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Effect of Entomopathogenic and Nematofagous Fungi on the behaviour of the Black Banana Weevil (*Cosmopolites sordidus*, Germar, 1824) (Coleoptera Curculionidae): Biological Control

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ABSTRACT

The present work has led to the detection of VOCs of fungal origin that acts as repellents against *Cosmopolites sordidus* (BW), pest-key of the banana tree (*Musa* spp.).

Initially the identification of the VOCs, produced by two different *Beauveria bassiana* strains (Bb203 and Bb1TS11), a *Metarhizium anisopliae* strain (Ma4TS04) and a *Pochonia clamydosporia* strain (Pc123), was made through the use of GC/MS-SPME (Gas Chromatography/Mass Spectrometry - Solid-Phase Micro Extraction). The GC/MS-SPME analysis led to the identification of 193 different VOCs, among which 5 repellent candidates were identified. The selected VOCs, together with two repellent compounds

of *Rhynchophorus ferrugineus* (C1 and C2) and two technical repellents (garlic and colloidal sulphur), were tested *in vitro* to evaluate their effect on the behaviour of *C. sordidus*, in laboratory conditions. Of the repellent candidates tested, only two (C1 and C2) were used for *in vivo* tests.

From the findings of *in vitro* data, *hard-repellents* (C7 and C5) can be considered effective repellents in the management of *C. sordidus*. *Soft-repellents* (C1, C2, C3, C4, C6 and garlic) are considered repellent, although milder, for BW under laboratory conditions.

In field tests, however, both tested VOCs did not exhibit the same repellent action shown in the laboratory. This phenomenon could be ascribed more to the more complex environmental conditions of *in vivo* tests than to those *in vitro*, but also the non-optimal VOCs dispersion technology used for field tests.

Therefore, *in vitro*, new VOCs have been identified that can be used as repellents for BW. The implementation of technologies associated with the dispersion of these repellents could produce progress in the agrobiotechnological sustainability of the world banana cultivation.

OBSERVACIONES

RESUMEN

El presente trabajo ha conducido a la detección de COVs de origen fúngica que actúan como repelentes contra *Cosmopolites sordidus* (BW), plaga de la platanera (*Musa* spp.). Inicialmente, la identificación de los COVs producidos por dos cepas diferentes de *Beauveria bassiana* (Bb203 y Bb1TS11), una cepa de *Metarhizium anisopliae* (Ma4TS04) y una cepa de *Pochonia clamydosporia* (Pc123), fue hecha mediante el uso de GC/MS-SPME (Cromatografía de Gases/Espectrometría de Masas – Micro Extracción en Fase Sólida). El análisis GC/MS-SPME llevó a la identificación de 193 VOC diferentes, entre los cuales se identificaron 5 candidatos repelentes. Los COVs seleccionados, juntos con dos compuestos repelentes de *Rhynchophorus ferrugineus* (C1 y C2) y dos repelentes técnicos (ajo y azufre coloidal), se probaron *in vitro* para evaluar sus efectos sobre el comportamiento de *C. sordidus*, en condiciones de laboratorio. De los candidatos repelentes probados, solo dos (C1 y C2) se usaron para pruebas *in vivo*.

A partir de los resultados de los datos *in vitro*, los repelentes rígidos (C7 y C5) pueden considerarse repelentes efectivos en el tratamiento de *C. sordidus*. Los repelentes blandos (C1, C2, C3, C4, C6 y ajo) se consideran repelentes, aunque más suaves, para BW en condiciones de laboratorio.

Sin embargo, en las pruebas de campo, ambos los COVs probados no exhibieron la misma acción repelente mostrada en el laboratorio. Este fenómeno podría atribuirse más a las condiciones ambientales más complejas de las pruebas *in vivo* que a las *in vitro*, pero también a la tecnología de dispersión de los COVs no óptima, utilizada para las pruebas de campo.

Por lo tanto, *in vitro*, se han identificado nuevos COVs que pueden usarse como repelentes para BW. La implementación de tecnologías asociadas con la dispersión de estos repelentes podría producir avances en la sostenibilidad agrobiotecnológica del cultivo mundial de platanera.

PALABRAS CLAVE

Musa spp.*, Cosmopolites sordidus, Pochonia chlamydosporia, Metarhizium anisopliae, Beauveria bassiana,* Compuestos Orgánicos Volátiles, repelentes, control biologico.

ABSTRACT

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KEY WORDS

Musa spp.*, Cosmopolites sordidus, Pochonia chlamydosporia, Metarhizium anisopliae, Beauveria bassiana,* Volatile Organic Compounds, repellents, biological control.

This Master Project (TFM) is subjected to confidentiality. Therefore, chemical names of compounds have been substituted by codes. However, information is given on the detection of the compounds by GC/MS-SPME.

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1. Introduction

1.1 Banana (*Musa spp.*)

Banana (*Musa spp.)* belongs to the Musaceae family (Nelson *et al.*, 2006). It is a monocotyledonous perennial that can grow up to 9 meters high or more (Turner, 1994, Nelson *et al.*, 2006). Despite its large dimensions, it does not have a real trunk; instead, it has leaf sheaths that develop a pseudocorm (Fig. 1) (Gold *et al.*, 2001; Turner *et al.*, 2007). The banana plant has an underground central axis, a sympodium, commonly called corm that holds the leaves (Turner *et al.*, 2007). It also presents a homorhyzic root system with ramified roots of different orders (Lecompte *et al.*, 2001).

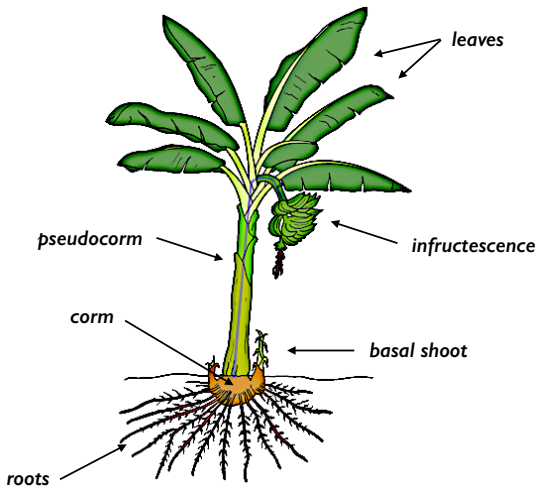

Figure 1: Morphology of the Musa sp. *plant*

The clones of the current cultivated banana plants derive from hybrids of the wild species: *Musa acuminata* Colla, 1820 (genome A) and *Musa balbisiana* Colla, 1820 (genome B) (Simmonds, 1962).

Bananas are essential for food security in a wide variety of tropical and subtropical countries, being one of the best-known fruits in industrialized countries (Lescot, 2011). People have been using bananas for at least 7,000 years in Papua New Guinea (Denham *et al*., 2003). Bananas are the most consumed and cultivated fruit trees worldwide (Ferri *et al*., 2012). They are the fourth crop, by production, after rice (*Oryza spp*.), wheat (*Triticum spp*.) and, corn (*Zea spp*.) (Nelson *et al*., 2006). Approximately 5.5 million hectares of land are devoted to banana production worldwide, according to the latest data available for 2015 (FAOSTAT). Therefore, given the growth of the human population in the last 50 years, a significant increase in food production is required (Pingali, 2012).

Therefore, improvement of crop management is an effective solution to address this problem.

The world production of bananas is 114 million tons (FAO, 2018). Asia is the continent with the highest production rate (50.82%). The global banana industry generates around 8 billion USD per year. However, only 15% of banana production is traded in the international market, the rest is consumed locally, especially in large producing countries such as India, China and Brazil, and some African countries where bananas highly contribute to human diet (FAO, 2016). The leading exporters of organic bananas are Colombia, Perú and the Dominican Republic (FAO, 2018).

The principal organisms that affect bananas are the Black Banana Weevil (BBW or BW) (*Cosmopolites sordidus* Germar 1824), plant-parasitic nematodes (*Pratylenchus goodeyi*, *Helicotylenchus multicintus*, *Radopholus similis* and *Meloidogyne spp*.) and pathogenic fungi such as *Fusarium oxysporum* f. sp. cubense (Foc) ((Smith) Snyder and Hansen) (Ostmark, 1974; Dubois *et al*., 2004; Waweru *et al*., 2014) and *Mycospharella fijiensis* Morelet (FAO 2013). These pests and pathogens affect the roots and corm, altering the absorption of water and nutrients. This causes a decrease in vigour, reduces the size and can lead to the death of the whole plant (Nankinga and Moore, 2000; Dubois *et al*., 2004; Waweru *et al*., 2014). Other important banana diseases are *Banana Bunchy Top Virus* (BBTV), *Banana Streak Virus* (BSV) and *Xanthomonas* wilt (*Xanthomonas campestris* pv. musacearum (Yirgou and Bradbury 1968) Dye 1978) (Blomme *et al*., 2014).

C. sordidus can cause losses to banana crops from 30% up to 90% total production (Carballo 1998; Musabyimana *et al*., 2001; Muñoz-Ruiz, 2007), in massive infestations.

Gros-Michel, Payo, Gran-dwarf and Little-dwarf banana cultivars are resistant to the Panama Disease caused by Focs but are sensitive to nematodes (Hwang and Ko, 2004). The Yangambi Km5 is also resistant to *Mycosphaerella* (Quénéhervé *et al*., 2009). However, there has been little information available on BW-resistant banana cultivars, making this pest a severe problem (Kiggundu *et al.*, 2003).

1.2 Black Banana Weevil (*Cosmopolites sordidus,* BW)

The Black Banana Weevil is a coleopteran that belongs to the Curculionoidea superfamily, the Curculionidae family and the Dryophthorinae subfamily. It has been known as *Curculio mendicus* (Olivier, 1791), but also as *Calandra sordida* (Germar,

1824). In 1885, Chevrolat changed it to its currently recognized name: *Cosmopolites sordidus* (Viswanath, 1976).

C. sordidus only have a single congenic species, *Cosmopolites pruinosus* (Heller, 1934) (Fig.2) which is associated with bananas in Indonesia, the Philippines and the Caroline Islands (Zimmerman, 1968 a, b).

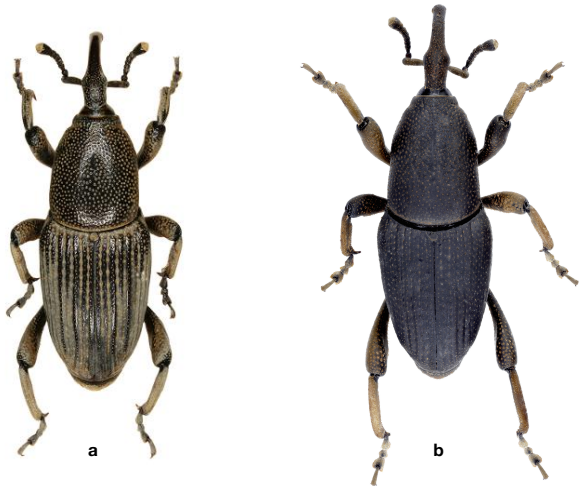

Figure 2: General view of Cosmopolites sordidus *(a) (https://inpn.mnhn.fr/espece/cd_nom/328477?lg=en) and* C. pruinosus *(b) (https://www.zin.ru/Animalia/Coleoptera/rus/curgshcc.htm)*

The Curculionoidea superfamily is the largest group in the most species abundant order of living organisms, the Coleoptera (Conord *et al.,* 2008). There are more than 62,000 species of weevils in the world which represent 15.5% of all known Coleoptera (Oberprieler *et al*., 2007). These organisms can be found in a wide variety of habitats where they are particularly abundant and diverse (Zimmerman, 1993). Some Coleoptera are main pests for agriculture and forestry, while others are beneficially used for the biological control of noxious weeds (Conord *et al.,* 2008). The family Curculionidae comprises more than 80% of all weevil species with approximately 4,600 genera and 51,000 described species (Oberprieler *et al.*, 2007). The Curculionidae feed most on the monocotyledons, and therefore these plants are probably their ancestral hosts and may have played a fundamental role in the diversification of the family (Marvaldi *et al.*, 2002, Oberprieler, 2004).

BW causes more crop destruction than other arthropod pests in all banana producing countries (Nankinga and Moore, 2000). It is native to the Indo-Malayan Region (Simmonds, 1966), coinciding with the area of origin of bananas (Stover and Simmons, 1987). BW has also been found in Central Africa, Central America, the Pacific Islands

and all regions where bananas grow between latitudes of 31° S and 30° N (Fig. 3) (Vilardebo, 1973). In Europe, its presence is limited only to Madeira (Portuguese territory) (Yuasa, 1939) and the Canary Islands (Spanish territory) (Carnero *et al*., 2002).

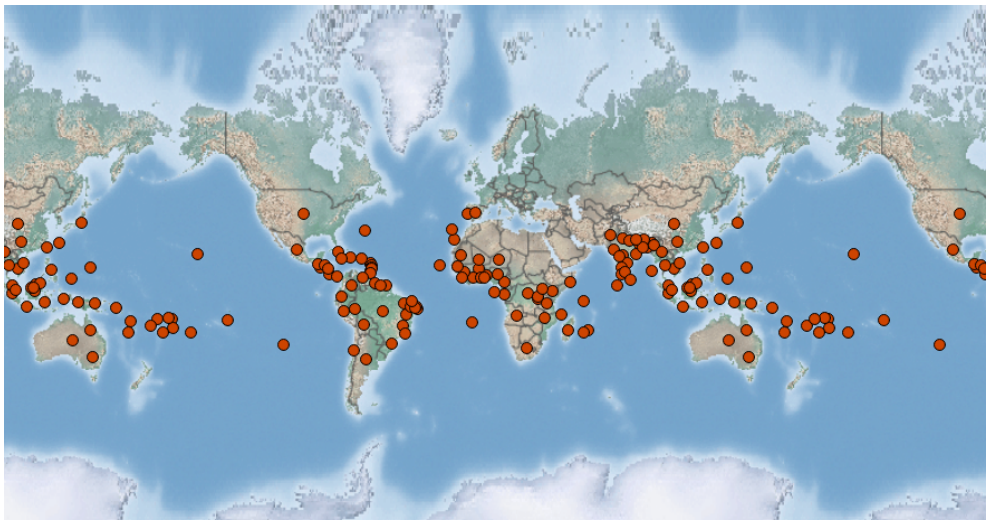

Figure 3: BW worldwide distribution map

The BW has a classic "*k-strategy*" life cycle with a long cycle of up to 2 years and low fertility. Adults are active at night and prefer crop residues and a humid environment. Although they have functional wings, their flight is limited, which makes the species relatively sedentary. Therefore, its dispersion mainly occurs through the transport of infested plant material.

The invasion and spread of the BWs are due to its behaviour to search and acquire food, mate, oviposition and breeding sites. BW can infest corm, pseudocorm and basal shoots of banana plants. In this process, BWs obtain resources are essential for growth and development and, to ensure the biological effectiveness of future generations. These efficient BWs search mechanisms are based on their antennas that function as specialized primary chemo- and mechanoreceptors. These precise environmental assessment mechanisms are crucial to ensure BWs survival and reproduction in the environment.

BW females have low reproductive potential, producing very few eggs (1-4 eggs/week) reaching 10-270 eggs in their whole lives (Lemaire, 1996). They also may not have oviposition for prolonged periods. BW have 5-8 larval stages in their life cycle (Fig. 4). The whole cycle from egg to adult takes 5-7 weeks under tropical conditions (Gold *et al*., 2001).

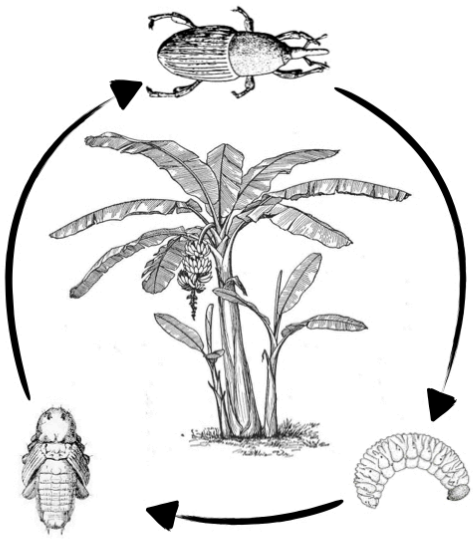

Figure 4: Life cycle of C. sordidus *in* Musa *sp. (original source)*

Plant damage is caused by the wounds produced by the larvae, in the corm. BW form tunnels of circular section which widen with larval growth. They are full of dark-coloured rubble (Franzmann, 1972). These tunnels reduce the absorption of nutrients and weaken plant stability. Damages and losses tend to increase over time (Gold *et al*., 2001) and destroy the root system. The tunnels lead to a slow and subside growth of the plants and the consequent reduction of fruit production. In most severe cases, plants can dry and die (Rukazambuga *et al*., 1998).

Tunnelling provides a point of entry for pathogens such as *Pseudomonas* (= *Ralstonia*) *solanacearum* (Moko disease) and other rot organisms (Gold *et al.*, 2001). The production and reduced growth of shoots (*keikis*) occur when mother plant is severely damaged. Relatively small damage is caused by adults who eat plant tissues (Franzmann, 1972).

BW can also act as vector of important banana pathogens such as *F. oxysporum* f.sp. *cubense* TR4 (Foc) (Meldrum *et al*., 2013). Several viable spores of Foc were found in the exoskeletons of 10% of the weevils, which implies that BW can be a vector. Therefore, control of vectors such as *C. sordidus* in banana plantations can help minimize the spread of the pathogens and its associated disease. Assosiated BW movement during oviposition and feeding on corm or pseudocorm infected with *Xanthomonas campestris pv. musacearum*, the BWs move and, therefore, can propagate the bacterium and infect healthy plants in the field (Were *et al*., 2015).

BW has several predators such as the coleopterans *Thyreocephalus interocularis* (Eppelsheim, 1895), *Plaesius laevigatus* Marseul, 1853, *Hololepta quadridentata* (Olivier, 1789), *Eutochia pulla* (Erichson, 1843), *Dactylosternum abdominale* (Erichson,

1840) and the ants *Tetramorium bicarinatum* (Nylander, 1846), *Pheidole guineensis* (Fabricius, 1793). Other antagonists are the entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* and entomopathogenic nematodes (e.g. *Steinerma spp*. and *Heterorhabditis spp*). Besides, there are occasional egg predators such as *Euborellia annulipes* (Lucas, 1847). The use of these natural enemies is not always easy. Sometimes, they are not established in BW infested regions, they have not been tested under field conditions, or their natural populations are not large enough to reduce BW damage (Gold and Messiaen, 2000).

1.3 Entomopathogenic and Nematophagous Fungi

The entomopathogenic fungi *Beauveria bassiana* (*Bb*) (Balsamo) Vuillemin and *Metarhizium anisopliae* (*Ma*) (Metschnikoff) Sorokin, belong to the Hypocreales (Ascomycota). They infect of a wide range of insects and arachnids and have a cosmopolitan distribution (Roberts and St. Leger, 2004; Rehner, 2005). Entomopathogenic fungi use their spores to infect their hosts directly through the exoskeleton (Huxham *et al*., 1989; La Forgia and Verheggen, 2018). Their spores are well adapted for dispersion (Evans and Hywel-Jones, 1997).

Bb is a cosmopolitan soilborne entomopathogenic fungus. *Bb* was the first microbe found to cause disease by Agostino Bassi in silkworm (*Bombyx mori*), long before Kock and Pasteur stated the basis of modern microbiology and axenic practices (Rolff and Reynolds, 2009). It is one of the most widely toward and easy to recognize of all entomopathogenic fungi. Morphologically, *Bb* is easy to distinguish because it is sympodial to whorled clusters of short-globose to flask-shaped conidiogenous cells that produce a succession of one-celled, sessile, hyaline, holoblastic conidia on a progressively elongating sympodial (zig-zag) rachis (Kirk *et al*. 2001). Unquestionably, considerable taxonomic confusion surrounds *B. bassiana*, for which many morphologically similar species have been described. Thus far, seven species have placed into synonymy with *B. bassiana*, including *B. dense*, *B. doryphorae*, *B. effusa*, *B. globulifera*, *B. shiotae*, *B. stephanoderis*, and *B. sulfurescens* (www.speciesfungorum.org, July 31, 2019).

Bb is well characterized in respect to pathogenicity to several insects, and has been used for the biological control of important agricultural pests worldwide (De Faria and Wright, 2001; Güerri-Agulló *et al.*, 2011; Vega and Kaya, 2012).

M. anisopliae is also a cosmopolitan species of soilborne entomogenous moulds, that can kill a broad spectrum of insects. It was the first fungus worldwide to be mass produced and utilized for insect-pest control (Steinhaus, 1975). Insects infected with *M. anisopliae* initially have a white mould that turns green early when conidia are produced (Freimoser *et al.*, 2003).

Ma is second only to *Bb*. They are the fungi most frequently used in the field against insects. *Bb* just as *Ma*, also appears to be a complex of morphologically similar species including *M. album*, *M. velutinum*, *M. guizhouense*, and *M. pinghaense* (www.speciesfungorum.org, July 31, 2019).

Pochonia chlamydosporia (*Pc*) (Goddard) Zare and Gams is a nematophagous fungus belonging to the Hypocreales (Ascomycota). *P. chlamydosporia* parasitizes egg masses and nematode females (Willcox and Tribe, 1974; De Leij *et al*., 1992; Bourne *et al*., 1996) in the rhizosphere but it is also a common soil fungus (Domsch *et al*., 1993; Zare *et al*., 2001). Therefore, it has been used as a biocontrol agent to manage populations of cyst and root knot nematodes (Manzanilla-Lopéz *et al*., 2013). *Pc* can grow in the soil as a saprophyte (Domsch *et al*., 1993) and can also colonize the roots as a true endophyte (Maciá-Vicente *et al*., 2009; Escudero and Lopez-Llorca, 2012).

1.4 VOCs (Volatile Organic Compounds)

Volatile organic compounds (VOCs) are solid and liquid carbon-based substances that enter the gaseous phase by vaporization at 20 °C and 0.01 kPa (Pagans *et al*., 2006).

Most VOCs are typically lipophilic liquids with high vapour pressure (Campos *et al*., 2010) and appear as intermediate and final products of various metabolic pathways. They are emitted into the atmosphere from natural sources in marine and terrestrial environments (Guenther *et al*., 1995). The surface flows of these compounds are interesting for their role in tropospheric chemistry and the global carbon cycle (Fehsenfeld *et al*., 1992).

The emission of volatile organic compounds plays essential ecological and physiological roles for many organisms, such as fungi which release a broad spectrum of VOCs (Splivallo *et al*., 2011; Kramer and Abraham, 2012). Fungal VOCs belong to different chemical groups such as monoterpenoids, sesquiterpenes, alcohols, aldehydes, aromatic compounds, esters, furans, hydrocarbons, ketones and others (Splivallo *et al*., 2007; Campos *et al*., 2010; Kramer and Abraham, 2012).

Fungi, such as the three species mentioned above, produce various volatile compounds (Crespo *et al*., 2006; Müller *et al*., 2013) through their metabolic pathways. Fungal VOCs derive from both primary and secondary metabolism (Korpi *et al*., 2009). Since they can spread through the atmosphere and the soil, they are ideal semiochemicals (Morath *et al*., 2012) and mediate interactions between organisms. They are essential for fungal-insect relations (Yanagawa *et al*., 2009). The role of many fungal VOCs is to act as attractants and/or repellents for insects and other invertebrates. Attractants are compounds that attract or lure insects or other arthropods, such as pheromones and kairomones. Instead, repellents are substances that deter insects or other invertebrates from approaching or settling in specific targets. The chemoreceptors of their antennae usually detect VOCs. These compounds may generate alerts in the insect about the presence of possible partners, food, suitable places to lay their eggs or dangers that should be avoided. Therefore, any chemical that could interrupt and modify the behaviour of the BW and in general its search ability for the host (*Musa sp.*) could serve as a tool for BW sustainable management.

Fungal-VOCs production is biologically dynamic. The profile of a particular species or strain varies according to the substrate, the duration of the incubation, nutrient type, temperature and other environmental parameters (Pasanen *et al*., 1997; Nilsson *et al*., 2004; Fiedler *et al*., 2005).

1.5 Objectives

The present Master Thesis has three main parts, each planned to achieve the main objective, namely the detection of VOCs of fungal origin that can act as repellents against BW.

