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METABOLIC DISCRIMINATION OF CITRUS SPECIES RESISTANT AND SUSCEPTIBLE TO BLACK SPOT DISEASE USING MULTI-PLATFORM-BASED METABOLOMIC APPROACH

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“Wisdom is brilliant, she never fades. By those who love her, she is readily seen, by those who seek her, she is readily found. She anticipates those who desire her by making herself known first. Whoever gets up early to seek her will have no trouble but will find her sitting at the door. Meditating on her is understanding in its perfect form, and anyone keeping awake for her will soon be free from care. For she herself searches everywhere for those who are worthy of her, benevolently appearing to them on their ways, anticipating their every thought”.

Bible, Wisdom 6:12-16.

(catholic.org)

List of Abbreviations

CBS	Citrus Black Spot
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-PDA	high-Performance Liquid Chromatography Coupled with Photodiode Array Detection
HPTLC	High-Performance Thin-Layer Chromatography
GC-MS	Gas Chromatography- Mass Spectrometry
HS-GC-MS	Headspace Gas Chromatography- Mass Spectrometry
UHPLC-DAD-MS	Ultra-High-Performance Liquid Chromatography–Diode Array Detector–Tandem Mass Spectrometry
HR-MS/MS	High Resolution Mass Spectrometry
CE-MS	Capillary Electrophoresis-Mass Spectrometry
1H NMR	Proton Nuclear Magnetic Resonance
1D and 2D NMR	One- and Two-Dimensional NMR Spectroscopy
COSY	Correlation Spectroscopy
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
MVDA	Multivariate Data Analysis
PCA	Principal Component Analysis
PLS-DA	Partial Least Squares- Discriminant Analysis
OPLS-DA	Orthogonal Partial Least Squares- Discriminant Analysis
ESEM	Environmental Scanning Electron Microscopy
HMDSO	Hexamethyldisiloxane
CH_3OH	Methanol

CH_3OD-d_4	Deuterated Methanol
EtOAc	Ethyl Acetate
DMSO	Dimethyl sulfoxide
ACN	Acetonitrile
QC	Quality Control
RT	Retention Time
RI	Retention Indices
VOCs	Volatile Organic Compounds
GLVs	Green Leaf Volatiles
OSMAC	One Strain Many Compounds
GNPS	Global Natural Product Social Molecular Networking
PD	Potato Dextrose
SB	Sabouraud
YMA	Malt + yeast
DKPs	Diketopiperazines
NPs	Natural Products
FA	Formic Acid

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ABSTRACT

METABOLIC DISCRIMINATION OF CITRUS SPECIES RESISTANT AND SUSCEPTIBLE TO BLACK SPOT DISEASE BY USING MULTI-PLATFORM-BASED METABOLOMIC APPROACH. Citrus black spot (CBS) caused by the fungus *Phyllosticta citricarpa*. CBS causes several economic damages to commercial citrus orchards, such as fruit blemishes and premature fruit drop, leading to yield loss. There are few studies related to the profile of compounds associated with the *Citrus*- *P. citricarpa* relationship. Thus, this study aimed to i) investigate the metabolic changes in leaves of two citrus species, *Citrus limon* (lemon) and *Citrus latifolia* (Tahiti lime), susceptible and resistant to citrus black spot disease, respectively, inoculated with the *P. citricarpa*; ii) have a general view of all metabolites present in citrus plants by using different metabolomics-based platforms, and iii) understand the mechanism that is supposed to be involved in citrus defense against this pathogen. Different analytical platforms, such as NMR, LC-MS, HPTLC and GC-MS were used. In addition, chemometrics tools were applied and as result, coumarins were observed at higher concentrations in *C. latifolia* specie after inoculation with the pathogen. Moreover, metabolomic of the pathogen was also investigated using *molecular networking*- GNPS platform which showed the diversity of compounds that may be produced by this fungus. This work provides a better understanding of the metabolic profile of resistant and susceptible citrus challenged by *P. citricarpa* as well as the compounds associated with the defense of plants against the pathogen.

Keys words: Coumarins, defense response, metabolomics, and volatile organic compounds.

RESUMO

DISCRIMINAÇÃO METABÓLICA DE ESPÉCIES DE CITROS RESISTENTES E SUSCETÍVEIS À PINTA PRETA, UTILIZANDO A ABORDAGEM METABOLÔMICA BASEADA EM MÚLTIPLAS PLATAFORMAS. Pinta preta dos citros (PPC) é causada pelo fungo *Phyllosticta citricarpa*. A PPC causa vários danos econômicos aos pomares comerciais de citros, como manchas nos frutos e queda prematura de frutas, levando à perda de rendimento. Existem poucos estudos relacionados ao perfil de compostos associados à interação *Citrus- P. citricarpa*. Portanto, este estudo teve como objetivo i) investigar as alterações metabólicas nas folhas de duas espécies de citros, *Citrus lemon* (limão verdadeiro) e *Citrus latifólia* (lima Tahiti), suscetíveis e resistentes à doença pinta preta dos citros, respectivamente, inoculadas com *P. citricarpa*; ii) obter uma visão geral de todos os metabólitos presentes nas plantas cítricas usando metabolômica baseada em diferentes plataformas e iii) entender o mecanismo que supostamente está envolvido na defesa de citros contra ao patógeno. Foram utilizadas diferentes plataformas analíticas como RMN, LC-MS, HPTLC e CG-MS. Além disso ferramentas quimiométricas foram aplicadas e como resultado, cumarinas foram observadas em altas concentrações em *Citrus latifólia* depois da inoculação com o patógeno. A metabolômica do patógeno também foi investigada, utilizando a plataforma GNPS de redes moleculares, nos quais os resultados mostraram a diversidade de compostos que podem ser produzidos por esse fungo. Este trabalho proporciona uma melhor compreensão do perfil metabólico de citros resistente e susceptível desafiados por *P. citricarpa*, bem como dos compostos associados à defesa contra o patógeno.

Palavras-chave: Cumarinas, resposta de defesa, metabolômica e compostos orgânicos voláteis.

CONTENT

1- GENERAL INTRODUCTION

1.1- Citrus production in Brazil and in the world

Citrus plants belonging to Rutaceae and originated in Asia are characterized by the attractive aroma and distinctive tastes, one of the most popular fruits in the world (WU et al., 2018; WANG et al., 2019). Many fruit species belong to the *Citrus* genus: oranges (*Citrus sinensis*), mandarins (*C. reticulata* and *C. deliciosa*), lemons (*C. limon*), acid limes (*C. latifolia* and *C. aurantiifolia*), sweet limes (*C. limettioide* and *Citrus paradise*), sour orange (*C. aurantium*) and grapefruits (*C. grandis*). New citrus varieties have been actively developed and introduced in the market to follow the trends of customers' tastes.

Citrus is the majority fruit tree crop in the world, which are highly consumed as fresh fruit and juice. The peel contains a wide variety of metabolites with vast antioxidant activity in comparison with other parts of the fruit. Citrus peel is a good source of molasses, pectin and limonene that are usually dried, mixed with dried pulps and sold as cattle feed (MANTHEY and GROHMANN, 2001; BOCCO et al., 1998; RAFIQ et al., 2018).

Due to the large demand for fruits, the world production of citrus rapidly increased from 34.9 million tons in 1968 to 146 million tons in 2017 at an average annual rate of 3.04%. Brazil is the biggest producer of orange fruit in the world with an annual production of around 20 million tons, followed by China (19.6 tons), United States (10 tons) and Mexico (6.8 tons). In 2018/2019 season, the orange production in São Paulo and Minas Gerais citrus belt was estimated in 285 million boxes of 40.8 kg. This production was 13.9% higher than the obtained in 2016/17 (245.31 million boxes); however, it was 28.2% less than that obtained in 2017/18 (398.35 million boxes), which was the fourth largest record in 30 years (CITRUSBR 2019; FUNDECITRUS 2019).

Particularly, around 80% of Brazilian orange production is destined for juice processing and the products are exported to many places such as European Union, United States, and Asia. The other 20% of the production is consumed as fresh fruit, mainly for the domestic market (AGRIANUAL 2019). Moreover, citrus production occupies a prominent place in the country, due to its great export value and its social importance, creating numerous jobs (NEVES et al., 2010).

Unfortunately, citrus production endures several problems that involve economic and market issues, such as tariff barriers that reduce its competitiveness in the international market, phytosanitary barriers, and technical requirements, as well as exchange rate that is unfavorable to Brazilian exporter. Furthermore, growers confront serious phytosanitary problems, for example, pests and diseases, which have affected many orchards worldwide (NEVES et al., 2010).

Several diseases are regarded as phytosanitary problems in citrus growing areas of São Paulo citrus belt, such as citrus black spot (CBS) (SILVA JUNIOR et al., 2016), citrus canker caused by the bacteria *Xanthomonas citri* subsp. *citri* (BEHLAU 2020), and huanglongbing (HLB), caused by *Candidatus* liberibacter spp. (BELASQUE JUNIOR et al., 2009).

1.2- Citrus black spot disease

Citrus black spot is an important disease that causes substantial losses in tropical and subtropical citrus-growing regions of Africa, Asia, Oceania, and the Americas (KOTZÉ 2000; EFSA 2014; SILVA JUNIOR et al., 2016). The disease is important in summer rainfall regions, where the weather conditions are suitable for disease establishment (EFSA 2014; YONOW et al., 2013).

Citrus black spot causes qualitative and quantitative damages. Qualitative damages are related to fruit appearance caused by the symptoms formed in the peel. CBS symptoms may reduce profitability or makes the fruits inadequate for the national and international market. Quantitative damages are related to the premature fruit drop, and consequently to the yield reduction. This damage is extremely relevant because around 70% of orange production is destined to fruit processing (SILVA JUNIOR. et al., 2016).

In Brazil, CBS was first reported in 1980s, causing significant losses in orchards of Rio de Janeiro (ROBBS et al., 1980) and Rio Grande do Sul states (ROBBS et al., 1995). In São Paulo state, CBS was detected in 1992 affecting lemon and sweet orange trees. In 2000s, CBS was observed in the states of Minas Gerais, Espírito Santo, Santa Catarina, Paraná, Mato Grosso, Mato Grosso do Sul, Goiás, Amazonas and Rondônia. In 2012, the disease was reported in Bahia state (SILVA JUNIOR. et al., 2016).

1.3- The pathogen and the cycle of disease

The causal agent of CBS was described by McAlpine (1889) in its asexual form and was initially designated as *Phoma citricarpa* and later as *Phyllosticta citricarpa* (FIGURE 1.1). The sexual form of the pathogen called *Guignardia citricarpa* was discovered by Kiely. The pathogen produces asexual spores called pycnidiospores or conidia and ascospores (sexual spores) (SPÓSITO et al., 2011). The pathogen infects a wide range of citrus hosts, including oranges, tangerines, and lemons (GOES, 1998, TRAN et al., 2018).

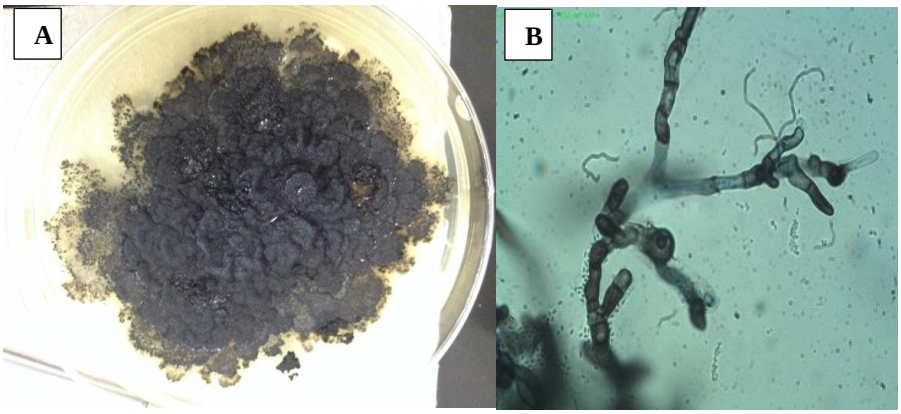


FIGURE 1.1- (A) *Phyllosticta citricarpa in potato dextrose culture medium*, (B) Microscopic structure of *P. citricarpa* (author's pictures).

The life cycle of *P. citricarpa* is divided in two stages: primary and secondary cycles (FIGURE 1.2); In the primary cycle (sexual phase), the ascospores are produced on the citrus leaf litter on the ground. These spores are produced in pseudothecia within 40 to 180 days after leaf fall. The production and release of ascospores depend on the alternation of wet and dry periods. Mature ascospores are ejected and dispersed by wind (FOURIE et al., 2013; KOTZÉ, 1981; MCONIE, 1964; SPÓSITO et al., 2007). In the secondary cycle (asexual phase), conidia are produced in pycnidia on fruit, leaves and dead twigs in the canopy and in leaf litter. These spores are washed down for a short distance during rainfall to the adjacent fruit, leaves and twigs (SPÓSITO et al., 2011). Although in South Africa conidia are not regarded as the important source of CBS epidemics, in Brazil conidia may play a significant role (REIS et al., 2006; SPÓSITO et al., 2011).

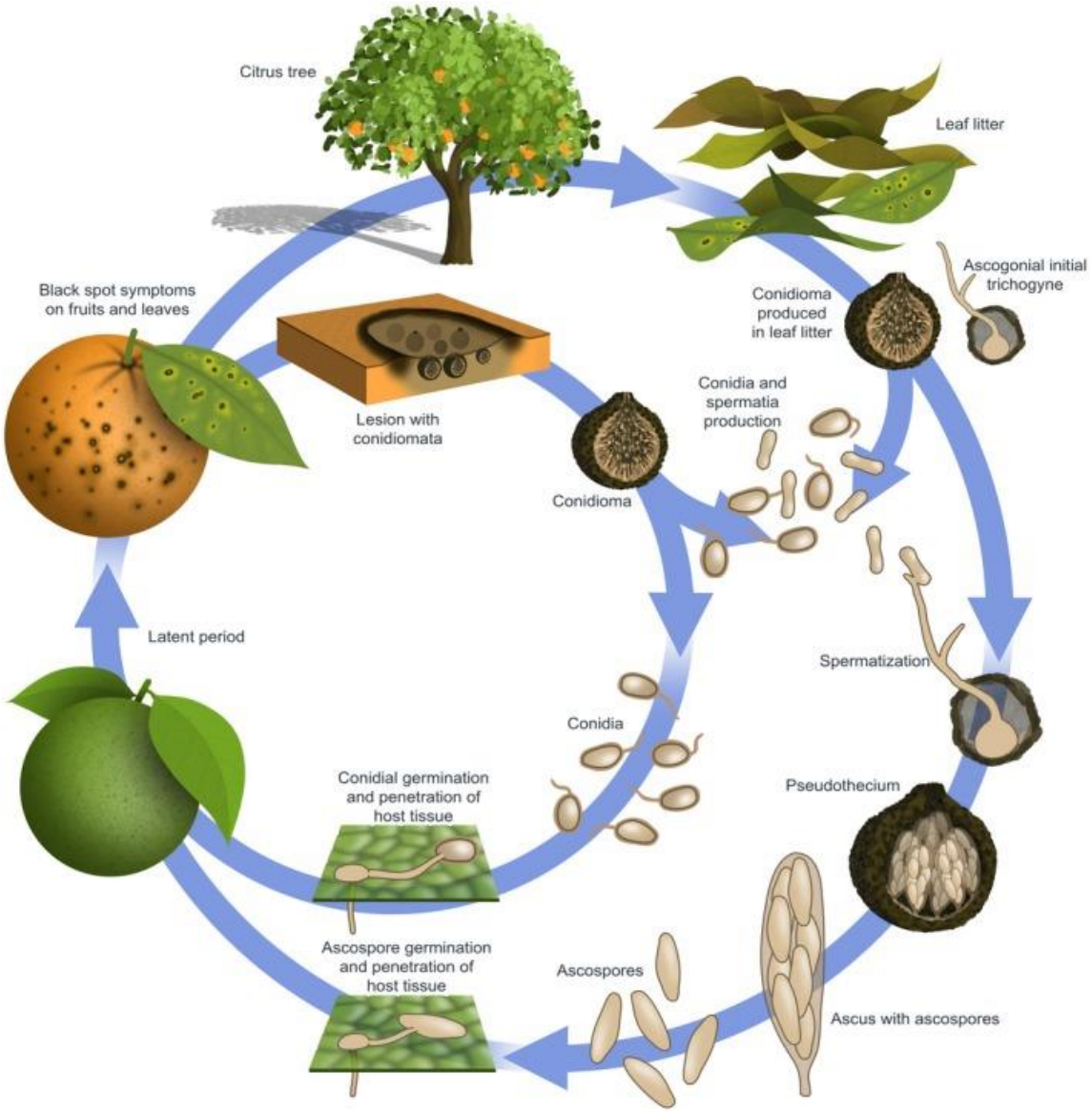


FIGURE 1.2- Cycle of citrus black spot, caused by *Phyllosticta citricarpa* (Source: GUARNACCIA et al., 2019).

1.4- The symptoms

The CBS symptoms are expressed on leaves, twigs and mainly on the fruit of all commercial varieties of sweet oranges, lemons, limes, and mandarins, except on *C. latifolia*. CBS symptoms are cosmetic and restricted to the flavedo, and there are no changes in juice quality (KOTZÉ, 1981).

Six types of symptoms are associated with the disease (FIGURE 1.3): (i) *hard spot*: the most typical CBS symptom on fruits and leaves appears as light brown to gray small lesions surrounded by a dark brown ring when the fruit color changes from green to orange/yellow; (ii) *freckle spot*: small red depressed lesions that appear on ripe fruit and during the postharvest stage. Both hard and freckle spots rarely occur on leaf and twig of orange, mandarin, and other commercial citrus species, but they are frequently present on lemons; (iii) *virulent spot*: characterized by coalescence of hard or freckle spots that may take the whole fruit area, appears as sunken necrotic lesions without defined borders mostly on mature fruit. These three kinds of symptoms may have pycnidia in the center of the lesion; (iv) *false melanose*: consisting of small black spots that are commonly observed on green fruit usually in a tear stain, without pycnidia; (v) *lacy spot*: small black lesions similar to the false melanose that affect the whole fruit surface, and (vi) *cracked spot*: appears in green fruits as irregular cracked spots associated to rust mite (KIELY 1948; KOTZÉ 1981; 2000; SPÓSITO et al., 2011, 2000; SPÓSITO et al., 2011; GUARNACCIA et al., 2019; SILVA JR et al., 2016; FRARE et al., 2018).

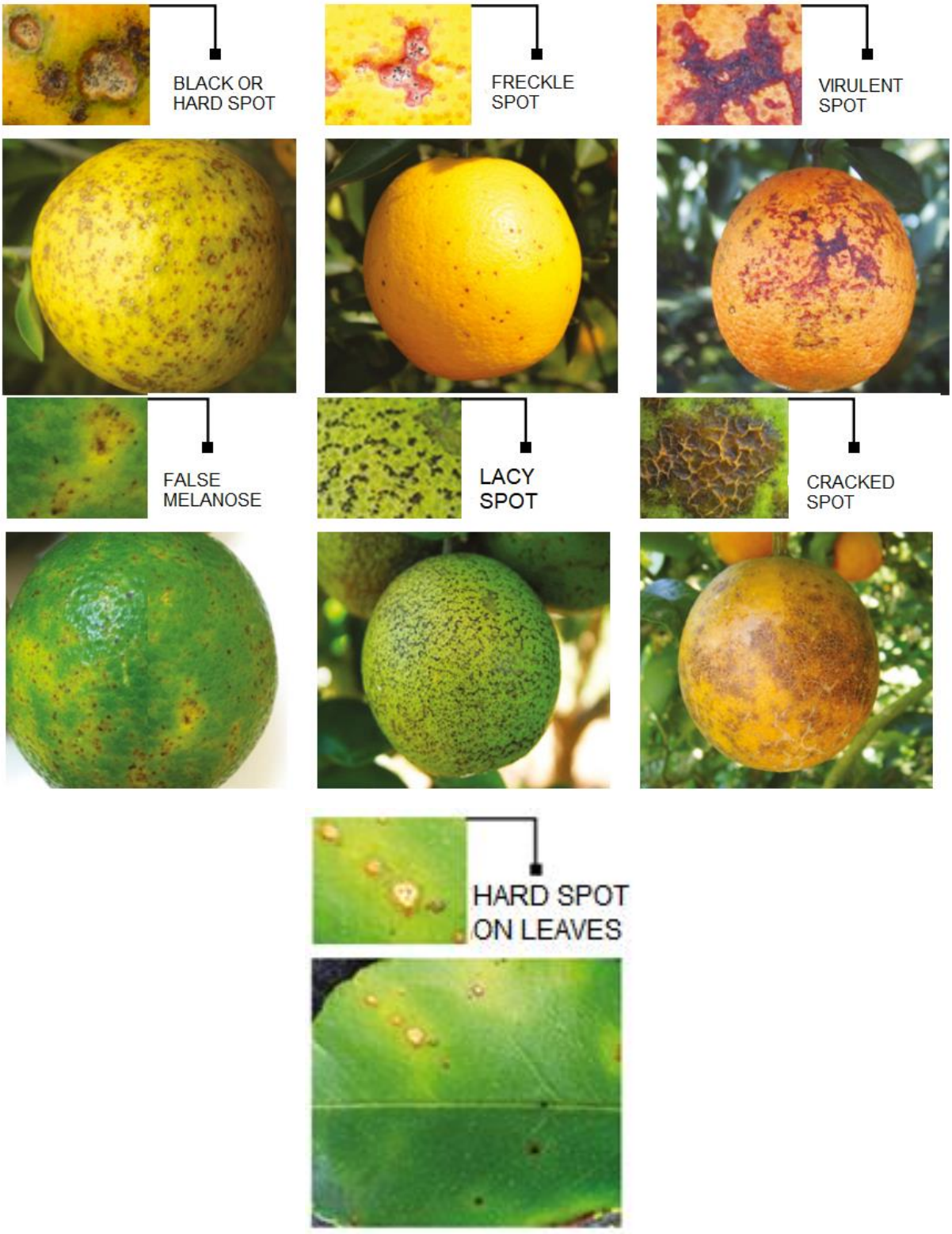


FIGURE 1.3- Symptoms of the black spot expressed on fruits and leaves of citrus plants (Source: FUNDECITRUS).

1.5- Control measures

Different control measures, such as exclusion practices (quarantine and sanitation), cultural, chemical, genetic, and biological methods have been used in an integrated approach to control the disease (SILVA JUNIOR et al., 2016; DEWDNEY et al., 2019; GUARNACCIA et al., 2019). The chemical control by using fungicide sprays has been the main CBS management strategy adopted during the critical period of infection (LANZA et al., 2018).

The challenge for CBS management is increasingly directed towards the use of sustainable substances that prevent disease damages. These pressures have been imposed by the organization of government policies that restrict the use of pesticides (GULLINO et al., 1994; RAGSDALE et al., 1994). In this way, alternative methods are also continuously investigated in order to improve the CBS management (PUNJA et al., 2003).

1.5.1- Exclusion practices

The main exclusion practice for CBS management is the planting of disease-free young citrus trees. Propagation material containing *P. citricarpa* propagules is consider an important via to introduce the pathogen in new areas. Phytosanitary legislations have been imposed by some countries in order to avoid the introduction of *P. citricarpa* in CBS-free areas (KOTZÉ 1981; EFSA 2014; GUARNACCIA et al., 2019). The removal of all sources of inoculum from the orchard as well as the restriction of machine movement containing infected leaves may be used as strategy to reduce spread of the pathogen among citrus areas (SILVA JUNIOR., 2016). As ascospores are formed only on leaf and spread by wind, citrus fruits is not considered an important source of inoculum for introduction of *P. citricapa* in CBS-free areas (SPÓSITO et al., 2011).

1.5.2- Cultural control

Cultural control measures are important for CBS management. The main cultural measures adopted are: removal or cover of leaf litter from the orchard ground by using specific machines to reduce ascospore release and dispersion; application of some compounds to increase the decomposition of leaf litter; pruning of dead twigs to eliminate this source of conidia, and; and early fruit harvesting to prevent overlapping fruit sets (KOTZÉ 1981; SCHUTTE and KOTZÉ 1997; BELLOTE et al., 2009, 2013; TRUTTER et al. 2007; SPÓSITO et al., 2011; SILVA JUNIOR et al., 2016; DEWDNEY et al., 2019; GUARNACCIA et al., 2019).

1.5.3- Chemical control

Chemical control by spraying fungicides is the main CBS management strategy. The number of application and the period of control depend on the level of infection in orchards and the destination of the production (fresh fruit or juice processing). The groups of fungicides available for CBS control are strobilurins (quinone outside inhibitors, QoI), benzimidazoles (methyl benzimidazole carbamate, MBC), dithiocarbamates and copper-based (multisite activity). These products may be applied singly or in mixtures with mineral oil as well as multisite associated with systemic fungicides (QoI or MBC) (DEWDNEY et al., 2018; KOTZÉ 2000; SILVA JUNIOR et al., 2016a, b; MILES et al., 2004; KOTZÉ et al., 1981, 2000; SCHUTTE et al., 2003).

Fungicides are sprayed only during the critical period for *P. citricarpa* infection, from petal fall stage (September/October) to the end of the rainy period (January/February) in South Africa and Australia (MILES et al., 2004; KOTZÉ et al., 1981, 2000; SCHUTTE et al., 1997, 2003) or March/April in Brazil (LANZA et al., 2018). Typically, two to five sprays may be required

depending on citrus variety, orchard age, weather conditions, and fruit destination. In São Paulo state, Brazil, the conventional CBS control is performed with two copper sprays after petal fall, using 21- to 28-day interval (September to November), followed by two to four QoI-fungicide applications at 35- to 42-day interval. The spray volumes and fungicide rates are calculated using the tree-row-volume (TRV) methodology that considers the canopy tree volume per hectare. The most effective spray volume is around 75 mL/m^3 of the canopy tree and the rate of copper is 30-50 mg of metallic copper/m^3 of canopy and the rate of QoI-fungicides is 2.8 mg active ingredient/m^3 of canopy (LANZA et al., 2018; SILVA JUNIOR et al., 2016a, b).

This risk for development of *P. citricarpa* isolates resistant to QoI-fungicides is considered low as a specific intron is present after the codon 143 in the cytochrome *b* gene (HINCAPIE et al., 2014; STAMMLER et al., 2013). However, no more than three or four sprays of QoI-fungicides are recommended per season for controlling fungal diseases in citrus orchards (DEWDNEY et al., 2019).

1.5.4- Biological and alternative control

Biological control is based on the suppression of phytopathogens by the action of non-pathogenic microorganisms to the plant. The literature reveals great diversity of microorganisms that may act as natural antagonists to several plant pathogens. These biocontrol agents have been studied as an alternative strategy to reduce the use of pesticides (RODRIGUES, 2006). Biological control by application of fungi and bacteria has grown in the last years as a demand for strategies free of residues.

Some fungi and bacteria have shown effect against *P. citricapa*, including *Trichoderma spp.* and *Bacillus* spp. as well as *P. capitalensis* commonly

found on citrus canopy (ALMEIDA 2009; KUPPER et. al, 2011; PENA et al., 2016; TRAN et al., 2019). However, none of these agents is used as an effective strategy to manage CBS under field conditions. On the other hand, combined application of biological control agents has been shown to be effective against some plant diseases (MATHELEMUSE and KENA, 2017).

Volatile compounds from plants have been tested and presented inhibitory effect against *P. citricarpa* (LOMBARDO et. al, 2016; RODRIGUEZ et al, 2018). However, field trials need to be performed in order to confirm the efficacy of these alternative compounds. Studies have been conducted to obtain transgenic plants with changes in volatile content that may reduce the infection by *P. citricarpa* (RODRIGUEZ et al., 2018).

