BIOACTIVITY STUDY AND ANALYSIS OF THE VOLATILE CONSTITUENTS AND ESSENTIAL OIL EXTRACTS OF *MAMMEA AMERICANA* L. FRUIT BY HS-SPME AND GC/MS

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I dedicate all this work with love to My Dear Mother!

Thanks for being always my support!

And to our creator God for being my light and guide on this journey!

ABSTRACT

Mammea Americana L. is a tropical fruit tree native from the West Indies with bark, seeds, leaves and flowers that have been reported to contain toxic compounds. However, it is the fruit pulp that results attractive for human consumption. The principal objective of this investigation was to apply the Headspace-Solid Phase Microextraction (HS-SPME) technique for the extraction of the aroma volatile constituents and to perform Microscale Soxhlet Extraction to obtain the essential oil of *Mammea Americana* L. fruit pulp from different municipalities in Puerto Rico. The aim was to determine the volatile compounds that give the fruit its characteristic aroma and to identify the mixture of compounds contained by the essential oil extracts from the fruit pulp. Gas Chromatography coupled to Mass Spectrometry (GC/MS) instrumental method was used for the separation and characterization of Mamey fruit chemical composition. Three different SPME fibers including the Polydimethylsiloxane (PDMS), Polydimethylsiloxane/Divinylbenzene (PDMS/DVB), and Divinylbenzene/Carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS) were subjected to a validation procedure of the HS-SPME technique to select the optimum equilibrium, extraction and desorption times, and to conclude which fiber coating material showed the highest sensitivity toward the extraction of the volatile composition of *Mammea Americana* L. fruit. In the HS-SPME/GC/MS analysis using the DVB/CAR/PDMS fiber, results showed that the most aromatic fruit was the one from Mayagüez municipality. The principal constituents found in the aroma composition were the woody β-ionone (24.34%) and the fruity hexanal (22.91%). Three different essential oil extracts of the fruit pulp were obtained during the Micro-Soxhlet Extraction procedure using polar to non-polar organic solvents. The Dichloromethane (DCM) extract contained the highest amount of reported bioactive compounds including 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-coumarin (16.16%) and Mammea A/AB (12.31%). Brine shrimp lethality bioassay results showed that the mixture of chemical compounds contained in the Mamey fruit pulp DCM extract was highly toxic or bioactive against brine shrimp (*Artemia salina* L.) larvae with a LC_{50} of 8.16 μg/mL.

RESUMEN

Mammea Americana L. es un árbol de fruto tropical nativo de las Indias Occidentales con corteza, semillas, hojas y flores que han sido reportadas por contener compuestos tóxicos. Sin embargo, es la pulpa de la fruta lo que resulta atractivo para el consumo humano. El objetivo principal de esta investigación fue aplicar la técnica de Microextracción en Fase Sólida-Espacio de Cabeza (HS-SPME) para la extracción de los constituyentes volátiles del aroma y realizar la Extracción de Soxhlet a Microescala para obtener el aceite esencial de la pulpa de la fruta de *Mammea Americana* L. de diferentes municipios de Puerto Rico. El propósito era determinar los compuestos volátiles que dan al fruto su aroma característico e identificar la mezcla de compuestos contenidos en los extractos de aceite esencial de la pulpa de la fruta. El método instrumental de Cromatografía de Gas acoplada a Espectrometría de Masas (GC/MS) fue utilizado para la separación y caracterización de la composición química de la fruta de Mamey. Tres diferentes fibras de SPME incluyendo la Polidimetilsiloxano (PDMS), Polidimetil-siloxano/Divinilbenceno (PDMS/DVB) y Divinilbenceno/Carboxeno/Polidimetilsiloxano (DVB/CAR/PDMS) fueron sometidas a un procedimiento de validación de la técnica de HS-SPME para seleccionar los tiempos óptimos de equilibrio, extracción, y desorción, así como para concluir cuál material de revestimiento de fibra demostraba la mayor sensitividad hacia la extracción de la composición volátil de la fruta de *Mammea Americana* L. En el análisis por HS-SPME/GC/MS utilizando la fibra DVB/CAR/PDMS, los resultados demostraron que la fruta más aromática era la del municipio de Mayagüez. Los constituyentes principales encontrados en la composición del aroma fueron la leñosa β-ionona (24.34%) y el frutoso hexanal (22.91%). Tres diferentes extractos de aceite esencial de la pulpa de la fruta se obtuvieron durante el procedimiento de Extracción Soxhlet a Microescala utilizando solventes orgánicos polares y no-polares. El extracto de Diclorometano (DCM) contenía la mayor cantidad de compuestos bioactivos reportados incluyendo 5,7-dihidroxi-6-isovaleril-8-(3-metil-2-butenil)-4-fenil-cumarina (16.16%) y Mammea A/AB (12.31%). Los resultados del bioensayo de letalidad con el camarón de salmuera mostraron que la mezcla de compuestos químicos contenidos en los extractos de DCM de la pulpa de la fruta de Mamey era altamente tóxica o bioactiva contra las larvas de camarón de salmuera (*Artemia salina* L.) con un LC_{50} de 8.16 μg/mL.

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LIST OF ABBREVIATIONS

amu	atomic mass units
A. salina	*Artemia salina*
BSLT	Brine Shrimp Lethality Test
$CHCl_3$	Chloroform
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DVB/CAR/PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane
EI	Electron Impact
EM	Electron Multiplier
GC/MS	Gas Chromatography coupled to Mass Spectrometry
HS-SPME	Head Space-Solid Phase Microextraction
$K_2Cr_2O_7$	Potassium Dichromate
LC_{50}	Lethal Concentration at 50%
MeOH	Methanol
Micro-Soxhlet	Microscale Soxhlet Extraction
%RSD	Percent Relative Standard Deviation
PDMS	Polydimethylsiloxane
PDMS/DVB	Polydimethylsiloxane/Divinylbenzene
rpm	revolutions per minute
Rt	Retention time
Std. Dev.	Standard Deviation
Std. Error	Standard Error
TIC	Total Ion Chromatogram

CHAPTER 1

INTRODUCTION

Mother Nature is a great source of natural chemical compounds which include materials derived from plants, microorganisms, invertebrates, and vertebrates (Bhat et al., 2005). Alkaloids, steroids, terpenoids, amino acids, carbohydrates, vitamins, natural dyes, among others, fall in this classification. The chemistry of natural products originated from the curiosity of human beings regarding their colors, taste, odors and their potential uses as an alternative to find cures for diseases (Bhat et al., 2005). It is known that many natural products extracted from plants have been used as pigments, dyes, insecticides, pheromones, and also for hunting and murder during centuries. Different civilizations around the world have used natural products in folkloric medicine playing a key role in reducing human suffering and helping them to heal (Bhat et al., 2005). New drugs derived from plant natural products have been discovered although the bioactivity of many others remains under investigation.

This work is focused in the chemical analysis of *Mammea Americana* L. fruit, commonly known as "mamey" or mammee-apple, which is a fruit tree from the family Guttiferae, native from the West Indies and northern South America. Mamey fruits are consumed raw or as marmalade, and according to literature, they are rich in proteins, carbohydrates, calcium, phosphorus, iron, and some vitamins including thiamine, riboflavin, niacin, ascorbic acid, and carotene (Nuñez-Meléndez, 1982). Mamey fruit pulp is described as light or golden yellow to orange, non fibrous, varying from firm to crisp, sometimes dry to tender and juicy. In Puerto Rico, the productivity of an individual tree is variable, yielding 150 to 200 fruits per crop (Morton, 1987).

Volatile compounds released from food matrices are monitored to determine the composition, quality and safety of the product and in some cases with the aim to synthesize its aroma for artificial production in the flavor and fragrances industry (Marsili, 1997). Aroma volatile compounds are commonly found in low concentrations in food matrices containing both organic and inorganic components. The aroma perceived by human olfaction and its quality are factors that make a fruit attractive for consumption (Marsili, 1997). Experimental research has found that the volatile components of the Mamey fruit are mainly composed of β-ionone and

other flowery or fruity odor compounds (Nellis, 1997). According to the literature, β-ionone, a precursor for carotenoids, exerts anticarcinogenic and antitumor activities in melanoma, meningioma and breast cancer, by inducing apoptosis in cancer and tumor cells (Janakiram et al., 2008).

On the other hand, essential oils have been used since ancient times as remedies for the treatment of diseases because of their healing properties. Morris et al. reported in 1952 that a concentrated extract of the fresh Mamey fruit proved fatally toxic to guinea pigs, and was also found poisonous to dogs and cats after ingestion (Morton, 1987). It is of particular interest that there are reports, from 1951, of the antibiotic activity of Mamey from an Agricultural Experimental Station in Río Piedras, Puerto Rico (Morton, 1987). Other texts report that Mamey has been used to treat skin infections and digestive problems, although, there are reports indicating that all parts of its fruit have properties that can be harmful to human health if they are ingested in large quantities or on a regular diet basis (Francis, 1989).

The analysis of volatiles compounds and essential oils from a fruit pulp is generally accomplished by an extraction step, followed by concentration, chromatographic separation, and finally the detection. Commonly used techniques for the extraction of volatile compounds involve solvent extraction, hydrodistillation or steam distillation and supercritical fluid extraction. The main disadvantage with extraction techniques such as hydrodistillation is that thermolabile compounds can be subjected to thermal transformation or decomposition, for example, hydrolysis of esters or polymerization of aldehydes, producing chromatographic profiles that are not representative of the sample (Sukkaew et al., 2014). For this reason, it is preferred to use techniques consisting in headspace analysis, or the direct analysis of the volatiles in the gas phase above the sample, in order to characterize the aroma volatile composition of a fruit (Marsili, 1997). Common headspace sampling techniques include: static headspace, dynamic headspace, and purge and trap. Static headspace and purge and trap are mostly used for the analysis of aqueous samples, while dynamic headspace is used for the analysis of the volatile constituents of solid samples matrices (Marsili, 1997). Solid Phase Microextraction (SPME) is an extraction technique for volatile and semivolatile organic compounds in which analytes are adsorbed or absorbed directly from the sample onto a fused silica fiber that is coated with an appropriate stationary phase. Compared to other extraction techniques, SPME has the advantages of pre-concentration, extraction and sample introduction procedures in one single step and can be

applied in headspace mode (Pawliszyn, 1997). SPME helps to have fewer variables during an extraction procedure due to less sample manipulation which results in representative chromatographic profiles (Sukkaew et al., 2014) and makes it the ideal extraction technique for the analysis of the volatile constituents of a food matrix.

Classical techniques used to extract essential oils from plant material include Soxhlet, hydrodistillation, and maceration with an alcohol-water mixture or hot fat (Wang, 2006). Novel extraction methods include ultrasound-assisted, microwave-assisted, supercritical fluid, or accelerated solvent extractions. Soxhlet extraction (solvent extraction) has been used for a long time and consists in the continuous extraction of a solid material by a solvent by placing a porous cellulose thimble, in where condensing solvent continuously percolate through it, and return to a round bottom or extraction flask. In other words, in this method the sample is always in contact with fresh solvent during the entire process. Soxhlet extraction can be applied using different types of organic solvents and do not require filtration after leaching. Since Soxhlet extraction is a very simple, cheap, and well established technique, have shown good reproducibility, and requires less extract manipulation compared to novel extraction methods, it has been considered as the preferred method for the extraction of essential oils from plant material (Wang, 2006).

Gas Chromatography (GC) is a modern separation technique which involves the analysis of volatile organic compounds at operating temperatures between 40 and 300°C (Bhat et al., 2005). Volatile compounds are generally excellent candidates for analysis by GC since they usually leave the sample matrix and escape to the air where their odor is perceived (Marsili, 1997). For the analysis of highly volatile essential oils, the instrument of choice also has been the GC. Specifically, Gas Chromatography coupled to Mass Spectrometry offers an excellent, fast and successful instrumental method for the analysis and characterization of the volatile chemical compounds and for the highly volatile essential oil extracts, since the Mass Selective Detector has a spectrum library database that allows the identification based on the mass to charge (m/z) ratio and the defragmentation pattern spectra (Yang, 1994).

Bioactive natural products are those chemical compounds produced by living organisms that exert a biological effect on other organisms (Colegate et al., 2008). The brine shrimp lethality test (BSLT) is a convenient general bioassay for active plant constituents used to determine the lethal concentration required to kill 50% of the population or LC_{50} values of natural product extracts. The eggs of brine shrimp *Artemia salina* that are used, hatch within 24

to 48 hours after being placed in a brine solution, providing a large number of larvae or nauplii (Meyer et al., 1982). It has been proven that BSLT is rapid, simple, reliable, inexpensive, and convenient as it uses small amounts of test material. This method provides a frontline screening, predictive of cytotoxicity and pesticidal activity, since a broad range of known active compounds is manifested as toxicity to the shrimp (Meyer et al., 1982).

In Puerto Rico and the Caribbean islands much of the knowledge on medicinal plants comes from folklore rather than scientific research and validated data. To our knowledge, in Puerto Rico there is no actual data that proves the medicinal properties that have been attributed to *Mammea Americana* L. fruit, neither knowledge about the aroma constituents nor the essential oil chemical composition of the fresh fruit pulp. For this reason, in this investigation it was of particular interest to analyze and characterize the chemical composition of the volatile constituents and the essential oil extracts of Mamey fruit pulp. *Mammea Americana* L. fruit samples were collected from trees located at 4 different municipalities in Puerto Rico. Headspace-Solid Phase Microextraction (HS-SPME) technique was applied for the extraction of the volatile constituents of *Mammea Americana* L., while Soxhlet Extraction technique was applied for the extraction of the essential oil from the fruit pulp. The chemical characterization and identification of the volatile constituents of the aroma and the essential oil extracts of the fruit was performed by Gas Chromatography coupled to Mass Spectrometry (GC/MS). Finally a brine shrimp lethality bioassay was carried out in order to determine if the mixture of the essential oil extracts had the potential to show toxicity to *A. salina* larvae.

CHAPTER 2

LITERATURE REVIEW

For several years, materials representing dried roots, stems, leaves, flowers, and seed collected from different species in Puerto Rico have been studied to determine their toxicity to insects (Sievers et al., 1949). Three types of tests were applied including: the turntable method using houseflies (*Musca domestica* L.); the dusted leaf section method using leaf-eating larvae; and a method using mosquito larvae (Sievers et al., 1949). In the research, all of the parts of Spurge nettle (*Cnidoscolus urens*), Nicaraguan cocoashade (*Gliricidia sepium*), Sandbox tree (*Hura crepitans*), Mamey (*Mammea Americana*), and Jamaica dogwood (*Piscidia piscipula*) tested showed appreciable insecticidal action. *Mammea americana* ranked highest as an insecticide, as all the parts tested showed considerable toxicity (Sievers et al., 1949). In Puerto Rico, no efforts have being done to actually know the chemical constituents that are responsible of the toxic properties showed by native plants.

The first study on the major volatile constituents from the fruit of *Mammea Americana* L. was done by Sagregro-Nieves et al. on 1989, where Gas Chromatography-Mass Spectrometry method was applied, identifying β-ionone (22%), 2-methylbutyric acid (12.5%), *E*-farnesol (4.6%), and nerilidol (3.8%) as the major constituents of the volatile fraction of the fresh fruit of *Mammea Americana* L. In this case, for the isolation of the volatile compounds, fresh fruit samples of *Mammea Americana* L. were stirred in distilled water, subjected to steam distillation, cooled in an ice bath, then subjected to liquid-liquid extraction with organic solvents and concentrated in a nitrogen steam prior to GC/MS analysis (Sagrero-Nieves et al., 1989).

On 1993, a research study was conducted by Morales and co-workers, where the volatile compounds of *Mammea Americana* L. were isolated by simultaneous distillation-extraction with organic solvent. The extract was fractionated by column chromatography on silica gel by a discontinuous pentane-diethyl ether gradient obtaining three fractions which were analyzed by High Resolution Gas Chromatography (HRGC) and High Resolution Gas Chromatography-Mass Spectrometry (HRGC-MS). Furfural (7281 µg/kg) and *E*-farnesol (2145 µg/kg) were found to be major components from 22 compounds identified (Morales et al., 1993).

The insecticidal effectiveness of *Mammea Americana* L. extracts was reexamined by Gallo et al. in 1996 to represent renewable sources of bioinsecticides. The results added western corn rootworm (*Diabrotica virgifera virgifera*) and cabbage looper (*Trichoplusia ni*) to the list of

insects which are susceptible to the insecticidal ingredients of *Mammea Americana* L. This confirmed previous reports of activity against German cockroach (*Blatella germanica*), American cockroach (*Periplaneta americana*), and diamondback moth (*Plutella xylostella*). It was also reported the LD_{50} for crude hexane extracts of *Mammea Americana* L. leaves and seeds against *Trichoplusia ni* (Gallo et al., 1996).

A study on plants from Puerto Rico with *Anti-Mycobacterium* tuberculosis properties was performed by Frame et al. on 1998. The purpose of the study was to assess the antitubercular potential of natural products obtained from plants reputed to have medicinal properties, which are part of the tropical flora of Puerto Rico. *Mammea Americana* L. showed the greatest inhibitory activity, even at 50 µg, suggesting that certain plant species yield valuable anti-Mycobacterium tuberculosis (MTB) substances (Frame et al., 1998). The bactericidal inhibitory pattern of MTB growth, exposed to *Mammea Americana* L. extract was comparable to streptomycin (Frame et al., 1998).

On the other hand, β-ionone, an end-ring analogue of β-carotenoid, which has been found in research studies as a major constituent of the volatile composition of the fruit of *Mammea Americana* L., has been analyzed for colon cancer chemoprevention and treatment. β-ionone induced cell growth inhibition and apoptosis in human colon cancer HCT116 cell line (Janakiram et al., 2008). HCT116 cells treated with subtoxic concentrations of β-ionone experienced dose-dependent cell growth suppression with G_1-S-phase growth arrest and significant induction of apoptosis. Administration of dietary 0.1% and 0.2% β-ionone to rats significantly suppressed total colonic aberrant crypt foci (ACF) formation up to 34% and 38% (Janakiram et al., 2008).

In more recent studies, the chemical composition, toxicity and insecticidal activity of essential oils of different plant species has been investigated. In a study conducted by Ebadollahi et al. in 2010, about the chemical composition of the essential oil from leaves and flowers of Spanish lavender (*Lavandula stoechas*), the Gas Chromatography-Mass Spectrometry (GC-MS) instrumental method was applied. Fumigant toxicity tests of the essential oil were carried out against adults of red flour beetle (*Tribolium castaneum*), cigarette beetle (*Lasioderma serricorne* F.) and lesser grain borer (*Rhyzopertha dominica* F.). It was found that mortality increased as the doses of essential oils and exposure period increased (Ebadollahi et al., 2010).

On another study, Fasola et al. obtained the essential oil from the stem bark of Nigerian species of guava (*Psidium guajava*) by hydro-distillation using an all-glass Clavenger apparatus (2011). Gas Chromatography-Mass Spectrometry (GC-MS) was carried out on the essential oil finding hydrocarbons, amines and esters as the major constituents. Brine shrimp lethality tests were carried out to determine the toxicity of the oils to living organisms by determining the LC_{50} value. An LC_{50} value of 1.0009 µg/mL obtained showed that the essential oil of *Psidium guajava* stem bark was toxic (Fasola et al., 2011). From these results, the authors stated that the toxicity of the *Psidium guajava* oil can be an advantage in the therapy of diseases involving cell or tumor growth.

In a research done by Sukkaew et al. on 2014, the volatile components of curry tree (*Murraya koenigii*) fresh leaves were studied by using HeadSpace (HS) Solid-Phase Microextraction (SPME) coupled with Gas Chromatography-Mass Spectrometry (GC-MS). In this case, four fibers were employed to extract the volatile compounds including Polydimethylsiloxane (PDMS), Polydimethylsiloxane-Divinylbenzene (PDMS-DVB), Carboxen-Polydimethylsiloxane (CAR-PDMS) and Divinylbenzene-Carboxen-Polydimethylsiloxane (DVB-CAR-PDMS) (Sukkaew et al., 2014). The volatile compounds in the fresh leaves of *Murraya koenigii* were also extracted by hydrodistillation and analyzed by GC-MS for comparison with the SPME/GC/MS method. The DVB-CAR-PDMS fiber was considered the best for trapping key volatile compounds of *Murraya koenigii* fresh leaves.

Studies have been carried out to investigate the toxic or insecticidal properties of several parts of *Mammea Americana* L., however little has been done to actually know which are the specific chemical constituents responsible of those properties. One of the major focuses of this research is to use Headspace-Solid Phase Microextraction (HS-SPME), with different types of SPME fibers, in order to extract the volatile compounds, and use Soxhlet Extraction to obtain the essential oil extracts of Mamey fruit, to finally analyze them by Gas Chromatography coupled to Mass Spectrometry (GC/MS). Knowing that previous studies report that *Mammea Americana* L. contain compounds with the potential to show toxicity or insecticidal activity, it is imperative to characterize the mixture of compounds that is present in both the volatile constituents and essential oil extracts of its fruit pulp.

CHAPTER 3

THEORY OVERVIEW

3.1 *Mammea Americana* L. Taxonomic Classification and Description

Taxonomic Hierarchy

Kingdom: Plantae (Plants)

Subkingdom: Tracheobionta (Vascular plants)

Superdivision: Spermatophyta (Seed plants)

Division: Magnoliophyta (Flowering plants)

Class: Magnoliopsida (Dicotyledons)

Subclass: Dilleniidae

Order: Theales

Family: Clusiaceae/Guttiferae (Mangosteen family)

Genus: *Mammea* L. (mammea)

Species: *Mammea Americana* L. (mammee apple, mamey) [Plants USDA, 2017]

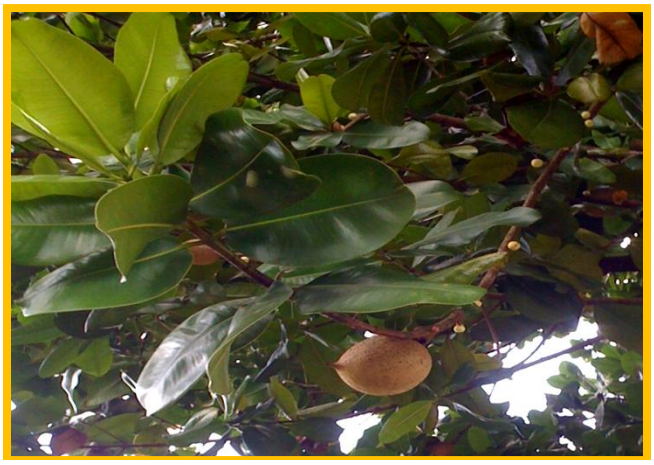


Figure 1. Fruit and leaves of *Mammea Americana* L. (taken at Mayagüez, Puerto Rico on May 31, 2014)

Mammea Americana L. is a tropical fruit tree, from the family Clusiaceae or Guttiferae and native from the West Indies (Nellis, 1997). It grows up to 25 m (85 ft) tall with a 60 cm (2 ft) diameter trunk and a dense round crown. Their leaves grow up to 20 cm (8 in) long having many parallel veins radiating from the central sunken midrib with a glossy dark green color, while their fragrant white flowers grow up to 5 cm (2 in) on twigs or branches behind the terminal leaf clusters normally having 6 petals (Nellis, 1997). Mamey fruits are 8 to 25 cm (3 to 10 in) in diameter and have a thick, rough, brown, bitter rind over a firm, yellow, juicy, apricot-flavored flesh. It is more or less free from the seed though bits of the seed covering usually adhere to the immediately surrounding wall of flesh. The ripe flesh is appetizingly fragrant and pleasantly subacid, but poor quality fruits may be too sour or slightly nauseating sweet (Morton, 1987). Mamey seeds are reddish-brown, rough, with an ellipsoid shape of about 6 cm (2 in) long. Small fruits usually have a single seed, while larger fruits may have 2 to 4 seeds. The tree thrives best on sites with rich, deep, moist soil being quite hardy since it survives at a smaller size on many soil types and in areas of moderate rainfall (Nellis, 1997).

3.2 *Mammea Americana* L. Origins, Distribution, and Climate

Mammea Americana L. grows very well in the Greater and Lesser Antilles, and also in the Bahamas, where it grows spontaneously along the roadsides. **Figure 2** shows the USDA nativity map of *Mammea Americana* L. There are records from 1514 of Mamey tree growing in Panama, while in 1529 it was included by Oviedo in his review of the fruits of the New World, and since 1735, it has been nurtured as a specimen in English greenhouses (Morton, 1987). In Mexico and Central America, it is sparingly grown, and in Colombia, Venezuela, Guyana, Ecuador and Northern Brazil its cultivation is scattered. In the United States, it was first introduced in Florida from the Bahamas. The tree was introduced into the tropics of the Old World being of very limited occurrence in West Africa, southeastern Asia, Philippines, and Hawaii.

Mammee apple is limited to tropical or near tropical climates and favors deep, rich, well drained soil, but it is adaptable to shallow and sandy terrain (Morton, 1987). Mamey seeds are the way of dissemination and usually germinate in 2 months or less. A slightly yellowing of the skin of the fruit may indicate ripeness, if it is green beneath it should not be picked. In Puerto

Rico, a high yielding Mamey tree can produce 1 to 2 crops per year, giving 150 to 200 fruits per crop, for an approximate total of 300 to 400 fruits annually (Morton, 1987). In some regions like in Barbados, the fruits begin to ripen in April, while in the Bahamas the season extends from May through July.

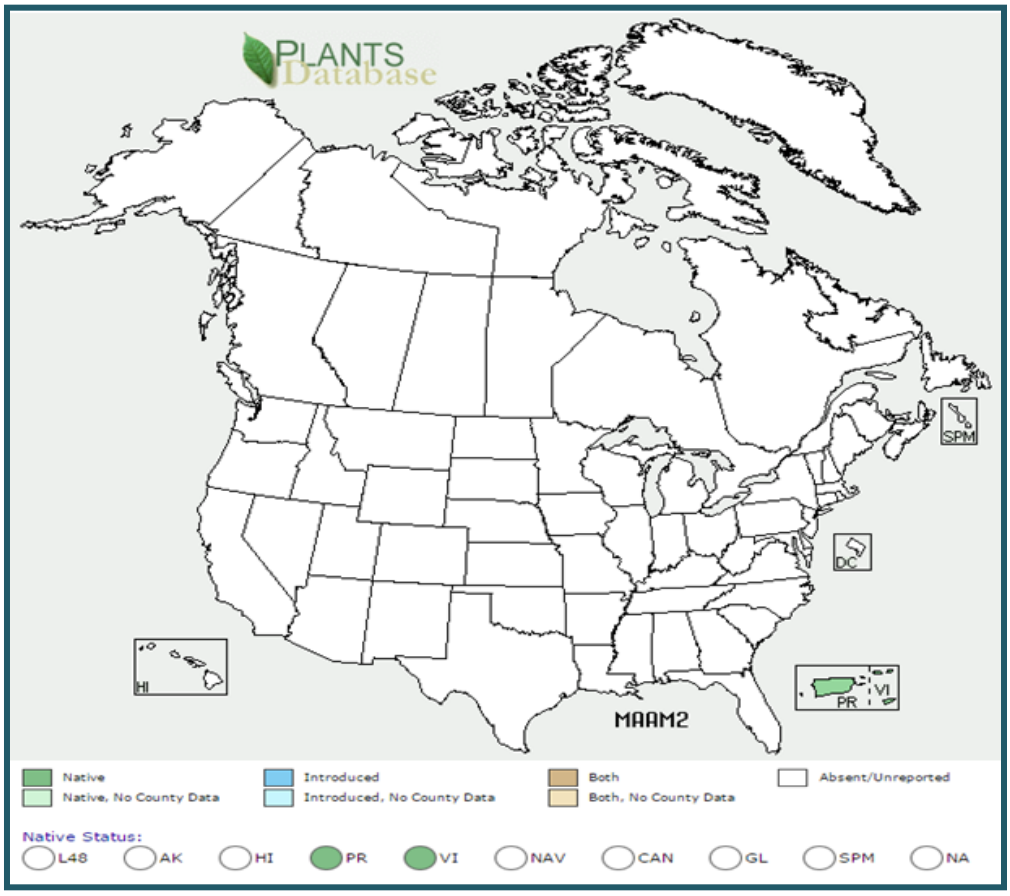


Figure 2. USDA Nativity Status Map of *Mammea Americana* L. (plants.usda.gov/maps/large/MA/MAAM2.png)

3.3 *Mammea Americana* L. Uses and Toxic Properties

Mamey fruits are eaten raw, in fruit salads or are made into marmalade. In some places, like in Jamaica and the Bahamas, the fruit is steeped in wine and sugar for a while prior eating it or the flesh is stand in lightly salted water to remove its bitterness before cooking with sugar. It is said that the practice of soaking the fruit pulp in salted water or steeping it in wine can be

safety precautions since the fruit can be poisonous to people with a weak digestive system (Morton, 1987).

The reddish-brown, strong, hard and heavy wood of *Mammea Americana* L. has been used for pilings, construction and carpentry and the bark is used in small scale tanning (Nellis, 1997). The thick yellow gum from the bark is melted with fat and applied to the feet to combat chiggers since it is strongly astringent. In the Antilles, Mammee apple flowers are distilled to produce an essential oil used in perfumes and an aromatic liqueur known as "eau de creole". There are reports which indicate that Mamey latex and powdered seeds have been used as insecticides (Morton, 1987). Toxic properties of various parts of Mamey tree were first reported in 1984 by Grosourdy in *El Médico Botánico Criollo* (Morton, 1987). There are no comments in the United States Department of Agriculture records on edible uses of Mamey fruit but only the insecticidal and medicinal uses of the species were noted (Morton, 1987).

According to Nellis, Mammein, one of many coumarins found in the seed with the molecular formula $C_{22}H_{28}O_5$, has shown antitumor activity against sarcomas and the essential oil of the seed contains compounds which have shown antifungal activity. The ground seeds have been used to confuse and capture fish, and also they have been mixed with coconut oil to kill lice. The leaf tea has been used as a remedy for high blood pressure and to cure malaria disease, while the bark tea has been employed to treat cough and skin diseases (Nellis, 1997).

In Puerto Rico, there was a practice of wrapping Mamey leaves like a collar around tomato plants to protect them from mole crickets and cutworms (Morton, 1987). In various tests performed at the Federal Experiment Station in Mayagüez, Puerto Rico, Mamey seeds appeared to be 1/5 as toxic as pyrethrum and less toxic to plant tests than nicotine sulfate and DDT (Morton, 1987).

CHAPTER 4

ANALYTICAL AND INSTRUMENTAL TECHNIQUES

4.1 Solid Phase Microextraction (SPME)

Sampling process for chemical analysis usually involves several steps including sampling, sample preparation, separation, quantitation, statistical evaluation and decision (Pawliszyn, 1997). Since most analytical instruments cannot handle a complex sample directly, the principal objective during the sample preparation step is to isolate all the components of interest and bring the analytes to a suitable concentration level for optimal detection. Traditional sample preparation methods are recognized for the use of toxic organic solvents, time and labor intensive, since they include multi step procedures that are prone to analyte loss.

Solid Phase Microextraction (SPME) is an innovative solvent free, rapid, economical, versatile and selective sample preparation technique used for the extraction of organic compounds. Specifically, SPME provides a reduction of 70% in sample preparation time, minimal use of solvents and their disposal, the fiber last for more than an average of 50 extractions, can be automated and done in-situ (Supelco, 2017). In SPME, analytes are adsorbed directly from the sample onto a fused-silica fiber coated with a 10-100 μm thick film of stationary phase, similar in composition to those used in Gas Chromatography (Harris, 2010). SPME process consists of two steps: partitioning of analytes between the sample matrix and the coating material, followed by desorption of the concentrated extracts into an analytical instrument (Pawliszyn, 1997). SPME is commonly coupled to GC or GC/MS for the analytical separation process but it has been coupled to HPLC instruments (Dean, 2003).

SPME fiber is attached to a holder that consists of a stainless steel barrel, a black polymeric plunger, an adjustable depth gauge with needle guide, and a stainless steel retaining nut (see **Figure 3**). During the sample extraction, the SPME fiber is retracted, and the needle passed through the sample vial septum. Then the plunger is depressed to expose the fiber to the sample solution for a fixed amount of time while stirring the sample. The extraction time, or the time required for the fiber to become saturated with analyte, should be determined experimentally since the concentration of the analyte in the fiber is likely to vary depending on the sample. Finally, the fiber is retracted into the needle, removed from the sample vial and

inserted, depending on the application, into the hot injection port of a GC or into the interface desorption chamber of a HPLC.

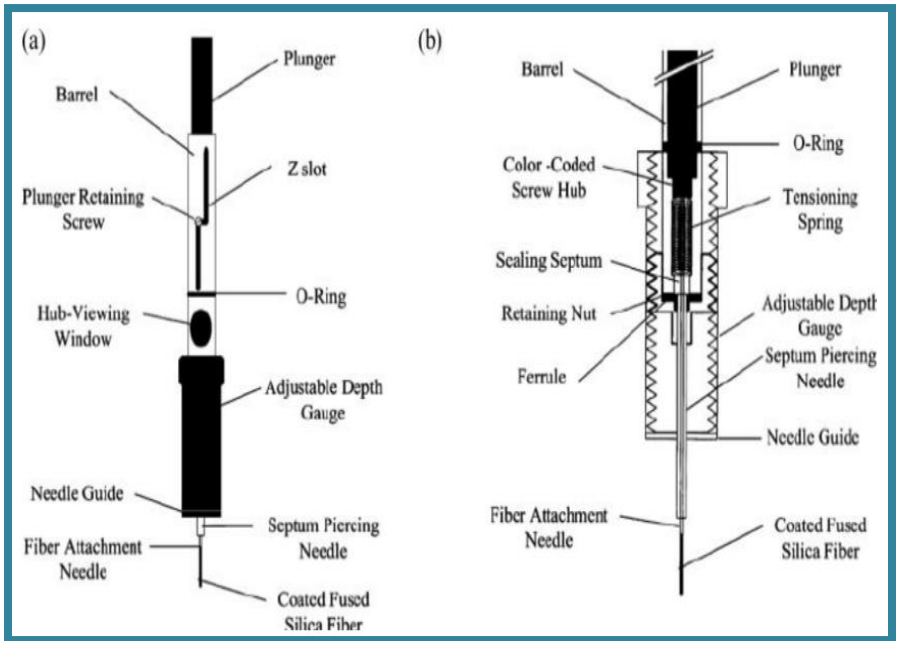


Figure 3. Manual SPME Fiber Holder Device: a) SPME Fiber Holder b) Cross-section of SPME Fiber Assembly (Supelco Data Sheet No. T713019A, 1998).

SPME technique is typically applied for the environmental analysis of water samples, headspace analysis of trace impurities in polymers and solid samples, flavor analysis of food products, toxicological analysis including alcohol in blood or drugs in urine, among other industrial applications (Supelco, 2017). There are different types of SPME fiber coating material and the one needed is determined according to the molecular weight and polarity of the analytes of interest. Polydimethylsiloxane (PDMS) 100 μm coated fiber is usually used for the extraction of low molecular weight or volatile compounds, while PDMS fibers of 7 μm or 30 μm are applied for the extraction of larger molecular weight or semivolatile compounds. An 85 μm polyacrylate-coated fiber is suitable for the extraction of polar analytes from polar samples but more polar analytes, such as alcohols and amines, are efficiently extracted and released faster with a 65 μm Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) coated fiber. For a volatile analysis performed at trace levels, it is recommended a 75 μm Carboxen®/PDMS (CAR/PDMS)

fiber, while for an expanded range of analytes including C_3-C_{20}, a 50/30 Divinylbenzene/Carboxen® on PDMS (DVB/CAR/PDMS) fiber is preferred (Supelco, 2017).