The first part deals with the identification of the VOCs produced by two different *Bb* strains (*Bb*203 and *Bb*1TS11), a *Ma* strain (*Ma*4TS04) and a *Pc* strain (*Pc*123) through the use of the GC/MS-SPME (Gas Chromatography/Mass Spectrometry - Solid-Phase Micro Extraction). The focus of the detection of the compounds produced is the metabolic characterization of the fungi, mentioned above, at different ages of mycelial development and, the identification of the characteristic VOCs for each species (or strain). The substances selected from this phase are then used for the laboratory tests (second part).

The second part concerns with the testing of volatile substances, selected formerly, in an olfactometer. This phase aims to test the effect of the selected VOCs, on the behaviour of *C. sordidus*, under laboratory conditions.

Of the VOCs compounds detected and tested in olfactometers, only two (C1 and C2) were used for field tests (third part).

These two substances were involved in the third part because they were already the subject of former experimentation and patenting of the laboratory of Plant Pathology of the Univesity of Alicante. Their repellent capacity has been tested on another species of weevil, namely the Red Palm Weevil (RPW) (*Rhynchophorus ferrugineus* Olivier, 1790) (Jalinas, 2016). The third part consists of the field testing (banana plant) on the island of Tenerife (Canary Islands) on commercial banana plantations naturally infested with BW.

2. Materials and Methods

2.1 *B. bassiana, M. anisopliae* and *P. chlamydosporia*

In order to identify the EF-VOCs (Entomopathogenic Fungi-VOCs) and the NF-VOCs (Nematophagous Fungi-VOCs), two species of entomopathogenic fungi (*B. bassiana* and *M. anisopliae*) and a species of nematophagous fungus (*P. clamydosporia*), have been used.

We used two different *Bb* strains, namely *Bb*203 and *Bb*1TS11. The first one was isolated from naturally infected *R. ferrugineus* adults in Spain (Daimès, Elche; CBS 121097; Güerri-Agulló *et al.*, 2010). Instead, *Bb*1TS11 is a strain isolated from soil samples from the rhizosphere of banana plantations located in the Canary Islands (CECT 21121; NCBI MK156717; Lozano-Soria, 2018). Therefore, this strain is supposed to have interacted directly with the Canarian population of the BW. *Bb*1TS11 is able to infect BW under laboratory conditions (A. Lozano-Soria, 2019 pers. com.). As far as *Ma* is concerned, a single strain was used: *Ma*4TS11 (CECT 21126; NCBI MK156715; Lozano-Soria, 2018). This strain has also been isolated from a banana plantation soil in the Canary Islands. *Ma*4TS04 is also able to infect BW (A. Lozano-Soria, 2019 pers. com.). The *Pc* strain was used (*Pc*123) was previously isolated from eggs of *Heterodera avenae* in Seville (SE Spain) (ATCC No. MYA-4875; CECT No. 20929; Olivares-Bernabeu and López-Llorca, 2002).

For the cultivation of the fungi, 250 ml flasks were used, to which were added, as a substrate, 75 g of rice (*Oryza sativa* cv Redondo), prepared and inoculated as reported by Güerri-Agulló *et al.* (2010)

2.2 Gas Chromatography-Mass Spectrometry (GC/MS)

GC/MS was carried out using SPME (Solid-Phase Micro Extraction). SPME uses a fused silica fibre (about 1 cm long and 0.110 mm ∅) covered with a small amount of extracting phase. The silica fibre (SPME fibre) is attached to a metal rod that acts as a piston and is made to slide inside a needle. The current version of this device (called *holder*), can be described as a modified syringe (Fig. 5) (Purcaro *et al*., 2014).

In a first extraction step, the previously thermo-conditioned fibre is exposed in the headspace of the sample to be analysed. In this phase, the analytes (VOCs) are divided between the sample and the fibre until an equilibrium is reached. After an appropriately optimized extraction time, the fibre is withdrawn into the needle and follows the desorption phase, which can be carried out in a GC injector.

This technique can eliminate or significantly reduce the consumption of solvent and allows VOCs extraction and purification in a single step. In fact, in the SPME, the final analytical determination can be considered incorporated, since the analytes are thermodesorbed directly in the GC injector.

The main advantages of SPME are reduced solvent use a minimum and small quantity of sample required for the analysis. Sample preparation is effortless and fast; furthermore, the analytes are not only extracted but also pre-concentrated from any matrix (culture mediums in our case).

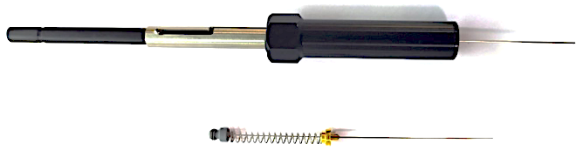

Figure 5: SPME holder and fibre needle

We prepared four samples, to analyse their VOCs, one for each fungus strain, plus uninoculated rice (control). For each sample 5 g were sampled and placed in a vial (HS, crimb, FB, 20 ml, clr, cert, 100 PK, Agilent Technologies) hermetically capped by a pressure plug with a plastic membrane (Fig 6d). Samples were then placed individually in a thermostatic bath, at 60 °C (Fig 6a, b).

Absorption of released VOCs occurred by exposing the fibre of the holder to the headspace (Fig 6c), for 15 min. Following this operation, the holder was inserted into the GC injector (Fig 7b) where it was left exposed at 150 °C desorption for 4 min with the spitless mode.

The equipment used is an Agilent 5973 Network Mass Spectrometer coupled with a gas chromatograph, also known as the Agilent model 6890N (Fig 7a, c).

The temperature program used for chromatography was initial temperature 35 ºC for 5 min and at 3 ºC/min at 150 ºC for 1 min. Afterwards at 5 ºC/min at 250 ºC until the end of the analysis. The chromatography run lasted 38 min.

The column used is a DB624 from J&W Scientific de 30 m, 0.25 mm ID 1.4 µm. The ionization source for electronic impact at 70 eV and 230 °C.

A simple quadrupole was used as a detector at 150 ºC. Wiley275 library was used for identifying VOCs.

Fungal cultures and controls were sampled and processed for GC-MS VOCs identification at 10, 20, 30, 40, 50 and 60 days after inoculation (dai).

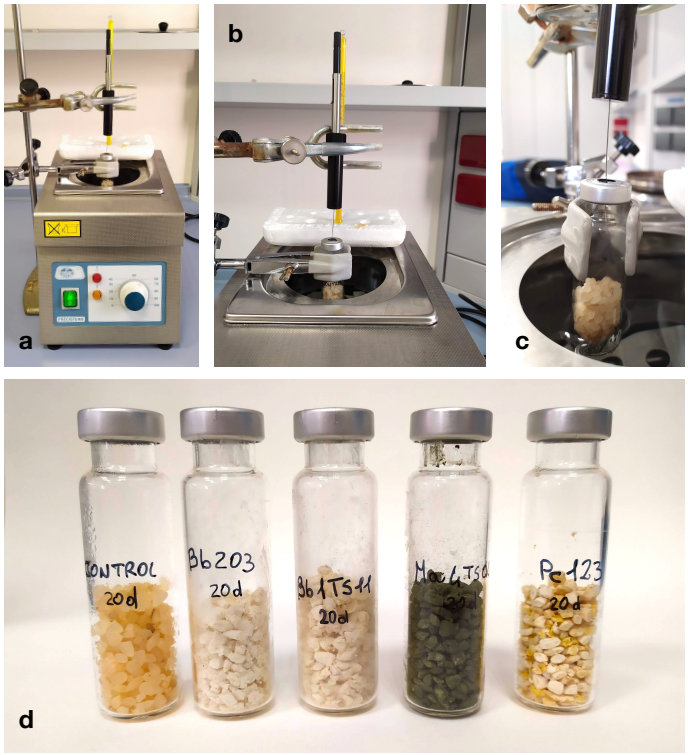

Figure 6: Handling for SPME sample preparation; a, b) Samples in the thermostatic bath; c) detail of fibre in headspace; d) Vials containing the tested samples (20 dai cultures)

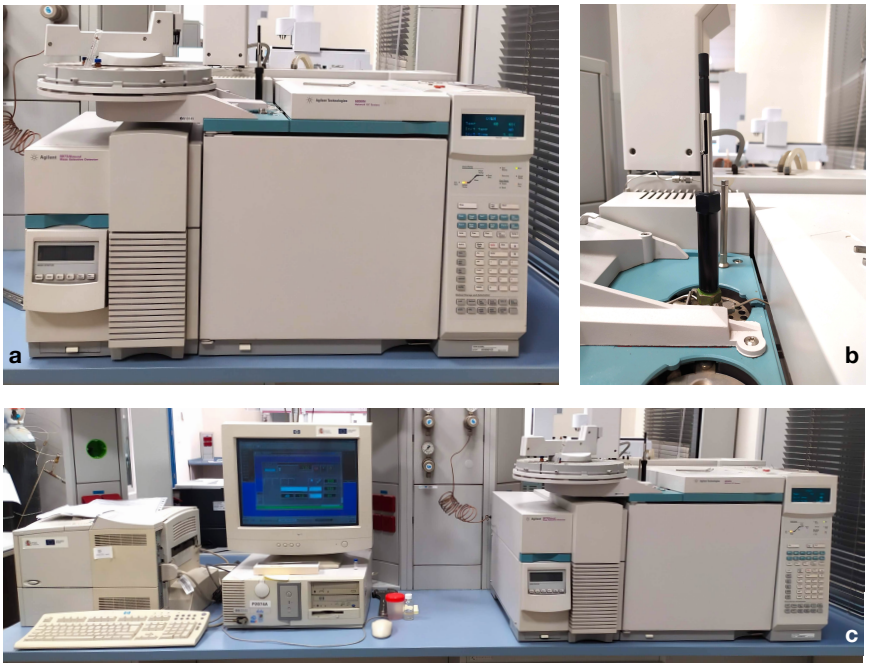

Figure 7: GC-MS equipment (SSTTII, University of Alicante); a) GC with holder inserted in the injector; b) detail of the holder inserted in the injector during the desorption; c) GC/MS-SPME system

After each chromatography run, the software generated a chromatogram and a detailed list of the VOCs detected compounds. Data obtained were processed in order to obtain, for every strain, different Venn diagrams. For each sample, there were three different diagrams. The first diagram is synoptic since lists all the VOCs detected characteristic of fungi (Total-VOCs – T-VOCs). A second set includes *major-VOCs* (M-VOCs), which match ≥ 50% with the database (DB) entries and a peak-height (abundance) > 100,000 ppm. Finally, the third set contains *minor-VOCs* (m-VOCs), i.e. those compounds that have a match ≥ 50% with DB entries and a peak-height between 100,000 and 20,000 ppm.

2.3 Two-way olfactometer

A Two-Way Olfactometer (TWO) (Fig. 8) was used to evaluate the behaviour of BW subjected to the action of different olfactory stimuli (VOCs attractive or repellent). The olfactometer is made up of a transparent Y-shaped glass tube with a length of the linear part of 30 cm; length of each arm 33 cm, Ø 6 cm and the angle between the two arms of 60°. At the end of the two arms, two "Odour Chambers" (OCs) (Bazzocchi and Maini, 2000) are placed, consisting of two glass flasks into which any olfactory stimulus to be tested, is inserted.

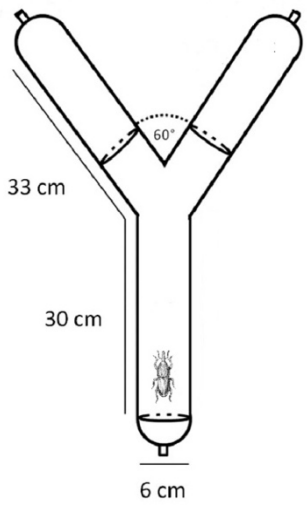

Figure 8: Two-way olfactometer structure design

Referring to the studies conducted by Jalinas (2016) on RPW, they were all conducted by placing a single BW adult at the centre of the straight arm. All insects tested had 10 min to move and make a choice. After each test, the BWs was eliminated, the TWO was rinsed with ethyl alcohol (Benito Parraga, S.L.), then with n-hexane (PanReac AppliChem) and finally with distilled water. Then it was dried with paper.

In TWO 21 trials were conducted, and a total of 120 BW individuals were used for each one. Each test consists of six replicas, each of 20 individuals.

The 21 tests carried out in this study can be grouped as following: Physio-Environmental Conditions Tests (TsPECs), Repellent Tests (TsR), Pheromone Tests (TsP) and Tests of Field Conditions (TsFCs).

BW adults were collected on the island of Tenerife (Canary Islands, Spain) using pheromone traps baited with ECOSordidina60 (ecobertura®, ref.: 019-FACS60), based on sordidine. The insects were kept in plastic boxes (40 cm x 30 cm x 21 cm) at 25 ± 0.5 °C in the dark. The plastic boxes contained a filter paper and a small container drilled with distilled water, to keep humidity ca. 80 ± 5%. Adults were not sexed since BW sexual characters are hard to visualise. Twenty healthy BW adults were selected at random from the stock population for experiments with bioassays in an olfactometer per stimulus tested.

Fresh pieces of banana corm/pseudocorm (*Musa sp*.) were collected from plants grown in the greenhouses of the University of Alicante for experiments and were discarded daily.

Pieces of 15.6 g of corm/pseudocorm were used for olfactometry bioassay experiments, from at least one-year-old plants.

2.3.1 Tests of the Physio-Environmental Conditions – TsPECs

In the first group, four TsPECs were conducted, in which the attractive activity of corm/pseudocorm was tested. Of the two OCs, the natural attractans was placed in one (right or left have been chosen randomly) while in the other OC, no attractants or repellents was placed. With this olfactory stimulation, two different physio-environmental conditions were tested. In these tests we wanted to evaluate the effect of light (L) and darkness (SL) on BW movement. BW mainly displays nocturnal habits (Gold *et al*., 2001).

Furthermore, hunger a physiological condition was also tested. Two BW populations were tested. The first consisted of individuals with an *ad libitum* food arrangement (SHa), while another was made up of individuals with at least a week of starvation (Ha). In this way, it was possible to define the physio-environmental conditions in which *C. sordidus* has the highest rate of movement.

2.3.2 Repellent Tests – TsR

In the second group, called Repellent Tests (TsR), nine tests were conducted with BW under SL-Ha conditions.

The tests were conducted with seven VOCs repellent candidates (C1 to C7) and with two BW repellents (garlic (Aj) and colloidal sulphur (Az)) used in banana commercial plantations (COPLACA, J. Cepero, pers. com.).

VOCs C1 and C2 are two BW repellent candidates, already known as repellents for RPW (Jalinas, 2016), both obtained from *Bb*203 and *Bb*1TS11. C1 was selected from a former metabolic profile of *Bb*203 (P201631534). VOCs C3 and C4 are two additional potential repellent candidates identified by the metabolomic profiles obtained with GC/MS-SPME from *Bb*203 and *Bb*1TS11 in this work. C3, unlike the others, was detected during preliminary tests conducted for the development of the appropriate protocol, in two samples of both *Bb* stains. C3 detection took place by subjecting 5 g of both *B. bassiana* strains solid formulates kept 15 min in a thermostatic bath to 100 °C. Given the its known repellent action this substance has on other insects, like mosquitoes (Diptera Culicidae) (Nerio *et al.*, 2010), C3 was selected as a potential repellent, despite not being a majoritarian compound. VOCs C5 and C6 were identified in the metabolomic profiles obtained from *Pc*123 and *Ma*4TS04. Finally, the C7 repellent candidate was identified by the metabolomic profiles of all four fungal strains tested.

In these tests, in an OC a fresh corm/pseudocorm sample was placed. The other OC contained a 0.5 ml dispenser (C1, C2, C5, C6 and C7) or 0.5 g (C3 and C4) of the repellent pure to be testedrehearse. The dispensers used were constructed in the laboratory using miracloth (Merck KGaA) to make (3.5 cm x 2.5 cm) envelopes each containing 2 g of silica gel 60A (70-200µ, Carlo Erba) inside. The volume of repellent was added directly to the silica. The volume weight of repellent was mixed with the silica. For Aj and Az, 15.6 g of each were used per test.

2.3.3 Pheromone Tests – TsP

In this group, called Pheromone Tests (TsP), three tests were conducted with BW under SL-Ha conditions.

The pheromone was compared in TWO with no stimulus, corm/pseudocorm, C1 and C2. The pheromone used was ECOSordidine60 (ecobertura®, ref.: 019-FACS60), based on sordidine. This substance is an aggregation pheromone isolated from BW males (Budenberg *et al*., 1993; Mori *et al*., 1996). It is, therefore, an attractant activity for both sexes. In TsP, the concentration at which the pheromone was used was equal to 1/16 ($1{,}7e^{-4}$ ml/cm^2) of the concentration of the commercial formulation (0,27 ml/cm^2). The use of ECOSordidina60 used as such, in *ex-ante* tests, produced an overstimulation of BWs, impeding their movement (*nda*: individuals reacted to this over-stimulation by turning over in supine position).

2.3.4 Tests of Field Conditions – TsFCs

In this last group, called Tests of Field Conditions (TsFCs), four tests were conducted with BW SL-Ha.

In the TWO, we reproduced *in vitro* the field conditions, which will be discussed in section 2.5. Two tests were carried out, to test these conditions, wherein the OCs 15.6 g of corm/pseudocorm were placed on one side, while in the other arm 1/16 of the concentration of ECOSordidine 60 were placed together with one of the potential repellents to be tested (C1 or C2). The repellents, as for the TsRs, were placed with the miracloth dispensers containing 2 g of silica gel and 0.5 ml of C1 or C2 (both liquids).

2.4 TWO data analysis

The ethological data shown by the BWs in the TWO tests were collected in an Excel spreadsheet. *C. sordidus* responded to the test in three different ways (Fig. 9). Some BW went to E1, which always included the attractants (corm/pseudocorm and pheromones). Other individuals turned to E2 stimulus; in this OC, all the repellents tested were placed

individually or no stimulus was placed. Still, others preferred not to choose and stood "home" (EC).

This triple ethological expression has been evaluated first with *ad hoc* constructed movement indexes, in order to have a quick and explanatory measure of the single tests conducted in TWO. Furthermore, data were analysed using the chi-square statistical test.

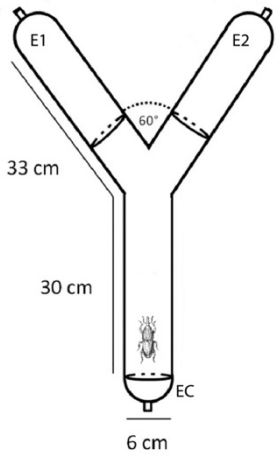

Figure 9: BW behavior in the olfactometer: E1 represents the individuals who have chosen the attractions; E2 represents all the individuals who have gone to the repellent candidates tested or alternatively the absence of stimulus; EC indicates individuals who have chosen not to move.

The movement indexes originated from the need to have a rapid and easily explanatory tool and, at the same time, summarize the ethological situation shown by the BWs in the tests.

We assumed that a BW population, placed in TWO in the absence of some stimuli would be uniformly distributed in all three possible choices maintaining a 1: 1: 1 ($n_{E1} = n_{E2} = n_{EC}$) *ratio* relationship. We also consider that the individuals remaining in the EC either did not respond to the stimulus (e.g. no stimulus placed in E2) or whom were repulsed by the substance tested (e.g. E2 had a repellent candidate).

Therefore, three indexes that took into consideration the relationship between individuals in EC, E1 and E2 and the total number of individuals tested (*N*) were formulated as follows.

$$I_{E1} = \frac{n_{E1}}{N}$$

$$I_{E2} = \frac{n_{E2}}{N}$$

$$I_{EC} = \frac{n_{EC}}{N}$$

where: n_{E1} = Number of individuals who have chosen E1, n_{E2} = Number of individuals who have chosen E2, n_{EC} = Number of individuals remaining in the EC, N = Total number of individuals tested.

Finally, to obtain an index that takes into consideration all these relationships between groups of individuals, the I_{E1}, I_{E2} and I_{EC} indices have been summarized in a single index: *IM* (*Index of Movement*).

$$IM = \frac{(I_{E1} + I_{E2})}{I_{EC}} = \frac{n_{E1} + n_{E2}}{n_{EC}}$$

where: $0 < IM < +\infty$, with $n_{EC} \neq 0$. The closer the *IM* is to zero, the more significant the portion of the population that has remained motionless, not reacting to a given stimulus.

IM was calculated for each of the six trials (N = 20 BWs), generating a series of data for each test. ANOVA tests were conducted on TsPECs, TsR, TsP and TsFCs data using the statistical software Rstudio.

2.5 Field tests

To validate the activity on the mobility of BW in the field, some substances from those selected were tested in commercial banana fields. Two consecutive tests were conducted in the same fields and following the same experimental scheme (detailed below). The first test was conducted from April 23, 2019 until June 4, 2019. The second test was conducted from June 4, 2019 until July 16, 2019. In this second test, unlike of the first, half of the dispenser (both pheromone and VOCs) were replaced three weeks after the start of the experiment.

Compounds C1 and C2 are the only repellent candidates tested in the field, in order to be compared with the data provided by J. Jalinas (2016), which evaluated their repellent action of these same compounds in the lab against another Curculionidae, the red palm weevil. These compounds, tested in the laboratory, have also been used in the field to check if they can modify the behaviour of *C. sordidus* even in agronomical conditions. Two banana plantations with natural BW infestation were identified to conduct these tests in the Island of Tenerife (Canary Islands). These two fields, a larger one (0.35 ha)

(28°23'39.72"N - 16°39'18.97" W; Z = 68 m above s.l.) (BF – Big Field) and a smaller one (0.06 ha) (28°23'40.14"N - 16°39'16.55" W; Z = 65 m above s.l.) (SF – Small Field) are adjacent (Fig. 10).

Figure 10: Banana fields selected for the VOCs tests, Tenerife - Canary Islands (Spain)

Twenty-nine traps were placed (Table 1) in a randomized block (Fig. 11). Of these, 10 contained BW pheromone only (Fig. 12). Further 10, in addition to the pheromone, each contained a C1 dispenser (2 g of silica gel and 0.5 ml of C1) (Fig. 11). The remaining 9 traps, in addition to the pheromone, each contained a C2 dispenser (2 g of silica gel and 0.5 ml of C2) (Fig. 12).

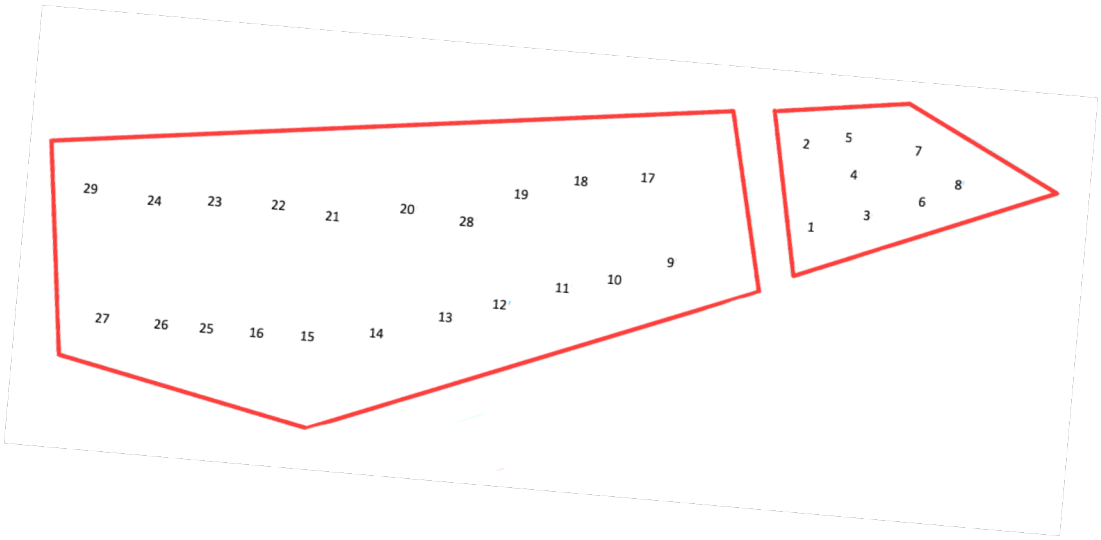

Figure 11: Arrangement of the BW traps in the two banana fields, according to the randomized block

Figure 12: BW traps used in the field: a) ECOSordidina60 dispenser; b) miracloth dispenser of compounds C1 and C2; c) base of the trap activated with ECOSordidina60 and C1/C2; d) trap positioned in the field

Table 1: Field treatments and position of the traps. *F* = pheromone; *FC1* = pheromone + C1; *FC2* = pheromone + C2.