2- NMR AND LC-MS METABOLOMIC APPROACH TO ESTABLISH A REPRESENTATIVE METABOLIC PROFILE IN CITRUS LEAVES INOCULATED OR NOT WITH *PHYLLOSTICTA CITRICARPA*

Abstract

In this chapter metabolic profile was assessed in leaves of *C. limon* (susceptible) and *C. latifolia* (resistant) inoculated by *P. citricarpa*. In addition, the chemical composition of *C. limon* and *C. latifolia* leaves after *P. citricarpa* inoculation in a dynamic approach, as chemometrics, was examined to clarify the relationships between the citrus hosts and the pathogen. Chemometrics data obtained by ^{1}H NMR and LC-MS demonstrated that coumarins are present at higher concentrations only in *C. latifolia* species after inoculation of the pathogen. Some of these coumarins were isolated and identified as 5,7-dimethoxycoumarin, 5-geranyloxy-7-methoxycoumarin, 7-geranyloxy-coumarin using chromatographic and spectroscopic systems. The significant increase of these coumarins in the inoculated leaves of resistant specie, suggesting that they play a role in the plant-pathogen interaction, probably as a phytoanticipin. Therefore, this study reported substances related to citrus defense against *P. citricarpa* that have potential to be tested as biofungicides in further investigations.

Key words: Biomarkers, defense mechanism, resistance, and target compounds.

2.1- INTRODUCTION

Plants are the most common source of biopesticides, producing a wide variety of secondary metabolites that are important in interactions between plants and their biotic environment. Some of these compounds may be involved on defense mechanisms of plants, including flavonoids and phenylpropanoids (CÉSPEDES et al., 2014; DEL RÍO et al., 2004). In some cases, essential oils are known and allowed for plant protection, for example: citronella, clove, spear, mint, tea tree and orange oil (ZARUBOVA et al., 2014). Citrus is one of the most studied plants for agriculture and traditional medicine – for example, the dried peels has a long history for controlling many diseases in traditional medicines (LV et al., 2015). Previous studies showed that some polymethoxyflavones, such as sinensetin, nobiletin, tangeretin and heptamethoxyflavone, as well as flavanones (hesperidin and isonaringin) commonly found in citrus revealed significant antifungal activity (DEL RÍO et al., 2004). In addition, the presence of phytoalexins and phytoanticipins in citrus tissue has been also reported and several coumarins and furanocoumarins are described as contributes to increasing resistance against phytopathogens. Several authors have reported that some coumarins, such as scoparone (5,6-dimethoxycoumarin), scopoletin (6-methoxy-7-hydroxycoumarin), xanthyletin (6,7-dimethylpyranocoumarin), umbelliferone (7-hydroxycoumarin) have shown antifungal activity in vitro against different fungus pathogens (RAMÍREZ-PELAYO et. al, 2019; KIM, 1991).

Despite citrus plants being a rich source of functional compounds, the defense mechanisms against pathogens involving these compounds are not yet clarified (MATSUKAWA et al., 2017). An investigation of metabolic citrus profile and their correlation with the pathogen is required to better understand these mechanisms. The metabolomic approach has been widely used as an

analysis in which all the metabolites of an organism (i.e., plant or pathogen) may be identified (BINO et al., 2004).

2.1.1- Metabolomics approach

Metabolomics, a tool introduced in the “omics” field and originated from metabolite profiling, corresponds to the study of a complete set of low-molecular-weight molecules (metabolites), varying according to the physiology, developmental or pathological state of the cell, tissue, organ, or organism. This strategy has been used for the scientific community in biological system studies, for example, in complex mixture analyses. In most cases, the approach consists of the metabolome study generated from different groups (control and test) in order to determine differences in their chemical profiles or metabolic biomarkers in response to external stimuli, such as pathologies, the effect of biochemical or environmental stresses (RODRIGUES, 2014; COURANT et al, 2014; SUMNER et al., 2003).

The first step in metabolomics studies is to determine the number of metabolites to be measured. In some cases, the use of target approach is interesting to examine a defined set of metabolites. Target approach consists of a specific metabolite measure, which is driven by a specific biochemical hypothesis that motivates the investigation towards a particular pathway. In other instances, an untargeted approach may be used, since as many metabolites as possible are simultaneously measured (MATHEW and PADMANABAN, 2013). In addition, untargeted metabolomics may be performed by using either NMR or mass spectrometry (MS) techniques, in which enable the detection of the most metabolites and have been the preferred techniques for global metabolite profiling efforts (WOLFENDER et al., 2013; KIM et al., 2011; MATHEW and PADMANABAN, 2013).

2.1.2- Nuclear Magnetic Resonance (NMR) based metabolomics

Nuclear magnetic resonance (NMR) spectroscopy has been a key technology used successfully in organic chemistry and natural products to identify and quantify products and impurities in complex mixtures. Besides, NMR has been successfully applied in several fingerprinting studies of biological systems as well as used extensively in evaluating and exploring the human metabolome for markers of disease states and the diversity of metabolic pathways in a variety of organisms (ELLINGER et al, 2013; FRÉDÉRICH et al., 2009).

The advantages of NMR spectroscopy in metabolic studies are the large variety of compounds that may be detected, the ability to quantify the concentrations of individual metabolites, speed, and the flexibility to handle many different sample types. On the other hand, the major limitations of NMR spectroscopy are its low sensitivity and spectral resolution. However, the increase of sensitivity may be reached through sample concentration and/or the use of cryogenic probes, while the resolution issue may be solved with two-dimensional NMR approaches by, increasing in field strength, and new experimental techniques (ELLINGER et al, 2013; WISHART, 2008; CUI et al, 2008).

A routine metabolomics study consists of different steps, such as sample preparation, identification, and quantification of metabolites by analytical techniques, statistical data analysis and biological interpretation of the resulting data. It is important to note that the biological interpretation as well as conclusive hypothesis depend on how accurately the metabolites are identified. NMR is considered one of the most selective analytical techniques, which gives unambiguous structural information about molecules. However, due to the complex biological sample matrix, metabolic identification needs application of advanced NMR techniques and analytical strategies for better accuracy (BHARTI and ROY, 2014).

NMR-based metabolomics has a wide range of applications in understanding the variety of biological mechanisms, diagnostics, biomedical research, biology, and medicine. Besides, NMR and fingerprinting profiling are also particularly relevant to investigations of plant-pathogen systems intended to explore the modification of plant metabolism, its response to disease, and to identify biomarkers of disease at its early stages (DEBORDE et. al 2017).

Indeed, NMR and mass spectrometry (MS) are complementary: the advantages of NMR include a high reproducibility and the access to unambiguous structural information, whereas the major advantage of MS is its high sensitivity (KOOY et al. 2008). Furthermore, signals from NMR spectrum are proportional to their molar concentration, making possible the direct comparison of concentrations of all compounds without a calibration curve of each compound. Moreover, NMR is a suitable technique for structure elucidation in which, through 2D NMR measurements, many signals may be identified without extract fractionation (KIM et al., 2010).

The quantitative nature of NMR spectroscopy makes it an ideal analytical method for comprehensive metabolite profiling. In addition, new software and rich NMR metabolite spectral databases are advancing the unique abilities of NMR spectroscopy to identify and quantify small molecules. Nowadays, commercial and non-commercial databases contain experimental 1D and 2D spectra and extracted spectral parameters for over a thousand compounds (EGHBALNIA et al., 2017).

2.1.3- Liquid Chromatography- Mass Spectrometry (LC-MS)-based metabolomics

LC-MS is another important technique used in metabolomics studies. This approach is expected to be of particular importance in plant studies, owing to the highly rich biochemistry of plants, which covers many semi-polar

compounds, including key secondary metabolite groups, which may better separate and detected by LC-MS (DE VOS et al., 2007). Besides, MS has become a method of choice to characterize the human metabolome (JING et al., 2019). MS technique is also highly selective and sensitive, providing important spectral information, such as the exact mass of the molecular ion and fragmentation patterns, which contribute to the identification of the metabolites (SCALBERT et al., 2009).

NMR spectroscopy provides a rapid, non-destructive, high-throughput method that requires minimal sample preparation. Moreover, the robustness of the NMR equipment is a key parameter that has not been equaled to date by other types of instruments. However, MS-based methods have been proved to be valuable for such studies, especially thanks to recent technological advances (COURANT et al, 2014).

Indeed, MS offers higher performance in terms of sensitivity than NMR, which is extremely useful for measuring species with low abundance analysis and facilitates elucidation of the chemical structures of interest metabolites (VILLAS-BÔAS et.al, 2004). However, MS-based metabolomics has its limitations and challenges that are constantly present in the continuous exploration of this field. Some of these challenges are highlighted here; (i) the concentration of metabolites in organisms varies widely and some compounds present in low concentrations may have important regulatory effects. The detection of metabolites in ultra-low concentration represents a challenge for MS-based metabolomics; (ii) with the continuous improvement of sample processing methods, chromatographic separation, and MS sensitivity, it may be possible to obtain large amounts of data. Thus, data processing becomes another challenge for metabolomics; (iii) considering the different forms of adduct ions, the large amount of data obtained after aligning the peaks, makes the identification of

metabolites considerably challenging (REN et al., 2018; SCALBERT et. al, 2009).

Despite all the challenges, MS-based metabolomics technology is widely used in plant metabolomics studies, which plays an important role in metabolites profiling due to its high sensitivity and high speed (DE VOS et al., 2007).

2.1.4- Multivariate Data Analysis (MVDA) in Metabolomics

Considering the large number of signals detected in metabolomics analysis, especially in metabolic fingerprinting, chemometrics appears necessary for extracting relevant information from the generated data (DEBORDE et al., 2017).

The chemometric analysis is a popular approach with a history of use in different scientific fields. In NMR, chemically related spectral pattern resolution may be enough for establishing the presence of differences in sample chemistry (BRERETON et al., 2018; BHARTI & ROY, 2014). The chemometrics tools are diverse and multivariate analysis methods, such as principal component analysis (PCA) and partial least squares (PLS), are widely used to compare data sets obtained through the respective spectra and identify groups of similarity or difference between them (FRÉDÉRICH et al., 2009).

Principal component analysis (PCA) is one of the most popular approach in the chemometric data analysis. PCA works by rotating the original coordinate axes of the data set which the first PCA component corresponds to the direction in the multi-dimensional space where the maximum variation is located. The second PCA component corresponds to the direction of the second highest variation with the additional constraint, being also orthogonal to the first component, and so on. This results in a small set of uncorrelated principal

components in which most of the variation of the original data set is kept intact (ELIASSON et. al, 2011).

On the other hand, Partial least squares (PLS), a regression-based method, builds a low-dimensional subspace based on linear combinations of the original X variables. It makes use of additional Y information by adjusting the model to capture the (Y)-related variation into the original X variables (SUGIMOTO et al., 2012). PLS is particularly useful when fewer samples are available than measured variables (metabolites). In metabolomics, PLS-based classification, and PLS-discriminant analysis (PLS-DA) have been widely used to sharpen the partition between groups or observations. In addition, Orthogonal PLS analysis and OPLS are recent extensions of the PLS method and several recent studies applied these techniques to metabolomic data. All these methods help to visualize and organize complex datasets based on their similar properties or affinity. Moreover, the S-plot (loading plot) helps to identify statistically significant and potentially biochemically significant metabolites, based both on contributions to the model and their reliability (BOCCARD et al., 2010; ELIASSON et al., 2011; WIKLUND et al., 2008; VILLA-RUANO et al., 2019).

2.1.5- High-Performance Thin-Layer Chromatography (HPTLC)

In recent years, most metabolomics studies were performed using the most popular analytical technologies, such as NMR, LC-MS, HPLC and GC-MS (JAMESON et al., 2011). The new developments reached, employing high chromatographic resolution/separation interfaced with high resolution mass spectrometry and sensibility, showed the power of this coupled technique in metabolomics. Nevertheless, High-Performance Thin-Layer Chromatography (HPTLC) is gaining more attraction metabolomics and it has been an alternative method for analysis of complex mixtures. Besides, HPTLC has been

acknowledged as a potent and suited analytical platform because it shows high accuracy, adaptability, flexibility, and reproducibility and it is also able to generate a quick fingerprint of diverse samples in a unique analysis (MALDINI, et al., 2016; 2019).

HPTLC is a tool in both qualitative and quantitative analyses of complex mixtures, fractions, and pure compounds and it has been considered a new opportunity for the application in natural product research. The studies involving HPTLC aims identification, isolation, and quantification of compounds, besides quality control and biological activity. Based on these uses, target analysis, fingerprint, and metabolomics based on HPTLC are classified as approaches applied to natural product research (WORSFOLD, et al., 2019).

In addition, HPTLC is expected to be a good candidate as a supplementary profiling tool. It has a wide detection range and preparative capability that allows the separation of compounds for further isolation and it may provide important benefits for a metabolomics study (SALOMÉ-ABARCA, et al., 2018).

2.1.6- Model of plant- pathogen systems employed in the project

The metabolomics approach is particularly relevant for investigation of plant-pathogen systems related to the modification of plant metabolism, plant response to disease, and biomarkers of disease response (DEBORDE et al., 2017), and it may provide means to the development of biopesticides. The success of plant metabolomics depends on the coverage of metabolites; however, metabolite profiling of crude extracts represents a challenging analytical task since these mixtures are composed of hundreds of compounds. In this context the use of different protocols and analytical techniques are necessary to obtain

comprehensive information about the large range of metabolites present in a crude extract (GAUDÊNCIO and PEREIRA, 2015).

With the purpose to analyze qualitatively all metabolites present in leaves of two citrus species (*C. limon* and *C. latifolia*), inoculated and non-inoculated with *P. citricarpa*, to have a general view of citrus plants metabolome and to identify the compounds that is supposed to be involved in citrus defense against this pathogen. Firstly, ^{1}H NMR technique was used, since it is a suitable method to carry out such analysis and allows the simultaneous detection of diverse groups of secondary metabolites, such as flavonoids, alkaloids, terpenoids and coumarins, in addition to abundant primary metabolites, like sugars, organic acids and amino acids. Despite the challenges, MS-based metabolomics technology plays an important role in metabolites profiling due to its high sensitivity and high speed. For that reason, LC-MS was selected as a second platform to a supplementary profiling tool, due to the complexity of citrus plant metabolome. Furthermore, HPTLC was employed in this study as a tool for the chemical fingerprinting targeting on phenolics and as preparative work to separation and isolation of targeted metabolites related with possible plant defense. The structure elucidation of target compounds was conducted by chromatographic and spectroscopic systems.

2.2- EXPERIMENTAL SECTION

2.2.1- Plant material and collection

The experiment was performed at Fundecitrus, Araraquara-SP, Brazil, under controlled conditions and the chemical analyses of dry extracts were conducted at Leiden University, the Netherlands. Young trees of *C. limon* and *C. latifolia*, both of six months old, were obtained from a commercial nursery. The leaves of young citrus tree were inoculated with *P. citricarpa* at the concentration of 10^5 conidia/mL. Non-inoculated leaves were used as controls. The inoculated and non-inoculated leaves were collected at the inoculation time (0) and 1, 15 and 60 days after inoculation (FIGURE 2.1). The sampled leaves were immediately frozen in liquid nitrogen and ground after the collected, and then dried using freeze-drying. A young tree for sample was used as replication. Five replicates were used per treatment. A total of 80 young trees were used (Table 2.1).

FIGURE 2.1- Young trees of *C. latifolia* and *Citrus limon* and the colony of pathogen *Phyllosticta citricarpa* used in the experiment performed to assess the leaf metabolic profile after inoculation with the pathogen spore suspension.

TABLE 2.1- Experimental design for inoculation experiments

SAMPLES	PLANT AGE	COLLECTION TIME (DAYS)	REPLICATE
C. limon (non-inoculated)	6 months old	0	5
C. limon (inoculated)	6 months old	1	5
C. latifolia (non-inoculated)	6 months old	15	5
C. latifolia (inoculated)	6 months old	60	5
			Total = 80 young trees

Leaves at the same age were collected from young citrus trees and rapid cooled in order to avoid chemical or enzymatic reactions of metabolites as well as to prevent or to reduce the degradation of compounds (KIM et al, 2010). Plant tissue was transferred immediately after removal from the original plant into a container filled with liquid nitrogen. All leaves were always collected at the same time as some primary metabolites vary throughout the day. The sample preparation step was included the following procedures: collecting, milling, drying, weighing, and extracting, as is shown in FIGURE 2.2.

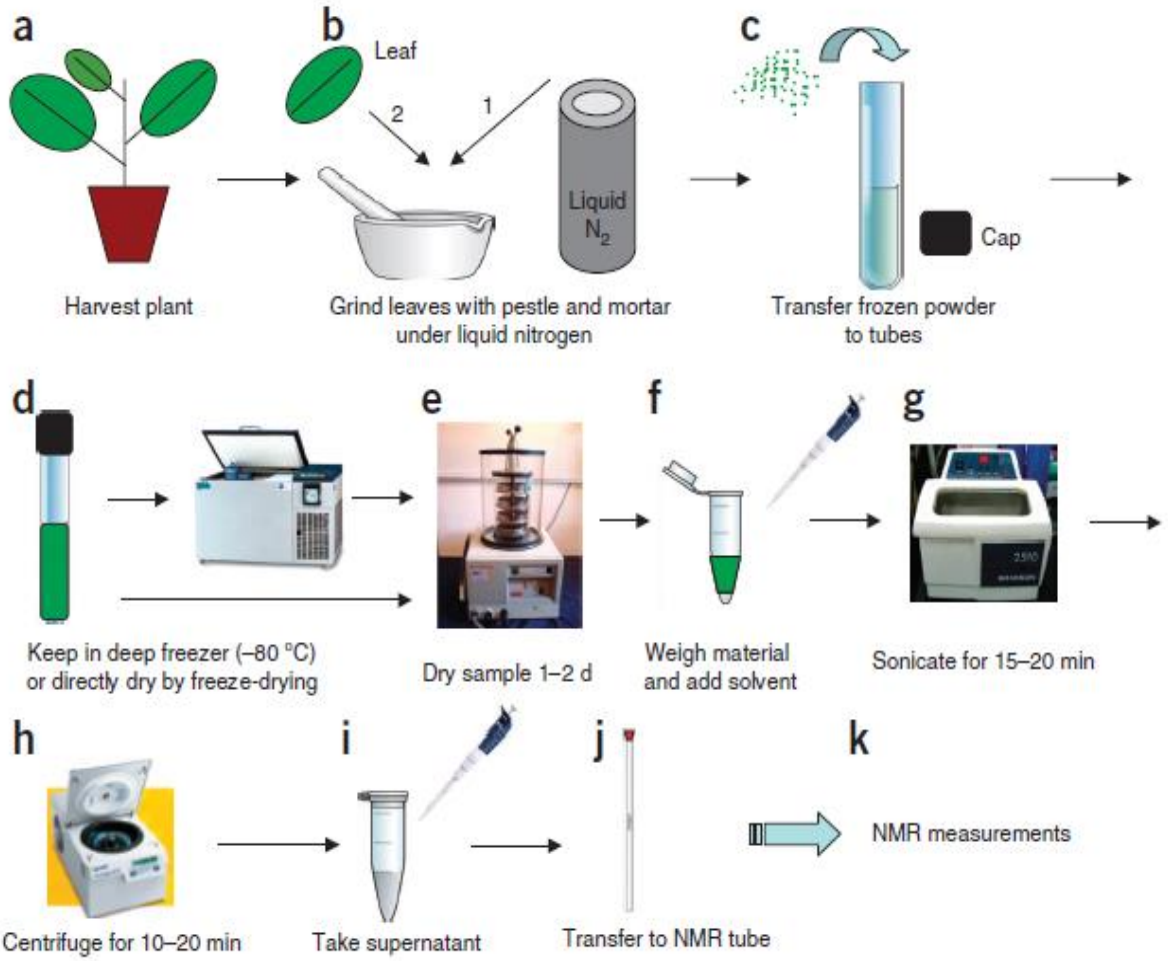


FIGURE 2.2- Overview of the experimental procedures for sample preparation. (**a**) Harvest plant. (**b**) Pre-cool pestle and mortar by adding liquid nitrogen first (1) and place the material in the pestle (2). Grind the materials under liquid nitrogen. (**c**) Transfer the frozen powder to plastic tubes. (**d**) Keep frozen samples at − 80 °C or directly dry using freeze-dryer. (**e**) Dry material. (**f**) Weigh dry samples (30 mg) and add extraction solvent (CH_3OH-d_4 + HMDSO). (**g**) Extract using ultrasonicator for 15 min. (**h**) Centrifuge at 13,000*rpm* at room temperature for 15 min to obtain clear supernatant. (**i**) Collect the supernatant. (**j**) Transfer 300 µL of supernatant to an NMR tube. (**k**) Analyze by NMR (Source: KIM et al, 2010).

2.2.3- Sample preparation for ^{1}H NMR analyses

Thirty milligrams of freeze-dried plant material were extracted in 1 mL of CH_3OH-d_4 containing 3.93 mM hexamethyldisiloxane (HMDSO) as an internal standard, followed by 15 min of ultrasonication. The mixtures were centrifuged at 13,000 rpm and 300 µL of the supernatant were transferred into 3 mm NMR tubes for ^{1}H NMR analyses.

The ^{1}H NMR analysis was performed in an AV-600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany), operating at the ^{1}H NMR frequency of 600.13 MHz. CH_3OH-d_4 was used as internal locking and the acquisitions parameters are shown in TABLE 2.2. A pre-saturation sequence was used to suppress the residual water signal, using low power selective irradiation at H_2O frequency during the recycle delay. The resulting spectra were manually phased, and then the baseline was corrected and calibrated to the hexamethyldisiloxane (HMDSO) signal at 0.06 ppm using TOPSPIN V. 3.0 (Bruker) program.

TABLE 2.2- Acquisition and processing parameters of ^{1}H NMR spectra used for metabolomic study of citrus plants.

PARAMETERS	**^{1}H NMR**
Pulse program (PULPROG)	zgpr30
Acquisition time (AQ)	2.72 sec
Recycle Delay (D1)	1.5 sec
Number of scans (NS)	64
Pulse width (P1)	7.5 μsec (90º)
Mixing time	0.010 sec
Receiver gain (RG)	287
Spectral width (SW)	20 ppm
Size of fid TD (F1)	65.53
Size of real spectrum (SI)	65.53
Exponential line broadening	0.3 Hz
Temperature	298 K

2.2.4- Sample preparation for LC-MS analyses

2.2.4.1- Metabolomics study

Thirty milligrams of freeze-dried plant material were extracted in 1 mL of CH_3OH, followed by 20 min of ultrasonication to analyze LC-MS. The

mixtures were centrifuged at 13,000 rpm and 100 µL of the supernatants were transferred into vial containing 900 µL CH_3OH for LC-MS analyses. An aliquot of 50 µL of each sample was mixed in CH_3OH to prepare the QC (quality control), and 1 mL was transferred to the vial for analysis.

The mass spectrometer parameters were set as follows: Nebulizer gas 2.0 bar, drying gas 10.0 mL min^{-1}, gas temperature 250°C, capillary voltage 3500 V, the mass spectrometer was operated in positive mode and data were collected between 100 and 1650 m/z. A UHPLC-DAD-MS was performed using Thermo Ultimate 3000 and a Bruker OTOF-Q II spectrometer with electrospray ionization (ESI). UHPLC separation was done on a Phenomenex, Kinetex, C18 column (2.1 × 150 mm, 2.6 µm) using a gradient mode with the solvent system containing (A) ultrapure water and (B) acetonitrile with 0.1 % formic acid, which is presented in TABLE 2.3. The column temperature was maintained at 35 ºC. The flow was of 0.3 mL min^{-1} and the injection volume was 1 µL. Analysis was performed randomly, and QC samples were injected between every ten runs of samples.

TABLE 2.3- Gradient system for UHPLC analyses

Time	% A	% B
0.0	95.0	5.0
1.0	95.0	5.0
25.0	2.0	98.0
32.0	2.0	98.0
34.0	95.0	5.0
35.0	95.0	5.0

2.2.4.2- Characterization of compounds

Phenolics compounds characterization, target analysis was performed by using liquid chromatography-QTOF in auto MS/MS mode using MASSHUNTER WORKSTATION software (version B.08, Agilent Technology).

Chromatography separation of the compounds was performed by using an SB ODS C-18 analytical column (3.0 x 50 mm id. 1.8 μm particle). The injection volume was 4 μL, and the mobile phase was composed of 0.1% of formic acid in both deionized water (phase A) and acetonitrile (phase B) at a constant flow rate 0.35 ml/min. The gradient used is presented in TABLE 2.4. After analysis, the column was equilibrated to the initial conditions within 5 min. The electrospray ionization source operated in both positive and negative ionization modes under the following conditions: nebulizer gas at 60 psi, drying gas flow rate at 13 ml/min and temperature 300ºC. The capillary voltage was set at 3000V. The data were acquired in centroid mode. The full scan was carried out at three spectra within the m/z range of 100-1700. MS/MS spectra was used as collision energy of 10, 15, 20, 25, 30 and 35 eV.

TABLE 2.4: Gradient system for HPLC-QTOF analyses

Time	% A	% B
0.0	95.0	5.0
1.0	95.0	5.0
25.0	0.0	100.0
30.0	0.0	100.0

2.2.5- High-Performance Thin-Layer Chromatography analysis

Thirty milligrams of freeze-dried plant material were extracted in 1 mL of CH_3OH, followed by 20 min of ultrasonication. The mixtures were centrifuged at 13,000 rpm and 100 µL of the supernatant were transferred into vial containing 900 µL CH_3OH for HPTLC analysis. Silica gel HPTLC plates (20 ×10 cm, F254) were purchased from Merck (Darmstadt, Germany). A CAMAG HPTLC system equipped with an automatic TLC sampler (version 4), derivatized (version 1.0 AT), TLC plate heater (version III) and TLC visualizer were used (CAMAG, Muttenz, Switzerland). A volume of 10 µL of each solution was spotted in 6mm bands. On each plate, 2 replicates from control and inoculated samples of *C. limon* and *C. latifolia* (times 1 and 60 days after inoculation), total of 16 samples, were applied at 10mm from the bottom edge and 20mm from the left and right borders of the plate. The distance between the bands was 8.8 mm. The mobile phase for HPTLC analysis was a mixture of EtOAc: formic acid: acetic acid: H_2O (100:1.1:1.1:2.7 v/v/v/v) as polar phase and toluene-EtOAc (8:2, v/v) as non-polar phase. The chamber saturation time was 20 min and the solvent migration distance was 80mm from the application point. Developed HPTLC plates were sprayed with 2 mL of 2-aminoethyl diphenylborinate solution using automatic derivatization and placed on a TLC plate heater at 100 °C for 3 min. Images of the derivatized plates were recorded using a TLC visualizer at 366 nm.

2.2.6- Data processing

2.2.6.1- NMR data

The NMR spectra were bucketed using AMIX 3.9.12 (Bruker BioSpin GmbH, Rheinstetten, Germany). Bucket data was obtained by spectra integration at every 0.04 ppm interval. The peak intensity of individual peaks was

scaled to the total intensity recorded from δ 0.30 to δ 11.50. Due to the residual signals of H_2O and CH_3OH-d_4, regions δ 4.7 – δ 4.9 and δ 3.28 – δ 3.34 were excluded from the analysis, respectively.

Metabolite characterization was assigned in accordance with databases, such as HMDB (Human Metabolome Database), BMRB (Biological Magnetic Resonance Data Bank), SDBS (Spectral Database for Organic Compounds) and a wide comparison with literature (LÓPEZ-GRESA et al., 2009, 2012; OLIVEIRA et al., 2014; FREITAS et al., 2005; BARTH et al., 2011; PÉREZ et al., 2010; SON et al., 2009; VILLA-RUANO et al., 2019). The assignment of some metabolites was also corroborated using a commercial database of ^{1}H-NMR spectra (Chenomx NMR).

Multivariate analysis was done using SIMCA P (version 14.1, Umetrics, Umeå, Sweden). Principal component analysis (PCA) was used to examine intrinsic variations in the dataset. All the raw data were Pareto-scaled. The variables were then subjected to an orthogonal projection to latent structures discriminant analysis (OPLS-DA) to identify differential components among samples. The quality of the model was estimated by R^2X and Q^2 values. R^2X indicated the fitness of the model and was defined as the proportional variance, whereas Q^2 was defined as the predictable variance (WAI et al., 2019; VILLA-RUANO et al., 2019). Furthermore, the models were validated for its robustness applying the permutation test (100 times) implemented in SIMCA, whose values of Q^2 greater than 0.40 are considered satisfactory (XIAOYING et al., 2011). Moreover, the analysis of variance testing of cross-validated predictive residuals (CV-ANOVA) was used to assess the reliability of the supervised models, p-values < 0.05 were considered as significant and $p < 0.01$ as highly significant SALOMÉ-ABARCA et al., 2020).

