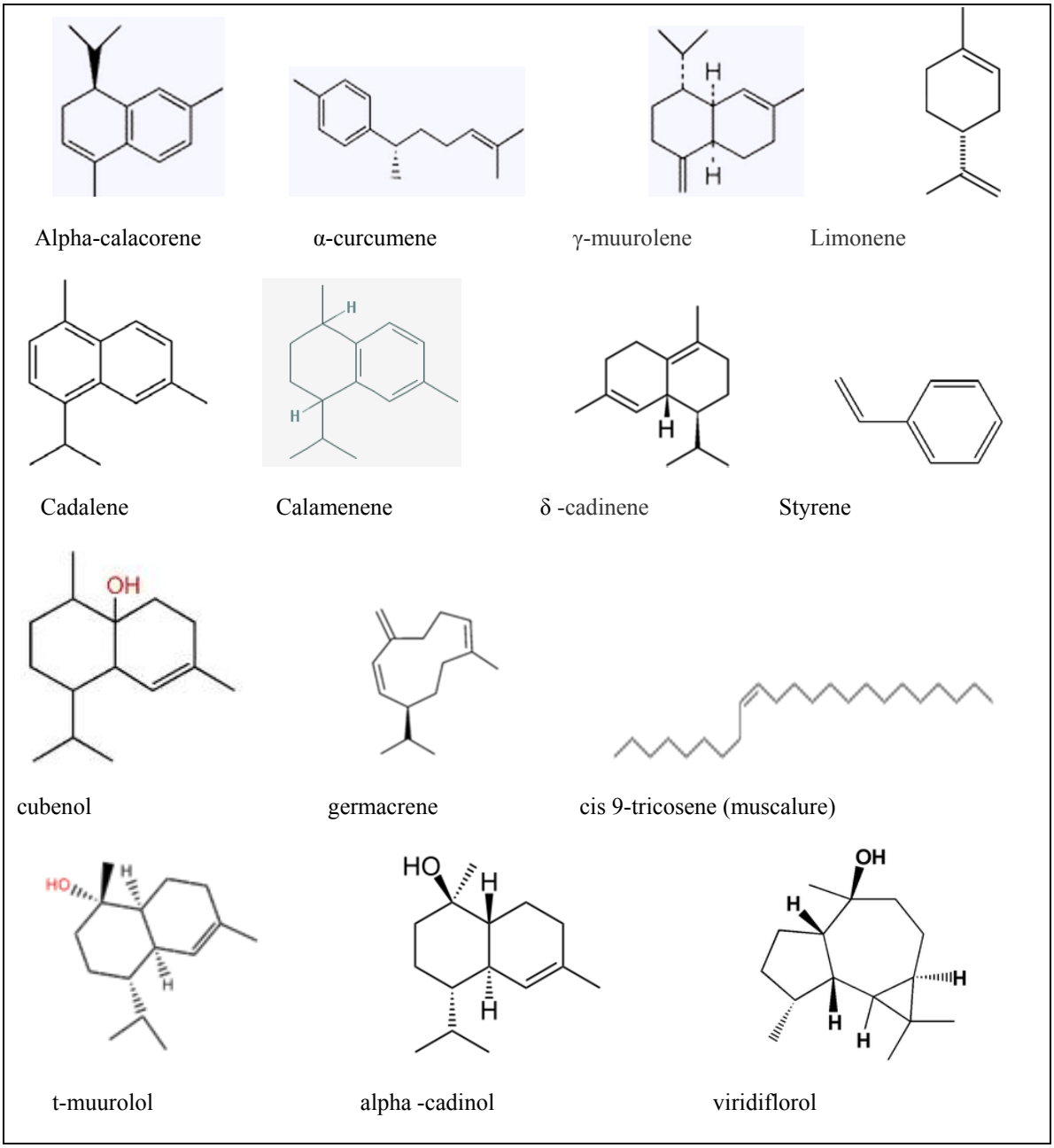


Figure 19. Sesquiterpenes found in *Vanilla pompona* extract.

Table 9. Compounds present in *Vanilla pompona* extract that are newly identified in vanilla and can be found naturally occurring.

Compound	**Selected natural sources***
trans 2-nonenal	Artichoke, asparagus, bread,carrot, coffee, ginger, grape, melon, olive, peas, peach, strawberry, sesame seed and tea.
cis 3-nonenal	Cucumber,watermelon.
trans 2-octenal	Acerola, apple, avocado, buckwheat, chamomile, cardamom, cherry, cocoa, citrus fruits, grape, berries, potato, rice, tomato, tea and walnut,
2-butenal	Apple, celery, citrus fruits, cocoa, carrot, cabbage, malt, date, guava, olive, peach, peanut, strawberry, peanut, potato and tomato.
heptanal	Apple, apricot, artichoke, carrot, celery, citrus fruits, cocoa, fig, grape.
4-methoxyphenol	Honey, licorice, sesame seed, and alpinia species.
2-methyl-3-buten-2-ol	Bilberry, cardamom, cherimoya, coffee, cranberry, mango, lemon, lavender oil, black currant and hops.
trans-3-penten-2-ol	Calvados, cognac, marasmius alliaceus
amyl formate	Strawberry, honey, strawberry, prickly pear and tomato.
p-cresyl methyl ether	Blue cheese, buckwheat, rooibos tea and starfruit.
methyl linoleate	Apple, buckwheat, gooseberry, elderberry, pineapple, plum and walnut.
Alpha curcumene	Guava, rosemary and ginger.
alpha-calacorene	Pepper, apple, cloves, guava, menthe, nutmeg, thyme and turpentine oil.
cadinene	Pistacia lentiscus.
cadalene	Calabash nutmeg, cloves, hop oil, lemon, rooibos tea and rosemary.
ethyl pyrazine	Asparagus, barley, beans, buckwheat, cocoa, coconut, coffee, otas, peanut, soybean, tamarind, wild rice.
2,3-octanedione	Coffee, mushroom, pear, peanut, soybean, tea, milk products.

*References:http://www.vcf-online.nl
http://www.thegoodscentscompany.com/docs/doc1192811.html#

6. Results: volatiles in *Vanilla pompona* beans extracted by DTD- GC-MS analysis

6.1. Results

A total of eighty volatiles and semi volatiles were identified in the cured beans of *Vanilla pompona* by DTD-GC-MS. The volatile profile is shown in Figure 20. The distribution by percentage of total area is presented in Figure 21.

The volatiles in cured beans are made up of the following 10 chemical classes by percentage of abundance (% of total area): 17 acids (38.51%), 14 heterocyclic compounds (mainly furans) (34.81%), 10 hydrocarbons (2.07%), 7 phenols (9.0%), 7 alcohols (5.32%), 7 carbonyl aldehydes (4.93%), 7 carbonyl ketones (3.39%) and 3 acetals (1.04%), and 8 esters (0.88%). The chemical compounds in this vanilla species are listed in descending order of abundance in Table 10 by chemical class, including the semi-quantification of the compounds estimated based on peak area integration comparisons to that of the internal standard. From Table 10, the most abundant volatiles, expressed in concentration units of g/100g of cured beans of *Vanilla pompona* were: linoleic acid (0.74%), hydroxy methyl furfural (0.66%), 5-hydroxy-5,6-dihydromaltol (0.43%), vanillin (0.28%), palmitic acid (0.20%), anisyl alcohol (0.14%), acetic acid (0.14%), 2-furfural (0.12%), the remainder of compounds were present at levels <0.1g/100g cured beans.

Fifteen compounds were already reported by Lee (2006). The remaining constituents have been previously reported in vanilla, except twelve compounds; ten of these compounds are presented in Table 11.

Finally, neither piperonal nor coumarin were found in the cured beans of *Vanilla pompona* under the conditions of this DTD-GC-MS method.

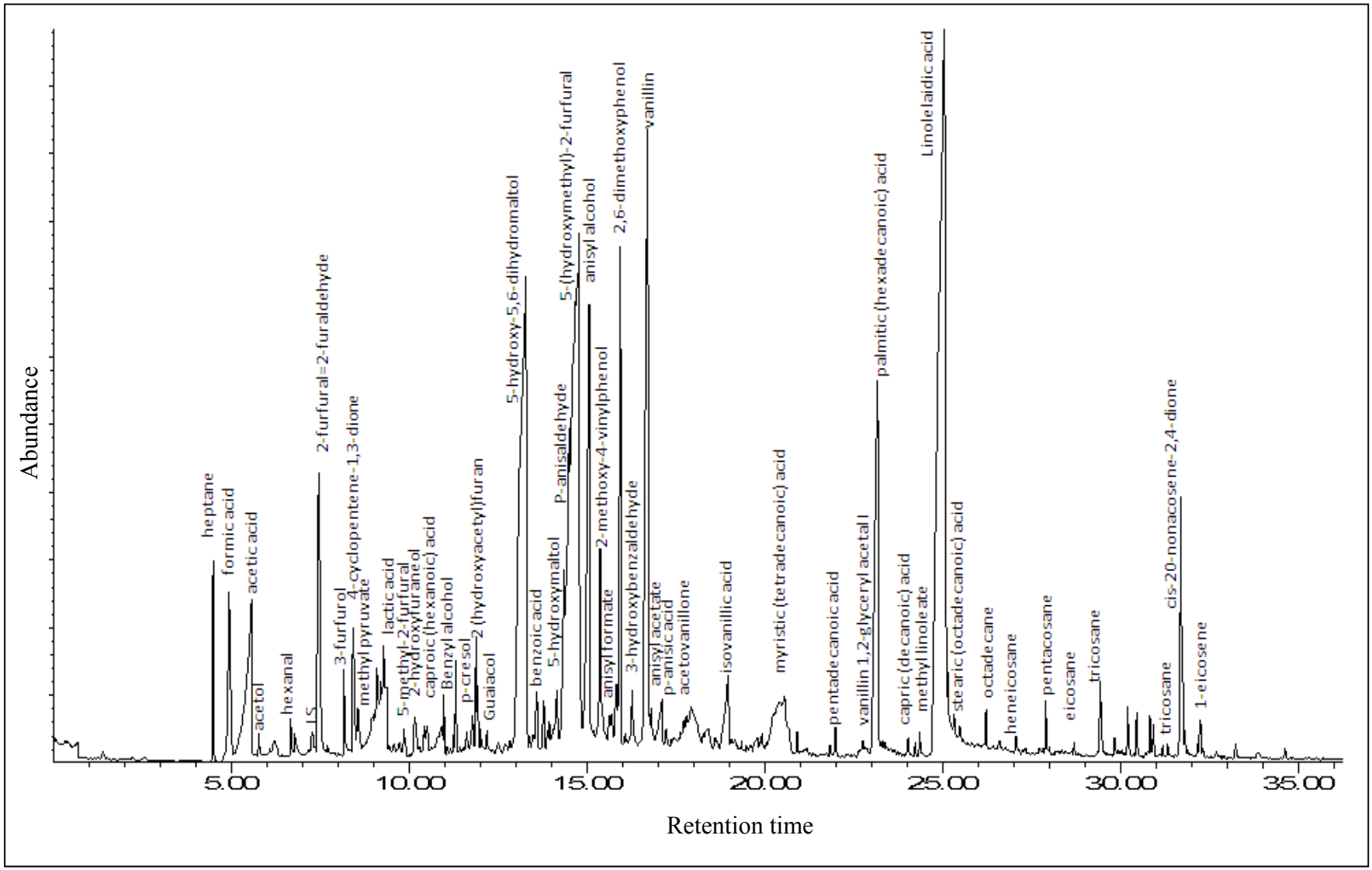


Figure 20. Chromatogram (TIC) of cured beans of *Vanilla pompona* using DTD-GC-MS method.

Table 10. Volatile compounds in cured beans of *Vanilla pompona* using GC column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No.	Ret.Time	Compound Name	CAS #	Area %	~ppm
Acids (38.51% of abundance)					
1	25.04	linoleic acid	60-33-3	21.57	7,428
2	23.17	palmitic (hexadecanoic) acid	057-10-3	5.81	2,000
3	5.57	acetic acid	64-19-7	4.13	1,423
4	9.37	lactic acid	50-21-5	2.32	797
5	4.95	formic acid	64-18-6	1.94	668
6	18.97	isovanillic acid	645-08-9	1.09	374
7	10.93	acetic acid	064-19-7	0.57	196
8	17.11	*p*-anisic acid = 4-methoxybenzoic acid	100-09-4	0.39	135
9	10.51	caproic (hexanoic) acid	142-62-1	0.28	98
10	21.99	pentadecanoic acid	1002-84-2	0.10	36
11	20.92	myristic (tetradecanoic) acid	544-63-8	0.07	25
12	24.03	capric (decanoic) acid	334-48-5	0.07	24
13	19.77	homovanillic acid	306-08-1	0.05	18
14	18.61	lauric (dodecanoic) acid	143-07-7	0.05	17
15	13.33	benzoic acid	65-85-0	0.03	12
16	13.40	caprylic (octanoic) acid	124-07-2	0.03	9
17	16.08	valeric (pentanoic) acid	109-52-4	0.01	4
Heterocyclics (Furans, pyrans) (34.81% of abundance)					
18	14.78	5-(hydroxymethyl)-2-furfural	67-47-0	19.18	6,606
19	13.29	5-hydroxy-5,6-dihydromaltol	28564-83-2	12.68	4,368
20	14.36	2(5H)-furanone	497-23-4	0.90	311

Table 10. Continued. Volatile compounds in cured beans of *Vanilla pompona* using GC column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No.	Ret.Time	Compound Name	CAS #	Area %	~ppm
21	13.60	3,4-dimethyl-2,5-furandione	766-39-2	0.50	173
22	10.17	2-hydroxyfuraneol = 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone	10230-62-3	0.47	162
23	13.79	5-hydroxymaltol	1073-96-7	0.27	93
24	9.10	2(5H)-furanone	497-23-4	0.21	71
25	11.93	5,6-dihydromaltol = 3-hydroxy-2-methyl-5,6-dihydropyran-4-one	38877-21-3	0.18	62
26	9.86	5-methyl-2-furfural= 5-methylfurfural	620-02-0	0.17	57
27	11.78	2,5-furandicarboxaldehyde	823-82-5	0.09	30
28	15.44	5-acetyl-2-furfurol	55087-82-6	0.05	16
29	8.95	2-acetylfuran	1192-62-7	0.04	14
30	12.51	maltol = corps praline	118-71-8	0.04	14
31	33.24	3-pentyl-5-methyldihydro-2(3H)-furanone		0.03	11
Phenols (9.0% of abundance)					
32	16.71	vanillin	121-33-5	8.18	2,816
33	15.37	2-methoxy-4-vinylphenol = 4-vinylguaiacol= "varamol 106"	7786-61-0	0.66	227
34	13.95	*p*-vinylphenol	2628-17-3	0.06	20
35	11.95	4-methoxyphenol (*p*-guaiacol)	150-76-5	0.06	19
36	13.86	catechol = 1,2-benzenediol	120-80-9	0.03	12
37	13.57	1,4-dimethoxybenzene = hydroquinone dimethyl ether	150-78-7	0.02	7
38	11.73	*p*-cresol = 4-methylphenol	106-44-5	0.02	6