Trap n.	Content	Coordinates	Trap n.	Content	Coordinates
1	F	28°23'39.9"N 16°39'16.6"W	16	FC1	28°23'40.1"N 16°39'18.0"W
2	FC1	28°23'40.4"N 16°39'16.9"W	17	FC2	28°23'40.0"N 16°39'18.4"W
3	FC2	28°23'40.0"N 16°39'16.3"W	18	F	28°23'39.9"N 16°39'19.0"W
4	FC1	28°23'40.3"N 16°39'16.5"W	19	FC2	28°23'39.9"N 16°39'19.4"W
5	F	28°23'40.1"N 16°39'16.1"W	20	FC1	28°23'39.8"N 16°39'19.7"W
6	F	28°23'40.4"N 16°39'16.2"W	21	F	28°23'39.9"N 16°39'20.0"W
7	FC1	28°23'40.2"N 16°39'16.0"W	22	FC2	28°23'39.8"N 16°39'20.2"W
8	FC2	28°23'39.7"N 16°39'17.4"W	23	FC1	28°23'39.1"N 16°39'19.8"W
9	F	28°23'39.8"N 16°39'17.8"W	24	F	28°23'39.2"N 16°39'20.1"W
10	FC1	28°23'39.4"N 16°39'18.1"W	25	F	28°23'39.2"N 16°39'20.4"W
11	F	28°23'39.3"N 16°39'18.4"W	26	FC1	28°23'39.9"N 16°39'18.7"W
12	FC2	28°23'39.1"N 16°39'19.1"W	27	FC2	28°23'40.5"N 16°39'16.6"W
13	FC1	28°23'39.1"N 16°39'19.3"W	28	FC1	28°23'39.3"N 16°39'18.7"W
14	F	28°23'39.1"N 16°39'19.6"W	29	FC2	28°23'39.8"N 16°39'20.6"W
15	FC2	28°23'40.2"N 16°39'17.6"W			

The count of the BW captures was scored weekly and removed from the traps.

On the collected data, histograms have been created with the accumulated value of the captures per treatment per day of both experimental fields. Furthermore, data were analysed with ANOVA tests using the Rstudio statistical software.

3. Results

3.1 VOCs production by Fungal Parasites of Invertebrates (*FPIs*)

One hundred and ninety-three VOCs were produced by the 4 strains fungal parasites of invertebrates tested during their 60 days growth period. Of these, only 5 DCs (Detected Compounds) were produced by all fungi tested (Fig. 13). These are DC002, DC027 (C7), DC078, DC081 and DC130. Furthermore, 16 DCs were found to be characteristic of *Bb*203, while 26 DCs were found to *Bb*1TS11 and as many to *Ma*4TS04. Instead, *Pc*123 presented 86 typical DCs.

Entomopathogenic fungi showed the largest VOCs production at 20 dai and 30 dai from the inoculum. Instead, *P. clamydosporia* recorded produced most VOCs from 30 to 50 dai.

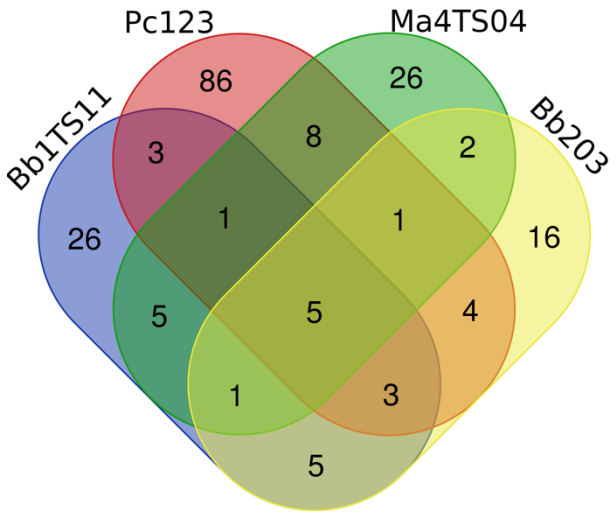

Figure 13: Venn diagram of the VOCs produced by 4 strains fungal pathogens of invertebrates

3.1.1 Entomopathogenic Fungi

*Bb*203 presented 37 different compounds characteristic of its metabolic profile (Fig. 13) in according to the Venn diagram. Of these, only three compounds, representing 8.1% of the total, were found to belong to the M-VOCs; while only 10 compounds, representing 27% of the total, were found to belong to the m-VOCs. Among the M-COVs, only the DC027 (C7) was found in the analyses (10-60 dai samples) made during the 60 days of growth of the fungus. DC130 was detected only in samples from 30 and 40 dai. Finally, the DC129 is the third M-VOC and was identified only 60 dai. Only DC141 (C4) was found among the m-VOCs in all six measurements made during the 60 days of growth of the fungus. The other m-VOCs of Bb203 are detailed in Table 2.

Table 2: M-VOCs and m-VOCs produced by *Bb*203 during 60 d of growth. Green indicates the individual M-VOCs and m-VOCs detected during fungal growth.

Bb 203								
	N. progressive	Compound Code	10d	20d	30d	40d	50d	60d
M-VOCs	1	DC027 (C7)						
	2	DC130						
	3	DC129						
	N. progressive	Compound Code	10d	20d	30d	40d	50d	60d
m-VOCs	1	DC141 (C4)						
	2	DC052						
	3	DC061						
	4	DC069						
	5	DC112						
	6	DC046						
	7	DC081						
	8	DC150						

Bb1TS11 presented 49 different T-VOCs, characteristic of its metabolic profile (Table 3). Of these, only three compounds, which represent 6.1% of the total, were found to belong to the M-VOCs category. Twelve compounds belonged to the category of m-VOCs and represent 24.5% of the total. Of the M-VOCs, DC027 (C7) was found in the six measurements made during the 60 days of fungal growth; whereas, DC130 was detected only in samples 30 and 40 dai. Finally, the DC026 a M-VOC was identified only 50 dai. Among the m-VOCs, only DC141 (C4) was detected at 10, 20, 40 and 50 dai. The other m-VOCs of Bb1TS11 are detailed in Table 3.

Table 3: M-VOCs and m-VOCs produced by *Bb*1TS11 during 60 d of growth. Green indicates the individual M-VOCs and m-VOCs detected during fungal growth.

Bb 1TS11								
	N. progressive	Compound Code	10d	20d	30d	40d	50d	60d
M-VOCs	1	DC027 (C7)						
	2	DC130						
	3	DC026						
	N. progressive	Compound Code	10d	20d	30d	40d	50d	60d
m-VOCs	1	DC141 (C4)						
	2	DC097						
	3	DC079						
	4	DC028						
	5	DC027 (C7)						
	6	DC072						
	7	DC150						
	8	DC166						
	9	DC154 (C2)						
	10	DC078						
	11	DC088						
	12	DC189						

Ma4TS04 showed 49 different T-VOCs characteristics of its metabolic profile (Table 4). Of these, only six compounds, representing 12.2% of the total, were found to belong to the category of M-VOCs. Eleven different compounds belonged to the category of m-VOCs and represented 22.4% of the total. Of the M-VOCs, DC027 (C7) was found 10 to 30 dai; whereas DC068 (C6) was detected as M-VOC only 20 and 30 dai. DC129 was detected in the measurements taken 20 and 60 days after substrate inoculation. DC127 was detected in measurements taken 10 and 50 dai. Other M-VOCs of Ma4TS04 are detailed in Table 4. Among the m-VOCs, DC189 was detected at 10, 20 and 40 dai. DC068 (C6) was found as m-VOCs only at 10 and 40 dai. The other m-VOCs of Ma4TS04 are detailed in Table 4.

Table 4: M-VOCs and m-VOCs produced by *Ma*4TS04 during 60 d of growth. Green indicates the individual M-VOCs and m-VOCs detected during fungal growth.

Ma 4TS04								
	N. progressive	Compound Code	10d	20d	30d	40d	50d	60d
M-VOCs	1	DC027 (C7)						
	2	DC127						
	3	DC068 (C6)						
	4	DC129						
	5	DC136 (C5)						
	6	DC130						
	N. progressive	Compound Code	10d	20d	30d	40d	50d	60d
m-VOCs	1	DC189						
	2	DC068 (C6)						
	3	DC153						
	4	DC076						
	5	DCO26						
	6	DC089						
	7	DC159						
	8	DC045						
	9	DC126						
	10	DC107						
	11	DC047						

3.1.2 Nematophagous Fungus

Pc123 showed 111 different T-VOCs characteristics of its metabolic profile (Table 5). Of these, only 14 compounds, representing 12.6% of the total, were found to belong to the category of M-VOCs. Forty-three different compounds belonged to the category of m-VOCs and represent 38.7% of the total. Of the M-VOCs, DC136 (C5) was found in the six measurements made during the growth of the fungus; while DC068 (C6) has been detected as M-VOCs only 10, 20, 30 and 60 dai. DC069 was identified 20, 40 and 50 dai; whereas DC027 (C7) was found only at 10 and 20 dai. Among the m-VOCs, DC014 was detected at 30, 50 and 60 dai. DC078 was detected at 30 and 40 dai. DC041, DC012 and DC091 were detected at 40 and 50 dai. Also, DC158 was detected at 50 and 60 days. The other m-VOCs of Pc123 are detailed in Table 5.

Table 5: M-VOCs and m-VOCs produced by *Pc*123 during 60 d of growth. Green indicates the individual M-VOCs and m-VOCs detected during fungal growth.

Pc 123																
	N. progressive	Compound Code	10d	20d	30d	40d	50d	60d	N. progressive	Compound Code	10d	20d	30d	40d	50d	60d
M-VOCs	1	DC136 (C5)							8	DC023						
	2	DC068 (C6)							9	DC056						
	3	DC069							10	DC103						
	4	DC027 (C7)							11	DC124						
	5	DC017							12	DC091						
	6	DC135							13	DC173						
	7	DC130							14	DC055						
	N. progressive	Compound Code	10d	20d	30d	40d	50d	60d	N. progressive	Compound Code	10d	20d	30d	40d	50d	60d
m-VOCs	1	DC014							23	DC033						
	2	DC108							24	DC184						
	3	DC078							25	DC094						
	4	DC012							26	DC042						
	5	DC041							27	DC165						
	6	DC091							28	DC076						
	7	DC158							29	DC051						
	8	DC034							30	DC163						
	9	DC140							31	DC036						
	10	DC039							32	DC038						
	11	DC110							33	DC171						
	12	DC072							34	DC155						
	13	DC105							35	DC077						
	14	DC153							36	DC050						
	15	DC139							37	DC095						
	16	DC018							38	DC071						
	17	DC074							39	DC081						
	18	DC192							40	DC043						
	19	DC032							41	DC109						
	20	DC029							42	DC046						
	21	DC016							43	DC085						
	22	DC044														

3.2 Effect of VOCs from FPIs on BW behaviour

3.2.1 Optimisation of BW mobility in two-way olfactometer

C. sordidus showed a different behaviour with respect to the physio-environmental conditions to which it was exposed to the *pabulum* (corm/pseudocorm), for 10 min (χ^2 = 17.952; *df* = 6; *p-value* = 0.006352). These conditions showed interference with phytophagous mobility (*F-value* = 3.305; *p-value* = 0.0413). Tukey's test (Fig. 14) shows how individuals exposed to SL-Ha conditions show greater mobility, presenting the highest *IM* (*IM* = 0.53).

The nocturnal aptitude of *C. sordidus* is evident from the higher mobility results shown by the tests with the SL (darkness) conditions, i.e. SL-Ha and SL-SHa (*IM* = 0.48), compared to those conducted with the L condition (presence of light), or L-Ha (*IM* = 0.36) and L-SHa (*IM* = 0.22). Moreover, a further reflection leads us to affirm that the condition Ha (starvation) stimulates more the movement of BW compared to SHa, both in the light.

For the reasons explained, darkness and starvation (SL-Ha) were the conditions selected to perform which all subsequent tests, as described below.

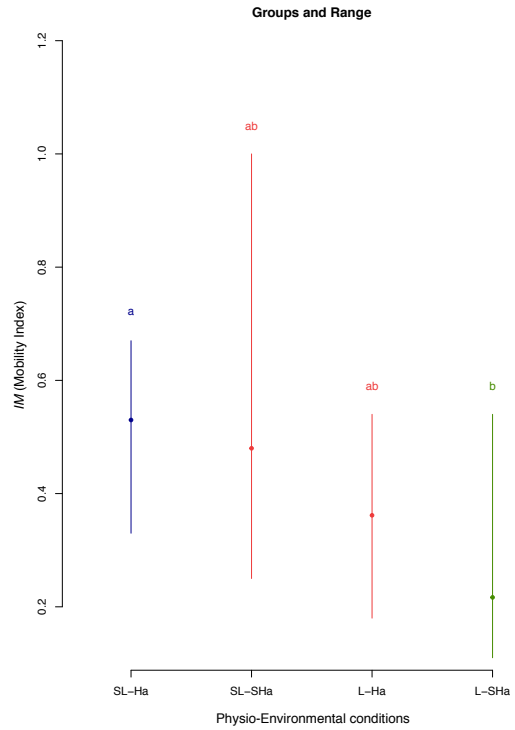

Figure 14: Effect of Physio-Environmental Conditions on BW mobility

3.2.2 Effect of technical repellents and VOCs on BW mobility

The behaviour of *C. sordidus* was influenced by VOCs and technical repellents tested for 10 min in a TWO (χ^2 = 60.881; *df* = 18; *p-value* = 1.473e^{-6}).

The stimulii to which BWs was exposed modified their mobility (*F-value* = 3.388; *p-value* = 0.00258). All olfactory stimulii (potential repellents) (Fig. 15), except for colloidal sulphur (Az), reduce the movement of BW compared to the SL-Ha condition (T4) (without repellents). Az showed the highest *IM* (*IM* = 0.64) compared to SL-Ha.

C7 turns out to be the compound that mostly reduced BW mobility (*IM* = 0.11). C5 also presented a low *IM* (*IM* = 0.18) for BW. Therefore, C7 and C5 can be considered *hard-repellents* for BWs.

C2 (*IM* = 0.26), C1 and C4 (*IM* = 0.28), Aj (garlic - *IM* = 0.35), C3 (*IM* = 0.45) and C6 (*IM* = 0.47), show a growing influence on BW movement. Despite this, the *IMs* recorded by them were inferior to those of the control conditions. These stimuli influence the movement, but without statistically appreciable differences between them. They can, therefore, be considered *soft-repellents*.

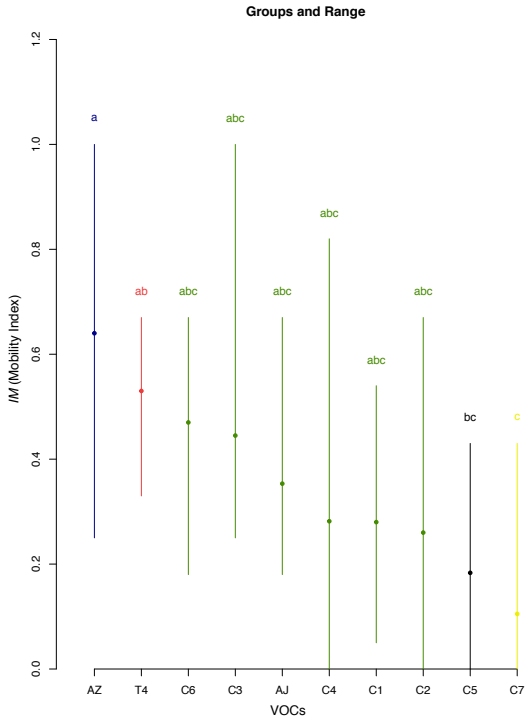

Figure 15: Effect of fungal VOCs and other compounds (technical repellents) on BW mobility; plot of the Tukey test on the TsR

3.2.3 Effect of VOCs on masking the pheromone attractiveness

The behavior of *C. sordidus* was influenced by the attractant (F) and repellents (C1 and C2), to which it was subjected for 10 min in TWO (χ^2 = 23.221; *df* = 4; *p-value* = 1.14e^{-4}).

The soft repellents tested modified the *C. sordidus* mobility with respect to the pheromone (*F-value* = 9.769; *p-value* = 0.00192). The presence of C1 and C2 generated a low *IM* (respectively IM_{FC1} = 0.03 and IM_{FC2} = 0.04), with respect to that of F (*IM* = 0.18) (Fig. 16).

A further reflection makes us state that the pheromone mobility rate is lower than the IM shown by the *pabulum* (control/ banana corm) ($IM_{SL\text{-}Ha}$ = 0.53), under the same physio-environmental conditions.

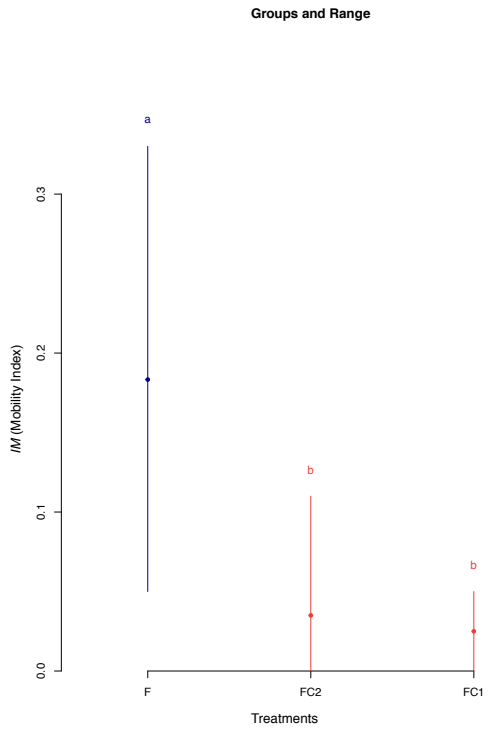

Figure 16: Effect of fungal VOCs on C. sordidus *pheromone attractiveness; F = Pheromone (control), FC1 = Pheromone + C1, FC2 = Pheromone + C2; Plot of the Tukey test on the TsP*

3.2.4 Field conditions tested in vitro

The field conditions, reproduced in vitro, also influenced *C. sordidus* mobility (χ^2 = 123.43; *df* = 4; *p-value* = 2.2e-16).

The repellents C1 and C2, together with the pheromone, modified the BW mobility with respect to the CF (corm-pheromone) conditions (*F-value* = 70.03; *p-value* = $2.46e^{-8}$). Also, in the presence of C1 (*IM* = 0.07) and C2 (*IM* = 0.02), the mobility of *C. sordidus* and the attractiveness of the *pabulum*-pheromone (IM_{CF} = 1.18) decrease drastically. These results are supported by the Tukey test (Fig. 17).

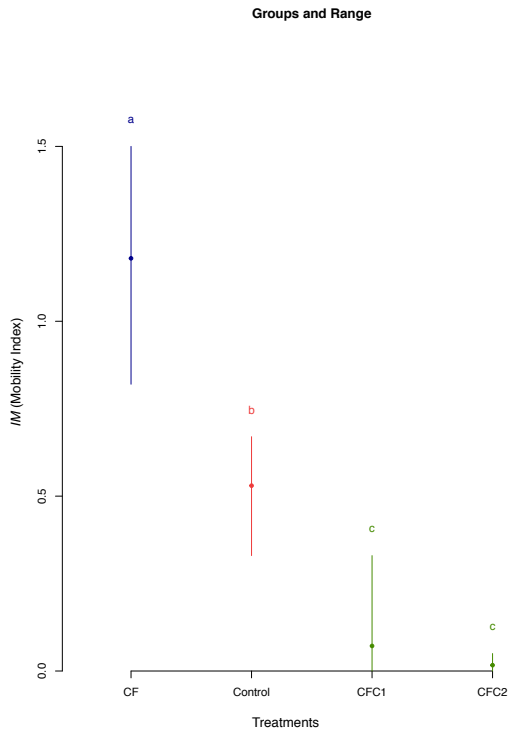

Figure 17: Effect of banana corm and BW pheromone and fungal VOCs attractiveness to C. sordidus; TsFCs and control (SL-Ha)

3.3 Effect of VOCs on BWs under field conditions

3.3.1 Captures of BWs in traps with VOCs

Compared to the control (F), in the first test it can be inferred that in the tests with C1 (FC1) the BW catches in the traps were fluctuating, showing a lower number of catches only in the second week (blue bar) (Fig. 18). In the rest of the surveys, the captures of this treatment are higher than the control.

As regards C2 (FC2), the catches were always lower than the control, except for the third catch (green bar) (Fig. 18). However, no significant difference was found for trap catches.

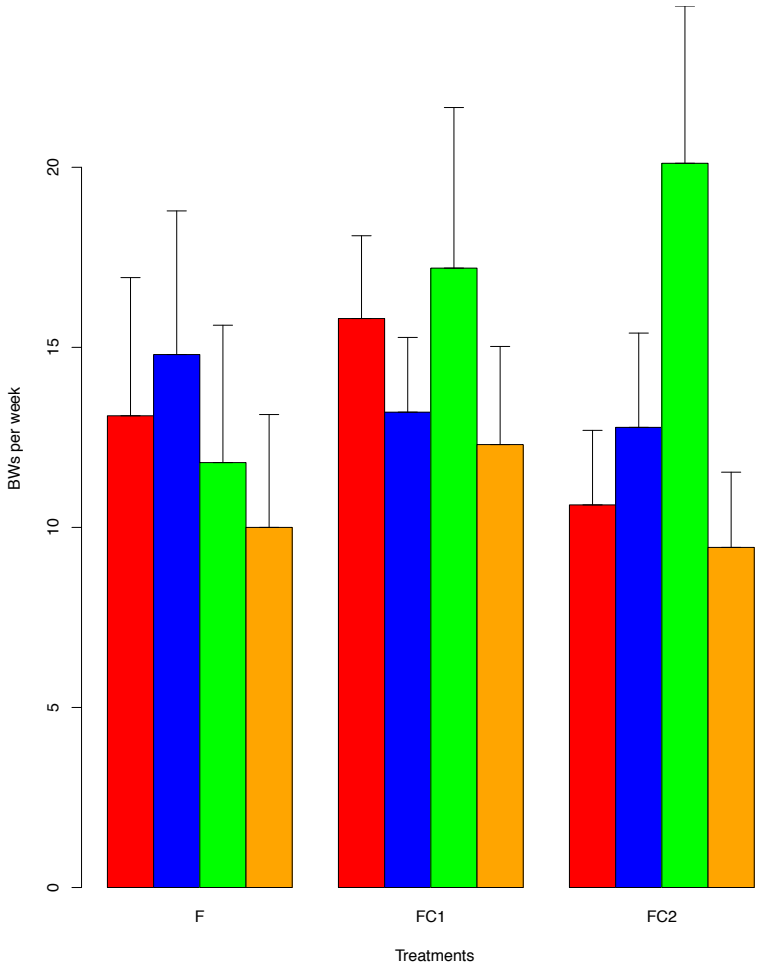

Figure 18: First test: trend of the catches for the treatments in the different weeks of the test. In red the catches are represented from 16 to 23 April 2019; in blue the catches from 24 to 30 April 2019; in green the catches from 1 to 15 May 2019; in orange the catches obtained from 16 to 22 May 2019

In the second trial, conducted from 4 June to 16 July 2019, catches were relatively lower than the previous test. This total decrease in catches is attributable to the rise in temperatures in this trapping period. High temperatures are known to reduce the mobility of BW in the field.

Compared to the control, FC1 showed lower catches in the first two weeks of the test and the last 3. Only in the third week, there was a higher capture than the control (Fig. 19). FC2, compared to the control, showed only lower catches compared to the control from the third to the fifth week of testing (Fig. 19).