2.2.6.2- LC-MS data

After analysis of citrus species by UHPLC-DAD-MS methods, the total ion chromatograms of all the samples were extracted. The acquired raw MS files were processed with the DataAnalysis (Bruker), which were automatically calibrated. XCMS software was used for data pre-treatments including peak identification, peak alignment, peak feature extraction, and peak area normalization, running separately under positive ionization mode.

The mass spectrometry matrix data containing sample names, m/z-retention time pairs, and ion intensity information were generated and exported. The background ions were removed with blank samples, and the quantitative results were normalized with QC samples. Finally, the identification and quantitative results from the data were obtained and used for subsequent multivariate analysis (MVDA) and performed using SIMCA P (version 14.1, Umetrics, Umeå, Sweden).

Targeted analysis of these metabolites from *C. latifolia* polar extract was performed, then the phenolics compounds have been tentatively identified without reference standards, based on their MS/MS spectra and the comparison with databases (METLIN MS and MSMS, MassBank MSMS) and literature (LEDESMA-ESCOBAR et al., 2015). Fragmentation patterns were useful for the identification of compounds.

2.2.7- Extraction and isolation of target compounds from *C. latifolia*

The target isolation of compounds from *C. latifolia* was performed by Pure Chromatography instruments (C-850 FlashPrep- Buchi). The plant material (leaves), dried and powdered (26 g) was extracted with 95% methanol at room temperature by ultrasound for 30 minutes, then filtered and dried (2.1 g).

Three steps were development to separation and isolation of compounds: In the first step was used the gradient 5-100 % $CHCl_3$: CH_3OH over 10 min followed by 100% CH_3OH (column: Ecoflex silica 80 g, 40x63 µm, flow 15 mL min^{-1} and 10 min of equilibration). After that, the fractions were analyzed by TLC and then repeated using the second step: gradient 5-100 % hexane: EtOAc over 5 min followed by 100% EtOAc (column: Ecoflex silica 4 g, 40x63 µm, flow 3 mL min^{1} and 2 min of equilibration) and the compound **1** was obtained (1.0 mg). To isolate the other compounds, the same fraction was separated again, using the last step with gradient 0-100 % toluene: EtOAc over 5 min followed by 100% EtOAc (column: Ecoflex silica 4 g, 40x63 µm, flow 3 mL min^{-1} and 2 min of equilibration), and the compound **2** (0.85 mg), compound **3** (0.6 mg) and compound **4** (1.8 mg) were obtained.

Due to the low amount of compounds isolated from leaves, and we did not have enough plant material to perform biological experiments, the compound **1** was obtained commercially and the other two were isolated from peels of citrus fruits commercially. The Tahiti lime fruits were obtained from a supermarket in Leiden in the Netherlands. The peels were removed, dried, and powdered (0.5 kg). The extraction was performed with 95% methanol at room temperature, soon after, the extract was filtered and dried (16.5 g). Due to a large amount of sugar, the methanolic extract was resuspended in H_2O with 10% of methanol and extracted by sonication for 30 minutes. After that, a partition liquid-liquid with ethyl acetate (3x) then butanol (3x) was carried out. The ethyl acetate extract was analyzed by HPTLC and compared with the leaves extract, which presented the same interest spots of target compounds.

Afterwards, the ethyl acetate extract (4.0 g) was performed to separation and isolation using the same methods from leaves, however, with some modifications: In the first step was used the gradient 0-100 % $CHCl_3$: CH_3OH over 10 min followed by 100% CH_3OH (column: Ecoflex silica 80 g, 40x63 µm,

flow 15 mL min^{-1} and 10 min of equilibration). After that, the fractions were analyzed by TLC and the fraction contain coumarins (1.6 g) was repeated isolated using the second step: gradient 5-100 % hexane: EtOAc over 5 min followed by 100% EtOAc (column: Ecoflex silica 12 g, 40x63 µm, flow 10 mL min^{-1} and 4 min of equilibration), and a clean fraction was obtained. The fraction was injected using the last step with gradient 0-100 % toluene: EtOAc over 5 min followed by 100% EtOAc (column: Ecoflex silica 12 g, 40x63 µm, flow 15 mL min^{-1} and 4 min of equilibration), and the compound **2** (68.4 mg) and compound **3** (5.4 mg) were obtained.

The compounds were dissolved in 300 µl of CH_3OH-d_4, transferred into 3 mm NMR tubes for ^1H NMR analysis. The ^1H NMR analysis was performed in an AV-600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany), operating at the ^1H NMR frequency of 600.13 MHz. The NMR spectra were compared with the spectra of compounds obtained from citrus leaves and then confirmed as the same compounds.

2.3- RESULTS AND DISCUSSION

In this study, two citrus species (one susceptible, *Citrus limon*, and one resistant, *Citrus latifolia*) were chosen to investigate the effect of metabolic variations in citrus plants in response to *P. citricapra,* the causal agent of citrus black spot (CBS) disease. The ^1H NMR spectra showed signals from sugars, organic acids and amino acids for both species. Signals from phenolic groups were also observed. Some phenolics compounds were identified as target compounds related with resistant specie, separated, and isolated using chromatography and spectroscopy systems.

C. limon and *C. latifolia* differed in their metabolome. The main differences between them are especially on aromatic (δ 6.0- δ 8.3), organic acids

and amino acids (δ 1.5- δ 4.3) regions, in which *C. latifolia* presents the largest number of signals on those regions (FIGURE 2.3).

The metabolic change was monitored during 60 days after the inoculation of the pathogen on leaves, as CBS presents a long period to symptom expression, depending on the type of symptom, inoculation concentration, phenotype stage and environmental conditions (SILVA JUNIOR et al., 2016).

The ^{1}H-NMR spectra obtained at 600 MHz exhibited three main regions. The first region was the high-field region (δ 0.6- δ 4.3) showing signals from organic acids and amino acids, although fumaric and formic acids were observed at δ 6.65 and δ 8.46. The organic acids identified in this high-field region were citric acid, succinic, tartaric, acetic, α- linolenic acid and malonic acid. Citric acid appeared as an AB system at δ 2.65 and δ 2.68 and was the most abundant organic acid in this region. The amino acid signals that appeared in the range at δ 0.89 to δ 1.74 revealed the presence of alanine, valine, leucine, and isoleucine, whereas proline was observed as a multiplet at δ 2.00- δ 2.15 and δ 4.01. Choline was observed as a singlet at δ 3.20.

In the second region (δ 4.53- δ 5.6), characteristic of sugars, signals associated with β-glucose (δ 5.54), α-glucose (δ 5.10), sucrose (δ 5.38) and fructose (δ 4.36) were identified. In the third region (δ 6.0- δ 11.10), characteristic flavonoids signals were observed within the δ 6.7- δ 7.90 range, whereas coumarins signals were observed as a doublet at δ 6.23, δ 6.29 and δ 8.07. The high degree of signal overlap in the aromatic region made it difficult to identify phenolic compounds due to the complexity of the chemical structures. Simple signals of methoxy groups were detected in the range δ 3.87- δ 3.93. Other compounds, such as HMF (δ 9.51) and trigonelline (δ 8.02 and δ 9.18) were also characterized. The metabolites identified and their chemical shifts are reported in TABLE 2.5.

Citrus latifolia *Citrus limon*

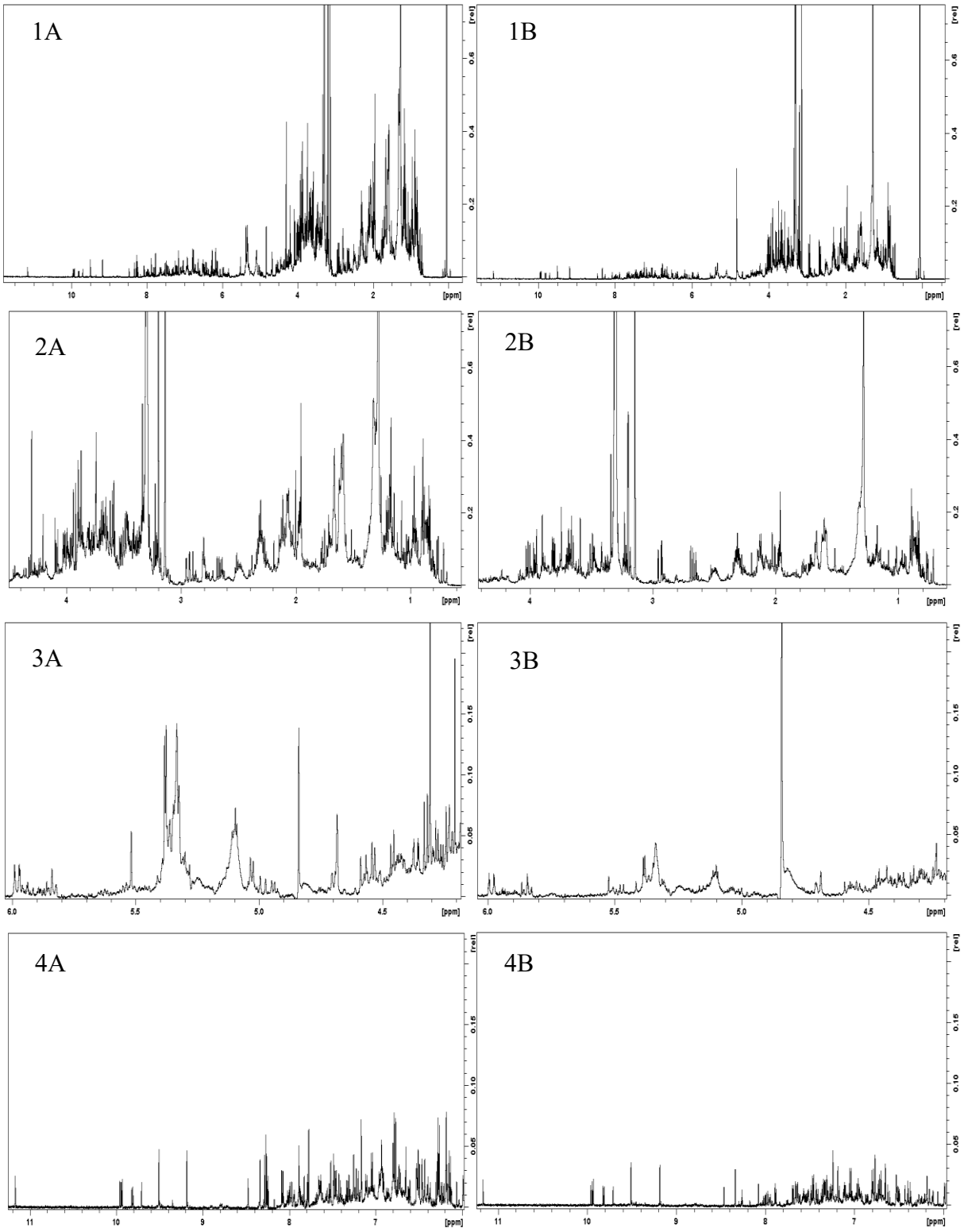


FIGURE 2.3- Typical ^{1}H NMR (600 MHz in CH_3OH-d_4) spectra of A: *Citrus latifolia* (1A) and B: *Citrus limon* (1B) species. Ranges δ 0.1- δ 4.3 (2A, 2B), δ 4.3- δ 6.0 (3A, 3B), δ 6.0- δ 11.1 (4A, 4B).

TABLE 2.5- ^{1}H NMR Chemical shift assignments of the compounds identified in extracts from leaves citrus species.

Nº	**Compound**	**Assignment**	**δ ^{1}H**	**Multiplicity [J(Hz)]**	***Species*** [a]	
					C. latifolia	***C. lemon***
			Carbohydrates			
1	β-glucose	1-CH	4.54	d (7,90)	D	D
2	α-glucose	1-CH	5.10	d (3.87)	D	D
3	Sucrose	1-CH	5.38	d (3.74)	D	D
		10-CH	3.41	m		
4	Fructose	1-CH	4.36	dd (1.9, 12.1)	D	D
			Organic Acids			
5	Citric acid	1-CH_2	2.65	d (9.46)	D	D
		2-CH_2	2.68	d (9.46)		
6	Tartaric acid	H-2, H-3	4.30	s	D	ND
7	Fumaric acid	H-2, H-3	6.65	s	D	D
8	Succinic acid	H-2, H-3	2.52	s	D	D
9	Formic acid	H-1	8.46	s	D	D
10	Acetic acid	H-1	1.96	s	D	D
11	Malonic acid	α-CH_2	3.14	s	D	D
12	α- Linolenic acid	α-CH_2	0.95	t (7.2)	D	D
13	γ-aminobutyric acid (gaba)	α-CH_2	2.31	m	D	D
		γ-CH3	2.95	t (7.24)	D	D
			Amino Acids			
14	Alanine	β-CH_3	1.45	d (7.27)	D	D
15	Valine	α-CH	3.68	d (4.35)	D	D
		β-CH	2.31	m		
		γ-CH_3	0.94	d (7.01)		
		γ`-CH_3	1.15	d (7.01)		
16	Leucine	α-CH_2	2.94	dd (16.7, 3.96)	D	D
		β-CH	1.74	m		
		γ-CH_3	1.74	m		
17	Isoleucine	α-CH_2	0.89	t (7.13)	D	D
		γ-CH_3	3.21	d (3.95)		

(Continued)

TABLE 2.5. *(Continued)*

Nº	**Compound**	**Assignment**	**δ ^{1}H**	**Multiplicity [J(Hz)]**	***Species*** [a]	
					C. latifolia	***C. lemon***
18	Threonine	γ-CH_3	1.31	d (6.6)	D	D
		β-CH	4.11	m		
19	Tyrosine	H-2	7.19	m	D	D
		H-3	6.87	m	D	D
20	Proline	α-CH	4.01	m	D	D
		β-CH_2	2.00-2.15	m	D	D
		γ-CH_2	2.25-2.34	m	D	D
		Other Compounds				
21	Choline	CH_3	3.20	s	D	D
22	5-hidroximetilfurfural) (HMF)	H- (CHO)	9.51	s	D	D
		H-6a, H-6b	4.68	s		
23	Trigonelline	H-5	9.18	s	D	D
		H-3	8.02	m		
24	Coumarins	H-3	6.23, 6.29	d (9.6)	D	ND
		H-4	8.07	d (9.6)		

D, detected; ND, non detected; m, multiplet; dd, double doublet; t, triplet; d, doublet; s, singlet.

2.3.1- Multivariate data analysis by ^{1}H NMR

Notwithstanding the goal of this study, to uncover the metabolites responsible for the resistance, the sample set of plants has more factors to influence metabolome than just resistance against the fungus, for example, age and species difference. A plethora of metabolites are affected by the factors in a complex manner and uncovering the individual effect on metabolites is no less hard to interpret, at least by direct visual spectra inspection. Therefore, ^{1}H NMR data set was collectively interpreted by a string of multivariate data analysis (MVDA) expecting to piece together a reasonable metabolic picture from a myriad of signals (FIGURE 2.4).

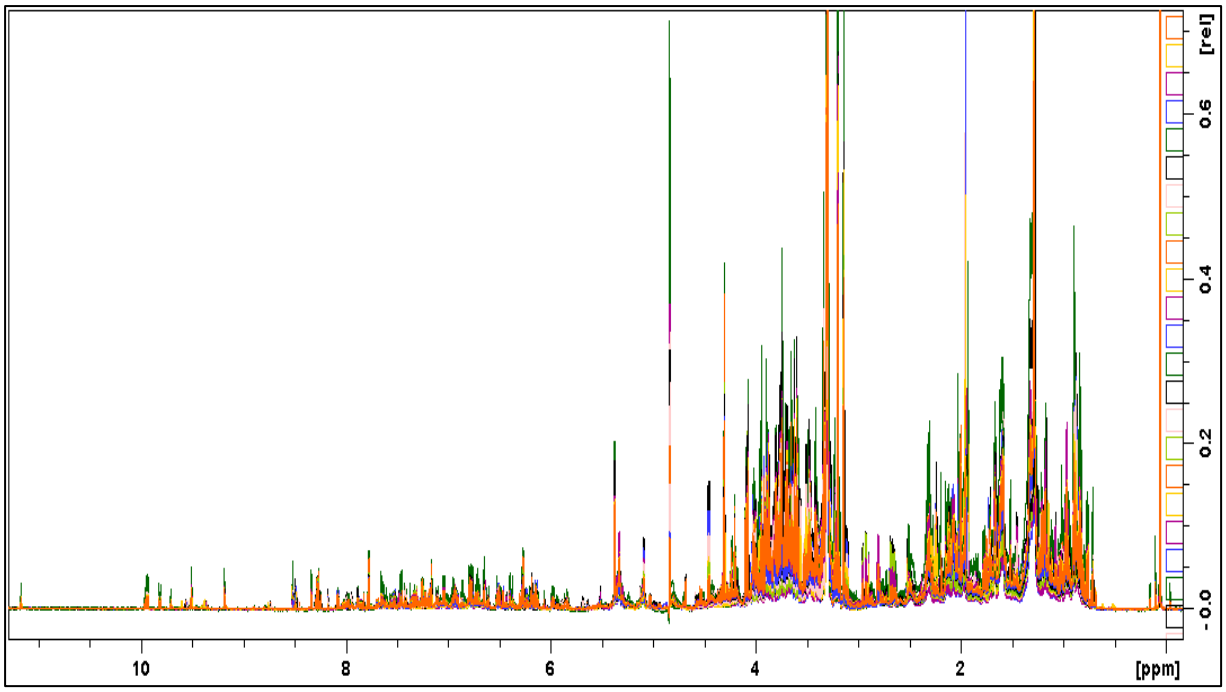


FIGURE 2.4- ^{1}H NMR spectra from *C. limon* and *C. latifolia* control and inoculated leaves.

Principal component analysis (PCA) was carried out for the dataset. With 8 PCs, the explained variation (R^2X) reached 0.96 (FIGURE 2.5). The PCA obtained with the resulting from inoculation degree did not provide an adequate separation between control and inoculated samples. The main separation factor was found to be by species. It would stand to reason that the degree caused by the fungal inoculation could be much smaller than that of the species effect. While PCA is an excellent tool for data reduction and grouping multivariate data, it has limitations, which must be considered. The separation by PCA is obtained only from maximum variations between samples, as an unsupervised method. However, when information on some classification is available, it may be useful to apply a type of discriminant analysis, i.e., a supervised method, in which the groups can be obtained by maximum covariance (e.g., metabolic difference related to classification) (DAO, 2010). Therefore, to overcome and supplement the PCA analysis, a present-day popular supervised MVDA, orthogonal partial least square – discriminant analysis (OPLS-DA) that has a crucial edge over PCA

in terms of the fortification of an interesting factor was applied to the same data set. The difference between *C. limon* and *C. latifolia* species was clearly seen in FIGURE 2.6 A (R^2X= 0.70, Q^2= 0.98 and p-value < 0.01). Besides, the S-plot (FIGURE 2.6 B) showed that main NMR signals related to the variation between species were correlated to carbohydrates (δ 4.54 – δ 5.38, α and β -glucose), organic acids (δ 2.52, succinic acid; δ 3.14, malonic acid; δ 2.65- δ 2.68, citric acid), amino acids (δ 1.31, threonine; δ 1.74- δ 3.68, leucine; δ 0.89, δ 3.21 isoleucine; δ 3.68, valine and δ 4.01, proline), some phenolics (δ 6.07- δ 7.98) and unknown compounds at δ 8.06- δ 8.30.

In order to understand the mechanism of infection and the compounds responsible for biochemical changes and identify the possible biomarker, as a next step, the ^{1}H NMR data set was analyzed by OPLS-DA with inoculation and control factors, in which each species was analyzed separated. To the analysis of inoculation and control samples from susceptible species, the OPLS-DA model applied to the sample set, was not validated, the model presented R^2 X value = 0.30, Q^2= 0.21 and p value > 0.05. However, to resistant specie, the same model using Pareto scaling showed a clear separation between control and inoculated samples and it was validated (R^2 X= 0.53, Q^2= 0.42 and p value < 0.01). The difference was clearly seen after 15 days of inoculation (FIGURE 2.7 A).

S-plot (FIGURE 2.7 B) from the factors of inoculation indicated the characteristic metabolites that were correlated of separation of the control and inoculated samples from resistant specie. Some signals were correlated to δ 9.51 (5-hidroximetilfurfural, HMF), δ 3.2 (choline), carbohydrates (δ 3.41 – δ 5.38, sucrose, fructose and δ 4.54 – δ 5.38, α and β -glucose) amino acids (δ 1.74- δ 3.68, leucine, valine and δ 4.01, proline), organic acids (δ 2.52, succinic acid; δ 6.65, fumaric acid), several phenolics signals (δ 6.06-7.94) that were difficult to

identify them, due to a large degree of signal overlap, besides unknown compounds at δ 8.02- δ 8.34.

In addition to the species and inoculated factors, the model plants had another effect, such time for the developmental stage. The plants present different stages of development, since the first stage of vegetation, growth of branches, the maturity of leaves, flowering, and fruiting, which also effects on the metabolome. During the experimental period, the tested citrus plants gradually grew, in this case, it should have a conspicuous effect on the metabolome.

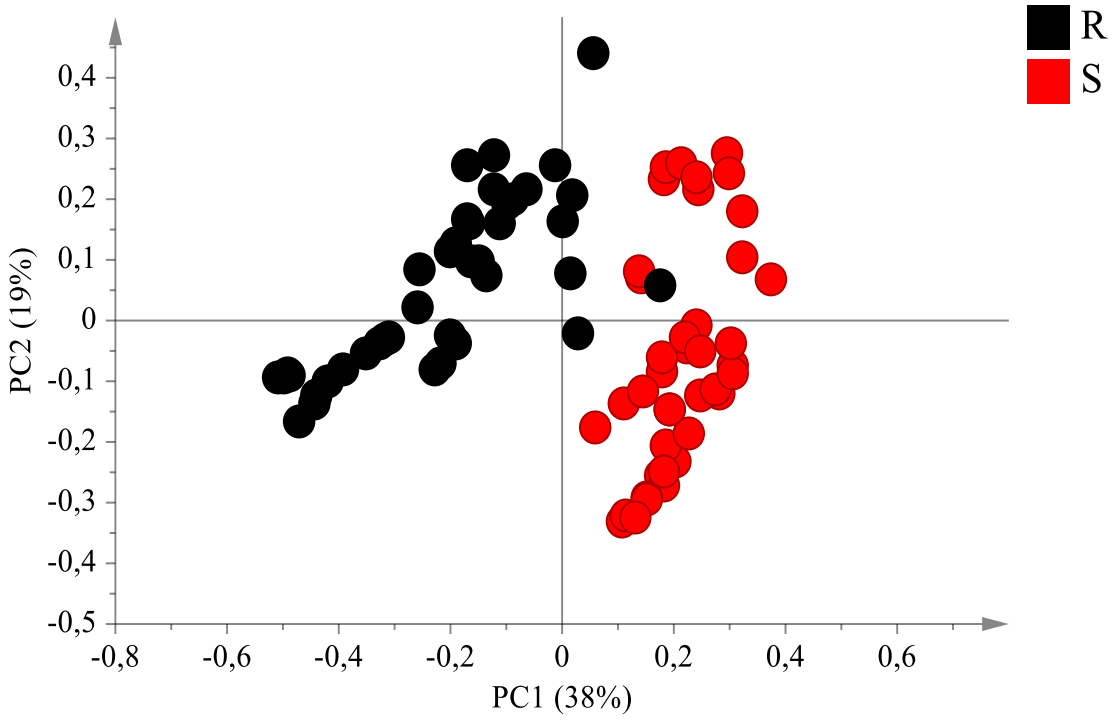


FIGURE 2.5- PCA score of *C. limon* (S) x *C. latifolia* (R).

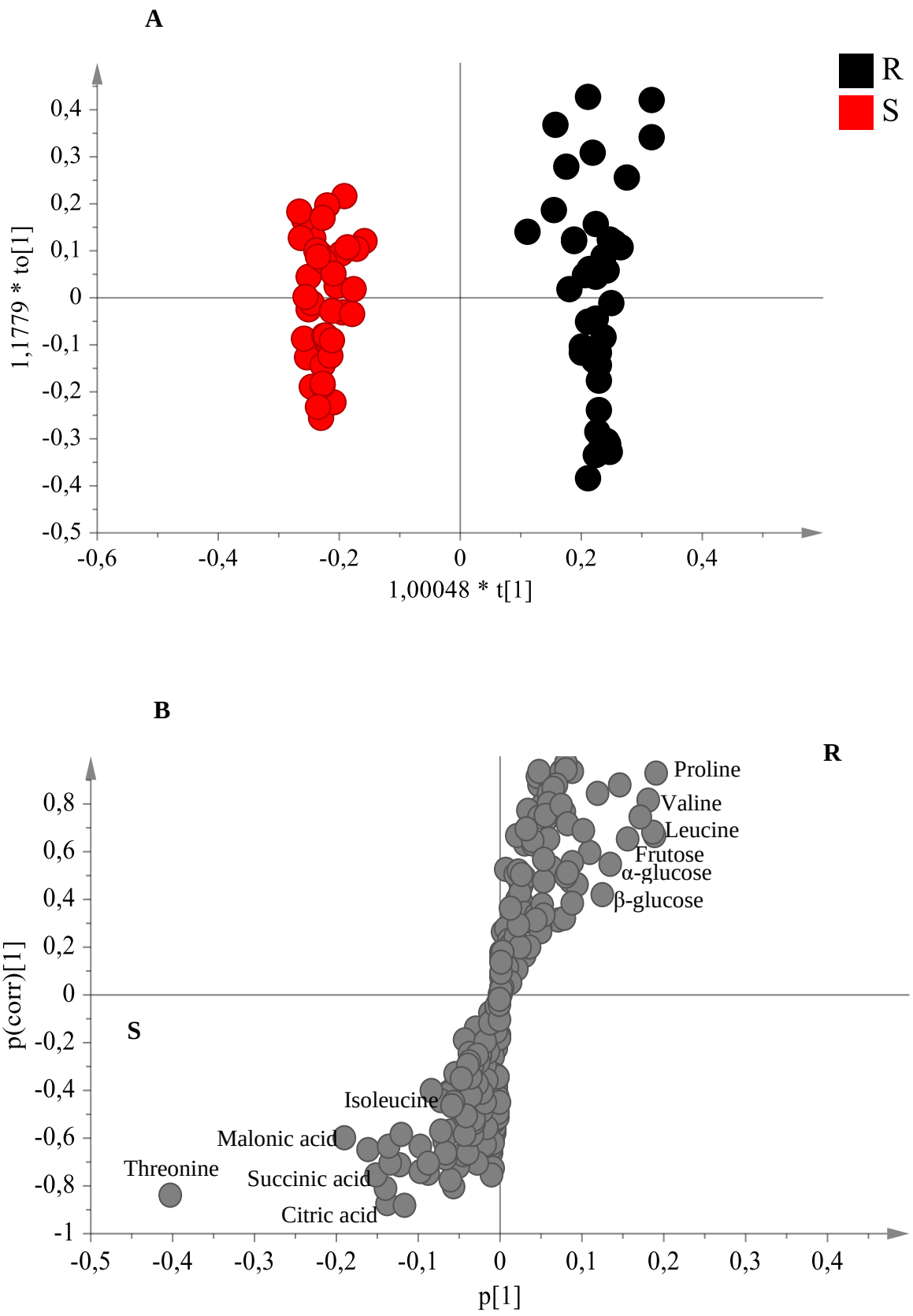


FIGURE 2.6- A: OPLS-DA and B: S-plot of *C. limon* (S) x *C. latifolia* (R).