Table 10. Continued. Volatile compounds in cured beans of *Vanilla pompona* using GC column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No.	Ret.Time	Compound Name	CAS #	Area %	~ppm
Alcohols (5.32% of abundance)					
39	15.06	anisyl alcohol	105-13-5	4.14	1,427
40	8.18	3-furfurol	4412-91-3	0.48	164
41	30.84	1-octadecanol = stearyl alcohol	112-92-5	0.28	96
42	10.98	2-furfurol	98-00-0	0.22	76
43	12.19	1-butanol	71-36-3	0.07	23
44	7.20	2,3-butanediol II	gkuzhy	0.04	15
45	11.07	benzyl alcohol	100-51-6	0.04	13
Carbonyls, aldehydes (4.93% of abundance)					
46	7.47	2-furfural = 2-furaldehyde	98-01-1	3.57	1,229
47	29.44	*cis*-9-octadecenal	56554-35-9	0.51	177
48	16.28	*p*-Hydroxybenzaldehyde	123-08-0	0.32	109
49	14.50	*p*-anisaldehyde = 4-methoxybenzaldehyde	123-11-5	0.23	78
50	6.79	hexanal	66-25-1	0.18	64
51	15.40	E2,E4-decadienal	25152-84-5	0.11	38
52	9.73	benzaldehyde	100-52-7	0.01	4
Carbonyls, ketones (3.39% of abundance)					
53	31.70	*cis*-20-nonacosene-2,4-dione	305805-40-7	1.52	524
54	8.44	4-cyclopentene-1,3-dione	930-60-9	1.23	422
55	9.29	2-hydroxy-2-cyclopenten-1-one	10493-98-8	0.23	80
56	30.21	nonadecane-2,4-dione	16577-69-8	0.23	79
57	7.28	4-hexen-3-one	2497-21-4	0.12	42

Table 10. Continued. Volatile compounds in cured beans of *Vanilla pompona* using GC column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No.	Ret.Time	Compound Name	CAS #	Area %	~ppm
58	17.80	apocynin = acetovanillone	498-02-2	0.05	17
59	15.44	2-acetyl-5-methylthiophene	13679-74-8	0.02	6
Hydrocarbons (2.07% of abundance)					
60	4.50	heptane	142-82-5	1.01	347
61	27.90	pentacosane	629-99-2	0.20	68
62	26.23	octadecane	593-45-3	0.19	64
63	30.47	squalene	111-02-4	0.16	56
64	29.46	tricosane	638-67-5	0.15	50
65	32.25	1-eicosene	567-04-0	0.14	48
66	30.93	tricosane	638-67-5	0.12	43
67	27.08	heneicosane	629-94-7	0.06	21
68	28.70	eicosane	112-95-8	0.04	13
69	20.03	cadalene = 4-isopropyl-1,6-dimethylnaphthalene	483-78-3	0.01	3
Other (Acetals, Unknown) (1.04% of abundance)					
70	11.31	acetoin propylene glycol ketal II (acetoin propyleneglycol acetal)	94089-23-3	0.44	152
71	31.75	benzaldehyde dimethyl acetal	1125-88-8	0.39	136
72	22.76	vanillin 1,2-glyceryl acetal I	85377-00-0	0.07	25
Esters (0.88% of abundance)					
73	8.57	methyl pyruvate	600-22-6	0.36	123
74	6.68	methyl acrylate = methyl 2-propenoate	96-33-3	0.20	68
75	30.45	anisyl palmitate		0.13	44
76	24.37	methyl linoleate = methyl cis-9,cis-12-octadecadienoate	112-63-0	0.09	32

Table 10. Continued. Volatile compounds in cured beans of *Vanilla pompona* using GC column Restex Rxi®-1ms (0.32mmX60mx1µm df).

No.	Ret.Time	Compound Name	CAS #	Area %	~ppm
77	16.81	anisyl acetate	104-21-2	0.07	24
78	6.23	butyl formate	592-84-7	0.07	24
79	15.62	anisyl formate	122-91-8	0.06	21
80	25.33	anisyl salicylate	72845-81-9	0.03	12

6.2. Discussion

The percent of vanillin in the cured beans of this Mexican *Vanilla pompona* using DTD-GC-MS analysis was 0.28% w/w, which is near the levels of commercial vanilla, between 0.3 and 3.4% w/w (Morison-Smith, D., 1964; Brodelius, 1994). However, this level of vanillin was lower than expected for this species, compared with previous results of 2.3% vanillin in *V. pompona* from Madagascar (Lee, 2006) and the levels of 2.33-5.71% reported in ripe fruits of Peruvian *Vanilla pompona* by Maruenda *et al.* (2013). The content of anisyl alcohol in these beans (0.14%) is in agreement with the level reported by Ehlers and Pfister (1997) in *Vanilla pompona* of unknown origin. Similarly, Maruenda *et al*. (2013) reported high levels of anisyl alcohol (1.05-7.13%) using nine months old fruits of Peruvian *Vanilla pompona.* In contrast, the level of this compound reported by Lee (2006) in *V. pompona* beans from Madagascar was much lower (0.041%) under similar analytical conditions.

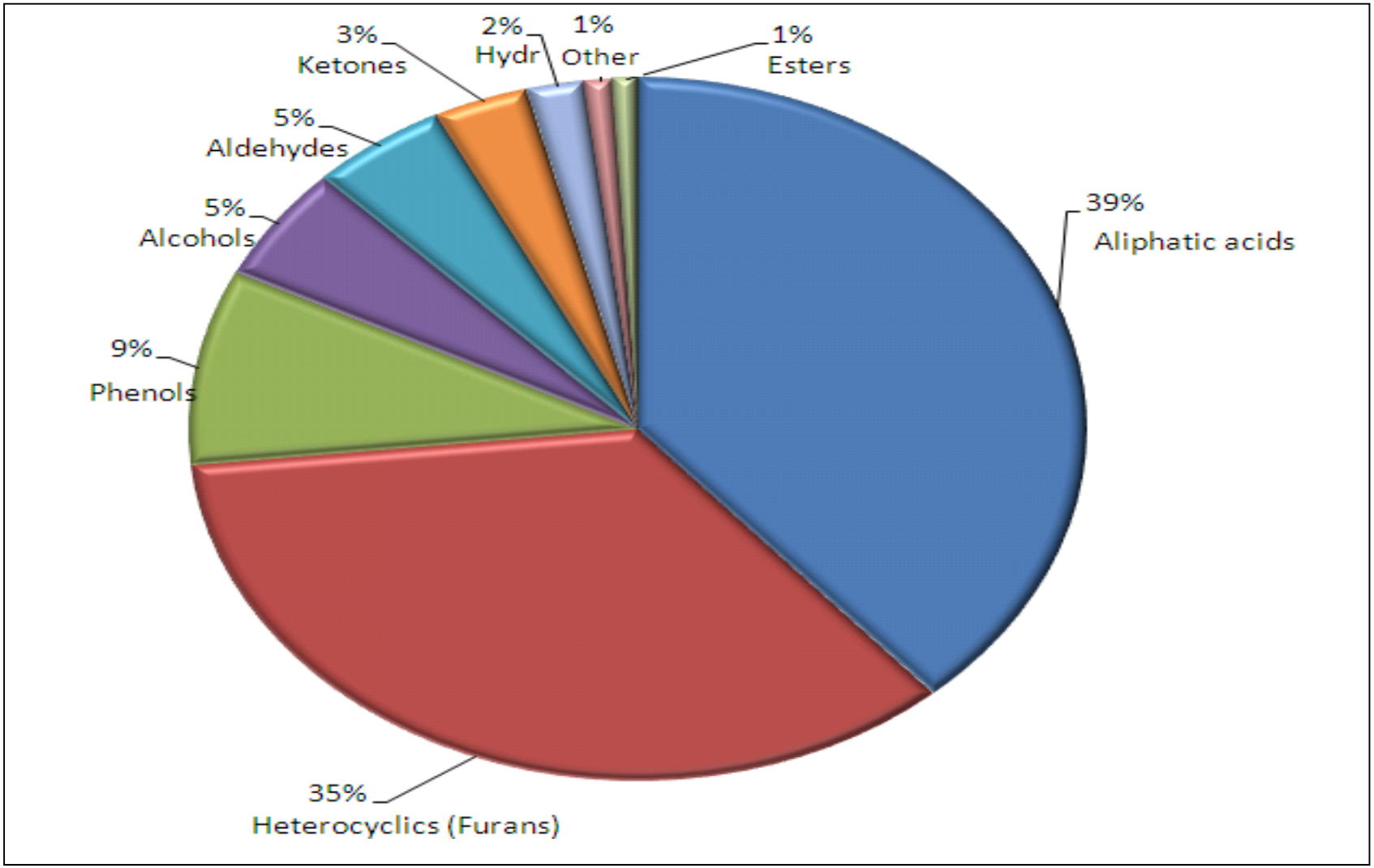


Figure 21. Percentage of area distribution of volatile compounds in cured *Vanilla pompona* beans using DTD-GC-MS with column: Rtx®-Wax column .32 mm ID x 1.00 µm DF, Restek).

Important phenols (Figure 5) such as *p*-hydroxybenzoic acid and vanillic acid, previously reported as present in *Vanilla pompona* (Ehlers and Pfister, 1997), were not identified in this study. Vanillic acid that was reported by Lee (2006) at a level of 1011ppm was not found, but two isomers were identified: 18 ppm of homovanillic acid (previously reported in vanilla) and 374 ppm of the newly reported compound in vanilla: isovanillic acid (3-hydroxy-4-methoxybenzoic acid) for which a standard was not available.

The compound *p*-hydroxybenzaldehyde was found present at a level of 0.01%, in contrast with the level of 0.35% reported in *V. pompona* from Madagascar (Lee, 2006).

Aliphatic acids were the most abundant chemical class in the cured beans of *Vanilla pompona*. These were eluting at the later section of the chromatogram (Figure 18). Long chain fatty acids, high molecular weight hydrocarbons, waxes, resins and tannins minimally contribute to the aroma, but serve as fixatives and help to control the release of volatiles (Adedeji, 1993).

Furan compounds, and particularly HMF, were the second most abundant chemical class identified in *Vanilla pompona* cured beans using this method. These types of compounds are generated by thermal degradation of the glucose as a result of the high thermal desorption temperature (Lee, 2006; Adedeji et al., 1993). Thermal degradation also produces a relatively high abundance of formic acid, acetic acid, furfurals and furfuryl alcohols (Hartman et al, 1992).

Despite *p*-anisaldehyde was found to be the most abundant aldehyde in the extract of *Vanilla pompona*, the level obtained using DTD-GC-MS method in cured beans was very low (0.0078%w/w). Also, this level is lower than the 0.03% w/w obtained by Ehlers

and Pfister (1997) using HPLC. The compound *p*-anisaldehyde was not mentioned in the Peruvian uncured fruits by HPLC-DAD (Maruenda *et al*., 2013) nor the previous work using cured beans from Madagascar (Lee, 2006). The difference in levels of *p*-anisaldehyde between the extract and the cured beans was probably due to reactions of aromatic aglycones with alcohol organic solvents that can lead to the formation of additional aromatic aldehydes in the extract, not occurring in the dry fruits.

Another characterizing compound *p*-anisic acid was present at level of 135 ppm in the cured beans. This level is higher than 39 ppm reported by Lee, (2006) in the Madagascar sample using DTD-GC-Ms; however, this is lower than the 400 ppm level reported by Ehlers and Pfister (1997) using HPLC.

The difference in the concentration of the volatiles reported previously in *Vanilla pompona* samples versus the results of the Mexican *Vanilla pompona* in this study is not surprising. The source of this variability can be explained by the different origin of the beans, associated with farming practices, harvesting time, curing method and the difference in sensitivity of the several analytical methods used in the studies (Hartman, 1992).

An additional cause of variability, specific to this species, was proposed by Maruenda et al., (2013) who found that *V. pompona* has a particular physiology of the formation of glycosides (aroma precursors). The glycosides in this species develop mostly at the late stage of ripening. This means that the fruits require at least 8-9 months before accumulating significant levels of aromatic glycosides while the aroma precursors in the fruits of *Vanilla planifolia* species build up progressively. It is well known that the common practice in Mexico is to pick all of the beans at the same time in December,

without considering the heterogeneity of ripeness of the beans (Hernández-Hernández, 2011).

Table 11. Selected compounds present in cured beans of *Vanilla pompona* that are newly identified in vanilla beans.

Compound	**Selected natural sources***
1,4-dimethoxybenzene	Cherimoya, menthe, papaya, tea.
dimethoxy-methylbenzene (Benzaldehyde dimethyl acetal)	Rhubarb
1-octadecanol	Acerola, apple, rice, cherimoya, gooseberry, guava, beef, cheese, milk and pork.
2-acetyl-5-methylthiophene	Beef, coffee, krill and pork.
butyl formate	Apple, cloudberry, cheese, strawberry, and bread.
methyl acrylate	Cashew apple and pineapple.
methyl pyruvate (Methyl 2-oxopropionate)	Artic bramble, honey and mangifera species.
1,2-benzenediol	Apple, barley, berry, cocoa, coffee, and honey.

*References:http://www.vcf-online.nl
http://www.thegoodscentscompany.com/docs/doc1192811.html#

6.2.1. Other characterizing compounds

Additional constituents of *Vanilla pompona* were identified directly from cured beans using this method (DTD-GC-MS) such as phenolic compound 1, 2 benzenediol and high molecular weight wax compounds, called β-Diketones: 20-nonacosen-2,4-dione and the tentative compound nonadecane-2,4-dione. These two constituents were not found using organic extraction, but were identified in the cured beans by DTD-GC-MS. The compound 4-cyclopentene-1, 3-dione was also found in the extract. β-Diketones are

common constituents of plant waxes and commonly found in commercial vanilla species (Lee, 2006; Hartman, 2010).

Two novel anisic compounds: anisyl palmitate and *p*-anisyl salicylate were identified in the beans at levels of 44 ppm and 12 ppm respectively. These were previously isolated from wild vanilla beans from a Peruvian rain forest using the same analytical method (Hartman, 2010; Lee, 2006). Spectra comparisons of these from both studies are shown in Figure 23 and Figure 24.

Among the compounds reported for the first time in vanilla, 8 were reported as naturally occurring (Table 11) besides the compounds isovanillic acid (3-Hydroxy-4-methoxybenzoic acid) and 3-furfural (3-hydroxymethyl furfural). Selected structures are shown in Figure 22.