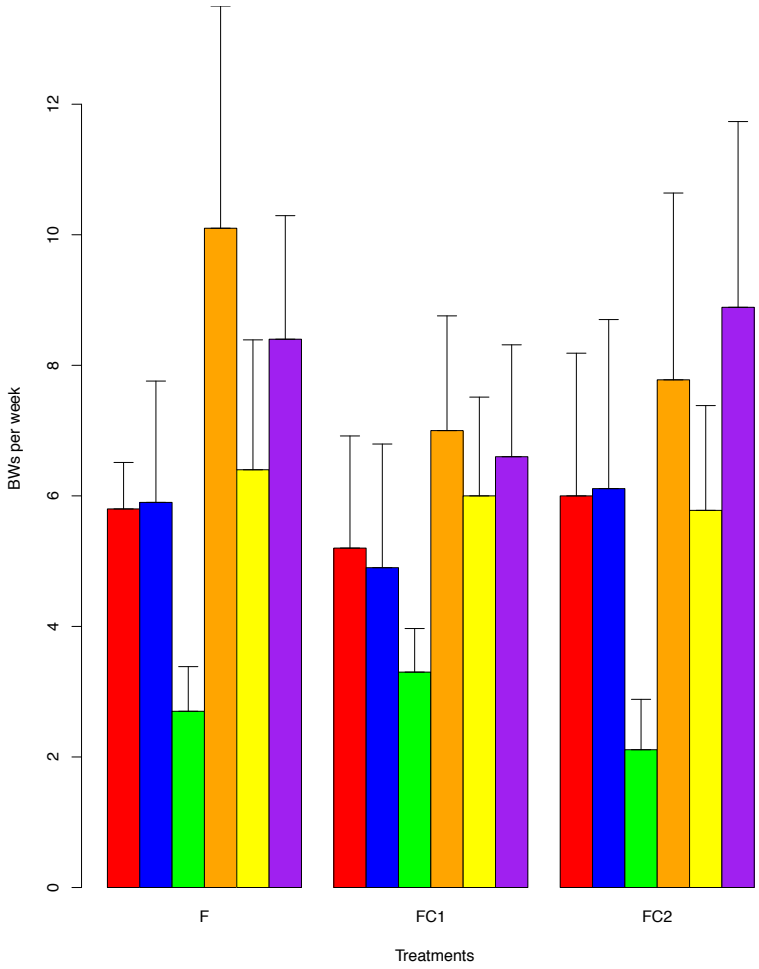

Figure 19: Second test: trend of the catches for the treatments in the different weeks of the test. In red the catches are represented from 4 to 11 June 2019; in blue the catches from 12 to 18 June 2019; in green the catches from 19 to 25 June 2019; in orange the catches from 26 June to 2 July 2019; in yellow from 3 to 9 July 2019; while in purple from 10 to 16 July 2019

In this test, as previously mentioned, the stimuli of half of the FC1 and FC2 treatments were renewed in the third week. As regards FC1, all the renewed traps captured a more (108 BWs) than those in which the stimuli were not changed (87 BWs). Instead, FC2 showed a different trend. All the renewed traps have captured less (94 BWs) than those with the stimuli not renewed (108 BWs). Like in the first test, in the second test no significant differences were for F vs FC1 or FC2 treatments.

The catches of both the first and second trials were compared with the trends of average temperature, average HR and the precipitations of the periods concerned to understand the progress and mobility of the BW population.

In the period of the first trial, the weekly average T has increased gradually. In Fig. 20 it is possible to see how, in the last week, the catches have decreased for all treatments together with a slight increase in average temperature. HR remained almost stable, between 73 and 75%. Therefore, the decrease found cannot in any way be ascribed to the recorded hygrometric trend (Fig. 21). Even in the case of precipitation, there seems to be no link with the decrease in catches. In fact, in the considered period, the precipitations were not almost wholly absent except for the date of traps laydown (Fig. 22).

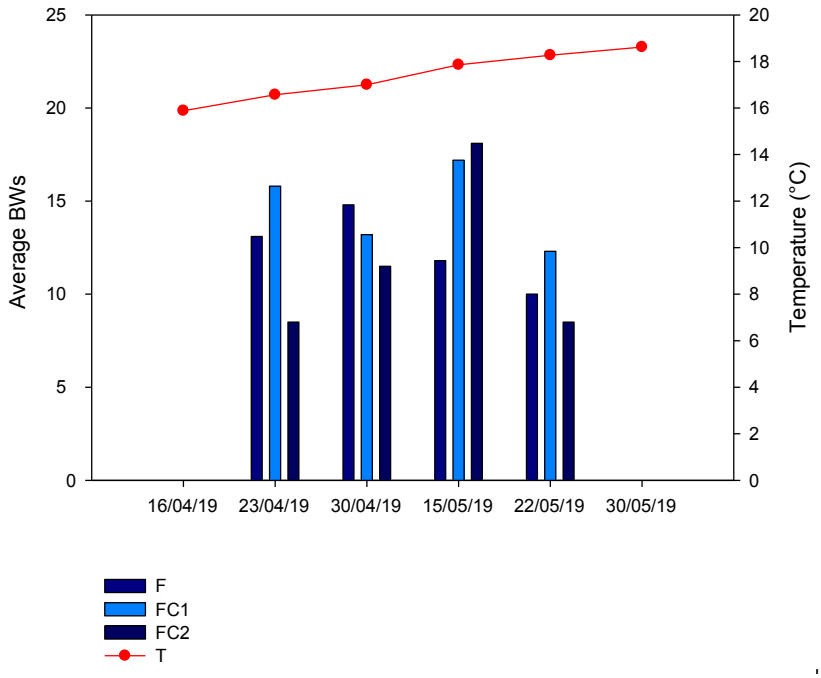

Figure 20: Comparison of data relating to BW catches of the first test and the average weekly temperature; the standard error is the same as shown in the Fig. 18

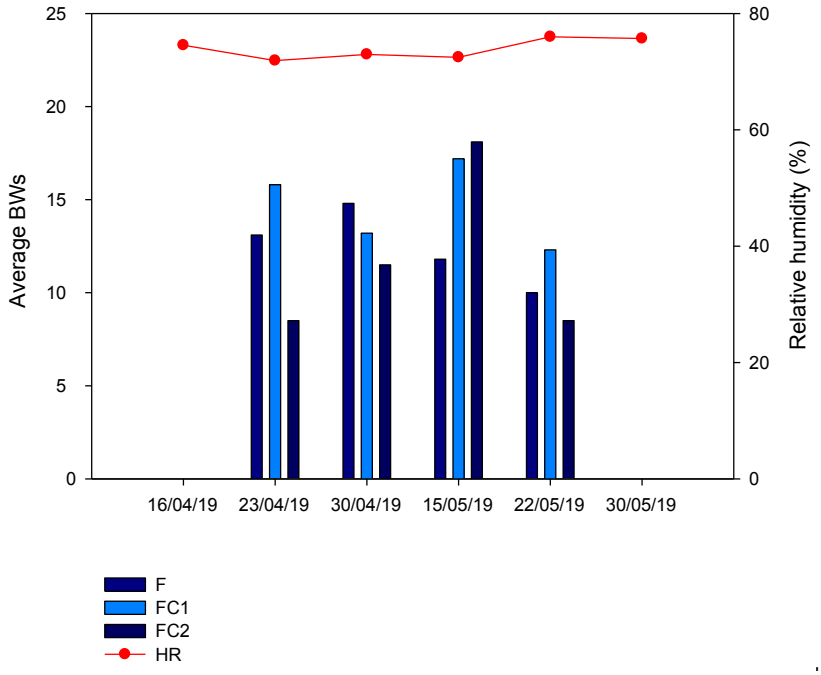

Figure 21: Comparison of the data relating to BW catches of the first test and the average weekly relative humidity; ; the standard error is the same as shown in the Fig. 18

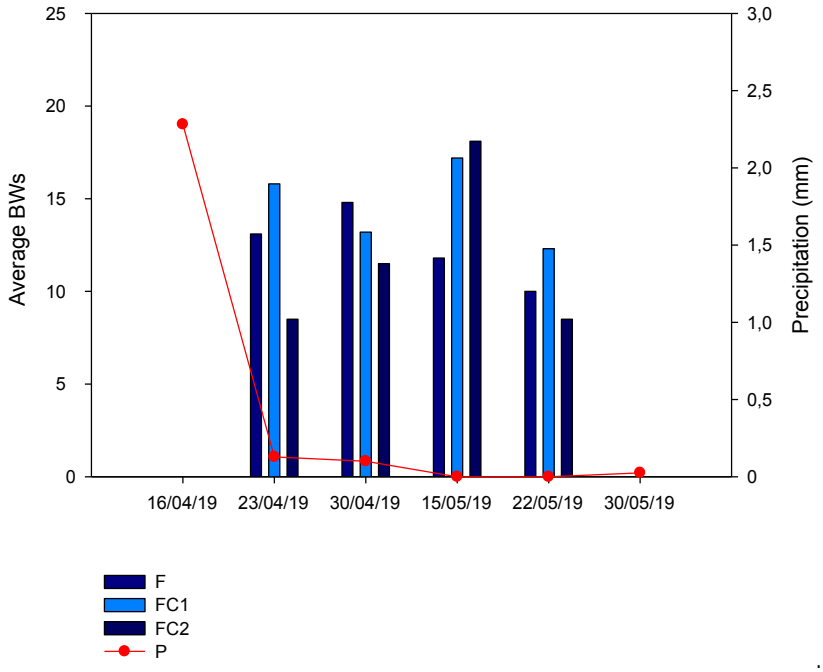

Figure 22: Comparison of the data relating to BW catches of the first test and the average weekly precipitation; ; the standard error is the same as shown in the Fig. 18

In the second trial, the BW weekly average T shows a slight increase in the capturing period (Fig. 23). BW catches during this period were lower than in the previous test. The most evident drop in BW catches occurred in the week between 19 and 25 June 2019. At the same time, there was also a considerable average rainfall (1.2 mm) (Fig. 25), which could have affected the BW ethology, concerning mobility in the field.

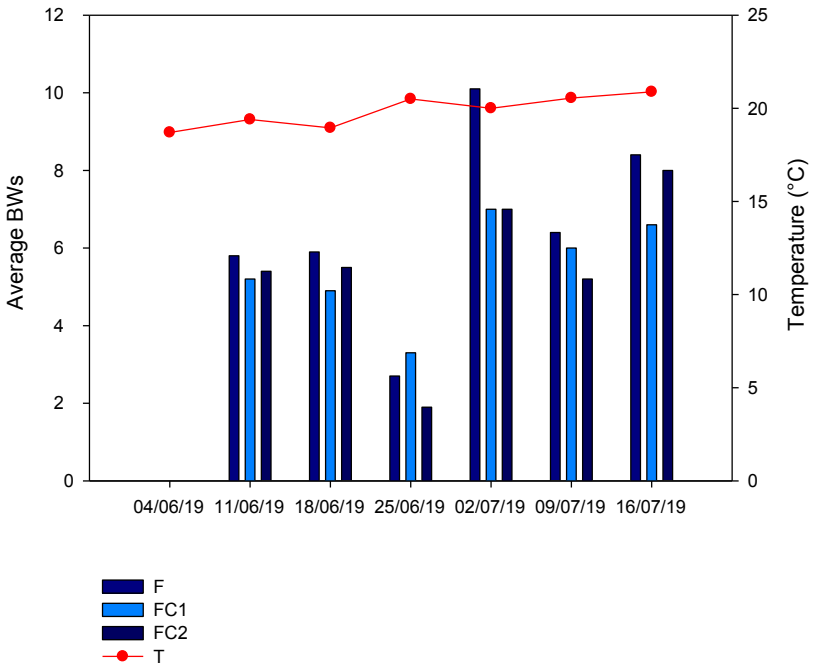

Figure 23: Comparison of data relating to BW catches of the second test and the average weekly temperature; the standard error is the same as shown in the Fig. 19

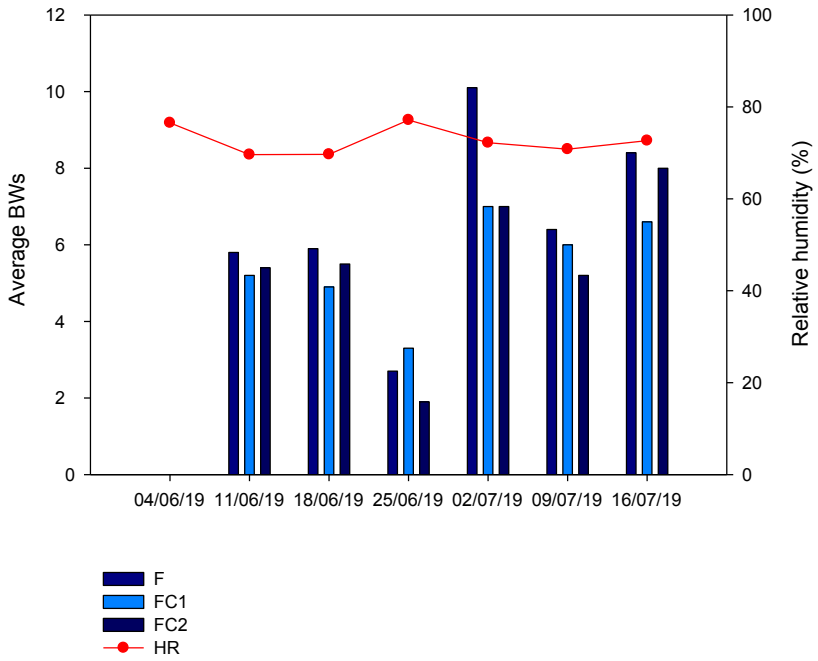

Figure 24: Comparison of data relating to BW catches of the second test and the average weekly relative humidity; the standard error is the same as shown in the Fig. 19

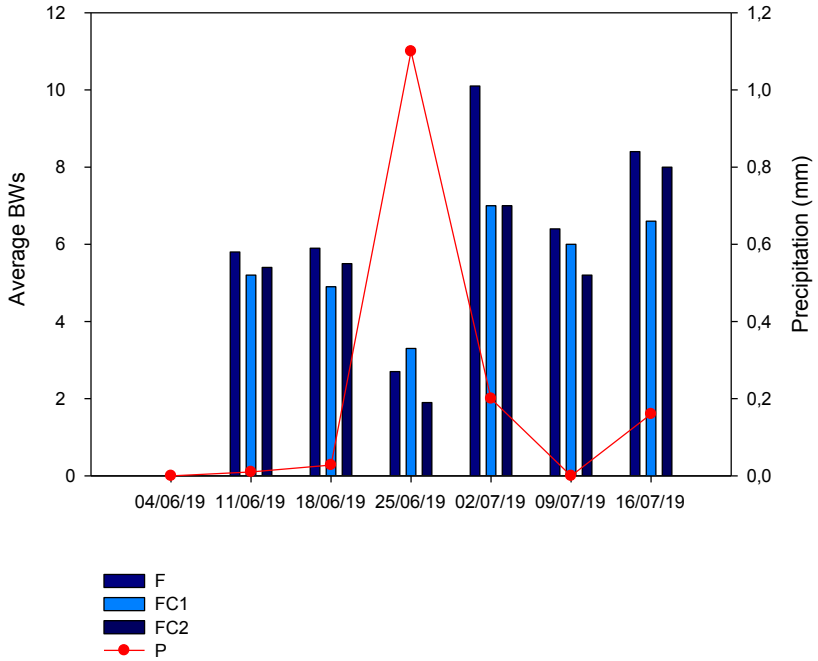

Figure 25: Comparison of data relating to BW catches of the second test and the average weekly precipitation; the standard error is the same as shown in the Fig. 19

3.3.2 Statistical analysis

The catches found in the first test do not appear to be conditioned by the stimuli placed in the traps. From the multifactorial ANOVA analysis, it also emerged that the recorded catches do not seem to be related to the time variable as the stimuli ($p\text{-}value_{VOCs} = 0{,}625$; $p\text{-}value_{weeks} = 0{,}226$; $p\text{-}value_{VOCs:weeks} = 0{,}658$).

As for the catches found in the second test, they also do not seem to be influenced by the stimuli with which the traps were loaded. However, these captures seem to be influenced by the time taken for the test ($p\text{-}value_{VOCs} = 0{,}63568$; $p\text{-}value_{weeks} = 0{,}00941$; $p\text{-}value_{VOCs:weeks} = 0{,}99735$).

3.3.3 Heat maps of BWs field distribution

With the data on BW catches per trap and the localization of traps, it was possible to generate heat maps to monitor the mobility of *C. sordidus* under field conditions.

In the temporal sequence of heat maps generated by the captures of the first trial (Fig. 26). BW movement during the experiment is apparent.

In the SF (small field) there is only one initial BW focus (trap 5-F), while in the BF (big field) there are two well-marked BW infestation foci (traps 23-FC1 and 11-F).
Following the temporal sequence, it is possible to observe how, over the weeks, the population of BWs present in the field has moved. The BW population gradually shifted, focusing more on the lower part of the BF. It can be seen from the colouration shown by the points of capture on the May 22nd heat map.

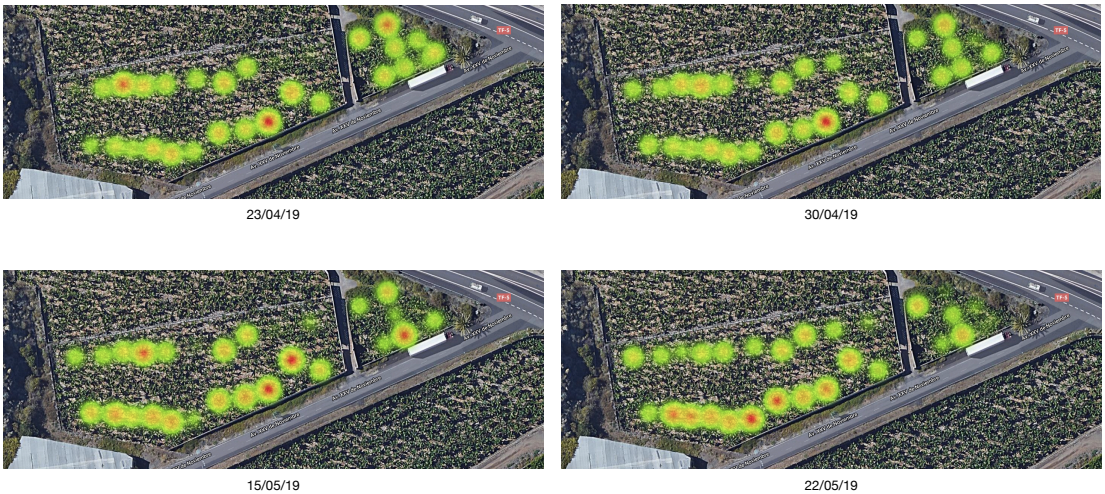

Figure 26: Weekly Heat Maps of the first test conducted in the field

With the accumulated data of all catches per trap, it was possible to generate an overall heat map (HM) of the test (Fig. 27). In this HM, it is possible to see a single outbreak that

is nothing but the trap that has recorded the most catches. This trap is number 11, loaded with pheromone only.

Figure 27: Total HM, cumulative catches of the first test conducted

HMs obtained with the weekly catches of the second test are shown in Fig. 28.

After three weeks, the data from the June 11th sampling show how the situation has changed. On this date, it is possible to note that in the SF there is an outbreak in correspondence of the trap 3 (FC2); while in the BF there is only one outbreak in correspondence of the trap 23 (FC1).

After two weeks, on June 22nd, it can be seen that the resident population has moved more towards the northern part of the BF. In this portion of the plantation, several hot foci are then observed.

Over time, the population has shown an increasingly intense polarization towards the north-west sector of the BF. On July 16th, we can see how the only hot spots are concentrated only in this area of the experimental field.

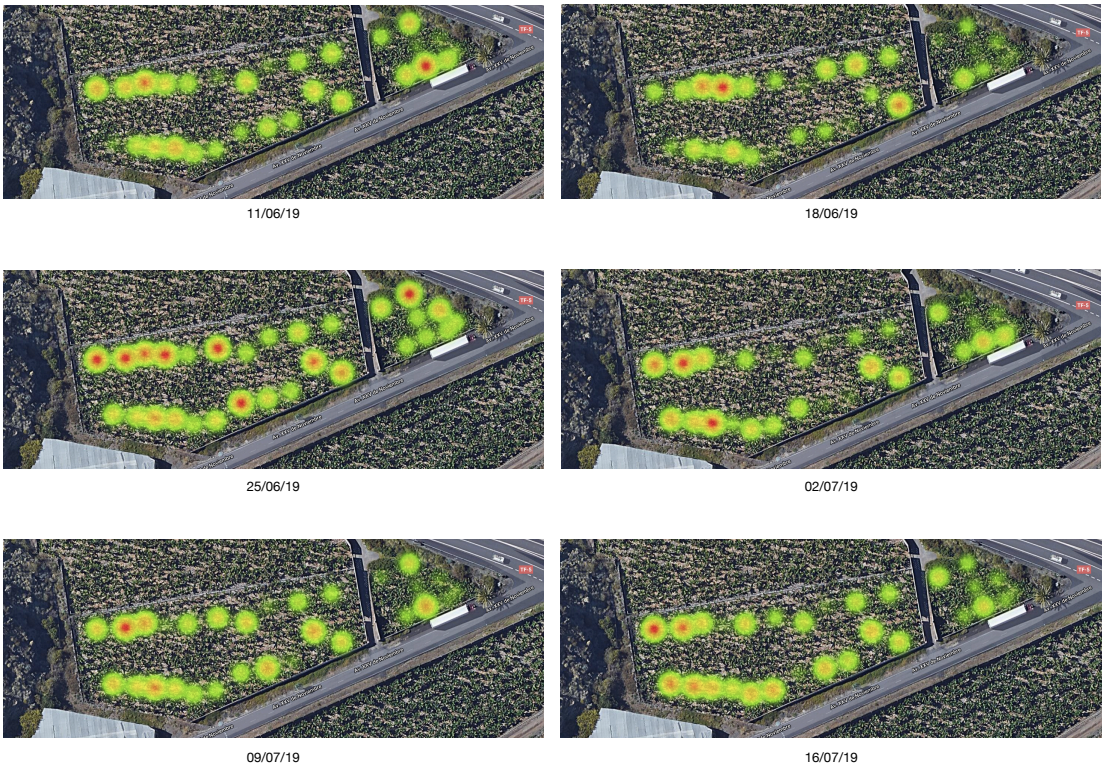

Figure 28: Weekly HMs of the second test conducted in the field

With the accumulated data of all catches per trap, it was also possible to generate an overall HM of this test (Fig. 29). The points with the highest amount of BWs captures are represented by different traps (29, 24, 23 and 25), loaded with the three treatments tested. Among these traps, the one with the hottest outbreak was number 24, again as for first test loaded with pheromone (F) only.

Figure 29: Total HM, cumulative catches of the second test conducted

4. Discussion

The fungal parasites of invertebrates (FPI) tested showed different VOCs metabolic profiles, only five compounds were found to be produced by all four selected strains. Of these, only DC027 (C7) was tested as a repellent against *C. sordidus*. This compound, also produced by other fungal species different from the one under study (Fischer *et al*., 1999), was found to be an M-VOCs in all cases. Belonging to this category makes C7 a compound of significant importance for the fungi under study. The function of this VOC is, however, still unknown. Based on the collected data, it is possible to state that DC027 is a compound incessantly produced during the whole period of study by both *B. bassiana* strains (it was found in all six measurements conducted). Instead, *M. anisopliae* and *P. clamydosporia* produce this compound only in the early stages of fungal growth (30 and 20 dai, respectively). From the tests conducted in an olfactometer, this M-VOCs turns out to be the one that mostly reduces the mobility of *C. sordidus* and therefore represents the most repellent molecule among those tested *in vitro*. Therefore, C7 was indicated as a *hard-repellent* of the black banana weevil.

C3 (identified during preliminary tests) and C4 (DC141), identified in the metabolic profiles of *B. bassiana*, have been selected since they are already known repellents of different insect taxa (Chokechaijaroenporn *et al*., 1994; Obeng-Ofori *et al*., 1999; Rozman *et al*., 2007). These two compounds showed a moderate action on the reduction of BW movement and therefore reduced the attractiveness of the *pabulum*. These compounds, given the moderate action, have been defined as *soft-repellents* of the BW. C4 (DC141), found in both *B. bassiana* strains tested, was found to be m-VOCs and present during early and late steps of fungal growth.

C5 (DC136) and C6 (DC068), were only detected in *P. clamydosporia* and *M. anisopliae* coltures. They were found to reduce the mobility of *C. sodidus in vitro*. The constant presence of these two compounds in the metabolic profile of these two FPIs is a further proof of the already known close relationship between them (Larriba *et al.*, 2014). Genomic studies support that *M. anisopliae* and *P. clamydosporia* have a single ancestral joint (Lin *et al*., 2015).