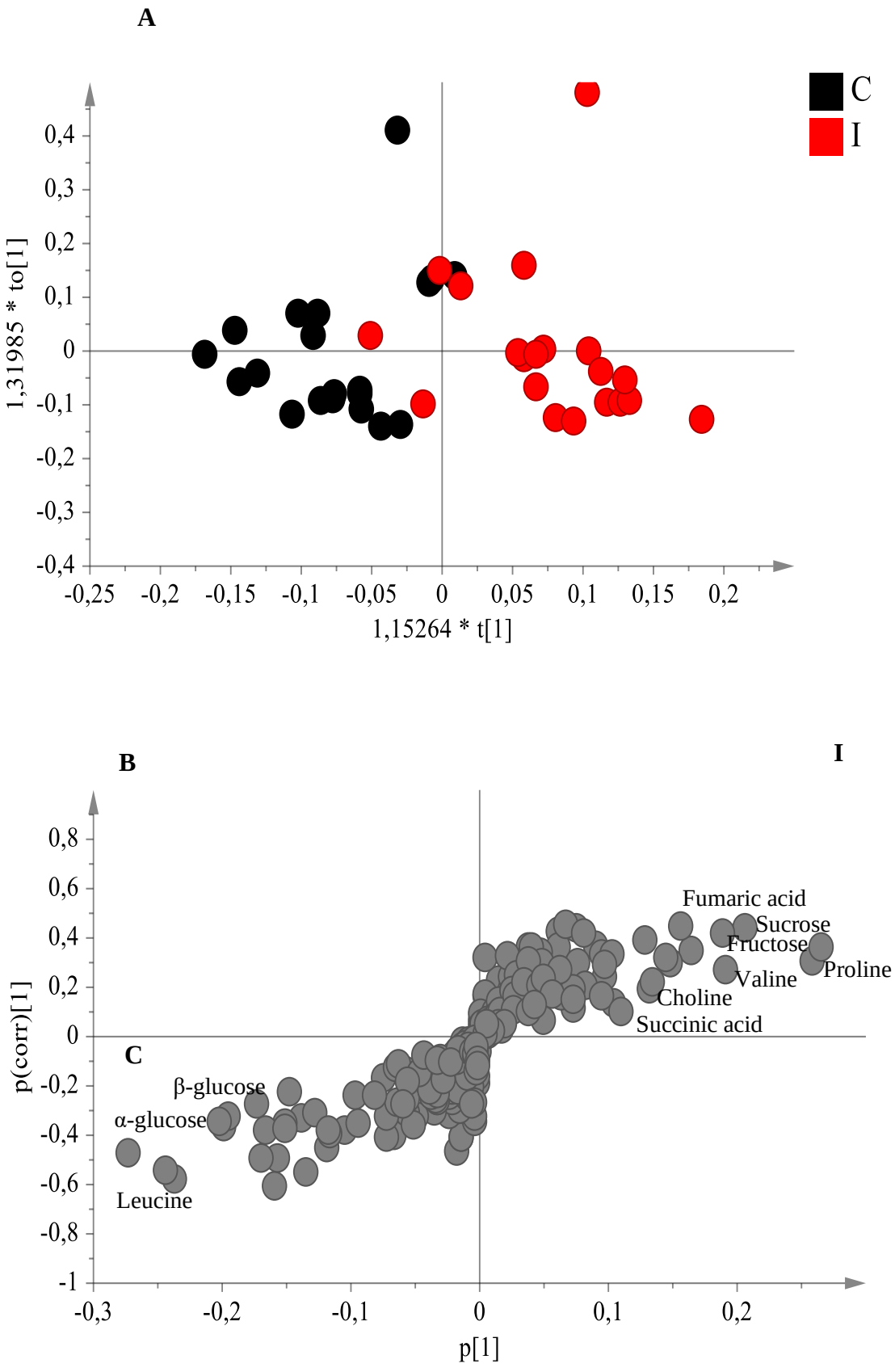


FIGURE 2.7- A: OPLS-DA model of *C. latifolia* (control and inoculated leaves) and B: S-plot.

For many years, it has been suggested that primary metabolism play an important role during plant-pathogen interactions, for example, the support cellular energy requirements for plant defense responses (ROJAS et al., 2014). It is known that some primary metabolites are correlated with biotic and abiotic stress condition in different plant species, therefore, metabolomic analysis of sugars, amino acids and organic acids are an important tool to correlate the metabolic changes with plant responses (KHAN et. al, 2020). On the ^{1}H NMR metabolomic profiling, supported by MVDA, sugars, amino acids and organic acids were correlated with the difference between susceptible and resistant species, besides, some of these compounds were also correlated with the difference between control and inoculated sample from resistant species.

Sugars are considered the major contributing factor in osmotic adjustment in most plant species, which the accumulation of soluble sugars has a profound effect on osmo-protection and scavenging of free radicles (KHAN et. al, 2018). Sugars are especially important in various metabolic events, work as a signal to regulate different gene expression that is involved in photosynthesis and sucrose metabolism. Sugars are also involved in many biological processes and structural constituents of the cell and act as a metabolic resource, may assist in stress and act directly as negative signals or modify the cell reactive pathways to induce the stress response signals and increase plant resistance to stress (ROSA et al., 2009; AHMAD et al., 2016).

On the other hand, amino acids are imitated as precursors of proteins and other organic compounds like nucleic acids that show an active role in plant reactions to many stresses. Proline, for example, is an important amino acid that plays crucial roles in stress tolerance of the plant, it is commonly found in the Rutaceae species and is traditionally considered nontoxic compatible osmolytes and are generally associated with the osmotic adjustment and protection of subcellular structures during abiotic and biotic stresses (FREITAS et. al, 2015).

Proline accumulation occurs in a large range of plant species in response to various kinds of environmental stresses and, a wide range of evidence suggests that a positive correlation occurs between proline accumulation and plant stress tolerance (DAR et. al, 2016).

Organic acids are also reported as responsible to increase the plant response to stress. The drought tolerance, for example, in plant species is attended through a change in the content of different organic compounds because of environmental stress conditions. Several organic acids are reported as important constituents that help in energy production, and they may increase or decrease in plants under severe stress conditions (KHAN et. al, 2020; SASSI et al., 2019).

Based on the ^{1}H NMR analysis supported by MVDA, a bunch of ^{1}H NMR resonances were found to be correlated to the resistance, mainly in the region of phenolics. However, due to the high degree of signal congestion was impossible to identify them. Hence, to overcome the limitation of ^{1}H NMR, a few experiments were performed, including LC-MS profiling targeting on phenolics compounds and multivariate analysis. A preparative work was also performed accoutered with high-performance thin-layer chromatography followed for further experiments.

2.3.2- Multivariate data analysis by LC-MS

The PCA model with 7 PCs presented explained variation (R^2 X) reached 0.90 and provide separation only by the species factor. The QC (quality control) used to the normalization of the data set, presented in the middle of the samples (FIGURE 2.8). The separation was confirmed by OPLS-DA (FIGURE 2.9 A). The OPLS-DA model used for two species (*C. limon* and *C. latifolia*) showed an evident separation between species and the model was highly validated (R^2 X = 0.90, Q^2 = 0.95 and p-value < 0.01). S-plot (FIGURE 2.9 B) presented

some ions correlated of separation like $[M+H]^+$ = 207.059, 339.159, 339.08, 217.049, 247.060 characteristic of coumarins and furanocoumarins and $[M+H]^+$ = 609.170, 610.181, 594.148, characteristic of flavonoids. The data obtained by LC-MS were not enough and hindered multivariate analysis according to the inoculation factor, in which it was not provided separation between control and inoculated for both species.

LC-MS is the most popular analytical platform for metabolomics together with ^{1}H NMR. Without highlighting its incomparable sensitivity and resolution, the easiness of chemical identification with the help of a conventional database is the attractive point to use it as a metabolomics tool, especially when applied to plant metabolites (SCALBERT et al., 2009). However, despite all the advantages, MS-based metabolomics has its limitations and challenges, therefore, as a result, the data obtained by LC-MS were used to identify the metabolites corroborating with the NMR analyses. Through new analysis from MS/MS, some of phenolics compound from resistant species were achieved by comparison between experimental MS/MS spectra and information from databases. The phenolics compounds identified by LC-MS/MS are shown in TABLE 2.6 and their MS/MS spectra are located in appendix A-1 (pages 118- 127) in the end of this thesis.

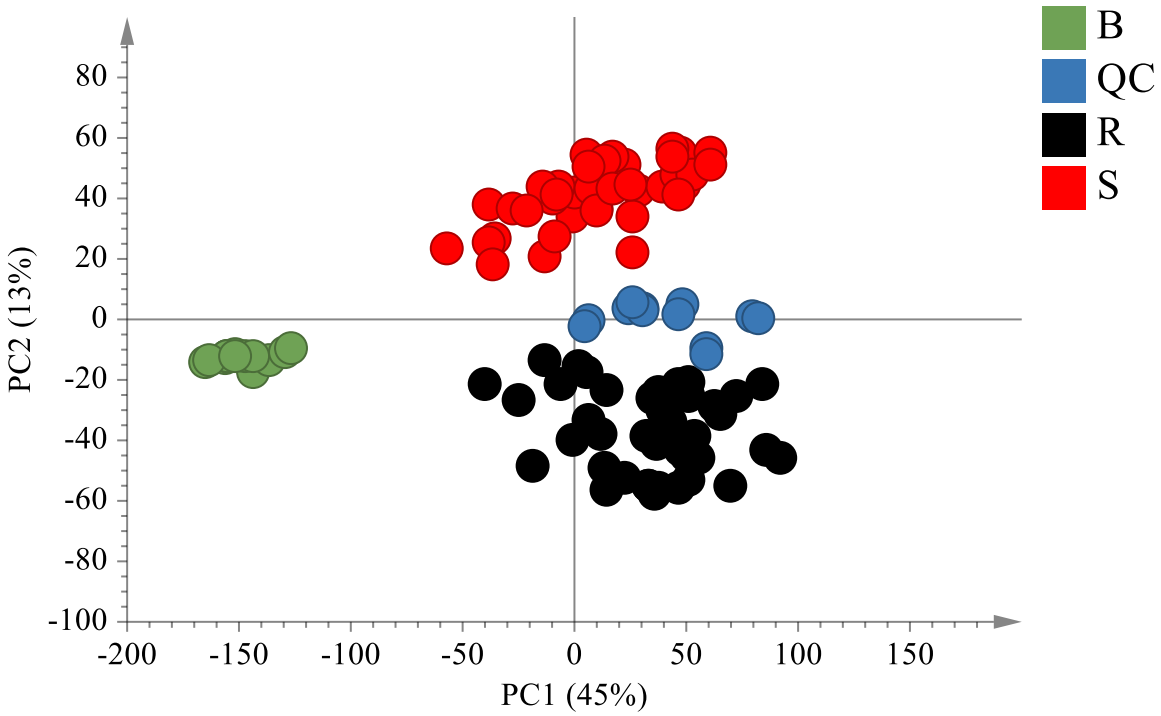


FIGURE 2.8- PCA (B: Blank, QC: Quality control, R: Resistant, S: Susceptible) of *C. limon* (S) x *C. latifolia* (R) and C: S-plot.

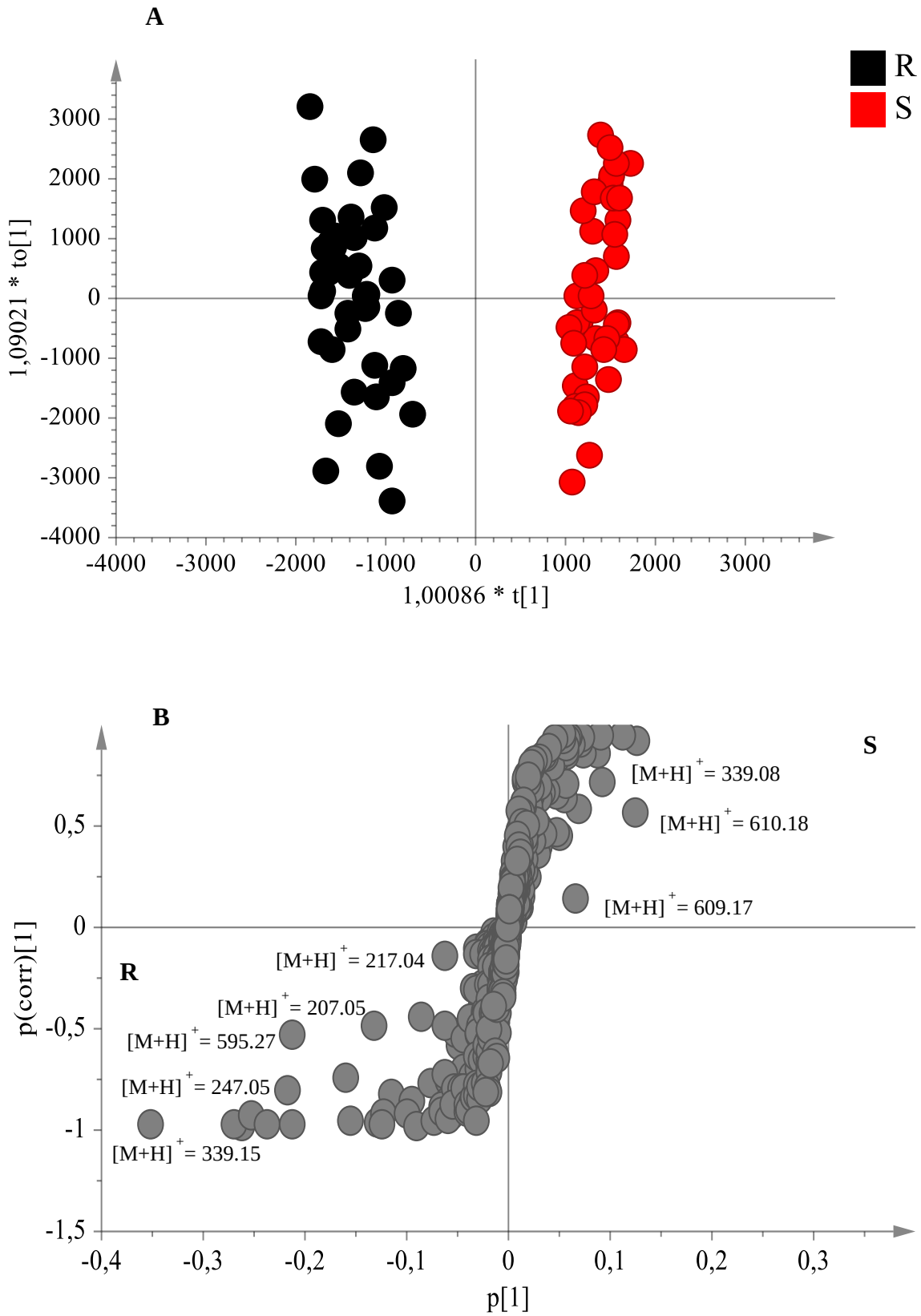


FIGURE 2.9- A: OPLS-DA models of *C. limon* (S) x *C. latifolia* (R) and B: S-plot.

TABLE 2.6- Parameters for identification of phenolics compounds from *Citrus latifolia.*

Compound name	**Formula**	**RT**	**Molecular Weight**	**Precursor ion (*m/z*)**	**Adduct**	**Main *m/z* fragments**		
Kaempferol-3-O-Rutinoside	C27H30O15	5.29	594.1584	595.1640	$[M+H]^+$	449.1065	287.0544	129.0546
Isovitexin 2''-O-xyloside	C26H28O14	5.68	564.1479	563.1388	$[M-H]^-$	413.0860	293.0440	-
Rutin	C27H30O16	5.82	610.1533	609.1441	$[M-H]^-$	300.0264	343.0447	271.0239
Quercetin 3,7-dirhamnoside	C27H3015	6.28	594.1584	593.1490	$[M-H]^-$	431.1027	232.9239	160.8419
Neoeriocitrin	C27H32O15	6.36	596.1742	595.1647	$[M-H]^-$	287.0555	443.0938	-
Genistin	C21H20O10	6.48	432.1056	431.0970	$[M-H]^-$	311.0551	413.0848	341.0652
Diosmetin-Glu-Rha	C28H32O15	6.69	608.1741	609.1799	$[M+H]^+$	463.1227	301.0704	129.0544
Isovitexin	C21H20O10	6.80	432.1087	431.0981	$[M-H]^-$	311.0553	283.0600	341.0656
Isorhoifolin	C27H30O14	6.91	578.1635	577.1542	$[M-H]^-$	269.0452	-	-
Hesperetin	C16H14O06	6.93	302.0790	303.0856	$[M+H]^+$	285.0748	177.0542	153.0177
Isorhamnetin 3-Glu- 4'-Rha	C28H32O16	7.10	624.1690	623.1599	$[M-H]^-$	315.0497	357.0601	479.1176
Diosmetin 7-O-beta-D-glucopyranoside	C22H22O11	7.18	462.1162	497.0842	$[M+Cl]^-$	461.1090	341.0657	191.0350

Continued

TABLE 2.6. *Continued*

Compound name	**Formula**	**RT**	**Molecular Weight**	**Precursor ion (*m/z*)**	**Adduct**	**Main *m/z* fragments**		
Hesperidin	C28H34O15	7.35	610.1897	609.1800	$[M-H]^-$	301.0709	325.0708	269.0444
Neohesperidin	C28H34O15	7.62	610.1898	609.1809	$[M-H]^-$	301.0704	343.0811	325.0714
Limocitrin-HMF-Glu	C29H32O17	7.72	652.1640	651.1558	$[M-H]^-$	548.1229	507.1125	345.0603
Citropten	C11H10O4	11.65	206.0579	207.0651	$[M+H]^+$	192.0411	163.0750	121.0645
8-methoxyfuranocoumarin	C12H8O4	12.02	216.0422	217.0493	$[M+H]^+$	202.0258	174.0308	161.0591
Isopimpinellin	C13H10O5	12.07	246.0582	247.0605	$[M+H]^+$	232.0367	217.0132	189.0185
Bergamottin	C21H22O4	20.91	338.1518	339.1591	$[M+H]^+$	203.0339	147.0446	103.0544
Coumarin (scopoletin derivative)	C20H24O4	21.23	328.1674	329.1743	$[M+H]^+$	193.0553	137.1223	-

RT, retention time; Glu, glucoside; Rha, rhamnoside; HMF, 3-hydroxyl-3methyl-glutaryl.

It is known that citrus plants accumulate a series of phenolic compounds and that they are involved to natural defense mechanisms. Besides, these compounds have been recognized as important due to their physiological and pharmacological role. Several studies mention that phenolic compounds like flavonoids and coumarins may play as phytoalexins in some citrus species. Some of them present antimicrobial activities against diverse citrus pathogens (GATTUSO et. al, 2007; ORTUÑO and DEL RÍO, 2009; RAMÍREZ-PELAYO et al., 2019).

In this first part of the study, NMR analyses supported by LC-MS and multivariate data analysis have identified some compounds belonging to the primary metabolism as well as secondary metabolism compounds. These phenolic compounds were characterized as flavonoids and coumarins, in which coumarins were identified as target compounds correlated to the resistant species. Since coumarins are widely known as a potential antifungal against pathogens, from this, a preparatory work including the targeted isolation of these metabolites and the elucidation of their structure was carried out. HPTLC (High-performance thin-layer chromatography) was employed, taking advantage of its preparatory excellence for the metabolic profile of plants.

2.3.3- Application of HPTLC as a supplementary tool for metabolomics

The optimised HPTLC conditions for phenolics analysis included the use of polar mobile phase (described on ITEM 2.2.5), which spots characteristic of glycosylated phenolic compounds were observed, however, little difference between species was noticed (FIGURE 2.10) and non-polar mobile phase (also described on ITEM 2.2.5), which it is possible to notice three major spots (RFs: 0.4, 0.55, 0.61), characteristics of coumarins and related to resistant specie (FIGURE 2.11).

These results corroborated with the signals previous detected from NMR and LC-MS analyses and it was important to confirm that target compounds

were correlated to resistant species. Afterwards, the target compounds were further isolated by Pure Chromatography instruments (C-850 FlashPrep- Buchi) and elucidated by 1D and 2D NMR. The optimized HPTLC conditions polar and nonpolar compounds analyses included the use of normal phase silica gel plates and the chemical derivatization with 2-Aminoethyl diphenylborinate for the visualization of phenolics compounds under UV 366 nm.

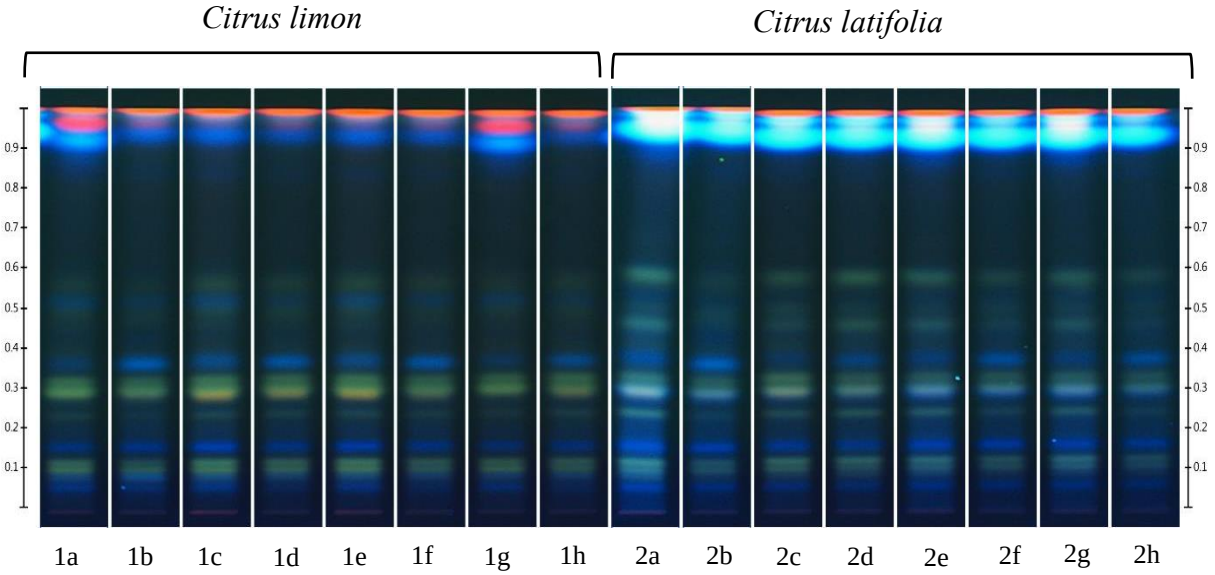


FIGURE 2.10- HPTLC chromatogram of the citrus leaves analyzed in this study, using EtOAc: formic acid: acetic acid: H2O (100:1.1:1.1:2.7 v/v/v/v) as the mobile phase. Plate was sprayed with 2-Aminoethyl diphenylborinate and viewed at 366 nm. Species/ control and inoculated samples; 1a/1b: *C. limon* control 1 day, 1c/1d: *C. limon* inoculated 1 day, 1e/1f: *C. limon* control 60 days, 1g/1h: *C. limon* inoculated 60 days, 2a/2b: *C. latifolia* control 1 day, 2c/2d: *C. latifolia* inoculated 1 day, 2e/2f: *C. latifolia* control 60 days, 2g/2h: *C. latifolia* inoculated 60 days.

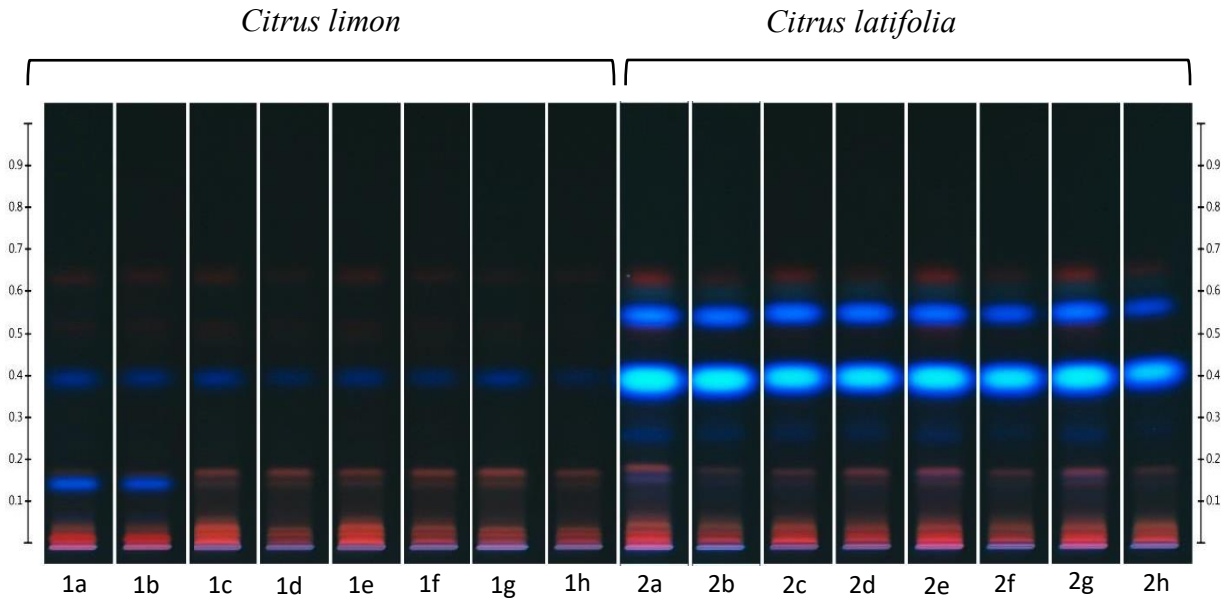
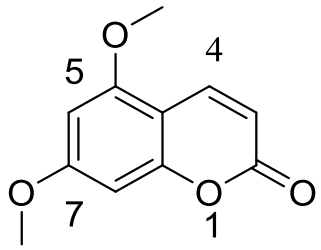


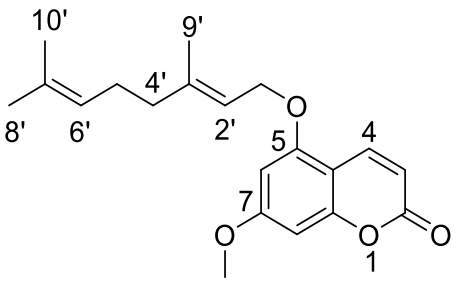
FIGURE 2.11- HPTLC chromatogram of the citrus leaves analyzed in this study, using toluene: EtOAc (8:2 v/v) as the mobile phase. Plates were viewed at 366 nm. Species/ control and inoculated samples; 1a/1b: *C. limon* control 1 day, 1c/1d: *C. limon* inoculated 1 day, 1e/1f: *C. limon* control 60 days, 1g/1h: *C. limon* inoculated 60 days, 2a/2b: *C. latifolia* control 1 day, 2c/2d: *C. latifolia* inoculated 1 day, 2e/2f: *C. latifolia* control 60 days, 2g/2h: *C. latifolia* inoculated 60 days.

2.3.6- Isolation of target compounds from *Citrus latifolia*



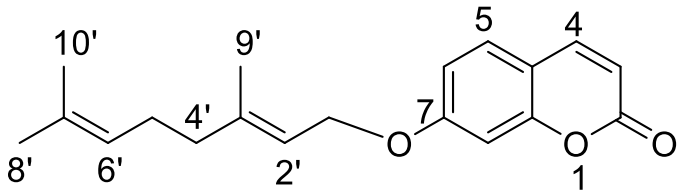
1

5,7-dimethoxycoumarin



2

5-geranyloxy-7-methoxycoumarin



3

5-geranyloxy-7-coumarin

The ^{1}H NMR spectra of the compound **01**, elucidated as 5,7-dimethoxycoumarin (citropten), presented signals at δ 6.15 (d, J= 9.6 Hz, H-3) and δ 8.09 (d, J= 9.6 Hz, H-4) were assigned to protons characteristic AB system signals of the lactone ring of a coumarin, correlations between these signals were observed on COSY. The signals at δ 6.47 (d, J= 2.2 Hz, H-6) and δ 6.52 (d, J= 2.2 Hz, H-8) were assigned to protons in the aromatic ring and the signals at δ 3.87 (s, 3H) and δ 3.93 (s, 3H) were assigned to be methoxy groups. HMBC correlations were observed at δ 157.4 (C5) and 164.46 (C-7), confirmed the methoxy groups (5-OCH_3 and 7-OCH_3) positions, respectively. The NMR spectra are located in appendix A-2 section (pages 128- 130) in the end of this thesis.