According to Pawliszyn (1997), other advantages of SPME include that coated fibers can be used to extract analytes from very small samples, the setup is small and convenient and the presence of a minute fiber is not likely to disturb chemical equilibria in a system. SPME being solvent free allows rapid extraction and transfer of sample to analytical instrument, and since sample preparation step is eliminated, the analytical process is accelerated and errors associated with analyte loss are prevented. The precision of the technique is subject to agitation conditions, sampling time, temperature, sample volume, headspace volume and vial shape, condition of the fiber coating material, sample matrix components, analyte losses, geometry and condition of the injector among others (Pawliszyn, 1997).

4.1.1 Basic Principles of SPME

SPME is considered a multiphase equilibration process in which the transport of analytes from the sample matrix into the coating material of the fiber begins in the moment where the coated fiber is placed in contact with the sample. The three phases considered in the process are the fiber coating, the gas phase or headspace, and the sample matrix (Pawliszyn, 1997). When the analyte concentration has reached distribution equilibrium between the sample matrix and the coating material, it is considered that the microextraction process has been completed and the distribution coefficient of the analyte is defined as:

$$K_{fs} = \frac{C_f^{\infty}}{C_s^{\infty}} \quad \textbf{(1)}$$

Following the law of conservation of mass, the total mass of an analyte should remain constant and is represented by equation (2):

$$C_0 V_s = C_f^{\infty} V_f + C_h^{\infty} V_h + C_s^{\infty} V_s \quad \textbf{(2)}$$

where C_0 is the initial concentration of the analyte in the matrix; C_f^∞, C_h^∞, and C_s^∞ are the equilibrium concentrations of the analyte in the fiber coating, the headspace, and the sample matrix, respectively; V_f, V_h, and V_s are the volumes of the fiber coating, the headspace, and the sample matrix, respectively. Assuming that the sample matrix can be represented as a single homogeneous phase and that no headspace is present in the system, the mass of the analyte absorbed by the coating material $n = C_f^\infty V_f$ at equilibrium conditions can be expressed as:

$$n = \frac{K_{fs} V_f V_s C_0}{K_{fs} V_f + V_s} \quad \textbf{(3)}$$

where n is the amount extracted by the coating material, K_{fs} is a fiber coating/sample matrix distribution constant, V_f is the fiber coating volume, V_s is the sample volume, and C_0 is the initial concentration of a given analyte in the sample. Equation (3) indicates that once equilibrium is reached, the extracted analyte amount is constant within the limits of experimental error and it is independent of further increase of extraction time (Pawliszyn, 1997). This means that there is a direct proportional relationship between sample concentration and the amount of analyte extracted. Also, this equation can be modified to account for the existence of other compartments in the matrix by considering the volumes of the individual phases and the appropriated distribution constants.

In SPME, careful timing of the extraction time and constant stirring conditions are necessary to obtain reproducible data. When the equilibrium point is reached, the amount of analyte extracted into the fiber coating is at a maximum, obtaining the highest sensitivity. The extraction time can be shortened if sensitivity is not a major concern of analysis. Because sample volumes tend to be very large compared to the fiber coating volume ($K_{fs}\ V_f \ll V_s$), equation (3) can be simplified into equation (4):

$$n = K_{fs} V_f C_0 \quad \textbf{(4)}$$

In other words, equation (4) states the usefulness of SPME technique when the sample volume is unknown since the amount of extracted analyte will correspond directly to its concentration in the sample matrix and is independent of the sample volume (Pawliszyn, 1997).

4.1.2 Extraction Modes in SPME

SPME technique can be applied using three basic types of extraction modes including direct extraction (DI), headspace configuration (HS), and membrane protection approach (Pawliszyn, 1997), as shown in **Figure 4**.

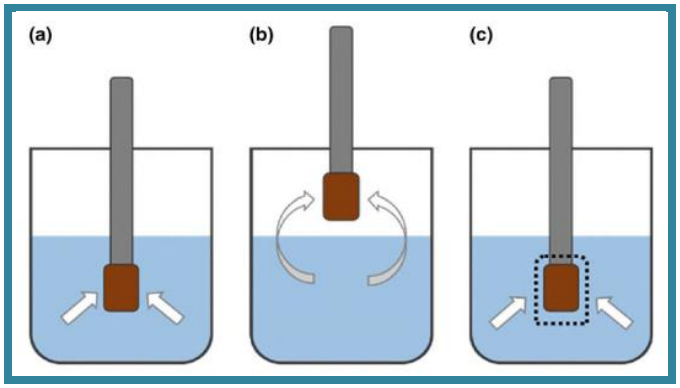


Figure 4. Extraction modes in SPME: (a) direct extraction, (b) headspace SPME, (c) membrane protected SPME (Ouyang et al., 2017)

In the direct extraction mode, the coated fiber is inserted directly into the sample and the analytes are transported directly from the sample matrix to the coating material. This allows analytes to partition between the coating material and the sample matrix (Pawliszyn, 1997).

In the headspace SPME mode, the fiber is exposed in the headspace above the sample matrix during the extraction procedure. The analytes are transported through the barrier of air before they can reach the coating material of the fiber (Pawliszyn, 1997). This works as a protection to the coating material from interferences caused by high molecular weight and nonvolatile species present in the sample matrix. As long as the sample and the gaseous headspace volumes remain the same at equilibrium, the amount of analyte extracted is identical using direct and headspace sampling. There are faster mass transfer rates through the headspace which makes volatile analytes to be extracted faster than semivolatiles.

On the other hand, in the membrane protection approach, a membrane barrier made of a selective material is used to protect the fiber against damage for extraction procedure on very dirty samples. This method is preferred for the determination of analytes with too low volatility to be suitable for the headspace approach (Pawliszyn, 1997).

4.1.3 Parameters involved in SPME performance

Some experimental parameters should be considered in order to develop a sensitive, selective and reproducible SPME method. Those parameters involve the coating material selection, sample volume optimization, sampling mode, optimization of the extraction conditions including the headspace/sample equilibrium time, agitation mode and temperature, extraction time, and desorption time into the analytical instrument. Matrix conditions such as temperature, pH, ionic strength, and the addition of an organic modifier also can be optimized (Ouyang et al., 2017).

4.2 Soxhlet Extraction

In 1879, German Chemist, Fran Von Soxhlet devised a liquid/solid extraction apparatus which bears his name and gave the foundation of today's automated solvent extraction systems (Luthria, 2004). Soxhlet extraction apparatus consists of a) solvent reservoir (round-bottomed flask), b) vapor tube, c) extraction chamber, d) water cooled reflux condenser, e) siphon, and it is placed on a heating source, which is commonly an isomantle (see **Figure 5**).

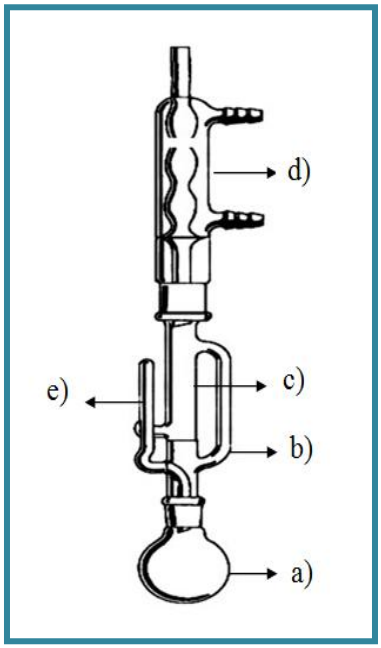


Figure 5. Soxhlet Extraction System (from www.v-pyrex.com)

In this liquid/solid extraction technique, a wide range of organic solvents is used to remove extractable materials from solid matrices based on the principle "like dissolves like" (Smith, 1999). Soxhlet extraction technique is widely used in the extraction of plant metabolites and has been used as a reference point for comparison with any new extraction technique. During the process, a solid sample is placed in a porous thimble, made of cellulose, glass or quartz, which in turn is located in the extraction chamber of the Soxhlet apparatus. Then the apparatus is fitted to a round-bottomed flask of appropriate volume containing the selected organic solvent, and to a reflux condenser. Using an isomantle, the solvent is boiled gently, their vapors pass up through the vapor tube to be condensed by the reflux condenser, and then the condensed solvent falls into the thimble to slowly fill the extraction chamber. At the time when the solvent fills completely the body of the Soxhlet apparatus, the organic solvent containing the analyte extracted from the sample in the thimble, siphons over into the round-bottomed flask, completing one extraction cycle (Dean, 2003). The fill and drain cycle is allowed to continue for 1 to 24 hours depending on the extraction conditions. When the sample has a lower density than the solvent, a glasswool plug is commonly placed on top of the solid in the cup (Smith, 1999). The extracted materials are then commonly analyzed by GC or HPLC method.

One of the main advantages of Soxhlet extraction is that it is a continuous process because when the saturated solvent empties into the round-bottomed flask, fresh solvent is recondensed and extracts the sample in the thimble yielding a greater recovery. This makes Soxhlet extraction the preferred method for extracting plant material since it is less solvent consuming and extraction time is shorter compared to maceration or percolation (Sarker et al., 2006). The extracted analyte normally have a higher boiling point than the organic solvent being retained in the flask while the fresh solvent recirculates. A limitation of this extraction technique is that the organic solvent is below its boiling point when it passes through the extraction chamber, although this is not a real problem since Soxhlet extraction is normally carried out for long time periods (Dean, 2003).

4.3 Gas Chromatography (GC)

Gas Chromatography is one of the most widely used techniques for qualitative identification and quantitative determination of separated chemical species. In this instrumental

method of analysis, the components of an organic vaporized sample are separated or partitioned between a gaseous mobile phase and a liquid phase immobilized on the surface of an inert solid packing material or on the walls of a capillary tubing (Skoog et al., 2007). A gas chromatograph consists of a carrier gas source, a hot injection port, a chromatographic column inside an oven, and a detector followed by a computer based data processing equipment. Common carrier gas sources used in gas chromatography include inert gases such as Helium, Argon, Nitrogen or Hydrogen. Chromatographic columns are divided in two general types including packed or capillary columns. For current applications, capillary columns replaced packed columns due to their improved efficiency (Harris, 2010). Capillary columns stationary phase consist of fused silica coated on the inside walls with bonded phases for more stability to high temperatures. Analytical separations in Gas Chromatography can be performed in isothermal or temperature programmed mode since column temperature is a key variable for an efficient analysis. For this purpose in Gas Chromatography, the chromatographic column is located inside a thermostated oven. The detector is maintained at a higher temperature than the column to maintain analytes in gaseous state (Harris, 2010). The ideal detector should have adequate sensitivity, good stability, reproducibility, a linear, short and selective response, nondestructive to sample, among others (Skoog et al., 2007). There are a lot of detectors that can be coupled to a gas chromatograph but not all of them meet the ideal characteristics.

In Gas Chromatography, the volatile liquid or gaseous sample is injected through the septum into the hot injection port, in which the sample rapidly evaporates. The vapor is swept through the chromatographic column by the carrier gas, which is hot enough to provide sufficient vapor pressure for analytes to be eluted in a reasonable amount of time. Then the separated analytes flow through a detector whose signal response is displayed on a computer (Harris, 2010).

4.4 Mass Spectrometry (MS)

Mass Spectrometry is the most widely applicable of all the analytical tools since it is capable of providing information on the elemental composition of samples of matter, the structures of inorganic, organic, and biological molecules, the qualitative and quantitative

composition of complex mixtures, the structure and composition of solid surfaces, and isotopic ratios of atoms in samples. A mass spectrometer is an instrument that produces ions and separates them according to their mass to charge ratios (m/z). The basic components of a mass spectrometer include the sample inlet, gaseous ion source, mass analyzer, detector or ion transducer and the signal processor (Skoog et al., 2007). Ions for mass analysis are commonly produced by electron impact (EI). This process consists in the ionization of a sample by bombarding with a beam of energetic electrons. The electrons are emitted from a heated tungsten or rhenium filament and accelerated by applying 70 volts between a filament and an anode. In this process a molecule undergoes the reaction:

$$M + e^- \rightarrow M^{\cdot +} + 2e^- \quad \textbf{(5)}$$

where M represents the analyte molecule, and $M^{\cdot +}$ is its molecular ion. Electron impact sources provide good sensitivity and an extensive fragmentation resulting in a large number of peaks making possible an unambiguous identification of analytes (Skoog et al., 2007).

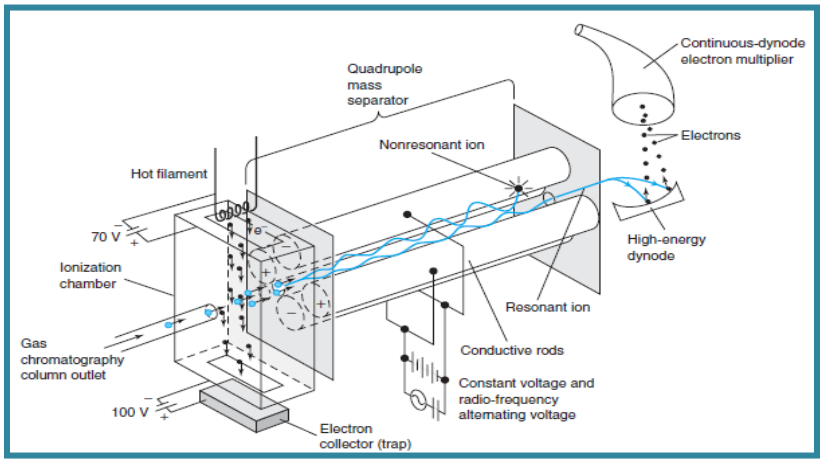


Figure 6. Quadrupole Mass Analyzer (from Harris D.C., 2010)

One of the most common types of mass spectrometers used is the quadrupole mass analyzer because it is compact, less expensive, and more rugged compared to other types (Skoog et al., 2007). The hearth of the quadrupole is the four parallel cylindrical rods that serve as the electrodes (see **Figure 6**). The mass spectrum of a chemical species is obtained when ions are accelerated into the space between the rods by a potential difference of 5 to 10 volts, the AC (alternating current) and DC (direct current) voltages on the rods are increased while maintaining

their ratio constant, and all the ions except those having a certain m/z value strike the rods and reach the transducer (Skoog et al., 2007). Thus, mass spectrometry is useful in the identification and characterization of chemical compounds since it provides important data including the molecular mass and molecular formula of a compound. Also, the fragmentation patterns in a mass spectrum reveal information of the presence or absence of some functional groups (Skoog et al., 2007).

4.5 Gas Chromatography coupled to Mass Spectrometry (GC/MS)

The Mass Spectrometer is one of the most powerful detectors for Gas Chromatography since it provides both qualitative and quantitative information (Harris, 2010). **Figure 7** shows a schematic of a typical capillary GC/MS instrument.

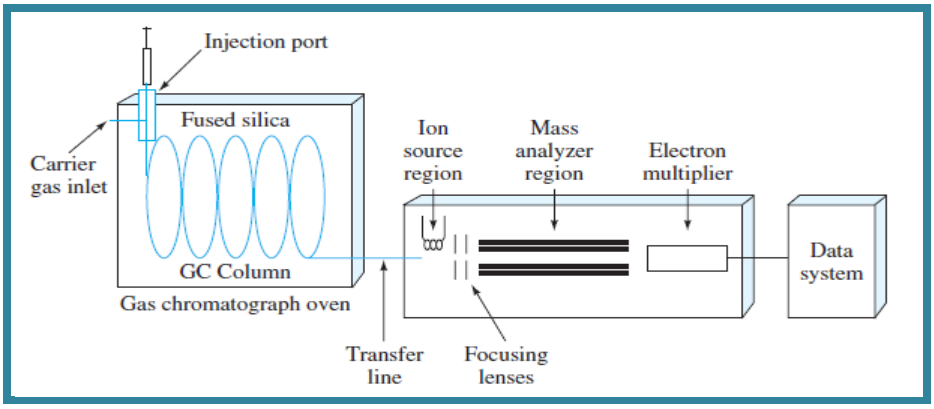


Figure 7. Schematic diagram of a GC/MS Instrument (from Skoog et al., 2007)

When the sample is injected into the gas chromatograph, it is separated in the chromatographic capillary column, and then the effluent enters the inlet of a quadrupole mass spectrometer. The molecules are fragmented and ionized by the source, are mass analyzed by the quadrupole and detected by the electron multiplier.

During a chromatographic separation using a GC/MS, the mass spectrometer scans the masses repetitively, and the total ion chromatogram (TIC), or a plot of the sum of ion abundances in each spectrum as a function of time is obtained. It is possible to display the mass spectrum at a particular time during the chromatographic separation to identify the chemical

species that is eluting at that specific retention time. In GC/MS, it is also possible to obtain information about unresolved peaks and look for the mass spectrum at the front edge, middle part or the trailing edge of a multi component peak. Also, selective ion monitoring (SIM) technique can help to select a single m/z value and monitor it throughout the chromatographic analysis (Skoog et al., 2007).

CHAPTER 5

OBJECTIVES

5.1 Principal Objective

Apply the Headspace-Solid Phase Microextraction (HS-SPME) technique for the extraction of the aroma volatile constituents and perform Soxhlet Extraction of the essential oil of *Mammea Americana* L. fruit for their analysis and characterization by Gas Chromatography coupled to Mass Spectrometry (GC/MS) instrumental method. Determine the biological activity of a selected essential oil extract of *Mammea Americana* L. fruit by means of a brine shrimp lethality bioassay.

5.1.1 Specific Objectives

- Optimize and validate important parameters involved in the HS-SPME technique for an efficient extraction of the volatile constituents of *Mammea Americana* L. fruit.
- Determine which type of SPME fiber coating material is the most suitable for the extraction of the volatile constituents of *Mammea Americana* L. fruit.
- Identify and characterize the chemical composition of the aroma volatile constituents of *Mammea Americana* L. fruit from different municipalities in Puerto Rico by Gas Chromatography coupled to Mass Spectrometry (GC/MS).
- Use Microscale Soxhlet Extraction with different types of organic solvents to obtain essential oil extracts of *Mammea Americana* L. fruit.
- Identify and characterize the chemical composition of the essential oil extracts of *Mammea Americana* L. fruit by Gas Chromatography coupled to Mass Spectrometry (GC/MS).
- Perform a brine shrimp lethality bioassay in order to prove the biological activity or toxicity of a selected essential oil extract of *Mammea Americana* L. fruit by means of the determination of the LC_{50} value.

CHAPTER 6

METHODOLOGY

6.1 *Mammea Americana* L. fruit Sample Collection

In order to have a better description of the genus, family and habitat of the tree, samples of the fresh leaves, branch, flowers and fruit of *Mammea Americana* L. were classified by Prof. Jeanine Vélez, taxonomist from the Biology Department, who deposited the voucher herbarium specimen at the repository of the Department of Biology Herbarium (MAPR) at the University of Puerto Rico-Mayagüez Campus. *Mammea Americana* L. fruit samples were collected at four different municipalities in Puerto Rico, including Rincón, Mayagüez, Aguada and Cayey, from trees located at different altitudes above sea level (see **Figure 8**).

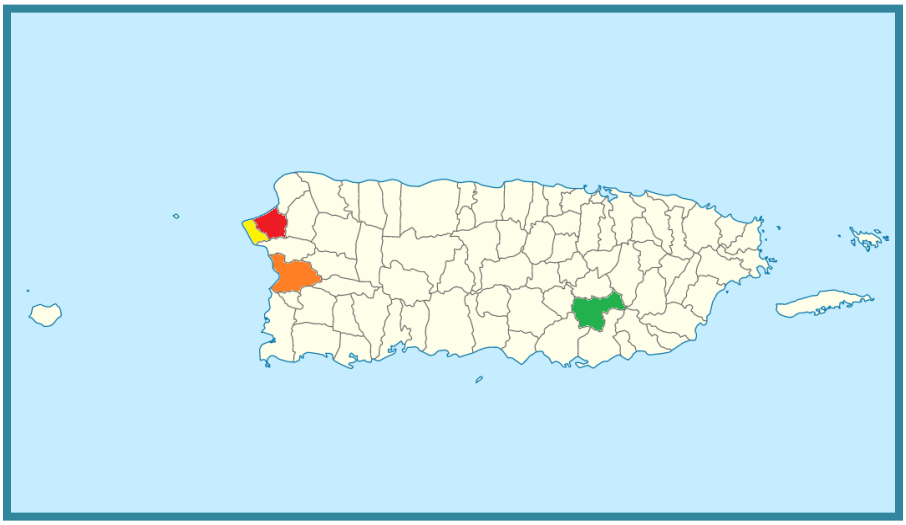


Figure 8. Geographical Location of Rincón (yellow), Mayagüez (orange), Aguada (red) and Cayey (green) municipalities in Puerto Rico

Rincón is located in the western coastal valley of Puerto Rico with an average annual temperature of 79.2°F (26.2°C) and average annual precipitation of 59.94 inches. Mayagüez is located in the center of the western coast of Puerto Rico having an average annual temperature of 78.3°F (25.7°C) and average annual precipitation of 85.38 inches. Aguada is located in the western coastal valley of Puerto Rico, east of Rincón, with an average annual temperature of 77.5°F (25.3°C) and average annual precipitation of 75.78 inches. Cayey is located in the central

mountain region of Puerto Rico, having an average annual temperature of 73.15°F (22.9°C) and average annual precipitation of 76.15 inches (www.weather.gov; www.en.climate-data.org; www.usclimatedata.com). **Table 1** summarizes the detailed addresses, coordinates, altitudes of the trees and average annual precipitation, where Mamey fruit samples were collected for this research.

Table 1. *Mammea Americana* L. fruit Sampling Areas

Municipality	Address	Geographic Coordinates		Altitude*	Average Annual Precipitation[a]
Rincon	**Bo. Córcega Calle 10**	**Lat. 18.31**	**Long. -67.24**	**3 ft**	**59.94 in**
Mayagüez	**Carr. 106 km 12.9 Camino Los Sole**	**Lat. 18.21**	**Long. -67.04**	**1193 ft**	**85.38 in**
Aguada	**Urb. Paseo Las Flores Calle Clavel**	**Lat. 18.36**	**Long. -67.19**	**118 ft**	**75.78 in**
Cayey	**Urb. Montellano E-53 Calle A**	**Lat. 18.11**	**Long. -66.15**	**1283 ft**	**76.15 in**

***Altitudes above the sea level**
[a]www.weather.gov; www.en.climate-data.org; www.usclimatedata.com

6.2 Sample Storage and Preparation before analysis

After sample collection, *Mammea Americana* L. fruit samples were transported to the Environmental Chemistry Research Laboratory Q-106 room, under the supervision of Professor Maritza De Jesús Echevarría, at the Chemistry Building in the University of Puerto Rico-Mayagüez Campus. Mamey fruits were weighed in a mechanical balance (OHAUS 700/800 Series Triple Beam Balance US. Pat.No. 2,729,439) and measured using a ruler (Fisher Scientific Cat. No. 09-016). Mamey fruits showed an average weight between 400-500 g, while measuring an average of 9.6 cm in length from the base to tip and an average of 9.4 cm at its widest side.

Figure 9. *Mammea Americana* L. fruit sample storage and preparation before analysis

Mamey fruit was peeled, the pulp was chopped into small pieces with a kitchen knife, the seed was removed and the pulp samples were homogenized. An amount of 10 g of *Mammea Americana* L. fruit pulp sample were weighed on an analytical balance (Acculab L Series LA-110), placed in a 25 mL THM flask (Supelco, Inc. Cat.No. 6-4716 Lot: 032498), sealed using a PTFE/silicone septum (Supelco, Inc. Cat.No. 2-7177 Lot. HD033 18 mm × 0.060in. × 5mil) with a hole cap and covered with parafilm and aluminum foil. All samples for SPME analysis were prepared in the same way to have consistency during the entire procedure. In order to preserve their integrity, the flasks prepared with fruit sample were immediately stored at -17°C in the freezer of a domestic refrigerator. The chopped fruit pulp samples for Soxhlet Extraction of essential oil were also stored at -17°C in the domestic refrigerator freezer but inside glass jars with lid and covered using parafilm, while seeds were stored in Ziploc bags for further analysis.

6.3 Analytical Instrumentation

6.3.1 GC Parameters

Gas Chromatography coupled to Mass Spectrometry (GC/MS) instrumental method was applied for the chromatographic separation and chemical characterization of *Mammea*

Americana L. fruit pulp volatile constituents and essential oil extracts composition. The instrument used was a Gas Chromatograph Model 6890 Series coupled to a Mass Selective Detector Model 5973 from Hewlett-Packard Company (HP) equipped with a fused silica capillary column (see **Figure 10**).

Figure 10. HP 6890 Series Gas Chromatograph (GC)/5973 Mass Selective Detector (MSD)

Specifically, the chromatographic column used was a non polar SPB-5 (Supelco, Inc. Cat.No. 24048) with a bonded poly(5% diphenyl/95% dimethyl siloxane) stationary phase composition with dimensions of 30 m × 0.32 mm i.d. × 0.25µm film thickness. The carrier gas used was ultra high purity Helium (99.999%).

For HS-SPME volatile compounds analysis, the GC was operated at a constant flow of 1.9 mL/min with an initial nominal pressure of 3.7 psi. The front injection port was equipped with a 0.75 mm internal diameter liner (Supelco SPME injection sleeve, Cat.No. 2-6375, Lot.No. 020498) and 11 mm pre-drilled septum (Supelco Thermogreen® LB-2, Cat.No. 23168, Lot.No. 16458C). The injection port temperature was at 250°C, in Splitless mode and a purge flow of 1.0 mL/min.

For essential oil analysis (applying Soxhlet Extraction) the GC was operated at a constant flow of 2.2 mL/min with an initial nominal pressure of 6.5 psi. The front injection port was equipped with a 1.5 mm internal diameter liner (Supelco Direction injection sleeve, Cat.No. 2-

0517, Lot.No. 062698) and 11 mm septum (Restek Corp. Thermolite®, Cat.No. 20364, Lot.No. ED1004). The injection port temperature was at 225°C, in Splitless mode and a purge flow of 50.0 mL/min. **Table 2** summarizes GC/MS critical parameters when applied to volatile composition analysis and to the essential oil extracts analysis of *Mammea Americana L.* fruit pulp.

Table 2. GC/MS Instrument Parameters

Parameter	HS-SPME/GC/MS Specifications	Soxhlet Extraction/GC/MS Specifications
Injection Port Temperature	250°C	225°C
Desorption Time	5 min	Direct Injection
Injector liner	SPME sleeve 0.75 mm ID	Direct sleeve 1.5 mm ID
GC Column	Capillary SPB-5 poly(5% diphenyl/95% dimethyl siloxane) 30 m × 0.32 mm × 0.25µm	Capillary SPB-5 poly(5% diphenyl/95% dimethyl siloxane) 30 m × 0.32 mm × 0.25µm
Oven Temperature Program	Initial: 40°C × 7.00 min Ramp 1: 8°C/min Final: 200°C × 5.00 min Ramp 2: 10°C/min Final: 250°C × 0.00 min	Initial: 70°C × 4.00 min Ramp 1: 10°C/min Final: 125°C × 5.00 min Ramp 2: 2°C/min Final: 250°C × 60.00 min
Flow	Constant 1.9 mL/min	Constant 2.2 mL/min
MS Interface Temperature	250°C	180°C
Ionization Mode	EI, 70eV	EI, 70eV
Ion Source Temperature	230°C	230°C
Quadrupole Temperature	106°C	106°C
Mass Range	33-400 amu	35-500 amu

6.3.2 MS Parameters

The Mass Selective Detector (MSD) of Hewlett-Packard Company (HP) Model 5973 that was coupled to the GC 6890 was working on Electron Impact (EI) ionization mode at 70eV. For HS-SPME volatile compounds analysis, the MS interface temperature was of 250°C, while the

MS ion source and MS quadrupole temperatures were at 230°C and 106°C, respectively. The scan mass range was of 33 to 400 amu with an Electron Multiplier EM voltage of 1600eV. For essential oil analysis (applying Soxhlet Extraction) the MS interface temperature was of 180°C while the MS ion source and MS quadrupole temperatures were at 230°C and 106°C, respectively. The scan mass range was of 35 to 500 amu with an Electron Multiplier EM voltage of 1647.1eV. The HP-Chem Program, Enhanced Chemstation, Version 4.03.00-1996 (Hewlett-Packard Company) for the Quadrupole Mass Analyzer was used during the analysis. The identification of the peaks and compound characterization in the obtained Total Ion Chromatograms (TIC) was done using the Wiley Registry of Mass Spectral Data 7th and 10th Editions.

6.4 Head Space-Solid Phase Microextraction Technique Validation and Optimization

In order to perform an efficient analysis and to select the most appropriate and sensitive SPME fiber coating material for the extraction of the volatile constituents of *Mammea Americana* L., three different types of SPME fibers with different types of coating material were selected for evaluation including the 100µm Polydimethylsiloxane (PDMS Supelco, Inc. Cat.No. 57300-U), 65µm Polydimethylsiloxane/Divinylbenzene (PDMS/DVB Supelco, Inc. Cat.No. 57310-U) and 50/30µm Stable Flex Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS Supelco, Inc. Cat.No. 57348-U). **Table 3** summarizes the different Supelco SPME fibers evaluated during the process, their film thickness, polarity, type of interaction and conditioning parameters.

Table 3. Supelco SPME Fiber coating materials

SPME Fiber Stationary Phase	Polarity	Type	Conditioning Temperature	Conditioning Time
100µm PDMS	Non-polar	Absorbent	250°C	30 min
65µm PDMS/DVB	Bi-polar	Adsorbent	250°C	30 min
50/30µm Stable Flex DVB/CAR/PDMS	Bi-polar	Adsorbent	270°C	1 hour

***Note: Conditioning times and temperatures according to Supelco Inc., an analytical division of Sigma-Aldrich Co.**

Critical parameters related to the HS-SPME technique including the flask headspace/sample matrix equilibrium time, extraction time and desorption time into the injection port of the Gas Chromatograph were validated in order to optimize the extraction conditions and to select which SPME fiber coating material was the most suitable for the extractive analysis of the volatile constituents of *Mammea Americana* L. fruit pulp (see **Figure 11**).

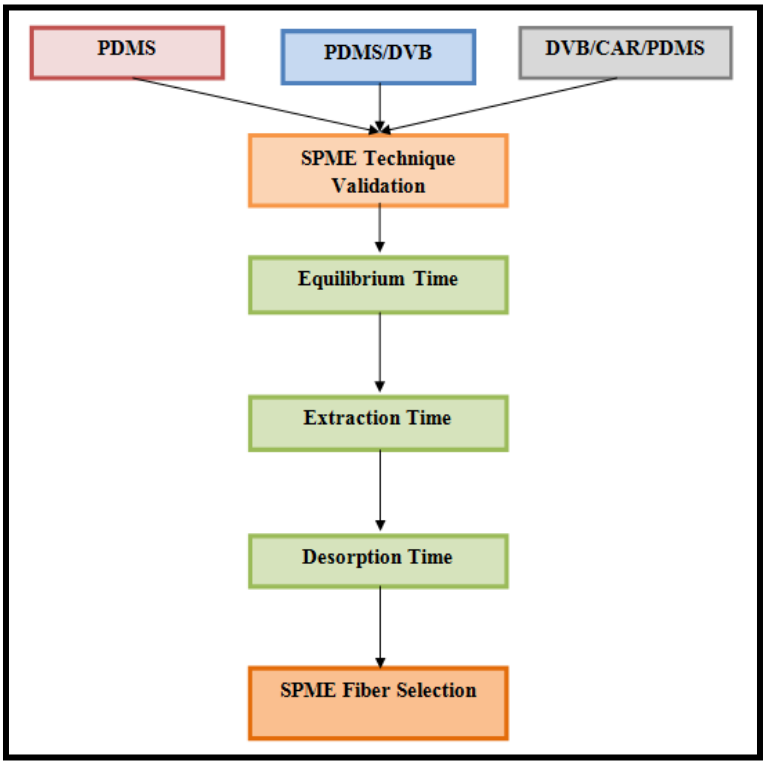


Figure 11. Flowchart for the SPME Technique Validation and Optimization

6.4.1 Fiber Conditioning System

The SPME fiber conditioning process helps to eliminate contaminants or impurities from the fiber coating material that could interfere with the extraction of the volatile compounds of interest. In this case, the conditioning process was performed in the back inlet of a Gas Chromatograph (GC) model 6890 of the Hewlett Packard Company that had a Flame Ionization Detector (GC/FID) at the back side following the conditioning times and temperatures established by Supelco, Inc. for each one of the three different SPME fibers.

6.4.2 Equilibrium Time Validation

The first parameter that was tested during the SPME technique validation was the equilibrium time between the headspace of the 25mL THM flask and the Mamey fruit pulp sample matrix under constant stirring conditions. For this purpose, the day of the analysis, the flask with fruit sample was put out of the freezer of the domestic refrigerator, the cap was removed to place a 2 cm length magnetic stirrer inside, the flask was capped again, and the sample was allowed to reach room temperature. The flask with fruit sample was then placed over a laboratory stirrer/hot plate (Corning Company Model PC-420 Serial No. 070596327895) that was set to a constant stirring of 250 rpm during the entire process. The headspace temperature of the flask was measured before and after the insertion of the SPME fiber by drilling the PTFE/silicone septum with a stainless steel needle of a microprocessor thermometer (Omega Engineering, Inc. Model HH21) consisting of a J-K-T type thermocouple. The equilibrium times tested in triplicate by using the three different SPME fibers were 0, 20, 30, 40 and 60 min, while the extraction time and desorption time at the injection port were maintained fixed at 40 min and 5 min, respectively.

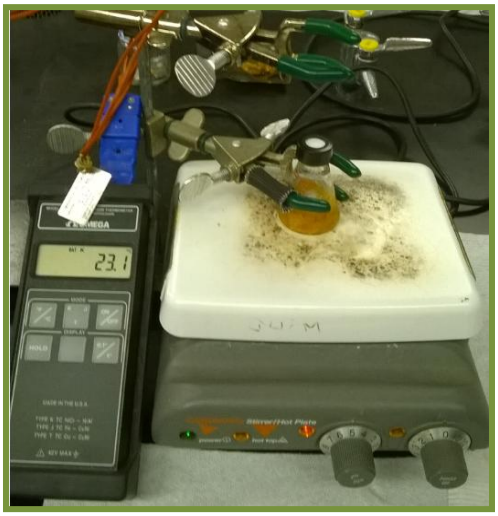


Figure 12. *Mammea Americana* L. fruit pulp at constant stirring reaching headspace/sample matrix equilibrium

6.4.3 Extraction Time Validation

The second parameter tested during the SPME technique validation was the extraction time or the time the SPME fiber was exposed to the headspace of the flask reaching an equilibrium distribution between the fiber coating material and the volatile sample matrix. For this purpose, the day of the analysis, the flask with fruit sample was removed from the freezer of the domestic refrigerator, the 2cm magnetic stirrer was placed inside, the flask was capped again and the sample was allowed to reach room temperature. The flask with fruit sample was placed over the laboratory stirrer/hot plate set to a constant stirring of 250 rpm during the entire process. After the optimum equilibrium time between the headspace and the sample matrix was reached, the headspace temperature of the flask was measured with the microprocessor thermometer, and then the SPME fiber was immediately inserted through the PTFE/silicone septum. In this case, the fiber was exposed to the headspace of the flask for sampling the volatile compounds of the Mamey fruit pulp varying the duration of exposure. The extraction times tested in triplicate were 5, 20, 30, 40 and 60 min, while the equilibrium time and desorption time at the injection port were maintained fixed at 30 min and 5 min, respectively.

After this step was completed, the PDMS fiber was discarded to follow the next step of the validation procedure since it did not showed effectiveness compared to the other two SPME fibers for the extraction of the volatile compounds of the Mamey fruit pulp.