In addition to C7 (DC027) and C5 (DC136) can also be considered a *hard-repellent* for *C. sordidus*. C5 was found to be one of the mostly reduced the mobility of the phytophagous towards the *pabulum*.

DC068 (C6), responsible for the typical odour of many fungi (Wnuk *et al*., 1983; Venkateshwarlu *et al*., 1999), was found to be the soft-repellent with the mildest action on movement reduction of *C. sordidus*.

Among the tested technical repellents, garlic (Aj) was found to belong to the *soft-repellent*s category, also reducing *in vitro* BW mobility. Several results for colloidal sulfur (Az), which, on the other hand, was not able to reduce the pabulum research of the phytophagous. His high *IM* indicates the inability to mask the attractiveness of the corm/pseudocorm. Therefore, it cannot, in any way, be considered as a repellent for *C. sordidus*. It should not them be used for this purpose in the field.

The compounds C1 and C2 (DC154), already known as repellents of *R. ferrugineus* (Jalinas, 2016), were also repellent for *C. sordidus* and are considered two *soft-repellents* for the latter. They can reduce the attractiveness of by *pabulum* (banana corm) and pheromone to BW. These two substances, tested *in vitro*, showed considerable repellent action. In the field tests, however, both do not seem to perform the same repellent action shown in the laboratory. In fact, in none of the two tests carried out in the banana plantations was there a clear reduction in catches in the presence of these two soft-repellents. Only initially, in the first two weeks of the two tests, slight drops in the catches were observed in the presence of both compounds. This phenomenon could be ascribed more to the more complex environmental conditions of the *in vivo* tests than those *in vitro*, but also to the non-optimal VOC dispersion technology used for the field tests. Furthermore, the presence of several BW foci and its distribution in the field may have influenced the activity of the compounds tested.

In this Master Thesis, new VOCs have been identified that can be used as BW repellents. The implementation of the technologies associated with the dispersion of these repellents could produce advancement in the agrobiotechnological sustainability of the world banana cultivation.

5. Conclusions

From what emerged from the in vitro data, the hard-repellents (C7 and C5) can be considered as effective repellents in the management of *C. sordidus*. The soft-repellents (C1, C2, C3, C4, C6 and Aj) are considered repellent, albeit milder, for the BW under laboratory conditions.

These compounds isolated from FPIs, are just some of the compounds to have a repellent function. Future experiments with additional compounds already identified in the metabolic profiles of the FPIs could produce an implementation of the list of repellents of the black banana weevil. Also, further in vivo tests will have to be conducted to prove that the selected VOCs are repellents technically usable even in the field.

The implementation of dispenser technologies, i.e. slow-release polymer matrices, would lead to an improvement in the performance and durability of these compounds in the field.

The economic importance of BW at a global level justifies the continuation of research in the identification of new molecules and technologies for the genesis of this new means for bio-management.

Managing *C. sordidus*, in an integrated way, could contribute to the increase in banana production, significantly contributing to the increase in global food production, given the extent and importance of this crop.

Besides since *C. sordidus* (BW) can act as rector of Foc, managing BW can help management of Panama disease. This is especially relevant in view of the current epidermis of Foc TR4 which threatening banana production worldwide.

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Annexes

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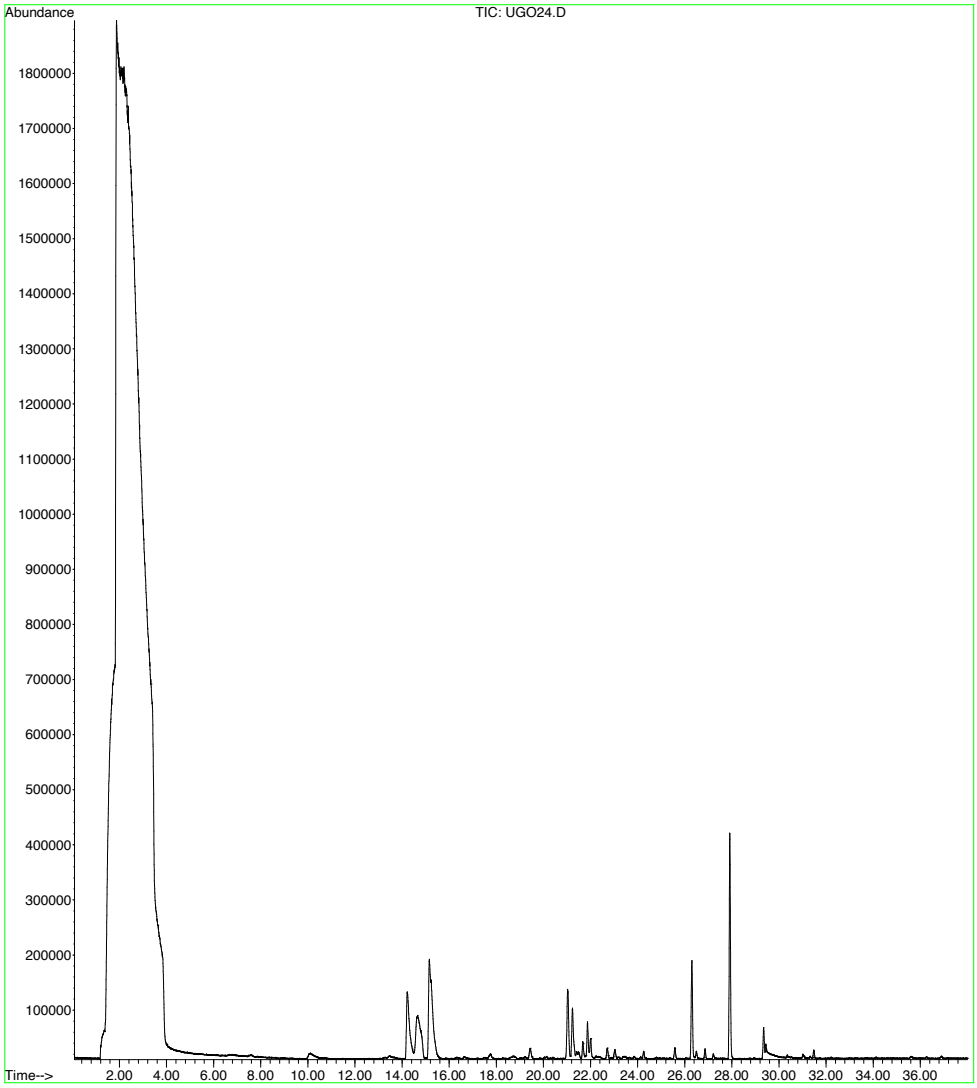

Annex 1: Chromatogram obtained with GC/MS-SPME of Bb203 - 10 dai

Code	Retention Time
DC027	15.16
DC037	19.44
DC046	21.23
DC072	22.71
DC052	25.58
DC141	29.35

Annex 2: Summary table of the Bb203 VOCs-peaks (10 dai) identified in Annex 1

```
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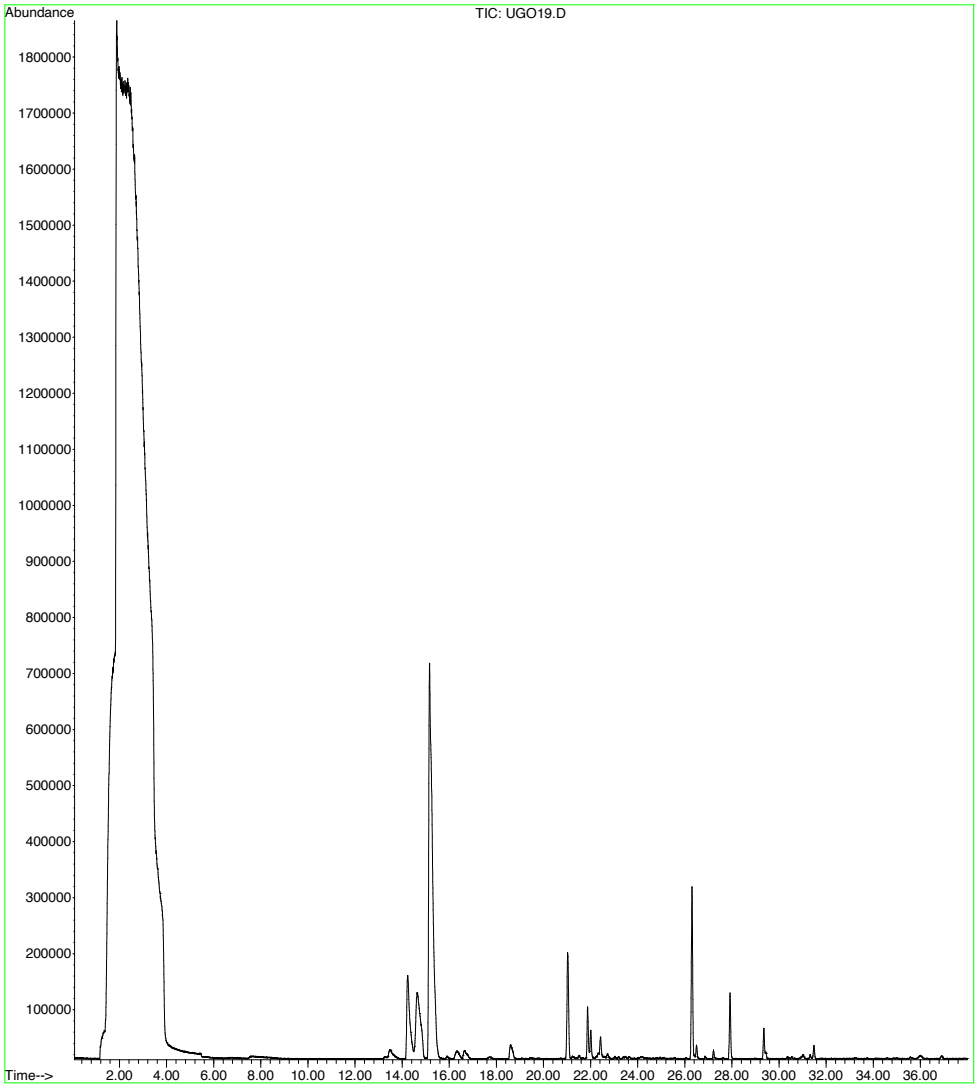

Annex 3: Chromatogram obtained with GC/MS-SPME of Bb203 - 20 dai

Code	Retention Time
DC025	13.48
DC027	15.16
DC032	16.64
DC069	22.42
DC141	29.36

Annex 4: Summary table of the Bb203 VOCs-peaks (20 dai) identified in Annex 3

```
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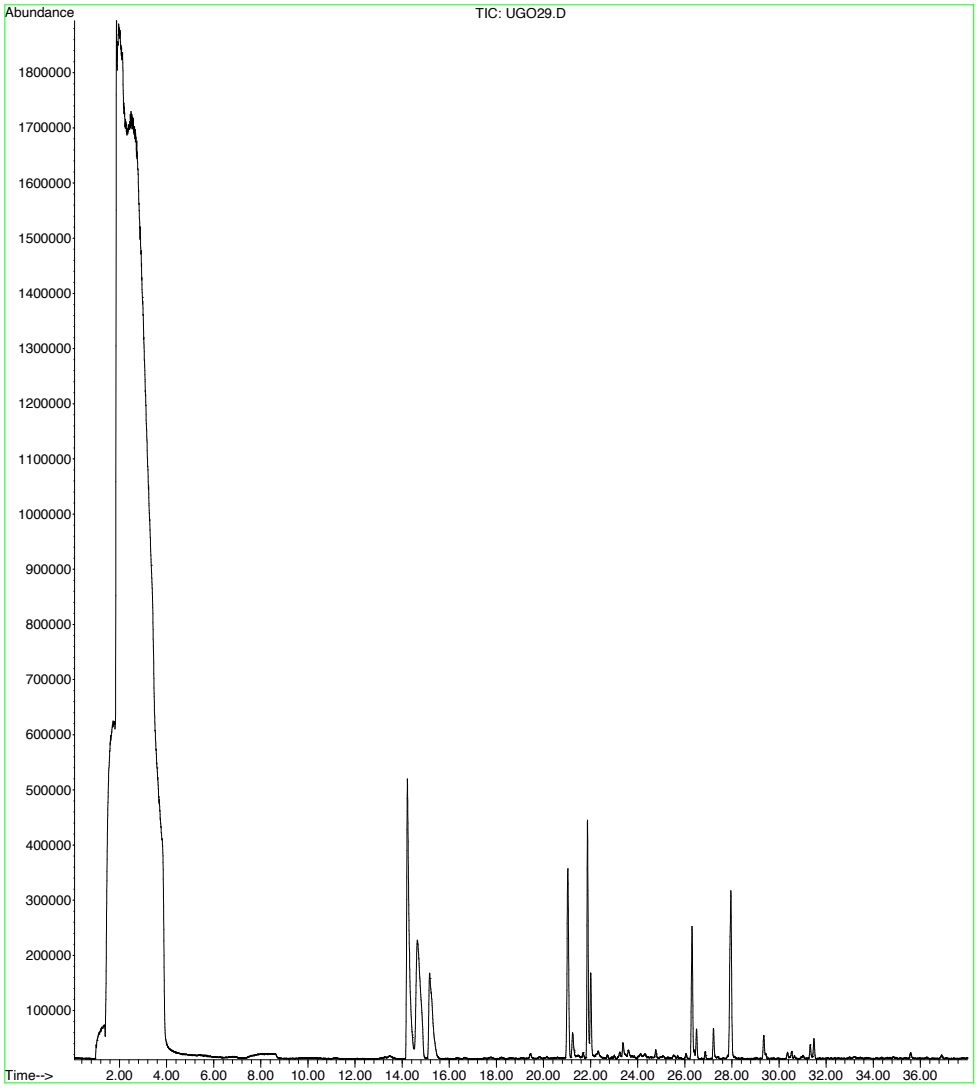

Annex 5: Chromatogram obtained with GC/MS-SPME of Bb203 - 30 dai

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DC046	21.25
DC067	22.32
DC078	23.61
DC130	27.95
DC141	29.36
DC149	30.54
DC150	31.49

Annex 6: Summary table of the Bb203 VOCs-peaks (30 dai) identified in Annex 5

```
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Vial Number: 1
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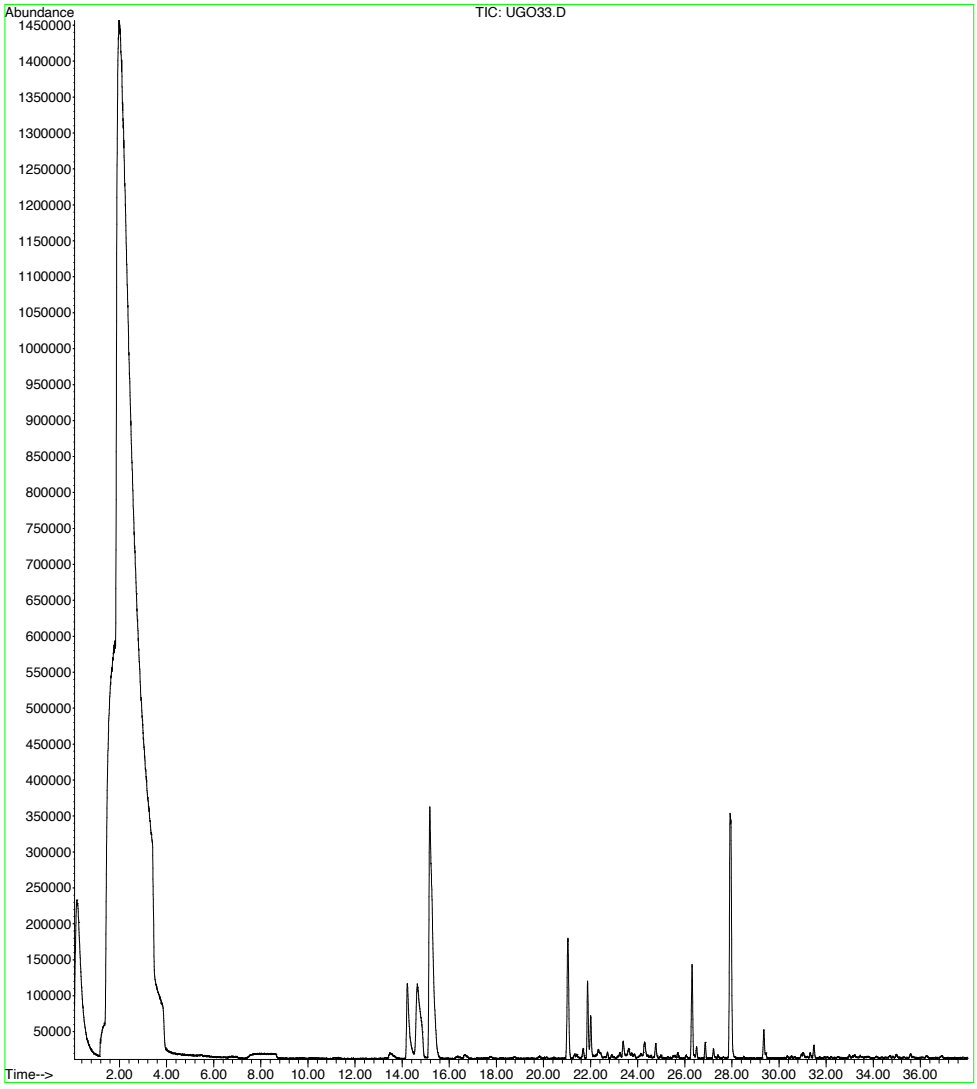

Annex 7: Chromatogram obtained with GC/MS-SPME of Bb203 - 40 dai

Code	Retention Time
DC027	15.19
DC081	24.30
DC130	27.94
DC141	29.36
DC150	31.49

Annex 8: Summary table of the Bb203 VOCs-peaks (40 dai) identified in Annex 7

```
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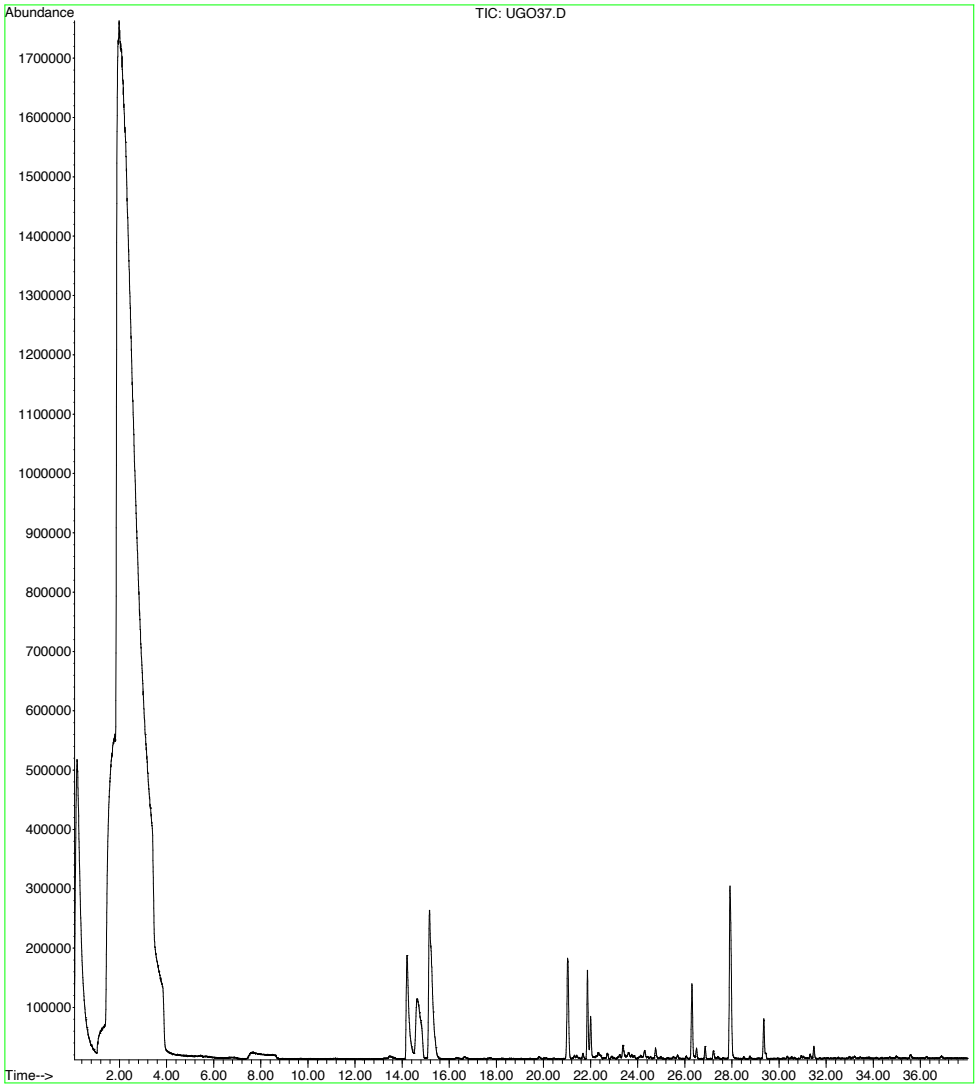

Annex 9: Chromatogram obtained with GC/MS-SPME of Bb203 - 50 dai

Code	Retention Time
DC027	15.17
DC141	29.36
DC150	31.48

Annex 10: Summary table of the Bb203 VOCs-peaks (50 dai) identified in Annex 9

```
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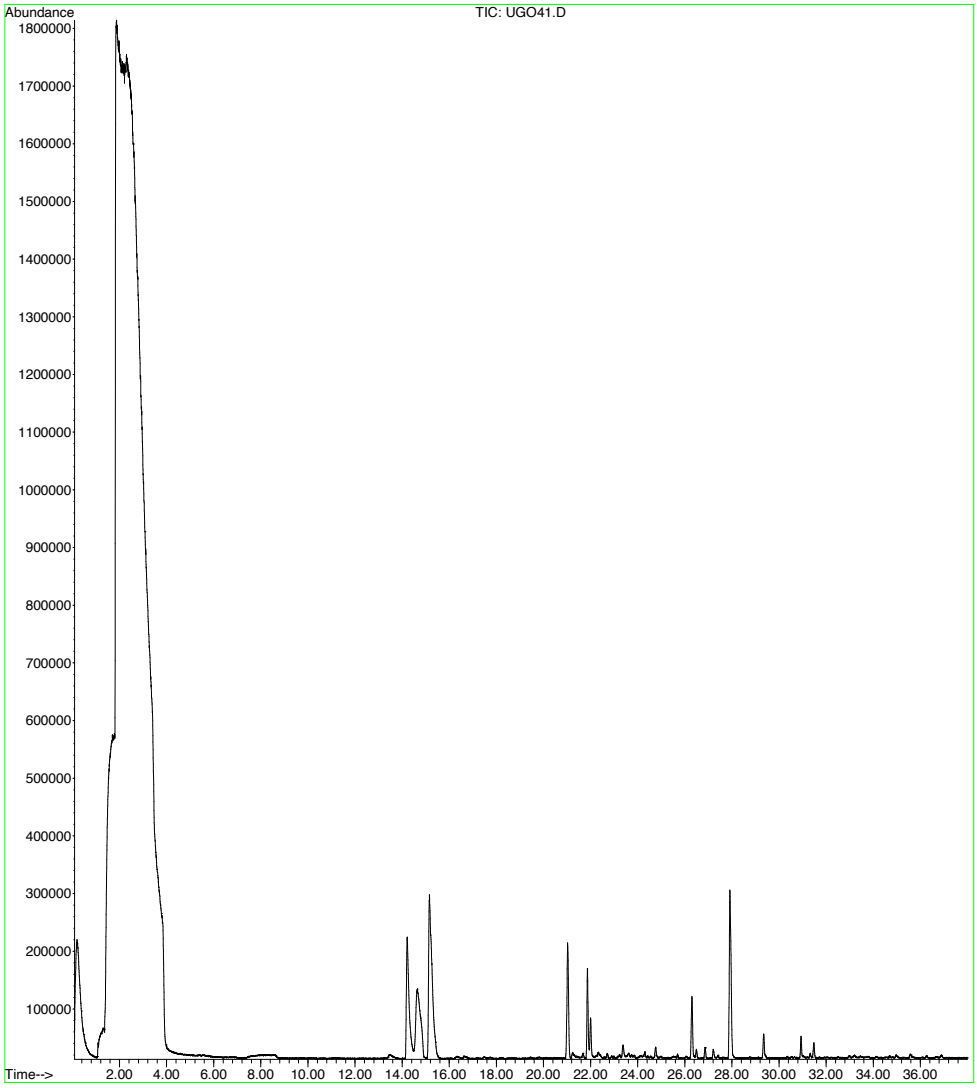

Annex 11: Chromatogram obtained with GC/MS-SPME of Bb203 - 60 dai

Code	Retention Time
DC027	15.17
DC109	26.49
DC129	27.93
DC141	29.35
DC150	31.48

Annex 12: Summary table of the Bb203 VOCs-peaks (60 dai) identified in Annex 11