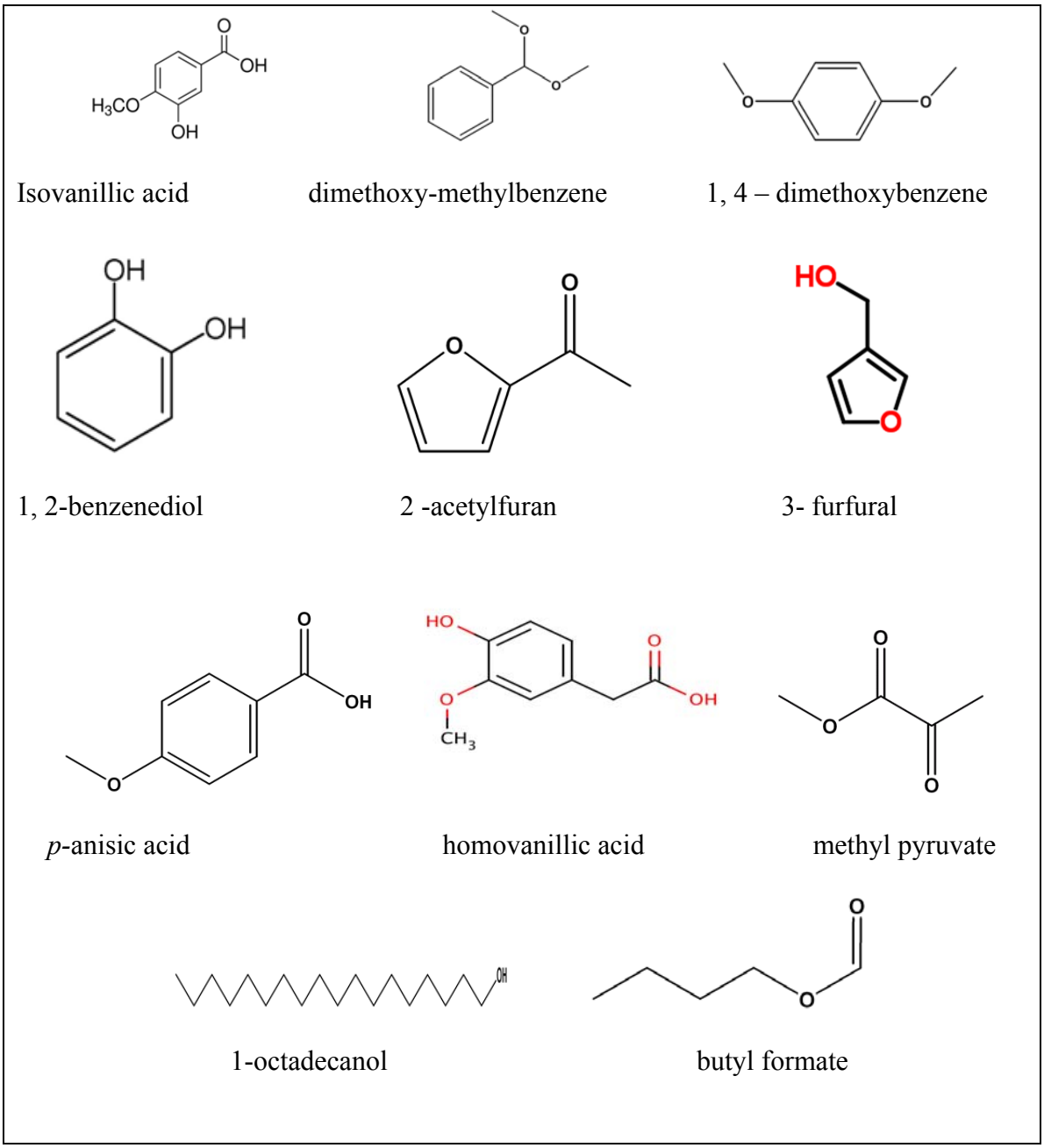
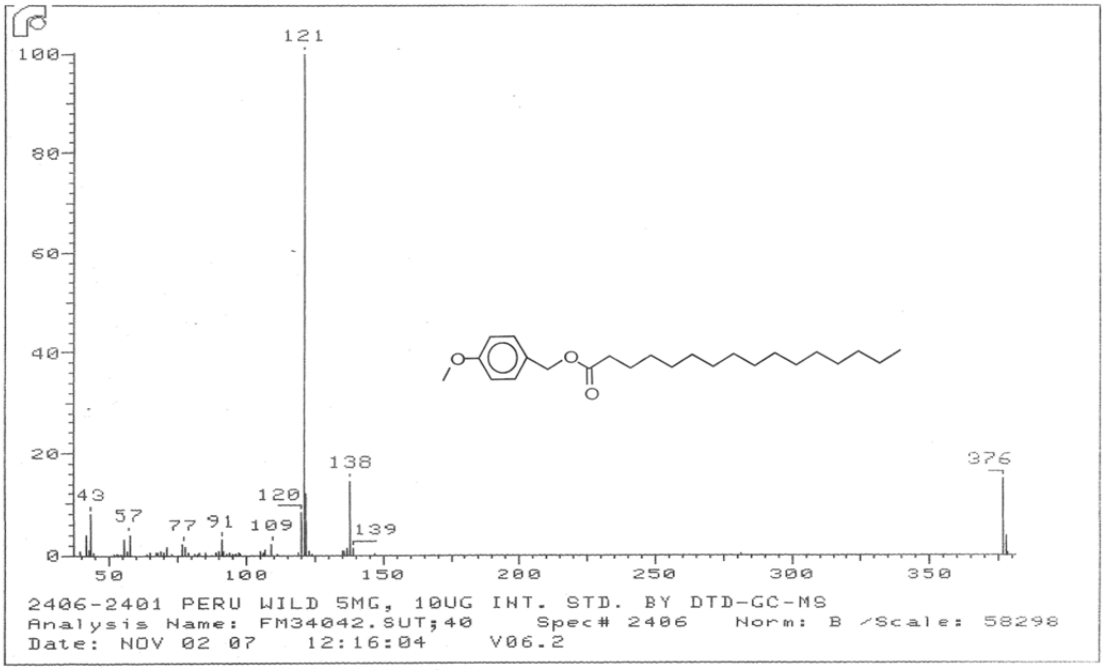


Figure 22. Compounds found in cured beans of *Vanilla pompona* using DTD-GC-MS.



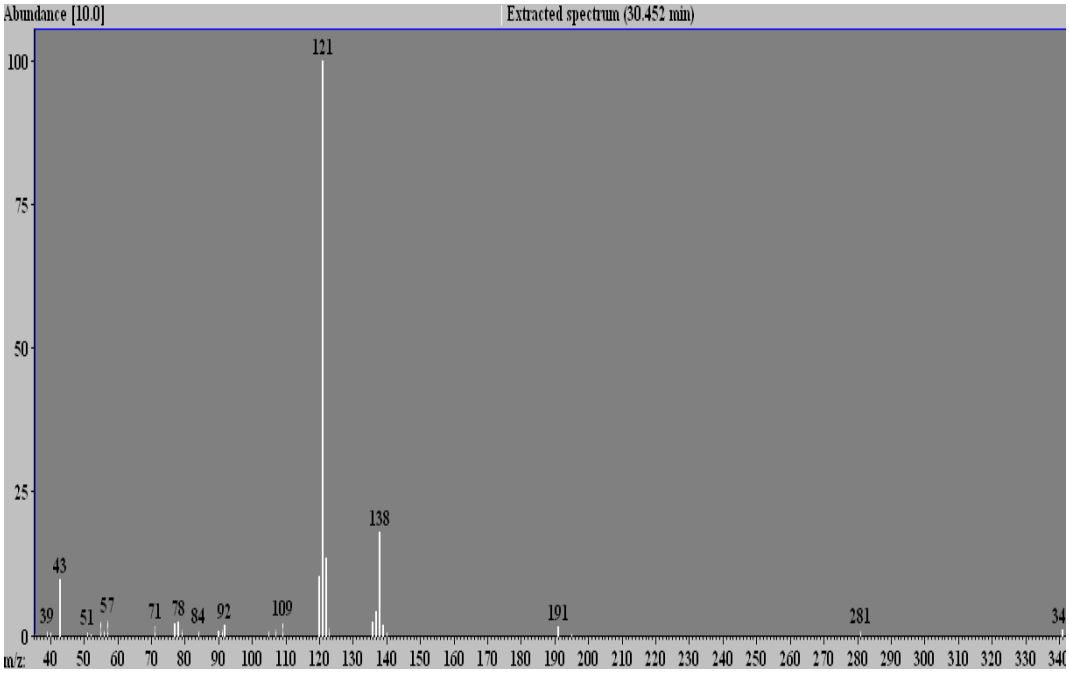


Figure 23. Spectra comparison of compound anisyl palmitate previously found (top) by DTD-GC-MS (Lee, 2006) and the Spectra deconvoluted by AMDIS from GC-MS of *Vanilla pompona* extract (bottom).

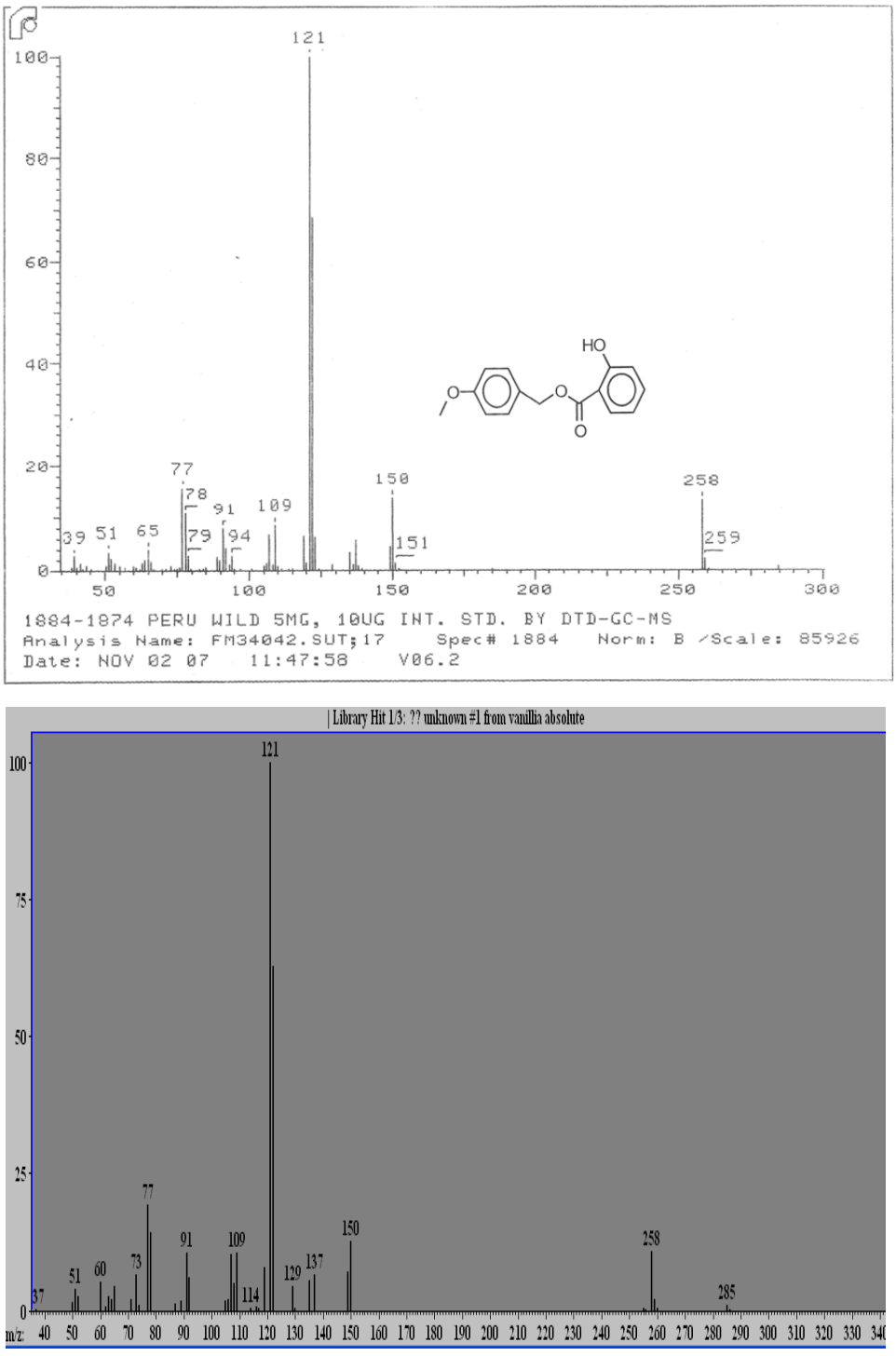


Figure 24. MS Spectra comparison of compound *p*-anisyl salicylate previously found (top) by DTD-GC-MS (Lee, 2006) and the Spectra deconvoluted by AMDIS from GC-MS of *Vanilla pompona* extract (bottom).

7. Results: Gas Chromatography-Olfactometry analysis of *Vanilla pompona* extract

The aroma extract of the *Vanilla pompona* was obtained using the ethanol extraction-CH_2Cl_2 back extraction method as described in the Materials and Method Section 3.4.4.2. The extract of the *Vanilla pompona* was described by the participating flavorists as vanillin, very sweet, dried fruit (pruny, raisin, and fig), strong phenolic, caramel and chocolate like, slightly floral, resinous, balsamic, and heliotropin. These are considered common descriptors of commercial vanilla aroma (Table 1).

The overall aroma of the extract used in this study was in agreement with these previous descriptions, except for woody nuances as indicated in the preliminary study. The absence of woody notes in this extract may be attributed, among other factors, to differences in the origin and the curing method applied to the beans. Both, origin of the beans and curing process, are considered key factors to define the final aroma of vanilla. In addition, experienced flavorists tend to breakdown single terms into several substituting descriptors. In this case, the term woody could have been substituted by resinous and balsamic.

7.2. Acquisition of GC-O/MS bulk data

Vanilla is well known for being a complex material that contains hundreds of odor active volatiles. This was evident during the GC-sniff sessions because the complexity of the volatile profile required recording olfactory data at very fast pace. It was noticed that sniffing vanilla was particular challenging during the most “crowded effluents section”, between 20 and 38 minutes of the GC run, where the effluents eluted

rapidly close to each other from the capillary column (See figure 25). The grade of difficulty for a person to simultaneously detect an odor, find a descriptor, and register the intensity was reported as a main cause of high variability recounted for a given sniffer (McDaniel *et al*., 1990; da Silva *et al*., 1994; Pollien *et al*, 1994; and Guichard *et al*., 1995). The consistency observed in the individual responses in the GC-sessions was fundamental to reduce complexity of the GC-O data analysis (decreasing noise), save time and minimize uncertainty of the results.

The aromagrams obtained from the GC-O sessions were very similar, but not exactly the same. It is well known that analytical and sensorial data cannot be presented with the same precision. In addition, differences of sensitivity within individuals of the GC-SNIF panel can be expected to reflect the normal differences among the population (Da Silva, 1997). According to Pollien *et al*. (1997), the hypothesis in the GC-SNIF technique is that panel reproducibility is comparable to intrapanel repeatability, in other words, independent panelits that are able to generate similar aromagrams of a given product can produce reproducible results. In this modified GC-NIF method, a trained panel comprised by individuals familiar with the methodology and a history of generating consistent olfactory information, was a resource equally as important as a suitable sampling method for *Vanilla pompona*.

7.3. Processing of GC-O raw data to determine aroma impact compounds

The bulk data that came from the GC-sniff sessions of fifty minutes was the building blocks for olfactometry analysis of *Vanilla pompona* extract. The raw data consisted of five hundred twenty three odor events recorded from the eight GC-sniff

sessions. Each odor event has a record of detection time, intensity value and aroma quality given by the panelists according to the procedure in Section 3.6.

The hyphenated system provided the GC-O/MS information of each session in an overaly diagram with the TIC (Total Ion Chromatogram) results at the top and the aromagram results at the bottom as seen in the single run diagram shown in Figure 25.

In this figure, it is interesting to highlight that some potent odor compounds such as compound 4 (Figure 25) characterized by high intensity values or peaks in the aromagram showed very little response in the GC-MS. In contrast, the most abundant chemical compounds identified by a Mass Spectrometer detector such as compound 44 (Figure 25) was not the most potent odor. The assumptions that an aroma zone in the aromagram would be proportionate to a chemical compound on the TIC diagram, or vice versa, was not always correct. It is a fact that most potent odorants in natural products are present at trace levels and can be difficult to detect in GC-MS data due to the presence of more abundant and less potent odor components.