The compound **02** (5-geranyloxy-7-methoxycoumarin), signals at δ 6.15 ppm (d, J= 9.5 Hz, H-3) and δ 8.08 (d, J= 9.5 Hz, H-4) were assigned to protons characteristic AB system signals of the lactone ring. The signals at δ 6.50 (d, J= 2.2 Hz, H-6) and δ 6.45 (d, J= 2.2 Hz, H-8) were assigned to protons in the aromatic ring and the signal at δ 3.87 (s, 3H) was assigned to be methoxy groups. The signals at δ 4.70 (d= 6.74 Hz, 2H) δ 5.49 (t= 6.74 Hz, 1H) and δ 5.09 (t= 6.03 Hz, 1H) were assigned to hydrogens 1a', 1b', 2' and 6' respectively, characteristic of a geranyloxy system signal. The other signals at δ 2.10 (m, 2H) and δ 2.15 (m, 2H) were also assigned to hydrogens from geranyloxy system: 4' and 5'. Finally, the methyl groups were assigned as singlets at δ 1.60 (H-10'); δ 1.64 (H-8') and δ 1.78 (H-9'). The

correlations of the signals assigned to protons characteristic AB system of the lactone ring δ 6.15 ppm (H-3) and δ 8.08 (H-4) were observed on COSY spectra. Besides, correlation between signals from geranyolxy group were also observed on COSY at δ 5.09 (1H) and δ 2.15 (2H), δ 4.70 (2H) and 5.49 (1H). Important HMBC correlations were observed at δ 8.08 (H-4) and the carbon at δ 156.6 from the geranyloxy group as well as δ 3.87 (OCH_3) and the carbon at δ 163.10, which confirm that the geranyloxy and methoxy groups were attached at C-5 and C-7, respectively. The methyl groups correlations were also observed, at δ 1.60 (H-10') and δ 1.64 were observed correlations with δ 5.09 (H-6') and at δ 1.78 (H-8) presented correlation with δ 5.49, δ 2.10 (H- 2' and H- 4', respectively). The spectra are on pages 131 and 132, in appendix A-2 section.

The compound **03**, was elucidated as 7-geranyloxy-coumarin (auraptene), which the signals characteristic AB system signals of the lactone ring of coumarin were assigned at δ 6.28 (d, J= 9.61 Hz, H-3) and δ 8.26 (d, J= 9.61 Hz, H-4). In addition, the signals at δ 7.16 (dd, J= 7.82 Hz, 2.64 Hz, H-6), δ 7.20 (d, J= 2.64 Hz, H-8) and δ 7.79 (m, H-5) were assigned to protons in the aromatic ring. The signals at δ 5.02 (d= 6.81 Hz, 2H) were attributed to 1a', 1b', the signal at δ 5.53 (t= 6.81 Hz, 1H) was attributed to H-2' and, the signal of H-6' presented in overlap at δ 5.02. The other signals at δ 2.05 (m, 2H) and δ 2.07 (m, 2H) were also assigned to hydrogens from geranyloxy system: 4' and 5'. The methyl groups were assigned as singlets at δ 1.67 (H-9'); δ 1.62 (H-10') and δ 1.57 (H-8'). Important correlations on COSY and HMBC spectra were observed among δ 6.28, δ 8.26 and δ 7.79, δ 7.16, δ 7.20 confirmed protons from aromatic ring. COSY correlations were also observed among H-6', H-4', H-5' and H-10' and between H-2' and H-9', which confirmed the signals overlap at δ 5.02. HSQC experiment also presented correlations and confirmed the signals overlap observed at δ 5.02 (appendix A-2 pages 133- 135).

Another compound was isolated by chromatography from leaves of *C. latifolia*. However, the compound was not definitively elucidated. Due to the signal overlapping, they did not show clear correlations in 2D NMR spectra. Nevertheless, the characteristic signals and correlations suggested furanocoumarin (a subclass of coumarins with an additional furan ring) or a mixture of two coumarins. As the signals characteristic AB system signals of the lactone ring of coumarin at δ 6.27 (d, J= 9.6 Hz, H-3); 8.27 (d, J= 9.6 Hz, H-4); 6.28 (d, J= 10.1 Hz) and 8.26 (d, J= 10.1 Hz). Signals from geranyloxy were also identified, that suggest prenylated coumarin (appendix A-2 page 136). The spectroscopic data of elucidated compounds are summarized in TABLE 2.7.

TABLE 2.7- NMR Chemical shift assignments of the target compounds isolated from *C. latifolia*.

Compound	Assignment	δ ^{1}H	COSY	HMBC	HSQC	Multiplicity [J(Hz)]
(1) Citropten	H-3	6.15	H-4	-	-	d (9.6)
	H-4	8.09	H-3	-	-	d (9.6)
	H-6	6.47	-	-	-	d (2.2)
	H-8	6.52	-	-	-	d (2.2)
	OCH_3 (H-7)	3.87	-	C-7	-	s
	OCH_3 (H-5)	3.93	-	C-5	-	S
(2) 5-geranyloxy-7-methoxy coumarin	H-3	6.15	-	-	-	d (9.5)
	H-3	6.15	H-4	C-5	-	d (9.5)
	H-4	8.08	H-3	C-5, C-2	-	d (9.5)
	H-6	6.50	-	C-5, C-7	-	d (2.2)
	H-8	6.45	-	C-7, C-5	-	d (2.2)
	OCH_3	3.87 (H-5)	-	C-7	-	s
	H-1a', 1b'	4.70	-	-	-	d (6.74)
	H-2'	5.49	-	-	-	t (6.74)
	H-4'	2.10	-	-	-	m
	H-5'	2.15	-	-	-	m
	H-6`	5.09	-	-	-	t (6.03)
	H-8` ($CH_{3)}$	1.64	-	C-6'	-	s
	H-9` (CH_3)	1.78	-	C-6'	-	s
	H-10' (CH_3)	1.60	-	C-2', C-4'	-	S
(3) Auraptene	H-3	6.28	H-4	-	-	d (9.6)
	H-4	8.26	H-3	C-2	-	d (9.6)
	H-5	7.79	H-6	C-6	-	d (2.6)
	H-6	7.16	H-5, H-8	C-5, C-7	-	dd (7.8, 2.6)
	H-8	7.20	H-6	C-7, C-9	-	s
	H-1a', 1b'	5.02	-	-	C-1' (δ 70.05)	d (6.81)
	H-2'	5.53	H-9'	-	-	t (6.81)
	H-4'	2.05	-	-	-	m
	H-5'	2.07	-	-	-	m

Continued

TABLE 2.7. *Continued*

Compound	Assignment	δ ^{1}H	COSY	HMBC	HSQC	Multiplicity [J(Hz)]
	H-6’	Overlap with H-1a’, 1b’	H-10’, H-4’, 5’	-	C-6’ (δ 124.6)	-
	H-8’ (CH_3)	1.57	-	-	-	s
	H-9` (CH_3)	1.67	-	-	-	s
	H-10` (CH_3)	1.62	-	-	-	S
(4) Coumarin	H-3	6.27	-	-	-	d (9.6)
	H-4	8.27	-	-	-	d (9.6)
	H-4`	6.28	-	-	-	d (10.1)
	H-5`	8.26	-	-	-	d (10.1)

2.3.4- Compounds with increased concentrations in the presence *Phyllosticta citricarpa* and their action mechanisms as inhibitors of fungal growth

Two groups of antimicrobial secondary metabolites receive the greatest attention in healing and defense against attack by microbes and herbivores: phytoalexins (induced) and phytoanticipins (constitutive). The phytoalexins are plant antibiotics synthesized and accumulated in plants as a response to diseases or stress. Meanwhile, phytoanticipins are present in plants prior to infection or may be also produced from pre-existing constituents after infection. These compounds act as the first chemical protection against a pathogen (RAMÍREZ-PELAYO et al., 2019; JOGAIAH and ABDELRAHMAN, 2019; VANETTEN et al., 1994).

Coumarins represent chemical classes that are present in different plant parts, such as roots, flowers, seeds, and fruits. They are widely distributed in plants, predominantly in angiosperms, whose simple structures are the most frequent (FERNANDES, et al., 2015). In addition, coumarins are highly active group of

molecules with a wide range of antimicrobial activity against both fungi and bacteria. It is believed that these cyclic compounds behave as natural pesticide defense compounds for plants, and they represent a starting point for the exploration of new derivatives possessing a range of improved antifungal activity (MAZID, et al., 2011), since they are considered as phytoanticipins serving as the basis of pest resistance (JOGAIAH and ABDELRAHMAN, 2019).

Antifungal evaluation of some coumarin derivatives against other pathogens, such as *Aspergillus fumigatus* and *A. flavus* are described by ARAÚJO et al, (2013). Moreover, some derivatives of coumarins showed significant antifungal activities, in which permitted conclusions that O-substitution is essential for antifungal activity and the presence of a short aliphatic chain (geranyl<prenyl) and/or electron-withdrawing groups (NO_2 and/or acetate) promotes activity. These results suggested derivatives of coumarins as promising compounds for the development of new antifungal agents. In addition, the geranyl substituent has been described to be related to the relative lipophilicity of the compounds, which promotes its more efficient permeation through the lipid layer of the fungi (RAMÍREZ-PELAYO et al, 2019).

In this study, the compounds 5,7-dimethoxycoumarin, 5-geranyloxy-7-methoxycoumarin and 7-geranyloxy-coumarin were identified and isolated as target compounds from C. *latifolia*. Therefore, our results suggest that these isolated compounds may be involved in the defense mechanisms of *C. latifolia* against *P. citricarpa.*

2.4- CONCLUSION

The data obtained by ^{1}H NMR and LC-MS associated MVDA provided satisfactory results and confirmed that these methods are extremely useful to provide

chemical information of citrus species leaves, chemical ecology interactions between the plant-pathogen and the identification of possible biomarker.

The effect of *P. citricarpa* on the variation of the metabolic profile in *Citrus limon* and *C. latifolia* was evaluated, which through of chemometrics analysis, coumarins were correlated and observed at higher concentrations in the resistant species (*C. latifolia*), after inoculation of the pathogen. It was interesting since coumarins present antimicrobial activity against diverse pathogens. Therefore, this study reported substances related to citrus defense mechanism against *P. citricarpa*. Moreover, these substances have potential to be tested as biofungicides in further investigations.

The conventional metabolomics tools, ^{1}H NMR and LC-MS that were supplemented with an HPTLC method were compared in terms of the separation of samples and identification capacity. The results revealed an attractive potential of these analytical platforms as a tool for metabolomics studies.

3- METABOLIC PROFILE CHANGES IN CITRUS LEAVES VOLATILES IN RESPONSE TO *PHYLLOSTICTA CITRICARPA*

Abstract

Citrus plants are known as a rich source of volatile organic compounds (VOCs) and several of them play important roles in defense responses. These data stimulated an investigation of the changes in the metabolic profile of the VOCs in citrus leaves in response to stress caused by *Phyllosticta citricarpa* using GC-MS. The advantages of used GC-MS were the high separation efficiency and reproducible retention times, which allowed the identification of some target compounds. The chemical compositions of the volatile from the samples of citrus were poorly differentiated on the evaluation of chemical compounds as markers following the static presence and absence criterion. However, chemometric techniques clarified the relationships between the citrus samples and, enabled us to understand their possible resistance to *P. citricarpa*. *Citrus latifolia* species is a variety with high resistance to the fungus in the field, nevertheless, through analysis by GC-MS, it was not observed significant changes in the metabolic profile after inoculation of fungus. The S-plot showed that the main differences between the two species was the presence of β-pinene in susceptible *Citrus limon* and D-limonene in resistant *Citrus latifolia.* These results may be related with the influences of mechanical infection/defense of citrus plants.

Key words: Alcohols, Headspace-GC-MS, defense mechanisms, terpenes.

3.1- INTRODUCTION

Many plants, including citrus plants are very known as a rich search of volatile organic compounds (VOCs) and several of these compounds present bioactivity functions including, antimicrobial (JING, et al 2014), anti-inflammatory (SANTOS, et al 2000, CHAVAN, et al 2010), anticancer (JAYAPRAKASHA, et al 2013), besides of antioxidant functions (CHOI, et al, 2000). However, the physiology functions of volatile organic compounds produced by citrus plants are few knows, as well as functions defense response them to environmental stress in citric plants (ASAI et al., 2016).

Metabolite measurements have been performed for decades owing to the fundamental regulatory importance of such molecules as components of metabolic pathways and an importance of some metabolites in the human diet. Historically, the measurement of metabolites was reached either by spectrophotometric analysis capable of detecting single metabolites or by chromatographic separation of mixtures of low complexity (LISEC et. al, 2006).

Nevertheless, in late 1940s and early 1950s the concept of a “metabolic pattern” was introduced to explain the results from a chromatography study that distinguished saliva and urine components among individuals (GATES and SWEELEY 1978; QIU and REED, 2014). This concept was not totally accepted until the 1960s, when gas chromatography (GC) was successfully used for profiling a complex biological matrix. In the early 1970s, the term “metabolic profiles” was conceived to describe the chromatographic pattern associated with bio-fluid analysis (QIU and REED, 2014).

Nowadays, numerous applications using GC-MS-based metabolomics have been used to investigate metabolites and pathways, in which are differentially regulated due to genetic or environmental perturbations. Extensive reports and reviews have been described how investigations of metabolite profiling using GC-

MS are employed to study plant metabolism (GENGA et. al, 2011; BEALE et. al, 2018). GC-MS-based metabolomics is a powerful tool for metabolite profiling of plants upon changing conditions, with diverse advantages over other analytical platforms, such as robustness, high separation power and reproducibility. The technology is well established for the analysis of volatiles, oil components of different origin, and fragrances (HILL and ROESSNER, 2013).

3.1.1- Volatile organic compounds in plant defense responses

Plants respond to wounding by induction of numerous defense reactions, such as accumulation of phytoalexin or pathogenesis-related protein and emission of VOCs. Among these reactions, emission of VOCs is involved not only in direct defenses, such as toxins and repellents against herbivores, but also in indirect defenses which include recruitment of natural enemies against pathogens and elicitation of defense mechanisms in intact receiver plants *(*MATSUKAWA et. al, 2017; ALLISON and HARE, 2009).

Plant VOCs consist of two major classes of compounds, i.e., terpenoids, such as monoterpene and sesquiterpene and their derivatives, and green leaf volatiles (GLVs). Terpenoids are formed from the biological precursors, isopentenyl pyrophosphate and its isomer dimethylallyl pyrophosphate. Terpenoids are a structurally diverse group of plant metabolites (ASAI, 2016). Meanwhile, GLVs consist of a family of C6 compounds, including aldehydes, alcohols and esters. GLVs are almost ubiquitously made by green plants and their increased release can be caused by abiotic stimuli, herbivores or pathogens. The compounds *trans*-2-hexenal and *cis*-3-hexenol, for example, are typically released in response to wounding and have been reported to mediate plant-plant signaling and intra-plant information transfer in indirect defenses (SCALA et al., 2013).

Terpenoids are one of the most structurally diverse groups of plant metabolites and play an important role in the interactions between specialized pathogens and fruits. It has been demonstrated that several terpenoids play roles as antimicrobial or antifeedant compounds in direct defense responses of plants (RODRIGUEZ et al, 2018; WANNER et. al, 2010).

Despite most of these VOCs have important roles in defenses responses against pathogens, little is known about physiological roles of these compounds released by leaves. Therefore, a comprehensive analysis of VOCs during wound defense responses may be useful to understand the roles of these volatile in plant defense *(*MATSUKAWA et. al, 2017).

3.1.2- Gas chromatography- mass spectrometry (GC-MS)-based metabolomics

GC-MS metabolomics approach has been extensively applicated in discovering the mode of action of drugs or herbicides and has helped to clear up the effect of altered gene expression on metabolism and organism performance in biotechnological applications. The key challenge of metabolite profiling is the fast, reliable, and unambiguous identification of hundreds of metabolites in overly complex preparations, such as blood plasma, microbial or plant extract (SCHAUER et. al, 2005). GC-MS is a technique widely used in plant metabolomics studies, especially for facilitating the identification and quantification of metabolites involved in the pathways of primary metabolisms, such as sugars, alcohols, amino acids, organic acids and polyamines (WANG et al., 2015).

A method capable rapidly to identify plants volatile constituents is a useful tool for a complete phytochemical analysis and the extraction technique is one of the most important factors that should be taken into consideration. There are different extractions techniques: the hydrodistillation, for example, is the most common extraction technique employed to obtain essential oil from aromatic plants.

However, hydrodistillation is a time-consuming and laborious process and needs large amounts of sample. On the other hand, solid phase microextraction (SPME) is a unique sample preparation technique, in which eliminates most disadvantages to extracting organics, including high cost and excessive preparation time; besides, SPME is a simple and fast modern tool used to characterize the volatile fraction of aromatic and medicinal plants (PELLATI et. al, 2005).

Headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography has been used successfully to determine the volatile profile of plant matrices and is most sensitive and useful method for comprehensive analysis of VOCs (NARDINI et. al, 2013). HS-SPME extraction technique can be rated in dynamic and static process. The dynamic headspace, known as "*purge and trap"*, is a continuous method of gas extraction which separates volatile sample constituents from the matrix by a continuous flow of an inert gas either above a solid or liquid sample. Meanwhile, the static headspace consists of vaporization of volatile compounds which the vial is thermostatted at a constant temperature until equilibrium. The volatiles components are extracted from the sample, by using a syringe, through of pressurization of the sample vial and time- or volume-controlled transfer of an aliquot of the headspace into the column (KOLB and ETTRE, 2006).

Among the diverse extraction phases commercially available to use in HS-SPME extractions, the more commonly employed of the volatile fraction of foods and plants are composed of divinylbenzene/polydimethylsiloxanes (DVB/PDMS) and/ or carboxen/polydimethylsiloxanes (CAR/PDMS) and fibers with a coating of only PDMS (ZANG et al., 1994; NARDINI et. al, 2013).

The use of the headspace technique for sampling volatile compounds has been also used in metabolomics studies of plants (PONTES et al., 2009) as well as human (ZANG and RAFTERY, 2014). Therefore, considering that citrus plants are an abundant source of volatile organic compounds and numerous of them can be related to defenses response, headspace combined with GC-MS, was applied to

investigate changes in chemical profile of the VOCs in citrus leaves, in response to stress caused by *Phyllosticta citricarp*a, pathogen causer of citrus black spot disease.

3.2- EXPERIMENTAL SECTION

3.2.1- Plant material and collection

The experiment was performed at Fundecitrus- Araraquara-SP, Brazil, under controlled conditions. Leaves of six-month-old *C. limon* and *C. latifolia* species, susceptible and resistant to the CBS, respectively, were inoculated with the *P. citricarpa* at the concentration of 10^5 conidia/mL. Non-inoculated leaves were used as controls. The inoculated and non-inoculated leaves were collected at 3, 4, 15, 30, 40 and 90 days after inoculation (FIGURE 3.1). The sampled leaves were immediately frozen in liquid nitrogen after the collected and kept in individual packages then stored at -80°C. The samples were done in 3 replicates.

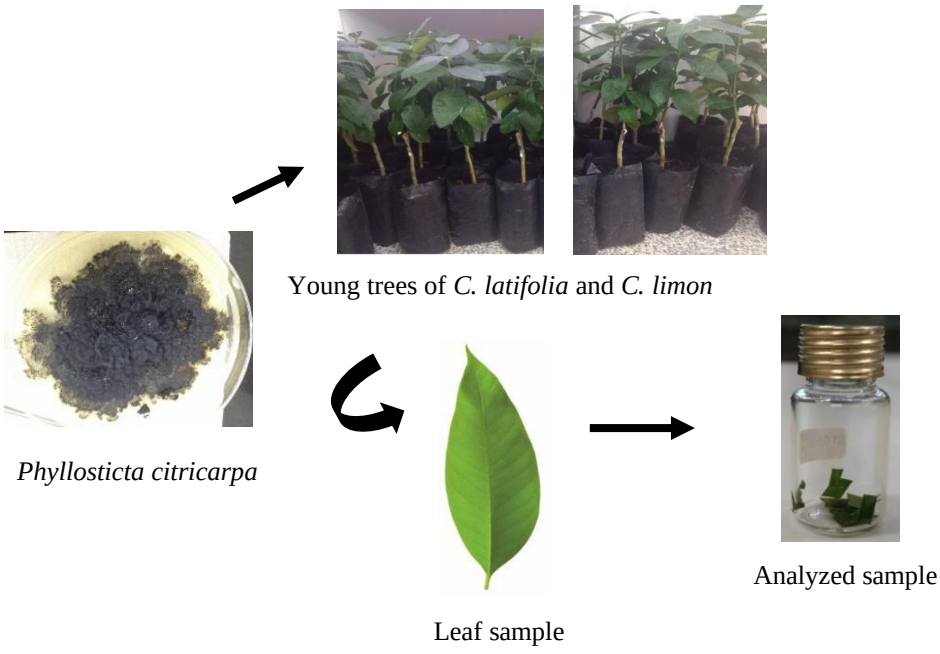


FIGURE 3.1- Young trees of *C. latifolia* and *Citrus limon,* collected sample and the colony of pathogen *Phyllosticta citricarpa* used in the experiment performed to assess the metabolic profile of the volatile after inoculation with the pathogen spore suspension.

3.2.2- Sample preparation for GC-MS and data acquisition

Experiments were performed to the optimization of the extracting condition. A full factorial 2^3 was carried out, which three independent variables were evaluated at a low and high level, such as weight of samples (50 and 100 mg), incubation temperature (60 and 70 ºC) and the use of Sodium sulfate ($NaSO_4$) as a drier (0 and 1 mg).

Eight different combinations of experiments were performed to select the best extracting method. After analyses by Headspace-GC-MS, four chromatography bands (identified as hexanal RT=4.63; 2-hexenal RT=6.17; β-pinene RT=10.76 and D-limonene RT=13.18) of larger areas were chosen to analyses of the optimum condition. The effect to each variable was calculated using the picks area of chromatograms of selected bands and the graphic was constructed.

The extraction condition was optimized to obtain the optimum and reproductive conditions to analyses by HS-GC-MS. The variables as the weight of samples (*w*), incubation temperature (*T*) and the use of Sodium sulfate as a drier (*s*) were evaluated. After that, the analyses were performed using the best extraction conditions, which portions (100 mg) of leaves of *C. limon* and *C. latifolia*, control and inoculated, were weighed, and placed in a headspace *viel* of 10 mL, sealed and were taken for GC-MS analyses, using a headspace static system. Aliquot of 6 µL of 1,4-cineol (used as internal standard) were added before capping the vial. The chromatography analysis was performed using a CG-MS TQ 8030 Shimadzu (Shimadzu Corporation), using a capillary column Rtx5-MS [30 m x 0,25 mm (0,25µm)]. The interface temperature was 280 ºC and helium (99.999%) was used as carrier gas at a constant flow of 1.2 mL min^{-1}. The oven temperature set at 70ºC and the incubation time was 40 minutes with rotation of 250 rpm. The agitation time was 5 seconds with stop in 2 seconds and the sample volume was 1.0 mL min^{-1}. The

mass spectra were obtained in electron ionization (EI), operated in positive mode at 70 eV with a scanning range of m/z 40e400 Da.

3.2.3- GC-MS data processing

GC-MS chromatograms were analyzed using *GCMS solution ver. 4.3 (Shimadzu, Kyoto, Japan)*. The mass spectrum data were compared against spectra in the NIST reference library (NIST11, NIST11s) of the GC-MS data system for identification of volatile compounds, besides, Kováts retention indexes were calculated and compared from literature (ADAMS, 2007, NIST/SRD/69). The chromatograms total peak areas (TIC) of compounds relative to that of the internal standard (2,3-cineol) were used for multivariate analysis.

SIMCA-P (15.0) software (Umetrics, Umeå, Sweden) was used to perform multivariate analysis, applying pareto and univariate scaling to the data. Loading plots, as well as VIP and S-plots were set up to visualize results and predict possible terpene biomarkers.

3.3- RESULTS AND DISCUSSION

The evaluated variables with the greatest effect were $s > T > w$ (FIGURE 3.2 A). The further away the points are from the y-axis, more effect the variable present. However, the variable *s* presented a negative effect, that is, the higher the concentration of Sodium sulfate, lower it is the signal. Variables *s, T* and *w* presented greater influence in the signal response (48.4, 38, and 9.6 %). These variables correspond to 96% of the response while their interactions correspond to approximately 4%. In short, to increase the signals, the variables *T* and *w* should be increased. However, the variable *s* must be reduced (FIGURE 3.2 B).

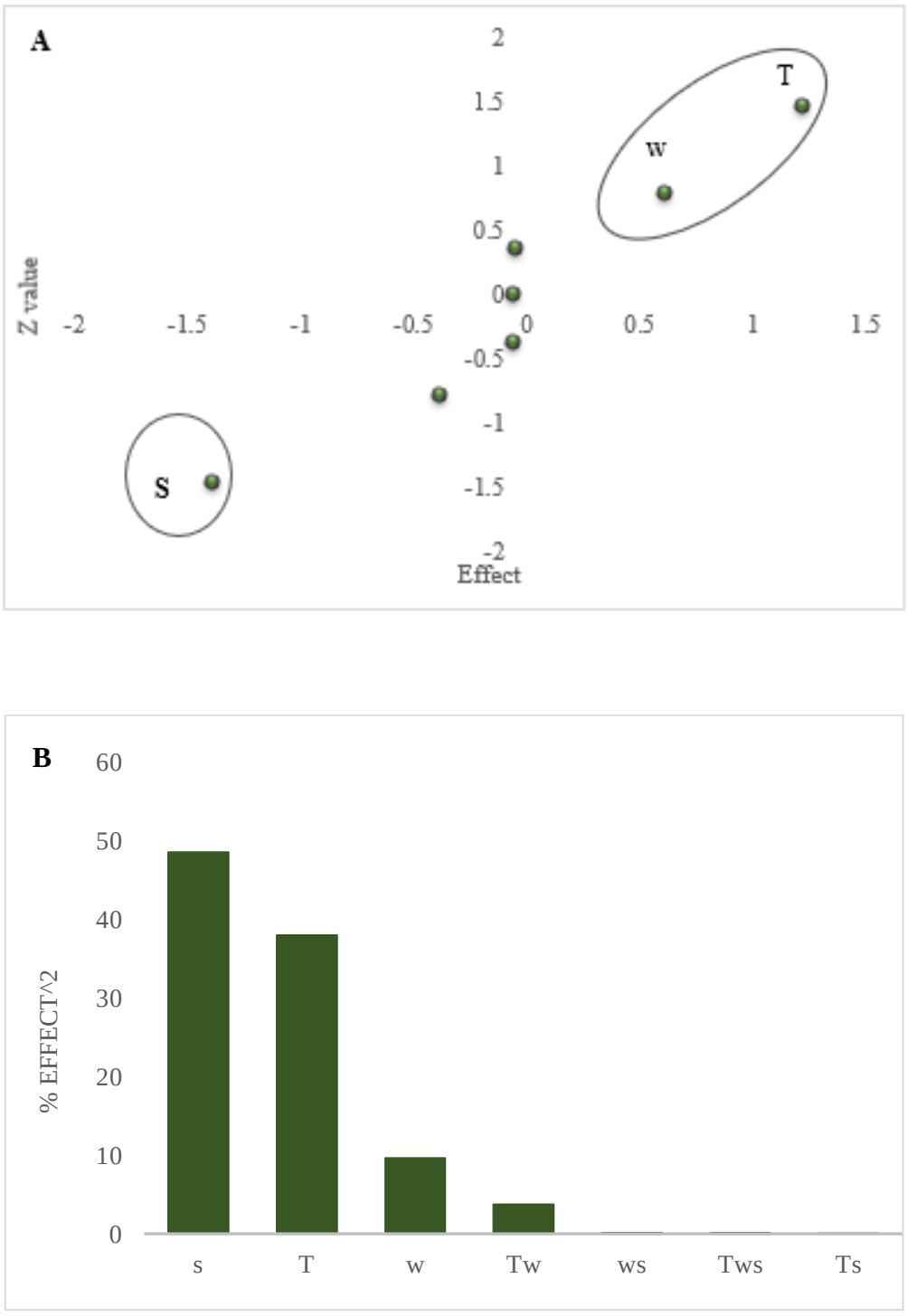


FIGURE 3.2- Effect (A) and percentage of effect (B) of variables in the extraction of the compounds. Salt (s), temperature (T), weight (w), temperature + weight (Tw), weight + salt (ws), temperature + weight + salt (Tws) and temperature + salt (Ts).

Among the eight experiments, one of them showed the best condition of extraction. The experiment 4 presented signals with higher intensity than other experiments, which made this condition more sensitive. The experiment 7 also showed high intensity signals; however, it presented a loss of intensity in the other signals, meanwhile the condition 4 was able to maintain the intensity for all peaks analyzed (FIGURE 3.3). Therefore, the condition 4 (w= 100 mg, T= 70ºC, NaSO4= 0 mg) was selected to HS-GC-MS metabolomic analyses of citrus leaves.

A comparison of the typical chromatograms of volatile compounds from *C. limon* and *C. latifolia* leaves is shown in FIGURE 3.4.