```
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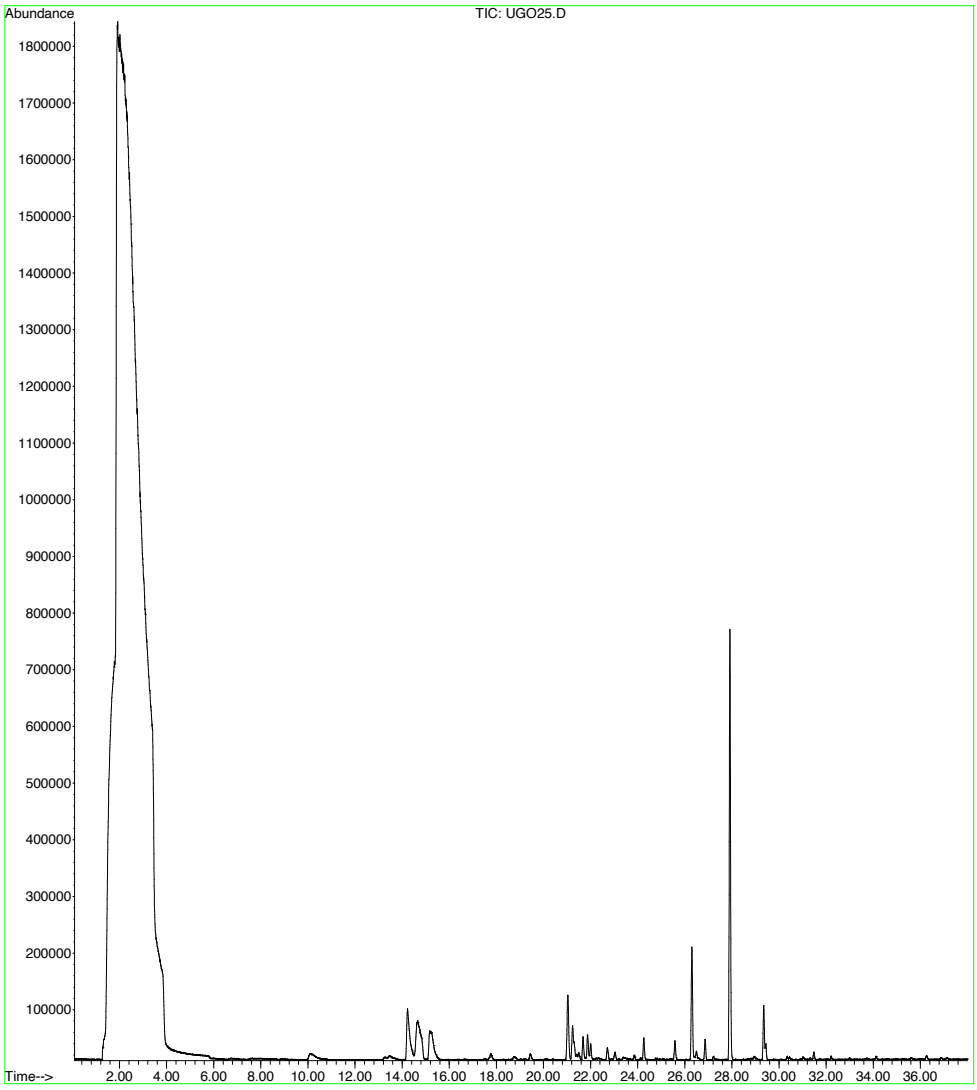

Annex 13: Chromatogram obtained with GC/MS-SPME of Bb1TS11 - 10 dai

Code	Retention Time
DC002	2.42
DC006	2.12
DC026	15.17
DC028	15.24
DC046	21.24
DC053	21.68
DC057	21.87
DC062	22.01
DC072	22.71
DC079	24.26
DC097	25.58
DC141	29.35
DC143	29.44

Annex 14: Summary table of the Bb1TS11 VOCs-peaks (10 dai) identified in Annex 13

```
File       : C:\MSDCHEM\1\DATA\UGO20.D
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Vial Number: 1
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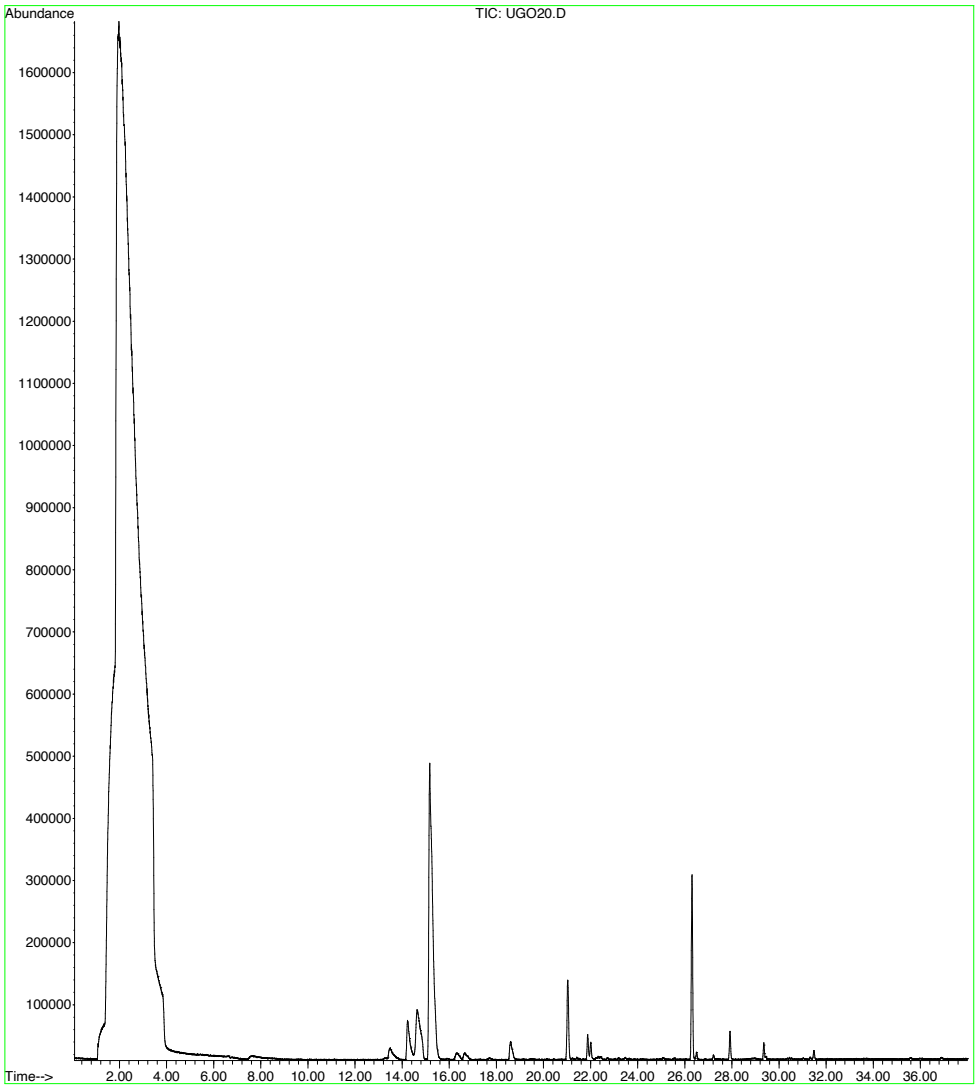

Annex 15: Chromatogram obtained with GC/MS-SPME of Bb1TS11 - 20 dai

Code	Retention Time
DC002	1.25
DC025	13.50
DC027	15.17
DC063	22.01
DC128	27.92
DC141	29.36

Annex 16: Summary table of the Bb1TS11 VOCs-peaks (20 dai) identified in Annex 15

```
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Vial Number: 1
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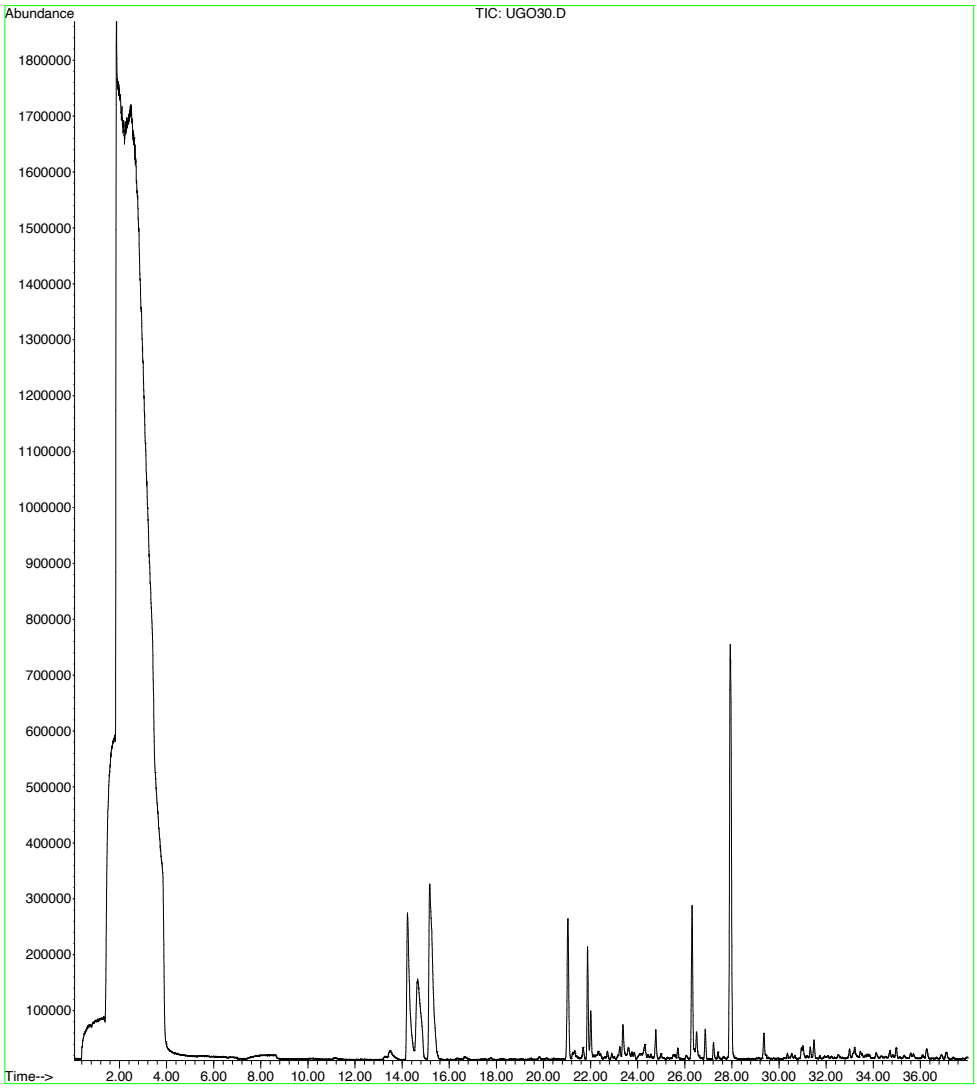

Annex 17: Chromatogram obtained with GC/MS-SPME of Bb1TS11 - 30 dai

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DC054	21.69
DC067	22.33
DC073	22.71
DC078	23.61
DC082	24.30
DC088	24.77
DC100	25.70
DC115	26.87
DC125	27.41
DC130	27.94
DC142	29.36
DC154	31.00
DC150	31.49
DC087	32.99
DC166	33.21
DC170	33.47
DC174	34.71
DC179	34.98
DC183	36.27
DC189	37.10

Annex 18: Summary table of the Bb1TS11 VOCs-peaks (30 dai) identified in Annex 17

```
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Vial Number: 1
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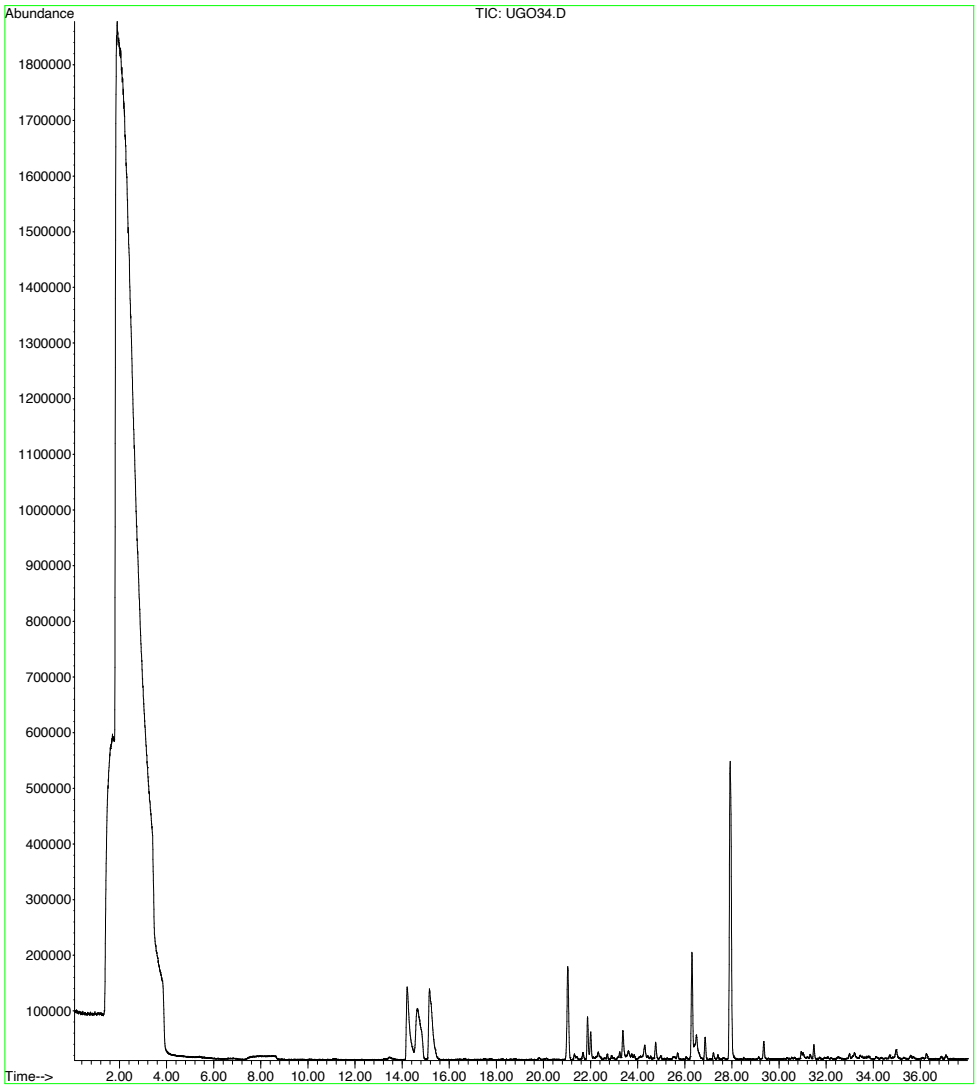

Annex 19: Chromatogram obtained with GC/MS-SPME of Bb1TS11 - 40 dai

Code	Retention Time
DC027	15.18
DC088	24.77
DC117	26.87
DC130	27.94
DC141	29.36
DC160	31.49

Annex 20: Summary table of the Bb1TS11 VOCs-peaks (40 dai) identified in Annex 19

```
File       : C:\MSDCHEM\1\DATA\UGO38.D
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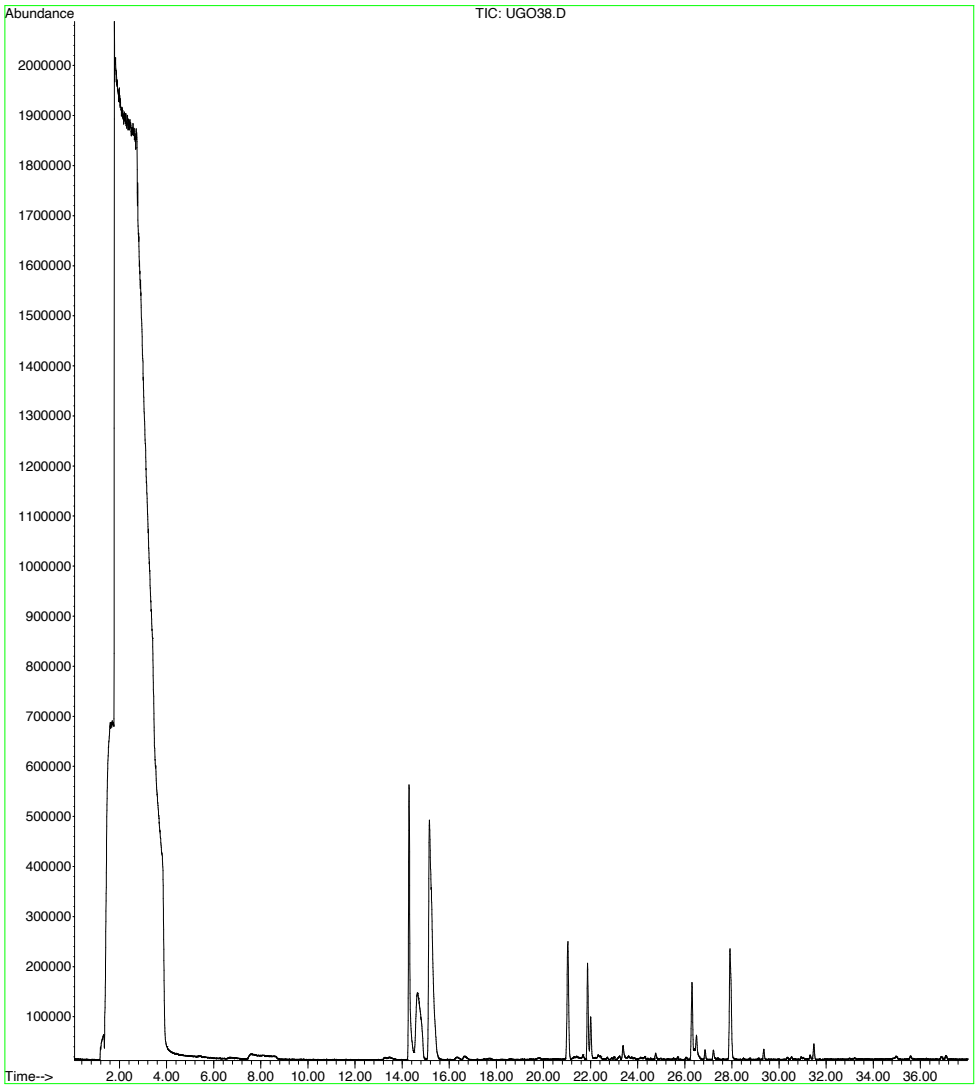
Acquired   : 27 Jun 2019  11:33     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: BB1TS11 50 DIAS
Misc Info  : GC= ANALUISVI
Vial Number: 1
```

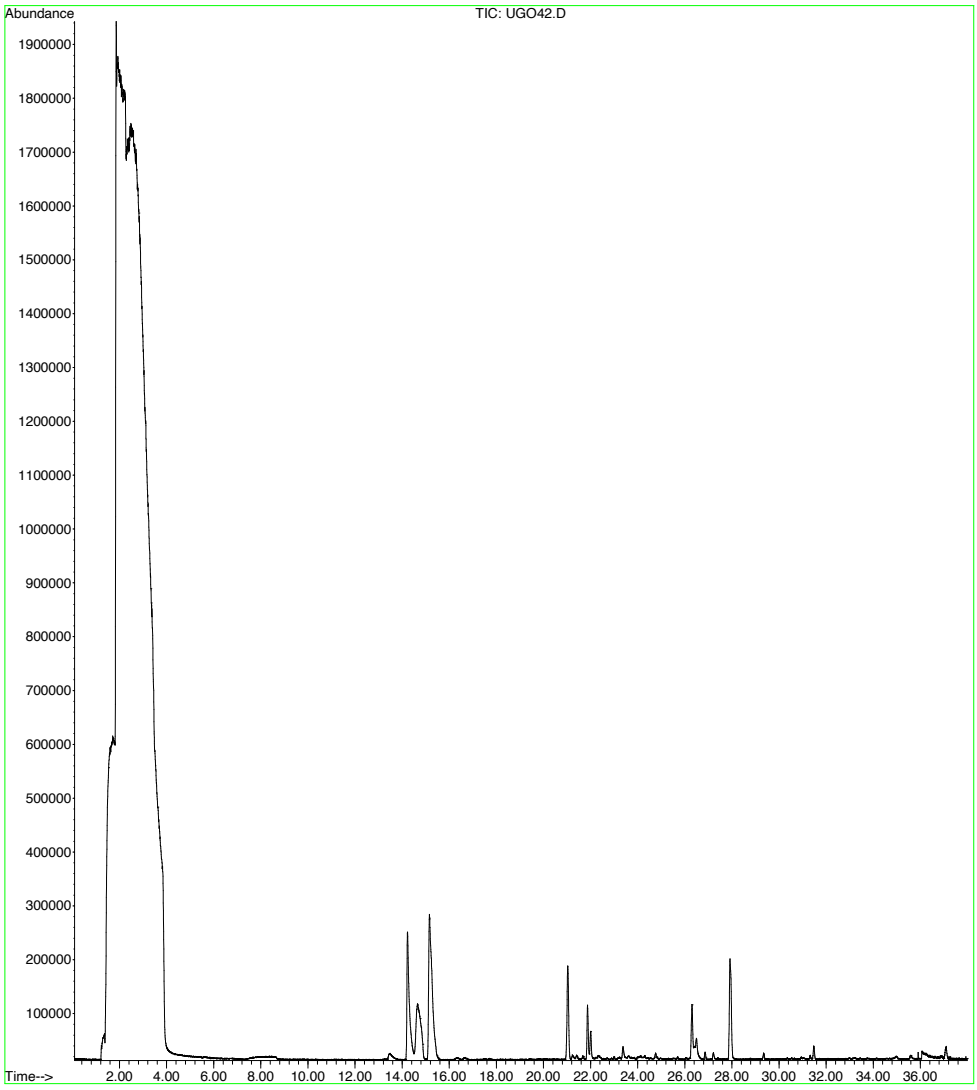


Annex 21: Chromatogram obtained with GC/MS-SPME of Bb1TS11 - 50 dai

Code	Retention Time
DC026	15.18
DC111	26.49
DC124	27.22
DC128	27.93
DC141	29.35
DC156	31.48

Annex 22: Summary table of the Bb1TS11 VOCs-peaks (50 dai) identified in Annex 21