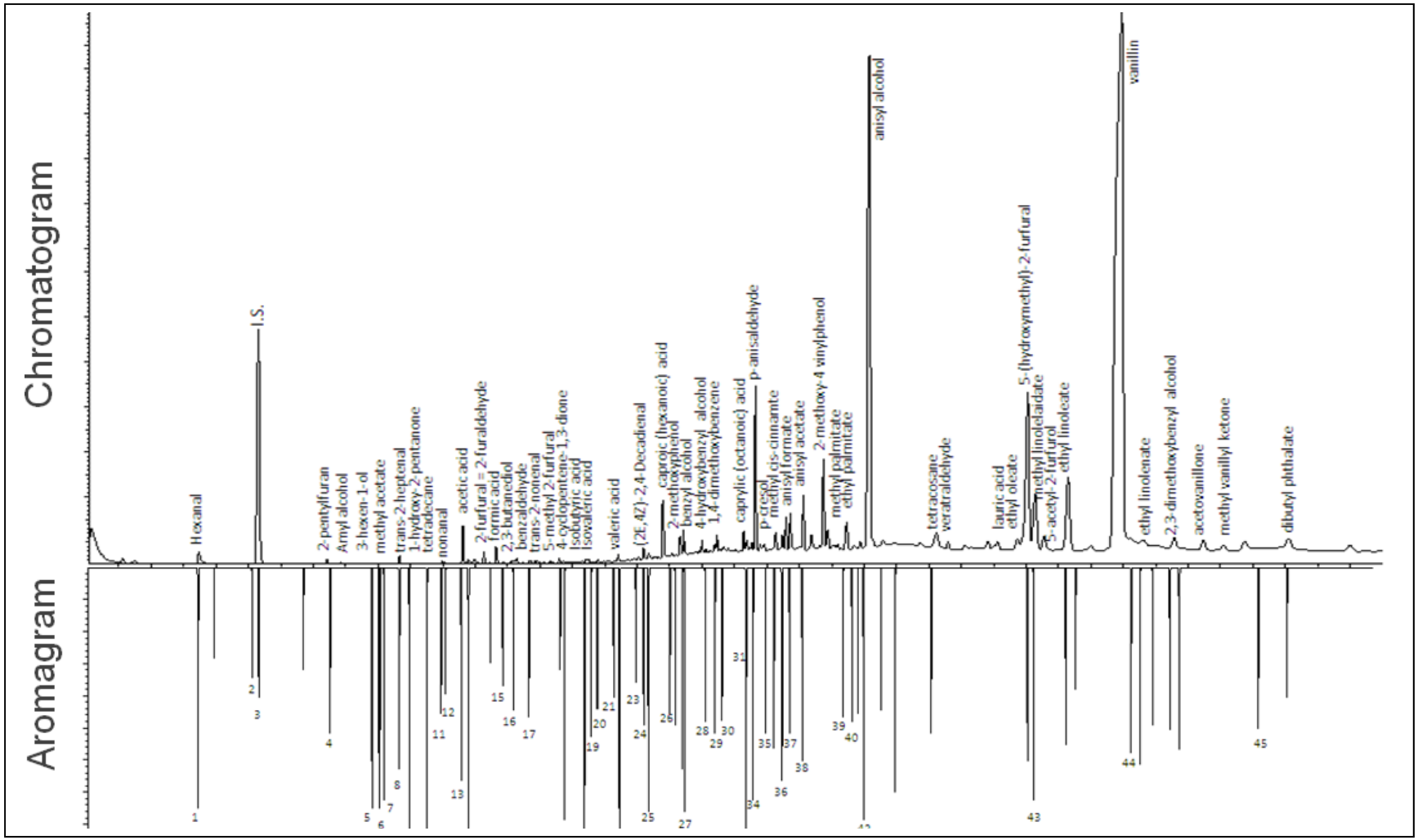


Figure 25. GC-MS chromatogram (top) and single aromagram (bottom) of *Vanilla pompona* aroma extract, separation performed on a Rtx®-Wax column (60 m x 0.32 mm ID x 1.00 µm DF, Restek).

7.3.1. Selection of aroma impact compounds in *V. pompona* extract

Aroma events with equivalent odor descriptors and detection times in the bulk GC-O data were combined, resulting in 198 unique aroma events. The total aroma intensity and the detection frequency were calculated for each of the aroma events (selected data shown in Appendix 2); they were then plotted against their retention time, resulting in a composite aromagram as shown in Figure 26.

The primary purpose of GC-O is to distinguish odor-active compounds from volatiles without odor impact. However, it would have been extremely difficult and time consuming to try and identify each one of these 198 combined events, considering that the underlying assumption of GC-O analysis is that only a small percentage of compounds are responsible for an aroma or odor of a sample (Grosch, 2001). Therefore, the first goal for the identification of chemicals responsible for these odors was to determine at what extent these volatiles contribute individually and to select those that contribute the most. The efforts would be directed to identify only those that have the most influence on the overall aroma of *Vanilla pompona* extract.

7.3.2. Reduced composite aromagram

The criteria for selection of aroma impact compounds as indicated in the Section 3.7.2. were applied to the combined odor events shown in Figure 26. The odor events in Figure 26 that had both a minimum detection frequency value of 4 (50% NIF value) and Intensity value over 200 were considered odor impact compounds.

It is reasonable to assume that aroma events with higher total intensity or detection frequency contribute more significantly to the overall aroma.

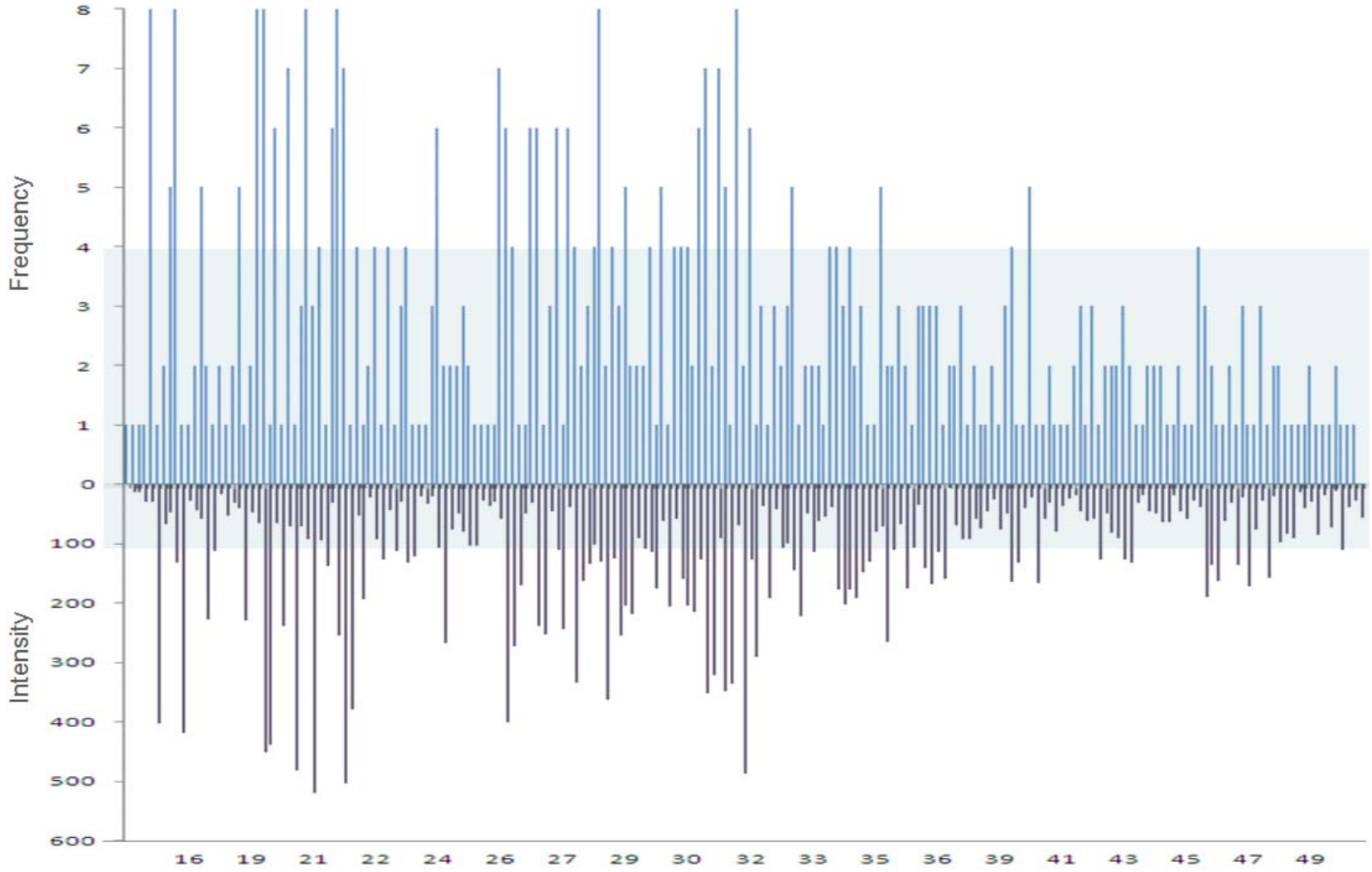


Figure 26. Composite Aromagram (Top: detection frequency. Bottom: total intensity) of combined odor events from GC-Sniff sessions of *Vanilla pompona* extract.

The aroma events in Figure 26 were therefore arbitrarily grouped into primary, secondary and background aroma-impact compounds, as well as noises, based on detection frequency. The primary aroma-impact compounds were those with detection frequencies of 7 and 8 (87.5% and 100% NIF value); the secondary aroma compounds had detection frequencies of 5 and 6 (62.5% and 75% NIF value); the background aroma-impact compounds corresponded to those with detection frequencies of 4 (50% NIF value). These categories represent the extent of contribution each compound to the overall aroma of the vanilla extract.

Finally, the aroma events with detection frequencies of 1, 2 or 3 were considered to have no aroma impact or noises (darkened in Figure 26). Removal of these noises from the composite aromagram led to a simplified aromagram called Reduced Composite Aromagram, shown in Figure 27, which contains only forty five peaks of aroma-impact.

A good linear correlation (R^2=0.8219) obtained from plotting the Frecuency value versus Intensity values for each of the 45 odor events indicated that the two independent criteria applied to select the aroma impact compounds, agreed with each other. This is in agreement with the results observed by Chaintreau (2002) for this method. The graph (Figure 41) was included in Appendix 5.

The next step in this GC-O analysis was to identify the chemical compounds responsible for the smell of these peaks. Although the efforts were directed to identify the primary and secondary categories, the role of the background compounds should not be neglected as these odorants with a typical note may also contribute to the balance and round up of the overall aroma (Blank, 2002).

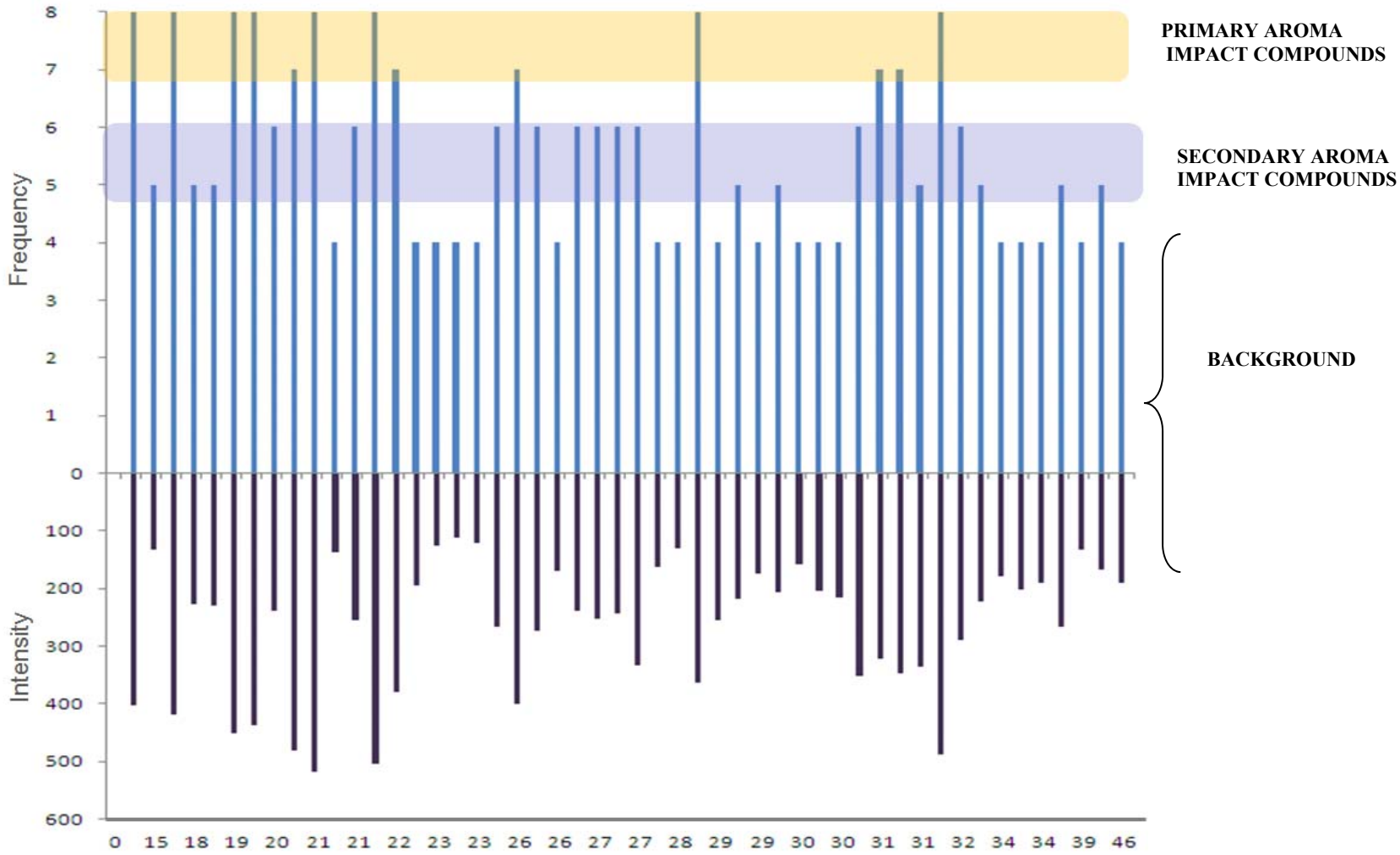


Figure 27. Reduced Composite Aromagrama (Top: detection frequency. Bottom: total intensity) and classification of the aroma impact compounds as primary, secondary and background of *Vanilla pompona* aroma extract.

7.4. Process of identification the aroma-impact compounds

In general, aroma impact compounds were identified based on retention indices, aroma qualities perceived at the sniff port, and mass spectra, or by retention indices, aroma qualities and injection of authentic compounds. Thirty-five of the 45 aroma-impact compounds were positively identified, four were tentatively identified, and six remain unknown. The results are summarized in Table 12. Since the most of the unknown compounds were part of the background, it was not considered a priority to investigate their identities.

During the process of identification of odorants, it was observed that the intent of identifying the chemical compounds responsible for the most impacting aroma events based solely on Mass Spectra data led in many cases to no identification or false identifications as a result of compound overlapping (more than one compound eluting at the same time) or because the intensities were below the instrument sensitivity. Therefore, the identification of such components was a challenging task. Examples of these were compounds #5 and #45 on the single run aromagram (Figure 25). Their absence from the GC-MS data can be explained by the low odor threshold of these compounds.

In the cases when the odorants did not have a MS counterpart or correspondent retention index, the process of searching chemicals based solely on odor quality led to hundreds of volatiles stored in olfactory databases. The paramater Olfactory Retention Indices ($RI_{(O)}$) (Section 3.7.3.1), based on the average detection time of the odor compounds, constituted a useful resourse that, along with the odor quality, allowed narrowing down the tentative compounds stored in olfactory databases.

After identifying the chemicals responsible for the most impacting odors, the Odor Retention indices ($RI_{(O)}$) were plotted versus the Retention Indices of the identified chemical compound ($RI_{(I),}$ $RI_{(MS)}$) obtaining a good linear relationship (R^2=0.9965), indicative that this approach was effective for the identification of the impacting odorants in agreement with the data obtained by Pollien et al., (1997) for this method. The graph is presented in Appendix 5 (Figure 42).

7.4.1. Aroma impact compounds identified using GC-MS data

As shown in Table 12, among the 45 aroma impact compounds, 32 were confirmed by their mass spectra. The retention index and olfactory quality were the main parameters used for characterization of aroma impact compounds (Section 3.7.3.1). Since the retention index value is proportional to the retention time, the closer the detection time of a given peak on the aromagram and its subsequent Odor Retention Index ($RI_{(O)}$) to the retention time of a GC-MS peak and its subsequent AMDIS Retention Index (RI_{MS} or $RI_{(I)}$), the more evident was the identity of a given odor active compound.

Despite the capability of AMDIS, additional steps were necessary when the mass signals were weak. This occurred for some compounds such as No. 3, 10, 12 and 15 (Figure 12). In these cases their mass spectra were still obtained by searching of the targets list in combination with manual deconvolution using AMDIS software.