6.4.4 Desorption Time Validation

The last parameter tested during the SPME technique validation procedure was the desorption time. The purpose was to determine the optimum time that takes to the SPME fiber coating material to completely desorb the extracted volatile compounds in the injection port of the GC without retaining them. The PDMS/DVB and DVB/CAR/PDMS SPME fibers were tested for desorption during 5, 7, and 9 min at an injection port temperature of 250°C, while the optimum equilibrium and extraction times were maintained fixed at 30 min and 40 min, respectively. This process was completed by analyzing a blank between each desorption at the injection port of the GC to test if the fiber coating material does not retain compounds adhered to its surface.

6.5 Head Space-Solid Phase Microextraction (HS-SPME) of the volatile constituents of *Mammea Americana* L. fruit from Different Municipalities

The next step of this research was to determine the volatile composition of *Mammea Americana* L. fruit pulp from different municipalities of Puerto Rico having optimized and validated important parameters related to the HS-SPME technique and using the selected 50/30μm Stable Flex DVB/CAR/PDMS bi-polar SPME fiber. For this purpose, Mamey fruit samples from Rincon, Mayagüez, Aguada and Cayey, were prepared in 25 mL THM flasks as described in section 6.2 of this chapter. The extraction of the volatile constituents was performed following the SPME optimum conditions of equilibrium time (between headspace/sample matrix), extraction time (fiber exposed at the headspace of the flask), and desorption time at the GC/MS injection port, determined during the validation procedure.

In order to have a better idea of how the Mamey fruit pulp volatile composition can vary when exposed to different factors, the HS-SPME was carried out by means of three different experimental conditions. The HS-SPME/GC/MS analysis was performed first by extracting the volatile compounds with the Mamey fruit sample at room temperature (23-25°C), then it was done by mixing fruit pulp sample with 5 mL of deionized water at room temperature, and finally by slightly heating the sample (flask headspace temperature between 30-32°C) with the laboratory stirrer/hot plate set at a temperature of 25±2°C (hotplate temperature dial setpoint at 2 according to Corning Company). All the analysis performed by the three different experimental conditions for each one of the Mamey fruit pulp samples from the different municipalities were carried out in triplicate to test the reproducibility of the experimental results. **Figure 13** shows an example of the HS-SPME procedure applied by slightly heating the Mamey fruit sample.

Figure 13. Head Space-Solid Phase Microextraction of the volatile constituents of *Mammea Americana* L. fruit pulp

6.6 Microscale Soxhlet Extraction of the Essential Oil of *Mammea Americana* L. fruit pulp

In order to determine the essential oil chemical composition of *Mammea Americana* L. fruit pulp from different municipalities in Puerto Rico (including Rincon, Mayagüez, Aguada and Cayey), the samples were subjected to a freeze drying process, followed by sample grinding and preparation, Micro-Soxhlet extraction of the essential oil and their posterior analysis by Gas Chromatography coupled to Mass Spectrometry (GC/MS).

6.6.1 Freeze Drying Process

The freezed chopped fruit pulp samples for Soxhlet Extraction of essential oil of *Mammea Americana* L. were removed from the freezer of the domestic refrigerator and transferred to safety coated wide mouth filter seal freeze drying flasks with rubber silicone top. The fruit samples were carefully transferred without filling the freeze drying flasks more than half of the flask total capacity. The freeze drying process was carried out using a VirTis Benchtop Freeze Dryer (The Virtis Company, Inc. Gardiner, N.Y. 12525) with a refrigeration temperature setpoint between -54.5°C to -55.5°C and vacuum pressure ranging from 52 millitorr

to 166 millitorr. The process was completed in a range from 24 to 28 hours, approximately, for each of the Mamey fruit samples from different municipalities. The freeze drying flask with dried Mamey fruit pulp sample was weighed, in an analytical balance, before and after the freeze drying process in order to determine the amount of water contained by the sample that was removed during the drying process. Finally, the dried fruit samples were transferred to glass jars with lid and covered with parafilm.

6.6.2 Dried Fruit Pulp Sample Grinding and Storage

Dried *Mammea Americana* L. fruit pulp samples were transported at ambient temperature in glass jars from the Chemistry Building-University of Puerto Rico, Mayagüez Campus to the Tropical Agricultural Research Station (TARS) of the United States Department of Agriculture (USDA) located in Mayagüez, Puerto Rico. The grinding process of the dried Mamey fruit pulp samples was carried out using an IKA MF10 Basic Analytical Grinder operating at 3250 rpm with a 1.00 mm sieve to achieve an appropriate and homogeneous granulated particle size. Although the Mamey fruit samples were subjected to freeze drying process, it was observed that the product obtained after grinding the samples was oily. Grinded samples were collected in plastic cups, and then transferred to 15 mL vials with Teflon lined caps (Supelco Cat. No. 2-3296), that were previously weighed. The grinding process was repeated with dried Mamey fruit pulp samples from each different municipality (see **Figure 14**).

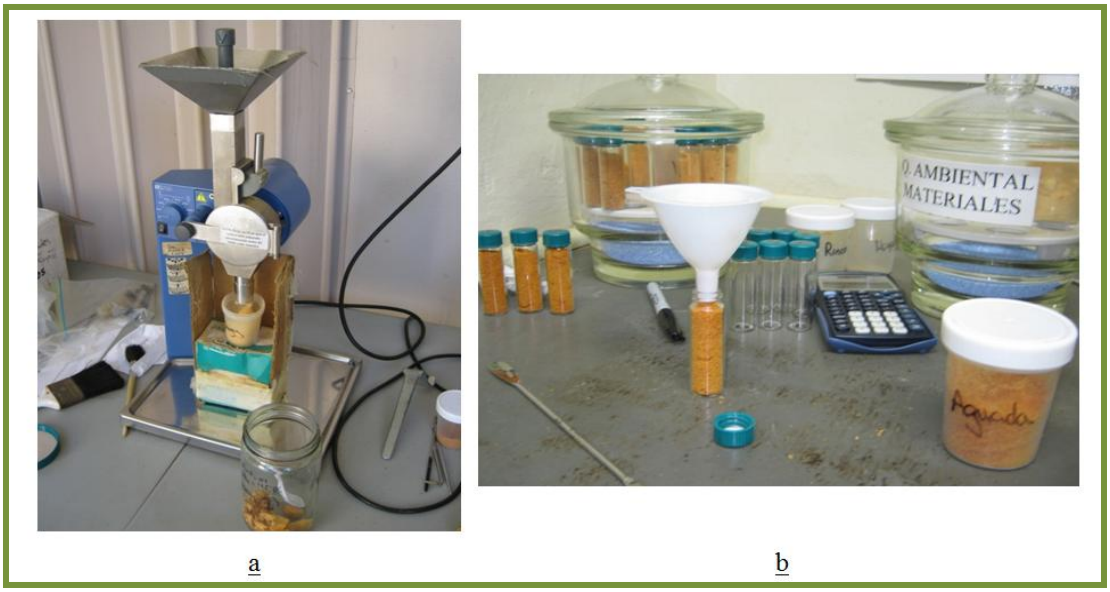


Figure 14. a) IKA MF10 Basic Analytical Grinder b) Grinded Mamey fruit samples stored in 15 mL vials inside desiccators

It was important to clean the grinder between grinding a different sample to remove residues from the previous one and to avoid cross contamination. For the cleaning process, the blades rotor was disassembled and all parts of the grinder were carefully cleaned using a vacuum cleaner, cleaner brushes, and premoistened deionized water cleanwipes (Fischer Scientific Catalog No. 06-665-23). It was important to wait a few minutes after cleaning the grinder to allow the surfaces of the blades to dry before grinding the next sample.

The characteristic aroma of Mamey fruit was perceived before, during, and after the entire procedure. When the grinding process was completed, the 15 mL vials with Mamey grinded fruit pulp samples were covered with parafilm, stored inside desiccators and transported at ambient temperature to the Environmental Chemistry Research Laboratory Q-106 room at the Chemistry Building-University of Puerto Rico, Mayagüez Campus. Finally, the samples inside the desiccators were stored at -17°C in the freezer of a domestic refrigerator.

6.6.3 Microscale Soxhlet Extraction Optimization

In order to determine the best conditions and perform an efficient extraction of the essential oil of *Mammea Americana* L. fuit pulp using Micro-Soxhlet Extraction, some parameters related to the technique were tested and optimized. Those parameters included testing different extraction solvents, varying extraction times and changing the volume at which the essential oil extract was concentrated. Dried and grinded Mamey fruit pulp samples were subjected to test using different organic solvents including Dichloromethane (Baker Analyzed HPLC Reagent Cat.No. 9315-03, Lot. F22260), Chloroform (Fisher Scientific HPLC Grade/ACS Cert. Cat.No. C-606, Lot. 744609), Methanol (Fisher Scientific Certified ACS ≥99.8% Cat.No. A412-20, Lot. 060896), Petroleum Ether (Fisher Scientific Optima^{TM} High Purity for GC Cat.No. E120-4, Lot. 903567), and Deionized Water. Tested extraction times included 1 hr, 2 hrs, 3 hrs and 4 hrs, while essential oil extracts were concentrated to different volumes including 0.1 mL, 0.3 mL, and 1.0 mL using DCM as solvent.

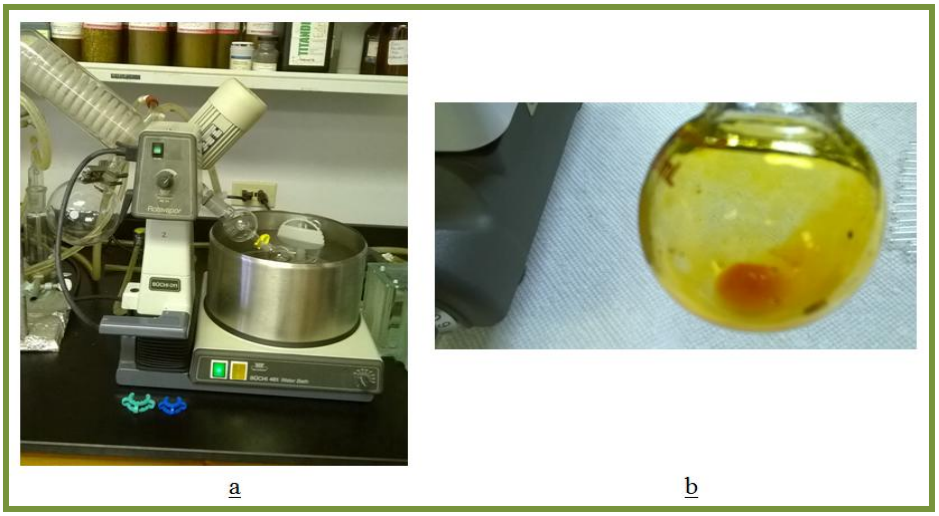
6.6.4 Micro-Soxhlet Extraction of the essential oil of *Mammea Americana* L. fruit from Different Municipalities

The next step was to determine the essential oil composition of *Mammea Americana* L. fruit pulp from Rincon, Mayagüez, Aguada and Cayey. For this purpose, three different organic solvents were selected during the Microscale Soxhlet Extraction optimization step, including Dichloromethane, Chloroform, and Methanol (mentioned in order of increasing polarity). The day of the extraction the dried and grinded Mamey fruit samples were taken out of the freezer of the domestic refrigerator and the samples were allowed to reach room temperature. An amount of 0.5000 g of dried and grinded *Mammea Americana* L. fruit pulp sample was weighed at an analytical balance (OHAUS PA214 Pioneer Analytical Balance 210g capacity, 0.1mg readability) and transferred to a cellulose extraction thimble (Whatman Schleicher &Schuell Cat.No. 2800105 Int.Dia. 10×50mm, Ext.Dia. 12×50mm). The thimble with grinded Mamey fruit pulp sample was placed inside the extraction chamber of a Micro-Soxhlet Extraction apparatus (Kontes Glass Company Cat.No. 292010-0000). The extraction apparatus was connected to an Allihn condenser (Kontes Glass Company Cat.No. 283250-0000) which had

cool water flowing through it from a recirculating bath (Fisher Scientific Water Bath Isotemp 3016 LR106974-4). An amount of 15.00 mL of organic solvent were transferred to a 25 mL round bottomed flask (Kontes Glass Company 14/20BW Cat. No. 294000-0025) which was then connected to the Micro-Soxhlet Extraction apparatus. The solvent was boiled gently using a heating mantle (Electrothermal Unimantle 115V Cat.No. UM0050B) that was connected to a rheostat (Superior Electric Powerstat 3PN116C Variable Transformer) to regulate the heating output. The cooling and heating temperatures were carefully controlled through the Soxhlet Extraction system to provide the appropriate conditions for continuous solvent condensation and siphoning cycles and to improve extraction yield (see **Figure 15**). The process was completed at an optimum extraction time of 4 hours.

Figure 15. Microscale Soxhlet Extraction of the Essential Oil of *Mammea Americana* L. fruit pulp

After the extraction process, the obtained sample was subjected to rotary evaporation using a Buchi RE111 rotary evaporator with a heating waterbath (Buchi 461) to remove the organic solvent from the essential oil extract of *Mammea Americana* L. fruit pulp (see **Figure 16**). The round bottomed flask with the extracted sample was weighed in an analytical balance before and after the rotoevaporation process in order to determine the exact amount of essential oil extracted.



a b

Figure 16. a) Buchi RE111 Rotavap with Buchi 461 Waterbath b) *Mammea Americana* L. fruit pulp essential oil extract after rotoevaporation process

After rotoevaporation process, the essential oil extract obtained was quantitatively transferred from the round bottomed flask to a 5 mL flat bottom mini vial with inner conical bottom using the original organic solvent applied during Soxhlet Extraction (see **Figure 17**). The essential oil extract was concentrated evaporating the solvent under pure nitrogen flux to an optimum volume of 0.1 mL when using Dichloromethane and Chloroform, and to a volume of 1.0 mL when using Methanol. Finally, the sample extract was analyzed by direct injection of 1.0 µL sample through the injection port of the GC/MS for chromatographic separation and chemical characterization.

Figure 17. *Mammea Americana* L. fruit pulp essential oil methanolic extracts concentrated to a volume of 1.0 mL in inner conical bottom vials with screw cap and silicone septum

All these processes, including the Micro-Soxhlet Extraction, followed by rotoevaporation of the solvent, quantitative transfer of the essential oil extract, solvent evaporation under nitrogen flux and their analysis by GC/MS were repeated with the grinded *Mammea Americana* L. fruit pulp samples from each different municipality using the three selected organic solvents.

6.7 Brine Shrimp Lethality Bioassay for *Mammea Americana* L. Fruit Pulp Essential Oil

6.7.1 *Mammea Americana* L. Essential Oil Extract Sample Preparation

Dichloromethane essential oil extract of *Mammea Americana* L. fruit pulp was assayed by a modified brine shrimp lethality bioassay (Meyer et. al., 1982). A stock solution of 1000 µg/mL was prepared by dissolving 10 mg of *Mammea Americana* L. fruit pulp Dichloromethane essential oil extract in 10 mL of DMSO 50% (Dimethyl Sulfoxide BP 231-100, Lot. 134698, CAS 67-68-5, EC 200-664-3).

6.7.2 Hatching *Artemia salina* Shrimp

Seawater used for the bioassay was collected at the open ocean, at an approximate distance of 200 to 300 ft from the west coast of Rincon, Puerto Rico and at a depth of 6 ft. In order to determine if the seawater collected had the appropriate conditions for brine shrimp egg hatching, several parameters concerned to seawater quality including pH, conductivity, salinity, and total dissolved solids were analyzed using a pH meter, with glass and reference electrode (Beckman Co. 110 Φ ISFET), and a conductivity meter, with platinum electrode type cell (HACH Company sensION5 Model 51800-10).

The eggs of *Artemia salina* (San Francisco Bay Brand, Inc. 8239 Enterprise Dr. Newark, CA 94560) were incubated in a homemade hatchery prepared using a 1.75 liter plastic bottle. The hatchery was filled with seawater and an air hose connected to an air pump (Tetra Whisper Air Pump 10 gal. 77851-900, United Pet Group, Inc. 3001 Commerce Street Blacksburg, VA 24060) was placed inside through the bottom of the reservoir for a constant aeration. The eggs were hatched at an average room temperature of 25°C under artificial light for a 24 hours period.

6.7.3 Brine Shrimp Bioassay

An amount of approximately 4 mL of seawater was added to 10 mL glass vials. The nauplii or harvested shrimps were attracted by an artificial light source, ten shrimps were counted using a disposable Pasteur pipette (VWR Scientific Products Cat.No. 14672-608 size: 5 3/4") and transferred to each vial. Seven concentrations (100, 50, 10, 7.5, 5, 2.5 and 1.25 µg/mL) were prepared in triplicate vials by delivering varying dose volumes from the 1000 µg/mL stock solution of Dichloromethane essential oil extract of *Mammea Americana* L. fruit pulp using an Eppendorf Micropipet (Adjustable Model 4810 Autoclavable Pipette 200-1000µL Cat.No. 22 44 020-9, 10-100 µL Cat.No. 22 44 010-1) . The vials were completed to the total volume of 10 mL with seawater. A 1000 µg/mL stock solution of Potassium Dichromate ($K_2Cr_2O_7$ Fisher Scientific Certified ACS ≥99% Cat.No. P188-3) and DMSO 40% were used for positive and negative controls, respectively. Positive control vials were prepared in triplicate and at the same concentrations as in the sample vials. Negative control vials were also prepared in triplicate but at three different concentrations (4%, 0.3% and 0.05%). After a 24 hour period, the vials were examined against a lighted background and the number of brine shrimps that survived in each vial was counted to determine the percent deaths at each applied concentration and controls.

Figure 18. Brine Shrimp Lethality Bioassay to predict the toxicity of *Mammea Americana* L. fruit pulp essential oil extract

6.7.4 LC_{50} Estimate Calculation for *Mammea Americana* L. Essential Oil Extract

The median lethal concentration or 50% concentration in which mortality of the brine shrimps occurs from toxicity (LC_{50}) was determined using Microsoft Excel 2007 with XLSTAT 2017 statistical analysis tool by probit analysis method. This program was used to construct a plot of shrimp's mortality against the logarithm of essential oil extract concentration obtaining a logistic regression or concentration-mortality response sigmoidal curve.

CHAPTER 7

RESULTS AND DISCUSSION

7.1 Head Space-Solid Phase Microextraction Technique Validation and Optimization

One of the principal objectives of this research work was to find the most appropriate and sensitive SPME fiber coating material to make an efficient extraction of the volatile constituents of *Mammea Americana* L. fruit pulp in order to characterize its chemical composition. For this reason three SPME fibers with different types of coating materials and different polarities were subjected to an optimization procedure including the non-polar absorbent PDMS, the bi-polar adsorbent PDMS/DVB and the bi-polar adsorbent DVB/CAR/PDMS fibers. According to the SPME theory discussed in Chapter 4, it is important to first investigate the behavior of the SPME fiber in terms of the flask headspace/sample matrix equilibrium time, extraction time and desorption time since when using the SPME technique careful timing and constant stirring conditions are essential to obtain reproducible and reliable data.

7.1.1 Equilibrium Time Optimization

The first critical parameter related to the HS-SPME technique that was validated and optimized was the flask headspace/sample matrix equilibrium time. According to Pawliszyn, in SPME it is important to reach equilibrium conditions to effectively extract a constant amount of analyte within the limits of experimental error. The PDMS, PDMS/DVB and DVB/CAR/PDMS SPME fibers were subjected to an equilibrium study by varying the equilibrium times between the flask headspace/sample matrix at 0, 20, 30, 40 and 60 min, while maintaining fixed other parameters such as the stirring at 250 rpm, 40 min of extraction time, 5 min of desorption time at the GC/MS injection port and at an average room temperature. One of the principal goals during this process was to find an optimum equilibrium time in where the highest amount of volatile analytes are extracted by the SPME fiber with high precision and in a reasonable amount of time. Plots of average number of chromatographic peaks (analytes extracted) vs. equilibrium time (min), or equilibrium time profiles, were constructed to analyze the behavior of the different SPME fibers at different equilibrium times.

7.1.1.1 PDMS Equilibrium Time Optimization

The PDMS fiber was subjected to study by varying the equilibrium times between the flask headspace/sample matrix before the insertion of the fiber through the headspace of the flask while maintaining fixed the extraction and desorption times and under constant stirring conditions. It was found that the PDMS fiber extracted an almost constant average amount of 9 analytes from 0 to 40 min of equilibrium time with a slightly decrease in the amount of analytes extracted at 60 min of equilibrium (see **Table 4**). At 30 min of equilibrium the PDMS fiber extracted an amount of 9±2 analytes with the highest precision, compared to the other equilibrium times tested, having a %RSD of 19.25 with a standard error of 1.00. It is important to note that all the %RSD values were quite high during this process being indicative of low precision of the experimental results with the PDMS fiber.

Table 4. PDMS fiber Equilibrium Time optimization for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

PDMS	Number of Chromatographic Peaks						
Equilibrium time (min)	**Trial #1**	**Trial #2**	**Trial #3**	**Average**	**Std. Dev.**	**%RSD**	**Std. Error**
0	10	12	4	9	4	48.04	2.40
20	6	6	14	9	5	53.29	2.67
30	10	10	7	9	2	19.25	1.00
40	7	8	11	9	2	24.02	1.20
60	6	8	7	7	1	14.29	0.58
Extraction time = 40 min							
Desorption time = 5 min							

In **Figure 19**, it was possible to confirm the behavior of the PDMS fiber toward the extraction of the volatile analytes from *Mammea Americana* L. fruit pulp at different equilibrium times. As shown in the plot, the extracted amount of analytes was almost constant from 0 to 40 min with a decrease in the amount of extracted analytes at 60 min of equilibrium which is indicative that with additional time the volatile analytes decompose. The highest amount of extracted analytes from Mamey fruit with the lowest uncertainty using the PDMS fiber was

observed at 30 min of equilibrium time as demonstrated by the error bars which are representative of the standard error values.

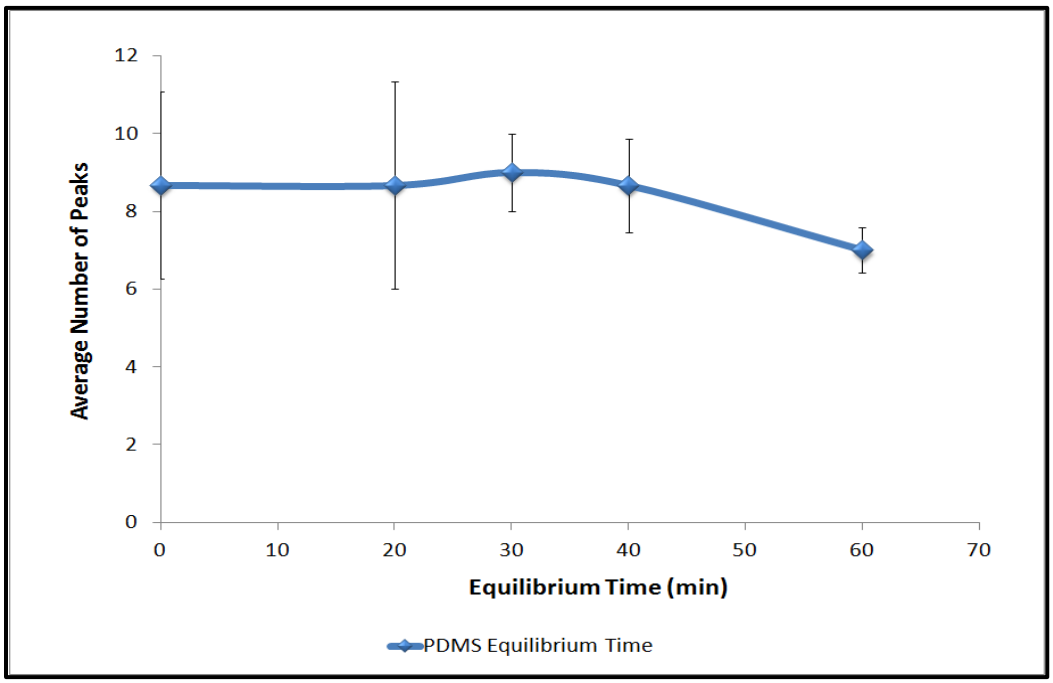


Figure 19. PDMS fiber Equilibrium Time profile for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

7.1.1.2 PDMS/DVB Equilibrium Time Optimization

The PDMS/DVB fiber was subjected to study by varying the equilibrium times between the flask headspace/sample matrix before the insertion of the fiber through the headspace of the flask while maintaining fixed the extraction and desorption times and under constant stirring conditions. It was found that the PDMS/DVB fiber extracted an almost constant amount of 23±0 to 24±1 analytes from 0 to 20 min of equilibrium time. It is important to note that at 30 min of equilibrium the PDMS/DVB fiber extracted 30±3 analytes, which was the highest amount of extracted volatile analytes with relatively good precision, compared to the other equilibrium times tested, having a %RSD of 10.00 with a standard error of 1.73. As shown in **Table 5**, there is a decrease in the amount of extracted analytes at 40 and 60 min of equilibrium. With the

PDMS/DVB fiber, all the %RSD values obtained with the different tested equilibrium times were relatively low being indicative of good precision of the experimental results.

Table 5. PDMS/DVB fiber Equilibrium Time optimization for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

PDMS/DVB	Number of Chromatographic Peaks						
Equilibrium time (min)	**Trial #1**	**Trial #2**	**Trial #3**	**Average**	**Std. Dev.**	**%RSD**	**Std. Error**
0	23	23	23	23	0	0.00	0.00
20	24	25	23	24	1	4.17	0.58
30	33	30	27	30	3	10.00	1.73
40	31	29	23	28	4	15.05	2.40
60	25	28	29	27	2	7.62	1.20
Extraction time = 40 min							
Desorption time = 5 min							

The behavior of the PDMS/DVB fiber at different equilibrium times can be confirmed in **Figure 20**. The extracted amount of analytes was almost constant from 0 to 20 min with an increase in the amount of extracted analytes at 30 min followed by a decrease in the amount of extracted analytes at 40 and 60 min of equilibrium. The decrease in the amount of analytes extracted at 40 and 60 min of equilibrium is indicative of that with additional time there are no optimum equilibrium conditions and the volatile analytes decompose.

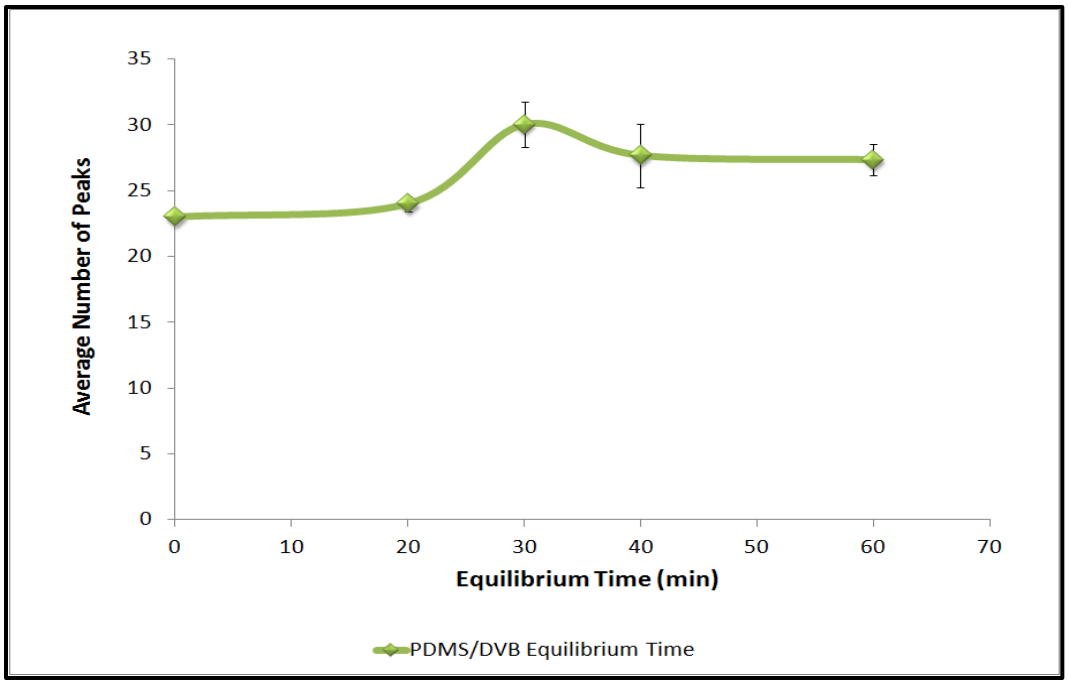


Figure 20. PDMS/DVB fiber Equilibrium Time profile for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

By analyzing the error bars from the equilibrium time profile for the PDMS/DVB fiber, the lowest uncertainty was observed at 0 and 20 min, although at 30 min of equilibrium of the flask headspace/sample matrix, it was possible to extract the highest amount of volatile analytes from *Mammea Americana* L. fruit pulp with relatively good precision.

7.1.1.3 DVB/CAR/PDMS Equilibrium Time Optimization

The DVB/CAR/PDMS fiber was subjected to study by varying the equilibrium times between the flask headspace/sample matrix before the insertion of the fiber through the headspace of the flask while maintaining fixed the extraction and desorption times and under constant stirring conditions. It was found that the DVB/CAR/PDMS fiber extracted an almost constant amount of 28±2 to 29±3 analytes from 0 to 20 min of equilibrium time. At 30 min and 40 min of equilibrium the DVB/CAR/PDMS fiber extracted the highest amount of volatile analytes from Mamey fruit. Specifically at 30 min of equilibrium, an amount of 36±1 analytes were extracted with a %RSD of 1.59 and a standard error of 0.33, and at 40 min of equilibrium

an amount of 39±1 analytes were extracted with a %RSD of 1.47 and a standard error of 0.33. However, there was a decrease in the amount of extracted analytes at 60 min of equilibrium as shown in **Table 6**. With the DVB/CAR/PDMS fiber, the %RSD and standard error values obtained were quite low in general, but the lowest uncertainty was found at 30 min and 40 min of equilibrium being indicative of good precision of the experimental results.

Table 6. DVB/CAR/PDMS fiber Equilibrium Time optimization for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

DVB/CAR/PDMS	Number of Chromatographic Peaks						
Equilibrium time (min)	**Trial #1**	**Trial #2**	**Trial #3**	**Average**	**Std. Dev.**	**%RSD**	**Std. Error**
0	29	25	29	28	2	8.35	1.33
20	27	28	33	29	3	10.96	1.86
30	37	36	36	36	1	1.59	0.33
40	39	40	39	39	1	1.47	0.33
60	31	37	31	33	3	10.50	2.00
Extraction time = 40 min							
Desorption time = 5 min							

Figure 21 shows the behavior of the DVB/CAR/PDMS at different equilibrium times. The extracted amount of analytes was almost constant from 0 to 20 min with an increase in the amount of extracted analytes at 30 and 40 min followed by a decrease in the amount of extracted analytes at 60 min of equilibrium. The decrease in the amount of analytes extracted at 60 min of equilibrium is indicative of that with additional time there are no optimum equilibrium conditions and the volatile analytes decompose.

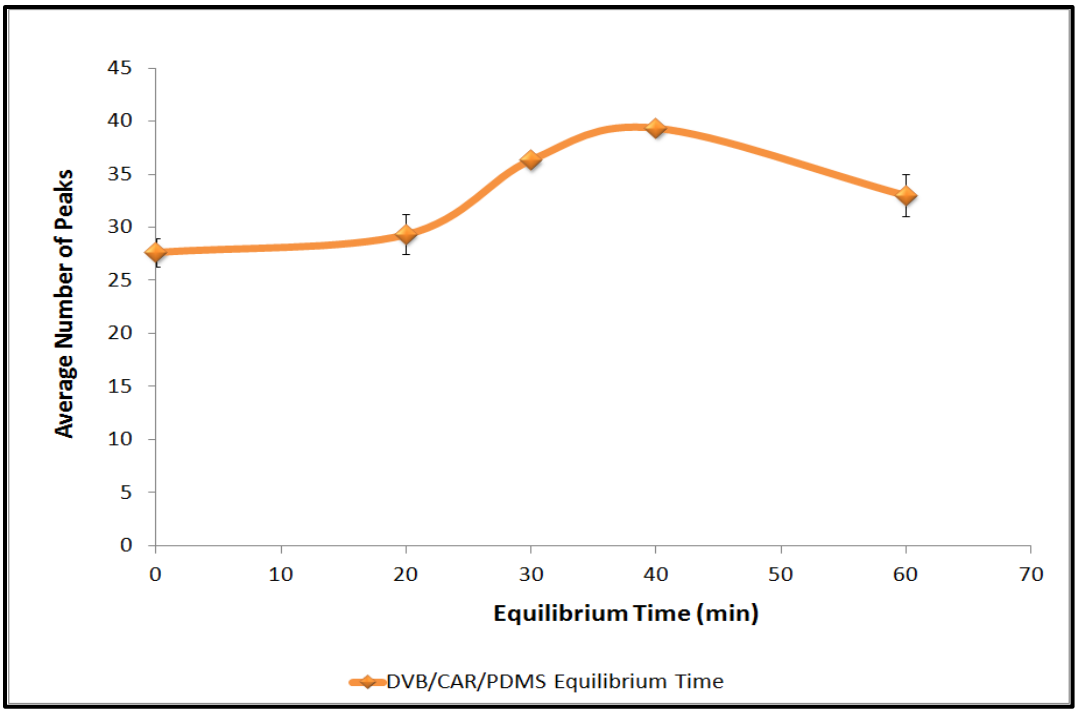


Figure 21. DVB/CAR/PDMS fiber Equilibrium Time profile for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

By analyzing the error bars from the equilibrium time profile for the DVB/CAR/PDMS fiber it was observed that at 30 and 40 min of equilibrium of the flask headspace/sample matrix it was possible to extract the highest amount of volatile analytes from *Mammea Americana* L. fruit pulp with a high precision.

7.1.1.4 Conclusion

During this validation and optimization process, it was possible to determine the optimum equilibrium time for each of the three different SPME fibers subjected to study maintaining fixed the extraction time at 40 min, the desorption time at 5 min and the fruit pulp sample under constant stirring conditions at 250 rpm during all the study. **Figure 22** shows the comparison of the equilibrium time profile for the three different SPME fibers tested.