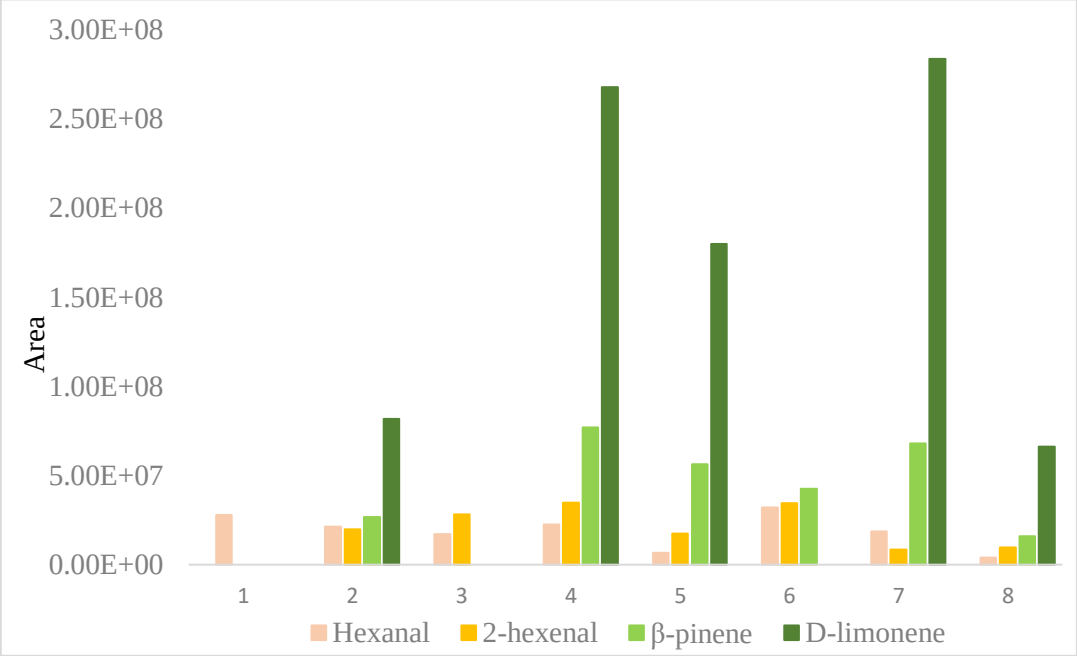


FIGURE 3.3- Experiments for the best condition of extraction. Exp 1: (w= 0.5 mg, T= 70ºC, $NaSO_4$= 0 mg), exp 2: (w= 0.5 mg, T= 60ºC, $NaSO_4$= 1 mg), exp 3: (w= 0.5 mg, T= 60ºC, $NaSO_4$= 0 mg), exp 4: (w= 100 mg, T= 70ºC, $NaSO_4$= 0 mg), exp 5: (w= 100 mg, T= 60ºC, $NaSO_4$= 0 mg), exp 6: (w= 100 mg, T= 60ºC, $NaSO_4$= 1 mg), exp 7: (w= 100 mg, T= 70ºC, $NaSO_4$= 1 mg), exp 8: (w= 0.5 mg, T= 70ºC, $NaSO_4$= 1 mg).

3.3.1- Identification of volatile organic compounds in citrus leaves

Thirty-five volatile organic compounds were identified from intact leaves of two citrus species, among them, alcohols, aldehydes, ketone, hydrocarbons, monoterpenes and sesquiterpenes, they are shown in TABLE 3.1. The aldehydes and monoterpenes constituted the largest part of the volatile compounds between the species and the proportion of sesquiterpenes in *C. latifolia* leaves were higher than *C. limon.* The identification of volatile compounds was based on comparison of their KIs and their mass fragmentation patterns to those in the NIST library and literature.

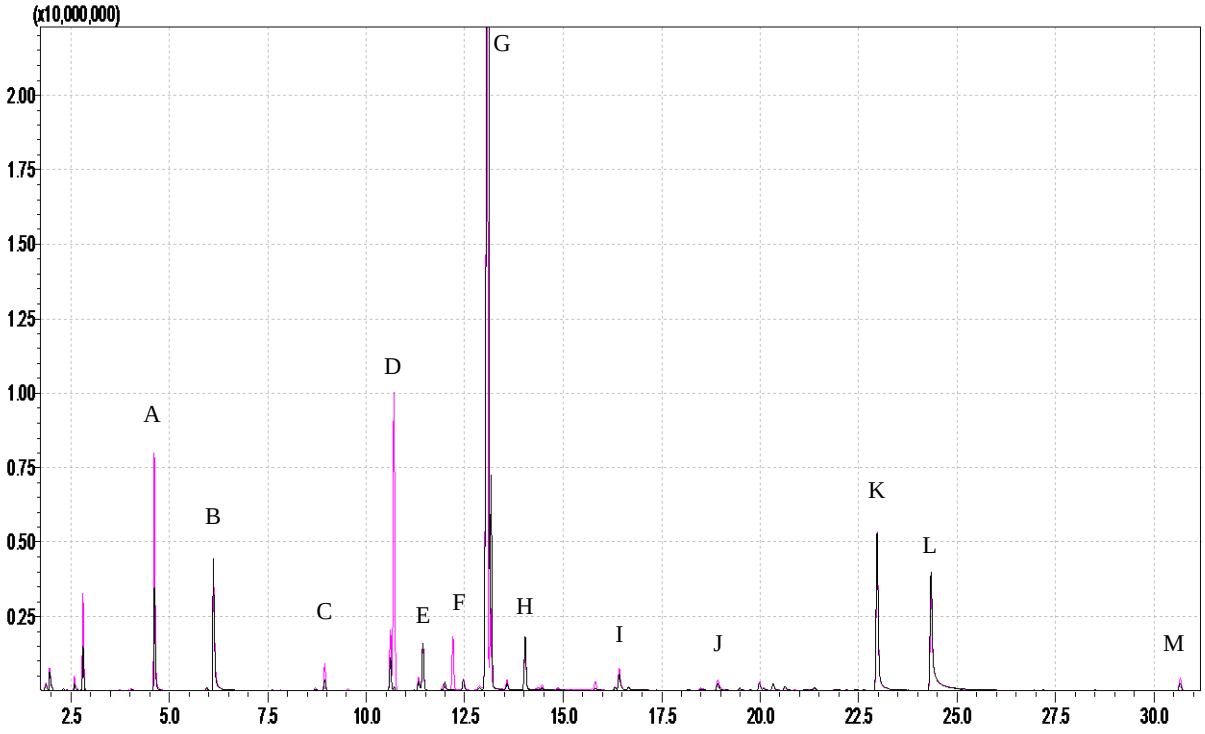


FIGURE 3.4- Comparison of the chromatograms from *C. limon* (pink) and *C. latifolia* (black) leaves. The compounds were separated/identified by GC-MS. (A) hexanal; (B) 2-hexenal; (C) alpha-pinene; (D) beta-pinene; (E) beta-myrcene; (F) 3-carene; (G) D-limonene; (H) beta-ocimene; (I) beta-linalool; (J) citronellal; (K) neral; (L) geranial; (M) caryophyllene.

TABLE 3.1- Volatile organic compounds in leaves of citrus species.

No	Chemical Group	Compounds	Molecular Weight	RI Exp.	RI Lit.	Mass Spectrum data *m/z* (relative intensities)	Identification by
1	Aldehyde	Hexanal	100	800	801	44(100) 56(97) 41(93)	RI, MS
2	Aldehyde	2-hexenal	98	848	846	41(100) 55(88) 69(77)	RI, MS
3	Monoterpene	α-pinene	136	929	932	93(100) 91(59) 92(42)	RI, MS
4	Monoterpene	Camphene	136	944	946	93(100) 121(55) 79(40)	RI, MS
5	Monoterpene	Sabinene	136	970	973	93(100) 91(79) 77(39)	RI, MS
6	Monoterpene	β-pinene	136	972	974	93(100) 41(38) 69(32)	RI, MS
7	ketone	Methyl-5-hepten-2one	126	987	981	43100) 41(57) 108(45)	RI, MS
8	Monoterpene	β-myrcene	136	990	988	93(100) 41(96) 69(81)	RI, MS
9	Monoterpene	α-phellandrene	136	1002	1002	93(100) 91(85) 77(49)	RI, MS
10	Aldehyde	Octanal	128	1003	1004	41(100) 56(76) 55(60)	RI, MS
11	Monoterpene	3-carene	136	1008	1008	93(100) 91(78) 92(32)	RI, MS
12	Monoterpene	D-limonene	136	1026	1024	68(100) 67(94) 93(78)	RI, MS
13	Alcohol	Eucalyptol	154	1028	1030	43(100) 81(72) 84 (43)	RI, MS
14	Monoterpene	cis-β-ocimene	136	1037	1032	93(100) 91(63) 92(39)	RI, MS
15	Monoterpene	trans-β-ocimene	136	1047	1044	93(100) 91(65) 80(40)	RI, MS
16	Monoterpene	ϒ-terpinene	136	1056	1054	93(100) 91(73) 136(31)	RI, MS

(Continued)

TABLE 3.1. *(Continued)*

No	Chemical Group	Compounds	Molecular Weight	RI Exp.	RI Lit.	Mass Spectrum data *m/z* (relative intensities)	Identification by
17	Hydrocarbon	Cyclohexene	136	1086	1085	93(100) 121 (72) 91(65)	RI, MS
18	Alcohol	β-linalool	154	1098	1095	93(100) 71(83) 55(62)	RI, MS
19	Aldehyde	Nonanal	142	1103	1100	57(100) 41(96) 56(62)	RI, MS
20	Monoterpene	Limonene oxide	152	1132	1132	67(100) 43(55) 93(48)	RI
21	Monoterpene	Isopulegol	154	1139	1144	67(100) 41(97) 69(86)	RI
22	Aldehyde	Citronellal	154	1147	1148	41(100) 69(92) 95(58)	RI, MS
23	Monoterpene	Cis-verbenol	152	1158	1137	67(100) 41(89) 91(71)	RI, MS
24	Alcohol	α-terpineol	154	1180	1186	93(100) 59(96) 136 (42)	RI, MS
25	Alcohol	Cis-geraniol (Nerol)	154	1217	1227	69(100) 41(94) 68(22)	RI, MS
26	Aldehyde	β-citral (Neral)	152	1230	1235	41(100) 69(94) 94(23)	RI, MS
27	Aldehyde	α-citral (Geranial)	152	1260	1264	69(100) 41(100) 84(24)	RI, MS
28	Sesquiterpene	β-elemene	204	1378	1385	93(100) 68(67) 81(42)	RI, MS
29	Sesquiterpene	Caryophyllene	204	1404	1408	91(100) 93(85) 133(79)	RI, MS
30	Sesquiterpene	β -bergamotene	204	1422	1436	93(100) 119(96) 41(53)	RI
31	Sesquiterpene	α-bergamotene	204	1422	1436	119(100) 93(97) 41(44)	RI, MS

(Continued)

TABLE 3.1. *(Continued)*

No	Chemical Group	Compounds	Molecular Weight	RI Exp.	RI Lit.	Mass Spectrum data *m/z* (relative intensities)	Identification by
32	Sesquiterpene	Humulene	204	1440	1452	93(100) 80(24) 121(18)	RI, MS
33	Sesquiterpene	Guaia-1(10),11-diene	204	1483	1442	91(100) 107(88) 133(80)	RI
34	Sesquiterpene	β-bisabolene	204	1498	1505	69(100) 93(84) 41(79)	RI, MS
35	Sesquiterpene	γ- elemene	204	1546	1434	121(100) 93 (92) 91(78)	MS

RI Exp.: Retention Index experimental; RI lit.: Retention Index from literature. MS: Mass Spectra; RI: Retention Index. Retention index on a RTX-5MS column;

3.3.2- Multivariate data analysis by GC-MS

Principal component analyses (PCA) using pareto scaling was applied to visualize the metabolic variations between susceptible and resistant species. With 4 PCs, the explained variation (R^2 X) reached 0.91 (FIGURE 3.5) and the separation between species were observed. In the loading plots, we could observe two well-distinguished metabolites for each group. However, to corroborate the correlation of these metabolites to species differences, a supervised model (OPLS-DA) was constructed using pareto scaling (FIGURE 3.6 A). The separation was more evident, and the model was highly validated (R^2 X= 0.91, Q^2= 0.96 and p-value < 0.01). The S-plot corroborated the previously detected metabolites in the PCA loading as discriminant compounds, which showed that the compounds more correlated to species differences were β-pinene in *C. limon* and D-limonene in *C. latifolia*. Moreover, other metabolites were less correlated than the two previous one. Caryophyllene, β-bergamotene, octanal and 2-hexenal, were correlated to resistant species while hexanal, sabinene, eucalyptol and 3-carene were correlated to susceptible species (FIGURE 3.6 B).

S-plots derived from OPLS are useful for the identification of biomarkers and are often used in metabolomics (WISHART, 2008). From the S-plot of susceptible *vs* resistant specie, D-limonene and β- pinene was identified as highlighted markers. The position of these terpenes on the S-plot describes the degree of influence of each terpene as a contributing factor in the model, indicates that the identification of D-limonene and β-pinene as markers can be made with confidence (ERIKSSON et al., 2006).

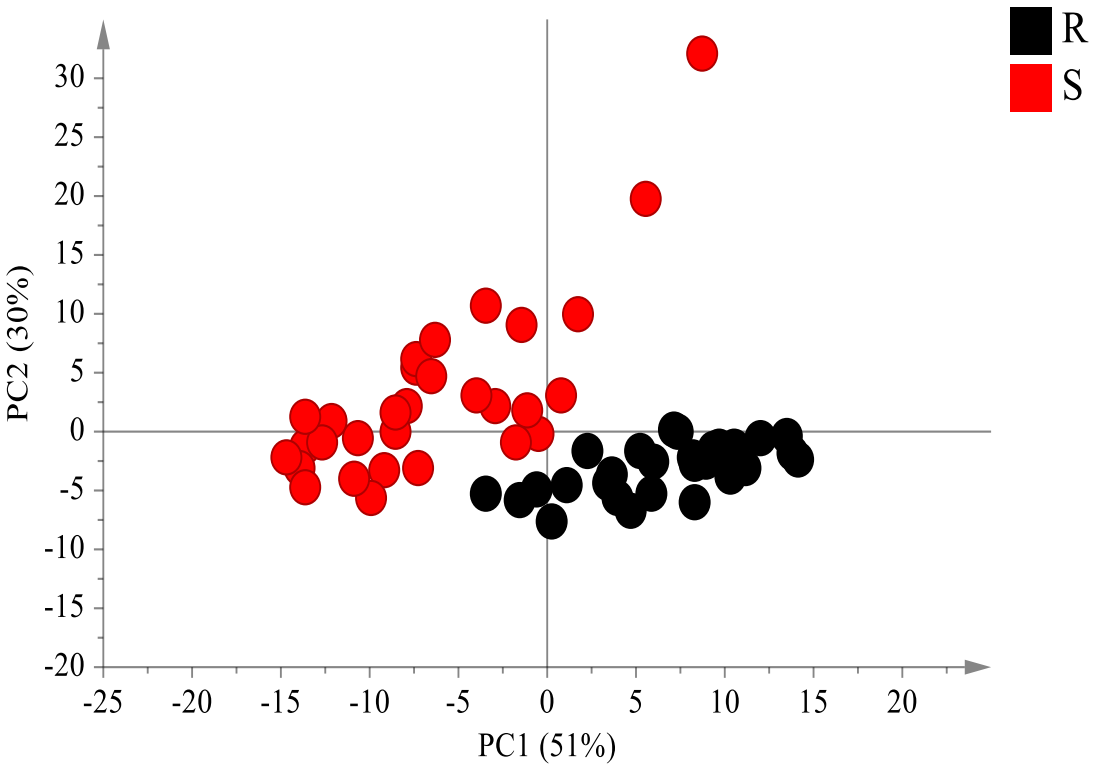


FIGURE 3.5- PCA score of *C. limon* (S) x *C. latifolia* (R).

In order to investigate the effect of fungal inoculation, the dataset was analyzed with inoculation and control factors and each species was analyzed separated. For *C. lemon* species, the PCA plot using UV scaling and with 8 PCs showed a slight trend in the sample separation driven by inoculation effects and the explained variation (R^2 X) reached 0.92 (FIGURE 3.7). To overcome this result, a PLS-DA model was constructed using UV scaling method (component 1= 33% and component 2= 24%), which showed a more evident separation by inoculation effects. Although the Q^2 value had presented a nearly validated (0.37) and R^2= 0.55, the p-value was highly significant ($p < 0.01$) (FIGURE 3.8).

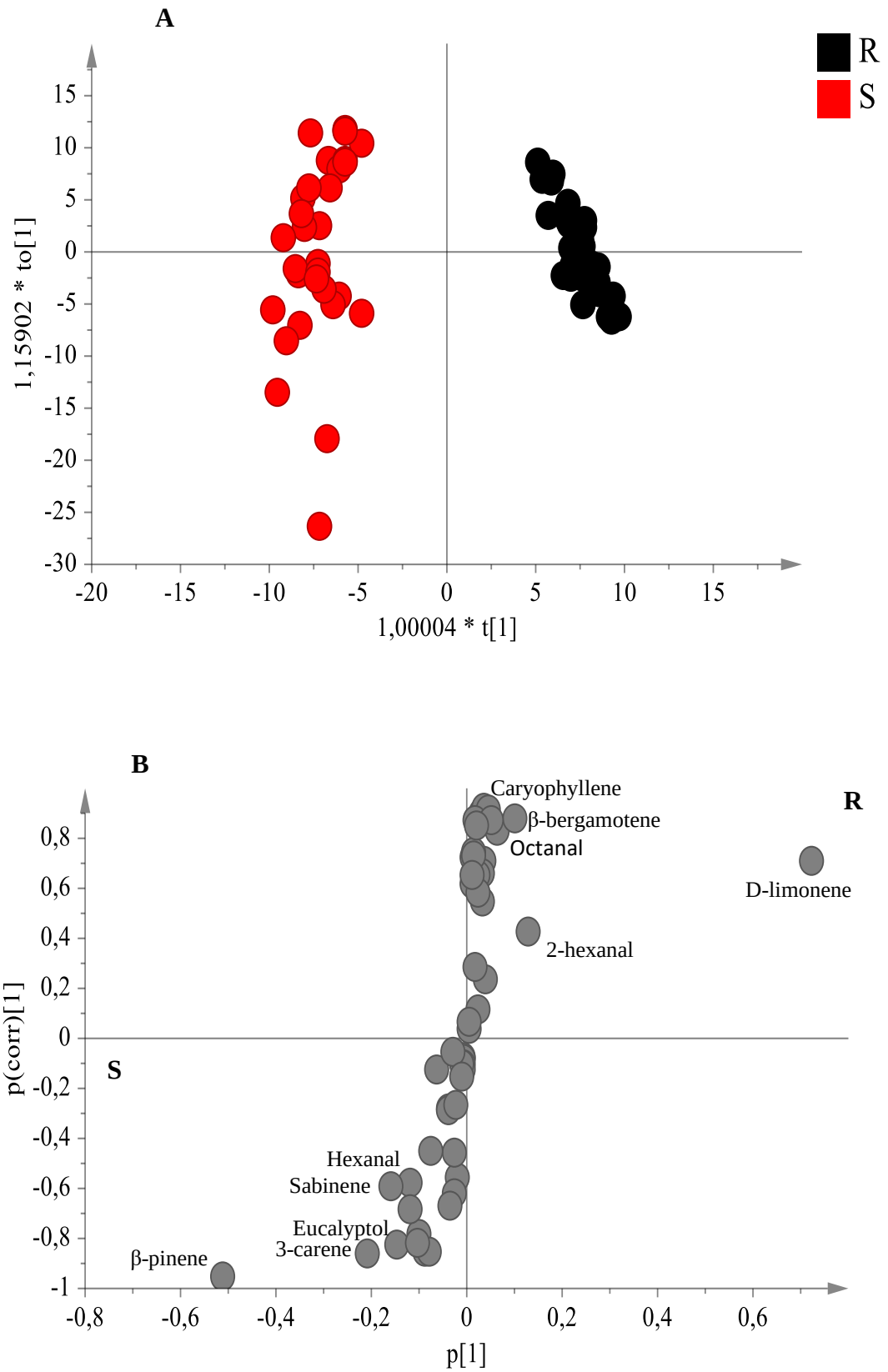


FIGURE 3.6- A: OPLS-DA model and B: S-plot of *C. limon* (S) x *C. latifolia* (R)

There are several approaches to judge whether the real model is characterized by chance correlation. A simple approach can be summarized as a set of decision inequalities based on the values of Q^2 and R^2 X. For example, Q^2 and R^2 X < 0.2 is considerate no chance of correlation. Q^2 < 0.3 and R^2 X < 0.4, the tolerable chance of correlation, and Q^2 and R^2 X greater than 0.4 is considerate recognized and satisfactory chance of correlation (KIRALJ and FERREIRA, 2009). Hence, this may mean that even the predictive power of the PLS-DA model was not high enough to predict unknown samples, there was an effect of the fungal infection over the volatile fraction of *C. limon*.

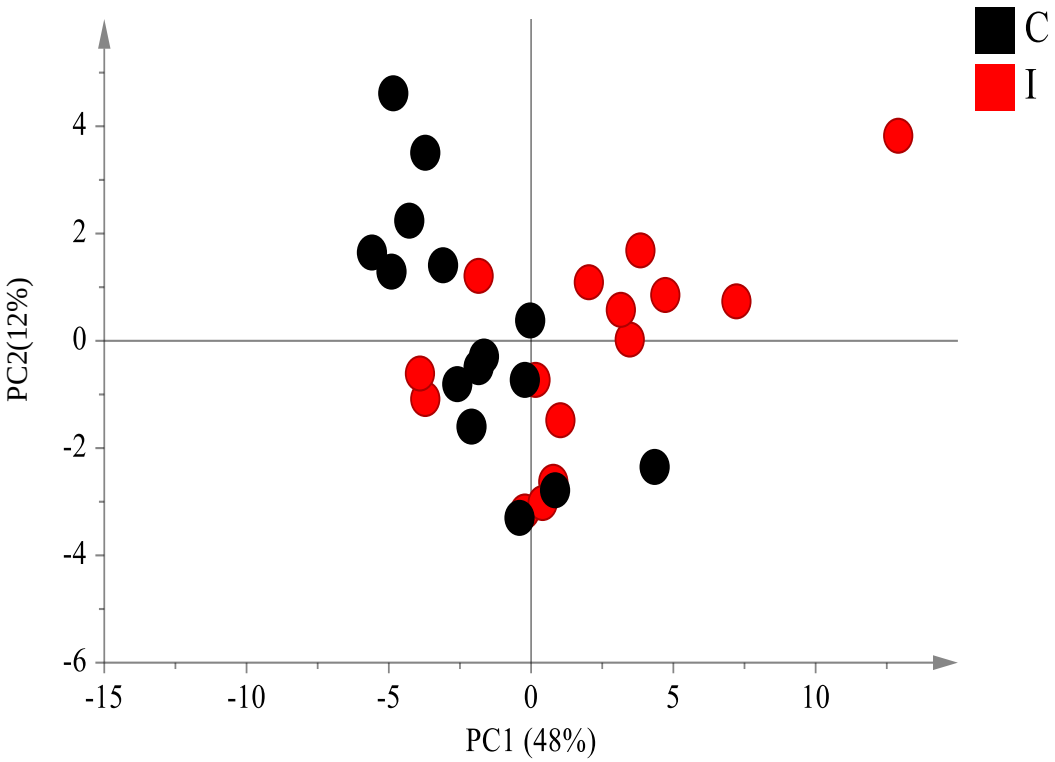


FIGURE 3.7- PCA score of *C. limon*, control (C) and inoculated (I) leaves.

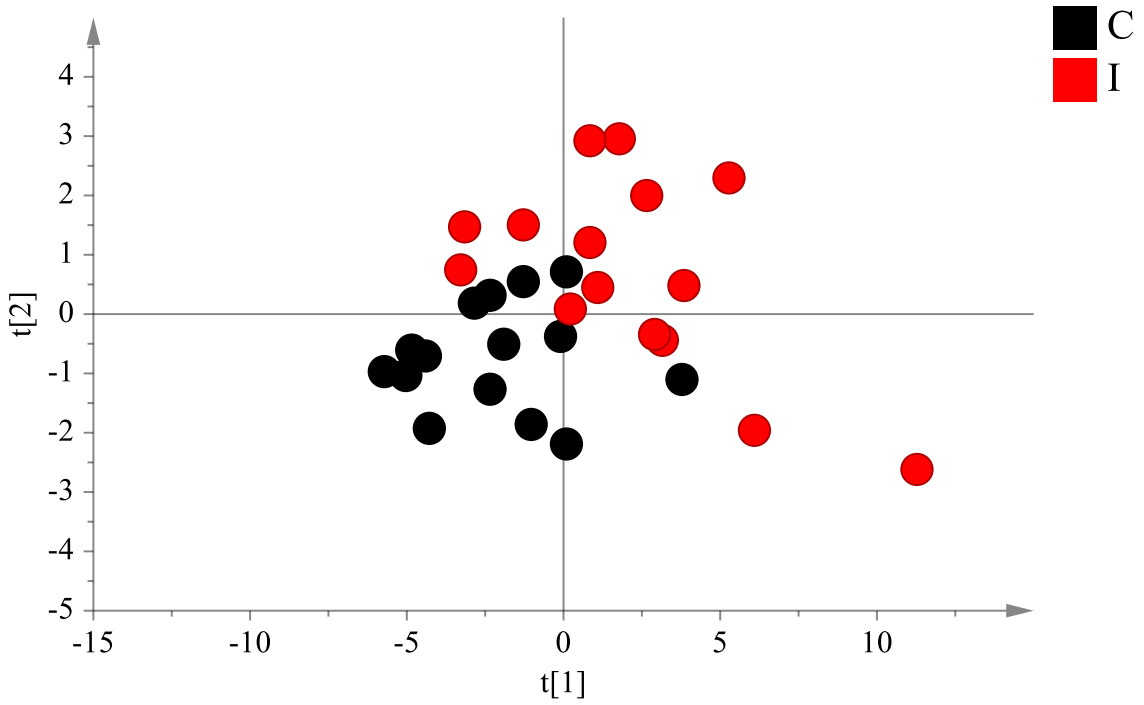
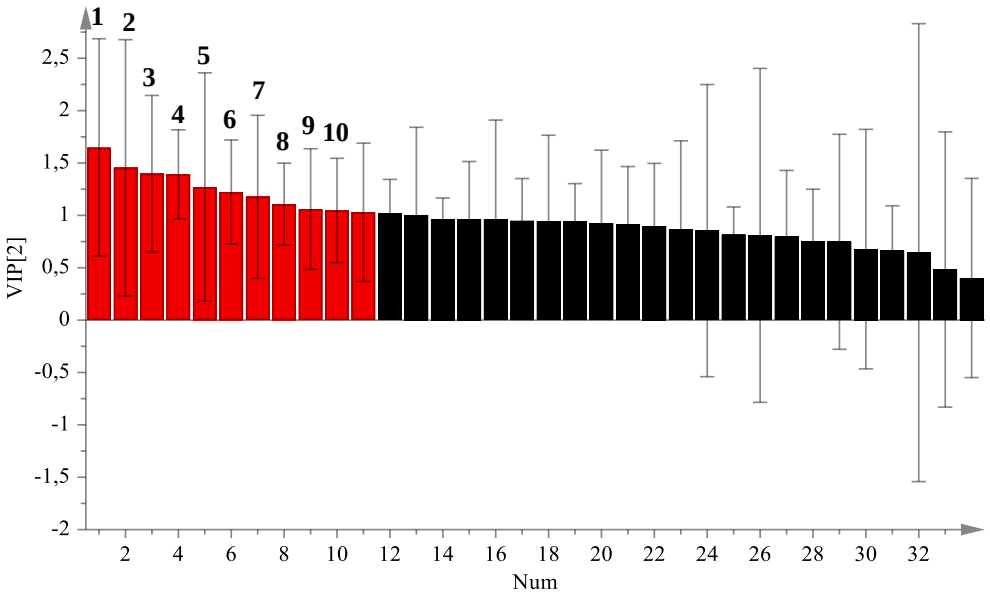


FIGURE 3.8- PLS-DA model of *C. limon*, control (C) and inoculated (I) leaves.