During the identification process of each compound, it was noticed retention time (or retention index) gaps between mass detection ($RI_{(MS)}$) and olfactory detection ($RI_{(O)}$) for later eluting compounds. To make things even more complicated, the retention time gaps were neither consistent nor had a clear trend. The highest deviation points

corresponded to the most crowded region of effluents from the GC column (during the retention time range of time 20-38 min).

The system had been setup to have identical retention times for mass detection and olfactory detection, which was the case as observed for early eluting compounds. However, it was discovered after the fact that high boiling compounds condensed at the end of the sniff port transfer line, where it was not well-heated. These condensed materials undoubtedly retained the compounds passing through the transfer line, resulting in unequal delays in retention times we observed. The solution to this problem was injecting authentic compounds to determine the retention time gaps whenever in doubt. As shown in Table 12, authentic compounds of No. 8, 12, 13, 20, 21, 22, 26, 29, 30, 37, 40 and 41 were injected for this purpose. The ID Criteria of these compounds includes the parameter "I" on Table 12.

7.4.2. Aroma impact compounds absent from GC-MS data

Several odor compounds with no presence in GC-MS data required additional steps for identification. In this case, the injection of standard compounds was critical to obtain the identities. This was the case for identification of compounds No. 5, 16, 23 and 27. Chemical structures were proposed for them based on their distinctive aroma quality and Retention Index ($RI_{(O)}$). The tentative compounds were then confirmed by injection of the authentic compounds.

Some standard compounds were neither present in GC-MS data with no available standard compounds for confirmation and the odorants were left with status "Tentative". This was the case for compounds such as No. 4, 9 and 24 (Table 12). Their identifications

were based on their familiar but unique aroma quality and retention index, however, their identification was not confirmed neither by mass spectra or injection of authentic compounds.

In the case of compounds 4 and 24 (Table 12), the potency of the odors had to be also considered to narrow down the tentative odorants. For instance, doing the crossreference of odor quality and tentative retention index ($RI_{(O)}$) of compound **4** (Table 12) from olfactory databases led to propose **2, 3-octanedione** (dill cooked broccoli buttery odors and $RI_{(lib)}$ of 1335). However, from previous work with this compound, 2, 3-octanedione lacks the high intensity that was reported for this odorant during the sessions. The high odor intensity and low threshold (detection frequency) indicates a more potent and well known sulfur compound **2-methyl-3-furanthiol** (cooked meat, fried, potato, roasted meat odors and $RI_{(lib)}$ 1319).

Similar criteria were applied to identify compound **24** (Table 12) which RI and olfactive quality led to tentatively indicate this was **diisopropyldisulfide (**RI of 1368 on DB-Wax column). This compound has characteristic medium-low intensity sulfury tonatily obtained from the internal database of previous studies.

8. Results: aroma impact compounds in *Vanilla pompona* extract by GC-O analysis

8.1. Results

The results of this GC-O analysis indicated that 45 components are responsible for the aroma of the *Vanilla pompona* extract. The aroma-impact compounds identified in this extract included the following chemical groups: 14 aldehydes, 7 phenolic compounds, 5 heterocyclic compounds, 3 esters, 4 ketones, 2 acids, 2 alcohol, 2 sulfur

compounds and 6 unknowns. Among these, thirteen were considered Primary aroma-impact compounds, eighteen were identified as Secondary aroma-impact compounds and 18 compounds were considered Background.

The most important contributors to the overall aroma of this vanilla extract are 1,5Z-octadien-3-one, Acetic acid, Trans-methyl cinnamate and 2-acetyl-1-pyrroline and 1-octen-3-one because these primary compounds had the highest aroma intensity. These potent odorants were the primary contributors to the sweet, floral, fruity, nutty, earthy and smoky profile of this extract (Figure 28). A summary of the Total Intensity values of the aroma impact odorants in the *Vanilla pompona* extract are presented in terms of chemical classes in Table 13.

It is worthwhile to note that the sweet and vanillic tonalities of the typical natural vanilla flavor were constant descriptors in most of the primary and secondary aroma impact compounds indicating that most of the odor compounds relevant to the overall aroma contributed at some extent to the strong sweet profile of this vanilla extract.

Sixteen compounds are newly identified as aroma impact compounds in vanilla by GC-O analysis. These are: ethyl pyrazine, 3Z-nonenal, 3-methyl butyric acid, trans-4, 5-epoxy-2E-decenal, 3-octen-2-one, (E,E)-2,6-nonadienal, 10-undecenal, anisyl formate, 3Z-hexenal, diisopropyldisulfide, (E,E)-2,4-nonadienal, 2E-undecenal, phenethanol, acetylpyrrole, trans-geranylacetone and 5-vinylguaiacol.

Table 12. Aroma Impact Compounds present in *Vanilla pompona* extract.

No.	Compound	Detection time (min.)	RI(O)	Selected Descriptors	Frequency	Total intensity	ID Criteria
1	hexanal	13.53	1093	Green, Fresh, Grassy, Ethereal	8	402	MS,O,RI
2	ethyl pyrazine	15.44	1164	Weird, Sweet, Veggie,Plastic,Phenolic, Fruity, Berry, Redfruit,Cherry, Solventy, Earthy	8	418	MS,RI,O,I
3	1-octen-3-one	19.15	1320	Mushroom, Earthy, Vegetable, Strong,Green, Herbaceous	8	452	MS,O,RI
4	2-methyl-3-furanthiol	19.33	1328	Alliaceous, Sulfury, Bready, Baked, Pyrazinic, Cracker like, Earthy, Savory, Meaty	8	438	O,RI^{T}
5	1,5Z-octadien-3-one	20.67	1393	Geranium, Pungent,Plastic, vial, Terpenic, Green,Fatty, Fruity,Familiar, Candy, Sweet, Powdery, Floral, Strong	8	519	O,RI,I^{1}
6	acetic acid	21.72	1446	Sour,Sulfury,Unknown, Acidic,Fatty,Sweet,Brown	8	503	MS,O,RI
7	guaiacol	28.48	1897	Phenolic, Sweet ,Smoky, Doughy, Fried, Playdough, Vanillic, Mushroom, Earthy, Cinnamic	8	363	MS,O,RI
8	*trans*-methyl cinnamate	31.60	2145	Sweet, Phenolic, Spicy, Benzaldehyde, Anisic, Powdery, Cherry, Woody	8	488	MS,O, RI,I

Table 12. Continuation. Aroma Impact Compounds present in *Vanilla pompona* extract.

No.	Compound	Detection time (min.)	RI(O)	Selected Descriptors	Frequency	Total intensity	ID Criteria
9	2-acetyl-1-pyrroline	20.11	1365	Pop corn, Hazelnut, Pretzel, Cooked, Baked, Roast	7	481	O, RI^T
10	3Z-nonenal	21.97	1460	Citrusy, Aldehyde, Sweet, Fruity, Green,Fatty, Mix of fruits, pyrazinic, Bread crust, Oily, Waxy oily, solventy	7	379	MS,O, RI
11	3-methyl butyric acid	25.57	1670	Butyric (long), Butyric, rancid, Skunky, Sour, Cheesy, Acidic	7	401	MS,O,RI
12	*trans*-4,5-epoxy-2E-decenal	30.99	2101	Green, Sweet, Fruity, Sugary, Sweet, Doughy, Burnt, Woody	7	321	MS,O,RI,I
13	4-anisaldehyde	31.23	2118	Eugenol, Grassy, Fresh, Cherry, Anisic, Metallic, Anisic, Sweet, Vanilla, Very sweet, Heliotropin	7	348	MS,O,RI,I
14	2E-heptenal	19.84	1352	Fatty, Oily, Nutty, Doughy, Earthy, Musty, Sweet, Woody, Vegetative	6	238	MS,O, RI
15	3-octen-2-one	21.36	1428	Cooked,Mushroom, Fruity,Green, Pyrazinic	6	255	MS,O, RI
16	2E,6E-nonadienal	23.95	1571	Musty, Smoky, Earthy, Oily, Fatty, Anisic, Weak	6	267	O, RI,I
17	10-undecenal	25.72	1680	Geranium, Aldehyde, Vegetative, Fruity, Floral, Sweet	6	273	MS,O,RI

No.	Compound	Detection time (min.)	RI(O)	Selected Descriptors	Frequency	Total intensity	ID Criteria
18	unknown	26.47	1733	Fatty Oily, Chocolaty, Sweet, Woody, Perfumy, Sweet, Pyrazine	6	238	O
19	2E,4Z-decadienal	27.29	1798	Sweet, Floral, Smoky, Floral, Honey, Bready, Honey	6	244	MS,O, RI
20	phenol	30.75	2082	Green, Sweet, Smoky, Phenolic, Sweet, Doughy, Pop corn, Burnt woody	6	352	MS, O, RI,I
21	anisyl formate	31.96	2170	Floral,Sweet, Spicy, Powdery, Candy	6	290	MS, O, RI,I
22	anisyl acetate	33.94	2240	Sweet, Earthy, Anisic, Candy, Cinnamic	6	273	MS, O, RI,I
23	3Z-hexenal	15.14	1152	Green, Woody, Grassy, Sandalwood, Sweet	5	133	O,RI,I
24	diisopropyldisulfide	17.62	1251	Tropical, Sulfury, Burnt, Fruity, Sweet	5	228	O,RI^{T}
25	octanal	18.90	1308	Fresh, Sweet, Cool, Fruity, Citrusy	5	229	MS, O, RI
26	2E, 4E-nonadienal	26.68	1750	Fatty, Oily, Green, Bready, Vanilla	5	239	MS, O, RI,I
27	2E-undecenal	27.50	1815	Metallic, Oily,Floral, doughy, Burnt	5	266	O,RI,I
28	phenethanol	29.00	1941	Fruity,Sweet, Woody	5	218	MS, O, RI

Table 12. Continuation. Aroma Impact Compounds present in *Vanilla pompona* extract.

No.	Compound	Detection time (min.)	RI(O)	Selected Descriptors	Frequency	Total intensity	ID Criteria
29	2-acetylpyrrole	29.74	2003	Sweet, Pyrazinic, Pop corn, Sweet, Rice, Nutty, Chocolate	5	206	MS,O,RI,I
30	gamma-nonalactone	31.36	2127	Sweet, Phenol, Bamboo, Cherry	5	335	MS,O,RI,I
31	anisyl alcohol	34.93	2342	Sweet, Floral, Sweet, Cinnamic, Odd, Electric	5	266	MS,O, RI
32	vanillin	40.44	2591	Peppery, Smoky, Weak, Powdery, Sweet, Spicy, Woody, Vanilla	5	260	MS,O,RI
33	nonanal	21.06	1412	Fruity, Vegetative, aldehyde, Metallic, Weak, Anisic, sweet, Powdery	4	137	MS,O,RI
34	unknown	22.90	1511	Fresh, Cool, Floral, Sandalwood, Sweet, Woody	4	112	O
35	unknown	23.37	1537	Meaty/Soapy,Hay Doughy, Warm, Woody	4	122	O
36	unknown	26.05	1702	Sweet, Creamy, Cheese, Butyric, Sour, Aldehyde, Waxy	4	162	O
37	2E,4E-decadienal	27.72	1834	Doughy, Powdery, Cereal like, Sandalwood, Nutty	4	163	MS,O,RI,I
38	*trans*-geranylacetone	28.24	1877	Phenolic, Sweet, Phenol, Floral, Cinnamic	4	130	MS, O,RI

Table 12. Continuation. Aroma Impact Compounds present in *Vanilla pompona* extract.

No.	Compound	Detection time (min.)	RI(O)	Selected Descriptors	Frequency	Total intensity	ID Criteria
39	4-methylguaiacol	29.48	1981	Sweet, Warm, Phenol, Hazelnut, Nutty, Fried, Fatty, Oily	4	175	MS,O, RI
40	maltol	30.36	2052	Sweet, Caramel, Vanilla, Phenolic, Cookie, Brown, Baked, Pyrazine	4	215	MS,O, RI,I
41	4-vinyl-guaiacol	34.01	2295	Smoky, Spicy, Sweet	4	178	MS, O, RI,I
42	5-vinyl-guaiacol	34.13	2302	Smoky, Strong, Long, Familiar, Floral, Weak, Caramel	4	202	MS, O, RI
43	unknown	34.45	2318	Meaty, Sulfury, Fatty	4	191	O
44	unknown	39.45	2515	Smoky, Earthy, Weak, Powdery, Burnt, Woody	4	133	O
45	acetovanillone	45.93	2693	Smoky, Sweet, Powdery, Fruity, Familiar, Spicy, Floral, Woody	4	190	MS,O,RI

[1] Retention Index ($RI_{(I)}$) injected in previous study under the same analytical conditions.
[T]Tentative odor compound.

8.2. Discussion

The chemical structures of the 13 Primary aroma-impact compounds of *Vanilla pompona* are shown in Figure 28, along with their aroma characteristic, total intensity and detection frequency obtained from GC-O analysis. The chemical structures of 18 Secondary aroma-impact compounds of *Vanilla pompona* are shown in Figure 29.

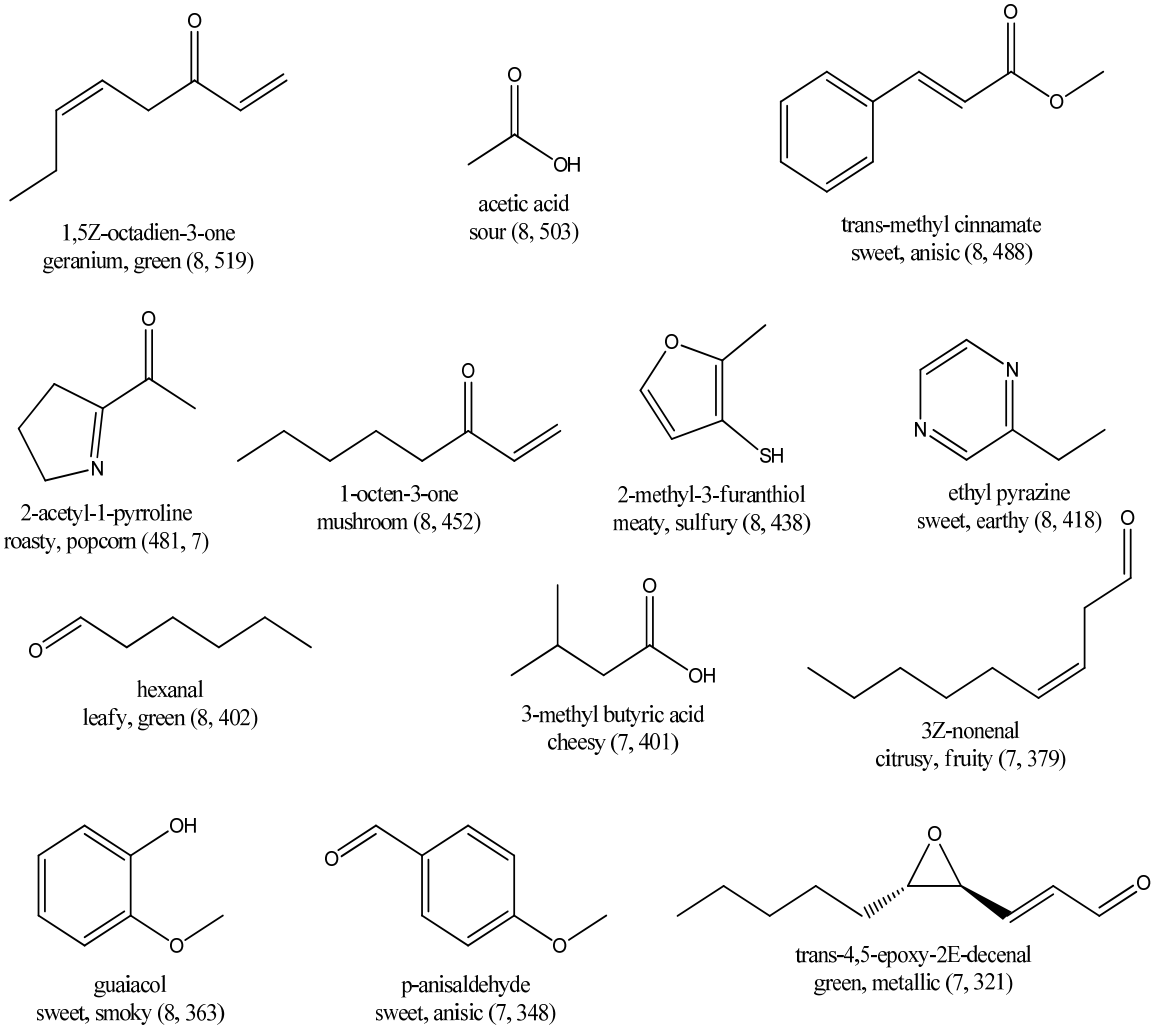


Figure 28. The chemical structures and aroma characteristics of the 13 Primary aroma-impact compounds *Vanilla pompona*, numbers (m, n) under the structures correspond to the detection frequency (m) and total intensity (n) from GC-O analysis as shown in Table 12.