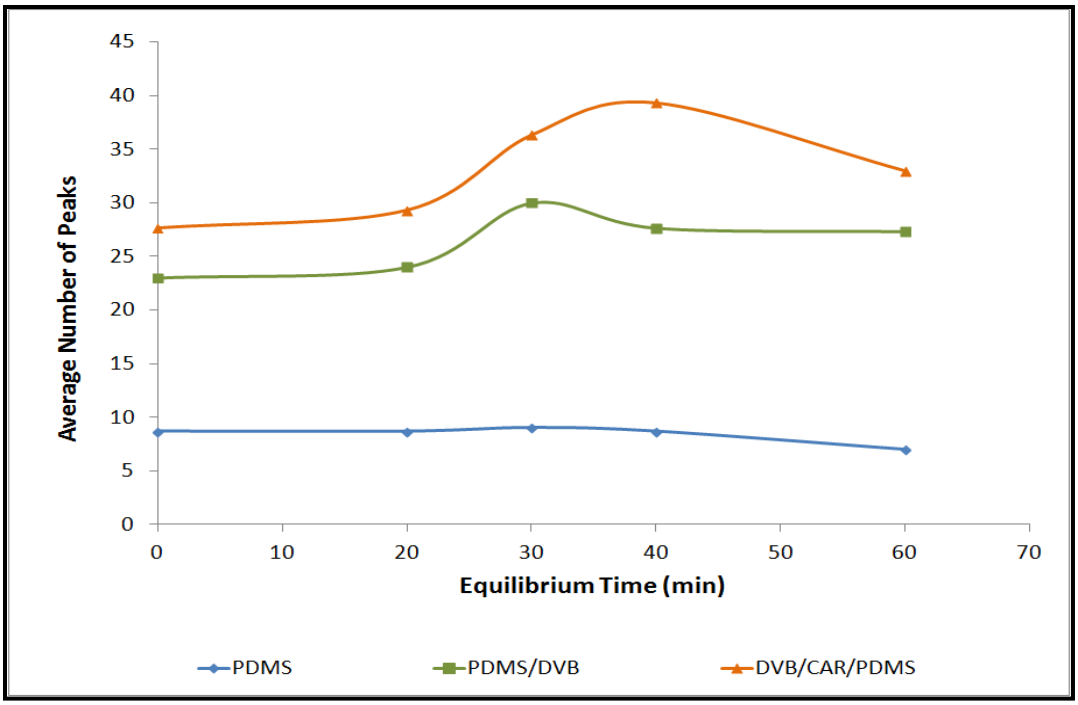


Figure 22. Different SPME fibers Equilibrium Time profile comparison for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

All of the SPME fibers showed a similar behavior toward the different equilibrium times tested extracting an almost constant amount of analytes at 0 to 20 min, having an increase in the extracted amount of analytes at 30 min, and a tendency to a decrease in the extracted amount of analytes at 40 and 60 min. For the PDMS fiber, it was found that the optimum equilibrium time was reached at 30 minutes of equilibrium of the flask headspace/sample matrix being capable to extract 9±2 volatile analytes having a %RSD of 19.25 and with a standard error of 1.00. On the other hand, the PDMS/DVB fiber equilibrium time profile showed that the optimum equilibrium time was reached at 30 minutes. The PDMS/DVB fiber with 30 minutes of equilibrium of the flask headspace/sample matrix was capable to extract an amount of 30±3 volatile analytes with relatively good precision having a %RSD of 10.00 and with a standard error of 1.73. While for the DVB/CAR/PDMS fiber, optimum equilibrium conditions between the flask headspace/sample matrix were reached at both 30 min and 40 min. The DVB/CAR/PDMS had the highest precision compared to the other fibers and was capable to extract an amount of 36±1 volatile analytes with a %RSD of 1.59 and a standard error of 0.33 at 30 minutes of equilibrium,

and an amount of 39±1 volatile analytes were extracted with a %RSD of 1.47 and a standard error of 0.33 at 40 minutes of equilibrium. Comparing the amount of extracted analytes by the DVB/CAR/PDMS fiber at 30 and 40 min of equilibrium there was no significant statistical difference. Since the principal objective during this process was to find an optimum equilibrium time in where the highest amount of volatile analytes can be extracted with high precision and in a reasonable amount of time it was concluded that the optimum equilibrium time for the DVB/CAR/PDMS fiber was 30 minutes. This conclusion can be sustained with the fact that when analyzing the behavior of the fiber toward the extraction of the volatile analytes of *Mammea Americana* L. fruit pulp, with an additional time of the required, the optimum equilibrium conditions are lost and the volatile analytes decompose. Also it is important to remark that with the DVB/CAR/PDMS at 30 min of equilibrium time, it was possible to extract the highest amount of volatile analytes from *Mammea Americana* L. fruit pulp compared to the PDMS and PDMS/DVB SPME fibers.

7.1.2 Extraction Time Optimization

The second critical parameter related to the HS-SPME technique that was validated and optimized was the extraction time or the time the SPME fiber was exposed to the headspace of the flask reaching an equilibrium distribution between the fiber coating material and the volatile sample matrix. As stated by Pawliszyn, SPME is considered a multiphase equilibration process in which the transport of the analytes from the sample matrix into the coating material of the fiber begins in the moment when the coated fiber is placed in contact with the sample. It is considered that the microextraction process has been completed in the moment when the analyte concentration has reached distribution equilibrium between the sample matrix and the coating material. The PDMS, PDMS/DVB and DVB/CAR/PDMS SPME fibers were subjected to an extraction study by varying the extraction times between at 5, 20, 30, 40 and 60 min, while maintaining fixed other parameters such as the previously determined optimum equilibrium time of 30 min for the three different tested fibers, the stirring was maintained at 250 rpm, 5 min of desorption time at the GC/MS injection port, and at an average room temperature. One of the principal goals was to find an optimum extraction time in where the amount of analyte extracted into the fiber coating was at its maximum, meaning that the microextraction process has been

completed, and obtaining the highest possible sensitivity and precision. Plots of average number of chromatographic peaks (analytes extracted) vs. extraction time (min), or extraction time profiles, were constructed to analyze the behavior of the different SPME fibers at different extraction times.

7.1.2.1 PDMS Extraction Time Optimization

The PDMS fiber was subjected to study by varying the extraction times or the time the PDMS fiber coating material was exposed to the headspace of the flask extracting the volatile analytes from *Mammea Americana* L. fruit pulp while maintaining fixed the desorption time, the determined optimum equilibrium time, and under constant stirring conditions. At 5 min of extraction, PDMS extracted a minimum amount of 2±0 analytes, while at 20 and 30 min it extracted an almost constant amount of 4±1 analytes with quite high %RSD value specifically at 30 min of extraction. The maximum amount of volatile analytes extracted by the PDMS fiber was 9±2 analytes obtained at 40 minutes of the fiber being exposed to the flask headspace absorbing the volatile analytes of the fruit sample with a %RSD of 19.25 and a standard error of 1.00 (see **Table 7**). The high %RSD value is indicative of poor precision of the experimental results. Also it is important to note that at 60 min there was an evident decrease in the amount of extracted analytes by the PDMS fiber.

Table 7. PDMS fiber Extraction Time optimization for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

PDMS	Number of Chromatographic Peaks						
Extraction time (min)	**Trial #1**	**Trial #2**	**Trial #3**	**Average**	**Std. Dev.**	**%RSD**	**Std. Error**
5	2	2	2	2	0	0.00	0.00
20	3	4	4	4	1	15.75	0.33
30	3	3	5	4	1	31.49	0.67
40	10	10	7	9	2	19.25	1.00
60	3	4	4	4	1	15.75	0.33
Equilibrium time = 30 min							
Desorption time = 5 min							

Figure 23 shows the extraction time profile for the PDMS fiber. It was possible to confirm the behavior of the PDMS fiber toward the extraction of the volatile analytes from *Mammea Americana* L. fruit pulp at the optimum equilibrium time of 30 minutes. As shown in the plot, PDMS fiber extracted a minimum amount of analytes at 5 min, then it extracted an almost constant amount of analytes from 20 to 30 min. An evident increase in the amount of analytes extracted was observed at 40 min, and this was followed by a marked decrease in the amount of extracted analytes at 60 min of extraction.

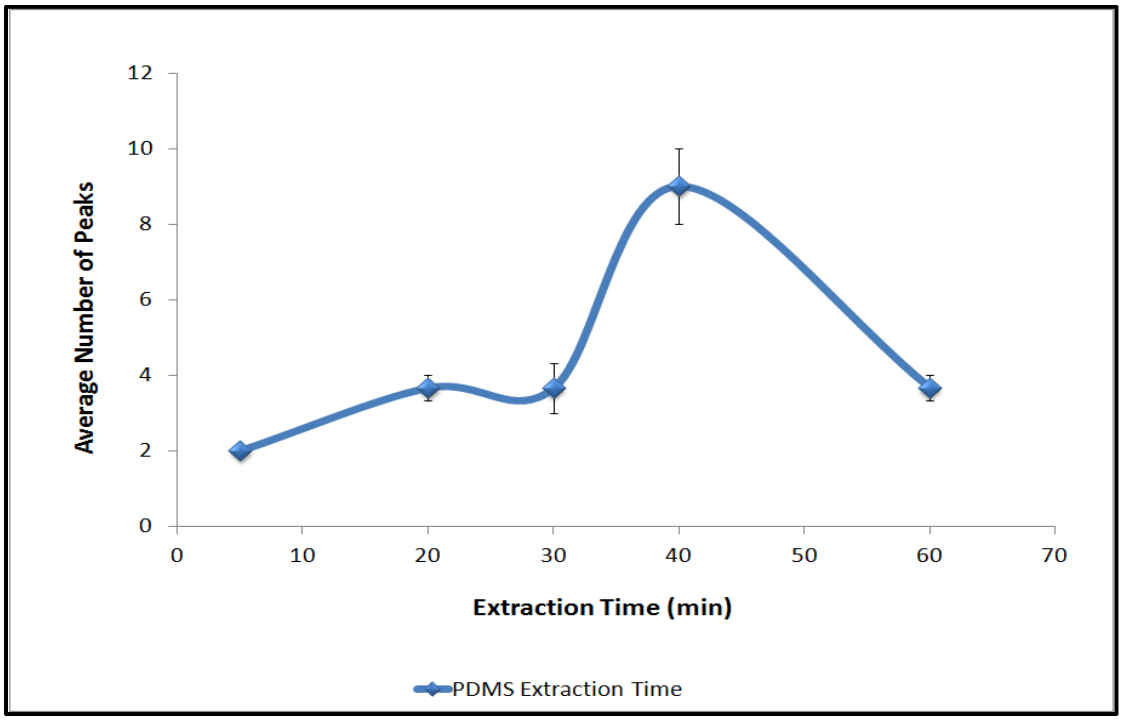


Figure 23. PDMS fiber Extraction Time profile for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

The decrease in the amount of extracted analytes at 60 min is indicative that with additional time the PDMS fiber releases the volatile analytes from its coating material again to the flask headspace or the volatile analytes decompose. It is important to remark that the highest uncertainty given by the error bars, which are representative of the standard error values, was

observed at 40 minutes of extraction which was when the PDMS fiber was capable to extract the maximum amount of volatile analytes from Mamey fruit pulp sample.

7.1.2.2 PDMS/DVB Extraction Time Optimization

The PDMS/DVB fiber was subjected to study by varying the extraction times or the time the PDMS/DVB fiber coating material was exposed to the headspace of the flask extracting the volatile analytes from Mamey fruit pulp while maintaining fixed the determined optimum equilibrium time, the desorption time, and under constant stirring conditions. At 5 min of extraction the PDMS/DVB fiber extracted a minimum amount of 7±1 volatile analytes. The amount of analytes extracted by the PDMS/DVB increased to 17±1 and 20±1 with %RSD values of 6.66 and 5.87 at 20 and 30 min of extraction, respectively, showing relatively good precision. However, as shown in **Table 8**, the highest amount of extracted analytes with the PDMS/DVB fiber was 30±3 obtained at 40 minutes of the fiber being exposed to the flask headspace adsorbing the volatile analytes of *Mammea Americana* L. fruit pulp. It is important to note that although the amount of extracted analytes increased at 40 min a lower precision was observed as indicated by the increase in the %RSD to 10.00. At 60 min of extraction a decrease in the amount of extracted analytes to 27±4 with an increase in the uncertainty of the experimental results was observed.

Table 8. PDMS/DVB fiber Extraction Time optimization for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

PDMS/DVB	Number of Chromatographic Peaks						
Extraction time (min)	**Trial #1**	**Trial #2**	**Trial #3**	**Average**	**Std. Dev.**	**%RSD**	**Std. Error**
5	6	6	8	7	1	17.32	0.67
20	16	18	18	17	1	6.66	0.67
30	19	19	21	20	1	5.87	0.67
40	33	30	27	30	3	10.00	1.73
60	23	26	31	27	4	15.16	2.33
Equilibrium time = 30 min							
Desorption time = 5 min							

It was possible to confirm the behavior of the PDMS/DVB fiber toward the extraction of the volatile analytes of Mamey fruit pulp at the optimum equilibrium time of 30 minutes by analyzing its extraction time profile in **Figure 24**. The PDMS/DVB fiber extracted a minimum amount of analytes at 5 min, and then a slight increase in the amount of extracted analytes was observed from 20 to 30 min followed by an evident increase at 40 min when it reached its maximum. Finally a decrease in the amount of extracted analytes was observed at 60 min of extraction.

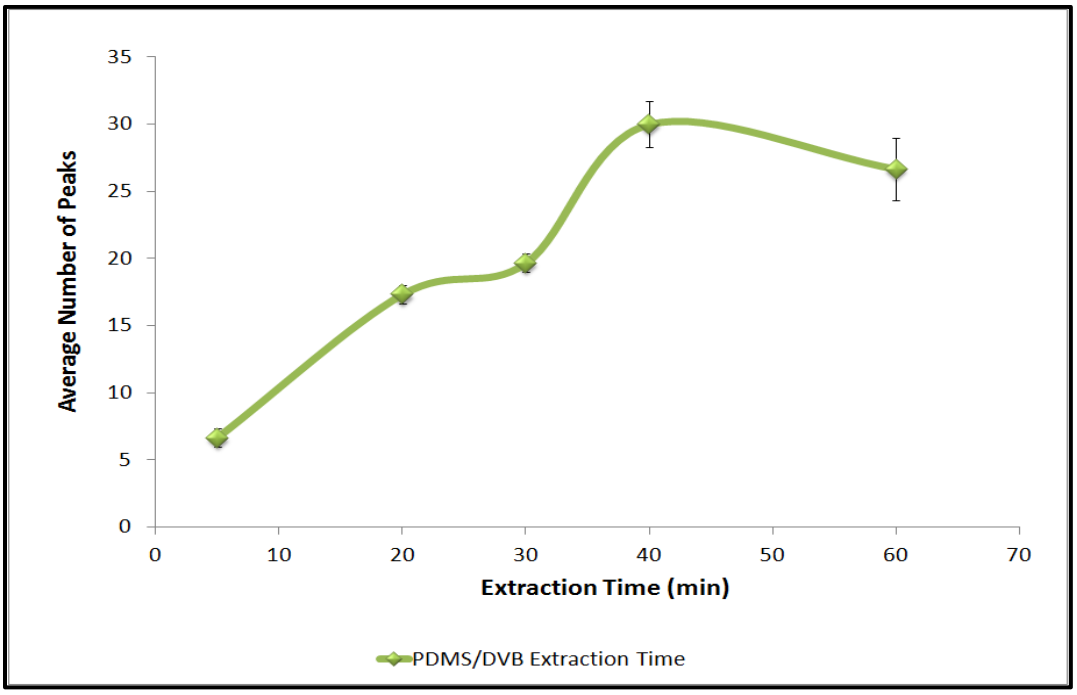


Figure 24. PDMS/DVB fiber Extraction Time profile for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

The decrease in the amount of extracted analytes at 60 min was attributed to volatile analytes decomposition or the release of the analytes by the PDMS/DVB fiber coating material. This coincides with the point of highest uncertainty in the PDMS/DVB extraction time profile given by the error bars. The lowest uncertainty in the extraction time profile for the PDMS/DVB fiber was observed at 20 and 30 min of extraction with a slightly increase in the uncertainty at 40 min, when the PDMS/DVB extracted the highest amount of volatile analytes.

7.1.2.3 DVB/CAR/PDMS Extraction Time Optimization

The DVB/CAR/PDMS fiber was subjected to study by varying the extraction times or the time the DVB/CAR/PDMS fiber coating material was exposed to the headspace of the flask extracting the volatile analytes from *Mammea Americana* L. fruit pulp while maintaining fixed the previously determined optimum equilibrium time, the desorption time, and under constant stirring conditions. As shown in **Table 9**, it was found that at 5 min of extraction, the DVB/CAR/PDMS fiber extracted a minimum amount of 11±1 volatile analytes with a %RSD of 5.09 and a standard error of 0.33. At 20 min of extraction, the amount of extracted analytes by DVB/CAR/PDMS increased to 24±3 volatile analytes with a slight loss in precision having a %RSD of 13.58 and a standard error of 1.86. Then at 30 min of extraction, the amount of extracted analytes increased to 27±2 with a %RSD of 5.59 and a standard error of 0.88 being indicative of relatively good precision at this extraction time. The increase in the amount of extracted analytes by DVB/CAR/PDMS fiber continued at 40 min of extraction with 36±1 volatile analytes, in this case with a remarkable improve in precision of the experimental results having a %RSD value of 1.59 and a standard error of 0.33. Finally, at 60 min the amount of extracted analytes had a slight increase to 39±7 volatile analytes although at this extraction time there was a significant loss in precision as demonstrated by the obtained %RSD of 16.83 and the standard error of 3.76.

Table 9. DVB/CAR/PDMS fiber Extraction Time optimization for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

DVB/CAR/PDMS	Number of Chromatographic Peaks						
Extraction time (min)	**Trial #1**	**Trial #2**	**Trial #3**	**Average**	**Std. Dev.**	**%RSD**	**Std. Error**
5	12	11	11	11	1	5.09	0.33
20	26	20	25	24	3	13.58	1.86
30	27	26	29	27	2	5.59	0.88
40	37	36	36	36	1	1.59	0.33
60	39	45	32	39	7	16.83	3.76
Equilibrium time = 30 min							
Desorption time = 5 min							

The behavior of the DVB/CAR/PDMS fiber toward the extraction of the volatile analytes of *Mammea Americana* L. fruit pulp at the optimum equilibrium time of 30 minutes was confirmed by analyzing its extraction time profile (see **Figure 25)**. The DVB/CAR/PDMS fiber extracted a minimum amount of analytes at 5 min. Then at 20 min there was an evident increase in the amount of analytes extracted, and as can be seen in the profile, an increasing pattern continued until the 60 min of extraction time.

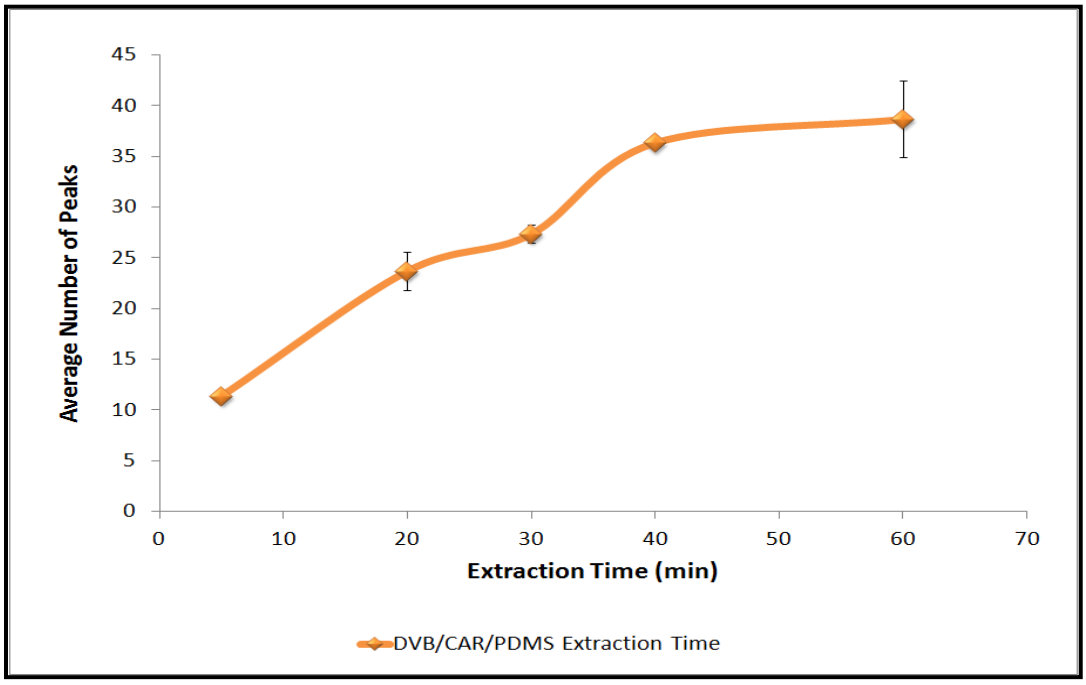


Figure 25. DVB/CAR/PDMS fiber Extraction Time profile for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

By analyzing the error bars from the extraction time profile for the DVB/CAR/PDMS fiber it was observed that at 5, 20, and 30 min of the fiber being exposed to the flask headspace adsorbing the volatile analytes of *Mammea Americana* L. fruit pulp there was low uncertainty in the fiber response. At 40 min of extraction, in where a higher response of the DVB/CAR/PDMS fiber to extract the volatile analytes was observed, a very low uncertainty was obtained as given by the error bars, which is indicative of an improved precision at that amount of time. Although at 60 min of extraction the fiber response was slightly higher compared to the 40 min of

extraction, a very high uncertainty was observed as given by the error bars, which in contrast is indicative of poor precision of the DVB/CAR/PDMS response at 60 min of extraction time.

7.1.2.4 Conclusion

During this validation and optimization process, it was possible to determine the optimum extraction time for each of the three different SPME fibers subjected to study maintaining fixed the optimum equilibrium time, which was 30 min for each of the fibers, the desorption time at 5 min and the Mamey fruit pulp sample under constant stirring conditions at 250 rpm during all the process. **Figure 26** shows the comparison of the extraction time profile for the three different SPME fibers tested.

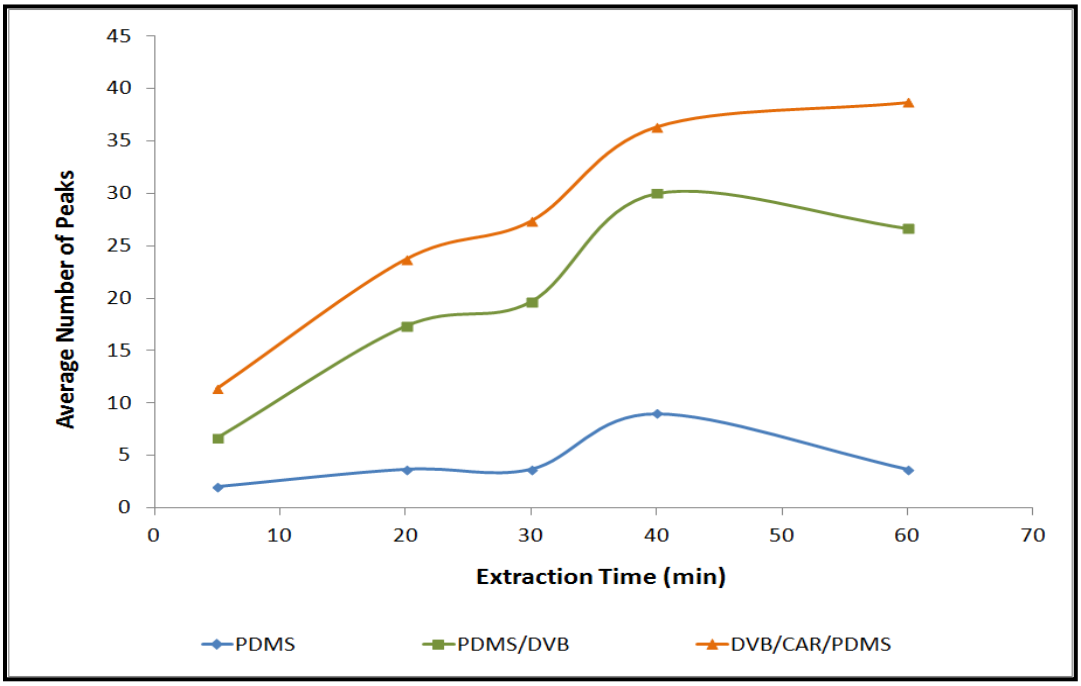


Figure 26. Different SPME fibers Extraction Time profile comparison for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

The PDMS fiber showed a somewhat different behavior toward the different extraction times tested compared to the PDMS/DVB and DVB/CAR/PDMS fibers. All of the SPME fibers tested extracted a minimum amount of volatile analytes at an extraction time of 5 min. At 20 and 30 min both the PDMS/DVB and DVB/CAR/PDMS fibers had an increase in the amount of extracted analytes. In contrast, the PDMS fiber extracted an almost constant amount of volatile analytes at 20 and 30 min of extraction. The PDMS and PDMS/DVB fibers showed their maximum capacity of extraction at 40 min followed by a decrease in the amount of extracted analytes at 60 min. This was attributed to the release of the volatile analytes by the PDMS and PDMS/DVB fiber coating material to the flask headspace or sample matrix with additional time of exposure or possible decomposition of the volatile analytes. On the other hand, the DVB/CAR/PDMS also had an evident increase in the amount of extracted analytes at 40 min but in contrast with the PDMS and PDMS/DVB at 60 min it showed a slight increase in the amount of extracted analytes.

For the PDMS fiber, it was found that the optimum extraction time was at 40 minutes of the fiber being exposed to the flask headspace absorbing the volatile analytes from *Mammea Americana* L. fruit pulp. PDMS was capable to extract a maximum amount of 9±2 volatile analytes with a %RSD of 19.25 and a standard error of 1.00. On the other hand, the determined optimum extraction time for the PDMS/DVB fiber was 40 minutes, time in which the fiber was capable to extract a maximum amount of 30±3 volatile analytes having a %RSD of 10.00 with a standard error of 1.73. The highest amount of volatile analytes extracted with the DVB/CAR/PDMS was observed at 60 min of extraction time in where 39±7 volatile analytes were extracted by the fiber with a %RSD of 16.83 and a standard error of 3.76. Those high values of standard deviation, standard error and %RSD are indicative of a high uncertainty which is indicative of poor precision in the fiber response at that extraction time. For this reason it was concluded that the optimum extraction time for the DVB/CAR/PDMS fiber was 40 minutes. This conclusion was sustained with the statistical facts which showed that at 40 min of extraction time, the DVB/CAR/PDMS was capable to extract 36±1 volatile analytes having a standard error of 0.33 and a %RSD of 1.59. Those low values of standard deviation, standard error and %RSD at 40 min of extraction are indicative of a very good precision in the DVB/CAR/PDMS response at that extraction time.

Finally, by comparing the amount of volatile analytes extracted by each of the three tested SPME fibers having optimized the equilibrium time and the extraction time, it was determined that PDMS did not show a good response or affinity toward the extraction of the volatile analytes of *Mammea Americana* L. fruit pulp. The amount of analytes extracted by PDMS was far below from the amount extracted by PDMS/DVB and DVB/CAR/PDMS which was indicative that the PDMS fiber coating material was not sensitive neither appropriate for the extraction of the volatile constituents of *Mammea Americana* L. fruit pulp. For this reason, the PDMS fiber was discarded to follow the next step of the validation/optimization process.

7.1.3 Desorption Time Optimization

The last critical parameter related to the HS-SPME technique that was validated and optimized was the desorption time or the optimum time that takes to the SPME fiber coating material to completely desorb the extracted volatile analytes in the hot injection port of the GC without retaining them on its surface. The PDMS/DVB and DVB/CAR/PDMS SPME fibers were subjected to a desorption study by varying the desorption times between at 5, 7, and 9 min, at a GC injection port temperature of 250°C while maintaining fixed other parameters such as the determined optimum equilibrium time of 30 min, the optimum extraction time of 40 min for both the PDMS/DVB and DVB/CAR/PDMS fibers, also the stirring was maintained constant at 250 rpm and the tests were performed at an average room temperature. One of the principal goals was to find a reasonable amount of time in where the SPME fiber completely desorbs all the extracted analytes and does not retain any residues adhered to its coating material. For that reason a blank was analyzed between each desorption at the GC injection port to test if the fiber effectively desorbs and do not retain compounds. Column charts of average number of chromatographic peaks (analytes desorbed) vs. desorption time (min), or desorption time profiles, were constructed to analyze the behavior of the PDMS/DVB and DVB/CAR/PDMS fibers at different desorption times.

7.1.3.1 PDMS/DVB Desorption Time Optimization

The PDMS/DVB fiber was subjected to study by varying the desorption times or the optimum time that takes to the PDMS/DVB fiber coating material to completely desorb the extracted volatile analytes in the hot injection port of the GC without retaining them on its surface, while maintaining fixed the determined optimum equilibrium and extraction times, and under constant stirring conditions. As shown in **Table 10**, at 5 minutes of desorption the PDMS/DVB was capable to completely desorb 29±2 volatile analytes at the GC injection port without retaining any residues as demonstrated by the subsequent analysis of a blank. The analytes desorption at 5 min was achieved with relatively good precision as demonstrated by a %RSD value of 7.44 and a standard error of 1.50.

Table 10. PDMS/DVB fiber Desorption Time optimization for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

PDMS/DVB	Number of Chromatographic Peaks					
Desorption time (min)	**Trial #1**	**Trial #2**	**Average**	**Std. Dev.**	**%RSD**	**Std. Error**
5	30	27	29	2	7.44	1.50
Fiber Residues	0	0	0	0	0.00	0.00
7	20	18	19	1	7.44	1.00
Fiber Residues	0	0	0	0	0.00	0.00
9	19	20	20	1	3.63	0.50
Fiber Residues	0	0	0	0	0.00	0.00
Equilibrium time = 30 min						
Extraction time = 40 min						

At 7 and 9 minutes of desorption, the PDMS/DVB fiber also desorbed completely the extracted volatile analytes with good precision, as demonstrated by the obtained %RSD and standard error values, although it was observed a decrease in the amount of desorbed volatile analytes identified by chromatographic separation which was attributed to thermal decomposition of the volatile analytes. PDMS/DVB also did not retained any residues at 7 and 9 min of desorption time (see **Figure 27**).

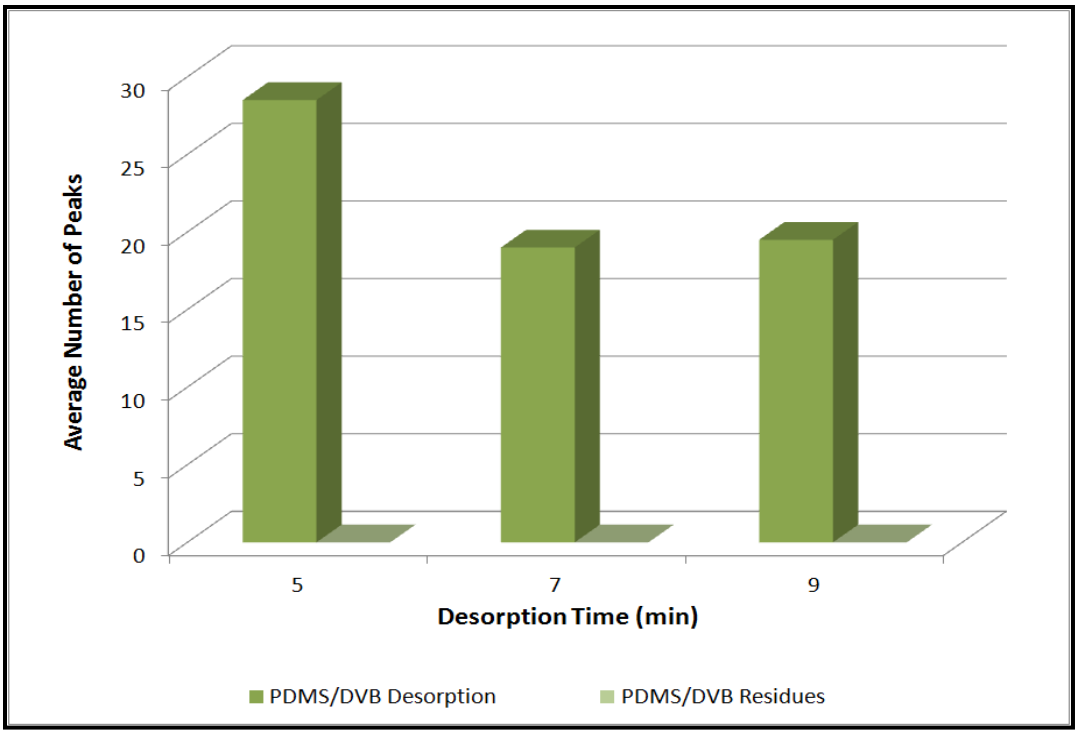


Figure 27. PDMS/DVB fiber Desorption Time profile for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

7.1.3.2 DVB/CAR/PDMS Desorption Time Optimization

The DVB/CAR/PDMS fiber was subjected to study by varying the desorption times or the optimum time that takes to the DVB/CAR/PDMS fiber coating material to completely desorb the extracted volatile analytes in the hot injection port of the GC without retaining them on its surface while maintaining fixed the determined optimum equilibrium and extraction times and under constant stirring conditions. As shown in **Table 11**, at 5 minutes of desorption the DVB/CAR/PDMS fiber was capable to completely desorb 37±1 volatile analytes at the GC injection port having a good precision as demonstrated by a %RSD value of 3.82 and a standard error of 1.00. At 5 min of desorption, DVB/CAR/PDMS did not retained any residues as demonstrated by the subsequent analysis of a blank.

Table 11. DVB/CAR/PDMS fiber Desorption Time Optimization for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

DVB/CAR/PDMS	Number of Chromatographic Peaks					
Desorption time (min)	**Trial #1**	**Trial #2**	**Average**	**Std. Dev.**	**%RSD**	**Std. Error**
5	36	38	37	1	3.82	1.00
Fiber Residues	0	0	0	0	0.00	0.00
7	27	31	29	3	9.75	2.00
Fiber Residues	0	0	0	0	0.00	0.00
9	29	29	29	0	0.00	0.00
Fiber Residues	0	0	0	0	0.00	0.00
Equilibrium time = 30 min						
Extraction time = 40 min						

DVB/CAR/PDMS fiber also completely desorbed the extracted volatile analytes at 7 and 9 min of desorption, although it was observed a decrease in the amount of volatile analytes identified by the chromatographic separation which was attributed to thermal decomposition of the volatile analytes. In this case a loss in precision in the amount of desorbed analytes at the GC injection port was observed at 7 min of desorption as demonstrated by the %RSD of 9.75 with a standard error of 2.00 which was higher compared to the ones obtained with 5 and 9 min of desorption. As can be seen in **Figure 28**, DVB/CAR/PDMS also did not retain any residues at 7 and 9 min of desorption time.

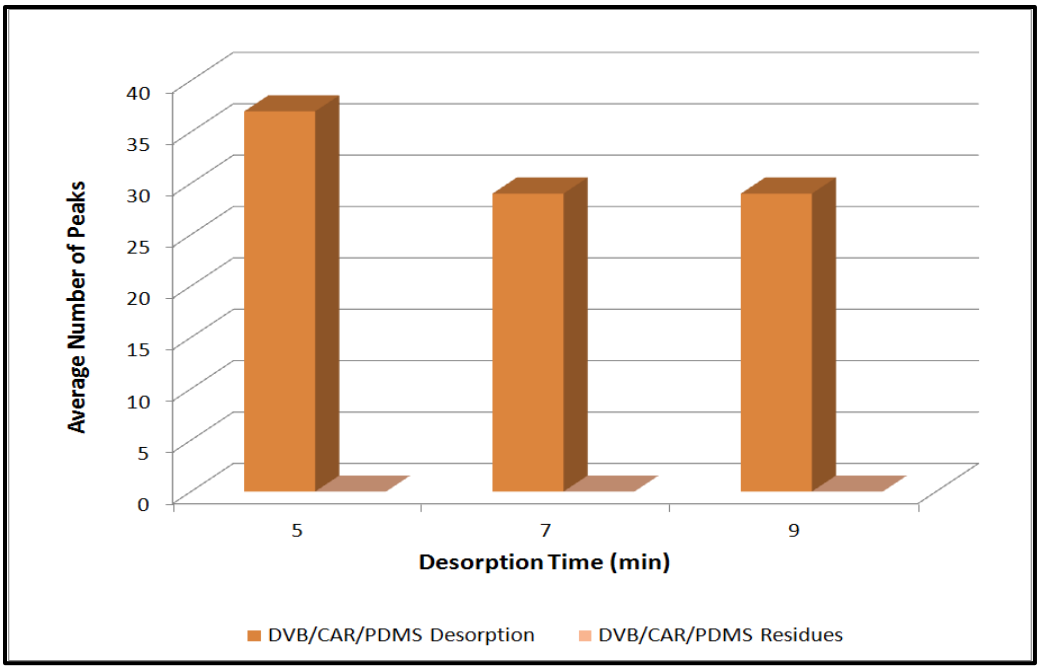


Figure 28. DVB/CAR/PDMS fiber Desorption Time profile for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

7.1.3.3 Conclusion

During this validation and optimization process, it was possible to determine the optimum desorption time for the PDMS/DVB and DVB/CAR/PDMS SPME fibers subjected to study maintaining fixed the equilibrium and extraction times at 30 and 40 min, respectively, with the Mamey fruit pulp sample under constant stirring conditions at 250 rpm during all the study. **Figure 29** shows the comparison of the desorption time profiles for the SPME fibers tested.