```
File       : C:\MSDCHEM\1\DATA\UGO42.D
Operator   : PILAR
Acquired   :  8 Jul 2019  11:37     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: BB1TS11 6OD
Misc Info  : GC= ANALUISVI
Vial Number: 1
```

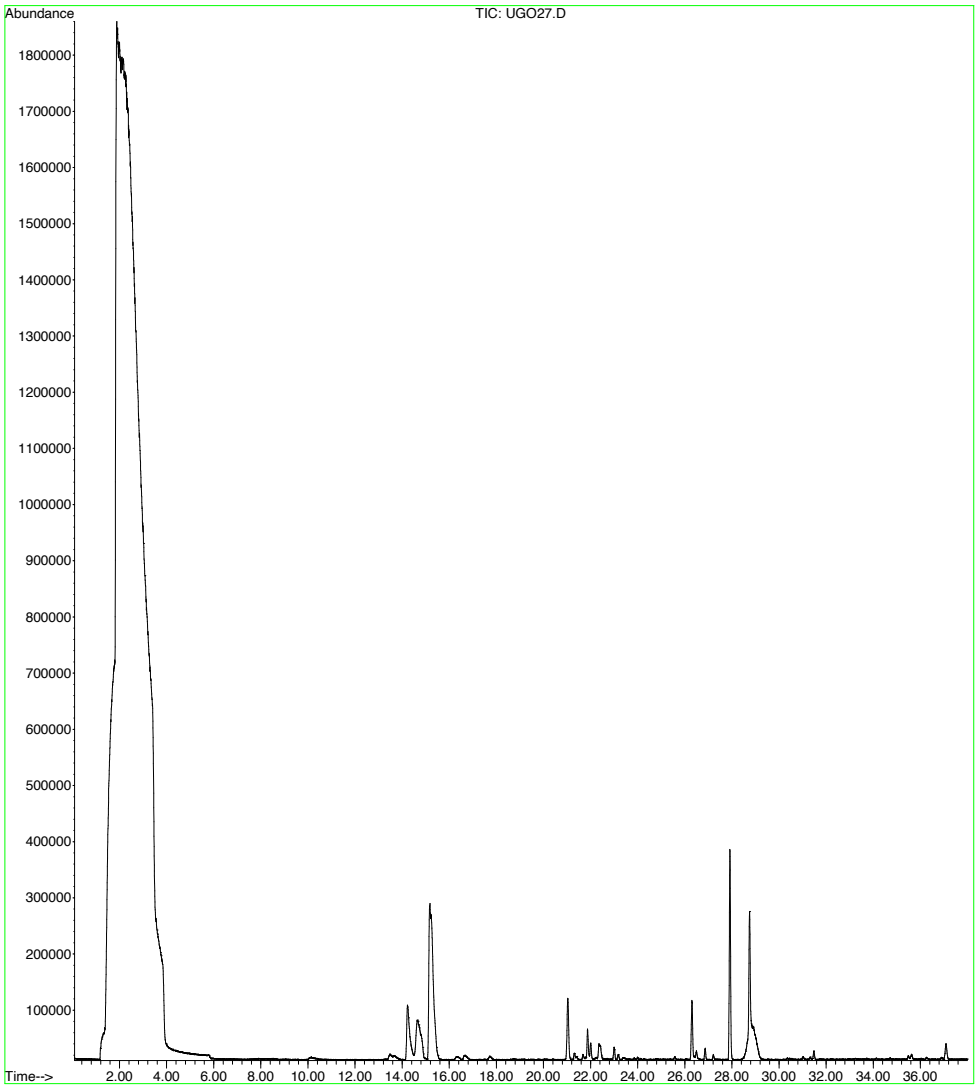


Annex 23: Chromatogram obtained with GC/MS-SPME of Bb1TS11 - 60 dai

Code	Retention Time
DC027	15.18
DC058	21.88
DC111	26.49
DC130	27.94
DC156	31.48
DC189	37.09

Annex 24: Summary table of the Bb1TS11 VOCs-peaks (60 dai) identified in Annex 23

```
File       : C:\MSDCHEM\1\DATA\UGO27.D
Operator   : PILAR
Acquired   : 30 May 2019  13:21     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: MA4TSO4
Misc Info  : GC=ANALUISVI
Vial Number: 1
```

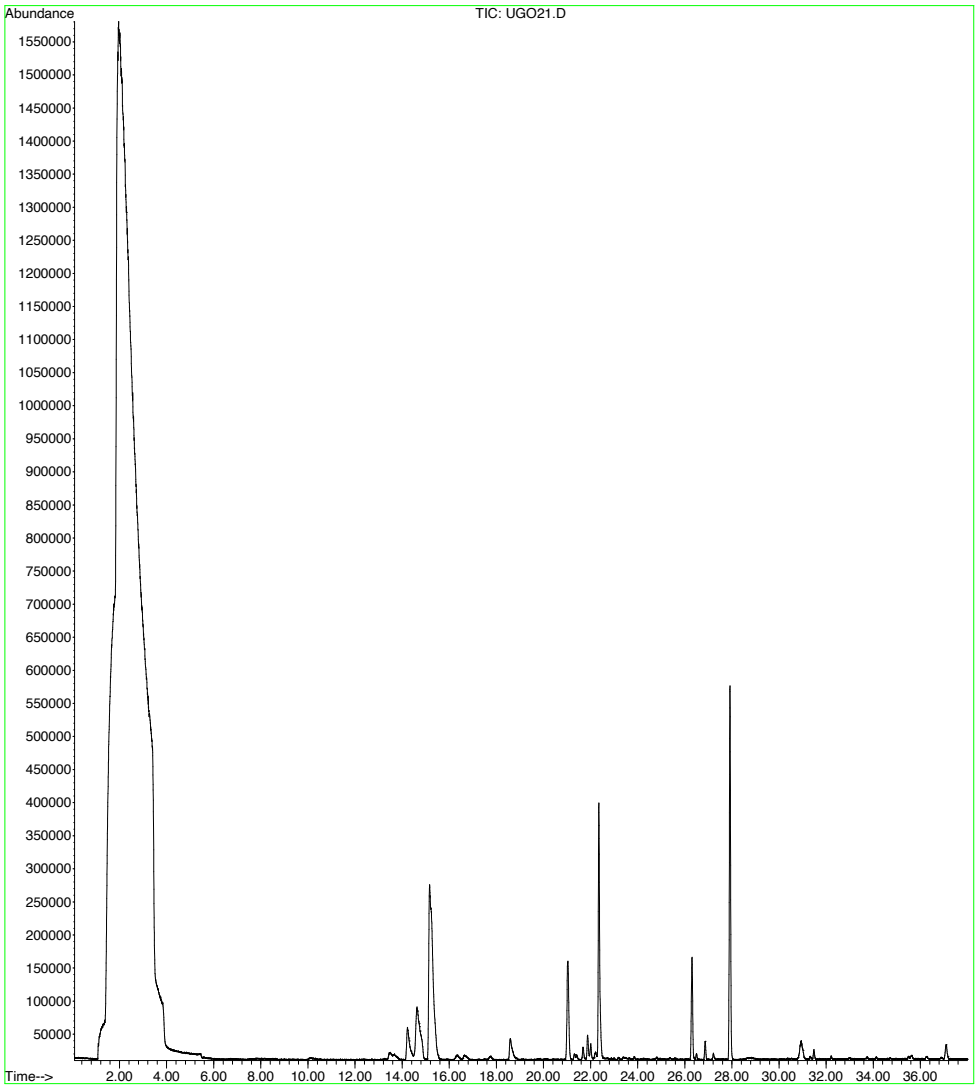


Annex 25: Chromatogram obtained with GC/MS-SPME of Ma4TS04 - 10 dai

Code	Retention Time
DC027	15.18
DC062	22.01
DC068	22.36
DC127	27.92
DC136	28.75
DC189	37.09

Annex 26: Summary table of the Ma4TS04 VOCs-peaks (10 dai) identified in Annex 25

```
File       : C:\MSDCHEM\1\DATA\UGO21.D
Operator   : PILAR
Acquired   : 27 May 2019  12:58     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: MA4TS04 20 DIAS
Misc Info  : GC=ANALUISVI
Vial Number: 1
```

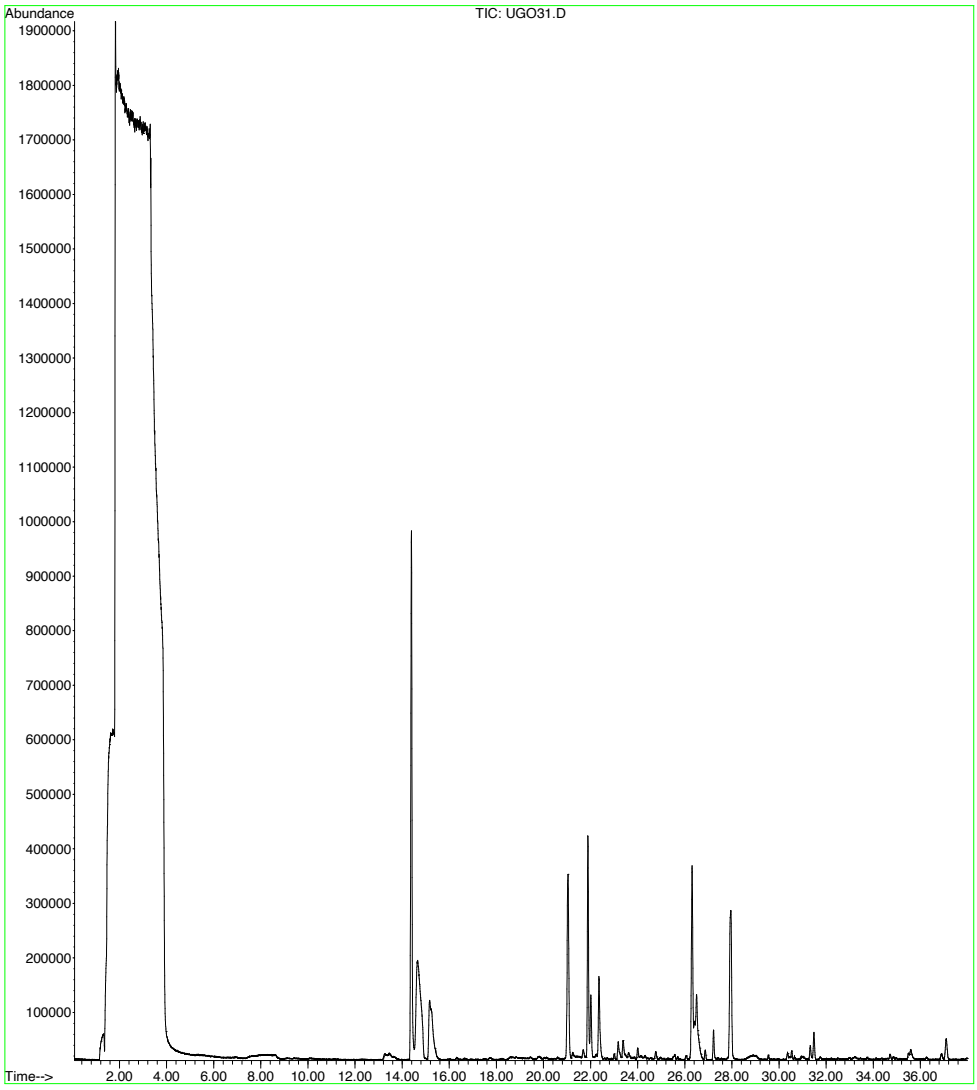


Annex 27: Chromatogram obtained with GC/MS-SPME of Ma4TS04 - 20 dai

Code	Retention Time
DC001	1.32
DC002	2.02
DC027	15.17
DC052	21.68
DC064	22.01
DC068	22.35
DC119	26.87
DC129	27.92
DC153	30.94
DC189	37.10

Annex 28: Summary table of the Ma4TS04 VOCs-peaks (20 dai) identified in Annex 27

```
File       : C:\MSDCHEM\1\DATA\UGO31.D
Operator   : PILAR
Acquired   :  7 Jun 2019  13:46     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: MA 4TS04 30d
Misc Info  : ANALUISVI, s
Vial Number: 1
```

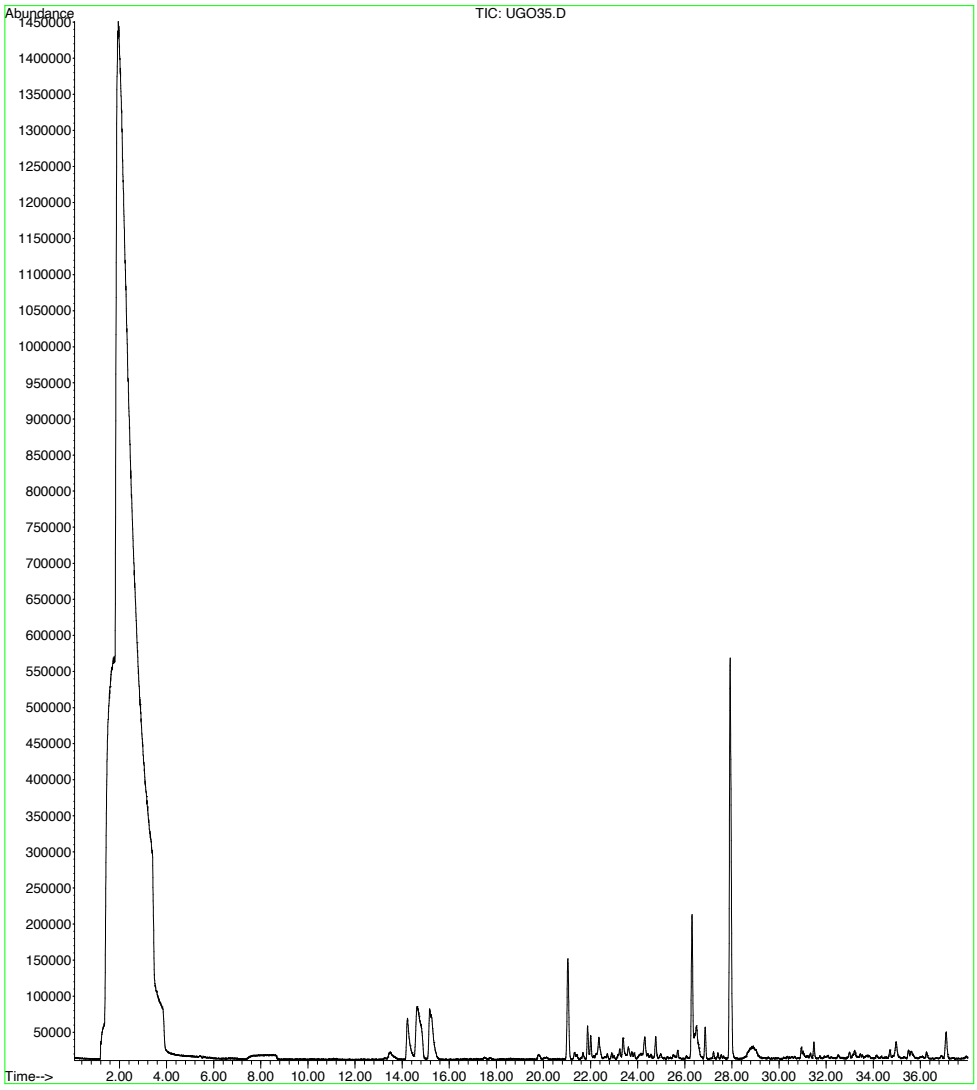


Annex 29: Chromatogram obtained with GC/MS-SPME of Ma4TS04 - 30 dai

Code	Retention Time
DC020	13.28
DC021	13.45
DC027	15.19
DC068	22.36
DC076	23.18
DC078	23.62
DC090	24.77
DC096	25.57
DC118	26.87
DC130	27.94
DC151	30.54
DC149	31.49
DC181	35.60
DC190	37.10

Annex 30: Summary table of the Ma4TS04 VOCs-peaks (30 dai) identified in Annex 29

```
File       : C:\MSDCHEM\1\DATA\UGO35.D
Operator   : PILAR
Acquired   : 17 Jun 2019  11:57     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: MA4TS04 40 DIAS
Misc Info  : GC= ANALUISVI
Vial Number: 1
```

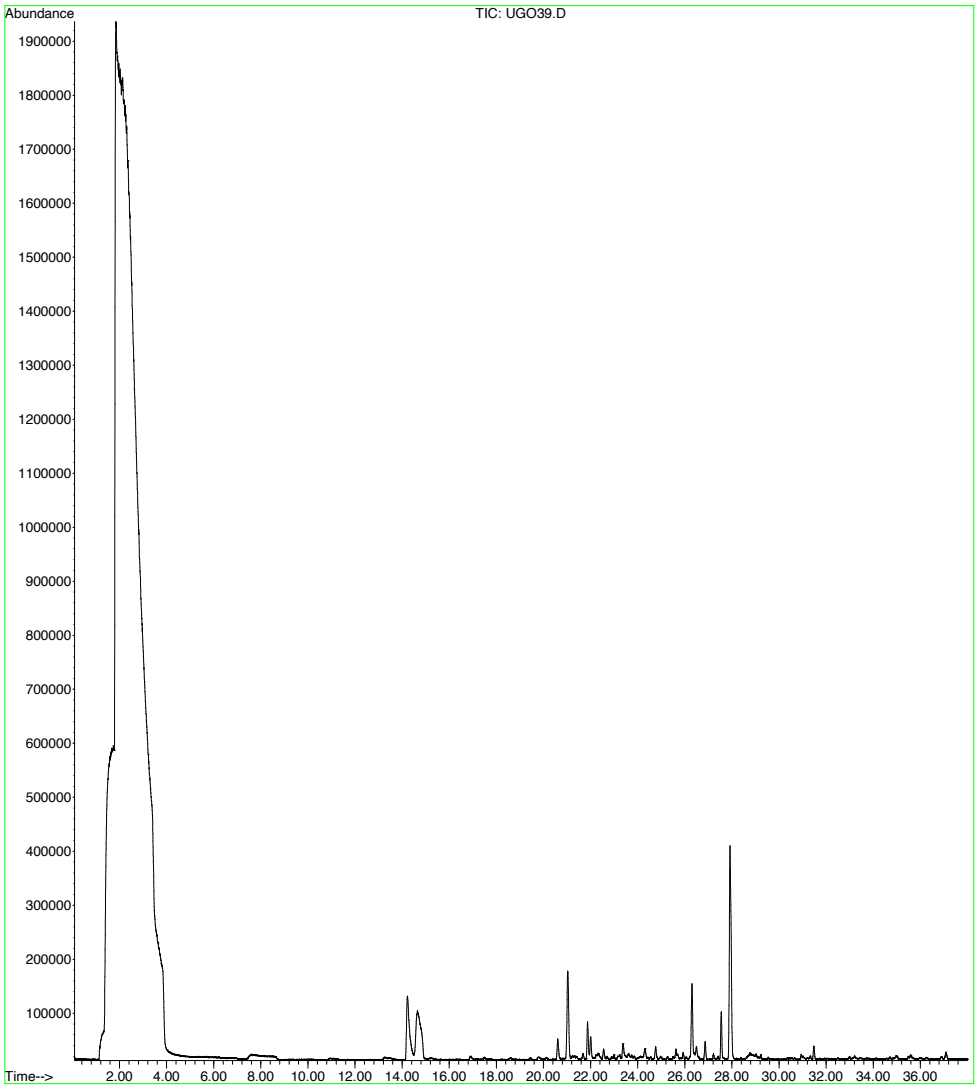


Annex 31: Chromatogram obtained with GC/MS-SPME of Ma4TS04 - 40 dai

Code	Retention Time
DC026	15.20
DC068	22.36
DC078	23.61
DC083	24.30
DC111	26.49
DC117	26.87
DC128	27.93
DC138	28.89
DC152	30.96
DC189	37.10

Annex 32: Summary table of the Ma4TS04 VOCs-peaks (40 dai) identified in Annex 31

```
File       : C:\MSDCHEM\1\DATA\UGO39.D
Operator   : PILAR
Acquired   : 27 Jun 2019  12:19     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: MA4TS04 50 DIAS
Misc Info  : GC= ANALUISVI
Vial Number: 1
```

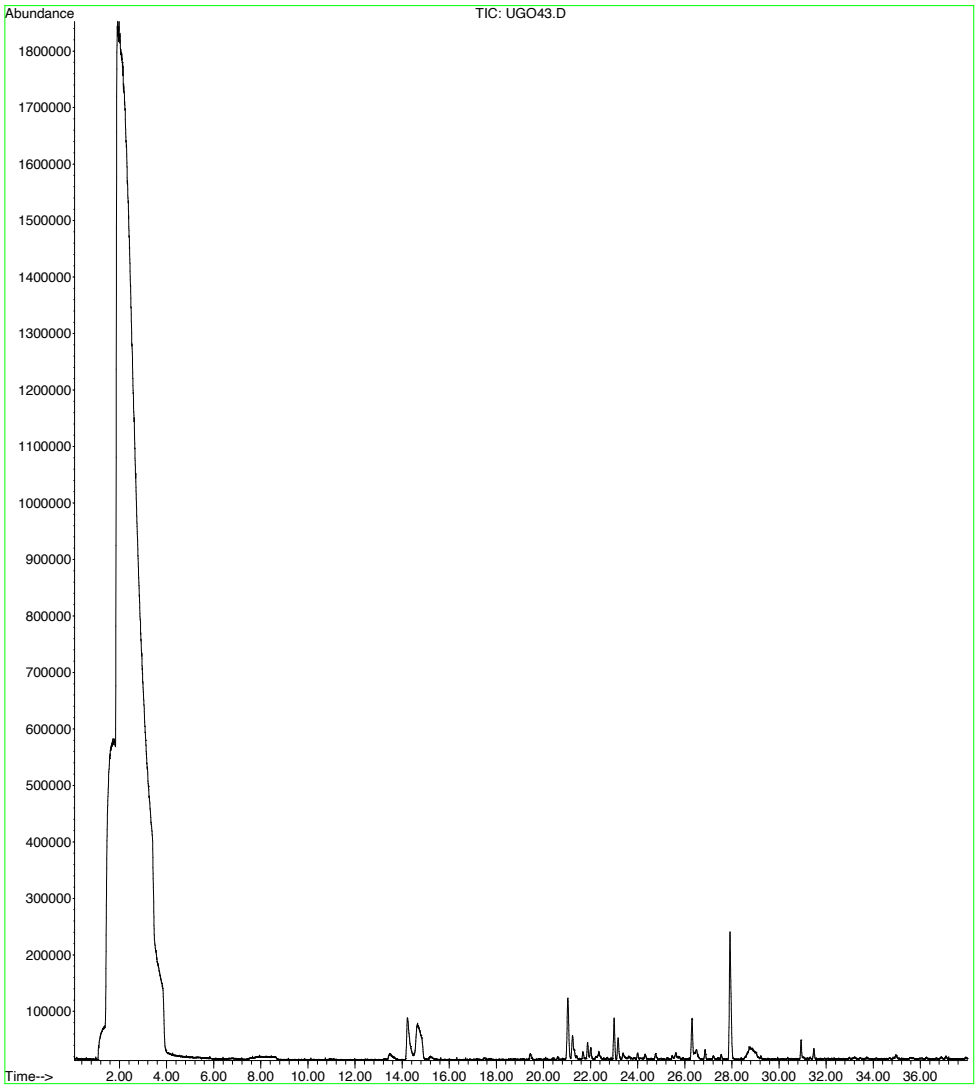


Annex 33: Chromatogram obtained with GC/MS-SPME of Ma4TS04 - 50 dai

Code	Retention Time
DC045	20.61
DC070	22.56
DC081	24.31
DC079	24.77
DC099	25.63
DC107	26.49
DC114	26.87
DC127	27.93
DC159	31.48

Annex 34: Summary table of the Ma4TS04 VOCs-peaks (50 dai) identified in Annex 33