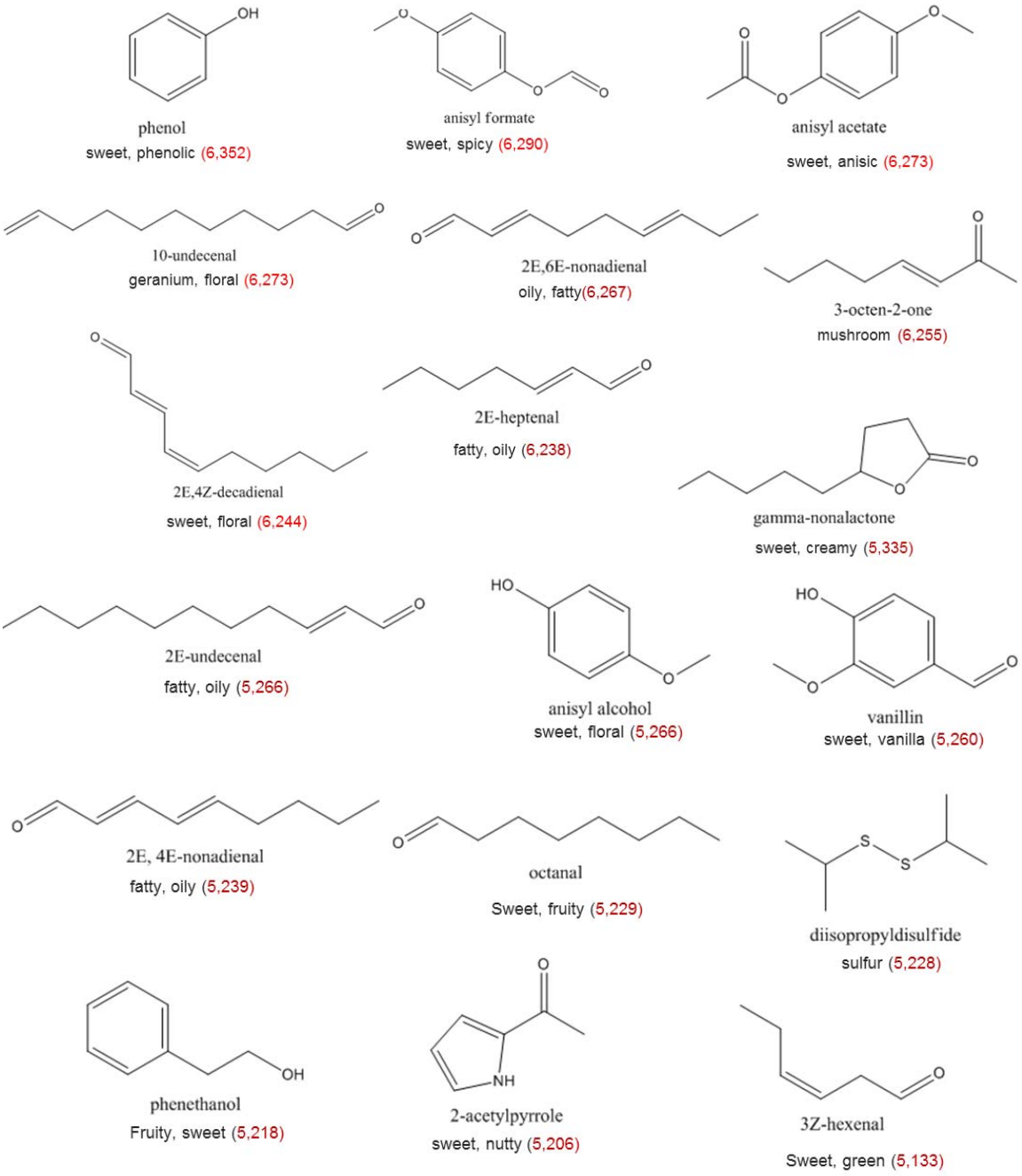


Figure 29. The chemical structures of 18 Secondary aroma-impact compounds of *Vanilla pompona.*

The different overall aroma between vanilla of different species are attributed to the various composition of aroma impact compounds. The aroma of *Vanilla pompona* appeared to involve a higher number of impacting odorants (45) compared with the

results of the GC-O analysis of the other two commercial species. Twenty six odorants has been reported in *Vanilla planifolia* extract (Perez Silva et al., 2006) and thirty eight odor active components from GC-O of *Vanilla tahitensis* extract (Brunschwig *et al*., 2012). Additional consituents that produce distinctive floral and spice-anise nuances were also found in *V. tahitensis* (Brunschwig et al., (2012). A significant overlap in aroma-impact compounds between *Vanilla pompona* and *Vanilla tahitensis* suggests that the two vanilla species might have very similar aroma characteristics.

The building block of the aroma of this vanilla based on the chemical classes of the aroma impact compounds is presented in Figure 30. Comparing this chemical distribution with the GC-MS results (Figure 16) with Figure 30, it is interesting that the aldehydic fraction of the odorants, which represents a small percentage in terms of abundance (5%) in the GC-MS data, contains the biggest number of odor impacting compounds (31%). In contrast, aliphatic alcohols that represent 21% of the total area abundance contributed in numbers only to 5% of the aroma of *Vanilla pompona* extract.

It is worthwhile to mention that the most abundant compounds according to the GC-MS analysis, vanillin and anisyl alcohol were not categorized as primary aroma-impact compounds in the GC-O analysis. This may be explained by the diluting effect caused by the elution in wide peaks that produced long lasting strong sweet notes difficult to pinpoint at given detection times.

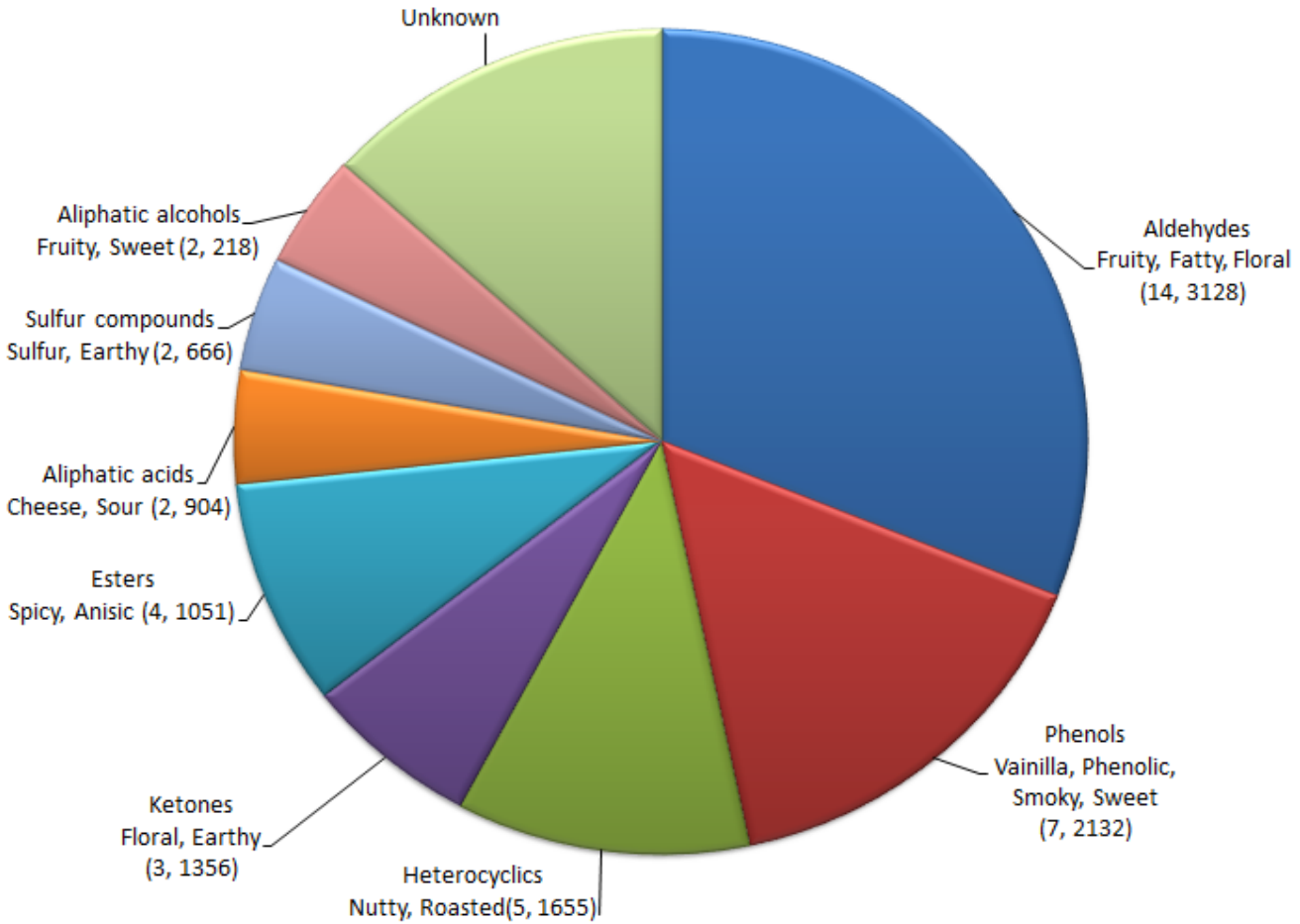


Figure 30. Diagram of the chemical distribution and selected odor qualities of aroma impact compounds present in *Vanilla pompona* Schiede extract (Number of compounds, Total Intensity).

Some compounds such as ethyl pyrazine and 3-methyl butyric acid that are present in the extract at very low concentrations (Table 8), are very important contributors to the overall aroma of *Vanilla pompona*. It is well known the relevance of trace compounds to the aroma of vanillas as it has been stressed by Adedeji (1993) and Perez Silva et al. (2006) regarding *V. planifolia*

8.3. Chemical distribution of the odor impact compounds in *Vanilla pompona*

The aroma of *Vanilla pompona* extract is primarily characterized by the volatile composition. Although the vast majority of these flavor volatiles arose during the fermentation, sunning, drying and conditioning stages of the curing process, it is relevant to note that volatile profiles vary to some degree depending on the sampling method. The odorants in this vanilla extract were organized by chemical group and each group showed distinctive aroma qualities and Intensities as described in below Table 13.

Table 13. Contribution of the aroma-impact compounds categories to the aroma of the *Vanilla pompona* extract.

Chemical group	General descriptor	No. Compounds	Total Intensity
Aldehydes	Fruity, Fatty, Floral	14	3128
Phenols	Vanilla, Phenolic, Smoky, Sweet	7	2132
Heterocyclics	Nutty, Roasted	5	1655
Ketones	Floral, Earthy	3	1356
Esters	Spicy, Anisic	4	1051
Aliphatic acids	Cheese, Sour	2	904
Sulfur compounds	Sulfur, Earthy	2	666
Aliphatic alcohol	Fruity, Sweet	2	218

8.3.1. Carbonyls, aldehydes (fatty, fruity and floral odors)

Odors described as "fatty" and "fruity" are high quality markers of vanilla aroma, and come from unsaturated and saturated aliphatic aldehydes. The most intense contributors to the "fatty" odors in the aroma of *Vanilla pompona* were 3Z-nonenal and trans-4, 5-epoxy-2E-decenal. It is well known that aldehydes such as hexanal, octanal and nonanal contribute significantly to the aroma of *V. planifolia* and *V. tahitensis*. These are also considered essential to the aroma of *Vanilla pompona*.

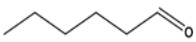
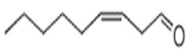
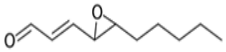
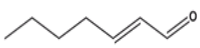
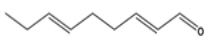
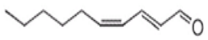
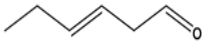
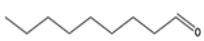
Compound	Structure	Selected Descriptor	F	I
Hexanal		Green, Fresh, Grassy, Ethereal	8	402
3Z-nonenal		Citrusy, Aldehyde, Sweet, Fruity, Green, Fatty, Mix of fruits, Oily, Waxy oily, Solventy	7	379
trans-4,5-epoxy-2E-decenal		Green, Sweet, Fruity, Sugary, Doughy, Burnt, Woody	7	321
2E-heptenal		Fatty, Oily, Nutty, Doughy, Earthy, Musty, Weak, Sweet, Woody, Vegetative	6	238
2E,6E-nonadienal		Musty,Smoky, Earthy,Oily, Fatty, Anisic, Weak	6	267
10-undecenal		Geranium,Metallic, Aldehyde, Vegetative, Fruity, Floral, Sweet	6	273
2E,4Z-decadienal		Sweet, Floral, Smoky, Floral, Honey, Bready	6	244
3Z-hexenal		Green, Woody, Grassy, Sandalwood, Sweet	5	133
Octanal		Fresh, Sweet, Cool,Fruity,Citrusy	5	229
2E, 4E-nonadienal		Fatty, Oily, Green, Bready, Vanilla	5	239
2E-undecenal		Metallic, Oily,Floral, Doughy, Burnt	5	266
Nonanal		Fruity, Vegetative, Aldehyde, Metallic, Weak, Anisic, sweet, Powdery	4	137

Figure 31. Carbonyl, aldehydes aroma-impact compounds in *Vanilla pompona* extract.

The compounds 10-undecenal, 2E-undecenal and 2E, 4Z-decadienal (Figure 31) are responsible for the heliotropin, floral or geranium like odors in the extract. The compound *p*-anisaldehyde, which is abundant in the extract, is also an important contributor to the floral fingerprint (Figure 8).

The total intensity of the aldehydic fraction of the aroma impact compounds is 3128. This Total intensity indicates that this odorant group, besides being the most numerous, is the strongest contributor to the overall aroma of *Vanilla pompona* extract.

The development of aldehydes responsible for these fruity and fatty odors is associated with the auto-oxidation of lipids using the substrates α-linolenic and linoleic acids which are abundant in the beans of Vanilla pompona (21.57% of area in Table 10). The medium-chain-length (C6-C12) volatiles that dominate come from unsaturation of long-chain fatty acids by α- and ß-oxidation catalyzed by the enzymes galactolipase, lypoxygenase and hydroperoxide lyase during the curing process (Dunphy & Bala, 2009, 2010).

8.3.2. Phenolics (vanilla, phenolic, smoky, sweet odors)

Phenolic compounds such as guaiacol, creosol and phenol were identified in this study as key components of *Vanilla pompona* aroma (Figure 32). These compounds nuanced the extract with strong smoky phenolic notes that have been also reported as characteristic of the other commercial vanilla species (Brunschwig *et al*., 2012; Perez Silva *et al*., 2006). Background compounds such as 4-vinyl-guaiacol, 5-vinyl-guaiacol and 4-methyl guaiacol are also contributors to the phenolic and smoky notes. The

compounds *p*-anisaldehyde and anisyl alcohol are strong contributors to the anisic, spicy and floral aroma of the extract.