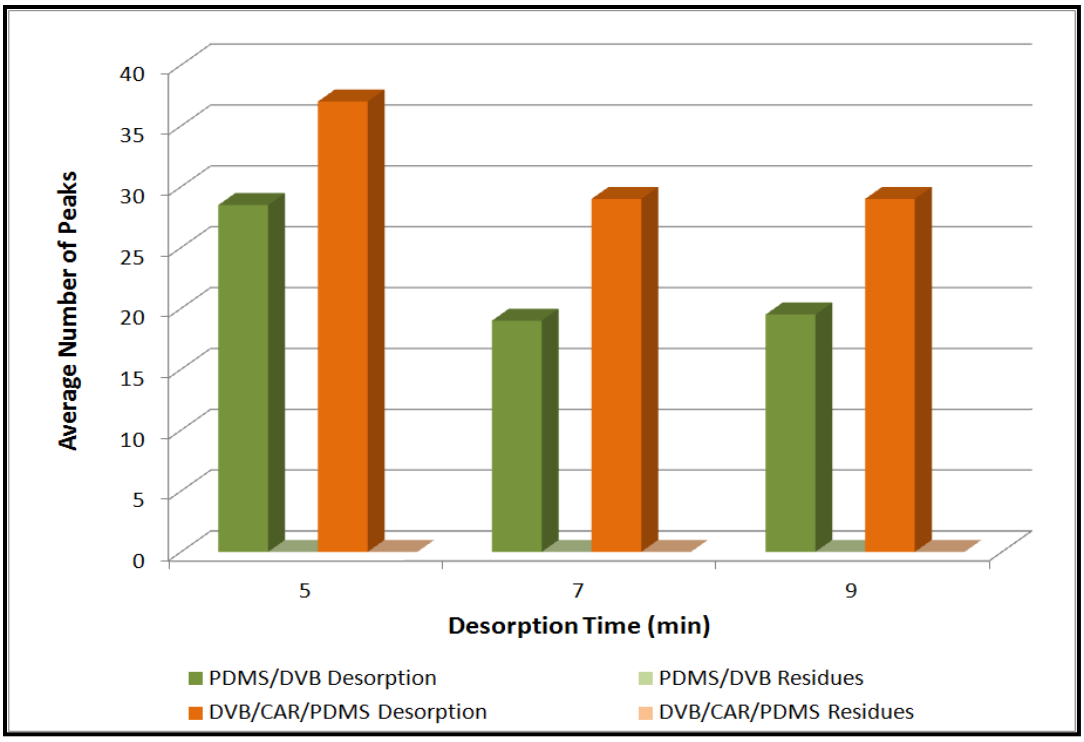


Figure 29. Different SPME fibers Desorption Time profile comparison for the analysis of the volatile constituents of *Mammea Americana* L. fruit pulp

Both the PDMS/DVB and DVB/CAR/PDMS SPME fibers showed that 5 minutes at the GC injection port were sufficient for their coating material to completely desorbs the extracted analytes from *Mammea Americana* L. fruit pulp sample without retaining any residues and in the case of the DVB/CAR/PDMS fiber clearly having a good precision. In other words, it was concluded that 5 minutes was the optimum desorption time for both fibers PDMS/DVB and DVB/CAR/PDMS since the purpose was to find a reasonable amount of time in where the SPME fiber completely desorbs all the extracted analytes, does not retain any residues adhered to its coating material, and avoiding thermal decomposition of the analytes at the GC injection port.

7.1.4 SPME Fiber Selection

In conclusion, the SPME technique validation and optimization steps were successfully completed. From this process it was concluded that the SPME fiber coating material that

demonstrated to be the most appropriated, efficient and suitable having showed the highest affinity, sensitivity and reproducibility toward the extraction of the volatile constituents of *Mammea Americana* L. fruit pulp was the bi-polar adsorbent DVB/CAR/PDMS fiber. In all the different optimization studies performed this fiber coating material was capable to extract and desorb the highest amount of volatile analytes from the fruit pulp sample with a remarkable improve in precision and in a reasonable amount of time compared to the other SPME fiber coating materials tested. The low uncertainty obtained in the experimental results by using the DVB/CAR/PDMS fiber was demonstrated statistically by the calculated values of standard deviation, percent relative standard deviation and standard errors.

It was validated that the optimum equilibrium time for the flask headspace/sample matrix was 30 minutes and that the optimum extraction time or the necessary amount of time for the DVB/CAR/PDMS SPME fiber to be exposed through the flask headspace adsorbing the maximum amount of volatile analytes from *Mammea Americana* L. fruit was 40 minutes. On the other hand, 5 minutes at the GC injection port are a reasonable amount of time for the fiber to desorb completely the volatile analytes into the GC carrier gas stream without leaving any residues in the fiber coating material. Since PDMS/DVB fiber showed good precision during the optimization process but it did not extract the highest amount of volatile analytes, it can be considered as a second alternative for the extraction of the volatile constituents from Mamey fruit or when the DVB/CAR/PDMS fiber is not available.

7.2 HS-SPME/GC/MS Analysis of the Volatile Constituents of *Mammea Americana* L. fruit

The volatile constituents of *Mammea Americana* L. fruit pulp from different municipalities in Puerto Rico were extracted using the Head Space-Solid Phase Microextraction (HS-SPME) technique with a DVB/CAR/PDMS fiber and analyzed through Gas Chromatography coupled to Mass Spectrometry (GC/MS).

7.2.1 HS-SPME/GC/MS of *Mammea Americana* L. fruit at different experimental conditions

The HS-SPME/GC/MS analysis was performed by different experimental conditions in order to determine if the Mamey fruit pulp volatile composition can vary when exposed to different environmental factors. The DVB/CAR/PDMS fiber was first exposed to the flask headspace with the Mamey fruit sample at average room temperature (23-25°C), the other analysis consisted in mixing Mamey fruit pulp sample with 5 mL of deionized water at room temperature, and last one consisted in slightly heating the fruit pulp sample (flask headspace temperature between 30-32°C). **Figure 30** shows the merged Total Ion Chromatograms (TIC) obtained by HS-SPME/GC/MS with Mamey fruit at room temperature and by heating the fruit sample.

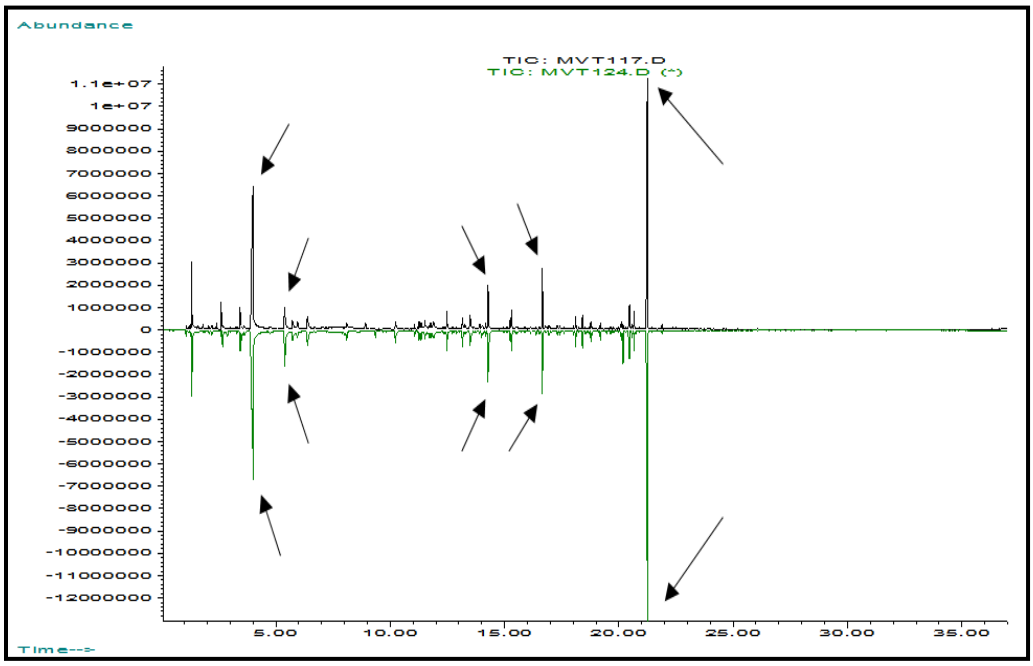


Figure 30. Merged Total Ion Chromatograms (TIC) obtained by GC/MS analysis of *Mammea Americana* L. fruit pulp applying HS-SPME technique at average room temperature (above) and by heating the fruit sample (below) using DVB/CAR/PDMS fiber

Both total ion chromatograms resemble mirror images and only a slight difference was observed in the abundance of some chromatographic peaks as can be seen in the major compounds indicated by black arrows on **Figure 30**. Specifically, the slight heating of the

Mamey fruit sample only caused an apparent increase in the abundance of the volatile compounds which contrasted with the chromatographic results obtained when the Mamey fruit sample was mixed with deionized water in where a decrease in the abundance of the chromatographic peaks was observed. This comparison is relative to the abundance of the chromatographic peaks obtained when HS-SPME was performed with the fruit sample at room temperature.

7.2.2 HS-SPME/GC/MS Analysis of *Mammea Americana* L. fruit pulp from different municipalities

The volatile composition of *Mammea Americana* L. fruit pulp from trees located at four different municipalities in Puerto Rico including Rincon, Mayagüez, Aguada and Cayey (3, 1193, 118 and 1283 ft of respective altitudes) was obtained and identified by HS-SPME/GC/MS with Mamey fruit samples at room temperature. The highest amount of volatile compounds was found in the Mamey fruit from Mayagüez with a total of 44 volatile compounds obtained by GC/MS analysis. Mamey from Mayagüez also had the highest amount of identified compounds with a 59.1% or 26 compounds with a match quality higher than 70 percent using the Wiley7.L and Wiley10.L mass spectral libraries. An amount of 40 volatile compounds were obtained by GC/MS analysis of Mamey fruit from Aguada in which a 32.5% or 13 compounds were identified with higher than 70 percent of match quality with the two mass spectral libraries. For Mamey fruit from Rincon, 29 volatile compounds were obtained from which a 51.7% or 15 compounds were identified with a high percent of match quality using the Wiley7.L and Wiley10.L mass spectral libraries. The least amount of volatile compounds was found in Mamey fruit from Cayey, with 26 compounds obtained by GC/MS analysis, from which 42.3% or 11 compounds were identified with a high match quality percent (see **Table 12**).

Table 12. Amount of chemical compounds in the volatile constituents of *Mammea Americana* L. fruit pulp from different municipalities obtained by HS-SPME/GC/MS analysis

Municipalities	Identified Compounds	Total Compounds	% Identified Compounds
Rincon	15	29	51.7
Mayagüez	26	44	59.1
Aguada	13	40	32.5
Cayey	11	26	42.3
Average	**16 ± 7**	**35 ± 8**	**46 ± 11**

Table 31 in **Appendix B** shows the complete list of chemical compounds obtained by HS-SPME/GC/MS analysis of the volatile composition of *Mammea Americana* L. fruit from Rincon, Mayagüez, Aguada and Cayey which were classified according to their chemical families. The volatile constituents responsible of the characteristic aroma of *Mammea Americana* L. included chemical compounds classified as alcohols, aldehydes, alkylphosphines, benzene derivatives, carboxylic acids, diazepines, esters, ethers, furans, hydrocarbons, ketones, lactones, monoterpenes, norisoprenoids, pyrazolines, pyridines, and sesquiterpenes.

Table 13. Percent relative amount of volatile constituents distributed by families found in *Mammea Americana* L. fruit pulp samples from the four different municipalities

	Relative Amount (%)				
Compound Classification	**Rincon**	**Mayagüez**	**Aguada**	**Cayey**	**Average**
Alcohols	6.9	4.5	7.5	7.7	6.7
Aldehydes	20.7	18.2	12.5	19.2	17.7
Alkylphosphines	0.0	0.0	2.5	0.0	0.6
Benzene derivatives	3.4	0.0	0.0	0.0	0.9
Carboxylic acids	6.9	4.5	10.0	0.0	5.4
Diazepines	0.0	0.0	2.5	0.0	0.6
Esters	20.7	9.1	7.5	23.1	15.1
Ethers	0.0	2.3	0.0	0.0	0.6
Furans	3.4	2.3	0.0	3.8	2.4
Hydrocarbons	3.4	13.6	10.0	3.8	7.7
Ketones	10.3	20.5	20.0	19.2	17.5
Lactones	3.4	2.3	2.5	0.0	2.1
Monoterpenes	6.9	4.5	10.0	7.7	7.3
Norisoprenoids	10.3	15.9	10.0	11.5	11.9
Pyrazolines	3.4	0.0	0.0	0.0	0.9
Pyridines	0.0	0.0	2.5	0.0	0.6
Sesquiterpenes	0.0	2.3	2.5	3.8	2.2
Total Number of Compounds	**29**	**44**	**40**	**26**	

From **Table 13**, it can be observed that in the volatile constituents of *Mammea Americana* L. fruit from Rincon, the most abundant compounds were aldehydes and esters, both with relative amounts of 20.7% of the volatile fraction. The most abundant compounds in Mamey fruit from Mayagüez were ketones with a relative amount of 20.5% of the volatile fraction followed by aldehydes with 18.2%. Ketones and aldehydes were the most abundant compounds in the volatile fraction of Mamey fruit from Aguada with relative amounts of 20.0% and 12.5%, respectively. In the Mamey fruit from Cayey, esters were the most predominant compounds with a relative amount of 23.1% of the volatile fraction followed by aldehydes and ketones both with relative amounts of 19.2%.

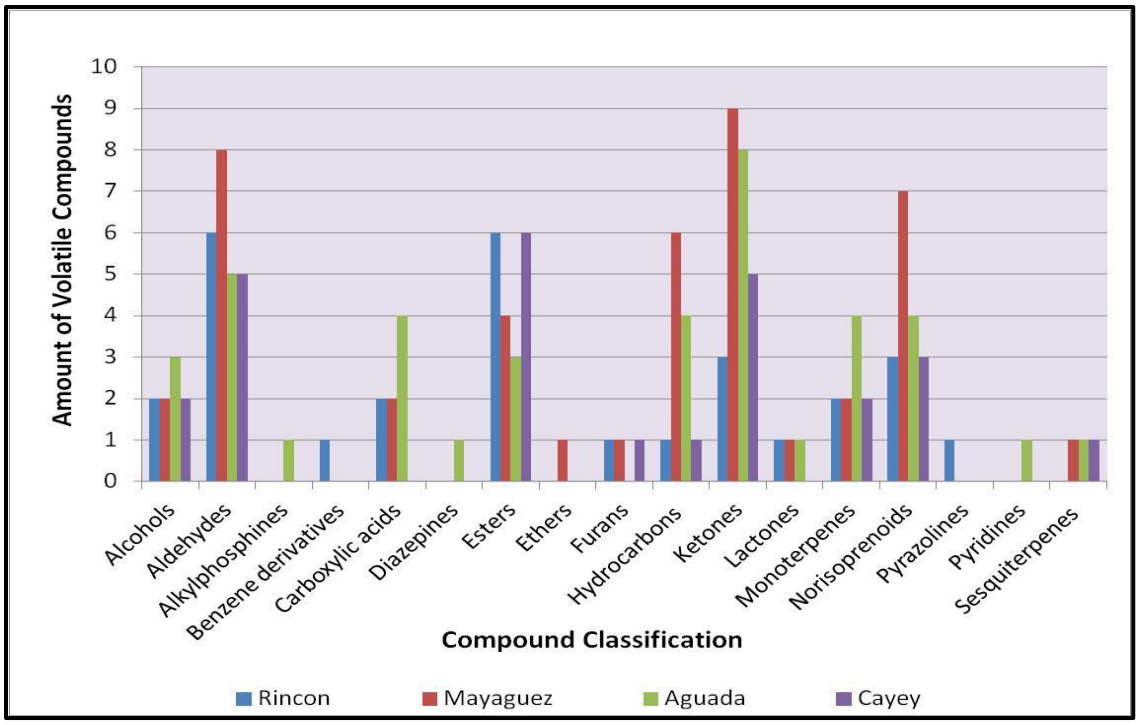


Figure 31. Distribution by families of the volatile constituents of *Mammea Americana* L. fruit pulp from the four different municipalities by HS-SPME/GC/MS analysis

Figure 31 confirms that the most abundant compounds in the volatile constituents of *Mammea Americana* L. fruit pulp from the different municipalities were aldehydes followed by ketones. Also it is important to note that Mamey fruit from Mayagüez has the highest amount of both classes of compounds in its volatile fraction.

7.2.3 Chromatographic Profile of the Volatile Constituents of *Mammea Americana* L. fruit from different municipalities

The chromatographic profiles of *Mammea Americana* L. fruit pulp from the different municipalities were carefully analyzed to identify which compounds can be responsible of the characteristic aroma of the fruit. The most abundant volatile fraction or major compounds can have a strong influence in the aroma of a fruit although it is known that other less abundant compounds can mask the characteristic odors that can be perceived by human olfaction (Sagrero-Nieves et al., 1989). **Table 14** shows the top ten compounds or major volatile constituents of Mamey fruit from Rincon. The first major compound identified was hexanal (**1**) which eluted

from the chromatographic column at a retention time of 3.97 min with a percent relative area of 21.24%, followed by methyl tiglate (**2**) at a retention time of 6.49 min with a percent relative area of 15.45%. Those compounds were followed by β-ionone (**3**) which eluted at a retention time of 21.22 min with a percent relative area of 8.78%, then butyric acid (**4**) with a percent relative area of 6.87% eluted at a retention time of 3.44 min, and β-Cyclocitral (**5**) at a retention time of 16.65 min with a percent relative area of 5.46%. The other major volatile compounds obtained were 6,6-dimethylhepta-2,4-diene with 2.70%, (2E,4E)-2,4-heptadienal with 2.68%, 4-methyl-6-hepten-3-one with 2.58%, 6-methyl-2-heptanone with 2.17%, and 1-isopropyl-5-methyl-2-pyrazoline with 1.86% of relative area. The total ion chromatogram obtained by GC/MS analysis of the volatile constituents of *Mammea Americana* L. fruit from Rincon can be seen in **Figure 32** in which the first five major compounds of the volatile fraction were identified with numbers.

Table 14. Major volatile constituents of *Mammea Americana* L. fruit pulp from Rincon obtained by HS-SPME/GC/MS

Rincon	Volatile Compound	Relative Area (%)*	Rt (min)	Molecular Formula	Aroma Profile [a,b]
1	Hexanal	21.24	3.97	$C_6H_{12}O$	fatty, green, grassy, powerful, penetrating, fruity
2	Methyl tiglate	15.45	6.49	$C_6H_{10}O_2$	ethereal
3	β-Ionone	8.78	21.22	$C_{13}H_{20}O$	violet-like, fruity and woody
4	Butyric acid	6.87	3.44	$C_6H_{12}O_2$	sweet, fruity, apple-like
5	β-Cyclocitral	5.46	16.65	$C_{10}H_{16}O$	minty
6	6,6-Dimethylhepta-2,4-diene	2.70	6.03	C_9H_{16}	-
7	(2E,4E)-2,4-heptadienal	2.68	11.91	$C_7H_{10}O$	fatty, green
8	6-hepten-3-one, 4-methyl	2.58	11.27	$C_8H_{14}O$	-
9	2-heptanone, 6-methyl	2.17	14.29	$C_8H_{16}O$	camphoreous
10	2-pyrazoline, 1-isopropyl-5-methyl	1.86	15.31	$C_7H_{14}N_2$	-

*percent relative to the total integrated peak areas of the chromatogram

[a] Burdock, G.A. Fenaroli's handbook of flavor ingredients

[b] www.thegoodscentscompany.com

The aroma profile of Mamey fruit from Rincon had the predominant fruity, fatty and green fragrances of hexanal, β-ionone and (2E,4E)-2,4-heptadienal but with the influence of the pungent ethereal smell characteristic of methyl tiglate masked by the sweet, minty and camphoreous odors of butyric acid, β-cyclocitral and 6-methyl-2-heptanone.

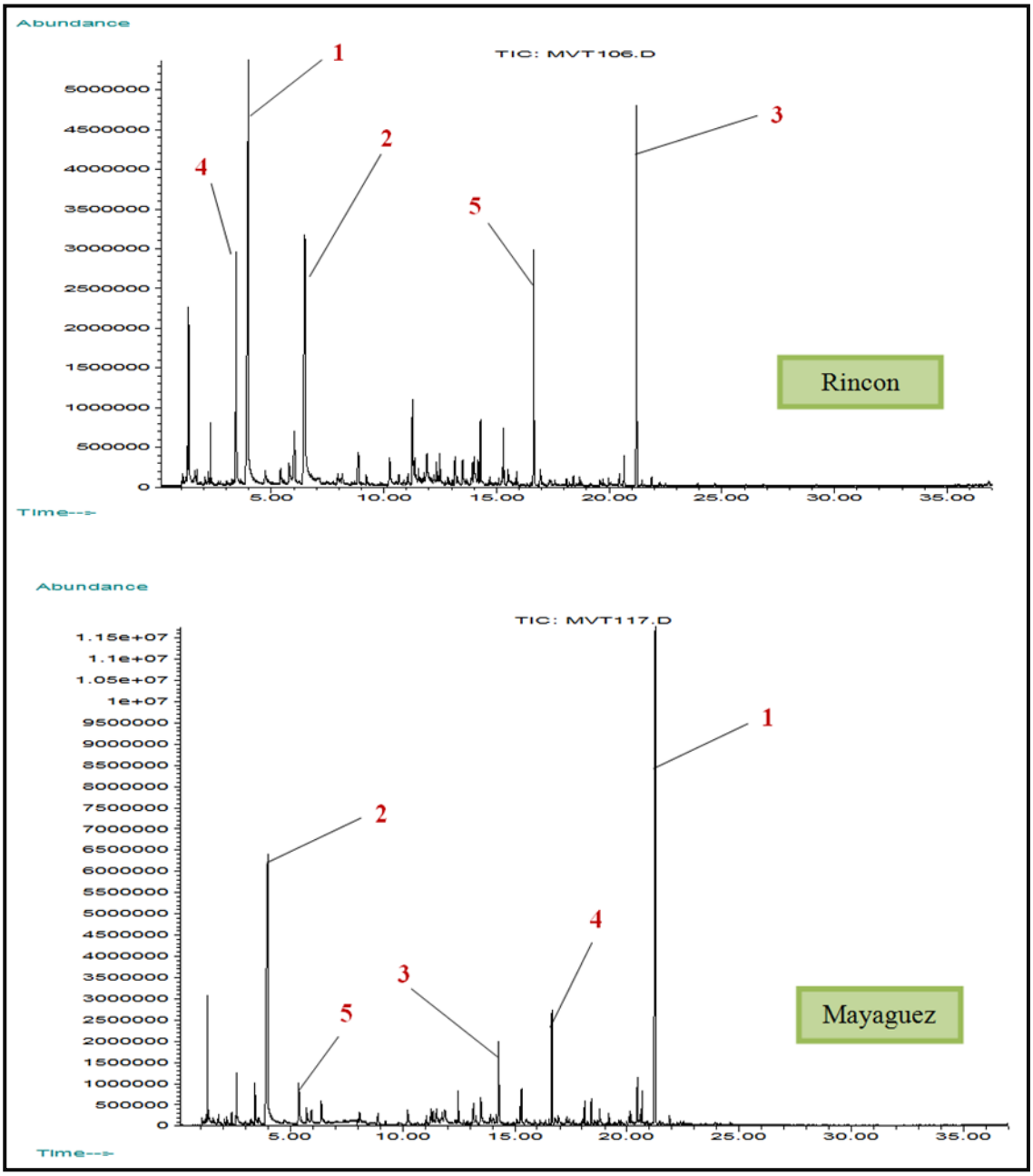


Figure 32. Total Ion Chromatograms (TIC) obtained by GC/MS analysis of *Mammea Americana* L. fruit pulp samples from Rincon (above) and Mayagüez (below) municipalities applying HS-SPME technique using the DVB/CAR/PDMS fiber

Top ten compounds or the major volatile constituents of Mamey fruit from Mayagüez are shown in **Table 15**. The first major compound identified was β-ionone (**1**) which eluted at a retention time of 21.25 min from the chromatographic column with a percent relative area of 24.34%, followed by hexanal (**2**) with a percent relative area of 22.91% at a retention time of 3.99 min. They were followed by 6-methyl-2-heptanone (**3**) which eluted at a retention time of 14.28 min with a percent relative area of 3.78%, then β-cyclocitral (**4**) with a percent relative area of 3.77% at a retention time of 16.65 min, and 3-methyl-2-butenoic acid methyl ester (**5**) at a retention time of 5.39 min with a percent relative area of 2.62%. The other major volatile compounds included butyric acid with 2.19%, cis-1,3,5-trimethyl-cyclohexane with 1.96%, 1,4-pentadiene with 1.93%, β-ionol with 1.75%, and (E,E) 3,5-octadiene-2-one with 1.59% of relative area. The total ion chromatogram for the volatile constituents of *Mammea Americana* L. fruit from Mayagüez can be seen in **Figure 32** in which the first five major compounds of the volatile fraction were identified with numbers.

Table 15. Major volatile constituents of *Mammea Americana* L. fruit pulp from Mayagüez obtained by HS-SPME/GC/MS

Mayagüez	Volatile Compound	Relative Area (%)*	Rt (min)	Molecular Formula	Aroma Profile [a,b]
1	β-Ionone	24.34	21.25	$C_{13}H_{20}O$	violet-like, fruity and woody
2	Hexanal	22.91	3.99	$C_6H_{12}O$	fatty, green, grassy, powerful, penetrating, fruity
3	2-Heptanone, 6-methyl	3.78	14.28	$C_8H_{16}O$	camphoreous
4	β-Cyclocitral	3.77	16.65	$C_{10}H_{16}O$	minty
5	2-Butenoic acid, 3-methyl, methyl ester	2.62	5.39	$C_6H_{10}O_2$	-
6	Butyric acid	2.19	3.43	$C_6H_{12}O_2$	sweet, fruity, apple-like
7	Cyclohexane, 1,3,5-trimethyl-, cis-	1.96	15.31	C_9H_{18}	-
8	1,4-Pentadiene	1.93	2.61	C_5H_8	-
9	β-Ionol	1.75	20.46	$C_{13}H_{22}O$	sweet, floral-balsamic, warm
10	(E,E) 3,5-Octadiene-2-one	1.59	13.49	$C_8H_{12}O$	fruity

*percent relative to the total integrated peak areas of the chromatogram

[a] Burdock, G.A. Fenaroli's handbook of flavor ingredients

[b] www.thegoodscentscompany.com

The aroma profile of Mamey fruit from Mayagüez had a strong influence of the fruity, woody, green and sweet fragrances of β-ionone, hexanal, (E,E) 3,5-Octadiene-2-one, β-Ionol and butyric acid but with a touch of the camphoreous and minty odors of 6-methyl-2-heptanone and β-cyclocitral.

Table 16 shows the top ten compounds or major volatile constituents of Mamey fruit from Aguada. The first major compound identified was hexanal (**1**) which eluted at a retention time of 3.97 min from the chromatographic column with a percent relative area of 22.10%, followed by β-ionone (**2**) with a percent relative area of 18.13% at a retention time of 21.23 min. Those were followed by α-methylbutyric acid (**3**) with a percent relative area of 4.94% which eluted at a retention time of 8.81 min, then α,β-dihydropseudoionone (**4**) at a retention time of 20.67 min with a percent relative area of 3.89%, and butyric acid (**5**) with a percent relative area of 3.16% which eluted at a retention time of 3.43 min. The other major volatile compounds included β-cyclocitral with 2.78%, 3,4-dimethylcyclopent-2-en-1-one with 2.42%, cyclopentene with 1.86%, 1-(1,2,2-trimethylcyclopent-1-yl)pent-2-ene-1,4-dione with 1.61%, and 2-ethyl-1-hexanol with 1.46% of relative area. The total ion chromatogram for the volatile constituents of *Mammea Americana* L. fruit from Aguada can be seen in **Figure 33** in which the first five major compounds of the volatile fraction were identified with numbers.

Table 16. Major volatile constituents of *Mammea Americana* L. fruit pulp from Aguada obtained by HS-SPME/GC/MS

Aguada	Volatile Compound	Relative Area (%)*	Rt (min)	Molecular Formula	Aroma Profile [a,b]
1	Hexanal	22.10	3.97	$C_6H_{12}O$	fatty, green, grassy, powerful, penetrating, fruity
2	β-Ionone	18.13	21.23	$C_{13}H_{20}O$	violet-like, fruity and woody
3	α-Methylbutyric acid	4.94	8.81	$C_5H_{10}O_2$	pungent, acrid, fruity
4	α,β-Dihydropseudoionone	3.89	20.67	$C_{13}H_{22}O$	floral, green, fruity, waxy, woody, pear, rosy
5	Butyric acid	3.16	3.43	$C_6H_{12}O_2$	sweet, fruity, apple-like
6	β-Cyclocitral	2.78	16.65	$C_{10}H_{16}O$	minty
7	3,4-dimethylcyclopent-2-en-1-one	2.42	14.28	$C_7H_{10}O$	-
8	Cyclopentene	1.86	2.62	C_5H_8	-
9	1-(1,2,2-Trimethylcyclopent-1-yl)pent-2-ene-1,4-dione	1.61	15.31	$C_{13}H_{20}O_2$	-
10	2-Ethyl-1-hexanol	1.46	12.49	$C_8H_{18}O$	mild, oily, sweet, slightly floral, fruity

*percent relative to the total integrated peak areas of the chromatogram

[a] Burdock, G.A. Fenaroli's handbook of flavor ingredients

[b] www.thegoodscentscompany.com

The aroma profile of Mamey fruit from Aguada had a predominant influence of the fruity, woody and green fragrances of hexanal, β-ionone, and α,β-dihydropseudoionone with a touch of the pungent and acrid smell of α-methylbutyric acid which was masked by the minty odor of β-cyclocitral and the sweet fragrance of butyric acid and 2-ethyl-1-hexanol.

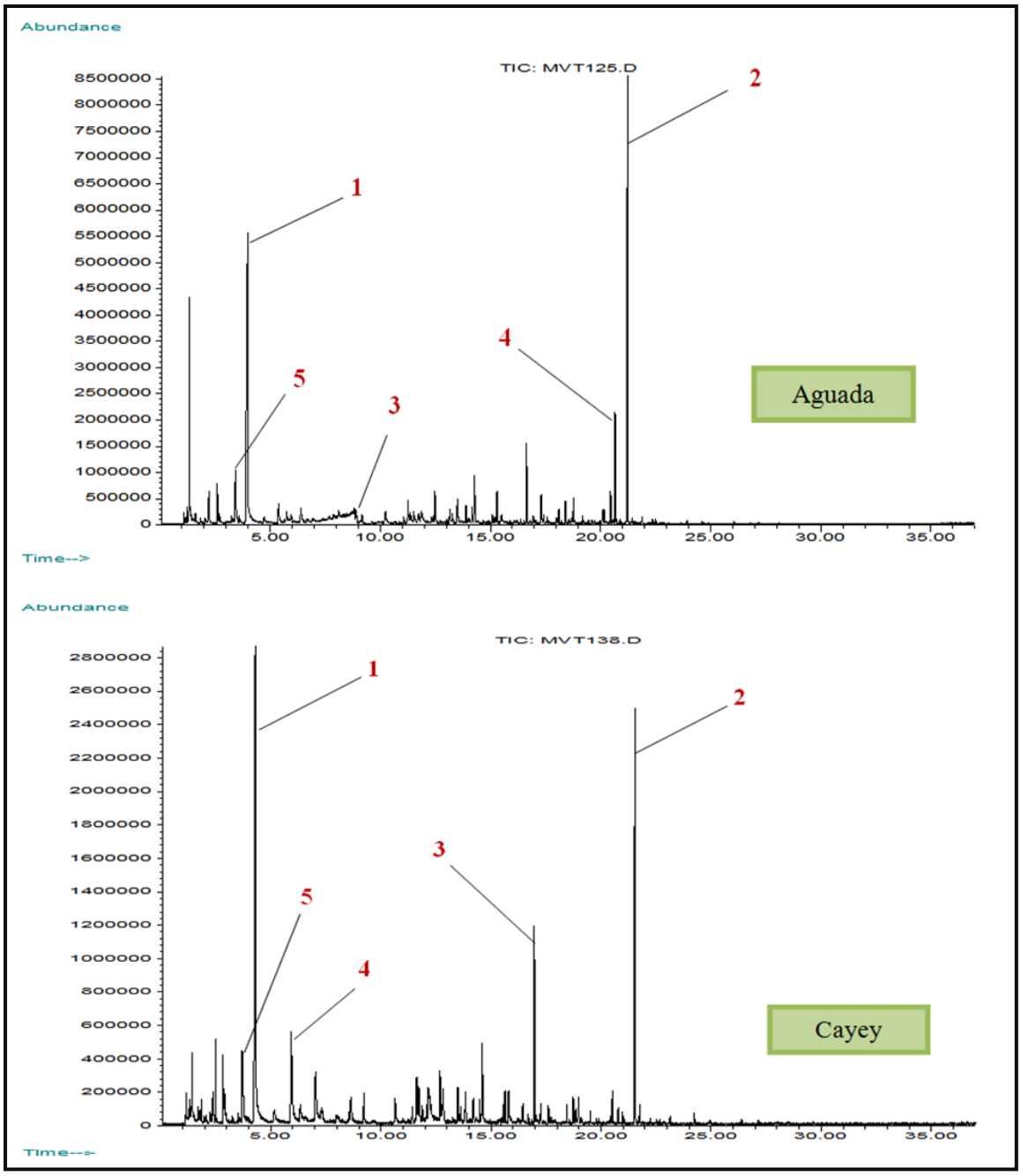


Figure 33. Total Ion Chromatograms (TIC) obtained by GC/MS analysis of *Mammea Americana* L. fruit pulp samples from Aguada (above) and Cayey (below) municipalities applying HS-SPME technique using the DVB/CAR/PDMS fiber

Top ten compounds or the major volatile constituents of Mamey fruit from Cayey are shown in **Table 17**. The first major compound identified was hexanal (**1**) which eluted at a retention time of 4.29 min from the chromatographic column with a percent relative area of

24.63%, followed by β-ionone (**2**) with a percent relative area of 11.24% at a retention time of 21.54 min. Those were followed by β-cyclocitral (**3**) which eluted at a retention time of 16.98 min with a percent relative area of 5.85%, then 3-methyl-2-butenoic acid methyl ester (**4**) with a percent relative area of 5.81% at a retention time of 5.93 min, and 3-methyl-2- buten-1-ol (**5**) which eluted at a retention time of 3.71 min with a percent relative area of 4.59%. The other major volatile compounds included nonanal with 3.70%, 3-methyl-3-buten-1-ol with 3.50%, methyl tiglate with 3.22%, heptanal with 2.04%, and 2,2,6-trimethyl cyclohexanone with 2.01% of relative area. The total ion chromatogram for the volatile constituents of *Mammea Americana* L. fruit from Cayey can be seen in **Figure 33** in which the first five major compounds of the volatile fraction were identified with numbers.