```
File       : C:\MSDCHEM\1\DATA\UGO43.D
Operator   : PILAR
Acquired   :  8 Jul 2019  12:22     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: MA4TS04 6OD
Misc Info  : GC= ANALUISVI
Vial Number: 1
```

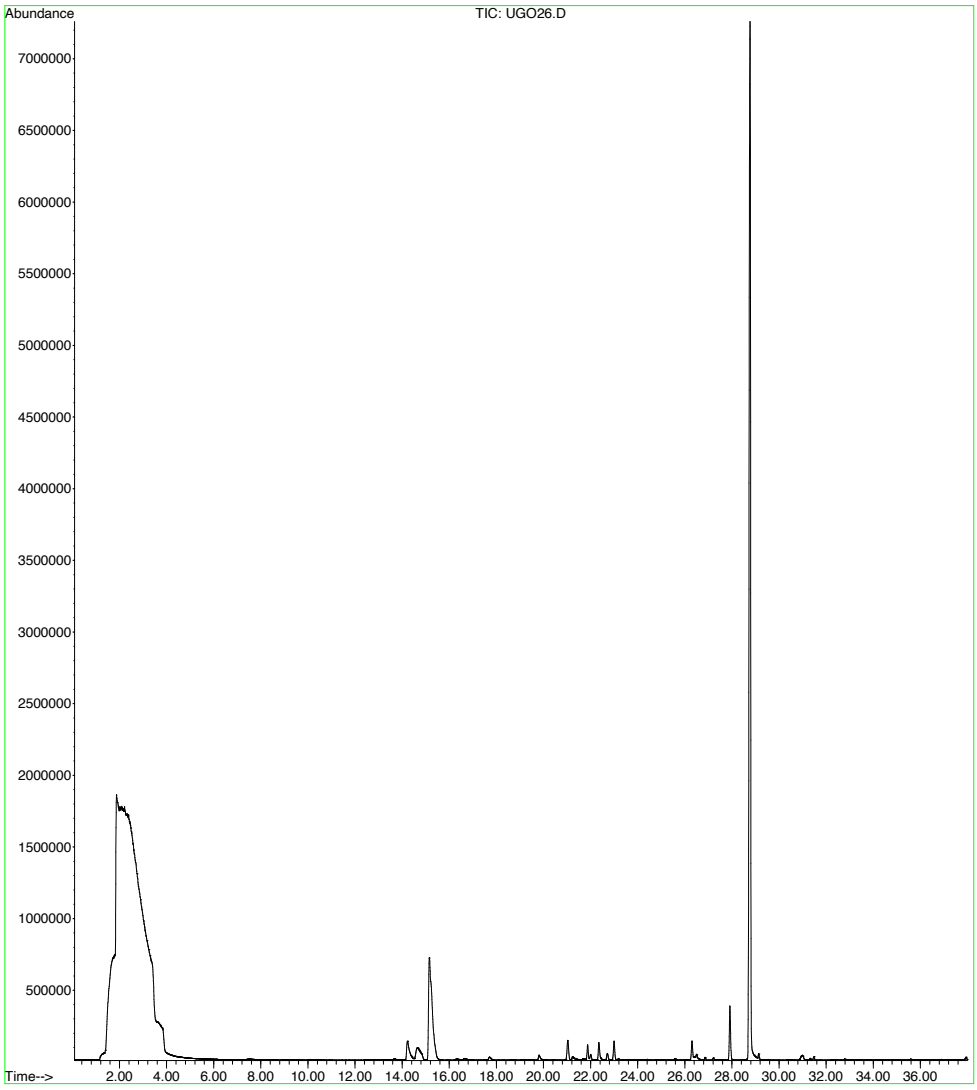


Annex 35: Chromatogram obtained with GC/MS-SPME of Ma4TS04 - 60 dai

Code	Retention Time
DC047	21.24
DC109	26.49
DC114	26.86
DC129	27.93
DC137	28.77
DC161	31.48

Annex 36: Summary table of the Ma4TS04 VOCs-peaks (60 dai) identified in Annex 35

```
File       : C:\MSDCHEM\1\DATA\UGO26.D
Operator   : PILAR
Acquired   : 30 May 2019  12:33     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: PC123
Misc Info  : GC=ANALUISVI
Vial Number: 1
```

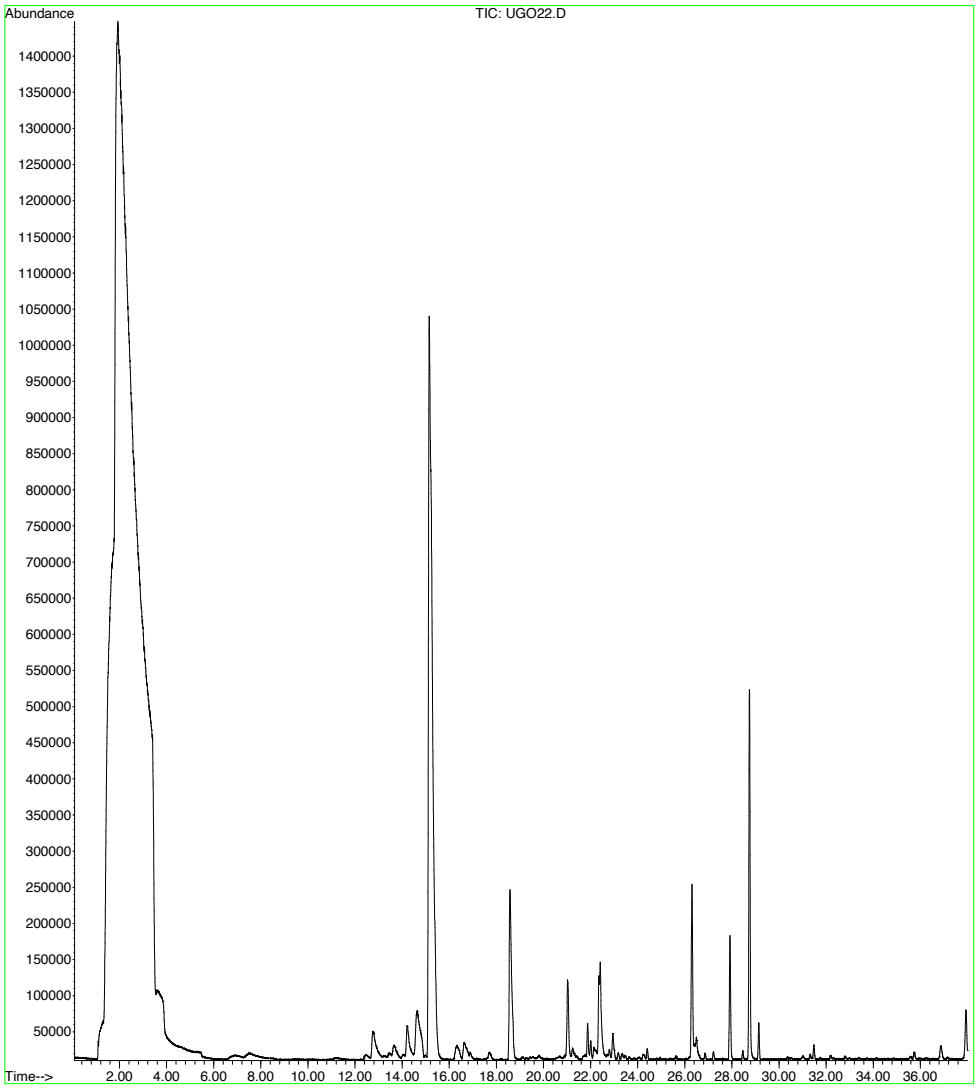


Annex 37: Chromatogram obtained with GC/MS-SPME of Pc123 - 10 dai

Code	Retention Time
DC001	1.36
DC002	2.16
DC027	15.16
DC034	17.71
DC039	19.81
DC046	21.24
DC068	22.36
DC072	22.71
DC105	26.41
DC110	26.49
DC120	26.87
DC136	28.77
DC140	29.14
DC153	31.00
DC193	37.94

Annex 38: Summary table of the Pc123 VOCs-peaks (10 dai) identified in Annex 37

```
File       : C:\MSDCHEM\1\DATA\UGO22.D
Operator   : PILAR
Acquired   : 27 May 2019  13:56     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: PC123 20 DIAS
Misc Info  : GC=ANALUISVI
Vial Number: 1
```

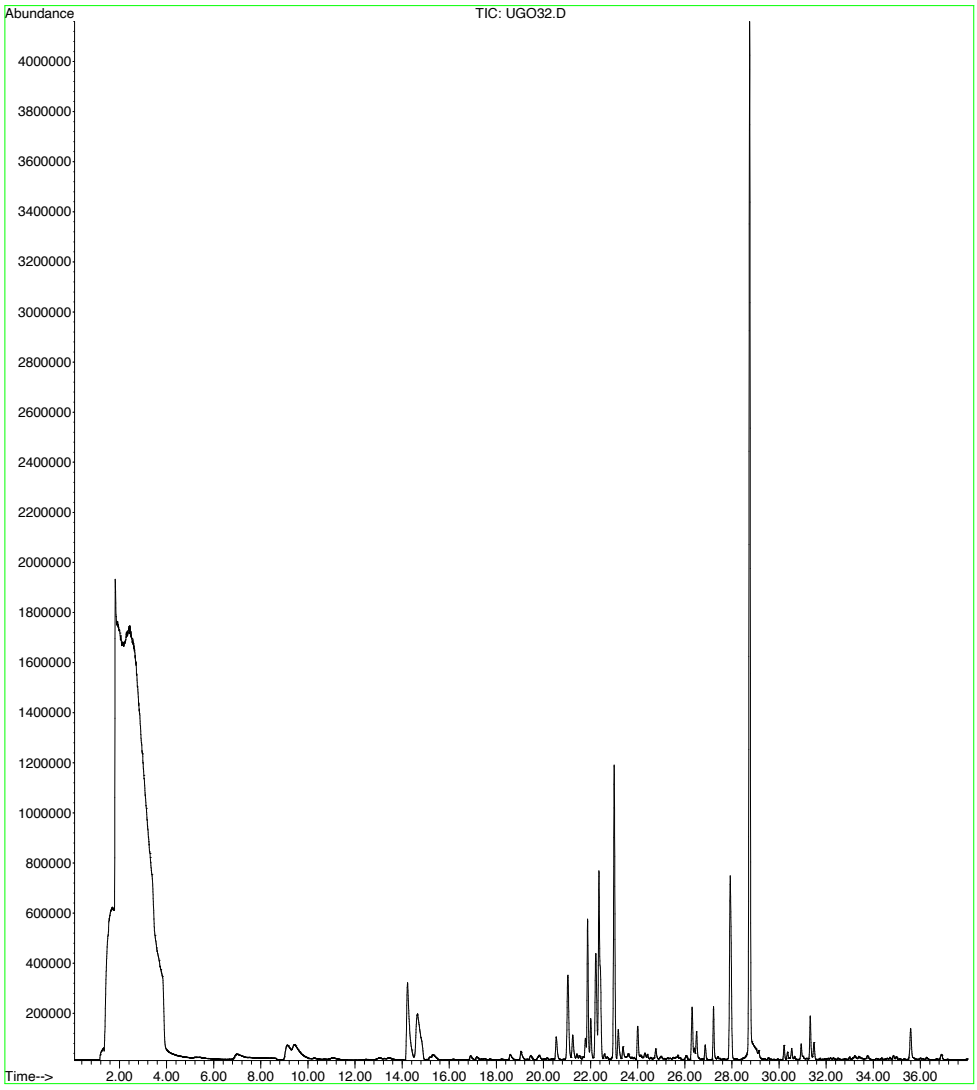


Annex 39: Chromatogram obtained with GC/MS-SPME of Pc123 - 20 dai

Code	Retention Time
DC001	1.24
DC006	2.17
DC018	12.76
DC027	15.15
DC032	16.64
DC066	22.14
DC068	22.36
DC069	22.41
DC074	22.95
DC104	26.40
DC108	26.49
DC127	27.92
DC136	28.75
DC139	29.14
DC161	31.49
DC192	37.94

Annex 40: Summary table of the Pc123 VOCs-peaks (20 dai) identified in Annex 39

```
File       : C:\MSDCHEM\1\DATA\UGO32.D
Operator   : PILAR
Acquired   :  7 Jun 2019  14:38     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: PC123 30d
Misc Info  : ANALUISVI, s
Vial Number: 1
```

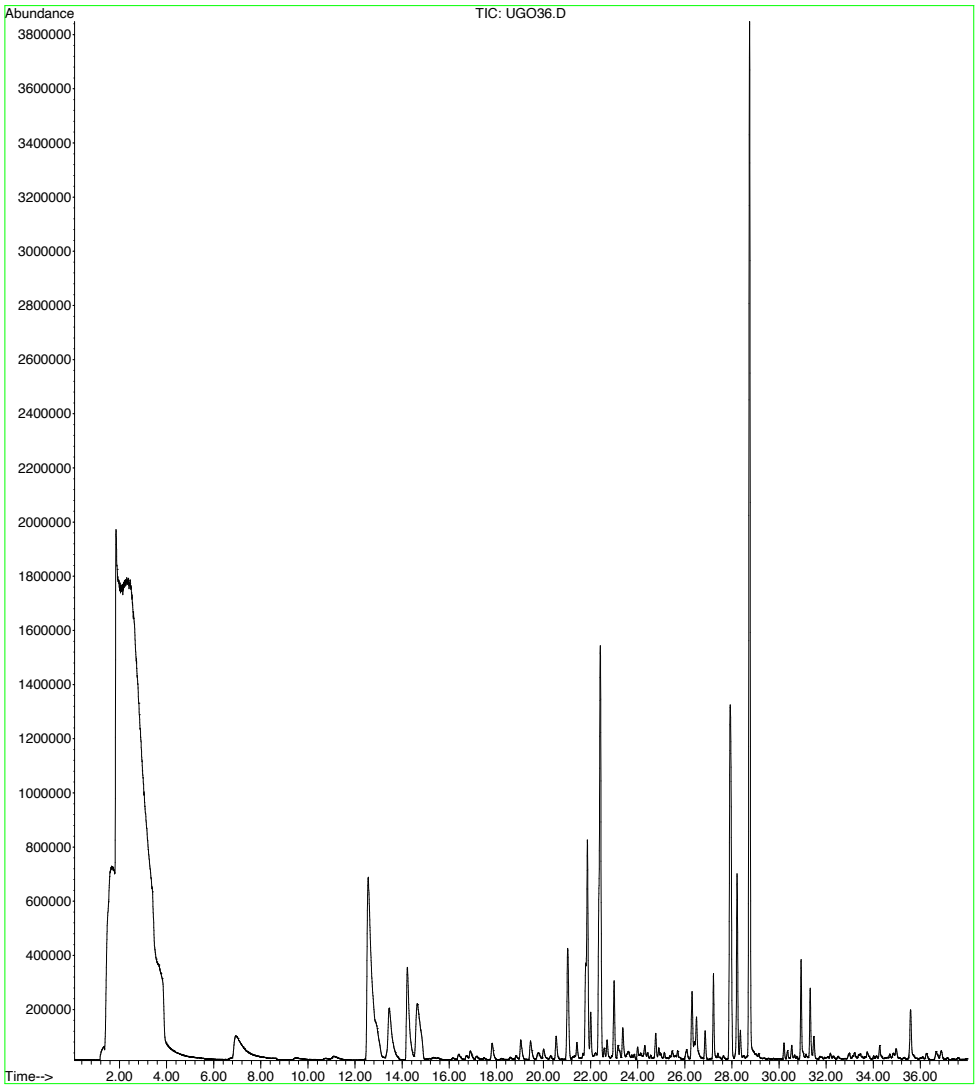


Annex 41: Chromatogram obtained with GC/MS-SPME of Pc123 - 30 dai

Code	Retention Time
DC009	2.42
DC012	6.99
DC014	9.14
DC016	9.43
DC019	13.04
DC033	16.92
DC035	17.18
DC037	19.46
DC040	19.82
DC044	20.55
DC046	21.25
DC059	21.88
DC068	22.36
DC078	23.60
DC084	24.31
DC089	24.77
DC092	24.99
DC102	25.70
DC160	26.06
DC123	26.87
DC124	27.22
DC128	27.94
DC136	28.75
DC145	30.22
DC150	30.54
DC167	33.22
DC168	33.40
DC171	33.77
DC175	34.86

Annex 42: Summary table of the Pc123 VOCs-peaks (30 dai) identified in Annex 41

```
File       : C:\MSDCHEM\1\DATA\UGO36.D
Operator   : PILAR
Acquired   : 17 Jun 2019  12:44     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: PC123 40 DIAS
Misc Info  : GC= ANALUISVI
Vial Number: 1
```

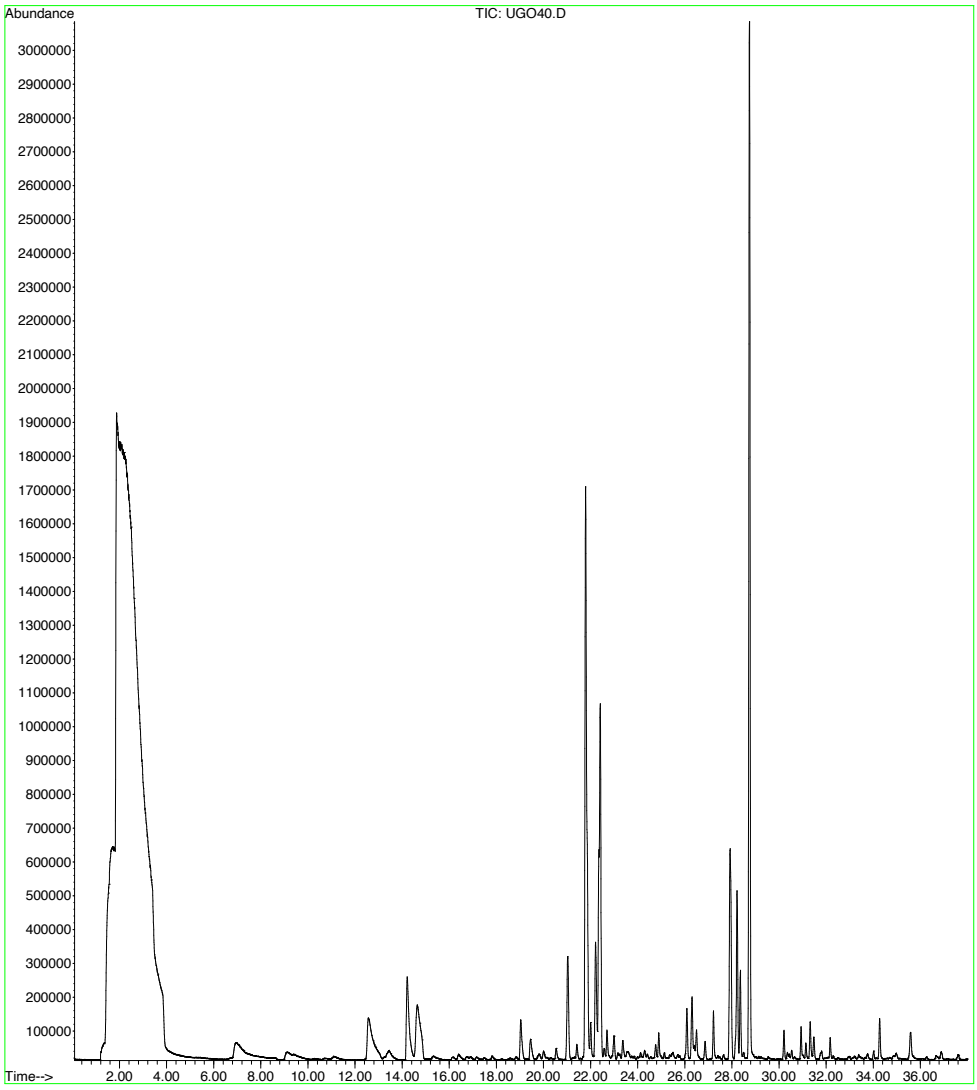


Annex 43: Chromatogram obtained with GC/MS-SPME of Pc123 - 40 dai

Code	Retention Time
DC012	6.95
DC017	12.57
DC019	13.46
DC031	16.41
DC033	16.91
DC036	17.83
DC038	19.79
DC041	20.01
DC042	20.54
DC051	21.42
DC069	22.41
DC076	23.18
DC078	23.60
DC080	24.30
DC051	25.13
DC101	25.70
DC103	26.08
DC106	26.49
DC116	26.87
DC130	27.94
DC135	28.22
DC136	28.75
DC145	30.22
DC148	30.54
DC163	32.19
DC164	32.98
DC165	33.21
DC169	33.44
DC171	33.75
DC091	34.28
DC178	34.97
DC182	36.27
DC184	36.68
DC187	36.89

Annex 44: Summary table of the Pc123 VOCs-peaks (40 dai) identified in Annex 43

```
File       : C:\MSDCHEM\1\DATA\UGO40.D
Operator   : PILAR
Acquired   : 27 Jun 2019  13:09     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: PC123 50 DIAS
Misc Info  : GC= ANALUISVI
Vial Number: 1
```

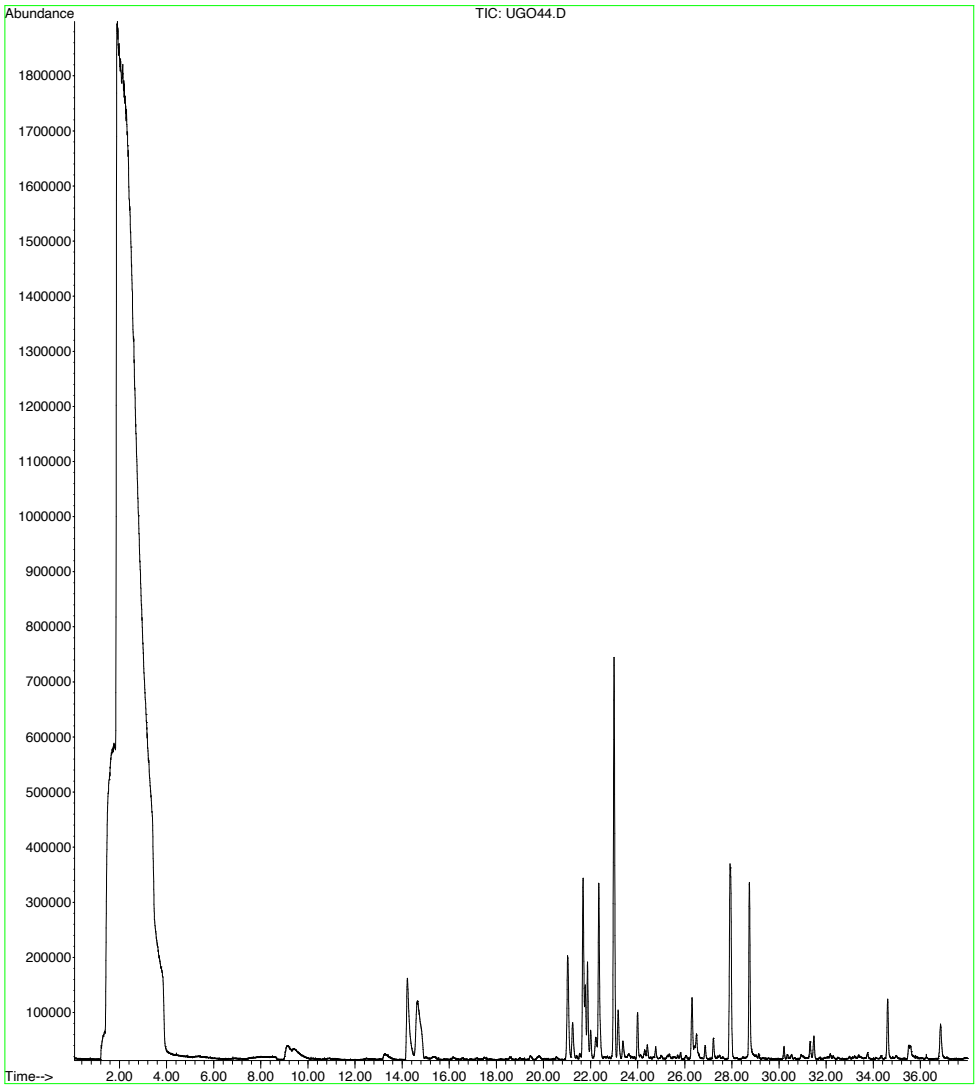


Annex 45: Chromatogram obtained with GC/MS-SPME of Pc123 - 50 dai

Code	Retention Time
DC012	6.96
DC014	9.13
DC017	12.58
DC022	13.45
DC030	16.41
DC039	19.82
DC041	20.01
DC043	20.55
DC050	21.42
DC056	21.79
DC069	22.40
DC076	23.18
DC027	23.58
DC081	24.30
DC091	24.90
DC093	25.13
DC095	25.49
DC103	26.09
DC108	26.49
DC124	27.22
DC134	27.93
DC135	28.22
DC136	28.75
DC146	30.21
DC155	31.15
DC158	31.48
DC162	31.80
DC172	33.76
DC091	34.02
DC173	34.97
DC191	37.60

Annex 46: Summary table of the Pc123 VOCs-peaks (50 dai) identified in Annex 45

```
File       : C:\MSDCHEM\1\DATA\UGO44.D
Operator   : PILAR
Acquired   :  8 Jul 2019  13:08     using AcqMethod ANALUISV
Instrument :   GSMSD
Sample Name: PC123 60 D
Misc Info  : GC= ANALUISVI
Vial Number: 1
```



Annex 47: Chromatogram obtained with GC/MS-SPME of Pc123 - 60 dai

Code	Retention Time
DC015	9.41
DC046	21.24
DC055	21.69
DC068	22.36
DC085	24.41
DC087	24.77
DC109	26.49
DC130	27.94
DC136	28.75
DC145	30.21
DC158	31.48
DC173	34.61
DC180	35.54
DC185	36.86

Annex 48: Summary table of the Pc123 VOCs-peaks (60 dai) identified in Annex 47