According to the VIP (*Variable Influence on Projection*), which is the most of significant influence on projection. It is a parameter used for calculating the cumulative measure of the influence of individual X-variables on the model. VIP values larger than 1 point to the most relevant variables, and generally VIP values below 0.5 are considered irrelevant variables (GALINDO-PRIETO et al., 2013). Thus, in the present analysis was considered VIP values > 1. Some of the variables that accounted more for the plot separation were correlated with isopulegol for the control samples and 3-carene, β-myrcene, β-linalool, α-terpineol and β-pinene for the inoculated samples, among other (FIGURE 3.9).



Compounds
1. 1-pentenol
2. 2-pentenol
3. 3-carene
4. Cyclohexane
5. Isopulegol (control samples)
6. β-myrcene
7.Trans-p-methadienol
8. β-linalool
9. α-terpineol
10. β-pinene

FIGURE 3.9- VIP (*Variable Influence on Projection*) model of *C. limon* (control and inoculated leaves), VIP> 1.

In *C. latifolia* species, the model could not be constructed, because the date did not reveal clustering or variation between samples, the PCA showed no differences between the inoculated and the control samples. This might mean there were no effect of the fungal interaction with this species, or at least they were not detectable by HS-GCMS. Furthermore, a PLS-DA model was constructed to further verify no differences between samples in this species. The model was not validated (R^2 X= 0.34, Q^2= 0.24 p > 0.05), thus confirming the results from PCA.

The foremost differences between susceptible and resistant species were the levels of β-pinene and D-limonene, which β-pinene was correlated with susceptible specie. ASAI et al., (2016) reported similar observations for citrus leaves from different stress treatments, in which D-limonene and β-pinene were identified as highlighted markers in *C. limon*. Moreover β-pinene has been associated with susceptibility of mango cultivars (AUGUSTYN et al, 2010). D-limonene is also reported as fungicide effect on *Alternaria spp*., *Fusarium spp.*, *Collethotricum spp*. and *Botrytis spp*. (SANTOS et al., 2010).

In summary, influences of mechanical infection/defense were observed. Our multivariate analyses data indicated that metabolic changes of volatiles in response to stress caused by inoculation of pathogen *Phyllosticta citricarpa* were different between citrus species and between control and inoculated samples of *C. limon.* Moreover, GLVs, hexanal, 2-hexenal, eucalyptol, β-linalool, α-terpineol, the monoterpenes 3-carene, isopulegol and β-myrcene were clearly affected, suggesting that these compounds may be candidates of the common stress biomarkers in citrus plants.

3.4- CONCLUSION

The advantages of used HS-GC-MS were the fast analyses, high separation efficiency and reproducible retention times, which allowed the identification of some target compounds. In addition, HS-GC-MS method developed and applied in this work, proved to be a simple, speed and convenient tool for the purpose of characterizing the volatile compounds from citrus species.

GC-MS associated MVDA provided satisfactory results and extremely useful to provide chemical information of citrus leaves. The main differences between the two species were the presence of β-pinene in susceptible *C. limon* and D-limonene in resistant *C. latifolia.* Moreover, significant changes were observed in the volatile metabolic profile of susceptible specie after inoculation of *P. citricarpa*, in which some VOCs were identified, and they may be candidates of the stress biomarkers in this species. On the other hand, *C. latifolia*, whose is a variety with high resistance to the fungus in the field, did not present significant changes in the metabolic profile after inoculation. This may be related with the influences of mechanical infection/defense of citrus plants. However, these experiments showed that several complex mechanisms may be involved in citrus plants defense.

4- MOLECULAR NETWORKING AS A TOOL OF SEARCH FOR NEW COMPOUNDS PRODUCED BY *PHYLLOSTICTA CITRICARPA*

Abstract

The study presents the development of *Phyllosticta citricarpa* in different culture media and molecular networking tools, in order to search for new compounds or that may be related to the control of citrus black spot. *P. citricarpa* was grown in four different culture media. The extracts were prepared and analyzed by UHPLC-MS. Afterwards, the data were exported and analyzed by molecular networking tools using the GNPS-platform. The preliminary results showed the molecular networking which contained over 2000 parent ions with a mass range of m/z 162-1264. Fifty-three putative compounds, including analogues, from different chemical classes, were annotated by the GNPS library. This result illustrated the diversity of this fungus metabolism and the potential for the possible discovery of new compounds produced by *P. citricarpa*.

Key words: GNPS-platform, Mass Spectrometry, metabolic profile.

4.1- INTRODUCTION

Microorganisms are excellent sources of bioactive compounds, which may be applied in different areas, such as pharmaceutical, agrochemical, food, and cosmetics (SHWAB and KELLER 2008, SUN et al., 2011). Understanding the mechanisms involved in a pathosystem is an important step to control diseases caused by *Phyllosticta* species. Experiments with pathogen culture filtrated have shown that tissue response *in vitro,* may correlate with disease reaction of the host species, and the isolated compounds produced by the phytopathogen, may allow to understand the virulence and pathogenicity mechanisms, as well as to select important traits to disease control (SAVI et al., 2018). Moreover, several bioactive metabolites and active extract are related as important biological activities (EVIDENTE et al., 2008; KUMARAN et. al, 2008; SAVI et al., 2019; SEVERINO, 2011).

4.1.1- OSMAC (One Strain Many Compounds) approach

One Strain Many Compounds (OSMAC) was proposed by BODE et al, (2002). This mechanism came about thanks to the observation of small changes in the growth conditions were able to alter the metabolic profile of many microorganisms. This approach is considered a powerful tool that may contribute significantly to elucidating the special metabolome of different microorganisms (GUBIANE, et. al, 2016). OSMAC consists of mimicking the environment, through changes in cultivation conditions, such as biotic or abiotic factors, variation in the concentration of carbon and nitrogen sources, changes in pH, salinity and the addition of precursors and inhibitors of metabolite biosynthesis of interest (GOGOI et al., 2008; AMARAL, 2014; MIAO et al., 2006). The biotic factor is based on the interactions between microorganisms in the environment,

which are related to the competition for space and nutrients and which is directly involved in the induction of special metabolites. In this way, the cultivation of one or more microorganisms in the same fermentation flask, for example, can lead to the production of new metabolites that were not previously observed in the individual cultures of each microorganism. In contrast, abiotic factors are related to chemical and/or physical changes in the growth medium, in which the changes related to physical parameters refer to temperature, aeration and light incidence (MIAO et al., 2006).

Although a systematic change in cultivation parameters may be applied to selected strains, the OSMAC approach is random and does not allow the development of common rules for all microorganisms, since each one will present a particular response defined by its genome. The subsequent objective of OSMAC is related to the identification of the genes responsible for the induction or alteration of the metabolic pattern, whose goal would be the evaluation and determination of which compounds in a given culture medium would be responsible for inducing the biosynthesis of unknown metabolites (BODE et al., 2002).

4.1.2- Molecular networking tool

Discovery of new bioactive metabolites has been a key role to achieve the holistic view of natural products chemistry. Analytical tools became useful in the bioprospecting programs to obtain accurate results, aiming mainly to increase the identification of new potentially active compounds. In this context, dereplication tools emerge as a rapid way of identifying known compounds in crude extracts, accelerating the selection of new and unknown compounds (SELEGATO et al., 2016).

The common and known way to performed replication of natural products is to establish the chemical profile of a single natural resource using a

hyphenated analytical system, as HPLC-MS, GC-MS, CE-MS and NMR, and direct comparison the obtained spectroscopic data with libraries of natural metabolites (HUBERT et al., 2015). However, the analytical approach remains challenging since the range of a plant or a fungal metabolome is still unknown and large differences in physicochemical properties exist among plant metabolites. Nowadays, the most powerful studies combine the advantages of MS and NMR spectrometry (WOLFENDER et al., 2009). Furthermore, natural product workflows typically incorporate LC-MS analysis, and MS/MS data because may be simultaneously acquired on most mass spectrometers and the introduction of molecular networking does not require significant adjustment of the current natural products discovery process (YANG et al., 2013).

Mass spectrometry-based molecular networking has been demonstrated for rapid chemical dereplication of complex crude fermentation extracts (PURVES et al., 2016). This approach relies on the observation that structurally similar molecules share similar MS/MS fragmentation patterns. Molecular networking is implemented in three fundamental steps: (i) MS/MS spectra are collected; (ii) a molecular network is generated using ‘cosine scores’ which measure relatedness in MS/MS spectra and can be visualized using Cytoscape, a popular bioinformatics package designed to visualize correlations of large datasets and finally (iii) the molecular network is analyzed through the visual representation of molecular relatedness, in other words, chemical similarity or match to the structurally related (YANG et al., 2013, SMOOT et al., 2011).

Global Natural Products Social Molecular Networking (GNPS) is an interactive online small molecule-focused MS/MS spectrometry data and analysis infrastructure. GNPS is currently widely used by scientific community in a wide variety of exploratory studies and has been used for the discovery of hundreds of new molecules in the past few years, ranging from immune regulators to

antimicrobials, including antiviral agents and protease inhibitors (ARON et al., 2020).

In this study mass spectrometry-based molecular networking- GNPS platform was used to investigate the metabolome of *P. citricarpa* grown in different culture media, in order to search for different metabolic profiles and to find new and possible bioactive compounds.

4.2- EXPERIMENTAL SECTION

4.2.1- Fermentation

Phyllosticta citricarpa strain, isolated from *Citrus sinensis* fruit at Graziela farm- Ibaté-SP, was provided by Fundecitrus. The cultivations were done in four different growth media: Potato Dextrose (PD), sabouraud (SB), malt + yeast (YMA) and rice. Tryptamine was used as source of nitrogen which 50 ml of solution was prepared in dimethyl sulfoxide (DMSO) at concentration of 5mM. The broth, PD, SB and YMA, were prepared separately and 300 mL of each medium and were transferred to 1 L Erlenmeyer. The culture media were dissolved, under constant agitation, in the appropriate proportion in distilled water. For the rice medium preparation, it was used 500 mL Erlenmeyer with 100g of the material in 75 mL of water. An aliquot of 2 mL from tryptamine solution was added to flasks with cultivation and culture media. All cultivations were done in triplicated, then all Erlenmeyer flasks were then autoclaved for 20 minutes, at 120 ° C and 1 atm of pressure to sterilize them. Afterwards, the flasks were transferred to the laminar flow hood, previously sterilized and, after the media reached room temperature, five discs of *P. citricarpa*, preserved in Petri dishes with potato dextrose agar, were transferred to the Erlenmeyer flasks, then incubated for 25 days.

4.2.2- Metabolite extraction

At the end of the incubation period, the PD, SB and YMA broth containing *P. citricarpa* mycelium (with and without tryptamine) and the control media, were crushed using an ultraturrax for 15 min. After trituration, the materials were vacuumed filtered to remove mycelium and extracted by a liquid-liquid partition with ethyl acetate (3 × 500 mL). To the flasks containing the rice cultivation, as well as the controls, were extracted in ethyl acetate (3 × 500 mL) and methanol (3 × 500 mL), respectively. After that, the solvent was evaporated and the extracts were then submitted to a clean-up process in cartridge filled with C-18 silica after reconstitution in methanol HPLC grade (Strata X, C18), followed by filtration in 0.22 μM membrane. In FIGURE 4.1 illustrates the experimental procedures for sample preparation.

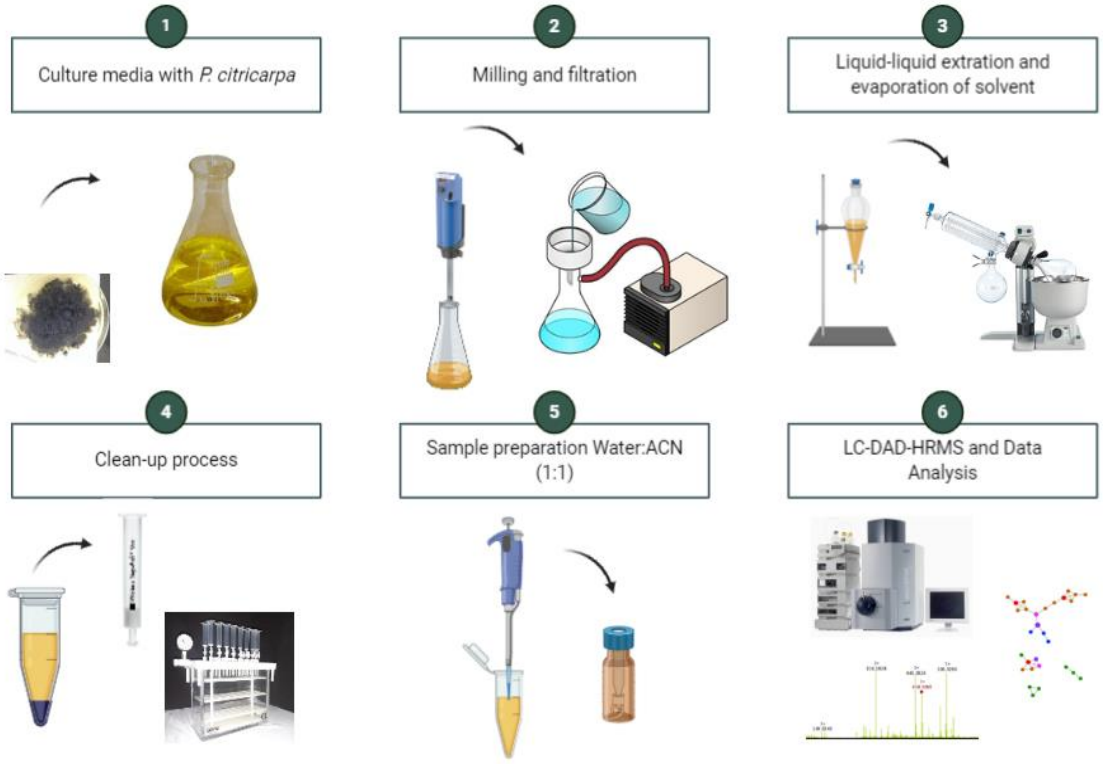


FIGURE 4.1- Overview of the experimental procedures for sample preparation. (**1**) Fungus in different culture media (**2**) Milling of liquid media containing *P. citricarpa* mycelium and filtration (3) liquid-liquid partition with ethyl acetate and evaporation of solvent (**4**) clean-up process with in SPE cartridge (**5**) sample preparation at concentration of 2 mg/mL in Water: ACN (1:1) (**6**) Analyzed by LC-HR-MS/MS and Data processing.

4.2.3- LC-MS data acquisition

To further investigate the metabolites produced by *P. citricapa* in different culture media, chromatography profiling of their crude extract was performed. All samples including the controls were prepared at concentration of 2 mg mL^{-1} in acetonitrile: water (1:1). High-resolution tandem mass spectrometry (HR-MS/MS) data were generated in Liquid chromatography with diode array detector model UFLC (Shimadzu), coupled to a mass spectrometer with electrospray ionization source (ESI) and Quadrupole-time-of-flight mass analyzer (Bruker Daltonics). The wavelength scan in the diode array detector was 190-400 nm. The races were acquired in a positive ionization mode. A m/z scan

50-1500 in centroid mode was used. The conditions of the ESI were capillary voltage of 3500 V, drying temperature of 220 ºC, 9 L min^{-1} of flow and 40 psi of the drying gas, a ramp of 20 to 75 eV, detecting signals above $1x10^3$. The chromatographic profiles were collected with a 150 mm x 3.0 mm column with 5 µm particles (Supelco Ascents Express C18) in gradient mode at a flow of 1.0 mL min^{-1} with the solvent system contain (A) ultrapure water and (B) acetonitrile, both with 0.1% formic acid, in which is presented in TABLE 4.1.

The temperature was maintained at 40 ºC. The flow was 0.7 mL min^{-1} and the injection volume was 10 µL. Analysis were performed randomly, and a solvent blank was included after every 10 runs of samples

TABLE: 4.1- Gradient system for HPLC-MS analyses.

Time	% A	% B
0.01	95	5
25	0	100
35	0	100
37	95	5
40.01	95	5

4.2.4- LC-MS data processing: Molecular networking analysis

HR-MS/MS raw data were acquired using DataAnalysis (Version 3.1). MS raw data files were converted from *.d* to *mzXML* file format using the *MSConvert* software (ProteoWizard). A molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu) (WANG et at., 2016). The data was uploaded using FileZilla. The data was filtered by removing all MS/MS peaks

within +/- 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 fragments ions in the +/- 50Da window throughout the spectrum. The data was clustered with MS-Cluster with a parent mass tolerance of 0.05 Da and an MS/MS fragment ion tolerance of 0.05 Da to create consensus spectra. A network was then created where edges were filtered to have a cosine score above 0.65 and more than 4 matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 0.8 most similar nodes. Finally, the maximum size of a molecular family was set to 100 and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were searched against GNPS spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.65 and at least 4 matched peaks. MolNetEnhancer tool was also used to classify the molecular families and subfamilies in the samples group according to the metabolite class (ERNST et. al., 2019). All data were imported into Cytoscape (Version 3.8.0) to visualization and displayed as a network of nodes and edges.

4.3- RESULTS AND DISCUSSION

4.3.1- Metabolic profile of *Phyllostica citricarpa* by HPLC-MS

The total ion chromatograms of representative *P. citricarpa* samples of each medium, revealed a greater number of chromatographic bands in the extracts produced of this fungus. The chromatograms presented different metabolic profile according to the type of culture medium. Little differences were observed between cultivation with and without tryptamine, however the extracts in rice contain the amino acid presented significant variation on the profile when compared with correspondent cultivations without amino acid (FIGURE 4.2).

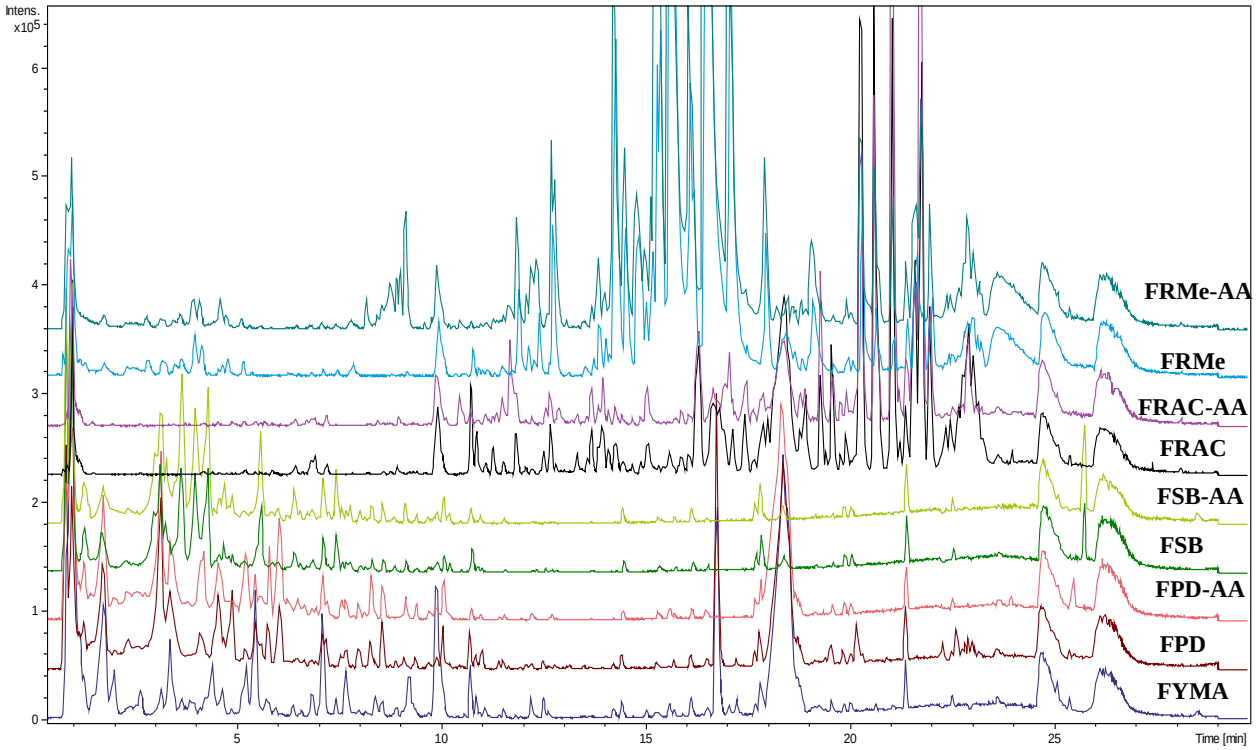


FIGURE 4.2- Total ion chromatograms of microbe extracts grown in different culture media. FYMA: Fungus in malt + yeast medium; FRMe: Fungus in rice extracted in MeOH; FRMe-AA: Fungus in rice and amino acid, extracted in MeOH, FRAc: Fungus in rice extracted in EtOAc; FRAc-AA: Fungus in rice and amino acid, extracted in EtOAc; FSB: Fungus in sabouraud medium; FSB-AA: Fungus in sabouraud medium and amino acid; FPD: Fungus in potato dextrose medium; FPD-AA: Fungus in potato dextrose medium and amino acid.

4.3.2- Molecular networking data analysis

Molecular networking of metabolite MS profiles from *P. citricarpa* grown up in different media, resulted in a molecular network of 2489 parent ions (nodes) and 3274 edges. The nodes are represented in different colors, which corresponds to the contribution of each culture media to produce the ion. Fifty-three putative compounds, including analogues, were annotated by the GNPS platform, they are presented in appendix B-1 of this thesis (pages 137 – 140). Twelve different chemical classes were annotated in the samples, by MolNetEnnhacer tool. Among them, alkaloids, and derivatives, benzenoids, lipids, organic nitrogen compounds, phenylpropanoids, among others (FIGURE 4.3).

Molecular networking resulted in dereplication of molecules, such as cholic acid, coproporphyrin I, some diketopiperazines, dipeptides, fatty acids, carboxylic acids, and derivatives. Analogues molecules of putative steroidal alkaloids were also annotated, among several others. Some compounds were characteristics from each culture media. Diketopiperazines, for example, were characteristics from PD, SB and YMA, while analogues compounds of steroidal alkaloids were annotated from rice medium with tryptamine (FIGURE 4.4).

The molecular network represents an overview of the chemical groups and their distribution among the samples. However, due to MS may generate different charge states and/or adducts with different ions for the same molecule, it should be considerate that the number of parent ions (nodes) in the network does not necessarily represent the number of metabolites present in the samples (BAUERMEISTER et. al, 2018). Three advantages of the molecular networking as a de-replication strategy are displayed here; (i) allows the simultaneous identification of known molecules and complex mixtures analogues; (ii) molecular networking is compatible with any MS ionization platform and (iii) it is easily incorporated into standard natural products discovery workflows (YANG et al., 2013). Furthermore, Comparison among MS/MS spectrum available in the GNPS platform is done by connecting spectra with an edge/line with statistical similarity, arranged them into groups, and separating groups that exhibited different fragmentation patterns. When the spectrum acquired present high similarity with an MS/MS spectrum from GNPS library, it is recognized and then annotated. Normally, molecules from the same chemical class or group with similar structures exhibit similar fragmentation patterns (BAUERMEISTER et al., 2018).

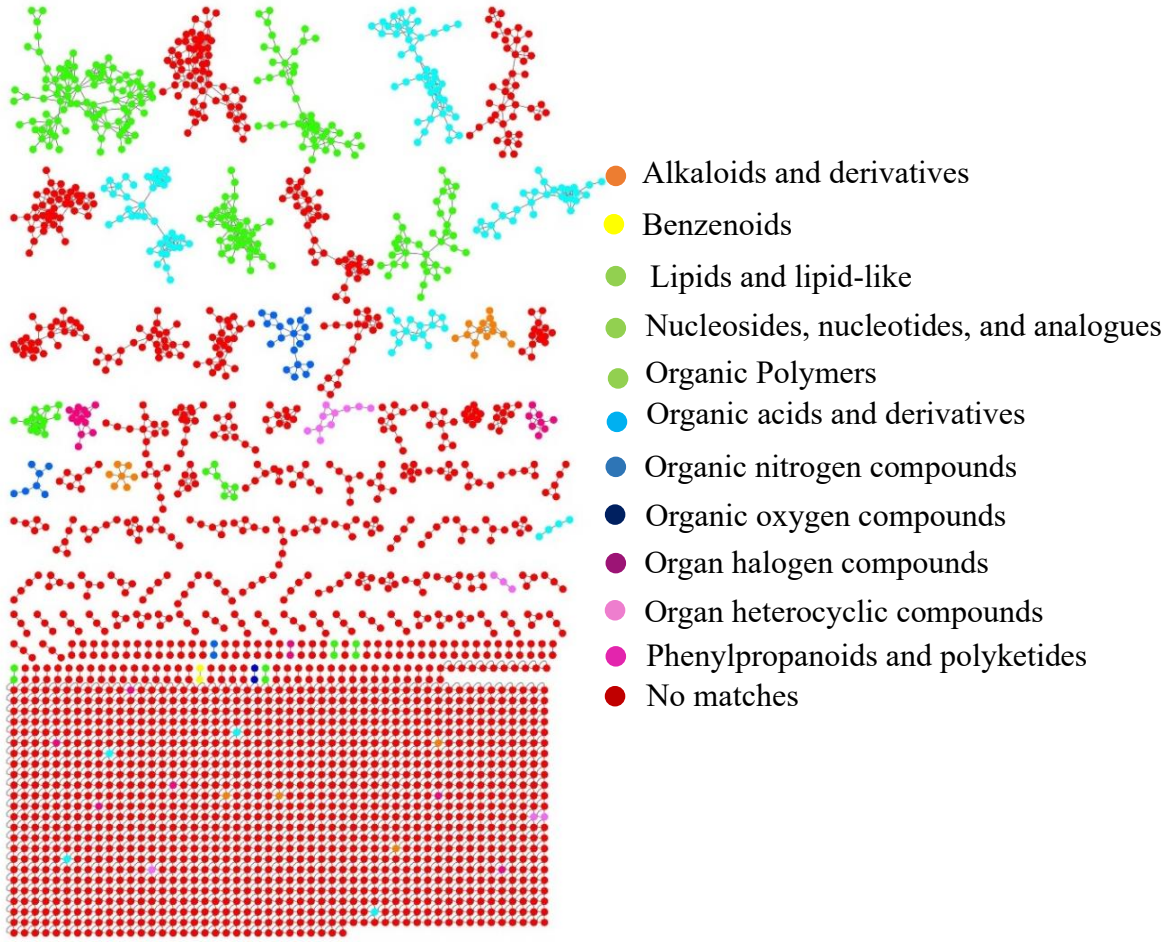


FIGURE 4.3- MolNetEnhancer used to classify chemical families of compounds produced by *Phyllosticta citricarpa*. Structural annotation was suggested based on MS fragments. The classes of metabolites are highlighted and the overview is summarized at this network.

Dipeptides and diketopiperazines (DKPs) such as cyclo (Phe-Leu) m/z 261.12, cyclo (Pro-Leu) m/z 211.14, cyclo (Phe-Tyr) m/z 311.13, cyclo (Pro-Phe) m/z 245.12, fruleuIle m/z 407.23, phenylalanylisoleucine m/z 279.11 and phenylalanylvaline m/z 265.14, were annotated and identified in the extracts, most of them from PDA, SB and YMA cultivations. The MS/MS spectra from dipeptides and DKPs are located on appendix section (Appendix B-2, pages 141-145).