Table 17. Major volatile constituents of *Mammea Americana* L. fruit pulp from Cayey obtained by HS-SPME/GC/MS

Cayey	Volatile Compound	Relative Area (%)*	Rt (min)	Molecular Formula	Aroma Profile [a,b]
1	Hexanal	24.63	4.29	$C_6H_{12}O$	fatty, green, grassy, powerful, penetrating, fruity
2	β-Ionone	11.24	21.54	$C_{13}H_{20}O$	violet-like, fruity and woody
3	β-Cyclocitral	5.85	16.98	$C_{10}H_{16}O$	minty
4	2-Butenoic acid, 3-methyl, methyl ester	5.81	5.93	$C_6H_{10}O_2$	-
5	2-Buten-1-ol, 3-methyl	4.59	3.71	$C_5H_{10}O$	phenolic, metallic
6	Nonanal	3.70	14.60	$C_9H_{18}O$	strong, fatty, citrus-like
7	3-buten-1-ol, 3-methyl	3.50	2.83	$C_5H_{10}O$	sweet fruity
8	Methyl tiglate	3.22	7.03	$C_6H_{10}O_2$	ethereal
9	Heptanal	2.04	8.64	$C_7H_{14}O$	very strong, fatty, harsh, pungent
10	Cyclohexanone, 2,2,6-trimethyl	2.01	12.82	$C_9H_{16}O$	thujonic

*percent relative to the total integrated peak areas of the chromatogram

[a] Burdock, G.A. Fenaroli's handbook of flavor ingredients

[b] www.thegoodscentscompany.com

The aroma profile of Mamey fruit from Cayey was mostly influenced by the fatty and fruity fragrances of hexanal, β-ionone with the presence of the ethereal and pungent smell of methyl tiglate and heptanal masked by the minty and sweet odors of β-cyclocitral and 3-methyl-3-buten-1-ol.

7.2.4 Conclusion

It was possible to determine and identify the volatile constituents that are responsible of the characteristic aroma of *Mammea Americana* L. fruit by HS-SPME/GC/MS analysis with the help of the Wiley7.L and Wiley10.L mass spectral libraries. Varying the experimental conditions in where the HS-SPME technique was applied did not show a significant difference in the obtained results since the volatile composition of the fruit practically remained the same. Major compounds found in the volatile fraction of *Mammea Americana* L.fruit pulp were very similar between the fruit samples of different municipalities analyzed. Volatile compounds such as hexanal, β-ionone and β-cyclocitral were common compounds between the major compounds at the Mamey fruit from each of the different municipalities. It is important to remark that β-ionone and α-methylbutyric acid were reported in previous works by Sagrero-Nieves et al. (1989) and Morales et. al (1993) as the most prominent volatile constituents of *Mammea Americana* L. fruit.

In general, the most aromatic *Mammea Americana* L. fruit sample resulted to be the one from Mayagüez, since it had the highest amount of extracted volatile analytes with the higher abundance of the chromatographic peaks obtained by GC/MS analysis compared to the other fruit samples analyzed. Mamey fruit from Cayey resulted to be the least aromatic fruit. It is important to note that the amount of precipitation in the sampling areas affects the quality and abundance of the aroma volatile compounds in the fruit samples. In weather stations reports, Mayagüez municipality has the highest average annual precipitation, with 85.38 inches, followed by Cayey with 76.15 inches. Therefore, it can be concluded that in a region with higher precipitation, the abundance of aromatic compounds in the fruit pulp is higher. The reason for that Mamey fruit from Cayey resulted to be the least aromatic fruit can be attributed to abnormally dry conditions in that region of Puerto Rico during the tree fruit production and sample collection period (June-August 2014), as reported by the U.S. Drought Monitor (droughtmonitor.unl.edu/MapsAndData/MapArchive.aspx).

7.3 Micro-Soxhlet Extraction of the Essential Oil of *Mammea Americana* L. fruit pulp

7.3.1 *Mammea Americana* L. fruit pulp freeze drying process

Mammea Americana L. fruit pulp samples were subjected to a freeze drying process in order to remove the moisture content that could interfere with the extraction of the essential oil from the fruit pulp material. **Table 18** shows the mass of *Mammea Americana* L. fruit pulp samples from the different municipalities before and after the freeze drying process.

Table 18. Weight amount of *Mammea Americana* L. fruit pulp sample from different municipalities before and after freeze drying process

	Mass of *Mammea Americana* L. fruit pulp (g)		
	Before Freeze Drying	**After Freeze Drying**	**Moisture Content (%)**
Rincon	191.00	22.56	88.2
Mayagüez	165.65	29.45	82.2
Aguada	171.07	31.10	81.8
Cayey	142.28	17.78	87.5
Average	**167.50**	**25.22**	**84.9**

Note: The freeze drying process was completed between 24 to 28 hours for all samples.

Mamey fruit pulp from Aguada had the lowest amount of moisture content removed with 81.8%, followed by Mamey fruit sample from Mayagüez with 82.2% of moisture removed. The freeze drying process removed an amount of 87.5% of water content from Mamey fruit of Cayey, but the highest amount of moisture content removed was for the Mamey fruit pulp of Rincon with 88.2% of moisture removed. From these results it was determined that *Mammea Americana* L. had an average of 84.9% of moisture content on its fruit pulp.

7.3.2 Micro-Soxhlet Extraction Optimization

The Micro-Soxhlet Extraction technique was optimized in order to perform an efficient extraction of the essential oil of *Mammea Americana* L. fruit pulp. The extraction times and the concentration volumes were varied in order to determine at which experimental conditions it was

possible to obtain the highest amount of volatile compounds in the essential oil extracts by GC/MS analysis. **Figure 34** shows the overlaid total ion chromatograms for the Micro-Soxhlet extraction of the essential oil of *Mammea Americana* L. fruit pulp at a constant concentration volume of 0.3 mL and varying the extraction times from 1hr to 4 hr by using Dichloromethane as the extraction solvent.

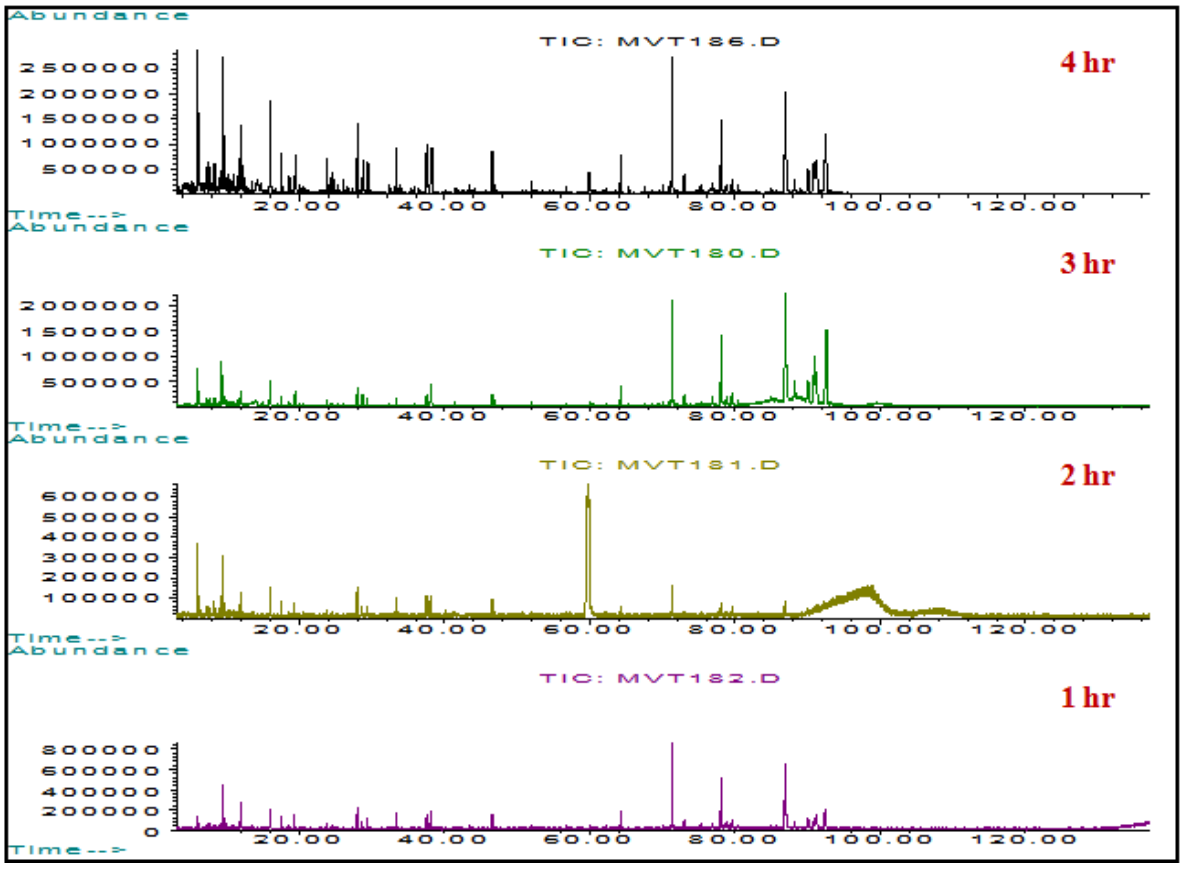


Figure 34. Overlaid Total Ion Chromatograms (TIC) obtained by GC/MS analysis of the DCM essential oil extracts of *Mammea Americana* L. fruit pulp concentrated to a volume of 0.3mL at different extraction times applying Micro-Soxhlet Extraction.

It was observed that the amount of volatile analytes in the DCM essential oil extracts of Mamey fruit was higher at 4 hours of extraction time with 30 volatile compounds obtained by GC/MS analysis compared to the amount obtained at 3 hr, 2 hr and 1 hr of extraction which were 9, 1, and 3 volatile compounds, respectively (see **Table 19**).

Table 19. Amount of chemical compounds obtained from *Mammea Americana* L. fruit pulp dried material at different extraction times and concentration volumes by Micro-Soxhlet Extraction and GC/MS

***Mammea Americana* L. dried fruit pulp**	**Total Compounds obtained by GC/MS analysis**	
Extraction time (hr)	**DCM Essential Oil Extract Concentrated to 0.3 mL**	**DCM Essential Oil Extract Concentrated to 0.1 mL**
1	3	0
2	1	23
3	9	38
4	30	57

On the other hand, **Figure 35** shows the overlaid total ion chromatograms for the Micro-Soxhlet Extraction of the DCM essential oil of *Mammea Americana* L. fruit pulp at a constant concentration volume of 0.1 mL again varying the extraction times from 1hr to 4 hr. It was observed that the amount of volatile analytes in the essential oil extracts of Mamey fruit was higher at 4 hours of extraction time with 57 volatile compounds obtained by GC/MS analysis compared to the amount obtained at 3 hr, 2 hr and 1 hr of extraction which were 38, 23, and 0 volatile compounds, respectively (see **Table 19**).

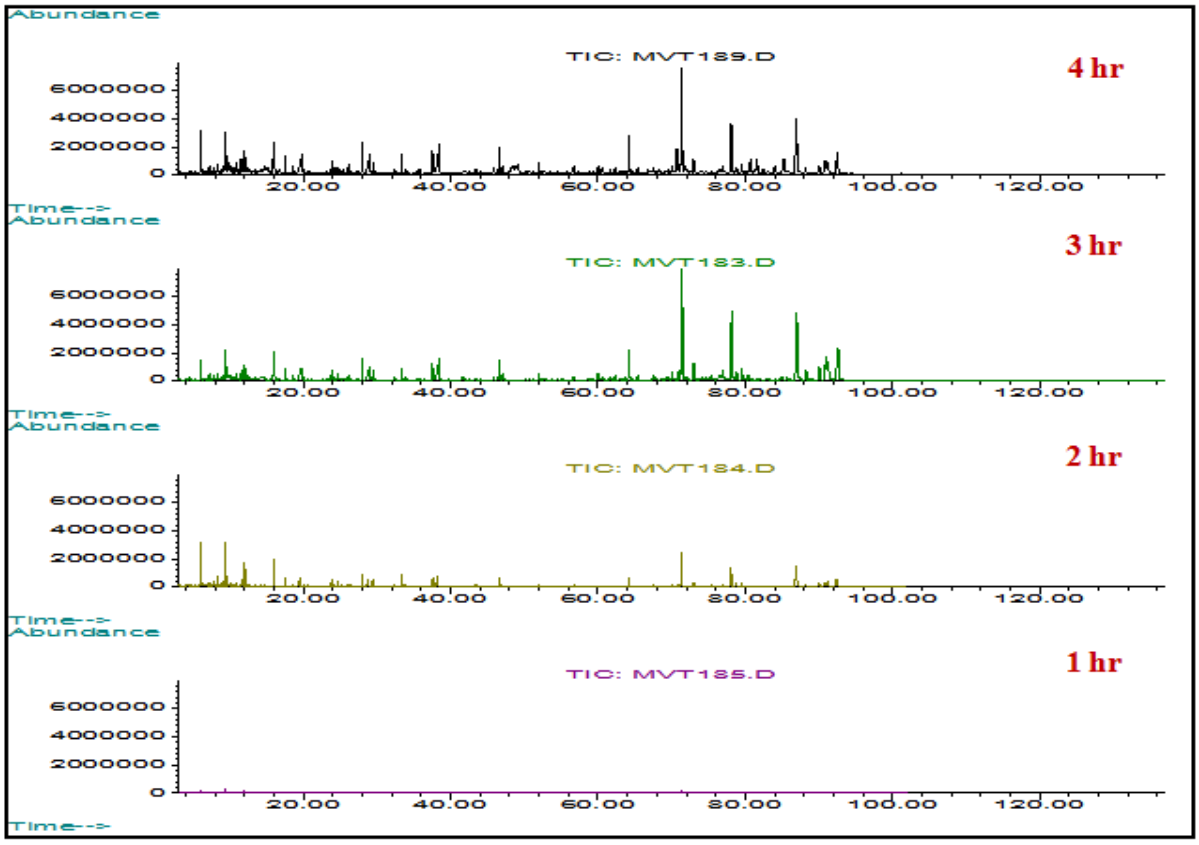


Figure 35. Overlaid Total Ion Chromatograms (TIC) obtained by GC/MS analysis of the DCM essential oil extracts of *Mammea Americana* L. fruit pulp concentrated to a volume of 0.1mL at different extraction times applying Micro-Soxhlet Extraction

By comparing the total ion chromatograms obtained by the GC/MS analysis of the DCM essential oil extracts of Mamey fruit at different concentration volumes it was observed that the abundance of the chromatographic peaks was significantly higher at a concentration volume of 0.1 mL compared to the essential oil extracts concentrated to a volume of 0.3 mL. Also there is a notable improvement in the amount of volatile analytes extracted and identified by the GC/MS from the essential oils extracted with 4 hr of extraction time. From the obtained results during this process it was concluded that the optimum extraction time for Micro-Soxhlet Extraction procedure of the essential oil *Mammea Americana* L. fruit pulp was 4 hours and that the essential oil extracts obtained should be concentrated to an optimum concentration volume of 0.1 mL.

7.3.3 Micro-Soxhlet Extraction of the Essential Oil of *Mammea Americana* L. fruit pulp using Different Organic Solvents

Different organic solvents with different polarities were used in order to determine *Mammea Americana* L. fruit pulp essential oil chemical composition. Since the principle of "like dissolve like" applies to these liquid/solid extractions (Smith, 1999), three different organic solvents were selected and used during this process including Dichloromethane, Chloroform and Methanol (mentioned in order of increasing polarity).

Table 20 shows the average amount of essential oil extracted from the fruit pulp of *Mammea Americana* L. by the different organic solvents used during 4 hours of extraction time. The highest amount of mass of essential oil was extracted using Methanol which extracted 570±28 mg of essential oil with an average extraction yield of 100±6%. This was considerably high compared to the amount of essential oil extracted by Chloroform which was 17±5 mg of essential oil with an average extraction yield of 3.0±0.7% and Dichloromethane which gave 13±2 mg of essential oil with an average extraction yield of 2.2±0.3%.

Table 20. Amount of extracted material and extraction yield for *Mammea Americana* L. fruit pulp essential oil obtained by Micro-Soxhlet Extraction using different organic solvents

Extraction Solvent	Extraction Time (hr)	Average Essential Oil Extracted (mg)	Average Extraction Yield (%)
Dichloromethane	4	13 ± 2	2.2 ± 0.3
Chloroform	4	17 ± 5	3.0 ± 0.7
Methanol	4	570 ± 28	100 ± 6

Figure 36 shows the overlaid total ion chromatograms for the GC/MS analysis of the essential oil extracts of *Mammea Americana* L. fruit pulp obtained using Dichloromethane, Chloroform and Methanol as extraction solvents during an extraction time of 4 hours. From these chromatograms it can be observed that the Methanolic essential oil extracts of *Mammea Americana* L. fruit pulp had the lowest amount of volatile analytes in its chemical composition, while the Chloroform essential oil extracts had the highest fraction of volatile analytes. This

contrasts with the amount of extracted material and the average extraction yield which was higher with Methanol. On the other hand, comparing the total ion chromatogram obtained by GC/MS analysis of the Dichloromethane essential oil extracts, the chromatographic peaks obtained were less overlapped and less asymmetrical compared to the chromatographic peaks of the total ion chromatograms obtained for the Chloroform and Methanol essential oil extracts.

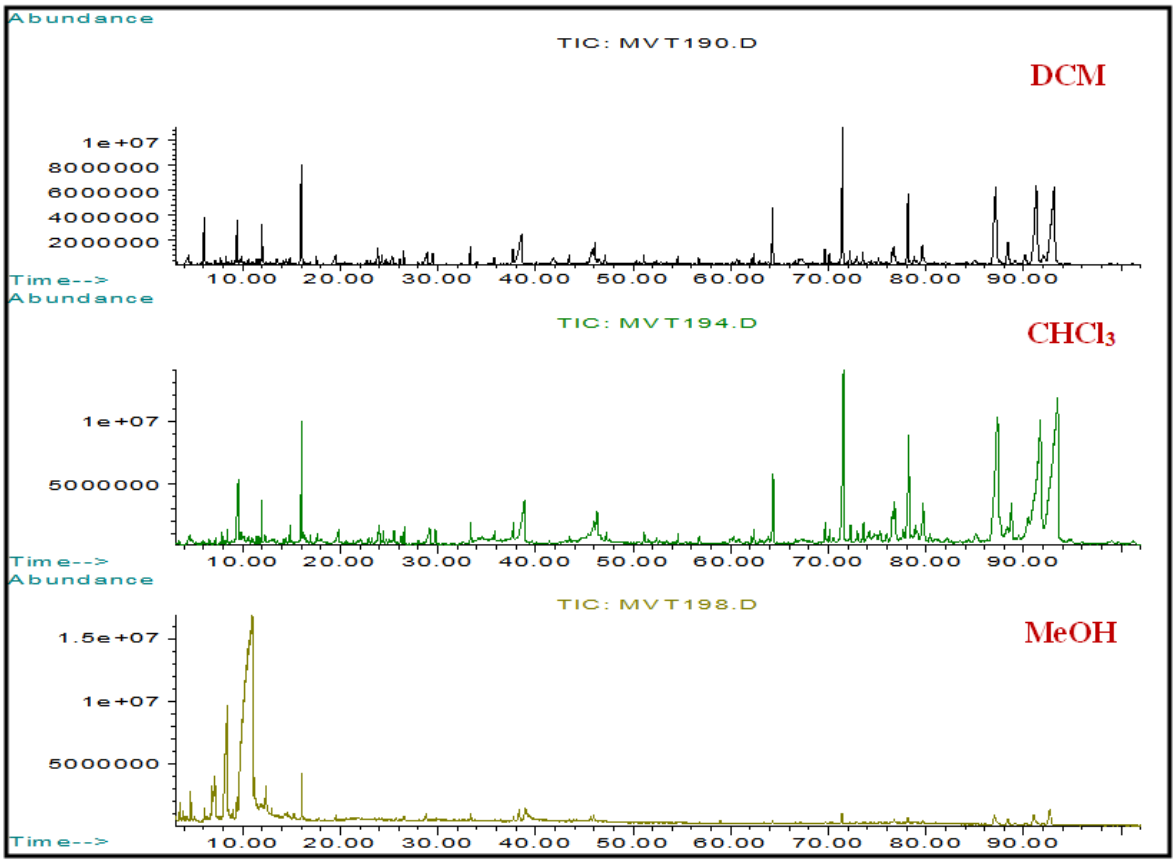


Figure 36. Overlaid Total Ion Chromatograms (TIC) obtained by GC/MS analysis of the essential oil extracts of *Mammea Americana* L. fruit pulp using different extraction solvents (DCM , $CHCl_3$, and MeOH) at 4 hrs of extraction applying Micro-Soxhlet Extraction

7.3.4 Micro-Soxhlet Extraction of the Essential Oil of *Mammea Americana* L. fruit pulp from different municipalities using different organic solvents

The essential oil of *Mammea Americana* L. fruit pulp from trees located at four different municipalities in Puerto Rico including Rincon, Mayagüez, Aguada and Cayey (3, 1193, 118 and 1283 ft of respective altitudes) was obtained by Micro-Soxhlet Extraction using the three different organic solvents and analyzed via GC/MS.

For the Dichloromethane essential oil extracts of *Mammea Americana* L., the highest amount of volatile compounds was found in the Mamey fruit from Mayagüez, with a total of 55 volatile compounds obtained by GC/MS analysis, in which a 56% or 31 compounds were identified with higher than 70 percent of match quality with the Wiley7.L and Wiley10.L mass spectral libraries. An amount of 51 volatile compounds were obtained by GC/MS analysis of the DCM essential oil of Mamey fruit from Rincon, in which a 37% or 19 compounds were identified with higher than 70 percent of match quality with the two mass spectral libraries. For the DCM essential oil extracts of Mamey fruit from Aguada, 35 volatile compounds were obtained from which a 60% or 21 compounds were identified with a high percent of match quality using the Wiley7.L and Wiley10.L mass spectral libraries. The least amount of volatile compounds was found in the DCM essential oil extracts of Mamey fruit from Cayey, in which 32 compounds were obtained by GC/MS analysis, with a 69% or 11 compounds identified with a high match quality percent (see **Table 21**).

Table 21. Amount of chemical compounds in the Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp from different municipalities obtained by Micro-Soxhlet Extraction and GC/MS analysis

Municipalities	Identified Compounds	Total Compounds	% Identified Compounds
Rincon	19	51	37
Mayagüez	31	55	56
Aguada	21	35	60
Cayey	22	32	69
Average	**23 ± 5**	**43 ± 11**	**56 ± 13**

From **Table 22**, it was observed that for the Chloroform essential oil extracts of *Mammea Americana* L. fruit pulp, the highest amount of volatile compounds was found in the essential oil of the fruit pulp from Rincon, with a total of 113 volatile compounds obtained by GC/MS analysis, from which a 46% or 52 compounds were identified with higher than 70 percent of match quality with the Wiley7.L and Wiley10.L mass spectral libraries. Similar amounts of volatile compounds were found in the $CHCl_3$ essential oil extracts of Mamey from Cayey and Aguada with 81 and 80 volatile compounds obtained by GC/MS analysis, respectively. Both differed in the percent of identified analytes which were 68% or 55 compounds and 55% or 44 compounds for Cayey and Aguada, respectively. The least amount of volatile compounds was found in the $CHCl_3$ essential oil extracts of Mamey fruit pulp from Mayagüez, in which 75 compounds were obtained by GC/MS analysis, but with a 60% or 45 compounds identified with a high match quality percent.

Table 22. Amount of chemical compounds in the Chloroform essential oil extracts of *Mammea Americana* L. fruit pulp from different municipalities obtained by Micro-Soxhlet Extraction and GC/MS analysis

Municipalities	Identified Compounds	Total Compounds	% Identified Compounds
Rincon	52	113	46
Mayagüez	45	75	60
Aguada	44	80	55
Cayey	55	81	68
Average	**49 ± 5**	**87 ± 17**	**57 ± 9**

For the Methanolic essential oil extracts of *Mammea Americana* L., the highest amount of volatile compounds was found in the essential oil extracts of Mamey fruit pulp from Cayey, with a total of 34 volatile compounds obtained by GC/MS analysis, from which a 44% or 15 compounds were identified with higher than high percent of match quality with the Wiley7.L and Wiley10.L mass spectral libraries. An amount of 31 volatile compounds were obtained by GC/MS analysis of the Methanolic essential oil extracts of Mamey fruit from Rincon, from which a 55% or 17 compounds were identified with higher than 70 percent of match quality with

the two mass spectral libraries. For the MeOH essential oil extracts of Mamey fruit from Mayagüez, 27 volatile compounds were obtained, from which a 48% or 13 compounds were identified with a high percent of match quality. The least amount of volatile compounds was found in Mamey fruit from Aguada, in which 21 volatile compounds were obtained by GC/MS analysis, with a 43% or 9 compounds identified with a high match quality percent using both mass spectral libraries (see **Table 23**).

Table 23. Amount of chemical compounds in the Methanol essential oil extracts of *Mammea Americana* L. fruit pulp from different municipalities obtained by Micro-Soxhlet Extraction and GC/MS analysis

Municipalities	Identified Compounds	Total Compounds	% Identified Compounds
Rincon	17	31	55
Mayagüez	13	27	48
Aguada	9	21	43
Cayey	15	34	44
Average	**14 ± 3**	**28 ± 6**	**47 ± 5**

In general, the highest average amount of volatile analytes was found in the Chloroform essential oil extracts of *Mammea Americana* L. with 87±17 compounds, while the lowest average amount of volatile analytes was found in the Methanolic essential oil extracts with 28±6 compounds. The Methanolic essential oil extracts of Mamey fruit from the different municipalities were mainly composed by 5-hydroxymethylfurfural and other structural isomers related to it. On the other hand, the Dichloromethane essential oil extracts had an average amount of 43±11 volatile compounds in its chemical composition. Although the highest amount of volatile analytes was found in the Chloroform essential oil extracts, chemical compounds reported in the literature with capacity to show biological or cytotoxic activity were found at a higher abundance and identified with a higher match quality percent of the mass spectral libraries in the Dichloromethane essential oil extracts of *Mammea Americana* L. For this reason in the next section the chemical composition and chromatographic profile of the DCM essential oil extracts of the Mamey fruit from different municipalities is further discussed.

7.3.5 Compounds distribution in the Dichloromethane Essential Oil extracts of *Mammea Americana* L. fruit pulp from different municipalities

Table 32 in **Appendix C** shows the complete list of chemical compounds obtained by Micro-Soxhlet Extraction and GC/MS analysis of the Dichloromethane essential oil extracts of *Mammea Americana* L. fruit from Rincon, Mayagüez, Aguada and Cayey which were classified according to their chemical families. The chemical composition of the DCM essential oil extracts of Mamey fruit pulp included alcohols, aldehydes, alkaloids, amides, benzene derivatives, carboxylic acids, coumarins, diazines, diazoles, esters, flavonoids, furans, hydrocarbons, ketones, lactones, monoterpenes, nitriles, norisoprenoids, organophosphorus compounds, phenols, pyridines, sesquiterpenes, steroids and triterpenes.

Table 24. Percent relative amount of volatile compounds distributed by families found in Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp from the four different municipalities

	Relative Amount (%)				
Compound Classification	**Rincon**	**Mayagüez**	**Aguada**	**Cayey**	**Average**
Alcohols	3.9	3.6	0.0	0.0	1.9
Aldehydes	5.9	1.8	5.7	3.1	4.1
Alkaloids	0.0	0.0	2.9	3.1	1.5
Amides	0.0	3.6	0.0	0.0	0.9
Benzene Derivatives	3.9	0.0	5.7	3.1	3.2
Carboxylic acids	7.8	10.9	14.3	15.6	12.2
Coumarins	3.9	5.5	2.9	6.3	4.6
Diazines	0.0	1.8	0.0	3.1	1.2
Diazoles	0.0	1.8	0.0	0.0	0.5
Esters	15.7	21.8	14.3	18.8	17.6
Flavonoids	0.0	0.0	2.9	3.1	1.5
Furans	3.9	1.8	2.9	3.1	2.9
Hydrocarbons	7.8	9.1	2.9	3.1	5.7
Ketones	17.6	9.1	8.6	6.3	10.4
Lactones	2.0	3.6	0.0	3.1	2.2
Monoterpenes	2.0	0.0	2.9	3.1	2.0
Nitriles	0.0	3.6	0.0	0.0	0.9
Norisoprenoids	7.8	9.1	17.1	6.3	10.1
Organophosphorus Compounds	0.0	1.8	0.0	3.1	1.2
Phenols	2.0	0.0	0.0	0.0	0.5
Pyridines	3.9	0.0	2.9	0.0	1.7
Sesquiterpenes	3.9	7.3	8.6	9.4	7.3
Steroids	7.8	1.8	2.9	6.3	4.7
Triterpenes	0.0	1.8	2.9	0.0	1.2
Total Number of Compounds	**51**	**55**	**35**	**32**	

From **Table 24**, it can be observed that in the chemical composition of the DCM essential oil extracts of *Mammea Americana* L. fruit pulp from Rincon the most abundant compounds were ketones with a relative amount of 17.6% followed by esters with 15.7% of the volatile fraction. The most abundant compounds in the DCM essential oil extracts of Mamey fruit pulp from Mayagüez were esters with a relative amount of 21.8% followed by carboxylic acids with

10.9% of the volatile fraction. Norisoprenoids were the most abundant compounds in the DCM essential oil extracts of Mamey fruit pulp from Aguada with a relative amount of 17.1% which were followed by carboxylic acids and esters, both with a relative amount of 14.3%. In the DCM essential oil extracts of Mamey fruit pulp from Cayey, esters were the most predominant compounds with a relative amount of 18.8% followed by carboxylic acids with a relative amount of 15.6% of the volatile fraction.

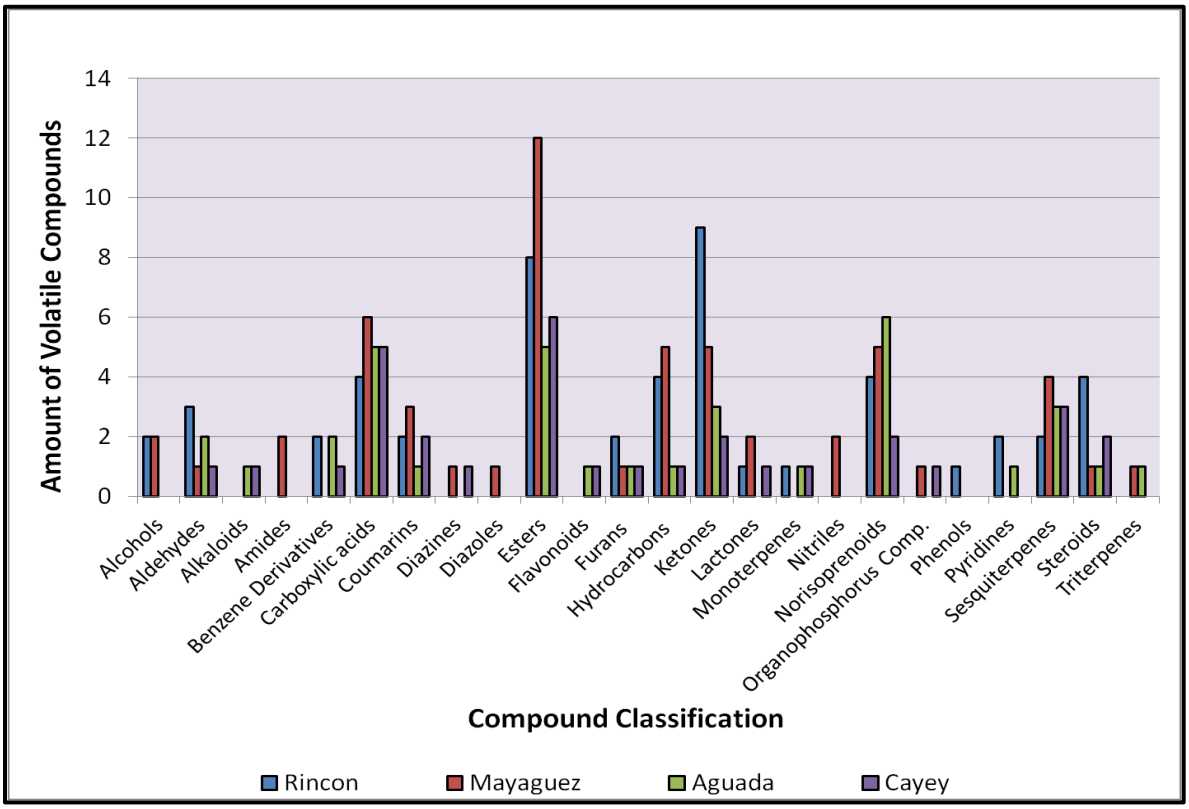


Figure 37. Distribution by families of the volatile composition of Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp from the four different municipalities by Micro-Soxhlet Extraction and GC/MS analysis

Figure 37 confirms that the most abundant compounds in the Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp from the different municipalities were esters followed by carboxylic acids and ketones. It is important to note that the DCM essential oil extracts of Mamey fruit from Mayagüez had the highest amount of esters and carboxylic acids in

its volatile fraction, although the highest amount of ketones was observed in the essential oil extracts of Mamey from Rincon.

7.3.6 Chromatographic Profile of the Chemical Composition of the Dichloromethane Essential Oil Extracts of *Mammea Americana* L. fruit pulp

The chromatographic profiles of the Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp from the different municipalities were carefully analyzed to identify their chemical composition. It is known that essential oils have demonstrated to be a source of biologically active compounds which may potentially have antibacterial, anti-tumor, cytotoxic and insecticidal activities among others (Ebadollahi et al. 2010; Gallo et al., 1996; Soares et al., 2012; Tripathi, et al., 2009).

Table 25 shows the top ten compounds or major volatile composition of DCM essential oil extracts of Mamey fruit pulp from Rincon. The first major compound identified was cis-caryophyllene (**1**) which eluted from the chromatographic column at a retention time of 71.41 min with a percent relative area of 17.11%, followed by 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-coumarin (**2**) at a retention time of 86.95 min with a percent relative area of 13.71%. Those compounds were followed by Palmitic acid (**3**) which eluted at a retention time of 38.50 min with a percent relative area of 6.45%, then Lauric acid (**4**) with a percent relative area of 3.50% eluted at a retention time of 19.82 min, and Myristic acid (**5**) at a retention time of 29.02 min with a percent relative area of 3.40%. The other major volatile compounds obtained were Propanoic acid, 2,2-dimethyl-, [(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl] ester with 3.06%, Mammea A/AB with 2.91%, 5-Hydroxymethylfurfural with 2.69%, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-cycloheptane with 2.68%, and 2,6,6-trimethyl-5-(3-methyl-2-butenyl)-1-Cyclohexene-1-methanol with 2.46% of relative area. The total ion chromatogram obtained by GC/MS analysis of the DCM essential oil extracts of *Mammea Americana* L. fruit pulp from Rincon can be seen in **Figure 38** in which the first five major compounds of the volatile fraction of the essential oil extracts were identified with numbers.