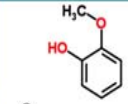
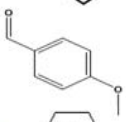
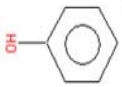
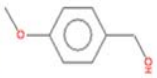
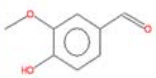
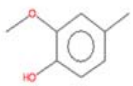
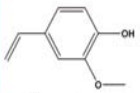
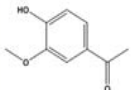
Compound	Structure	Selected Descriptor	F	I
Guaiacol		Phenolic, Sweet ,Smoky, Doughy, Vanillin, Cinnamic.	8	363
p-anisaldehyde		Eugenol, Grassy, Fresh, Cherry, Anisic, Vanilla, Very sweet, Heliotropin	7	348
Phenol		Smoky, Phenolic, Doughy, Burnt woody	6	352
Anisyl alcohol		Sweet, Floral, Cinnamic	5	266
Vanillin		Peppery,Smoky, Weak, Powdery, Sweet, Spicy, Woody,Vanilla	5	260
4-methylguaiacol = creosol		Sweet, Warm, Phenol, Hazelnut, Nutty,Fried, Fatty, Oily	4	175
4-vinyl guaiacol		Smoky, Spicy, Sweet	4	178
Acetovanillone		Smoky, Sweet, Powdery, Fruity, Familiar, Spicy, Floral, Woody	4	190

Figure 32. Phenolics identified as aroma-impact compounds in *Vanilla pompona* extract.

The potency of the aromas from the phenolic fraction of the odorants is expressed by a Total Intensity of 2132, which represents an important contribution to the overall aroma of the extract.

Phenolic compounds are known to be bound as glycosides in the pods, formed during the early sages of fermentation of vanilla beans, and accumulate over time. The most studied pathway in vanilla research has been the formation of vanillin and the

factors that affect its final yield. It is generally accepted that vanillin is formed by ß-glucosidase-catalyzed hydrolytic cleavage of the glucoside (Glucovanillin). In general, the production of the phenolic aromatic aglycones is associated with the hydrolysis of these glucosides by Shikimic acid pathway or from interconversions of Shikimate derivatives into others (Perez Silva *et al*., 2006; Dunphy & Bala, 2009).

8.3.3. Heterocyclics (nutty, roasted odors)

Compounds that produced popcorn, roast, and cheese notes such as 2-acetyl-1-pyrroline and 2-acetylpyrrole (Figure 33) were also high odor impacting in the aroma of this extract with a Total Intensity value of 1655.

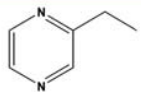
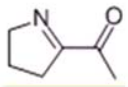
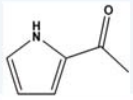
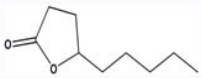
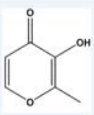
Compound	Structure	Descriptor	F	I
ethyl pyrazine		Sweet, Veggie, Phenolic, Fruity, Berry, Redfruit,Cherry, Solventy, Earthy	8	418
2-acetyl-1-pyrroline		Pop corn, Hazelnut, Pretzel, Cooked, Baked, Roast	7	481
2-acetylpyrrole		Sweet, Pyrazinic, Pop corn, Sweet, Rice, Nutty, Chocolate	5	206
gamma-nonalactone		Sweet, Cherry	5	335
3-hydroxy-2-methyl-4-pyrone= maltol		Sweet, Caramel, Vanilla, Phenolic, Cookie, Brown, Baked, Pyrazine, Strong	4	215

Figure 33. Heterocyclics identified as aroma-impact compounds in *Vanilla pompona*.

The compound Ethyl pyrazine, which structure can be seen in Figure 33 was not only reported in vanilla for the first time, but also is a high intensive Primary impact-aroma compound that contributes with phenolic, red fruits and earthy notes to the aroma of this extract. The formation of pyrazines is associated with nonenzymatic browning and the result of microbial action.

Heterocyclic compounds are mostly products of Maillard reactions and non-enzymatic browning occurred during the process of curing, particularly during the later part of the curing process when chemical reactions take place in the presence of reducing sugars and sources of nitrogen to favor the formation of volatile products via the Maillard reactions. A substantial amount of work has been done to determine the pathways for the formation of individual odor compounds involved in Maillard reactions (Reineccius, 2006; Damodaran *et al.*, 2008).

8.3.4. Carbonyl, ketones (floral, earthy odors)

Several types of compounds were identified as responsible of nuanced floral notes in the extract. However, the compound 1-5Z-octadien-3-one (Figure 34) was attributed with the highest intensity among all the aroma-impact compounds (519). This compound produced strong floral and geranium nuances. The compound trans-geranylacetone also contributed to the floral background.

Carbonyl, Ketones such as 1-octen-3-one and 3-octen-2-one (Figure 34) nuanced the aroma of *Vanilla pompona* extract with mushroom and earthy notes. These odors were also characteristic of *Vanilla tahitensis* according to the report by Brunschwig *et al.* (2012). A total intensiy of the odorants in this chemical fraction is 1356.

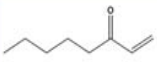
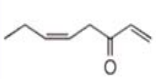
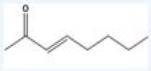
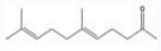
Compound	Structure	Selected Descriptor	F	I
1-octen-3-one		Mushroom, Earthy, Vegetable, Strong,Green, Herbaceous	8	452
1,5Z-octadien-3-one		Geranium, Pungent, Terpenic, Green, Fatty, Fruity, Familiar, Candy, Sweet, Powdery, Floral, Strong	8	519
3-octen-2-one		Cooked, Mushroom, Fruity, Green, Pyrazinic	6	255
trans-geranylacetone		Sweet, Floral, Cinnamic	4	130

Figure 34. Carbonyl, Ketones identified as aroma-impact compounds in *Vanilla pompona* extract.

8.3.5. Esters (spicy, anisic odors)

Strong spicy anise cinnamic odors come from the presence of abundant anisic compounds such as *p*-anisaldehyde and anisyl alcohol. However, Ketones such as trans-methyl cinnamate, anisyl formate and anisyl acetate are important odor contributors.

Anisic notes are not present in *Vanilla planifolia* flavor and these have been previously pointed out as a fingerprint of Tahitian vanilla flavor (Brunschwig *et al*, 2012). It is now confirmed that anisic compounds are also backbone of *Vanilla pompona* aroma. The chemical structures and mass spectra of anisic compounds can be seen in Figure 35 and their mass spectra were presented in Figure 17. The Total Intensity of this aroma impacting chemical fraction is 1051 which still represents a significant contribution.

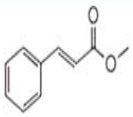
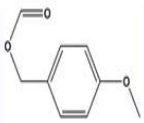
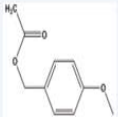
Compound	Structure	Selected Descriptor	F	I	ID
trans-methyl cinnamate		Sweet, Phenolic, Spicy, Benzaldehyde, Anisic, Powdery, Sweet, Cherry, Woody	8	488	RI,MS,O
anisyl formate		Floral,Sweet, Spicy, Powdery, Candy	6	290	RI,MS,O
anisyl acetate		Sweet, Earthy, Anisic, Candy, Cinnamic	6	273	RI,MS,O

Figure 35. Esters identified as aroma-impact compounds in *Vanilla pompona* extract.

The formation of aromatic esters has been associated with the oxidation of lipids in the presence of specific enzymes such as lypoxygenase; lipid oxidation contributes to flavor formation by the classical oxylipin pathway. Acids and ketones among other intermediates in the oxidation process are ready converted to alcohols, aldehydes, and esters by enzymatic process during the curing process.

8.3.6. Aliphatic acids (cheese, sour odors)

Also identified in this vanilla, short branched fatty acids that bring cheese notes such as 3-methylbutyric acid and 2-methyl butyric acid have been found to be contributors to the aroma of the other two commercial vanilla species (*V. planifolia* and *V. tahitensis*). The potency of these odors was expressed by a Total Intensity of 904. Similar to the other two commercial vanilla species, acetic acid was also identified as a potent odorant and one of the major contributors to the aroma of *Vanilla pompona.*

The formation of aliphatic acids in vanilla is associated with two alternative pathways. Branched chain acids can be biochemically generated from the corresponding C_{n+1} aminoacids. The route is via oxidative deamination of the corresponding amino acid. For instance, acetic acid, a major odor contributor, can arise either by hydrolysis of acetyl SCoA formed via glycolysis or as a product of ß-oxidation of even-numbered-long chain fatty acids (Dunphy & Bala, 2009).

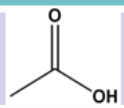
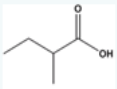
Compound	Structure	Descriptor	F	I
acetic acid		Sour, Sulfury, Acidic, Fatty	8	503
methyl butyric acid		Butyric (long), Rancid, Skunk, Sour, Cheesy, Acidic	7	401

Figure 36. Aliphatic acids that highly contribute to the aroma of *Vanilla pompona* extract.

8.3.7. Sulfur compounds (sulfur, earthy odors)

Two compounds with sulfur odors were identified as aroma impacting in this *V. pompona* extract and perceived with a total intensity of 666. 2-methyl-3-furanthiol is a primary aroma-impact compound (Figure 37) that has been previously identified as odor impacting in *Vanilla tahitensis* contributing to the "earthy" aroma. The compound diisopropyldisulfide is being reported for the first time in vanilla pods and it is a secondary contributor to the aroma. According to Sarter (2011), the presence of these compounds may be due to methional development on the surface of the beans induced by thermal degradation in presence of micro flora during early stages of curing process

which should be controlled (Brunschwig *et al*., 2012). The aminoacid methionine contains a sulfur atom that is involved in the formation of methional by the Strecker degradation reaction. The methional subsequently oxidizes to yield disulfides and thiols.

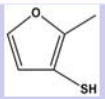
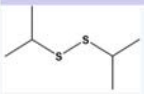
Compound	Structure	Descriptor	F	I
2-methyl-3-furanthiol		Alliaceous, Sulfury,Bready,Baked, Pyrazinic, Cracker like, Earthy, Savory, Meaty	8	438
diisopropyldisulfide		Tropical, Sulfury, Burnt, Fruity, sweet, weak	5	228

Figure 37. Sulfur aroma impact compounds identified in *Vanilla pompona* extract.

9. Conclusions

- Ethanol extraction method was selected to generate aroma extract for GC-MS and GC-O analysis, because it produced the most representative aroma extract of *Vanilla pompona*.
- One hundred and twenty three volatile and semi volatile compounds were identified in *Vanilla pompona* extract by GC-MS.
- Eighty volatiles were identified using the sampling method DTD-GC-MS. Additional phenol compound, β-diketones and two novel anisyl esters were observed only by DTD-GC-MS.
- Twenty six compounds were newly identified in vanilla in this study.
- Forty five aroma-impact compounds were identified in extract of *V. pompona* using a modified GC-NIF method. The aroma profile of the *Vanilla pompona*

extract consisted of thirteen primary aroma-impact compounds, eighteen identified as secondary aroma-impact compounds and thirteen odorants considered background.

- The chemical profile of the aroma impacting compounds in *Vanilla pompona* extract included 14 aldehydes, 7 phenolic compounds, 5 heterocyclic compounds, 3 esters, 4 ketones, 2 acids, 2 alcohol, 2 sulfur compounds and 6 unknown aroma impacting compounds.
- *Vanilla pompona* aroma is complex and rich with typical vanilla characteristics. It could be a valuable source for perfumery applications.

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11. Appendices

Appendix 1.

Table 14. Example of the bulk Olfactometry data compiled for the GC-O analysis of *Vanilla pompona* Schiede.

#	RT	Descriptor	Intensity								F	Total Intensity	RI(O)
			AJL	AJL	USH	USH	JMS	JMS	FRD	FRD			
1	11.28	Woody							13		1	13	1014
2	11.61	Spicy, Woody								14	1	14	1026
3	12.92	Cinnamic								29	1	29	1071
4	13.28	Musty			29						1	29	1084
5	13.53	Green, Fresh, Grassy, Ethereal	57	61	59	59	48	38	39	41	8	402	1093
6	13.75	Grassy			68						1		1101
7	14.00	Burnt bone	24	23							2	47	1110
8	15.14	Green, Woody, Grassy, Sandalwood,Sweet	25	28		51			8	21	5	133	1152
9	15.44	Weird, Sweet, Veggie,Plastic,Phenolic, Fruity, Berry, Redfruit,Cherry, Solenty, Earthy	28	33	79	66	60	39	62	51	8	418	1164
10	16.13	Overripe	28								1	28	1190
11	16.51	Vanilla, Sweet								43	1	43	1206
12	16.75	Pungent, Fruity, Sweet	32	26							2	58	1215
13	17.62	Tropical, Sulfury, Burnt, Fruity,sweet, weak	47	42		70	40	29			5	228	1251
14	17.71	Musty, earthy,Onion			42	70					2	112	1255

Appendix 2

Table 15. Example of the combined GC-Olfactometry data and reduced using NIF criteria

No.	Detection time (min)	Descriptor	Frequency	Total intensity	RI(cal.)
1	13.53	Green, Fresh, Grassy, Ethereal	8	402	1093
2	15.44	Weird, Sweet, Veggie,Plastic,Phenolic, Fruity, Berry, Redfruit,Cherry, Solenty, Earthy	8	418	1164
3	19.15	Mushroom, Earthy, Vegetable, Strong,Green, Herbaceous	8	452	1320
4	19.33	Alliacus, Sulfury,Bready,Baked, Pyrazinic, Cracker like, Earthy, Savory, Meaty	8	438	1328
5	20.67	Geranium, Pungent,Plastic, vial, Terpenic, Green,Fatty, Fruity,Familiar, Candy, Sweet, Powdery, Floral, Strong	8	519	1393
6	21.72	Sour,Sulfury,Unknown, Acidic,Fatty,Sweet,Brown	8	503	1446
7	28.48	Phenolic, Sweet ,Smoky, Doughy, Fried,Playdough, Vanillic,Mushroom, Earthy, Cinnamic	8	363	1897
8	31.60	Sweet, Phenolic, Spicy,Benzaldehyde,Anisic, Powdery, Cherry, Woody	8	488	2145
9	20.11	Pop corn, Hazelnut, Pretzel,Cooked, Baked,Roast	7	481	1365
10	21.97	Citrusy,Aldehyde,Sweet,Fruity, Green,Fatty, Mix of fruits, pyrazinic, Bread crust, Oily,Waxy oily, solventy	7	379	1460
11	25.57	Butyric (long), Butyric, rancid,Skunky,Sour, Cheesy, Acidic	7	401	1670
12	31.00	Green, Sweet, Fruity, Sugary, Sweet, Doughy,Burnt, Woody	7	321	2101