Table 25. Major volatile composition of Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp from Rincon obtained by Micro-Soxhlet Extraction and GC/MS

Rincon	Compound	Relative Area (%)*	Rt (min)	Molecular Formula	Biological and Toxicity Profile [a,b,c]
1	cis-Caryophyllene	17.11	71.41	$C_{15}H_{24}$	anti-inflammatory, analgesic, antipyretic, platelet-inhibitory
2	Coumarin, 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-	13.71	86.95	$C_{25}H_{26}O_5$	activity against Gram-positive bacteria, cytotoxic against human cancer cells
3	Palmitic acid	6.45	38.50	$C_{16}H_{32}O_2$	antimycobacterial and anti-inflammatory properties
4	Lauric acid	3.50	19.82	$C_{12}H_{24}O_2$	antibacterial, anti-inflammatory properties, induction of apoptosis in a CRC cell line
5	Myristic acid	3.40	29.02	$C_{14}H_{28}O_2$	-
6	Propanoic acid, 2,2-dimethyl-, [(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl] ester	3.06	70.73	$C_{20}H_{34}O_2$	-
7	(.+-.)-Mammea A/AB	2.91	85.26	$C_{25}H_{26}O_5$	insecticide, inhibitor of IKBα kinase
8	5-Hydroxymethylfurfural	2.69	9.34	$C_6H_6O_3$	shows antibacterial activity
9	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	2.68	49.14	$C_{15}H_{24}$	-
10	1-Cyclohexene-1-methanol, 2,6,6-trimethyl-5-(3-methyl-2-butenyl)-, (.+-.)	2.46	48.49	$C_{15}H_{26}O$	-

[a] Dictionary of Natural Products/Dictionary of Organic Compounds - www.chemnetbase.com
[b] Open Chemistry Database - pubchem.ncbi.nlm.nih.gov
[c] Toxicology Data Network - toxnet.nlm.nih.gov
*percent relative to the total integrated peak areas of the chromatogram

Between the major compounds found in the DCM essential oil extracts of Mamey fruit from Rincon, there are six compounds that are reported in the literature as bioactive compounds. Chemical compounds such as the 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-coumarin and Lauric acid are recognized by their antibacterial and cytotoxic activity against human colon cancer cells. Palmitic acid, 5-Hydroxymethylfurfural and cis-caryophyllene are

recognized by their antibacterial and anti-inflammatory properties, while Mammea A/AB is classified as a natural insecticide (Gallo et al., 1996).

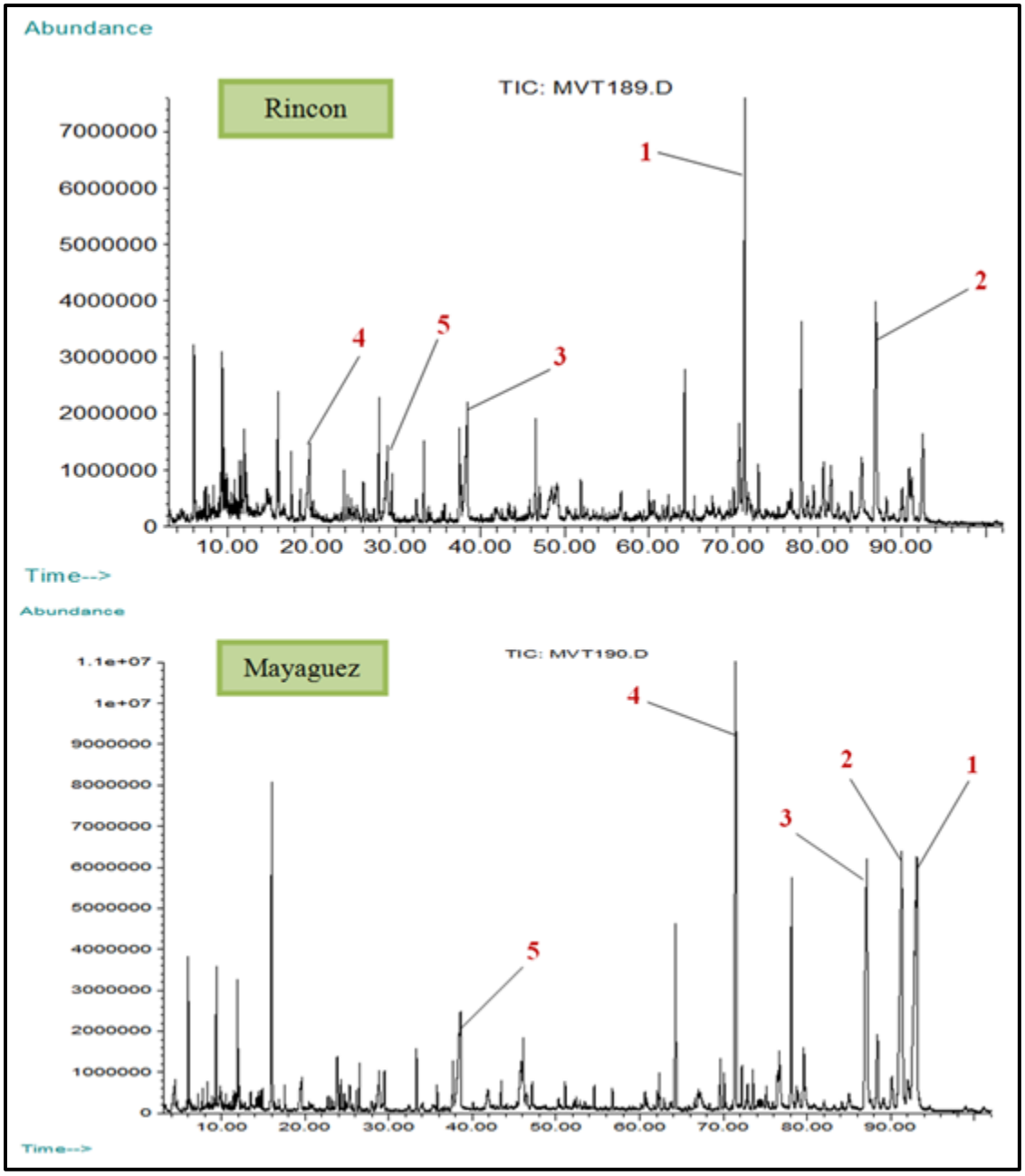


Figure 38. Total Ion Chromatograms (TIC) obtained by GC/MS analysis of Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp from Rincon (above) and Mayagüez (below) municipalities applying Micro-Soxhlet Extraction

Top ten compounds or the major volatile composition of DCM essential oil extracts of Mamey fruit pulp from Mayagüez are shown in **Table 26**. The first major compound identified was 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-coumarin (**1**) which eluted at a retention time of 93.12 min from the chromatographic column with a percent relative area of 16.16%, followed by Mammea A/AB (**2**) with a percent relative area of 12.31% at a retention time of 91.29 min. Those were followed by Carbonic acid, methyl ester, [(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl] ester (**3**) which eluted at a retention time of 87.14 min with a percent relative area of 10.80%, then γ-Gurjunene (**4**) with a percent relative area of 9.49% at a retention time of 71.48 min, and Palmitic acid (**5**) which eluted at a retention time of 38.58 min with a percent relative area of 4.94%. The other major volatile compounds included α-trans-sequicyclogeraniol with 4.35%, β-Ionone with 4.29%, cis-Caryophyllene with 3.01%, Isomammeisin with 2.38%, and 2-Methoxycarbonyl-5-methyl-3,4-diphenyltricyclo[4.4.1.1(2,5)] dodeca-3,7,9-trien-12-one with 1.98% of relative area. The total ion chromatogram obtained by GC/MS analysis of the DCM essential oil extracts of *Mammea Americana* L. fruit pulp from Mayagüez can be seen in **Figure 38** in which the first five major compounds of the volatile fraction of the essential oil extracts were identified with numbers.

Table 26. Major volatile composition of Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp from Mayagüez obtained by Micro-Soxhlet Extraction and GC/MS

Mayagüez	Compound	Relative Area (%)*	Rt (min)	Molecular Formula	Biological and Toxicity Profile [a,b,c]
1	Coumarin, 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-	16.16	93.12	$C_{25}H_{26}O_5$	activity against Gram-positive bacteria, cytotoxic against a range of human cancer cell lines
2	(.+-.)-Mammea A/AB	12.31	91.29	$C_{25}H_{26}O_5$	insecticide, inhibitor of IKBα kinase
3	Carbonic acid, methyl ester, [(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl] ester	10.80	87.14	$C_{17}H_{28}O_3$	-
4	γ-Gurjunene	9.49	71.48	$C_{15}H_{24}$	-
5	Palmitic acid	4.94	38.58	$C_{16}H_{32}O_2$	antimycobacterial and anti-inflammatory properties
6	α-trans-sequicyclogeraniol	4.35	78.16	$C_{15}H_{26}O$	-
7	β-Ionone	4.29	16.01	$C_{13}H_{20}O$	induces cell growth inhibition and apoptosis in human colon cancer cells
8	cis-Caryophyllene	3.01	64.29	$C_{15}H_{24}$	anti-inflammatory, analgesic, antipyretic, platelet-inhibitory activity
9	Isomammeisin	2.38	88.42	$C_{25}H_{26}O_5$	cytotoxic against human tumour cell lines and cultured mammalian cancer cell lines, antibacterial activity against Gram-positive bacteria
10	2-Methoxycarbonyl-5-methyl-3,4-diphenyltricyclo[4.4.1.1(2,5)]dodeca-3,7,9-trien-12-one	1.98	79.60	$C_{27}H_{24}O_3$	-

[a] Dictionary of Natural Products/Dictionary of Organic Compounds - www.chemnetbase.com
[b] Open Chemistry Database - pubchem.ncbi.nlm.nih.gov
[c] Toxicology Data Network - toxnet.nlm.nih.gov
*percent relative to the total integrated peak areas of the chromatogram

There are six compounds that are reported in the literature as bioactive compounds between the top ten compounds found in the DCM essential oil extracts of Mamey fruit from Mayagüez. Chemical compounds such as the 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-

4-phenyl-coumarin, β-Ionone and Isomammeisin are recognized by their antibacterial and cytotoxic activity against human colon cancer cells (Janakiram et al., 2008). Mammea A/AB is classified as a natural insecticide (Gallo et al., 1996), while Palmitic acid and cis-caryophyllene are recognized by their antibacterial and anti-inflammatory properties.

Table 27 shows the top ten compounds or major volatile composition of DCM essential oil extracts of Mamey fruit pulp from Aguada. The first major compound identified was 1-ethyl-3-(4-methylphenyl)-4a,10b-dihydro-1H-chromeno[3,4-c]pyridine-2,4,5-trione (**1**) which eluted at a retention time of 91.12 min from the chromatographic column with a percent relative area of 13.76%, followed by 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-coumarin (**2**) with a percent relative area of 12.59% at a retention time of 92.77 min. Those were followed by dimethyl 11b-(2-methoxy-2-oxoethyl)-1-oxo-5,6,11,11b-tetrahydro-1H-indolizino[8,7-b]indole-2,3-dicarboxylate (**3**) with a percent relative area of 10.98% which eluted at a retention time of 86.96 min, then Palmitic acid (**4**) at a retention time of 38.49 min with a percent relative area of 7.96%, and trans-β-Farnesene (**5**) with a percent relative area of 7.50% which eluted at a retention time of 71.39 min. The other major volatile compounds included Carbonic acid, methyl ester, [(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl] ester with 3.13%, 1,3-dimethoxy-2,4-bis(3-methyl-2-cyclohexen-1-yl)-5-pentylbenzene with 2.90%, Lauric acid with 2.73%, Myristic acid with 2.44%, and γ-Sitosterol with 2.25% of relative area. The total ion chromatogram obtained by GC/MS analysis of the DCM essential oil extracts of *Mammea Americana* L. fruit pulp from Aguada can be seen in **Figure 39**, in which the first five major compounds of the volatile fraction of the essential oil extracts were identified with numbers.

Table 27. Major volatile composition of Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp from Aguada obtained by Micro-Soxhlet Extraction and GC/MS

Aguada	Compound	Relative Area (%)*	Rt (min)	Molecular Formula	Biological and Toxicity Profile [a,b,c]
1	1-ethyl-3-(4-methylphenyl)-4a,10b-dihydro-1H-chromeno[3,4-c]pyridine-2,4,5-trione	13.76	91.12	$C_{21}H_{19}NO_4$	-
2	Coumarin, 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-	12.59	92.77	$C_{25}H_{26}O_5$	activity against Gram-positive bacteria, cytotoxic against a range of human cancer cell lines
3	dimethyl 11b-(2-methoxy-2-oxoethyl)-1-oxo-5,6,11,11b-tetrahydro-1H-indolizino[8,7-b]indole-2,3-dicarboxylate	10.98	86.96	$C_{21}H_{20}N_2O_7$	-
4	Palmitic acid	7.96	38.49	$C_{16}H_{32}O_2$	antimycobacterial and anti-inflammatory properties
5	trans-β-Farnesene	7.50	71.39	$C_{15}H_{24}$	-
6	Carbonic acid, methyl ester, [(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl] ester	3.13	78.10	$C_{17}H_{28}O_3$	-
7	1,3-dimethoxy-2,4-bis(3-methyl-2-cyclohexen-1-yl)-5-pentylbenzene	2.90	79.57	$C_{27}H_{40}O_2$	-
8	Lauric acid	2.73	19.60	$C_{12}H_{24}O_2$	antibacterial and anti-inflammatory properties, induction of apoptosis in a CRC cell line
9	Myristic acid	2.44	28.88	$C_{14}H_{28}O_2$	-
10	γ-Sitosterol	2.25	90.06	$C_{29}H_{50}O$	used to treat hyperlipidemias

[a] Dictionary of Natural Products/Dictionary of Organic Compounds - www.chemnetbase.com
[b] Open Chemistry Database - pubchem.ncbi.nlm.nih.gov
[c] Toxicology Data Network - toxnet.nlm.nih.gov
* percent relative to the total integrated peak areas of the chromatogram

Between the major compounds found in the DCM essential oil extracts of Mamey fruit from Aguada there are four compounds that are reported in the literature as bioactive compounds. Chemical compounds such as the 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-coumarin and Lauric acid are recognized by their antibacterial and cytotoxic

activity against human colon cancer cells. Palmitic acid is recognized by its antibacterial and anti-inflammatory properties and γ-Sitosterol has been used in the treatment of hyperlipidemias.

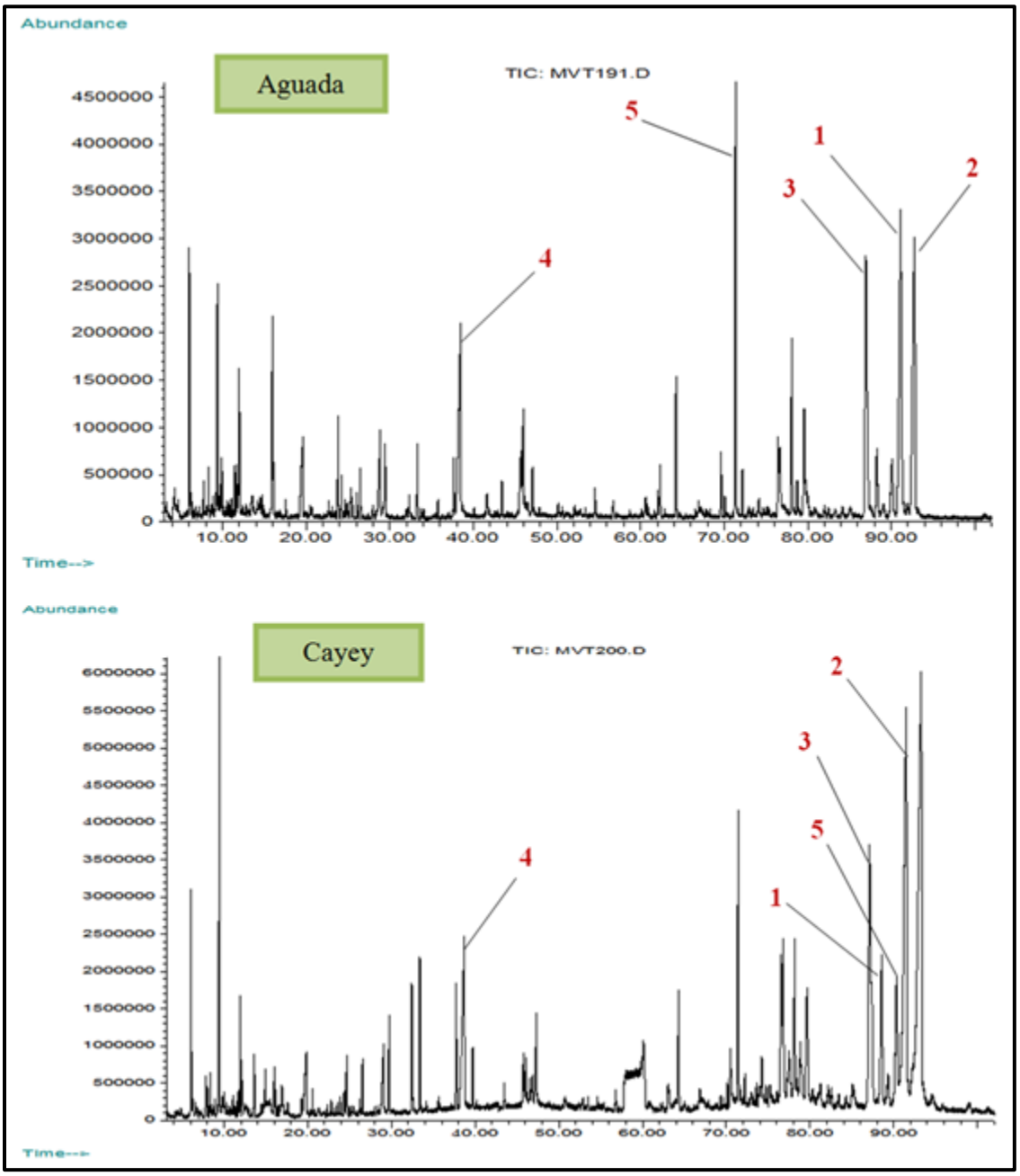


Figure 39. Total Ion Chromatograms (TIC) obtained by GC/MS analysis of Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp from Aguada (above) and Cayey (below) municipalities applying Micro-Soxhlet Extraction

Top ten compounds or the major volatile composition of DCM essential oil extracts of Mamey fruit pulp from Cayey are shown in **Table 28**. The first major compound identified was 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-coumarin (**1**) which eluted at a retention time of 88.56 min from the chromatographic column with a percent relative area of 25.03%, followed by Mammea A/AB (**2**) with a percent relative area of 15.45% at a retention time of 91.47 min. They were followed by 3-(3,7,11,15-tetramethyleicos-2,6,10,14-tetraen-1-yl)-9-oxabicyclo[4.3.0]nona-trien-8-one (**3**) which eluted at a retention time of 87.12 min with a percent relative area of 9.15%, then Palmitic acid (**4**) with a percent relative area of 5.14% at a retention time of 38.71 min, and β-Sitosterol (**5**) at a retention time of 90.34 min with a percent relative area of 3.70%. The other major volatile compounds included Farnesyl acetate with 3.62%, trans-β-Farnesene with 3.29%, ethyl 6-hydroxy-5-methoxy-1-(4-methoxyphenyl)-2-methyl-2,3-dihydro-1H-benzo[de][1,8]naphthyridine-8-carboxylate with 2.79%, 5-Hydroxymethylfurfural with 2.78%, and 1,3-dimethoxy-2,4-bis(3-methyl-2-cyclohexen-1-yl)-5-pentylbenzene with 2.77% of relative area. The total ion chromatogram obtained by GC/MS analysis of the DCM essential oil extracts of *Mammea Americana* L. fruit pulp from Cayey can be seen in **Figure 39** in which the first five major compounds of the volatile fraction of the essential oil extracts were identified with numbers.

Table 28. Major volatile composition of Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp from Cayey obtained by Micro-Soxhlet Extraction and GC/MS

Cayey	Compound	Relative Area (%)*	Rt (min)	Molecular Formula	Biological and Toxicity Profile [a,b,c]
1	Coumarin, 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-	25.03	88.56	$C_{25}H_{26}O_5$	activity against Gram-positive bacteria, cytotoxic against a range of human cancer cell lines
2	(.+-.)-Mammea A/AB	15.45	91.47	$C_{25}H_{26}O_5$	insecticide, inhibitor of IKBα kinase
3	3-(3,7,11,15-tetramethyleicos-2,6,10,14-tetraen-1-yl)-9-oxabicyclo[4.3.0]nona-trien-8-one	9.15	87.12	$C_{28}H_{38}O_2$	-
4	Palmitic acid	5.14	38.71	$C_{16}H_{32}O_2$	antimycobacterial and anti-inflammatory properties
5	β-Sitosterol	3.70	90.34	$C_{29}H_{50}O$	used to treat hyperlipidemias
6	Farnesyl acetate	3.62	64.29	$C_{17}H_{28}O_2$	-
7	trans-β-Farnesene	3.29	71.42	$C_{15}H_{24}$	-
8	ethyl 6-hydroxy-5-methoxy-1-(4-methoxyphenyl)-2-methyl-2,3-dihydro-1H-benzo[de][1,8]naphthyridine-8-carboxylate	2.79	76.77	$C_{23}H_{24}N_2O_5$	-
9	5-Hydroxymethylfurfural	2.78	9.43	$C_6H_6O_3$	shows antibacterial activity
10	1,3-dimethoxy-2,4-bis(3-methyl-2-cyclohexen-1-yl)-5-pentylbenzene	2.77	79.64	$C_{27}H_{40}O_2$	-

[a] Dictionary of Natural Products/Dictionary of Organic Compounds - www.chemnetbase.com
[b] Open Chemistry Database - pubchem.ncbi.nlm.nih.gov
[c] Toxicology Data Network - toxnet.nlm.nih.gov
[*]percent relative to the total integrated peak areas of the chromatogram

There are five compounds that are reported in the literature as bioactive compounds between the top ten compounds found in the DCM essential oil extracts of Mamey fruit from Cayey. The chemical compound 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-coumarin is recognized by its antibacterial and cytotoxic activity against human colon cancer cells. Other compounds such as Palmitic acid and 5-Hydroxymethylfurfural are recognized by

their anti-inflammatory and antibacterial activity, while β-Sitosterol has been used in the treatment of hyperlipidemias.

7.3.7 Conclusion

It was possible to determine the essential oil chemical composition of *Mammea Americana* L. fruit pulp applying Micro-Soxhlet Extraction procedure and using GC/MS analysis for the identification of the chemical constituents with the Wiley7.L and Wiley10.L mass spectral libraries. Parameters related to the Micro-Soxhlet Extraction procedure such as the extraction time and concentration volumes were optimized. The chemical composition of the Dichloromethane essential oil extracts was further studied because it was found that in this extracts there was a higher abundance of compounds reported in the literature to have a biological or toxicological activity. Major compounds found in the volatile fraction of the DCM essential oil extracts of Mamey fruit pulp were very similar between the essential oil extracts of fruit pulp samples from different municipalities. Volatile compounds such as 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-coumarin and Palmitic acid were common compounds between the major compounds in the essential oil of the Mamey fruit from different municipalities, while Mammea A/AB was found in 3 out of 4 of the DCM essential oil extracts. It is important to remark that 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-coumarin, Mammea A/AB and β-ionone were reported in previous studies by Gallo et al. (1996) and Janakiram et al. (2008) to have certain biological, insecticidal or cytotoxic activities.

7.4 Brine Shrimp Lethality Bioassay for *Mammea Americana* L. Fruit Pulp Essential Oil Extract

The Brine Shrimp Lethality Test represents a simple, rapid, reliable, convenient and inexpensive bioassay for testing natural products bioactivity (Meyer et. al., 1982). The pH and temperature of the seawater used for the bioassay are very important factors to be considered for the *A. salina* egg hatching. In order to determine if the seawater used had the optimal experimental conditions for *A. Salina* egg hatching some parameters were tested and their results are shown in **Table 29**.

Table 29. Seawater parameters tested for *A. Salina* egg hatching

Parameters Tested	Results
pH	8.15
Temperature	22°C
Conductivity	61.2 mS/cm
Salinity	41.0 ppt
Total Dissolved Solids (TDS)	39.5 g/L

It was determined that the seawater used had a pH of 8.15 which is between the optimal pH range of 8.0±0.5 described by Hamidi et al. (2014). On the other hand, seawater had a temperature of 22°C which is considered optimal for hatching *A. salina* eggs in a 24 hours period.

Brine Shrimp Lethality Bioassay was conducted using a modified procedure according to the methods of Meyer et. al. (1982) in order to predict the toxicity of the Dichloromethane essential oil extract of *Mammea Americana* L. fruit pulp after 24 hours of exposure. In most cases this type of bioassay correlates reasonably well with cytotoxic and anti-tumor properties (Baravalia et. al., 2012). **Table 30** shows the brine shrimp lethality activity of the *Mammea Americana* L. Dichloromethane essential oil extracts at different concentrations against the brine shrimp and that of the positive control, Potassium Dichromate.

Table 30. Results of Brine Shrimp Lethality Bioassay for *Mammea Americana* L. fruit pulp Dichloromethane Essential Oil Extract

Test Material	%Mortality under the studied concentration (µg/mL)							LC_{50} (µg/mL)	95% Confidence Interval	Toxicity Profile
(w/v)	**1.25**	**2.5**	**5**	**7.5**	**10**	**50**	**100**			
***Mammea Americana* L. Dichloromethane extract**	0	3.3	26.7	40.0	73.3	96.7	100.0	8.16	6.18-10.57	Highly Toxic
(w/v)	**1.25**	**2.5**	**5**	**7.5**	**10**	**50**	**100**			
$K_2Cr_2O_7$ (Positive Control)	0	0	0	0	0	20.0	40.0	118.69	76.90-293.93	Toxic
(v/v)	**0.05%**			**0.3%**			**4%**			
DMSO (Negative Control)	0			0			0	0	0	Non-Toxic

Note: Values reported are the average of triplicate studies, N = 10 (no. of shrimps). Score for LC_{50}: Highly-toxic < 20 µg/mL, Toxic - up to 1000 µg/mL, Non-toxic > 1000µg/mL
***(Baravalia et al., 2012)**

At a concentration of 100µg/mL the fruit pulp essential oil extract showed an average of 100% mortality compared to the Potassium Dichromate positive control which showed only an average 40% mortality at the same concentration, while negative controls with DMSO showed an average of 0% mortality at a concentration of 4%. The least percent mortalities were observed at 1.25µg/mL concentration of the extract. As shown in **Figures 40** and **41**, using Microsoft Excel 2007 with XLSTAT 2017 statistical analysis tool, a dose-response curve of the percent of brine shrimp killed against the logarithm of the concentration with a logistic regression using the probit analysis method was constructed to determine the LC_{50} or median lethal concentration at which mortality of the brine shrimp occurs from toxicity. It was found that the *Mammea Americana* L. Dichloromethane essential oil extract had a LC_{50} value of 8.16µg/mL, with a 95% confidence interval of 6.18-10.57, which is considered as highly toxic or bioactive. On the other hand, the positive control Potassium Dichromate showed a LC_{50} value of 118.69µg/mL, while no mortality was observed in the negative control with DMSO. The significant lethality of the DCM essential oil extract of *Mammea Americana* L. fruit pulp to brine shrimp is an indicative of the presence of a mixture of bioactive or potent cytotoxic components which need further investigation.

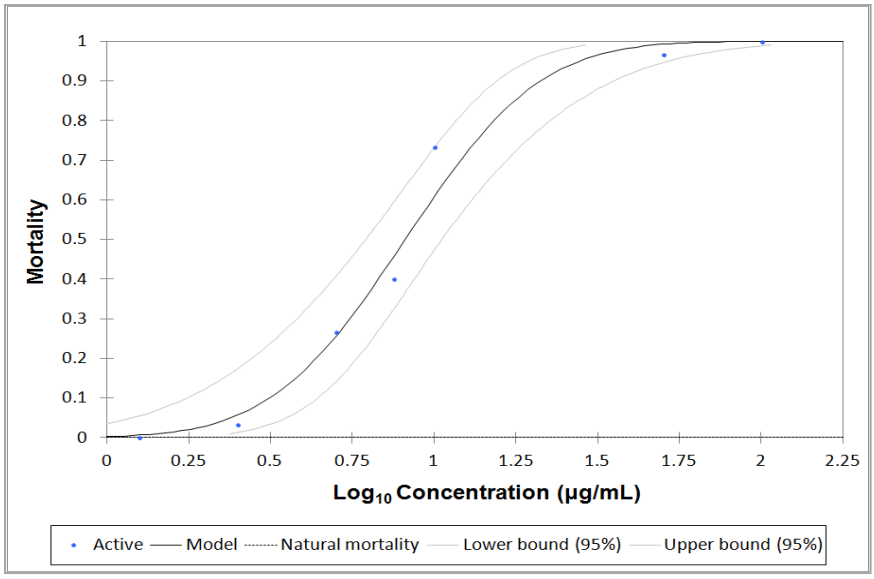


Figure 40. Dose-Response Curve of Brine Shrimp Lethality Bioassay for *Mammea Americana* L. fruit pulp Dichloromethane Essential Oil Extract

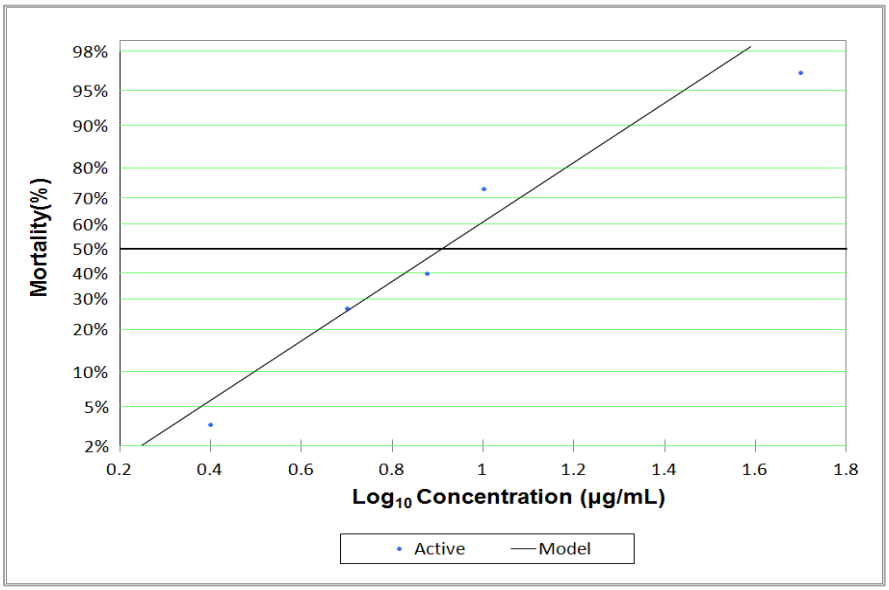


Figure 41. Linear Scale for Logistic Regression of Brine Shrimp Lethality Bioassay for *Mammea Americana* L. fruit pulp Dichloromethane Essential Oil Extract

CHAPTER 8

CONCLUSIONS

The experimental results obtained during this research proved that:

HS-SPME Technique Validation and Optimization

- ✓ HS-SPME technique was an efficient, fast, reliable and reproducible method for the extraction of the volatile composition of *Mammea Americana* L. and their subsequent analysis using the GC/MS instrumental method.
- ✓ The optimization and validation procedure of the HS-SPME technique allowed to determine the optimum 30 min of equilibrium, 40 min of extraction and 5 min of desorption time for the extraction and analysis of the volatile constituents of Mamey fruit pulp using the DVB/CAR/PDMS and PDMS/DVB SPME fiber coating materials.
- ✓ DVB/CAR/PDMS fiber was selected to perform the extraction and analysis of the volatile constituents of *Mammea Americana* L. from different municipalities since it proved to be the most sensitive, efficient, appropriate and suitable SPME fiber coating material.

HS-SPME/GC/MS Analysis of the Volatile Constituents of *Mammea Americana* L. Fruit

- ✓ HS-SPME optimized parameters, the fiber coating material selected, and GC/MS analysis allowed a precise determination and identification of the volatile compounds that give *Mammea Americana* L. its characteristic aroma profile.
- ✓ There is no variation in the characteristic aroma profile of Mamey fruit pulp from the different municipalities that were analyzed.
- ✓ The most abundant compounds in the volatile constituents of Mamey fruit pulp from different municipalities in Puerto Rico were classified as aldehydes and ketones, while the major compounds that were common among the different samples analyzed were hexanal, β-ionone, and β-cyclocitral.
- ✓ The most aromatic fruit was the Mamey from Mayagüez, which is the municipality with the highest average annual precipitation, as reported by the National Weather Service Stations, and the second highest tree in altitude.

Micro-Soxhlet Extraction of the Essential Oil of *Mammea Americana* L. Fruit Pulp

- ✓ Micro-Soxhlet Extraction resulted to be an efficient technique since it allowed to extract an appropriate amount of essential oil from *Mammea Americana* L. fruit pulp using organic solvents with different polarities.
- ✓ The optimization of the extraction time and the concentration volume when using Micro-Soxhlet Extraction improved the amount of analytes separated and identified in the essential oil extracts by the GC/MS.
- ✓ Some compounds identified in the obtained Dichloromethane, Chloroform, and Methanol essential oil extracts from *Mammea Americana* L. have been reported in the literature to have the potential to show biological activity.
- ✓ The chemical composition of the obtained essential oil extracts using different organic solvents did not show a significant variation among the Mamey fruit pulp essential oil extracts from different municipalities that were analyzed.
- ✓ Dichloromethane essential oil extracts from *Mammea Americana* L. had the highest amount and abundance of chemical compounds that, according to the literature, have antibacterial, anti-inflammatory, anti-tumor, and insecticidal properties, and also show cytotoxic activity against human colon cancer cells.
- ✓ The most abundant compounds in the chemical composition of the DCM essential oil extracts of Mamey fruit pulp from different municipalities in Puerto Rico were classified as carboxylic acids and ketones, while the major compounds that were common among the different samples analyzed were 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-coumarin and Palmitic acid.

Brine Shrimp Lethality Test for *Mammea Americana* L. Fruit Pulp Essential Oil Extract

- ✓ Brine Shrimp Lethality Bioassay resulted to be a simple, rapid, reliable, convenient and inexpensive bioassay for testing the bioactivity of *Mammea Americana* L. DCM essential oil fruit pulp extracts.
- ✓ Results obtained from the bioassay showed that the DCM essential oil extracts of *Mammea Americana* L. fruit pulp were highly toxic to the brine shrimp larvae, with an LC_{50} = 8.16μg/mL, which is indicative of the presence of a potent mixture of bioactive and/or cytotoxic compounds.

CHAPTER 9

RECOMMENDATIONS

The following recommendations are given after the completion of this study:

- ✓ The GC/MS oven temperature program used for the chromatographic separation of the essential oil extracts can be optimized and a 60 meters SPB-5 capillary column could be used in order to improve the resolution of the chromatographic peaks when analyzing the essential oil extracts of *Mammea Americana* L. obtained by using Chloroform and Methanol as the extraction solvents.

- ✓ Chloroform and Methanol essential oil extracts of *Mammea Americana* L. should be subjected to Brine Shrimp Lethality Test in order to determine their bioactivity.

- ✓ Further investigation of the Dichloromethane essential oil extracts of *Mammea Americana* L. fruit pulp should be performed in order to find which are the chemical compounds responsible for the high bioactivity observed during the Brine Shrimp Lethality Bioassay.

- ✓ The cytotoxic activity of the Dichloromethane essential oil extracts of *Mammea Americana* L. should be investigated in human colon cancer cell lines.

- ✓ The insecticidal activity of the essential oil extracts should be investigated using dose-response bioassays with insects.

- ✓ The Dichloromethane essential oil extracts from Mamey fruit should be fractionated using column chromatography to purify the individual chemical compounds from the mixture of compounds that is contained in the essential oil and analyzed via GC/MS in order to identify their specific chemical composition.

- ✓ The antioxidant activity of the obtained fractions should be evaluated by DPPH free radical scavenging assays.

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Appendix A

a) Standard Deviation (Std. Dev.)

$$s = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N - 1}}$$

b) Percent Relative Standard Deviation (%RSD)

$$\%\text{RSD} = \frac{s}{\bar{x}} \times 100$$

c) Standard Error (Std. Error)

$$\text{Std. Error} = \frac{s}{\sqrt{n}}$$

Appendix B

Table 31. Volatile constituents of *Mammea Americana* L. fruit pulp by HS-SPME/GC/MS

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
	Alcohols								
1	3-buten-1-ol, 3-methyl	$C_5H_{10}O$	86	000763-32-6	726				X
2	1-Butanol, 3-methyl	$C_5H_{12}O$	88	000123-51-3	732			X	
3	2-Buten-1-ol, 3-methyl	$C_5H_{10}O$	86	000556-82-1	779				X
4	1-Octen-3-ol	$C_8H_{16}O$	128	003391-86-4	974	X	X	X	
5	2-Ethyl-1-hexanol	$C_8H_{18}O$	130	000104-76-7	1028		X	X	
6	3,6-dimethoxy-9-(2-phenylethynyl)-9-fluorenol	$C_{23}H_{18}O_3$	342	2000471-86-0	-	X			
	Total Number of Alcohols					**2**	**2**	**3**	**2**
	Relative Amount (%)					**6.9%**	**4.5 %**	**7.5 %**	**7.7 %**
	Aldehydes								
1	β-Methylbutanal	$C_5H_{10}O$	86	000590-86-3	652	X	X		
2	Pentanal	$C_5H_{10}O$	86	000110-62-3	700				X
3	β-Methylcrotonaldehyde	C_5H_8O	84	000107-86-8	796		X		
4	Hexanal	$C_6H_{12}O$	100	000066-25-1	786	X	X	X	X
5	2-Hexenal	$C_6H_{10}O$	98	000505-57-7	848	X			
6	n-Heptanal	$C_7H_{14}O$	114	000111-71-7	902		X		X
7	2-Heptenal, (E)	$C_7H_{12}O$	112	018829-55-5	978	X	X	X	
8	o-Carboxybenzaldehyde	$C_8H_6O_3$	150	000119-67-5	-				X
9	2,4-Heptadienal, (E,E)	$C_7H_{10}O$	110	004313-03-5	1003	X	X	X	

Table 31. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
10	(E)-oct-2-enal	$C_8H_{14}O$	126	002548-87-0	1066		X	X	
11	3-Cyclohexene-1-carboxaldehyde	$C_7H_{10}O$	110	000931-96-4	-			X	
12	Nonanal	$C_9H_{18}O$	142	000124-19-6	1104				X
13	4-methyl-3-pentenal	$C_6H_{10}O$	98	005362-50-5	-	X			
14	2-furancarboxaldehyde	$C_6H_6O_3$	126	000067-47-0	1224		X		
	Total Number of Aldehydes					**6**	**8**	**5**	**5**
	Relative Amount (%)					**20.7 %**	**18.2 %**	**12.5 %**	**19.2 %**
	Alkylphosphines								
1	Trimethylphosphine oxide	C_3H_9OP	92	000676-96-0	-			X	
	Total Number of Alkylphosphines					**0**	**0**	**1**	**0**
	Relative Amount (%)					**0 %**	**0 %**	**2.5 %**	**0 %**
	Benzene derivatives								
1	2-Methoxymesitylene	$C_{10}H_{14}O$	150	2000042-93-1	-	X			
	Total Number of Benzene Derivatives					**1**	**0**	**0**	**0**
	Relative Amount (%)					**3.4 %**	**0 %**	**0 %**	**0 %**
	Carboxylic acids								
1	Acetic acid	$C_2H_4O_2$	60	000064-19-7	645	X	X		
2	Butyric acid	$C_6H_{12}O_2$	116	000868-57-5	771	X	X	X	
3	α-Methylcaproic acid	$C_7H_{14}O_2$	130	004536-23-6	-			X	
4	α-Methylbutyric acid	$C_5H_{10}O_2$	102	000116-53-0	867			X	
5	3-(aminomethyl)-2-furancarboxylic acid	$C_6H_7NO_3$	141	2000032-12-9	-			X	

Table 31. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
	Total Number of Carboxylic acids					**2**	**2**	**4**	**0**
	Relative Amount (%)					**6.9 %**	**4.5 %**	**10.0 %**	**0 %**
	Diazepines								
1	1H-1,4-Diazepine, hexahydro-1-(2-pyridinyl)-	$C_{10}H_{15}N_3$	177	2000086-53-9	-			X	
	Total Number of Diazepines					**0**	**0**	**1**	**0**
	Relative Amount (%)					**0 %**	**0 %**	**2.5 %**	**0 %**
	Esters								
1	Isopropenyl acetate	$C_5H_8O_2$	100	000108-22-5	-				X
2	Isopropoxycarbamic acid, ethyl ester	$C_6H_{13}NO_3$	147	2000038-89-3	-	X	X	X	X
3	2-Butenoic acid, 3-methyl, methyl ester	$C_6H_{10}O_2$	114	000924-50-5	842	X	X	X	X
4	Methyl tiglate	$C_6H_{10}O_2$	114	006622-76-0	868	X	X	X	X
5	(S)-(+)-Methyl 3-hydroxy-2-methylpropionate	$C_5H_{10}O_3$	118	080657-57-4	-	X			
6	Oxime-, methoxy-phenyl	$C_8H_9NO_2$	151	2000043-74-8	-	X	X		X
7	Benzoic acid, methyl ester	$C_8H_8O_2$	136	000093-58-3	-	X			
8	8,12-Tetradecadienoic acid, 5-ethenyl-3,5,9,13-tetramethyl-, methyl ester	$C_{21}H_{36}O_2$	320	036237-73-7	-				X
	Total Number of Esters					**6**	**4**	**3**	**6**
	Relative Amount (%)					**20.7 %**	**9.1 %**	**7.5 %**	**23.1 %**
	Ethers								
1	2-Ethyl-3-vinyloxirane	$C_6H_{10}O$	98	034485-78-4	-		X		
	Total Number of Ethers					**0**	**1**	**0**	**0**

Table 31. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
	Relative Amount (%)					**0 %**	**2.3 %**	**0 %**	**0 %**
	Furans								
1	Furan, 2-pentyl	$C_9H_{14}O$	138	003777-69-3	989	X	X		
2	cis-linaloloxide	$C_{10}H_{18}O_2$	170	2000075-20-3	-				X
	Total Number of Furans					**1**	**1**	**0**	**1**
	Relative Amount (%)					**3.4 %**	**2.3 %**	**0 %**	**3.8 %**
	Hydrocarbons								
1	1,4-Pentadiene	C_5H_8	68	000591-93-5	-		X		
2	1-cyclopentene	C_5H_8	68	000142-29-0	-			X	
3	Ethylcyclopropane	C_5H_{10}	70	001191-96-4	-		X		
4	6,6-Dimethylhepta-2,4-diene	C_9H_{16}	124	2000017-13-5	847	X	X	X	
5	Cyclopentane, 1-isobutylidene-3-methyl-	$C_{10}H_{18}$	138	2000029-56-2	-				X
6	Cyclohexane, 1,3,5-trimethyl-, cis-	C_9H_{18}	126	001795-27-3	-		X		
7	(3Z)-3-methyl-1,3-nonadiene	$C_{10}H_{18}$	138	128838-77-7	-		X		
8	2,3-Dimethyl-1,4-hexadiene	C_8H_{14}	110	018669-52-8	-			X	
9	3,4-Heptadiene	C_7H_{12}	96	002454-31-1	-		X		
10	2,3,4,5,7,7-Hexamethyl-1,3,5-cycloheptatriene	$C_{13}H_{20}$	176	074779-68-3	-			X	
	Total Number of Hydrocarbons					**1**	**6**	**4**	**1**
	Relative Amount (%)					**3.4 %**	**13.6 %**	**10.0 %**	**3.8 %**
	Ketones								
1	5-methylene-2-pyranone	$C_6H_6O_2$	110	2000008-85-3	-		X		

Table 31. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
2	6-Bromo-4,5-dichloro-2-benzoxazolinone	$C_7H_2BrCl_2NO_2$	281	2000332-82-8	-			X	
3	6-Hepten-3-one, 4-methyl	$C_8H_{14}O$	126	026118-97-8	938	X	X	X	X
4	3-Octen-2-one	$C_8H_{14}O$	126	001669-44-9	1046		X		
5	Cyclohexanone, 2,2,6-trimethyl	$C_9H_{16}O$	140	002408-37-9	1035				X
6	Acetophenone	C_8H_8O	120	000098-86-2	1071		X	X	
7	(E,E) 3,5-Octadiene-2-one	$C_8H_{12}O$	124	2000016-90-5	1068	X	X		
8	3,5-ciss-3-Hydroxy-5-[(E)-3-methyl-1-butenyl]-4,5-dihydro-2(3H)-furanone	$C_9H_{14}O_3$	170	2000074-32-8	-				X
9	2H-8'-oxaspiro[acenaphthylene-1,7'-bicyclo[4.2.0]octane]-2-one	$C_{18}H_{16}O_2$	264	2000292-50-8	-				X
10	3,4-dimethylcyclopent-2-en-1-one	$C_7H_{10}O$	110	030434-64-1	-			X	
11	2-Heptanone, 6-methyl	$C_8H_{16}O$	128	000928-68-7	-	X	X		
12	4-Ketoisophorone	$C_9H_{12}O_2$	152	001125-21-9	-		X	X	
13	(2E)-1-(1,2,2-trimethylcyclopentyl)-2-pentene-1,4-dione	$C_{13}H_{20}O_2$	208	2000152-89-9	-			X	
14	2-Methyl-2-nonen-4-one	$C_{10}H_{18}$	154	002903-23-3	1215				X
15	3-Methyl-3-(4'-methylfuran-1'-yl)cyclohexanone	$C_{12}H_{16}O_2$	192	2000116-33-3	-			X	
16	9H-pyrrolo[3',4':3,4]pyrrolo[2,1-a]phthalazine-9,11(10H)-dione,10-ethyl-8-phenyl	$C_{21}H_{15}N_3O_2$	341	095647-39-5	-			X	
17	1-isopropyl-5-methylbicyclo[3.2.2]non-3-en-2-one	$C_{13}H_{20}O$	192	101968-43-8	-		X		
18	2,6,6-Trimethyl-4-butylidenecyclohex-2-en-1-one	$C_{13}H_{20}O$	192	2000117-15-9	-		X		
	Total Number of Ketones					**3**	**9**	**8**	**5**
	Relative Amount (%)					**10.3 %**	**20.5 %**	**20.0 %**	**19.2 %**

Table 31. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
	Lactones								
1	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	$C_{11}H_{16}O_2$	180	015356-74-8	1537	X	X	X	
	Total Number of Lactones					**1**	**1**	**1**	**0**
	Relative Amount (%)					**3.4 %**	**2.3 %**	**2.5 %**	**0 %**
	Monoterpenes								
1	Limonene	$C_{10}H_{16}$	136	000138-86-3	1026	X		X	X
2	β-Cyclocitral	$C_{10}H_{16}O$	152	000432-25-7	1222	X	X	X	X
3	Geraniol	$C_{10}H_{18}O$	154	000106-24-1	-			X	
4	cis-Geraniol	$C_{10}H_{18}O$	154	000106-25-2	1228		X		
5	Citral	$C_{10}H_{16}O$	152	005392-40-5	1240			X	
	Total Number of Monoterpenes					**2**	**2**	**4**	**2**
	Relative Amount (%)					**6.9 %**	**4.5 %**	**10.0 %**	**7.6 %**
	Norisoprenoids								
1	Theaspirane A	$C_{13}H_{22}O$	194	2000121-77-0	1305	X	X		X
2	Theaspirane B	$C_{13}H_{22}O$	194	2000121-77-3	1331				X
3	Megastigma-4,6(E),8(Z)-triene	$C_{13}H_{20}$	176	071186-24-8	1354		X	X	
4	β-Ionol	$C_{13}H_{22}O$	194	022029-76-1	1428		X		
5	Dihydro-β-ionone	$C_{13}H_{22}O$	194	017283-81-7	1433	X	X	X	
6	β-dihydroionol	$C_{13}H_{24}O$	196	2000126-59-7	1455		X		
7	α,β-Dihydropseudoionone	$C_{13}H_{22}O$	194	000689-67-8	1456		X	X	

Table 31.Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
8	β-Ionone	$C_{13}H_{20}O$	192	014901-07-6	1493	X	X	X	X
	Total Number of Norisoprenoids					**3**	**7**	**4**	**3**
	Relative Amount (%)					**10.3 %**	**15.9 %**	**10.0 %**	**11.5 %**
	Pyrazolines								
1	1-Isopropyl-5-methyl-4,5-dihydro-1H-pyrazole	$C_7H_{14}N_2$	126	026964-54-5	-	X			
	Total Number of Pyrazolines					**1**	**0**	**0**	**0**
	Relative Amount (%)					**3.4 %**	**0 %**	**0 %**	**0 %**
	Pyridines								
1	6-(1-Methylhydrazino)isocytosine hemihydrate	$C_8H_9N_5O_2$	207	000000-00-0	-			X	
	Total Number of Pyridines					**0**	**0**	**1**	**0**
	Relative Amount (%)					**0 %**	**0 %**	**2.5 %**	**0 %**
	Sesquiterpenes								
1	trans-Caryophyllene	$C_{15}H_{24}$	204	000087-44-5	1423		X	X	
2	Bicyclo[5.2.0]nonane, 2-methylene-	$C_{15}H_{24}$	204	242794-76-9	-				X
	Total Number of Sesquiterpenes					**0**	**1**	**1**	**1**
	Relative Amount (%)					**0 %**	**2.3 %**	**2.5 %**	**3.8 %**
	Total Number of Identified Compounds*					**15**	**26**	**13**	**11**
	Total Compounds					**29**	**44**	**40**	**26**

[a]Chemical Abstract Service Registry Number
[b]Literature Retention Index from NIST Standard Reference Database

Appendix C

Table 32. Volatile composition of Dichloromethane essential oil extracts from *Mammea Americana* L. fruit pulp by Micro-Soxhlet Extraction and GC/MS

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
	Alcohols								
1	2-Methyl-2,6-heptadiene-4-ol	$C_8H_{14}O$	126	2000018-56-4	-	X			
2	(2RS,8aSR)-1,2,3,5,6,7,8,8a-octahydro-5,5,8a-trimethylnaphthalen-2-ol	$C_{13}H_{22}O$	194	126090-85-5	-		X		
3	[2,6,6-trimethyl-4-(3-methyl-2-butenyl)-1-cyclohexen-1-yl]methanol	$C_{15}H_{26}O$	222	108287-13-4	-	X			
4	.delta.1,.beta.-Cyclohexaneethanol, 3,3-dimethyl-	$C_{10}H_{18}O$	154	041370-29-0	-		X		
	Total Number of Alcohols					**2**	**2**	**0**	**0**
	Relative Amount (%)					**4.0 %**	**3.6 %**	**0 %**	**0 %**
	Aldehydes								
1	5-Hydroxymethylfurfural	$C_6H_6O_3$	126	000067-47-0	1224	X	X	X	X
2	2,4-Decadienal	$C_{10}H_{16}O$	152	002363-88-4	1323	X			
3	Benzaldehyde, 2-hydroxy-	$C_7H_6O_2$	122	000090-02-8	1047	X		X	
	Total Number of Aldehydes					**3**	**1**	**2**	**1**
	Relative Amount (%)					**5.9 %**	**1.8 %**	**5.7 %**	**3.1 %**
	Alkaloids								
1	6-Methoxy-12,12,15-trimethyl-12,15-dihydropyran[3,2-b]quino[4,3,2-mn]acridine	$C_{26}H_{22}N_2O_2$	394	2000556-16-3	-				X
2	2,3-Dicarbomethoxy-11b-carbomethoxymethyl-1-oxo-1,5,6,11b-tetrahydroindolizino[8,7-b]indole	$C_{21}H_{20}N_2O_7$	412	2000577-69-1	-			X	
	Total Number of Alkaloids					**0**	**0**	**1**	**1**
	Relative Amount (%)					**0 %**	**0 %**	**2.9 %**	**3.1 %**

Table 32. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
	Amides								
1	1-Ethyl-3,5-bis-[1-thiophen-2-yl-meth-(E)-ylidene]-piperidin-4-one	$C_{17}H_{17}NOS_2$	315	2000414-23-2	-		X		
2	N-(10-Oxo-3-thiophen-2-yl-10H-9-oxa-2,4-diaza-phenanthren-1-yl)-propionamide	$C_{18}H_{13}N_3O_3S$	351	2000487-66-6	-		X		
	Total Number of Amides					**0**	**2**	**0**	**0**
	Relative Amount (%)					**0 %**	**3.6 %**	**0 %**	**0 %**
	Benzene Derivatives								
1	1,2,3,4-tetramethyl-5-prop-1-en-2-yl-benzene	$C_{13}H_{18}$	174	061142-76-5	-	X			
2	3,8,8-Trimethyltetrahydronaphthale	$C_{13}H_{18}$	174	000000-00-0	-			X	
3	1,4-Dihydroxy-2-tetrakis(3'-methylbut-2'-en-1'-yl)-5-methylbenzene	$C_{27}H_{40}O_2$	396	2000558-82-4	-	X			
4	1,3-dimethoxy-2,4-bis(3-methyl-2-cyclohexen-1-yl)-5-pentylbenzene	$C_{27}H_{40}O_2$	396	2000558-81-7	-			X	X
	Total Number of Benzene Derivatives					**2**	**0**	**2**	**1**
	Relative Amount (%)					**3.9 %**	**0 %**	**5.7 %**	**3.1 %**
	Carboxylic acids								
1	β-Hydroxyisovaleric acid	$C_5H_{10}O_3$	118	000625-08-1	-		X		
2	Lauric acid	$C_{12}H_{24}O_2$	200	000143-07-7	1578	X	X	X	X
3	Myristic acid	$C_{14}H_{28}O_2$	228	000544-63-8	1768	X	X	X	X
4	Palmitic acid	$C_{16}H_{32}O_2$	256	000057-10-3	1958	X	X	X	X
5	Linoleic acid	$C_{18}H_{32}O_2$	280	000060-33-3	2152		X	X	X

Table 32. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
6	Stearic acid	$C_{18}H_{36}O_2$	284	000057-11-4	2172	X	X	X	X
	Total Number of Carboxylic acids					**4**	**6**	**5**	**5**
	Relative Amount (%)					**7.8 %**	**10.9 %**	**14.3 %**	**15.6 %**
	Coumarins								
1	(.+-.)-Mammea A/AB	$C_{25}H_{26}O_5$	406	111761-54-7	-	X	X		X
2	Isomammeisin	$C_{25}H_{26}O_5$	406	005224-54-4	-		X		
3	Coumarin, 5,7-dihydroxy-6-isovaleryl-8-(3-methyl-2-butenyl)-4-phenyl-	$C_{25}H_{26}O_5$	406	018483-64-2	-	X	X	X	X
	Total Number of Coumarins					**2**	**3**	**1**	**2**
	Relative Amount (%)					**3.9 %**	**5.5 %**	**2.9 %**	**6.3 %**
	Diazines								
1	Trimethylpyrazine	$C_7H_{10}N_2$	122	014667-55-1	1005				X
2	2-(1,3-benzodioxol-5-yl)-3-methoxy-6-(3-pyridinylmethylthio)imidazo[1,2-b]pyridazine	$C_{20}H_{16}N_4O_3S$	392	2000552-75-6	-		X		
	Total Number of Diazines					**0**	**1**	**0**	**1**
	Relative Amount (%)					**0 %**	**1.8 %**	**0 %**	**3.1 %**
	Diazoles								
1	2,2-Dimethyl-5-methoxy-2H-benzimidazole-1,3-Dioxide	$C_{10}H_{12}N_2O_3$	208	2000151-24-7	-		X		
	Total Number of Diazoles					**0**	**1**	**0**	**0**
	Relative Amount (%)					**0 %**	**1.8 %**	**0 %**	**0 %**
	Esters								

Table 32. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
1	dimethyl α-D-glucopyranosiduronate	$C_8H_{14}O_7$	222	018486-51-6	-	X			
2	3-(4-N,N-Dimethylaminophenyl)propenoic acid, 2-(diethoxyphosphinyl)-, ethyl ester	$C_{17}H_{26}NO_5P$	355	066564-08-7	-				X
3	2-oxo-3-phenoxy-3-phenylpropyl acetate	$C_{17}H_{16}O_4$	284	087385-72-6	-	X			
4	Diisobutyl phthalate	$C_{16}H_{22}O_4$	278	000084-69-5	1877	X	X	X	X
5	Butyl isobutyl phthalate	$C_{16}H_{22}O_4$	278	017851-53-5	1924	X	X	X	X
6	2-azidoacetic acid methyl ester	$C_3H_5N_3O_2$	115	2000012-04-9	-		X		
7	Methyl 7,10,13-hexadecatrienoate	$C_{17}H_{28}O_2$	264	056554-30-4	-		X	X	
8	Methyl hexadecatrienoate	$C_{17}H_{28}O_2$	264	037822-81-4	-				X
9	3-Butenoic acid, 3-methyl-, (3-methyl-3-butenyl) ester	$C_{10}H_{16}O_2$	168	084817-90-3	-		X		
10	Geranyl hexanoate	$C_{16}H_{28}O_2$	252	010032-02-7	1711		X		
11	ethyl 2-(1-pyrrolidinyl)cyclopentanecarboxylate	$C_{12}H_{21}NO_2$	211	082297-61-8	-	X			
12	1-(8-methylnonyl) 2-octyl phthalate	$C_{26}H_{42}O_4$	418	001330-96-7	-		X		
13	Geranyl isobutyrate	$C_{14}H_{24}O$	208	002345-26-8	1518		X		
14	Farnesyl acetate	$C_{17}H_{28}O_2$	264	029548-30-9	1820	X			X
15	Neryl propionate	$C_{13}H_{22}O_2$	210	000105-91-9	-			X	
16	Geranyl propionate	$C_{13}H_{22}O_2$	210	000105-90-8	1475		X		
17	(2E,6E)-3,7,11-Trimethyl-2,6,10-dodecatrienyl pivalate	$C_{20}H_{34}O_2$	306	2000394-73-2	-	X			

Table 32. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
18	1H-Benzo[de][1,8]naphthyridine-8-carboxylic acid, 2,3-dihydro-6-hydroxy-5-methoxy-1-(4-methoxyphenyl)-2-methyl-, ethyl ester	$C_{23}H_{24}N_2O_5$	408	127983-55-5	-		X		X
19	Carbonic acid methyl[(2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienyl]ester	$C_{17}H_{28}O_3$	280	2000331-90-0	-		X	X	
20	Methyl 5-methyl-12-oxo-3,4-diphenyltricyclo[4.4.1.1(2,5)]dodeca-3,7,9-triene-2-carboxylate	$C_{27}H_{24}O_3$	396	2000558-78-3	-		X		
21	Methyl 1-(2,2-dimethoxyethyl)-5-hydroxy-2-methyl-1H-indole-3-carboxylate	$C_{15}H_{19}NO_5$	293	092827-50-4	-	X			
	Total Number of Esters					**8**	**12**	**5**	**6**
	Relative Amount (%)					**15.7 %**	**21.8 %**	**14.3 %**	**18.8 %**
	Flavonoids								
1	5-hydroxy-6-isopentyl-2,3-dimethyl-10-phenyl-2,3,9,10-tetrahydro-4H,8H-pyrano[2,3-f]chromene-4,8-dione	$C_{25}H_{28}O_5$	408	081559-57-1	-				X
2	Lupalbigenin	$C_{25}H_{26}O_5$	406	2000571-48-0	-			X	
	Total Number of Flavonoids					**0**	**0**	**1**	**1**
	Relative Amount (%)					**0 %**	**0 %**	**2.9 %**	**3.1 %**
	Furans								
1	(2R,4R,5R)-2-(3-furyl)-4,5-isopropylidenedioxytetrahydrofuran	$C_{11}H_{14}O_4$	210	124752-99-4	-	X			
2	2-Methyl-5-(1,1,5-trimethyl-5-hexenyl)furan	$C_{14}H_{22}O$	206	077143-15-8	-	X			

Table 32. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
3	6-Bis(4-cyanophenyl)amino-2,3,4,7-tetramethylbenzo[b]thiophene	$C_{26}H_{21}N_3S$	407	2000572-51-1	-		X	X	X
	Total Number of Furans					**2**	**1**	**1**	**1**
	Relative Amount (%)					**3.9 %**	**1.8 %**	**2.9 %**	**3.1 %**
	Hydrocarbons								
1	4-ethyl-2-methyl-2,3-hexadiene	C_9H_{16}	124	017530-19-7	-		X		
2	ethylidenecyclopentane	C_7H_{12}	96	002146-37-4	-	X			
3	3,5,7-trimethyl-2E,4E,6E,8E-decatetraene	$C_{13}H_{20}$	176	2000085-76-6	-				X
4	Dispiro[cyclopropane-(1,3')-tricyclo[2.2.1.0(2,6)]heptane-(5',1'')-cyclopropane-(7',1'')-cyclopropane]	$C_{13}H_{16}$	172	2000079-20-1	-	X			
5	Octa-2,4,6-triene	C_8H_{12}	108	2000008-30-1	-			X	
6	1-Methyl-2-methylene-4-isopropenylcyclohexane	$C_{11}H_{18}$	150	125673-03-2	-		X		
7	4-Isopropyl-1-methyl-3-methylenecyclohexane	$C_{11}H_{20}$	152	2000046-59-5	-		X		
8	2,6,10-Dodecatriene, 3(E),7(E),11-trimethyl-1-methoxy-	$C_{16}H_{28}O$	236	2000222-05-4	-		X		
9	Cyclohexadecane	$C_{16}H_{32}$	224	000295-65-8	1880		X		
10	1-[(1E)-1-octenyl]-3-vinylcyclopentane	$C_{15}H_{26}$	206	2000148-86-9	-	X			
11	(6Z)-3,7,11-trimethyl-1,6,10-dodecatriene	$C_{15}H_{26}$	206	076164-12-0	-	X			
	Total Number of Hydrocarbons					**4**	**5**	**1**	**1**
	Relative Amount (%)					**7.8 %**	**9.1 %**	**2.9 %**	**3.1 %**

Table 32. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
	Ketones								
1	3-Methyl-4-heptanone	$C_8H_{16}O$	128	015726-15-5	932	X			
2	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	$C_6H_8O_4$	144	028564-83-2	1130	X			X
3	1-Methylspiro[2.4]heptan-4-one	$C_8H_{12}O$	124	2000017-08-7	-	X			
4	1,3-Indandione, 2-diazo-	$C_9H_4N_2O_2$	172	001807-49-4	-		X		
5	10H-indeno[2,1-e]tetraazolo[1,5-b][1,2,4]triazin-10-one	$C_{10}H_4N_6O$	224	329710-24-9	-			X	
6	3-Methyl-4-methylenecycloheptanone	$C_9H_{14}O$	138	2000029-27-9	-	X			
7	4-(4-Methoxyphenyl)but-3-yn-2-one	$C_{11}H_{10}O_2$	174	2000081-60-7	-	X			
8	1,4-Oxathian-2-one, 3-methyl-	$C_5H_8O_2S$	132	035562-74-4	-			X	
9	trans-Geranylacetone	$C_{13}H_{22}O$	194	003796-70-1	1455		X		
10	3,4,5,6,7,8-hexahydro-1H-naphthalen-2-one	$C_{10}H_{14}O$	150	013837-12-2	-	X			
11	Farnesyl acetone C	$C_{18}H_{30}O$	262	2000287-52-7	-		X		
12	Cyclooctanone	$C_8H_{14}O$	126	000502-49-8	-	X			
13	2-Oxecanone, 10-methyl-, (.+-.)-	$C_{10}H_{18}O_2$	170	065371-24-6	1308	X			
14	5-Methyl-7-fluoro-1-tetralone	$C_{11}H_{11}FO$	178	2000088-11-4	-	X			
15	(20R)-3.beta.,5.alpha.,14.alpha.,20-Trihydroxy-24-norchol-7-en-22-yn-6-one	$C_{23}H_{32}O_4$	372	2000524-57-5	-		X		
16	Herqueinone	$C_{20}H_{20}O_7$	372	026871-30-7	-		X		
17	1-[3-(1H-indol-3-yl)-2,4-bis(4-methylphenyl)-3H-1,2,4-triazol-5-yl]ethanone	$C_{26}H_{24}N_4O$	408	2000573-85-4	-			X	

Table 32. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
18	3-(3,7,11,15-tetramethyleicos-2,6,10,14-tetraen-1-yl)-9-oxabicyclo[4.3.0]nona-1,3,5-trien-8-one	$C_{28}H_{38}O_2$	406	2000571-78-7	-				X
	Total Number of Ketones					**9**	**5**	**3**	**2**
	Relative Amount (%)					**17.6 %**	**9.1 %**	**8.6 %**	**6.3 %**
	Lactones								
1	ε-Caprolactone	$C_6H_{10}O_2$	114	000502-44-3	-		X		X
2	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	$C_{11}H_{16}O_2$	180	017092-92-1	1537	X	X		
	Total Number of Lactones					**1**	**2**	**0**	**1**
	Relative Amount (%)					**2.0 %**	**3.6 %**	**0 %**	**3.1 %**
	Monoterpenes								
1	Geranyl linalool isomer B	$C_{20}H_{34}O$	290	000000-00-0	-				X
2	γ-Terpineol	$C_{10}H_{18}O$	154	000586-81-2	1192	X			
3	α-Fenchene	$C_{10}H_{16}$	136	000471-84-1	953			X	
	Total Number of Monoterpenes					**1**	**0**	**1**	**1**
	Relative Amount (%)					**2.0 %**	**0 %**	**2.9 %**	**3.1 %**
	Nitriles								
1	2-(3-cyano-4-[(E)-2-(2-furyl)ethenyl]-5,5-dimethyl-2(5H)-furanylidene)malononitrile	$C_{16}H_{11}N_3O_2$	277	2000323-85-2	-		X		
2	2-(4-methoxyphenoxy)acetonitrile	$C_9H_9NO_2$	163	022446-12-4	-		X		

Table 32. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
	Total Number of Nitriles					**0**	**2**	**0**	**0**
	Relative Amount (%)					**0 %**	**3.6 %**	**0 %**	**0 %**
	Norisoprenoids								
1	(E)-.alpha.-ionone	$C_{13}H_{20}O$	192	2000117-32-9	-			X	
2	Megastigma-4,6(E),8(Z)-triene	$C_{13}H_{20}$	176	071186-24-8	1354		X		
3	α-Ionene	$C_{13}H_{18}$	174	000475-03-6	1266			X	
4	β-Ionone	$C_{13}H_{20}O$	192	014901-07-6	1493	X	X	X	X
5	3-Buten-2-one, 4-(3-hydroxy-2,6,6-	$C_{13}H_{20}O_2$	208	015401-34-0	-			X	
6	(E)-4-Oxo-β-ionone	$C_{13}H_{18}O_2$	206	027185-77-9	-	X			
7	3,5,5-Trimethyl-4-(3-oxobutyl)cyclohex-2-enone	$C_{13}H_{20}O_2$	208	074233-41-3	-		X		
8	2,4,4-trimethyl-3-[(E)-3-oxidanylbut-1-enyl]cyclohex-2-en-1-one	$C_{13}H_{20}O_2$	208	029790-30-5	-	X	X	X	
9	Vomifoliol	$C_{13}H_{20}O_3$	224	023526-45-6	1806	X	X	X	X
	Total Number of Norisoprenoids					**4**	**5**	**6**	**2**
	Relative Amount (%)					**7.8 %**	**9.1 %**	**17.1 %**	**6.3 %**
	Organophosphorus Compounds								
1	1-tert-butylphospholane	$C_8H_{17}P$	144	108568-39-4	-		X		
2	Triphenylphosphine oxide	$C_{18}H_{15}OP$	278	000791-28-6	2561				X
	Total Number of Organophosphorus Compounds					**0**	**1**	**0**	**1**
	Relative Amount (%)					**0 %**	**1.8 %**	**0 %**	**3.1 %**

Table 32. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
	Phenols								
1	2-allyl-4-tert-butyl-phenol	$C_{13}H_{18}O$	190	2000112-79-9	-	X			
	Total Number of Phenols					**1**	**0**	**0**	**0**
	Relative Amount (%)					**2.0 %**	**0 %**	**0 %**	**0 %**
	Pyridines								
1	2-acetyl-3,4,5,6-tetrahydropyridine	$C_7H_{11}NO$	125	027300-27-2	-	X			
2	6,6'-Bis[1,3-nonadiynyl]-2,2'-bipyridine	$C_{28}H_{28}N_2$	392	2000553-79-7	-	X			
3	1-ethyl-3-(4-methylphenyl)-4a,10b-dihydro-1H-chromeno[3,4-c]pyridine-2,4,5-trione	$C_{21}H_{19}NO_4$	349	2000484-64-9	-			X	
	Total Number of Pyridines					**2**	**0**	**1**	**0**
	Relative Amount (%)					**3.9 %**	**0 %**	**2.9 %**	**0 %**
	Sesquiterpenes								
1	trans-β-Caryophyllene	$C_{15}H_{24}$	204	2000143-74-7	-				X
2	β-Sesquiphellandrene	$C_{15}H_{24}$	204	020307-83-9	1526		X		
3	α-Bisabolene	$C_{15}H_{24}$	204	025532-79-0	1504				X
4	(E)-Nerolidol	$C_{15}H_{26}O$	222	040716-66-3	1569			X	
5	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	$C_{15}H_{24}$	204	2000143-89-9	-	X			
6	α-trans-sequicyclogeraniol	$C_{15}H_{26}O$	222	108287-12-3	-		X	X	
7	cis-Caryophyllene	$C_{15}H_{24}$	204	013877-93-5	1467	X	X		
8	trans-β-Farnesene	$C_{15}H_{24}$	204	000502-60-3	-			X	X

Table 32. Cont.

#	Compound ID	Molecular Formula	Molecular Weight	CAS [a]	RI [b]	Municipalities			
						Rincon	Mayagüez	Aguada	Cayey
9	γ-Gurjunene	$C_{15}H_{24}$	204	022567-17-5	1470		X		
	Total Number of Sesquiterpenes					**2**	**4**	**3**	**3**
	Relative Amount (%)					**3.9 %**	**7.3 %**	**8.6 %**	**9.4 %**
	Steroids								
1	6-Methyl-16.alpha.,17.alpha.-cyclohexano-19-nor-pregn-4-ene-3,20-dione	$C_{25}H_{36}O_2$	368	2000518-10-1	-				X
2	Stigmasterol	$C_{29}H_{48}O$	413	000083-48-7	-	X			
3	Cichosterol (13,14-seco-stigma-5(6),14(15)-diene-3.beta.-ol)	$C_{29}H_{50}O$	414	2000581-27-8	-	X			
4	24.xi.-Stigmast-5-en-3.beta.-ol	$C_{29}H_{50}O$	414	019044-06-5	-	X	X		
5	γ-Sitosterol	$C_{29}H_{50}O$	415	000083-47-6	-			X	
6	β-Sitosterol	$C_{29}H_{50}O$	415	000083-46-5	3197				X
7	Fucosterol	$C_{29}H_{48}O$	413	017605-67-3	-	X			
	Total Number of Steroids					**4**	**1**	**1**	**2**
	Relative Amount (%)					**7.8 %**	**1.8 %**	**2.9 %**	**6.3 %**
	Triterpenes								
1	Squalene	$C_{30}H_{50}$	411	007683-64-9	2808		X	X	
	Total Number of Sesquiterpenes					**0**	**1**	**1**	**0**
	Relative Amount (%)					**0 %**	**1.8 %**	**2.9 %**	**0 %**
	Total Number of Identified Compounds*					**19**	**31**	**21**	**22**
	Total Compounds					**51**	**55**	**35**	**32**

[a]Chemical Abstract Service Registry Number
[b]Literature Retention Index from NIST Standard Reference Database