Appendix 3

Reproducibility of the olafactory data

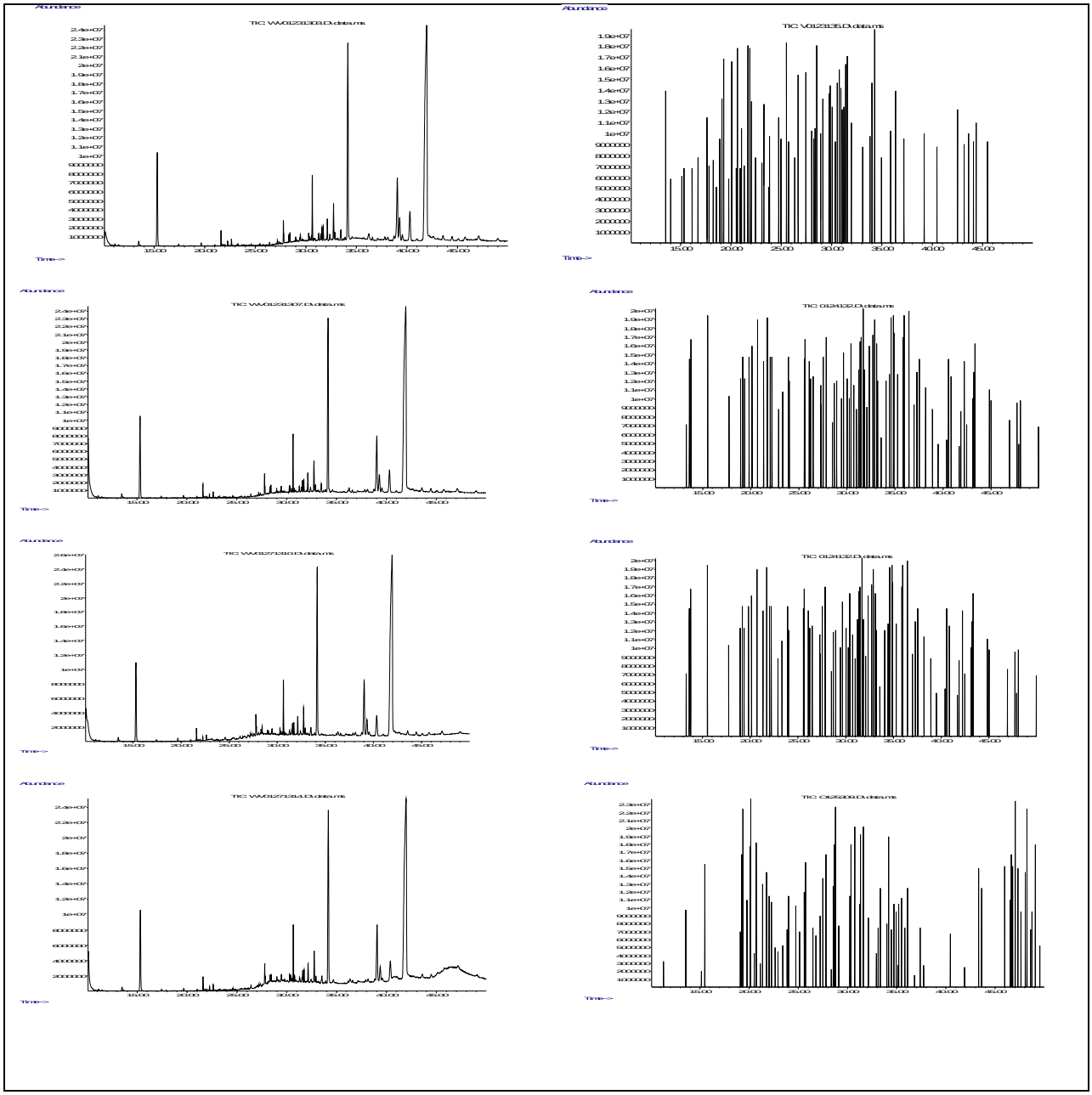

Figure 38. Selected TIC graphs (Left) and Aromagrams (Right) for GC-MS/O analysis of *Vanilla pompona* extract.

Repeatibility of detection times in olfactory data

The averaged standard deviation of the detection times was ±0.05 minutes. Polline *et al*. (1997) reported a difference in sensitivity of panelists during GC-Sniff sessions, the author suggested that a fraction of panelists can detect stimulus earlier than the others, calling them early "clickers" who perceive the odor at a retention time at a stage corresponding to the beginning of the MS peak. This difference in sensitivity among the panelists during the data acquisition is the reason why the standard deviation cannot be zero. No publications have been identified that measure of panel sensitivity using the parameter detection time in GC-O analysis

Reproducibility of intensity values

Another relevant parameter used in GC-O analysis to select the odor active compounds in a food sample is Intensity value. Large variations of intensity values produced by a single sniffer (up to 108%) and high variability within and between panelists have been reported. For instance, Guichard *et al.* (1995) published an average standard deviation of 30.9% using a panel of 10 sniffers. In this study, the average standard deviation of intensity values assigned by individual panelists to each odor impacting compounds in the duplicated sniffing sessions was 9.39%. In addition, the average of the standard deviation of all the intensity values assigned to each aroma compound was 14.2%. These two values indicate a higher individual reproducibility of intensity values individually and combined.

Appendix 4

N-Alkanes Calibration Curves to estimate the Retention Indices (RI_O) of odor compounds

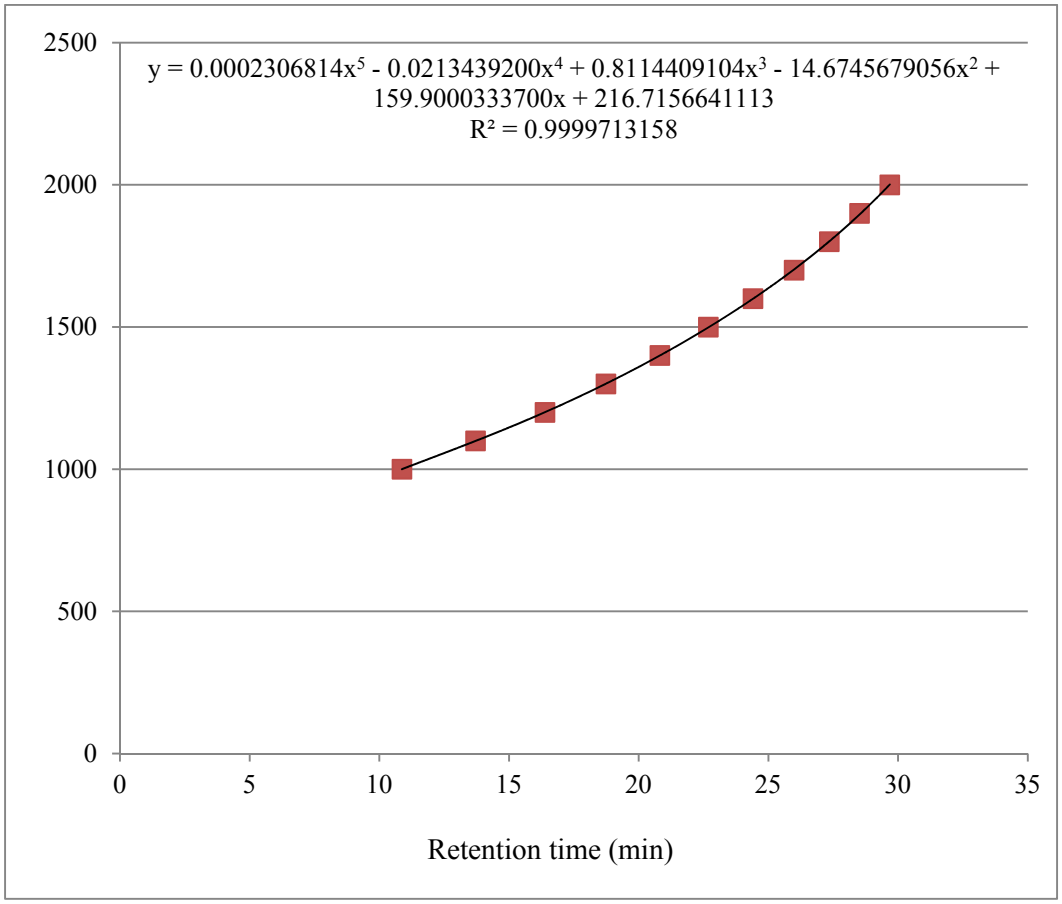

Figure 39. Retention Indices produced by means of injecting standard alkanes (C10 – C27) and linear equation to calculate olfactory retention indices from 10.0 to 25.9 min.

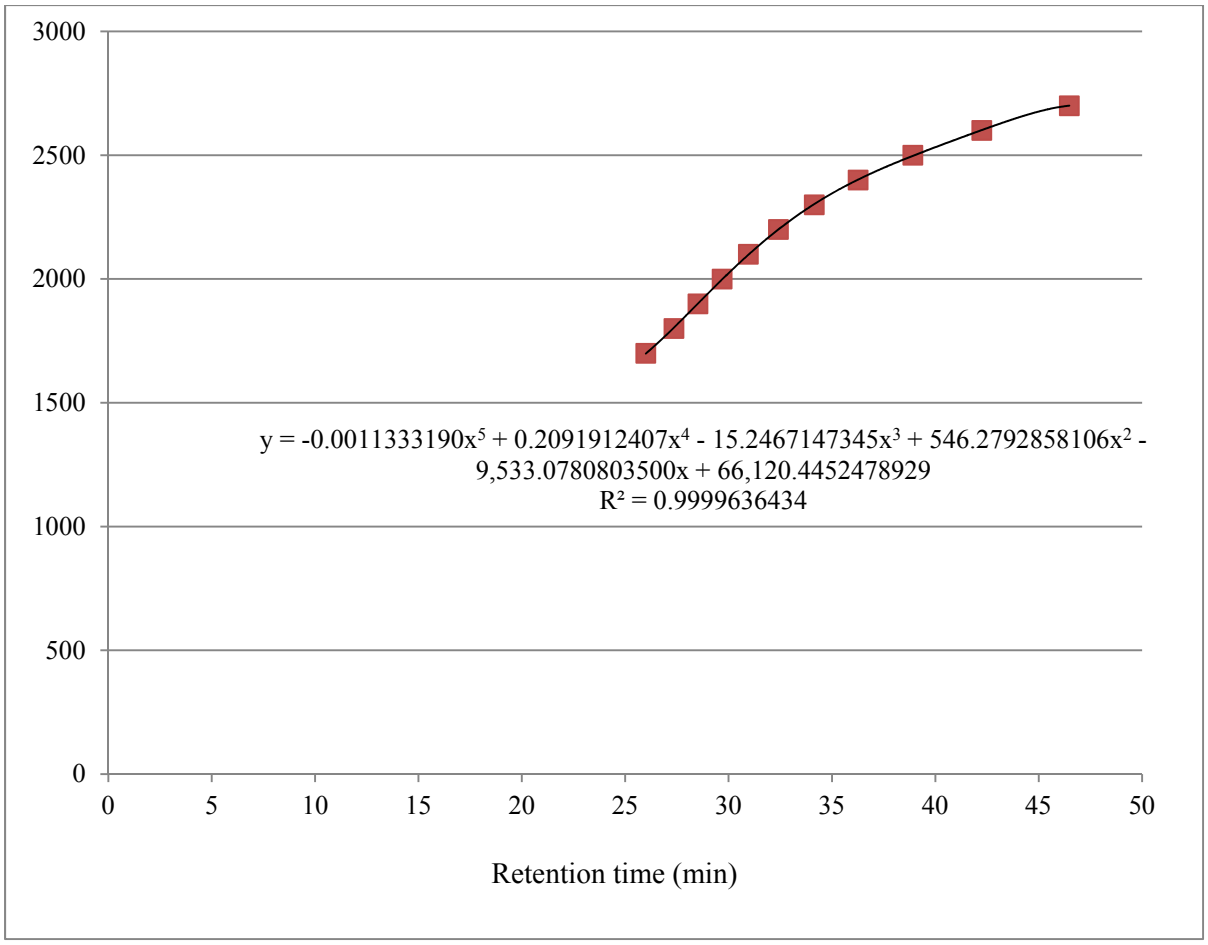

Figure 40. Retention Indices produced by means of injecting standard alkanes (C10 – C27) and linear equation to calculate olfactory retention indices from 25.9 to 50 min.

Appendix 5

Correlation of GC-O Parameters

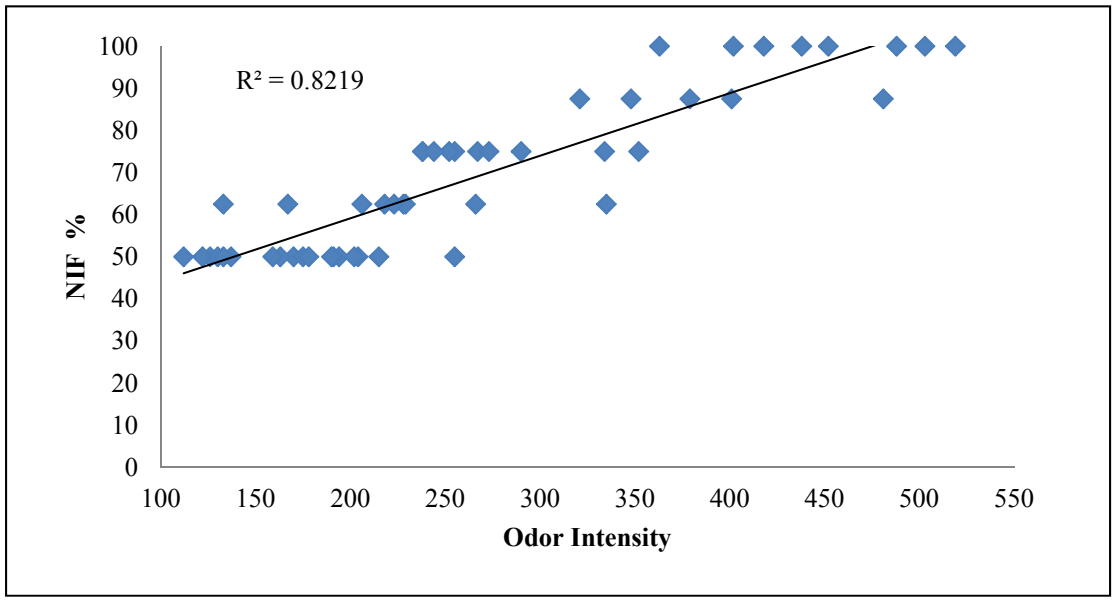

Figure 41. Comparison of NIF (Nasal Impact Frequency) in percentage and values of odor intensity responses (I) for the most important aroma impacting compounds found in *Vanilla pompona* extract.

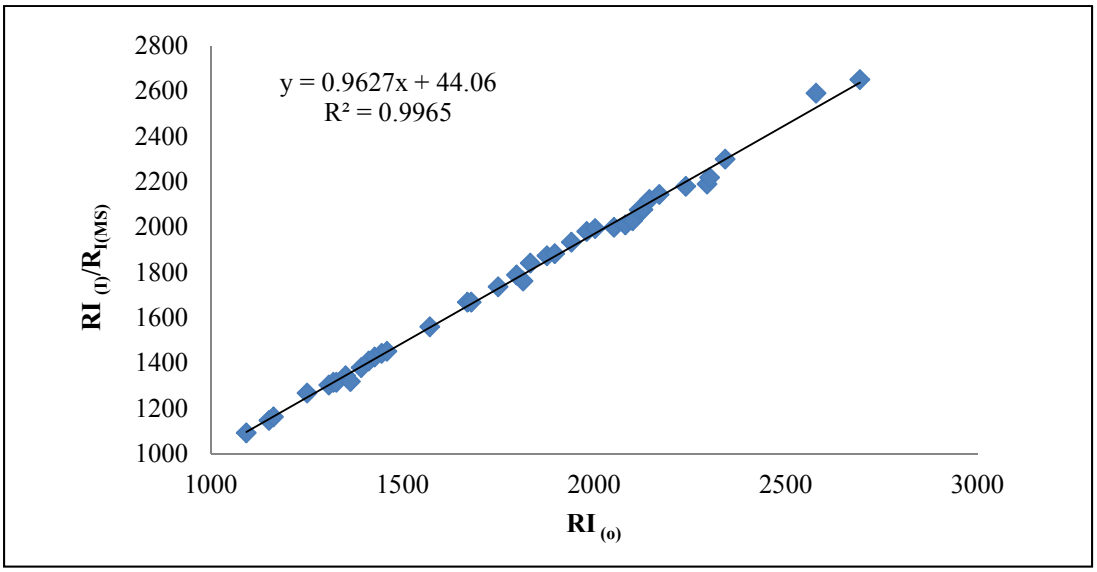

Figure 42. Correlation between Retention Indices of *Vanilla pompona* odorants from the GC/O and GC-MS analysis.

Appendix 6

Figure 43. Mexican Curing Process (Beneficiado) workflow. Diagram and pictures cortesy of Desarrollo Agroindustrial Gaya, S.A. de C.V (www.vanillamexico.com).