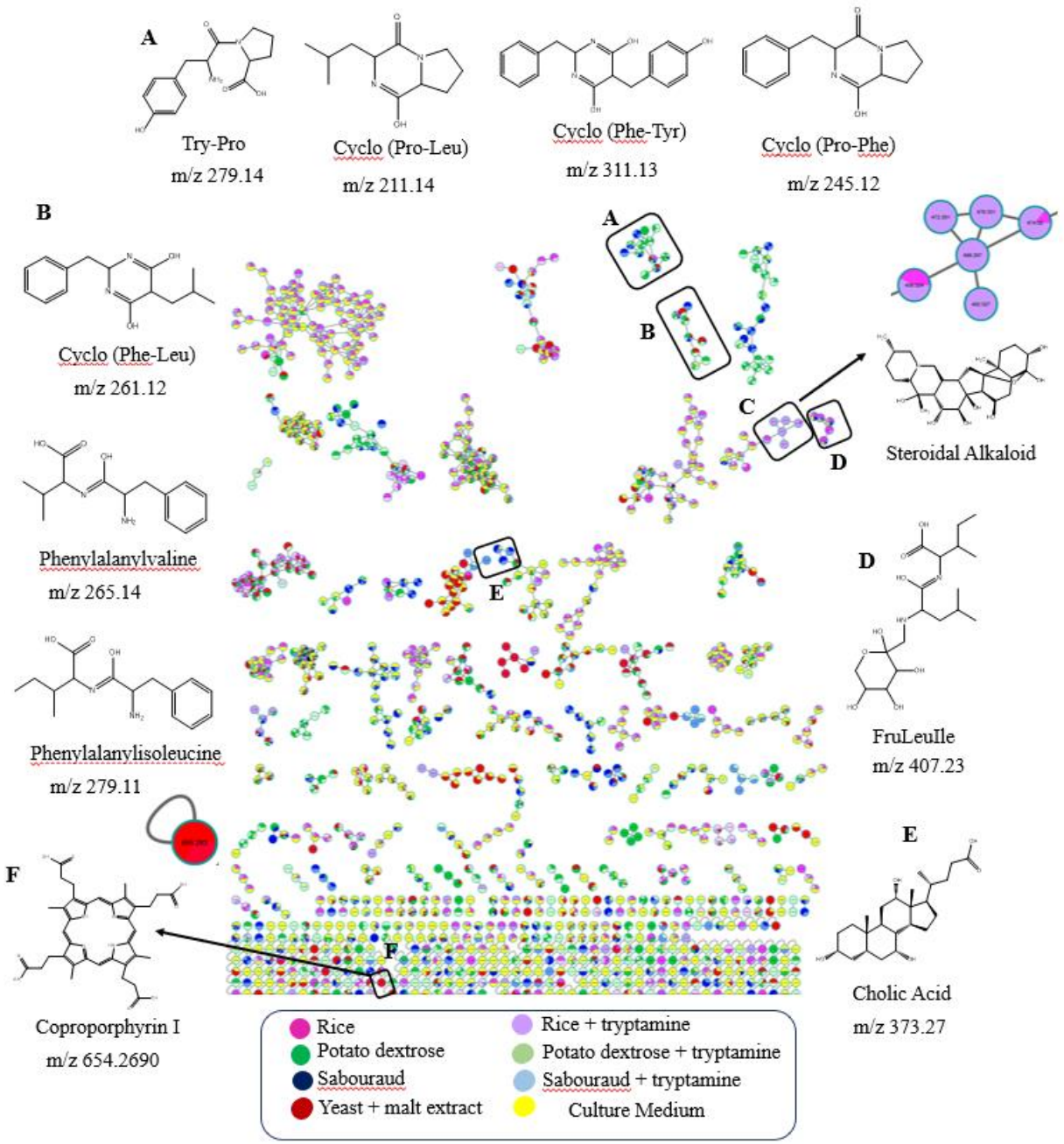


FIGURE 4.4- Molecular networking of extracts from *Phyllosticta citricarpa* and some chemical compounds GNPS matches.

DKPs are cyclic dipeptides that have been isolated from microorganisms (STROM et al., 2002), sponges (MITOVA et al., 2004) and from body fluids (KIBRICK et al., 1965). These heterocyclic compounds have several biological properties, such as antibacterial, antifungal, antitumor, antiviral and plant growth regulation (FURTADO et al., 2005). However, the origin of DKPs

has been questioned once several cyclic dipeptides have been found in fermentation broths and cultures of yeast. DKPs may be generated via non-enzymatic cyclization of linear dipeptides at extremes of temperatures (HOLDEN et al., 1999; FURTADO et al., 2005). Nonetheless, these compounds were detected in clusters from culture media with the fungus. The cyclic dipeptides cyclo (Phe-Tyr), cyclo (Pro-Phe) and cyclo (Pro-Leu) have been previously isolated from *G. citricarpa* (sexual phase) (SEVERINO, 2011).

The annotation of putative alkaloids could be from compounds of the same group or chemical family that present similar structures and fragmentation, since the MS/MS spectra presented similar fragmentation, with a cosine score of 0.86 and a gold match in GNPS platform (FIGURE 4.5). Alkaloids such as Taxol was already reported from *P. citricarpa* (KUMARAN et al., 2008). These compounds may be a new group of compounds synthetized by *P. citricarpa*.

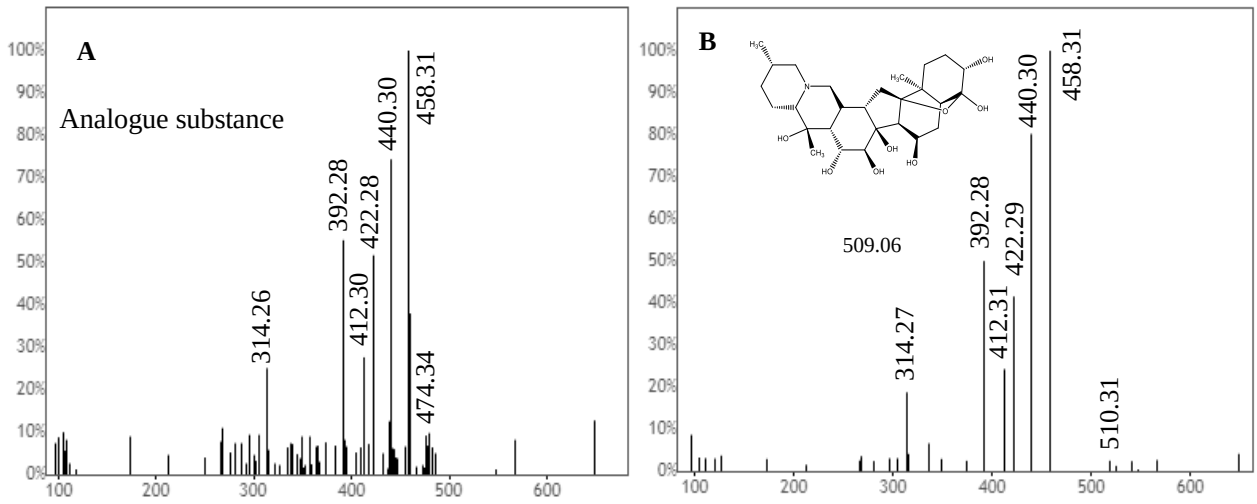


FIGURE 4.5- Steroidal alkaloids: (**A**) MS/MS parent ion fragmentation for this study (**B**) MS/MS parent ion fragmentation for the molecular networking standard.

Bioactive metabolites have been reported from *Phyllosticta* species. Compounds, such as phyllostoxin and phyllostine were isolated from *P. cirtsii*

showing potential mycoherbicide to *Cirsium arvense* biocontrol (EVIDENTE et al. 2008). Promising MIC values from the extract and fractions were obtained from *G. citricarpa* against *Xyllela fastidiosa* which the extract active contained compounds such as DKPs that were related to bactericides and antitumoral activities (SEVERINO, 2011). Therefore, the current study was useful to show new metabolic profiles from *P. citricarpa* and the possibility to find new compounds produced by this fungus.

In addition, in the GNPS database, many clusters in the networking were not annotated by the GNPS library (FIGURE 4.6), suggesting that there are many metabolites produced by *P. citricarpa* that have not been deposited in the GNPS library or are novel metabolites that have not been characterized yet. Therefore, new studies in this regard could be carried out, in order to search for new compounds and also, to contribute to the understanding of the phytopathogen/plant relationship.

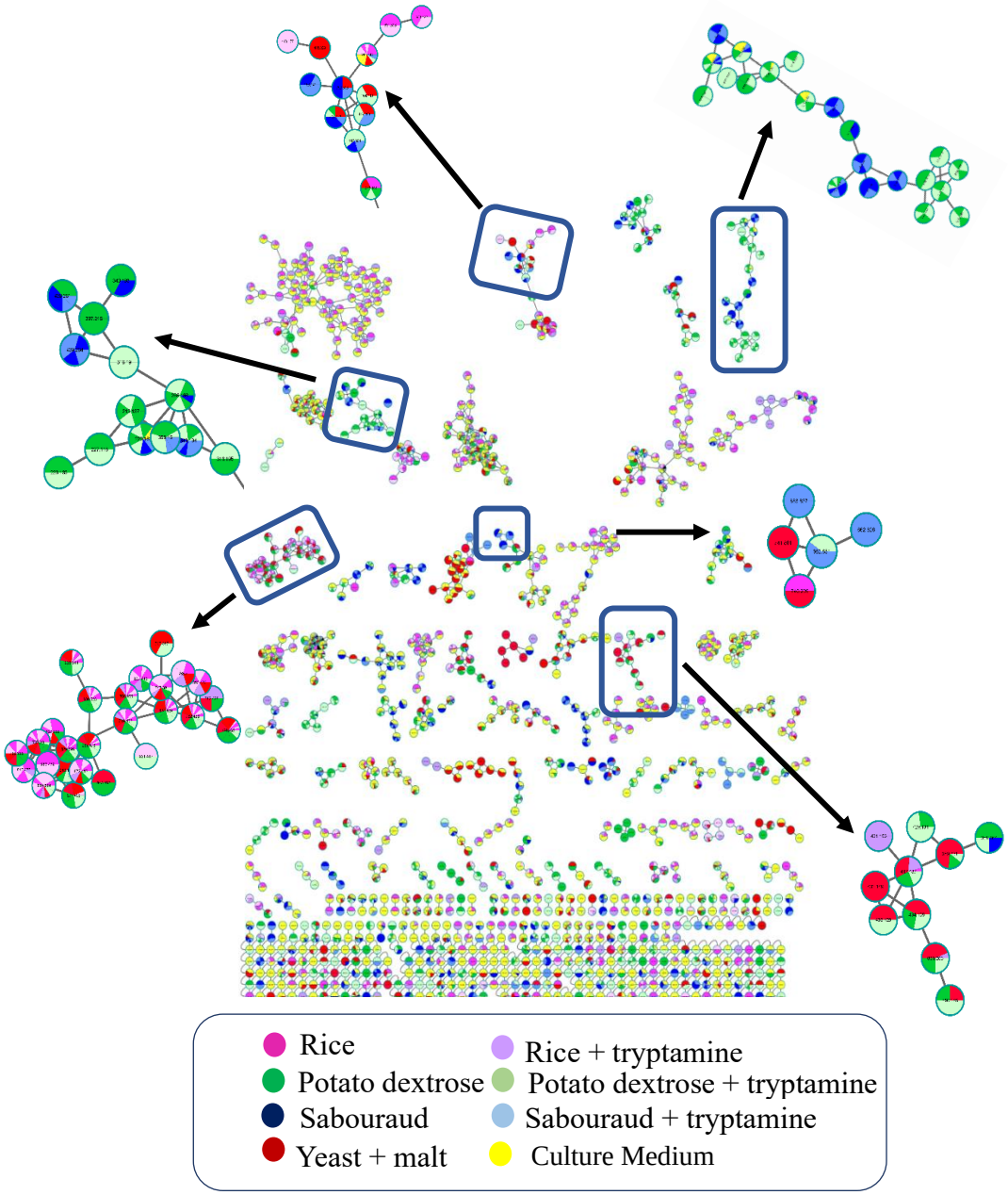


FIGURE 4.6- Clusters from molecular networking of extracts from *P. citricarpa* non annotated by GNPS library.

4.4- CONCLUSION

This chapter showed a brief search of different metabolic profile from *P. citricarpa*, in order to find new and possible bioactive compounds. In the current study, it was dereplicated fifty-three molecules including analogues, and

to our knowledge, it is the first to use molecular networking to find new metabolic profiles from this fungus.

Mass spectrometry-based molecular networking was a useful tool applied to this study and the methodologies developed for the analyses showed promising results, whose preliminary results were satisfactory for the purpose presented here. Since several compounds were annotated by the GNPS library, this enabled to know the diversity of structural classes that can be produced by *P. citricarpa*, besides the perspectives of the discovery of new compounds produced by this pathogen.

Therefore, this study brings a new approach to search in natural products focused on different methodology finding new compounds from *P. citricarpa*.

5- GENERAL DISCUSSION

It is known that, throughout their life cycles, plants react differently to various biotic and abiotic stresses, such as salinity, drought, extreme temperatures, heavy metal, nutrient deficiency, UV radiation and infection by pathogens (DAR et al., 2016). However, changes in primary metabolism are the commonest response, it includes changes in the levels of sugars, alcohols, organic acids, and amino acids, exhibiting common characters in stress responses. (KHAN et al., 2020).

The amino acid proline, for example, plays a key role in osmoregulation and bioenergetic of the cell (KHAN et al., 2018), it is generally associated with the osmotic adjustment and protection of subcellular structures during stress suffered by the plant, besides, the accumulation of proline has been observed in response to the abiotic and biotic stress in many plant species, including citrus plants in response to stress caused by pathogens (CEVALLOS-CEVALLOS et al., 2010; FREITAS et al., 2015; KILLINY et al, 2017).

In Chapter 2, our results indicated that some primary metabolites as proline might be involved in the defense mechanism of *Citrus latifolia* against *P. citricarpa*. Through our multivariate data analysis, proline and other primary metabolites were correlated to the metabolic alteration in inoculated samples from *C. latifolia* (resistant species). These metabolic changes in some amino acids, like proline amount in the inoculated leaves of *C. latifolia* remain uncertain and may indicate a specific plant-pathogen interaction that may specifically modifies this metabolic pathway. Therefore, further studies on the precursors and activities of the key enzymes in the metabolism of proline, are necessary to understand and describe the role of these amino acids in the response to citrus black spot, as well as other citrus disease (FREITAS et al., 2015).

Furthermore, plants also show alterations in the level of secondary metabolites when exposed to stresses and these alterations vary according to

species and to stress type (KHAN et al., 2018). These metabolites play an important role in defense activities or may act as precursors to physical defense systems. In plant–microbe interactions, phenolic compounds, for example, also play a role in signaling, in which, several studies have reported that flavonoids and polymethoxylated flavones may act as phytoanticipins and be involved in the natural defense of citrus fruit against pathogen infection (ORTUÑO et al., 2008; Del RÍO et al., 2004; BALLESTER et al., 2012). Moreover, citrus plants may accumulate compounds such as the coumarins, in response to a pathogen attack (BALLESTER et al., 2012), and they may also act as phytoanticipins, that are plant antibiotics presented in tissue before infection, serving as the basis of pest tolerance (JOGAIAH and ABDELRAHMAN, 2019).

Despite the accumulation of primary metabolites, our results also indicated that coumarins and flavonoids might be involved in the defense mechanism of *Citrus latifolia* against *P. citricarpa*. Some coumarins were identified as target compounds correlated to resistant species and may be related to citrus defense mechanism.

Coumarins and furanocoumarins have been correlated to the defense against various pathogens in citrus plants (BOURGAUD et al., 2006). Scoporane, for example, has been reported as the main phytoanticipins of citrus in response to various plant pathogenic fungi, such as *Phytophthora citrophthora* (AFEK and SZTEJNBERG, 1988), *Guignardia citricarpa* Kiely (DE LANGE et al., 1976), *Penicillium digitatum* Sacc., and *P. italicum* (KIM et al., 1991; ARRAS et al., 2006), *Diaporthe citri* (Faw.) Wolf (ARIMOTO et al., 1986), and *Botrytis cinerea* (Persoon) (KUNIGA et al., 2015).

Other coumarins, such as scopoletin (6-methoxy-7-hydroxycoumarin), xanthyletin (6,7-dimethylpyranocoumarin) and umbelliferone (7-hydroxycoumarin) were also found in citrus tissues induced by pathogens (like *Phytophthora* spp and *P. digitatum*) (KHAN et al., 1985; AFEK et al., 1999).

RAMÍREZ-PELAYO et al. (2019), reported that bergaptene and limettin (citropten) exhibited the highest inhibitory effects against *Colletotrichum* sp., which was even greater than those of known scoparone and umbelliferone. They also showed that the mixture of bergaptene and limettin displayed even greater antifungal effect than the individual compounds. Auraptene has also shown growth inhibitory effects against *Pseudomonas aeruginosa*, *Escherichia coli* B and *Aspergillus niger* (NAKATANI et al., 1987). Therefore, our results described in Chapter 2 are promising, since that isolated coumarins could be involved in the defense mechanisms of *C. latifolia*. The results are hopeful for searching biopesticides.

Currently, the search for alternative solutions to the use of chemical fungicides is of major interest. As such, several studies have focused on attempts to use Volatile organic compounds (VOCs) or plant essential oils (EOs) for the control of fungal development to maintain the fruit quality during storage. In parallel, in plants that accumulate natural VOCs at high concentrations, it is important to understand the action of these compounds in the interaction with plant-pathogens (RODRIGUES et al., 2018). VOCs are widely emitted from plant organs and have a potential application in agriculture, these compounds play an important role in plants defense against abiotic and biotic stress. Some VOCs are also related as antimicrobial that protects the plants against pathogens attack (RIFFEL and COSTA, 2015). Besides, plant volatiles compounds may provide important clues to the biochemical processes of a plant and more importantly, they may be critical chemical cues that enable a plant to communicate with other plants, insects and/or microbes (BECK et. al, 2014).

In citrus plants, the volatile constituents are a mixture of monoterpene (mainly limonene) and sesquiterpene hydrocarbons and their oxygenated derivatives including aldehydes, ketones, acids, alcohols, and esters. Among these components, limonene is the major compound, and its content varies

significantly among the citrus species and/or varieties. In addition, citrus essential oil also presents a mixture of VOCs, and it has been shown significant antifungal activities against several microorganisms, such as *Aspergillus niger, Penicillium chrysogenum*, *Fusarium proliferatum*, *Alternaria alternata, Trichoderma viride* and others (JING et al., 2014).

In that context, in Chapter 3 we presented the chemical investigation of volatile compounds of citrus plants in response to pathogen *P. citricarpa,* to aim source for putative biomarkers that are involved in the defense mechanism of citrus plants against the fungus. The metabolic changes of VOCs, by inoculation factor, were observed mainly in the susceptible species, *C. limon,* which through of chemometric tools, various compounds, such as alcohols, monoterpenes and sesquiterpenes were correlated to differences between inoculated and control samples, suggesting that they may be candidates to biomarkers. In contrast, the resistant specie, *C. latifolia,* did not show significant differences. When compared between species, the results showed that β-pinene and D-limonene were the main compounds correlated with species differences, besides other compounds, such as 3-carene, eucalyptol, sabinene, caryophyllene and β-bergamotene, which may be associated with susceptibility and resistance of these species.

Nevertheless, in plants, a complex array of defense response is induced after the detection of microorganism via recognition of elicitor molecules released during plant–pathogen interaction. Hence, plant-microbe interaction network-based analysis is a holistic approach that can enable a detailed understanding of the relationships between plants and microbes (PEYRAUD et al. 2017; JOGAIAH and ABDELRAHMAN, 2019).

Our results provide information that bring perspectives in new studies of compounds employed in defense of citrus plants against *Phyllosticta citricarpa,* whose compounds (related in this study) may be possible biomarkers with a potential to become biopesticides. Furthermore, the application of ^{1}H

NMR, LC-MS, HPTLC and GC-MS metabolic profile on the same biological sample provided complementary information illustrating the value of this integrated approach in the study of metabolic changes in plant-pathogen interactions.

In the last chapter, we described a brief metabolomic study of *Phyllosticta citricarpa* using molecular networking approach. In order to investigate the metabolites produced by *P. citricarpa* grown different culture media, we employed metabolomic tools such as molecular networking, an online molecular tool at Global Natural Products Social Molecular Networking (GNPS), to aim to search new metabolic profile and possible bioactive compounds.

MS-based metabolomics has been widely employed in the investigation of microbial metabolite content owing especially to its high sensitivity, which enables the detection of molecules even at low concentrations in complex samples, besides allowing the rapid analysis of a large range of chemical classes in complex samples. Since that similar chemical structures may present a similar fragmentation pattern, molecular networking tool allows the clustering of such related chemical structures based on their spectral similarities. Furthermore, by comparison with a library, this tool also helps to accelerate the dereplication process of complex samples such as microbial crude extracts (WANG et al., 2016, BAUERMEISTER et al., 2018).

The study showed different chromatographic profile to different extract containing *P. citricarpa* and culture media and significant differences were observed in the rice extract with tryptamine, which the cluster was annotated by GNPS library as putative analogues of steroidal alkaloids. In addition, several compounds, such as cholic acid, coproporphyrin I, some fatty acids and carboxylic acids were also annotated by GNPS library. Diketopiperazines were also annotated and identified in most of the culture media. Diketopiperazines are a group of biologically activity compounds produced by several microbes. They

consist of residual of two amino acids and mevalonic acid and some of them present important biological activity, such as antibiotics and antitumor activities (KUMAR et al. 2018). Moreover, different chemical classes were classified in extracts samples, showing the metabolic diversity of this pathogen. Therefore, these preliminary results may provide new proposals of research into bioactive molecules.

Nevertheless, the metabolomic study of *P. citricarpa* using OSMAC approach and molecular networking enabled to know the diversity of structural classes produced by this fungus and this study may open the way to new research ideas into natural products and, as well, contribute to the understanding of the phytopathogen/plant interaction.

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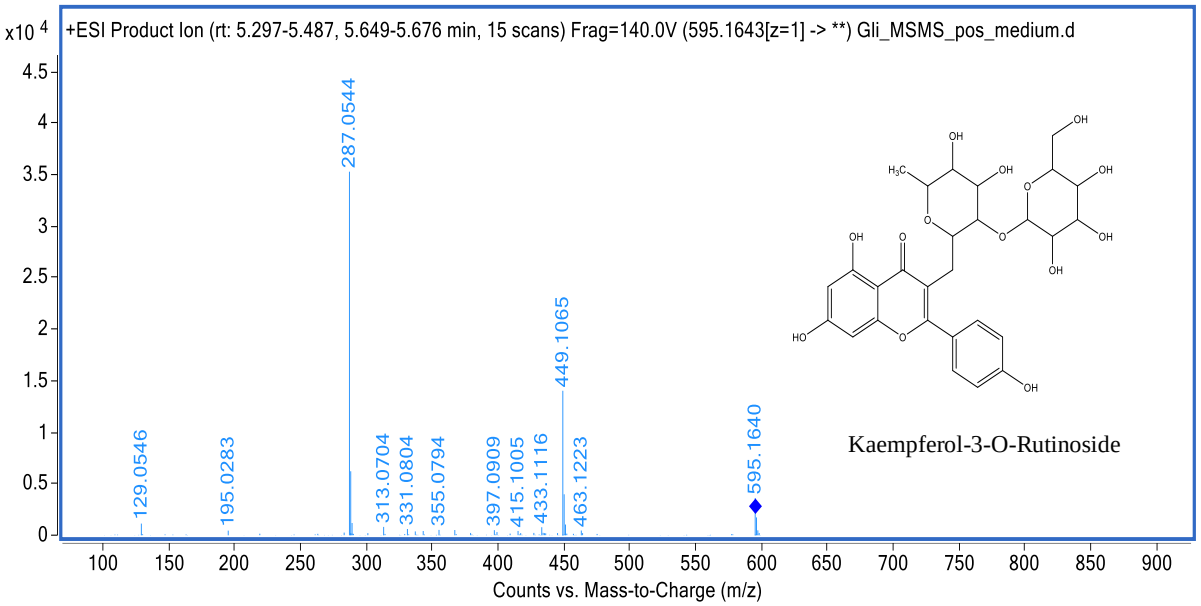
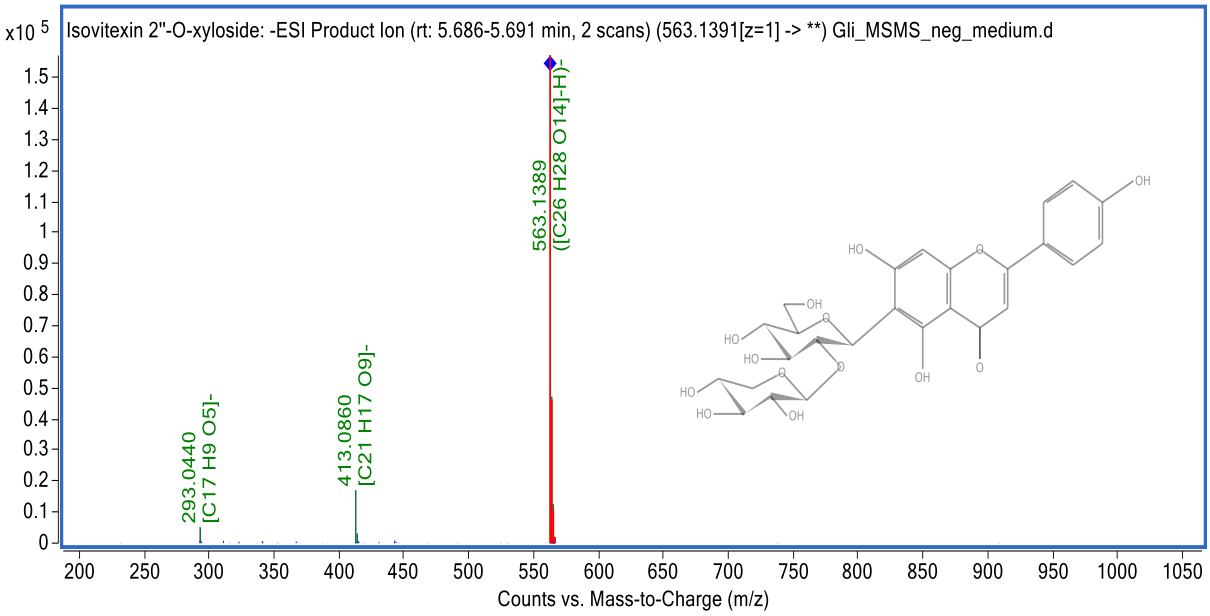
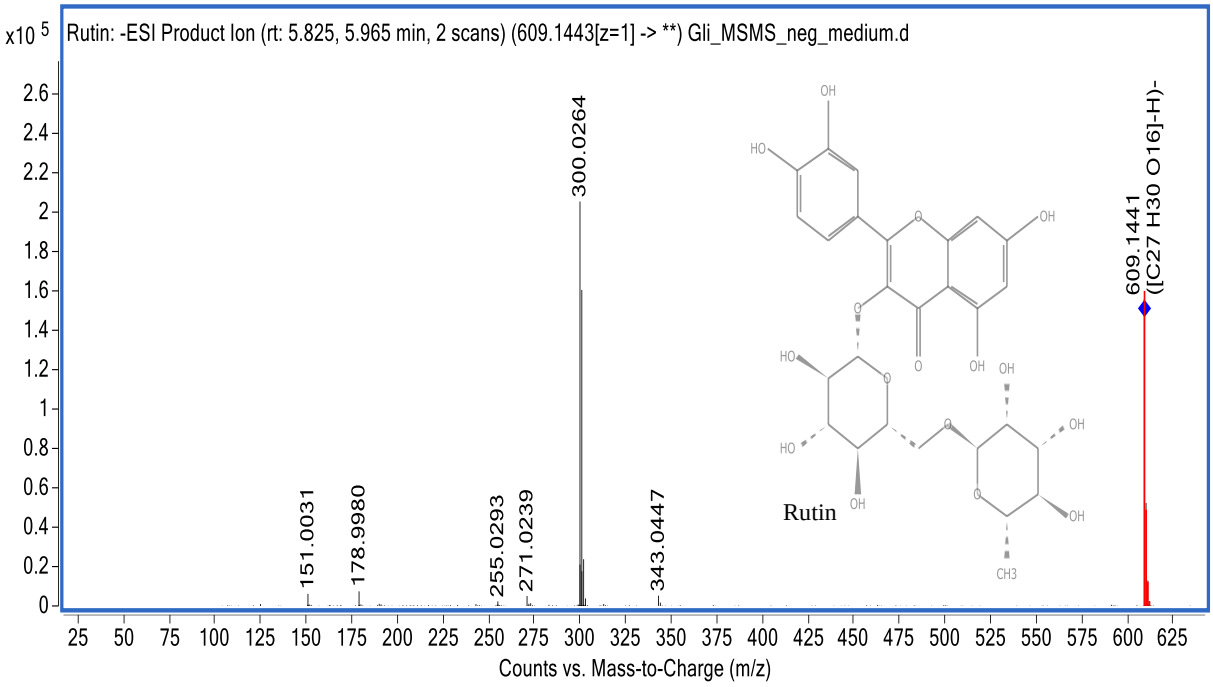
Appendix

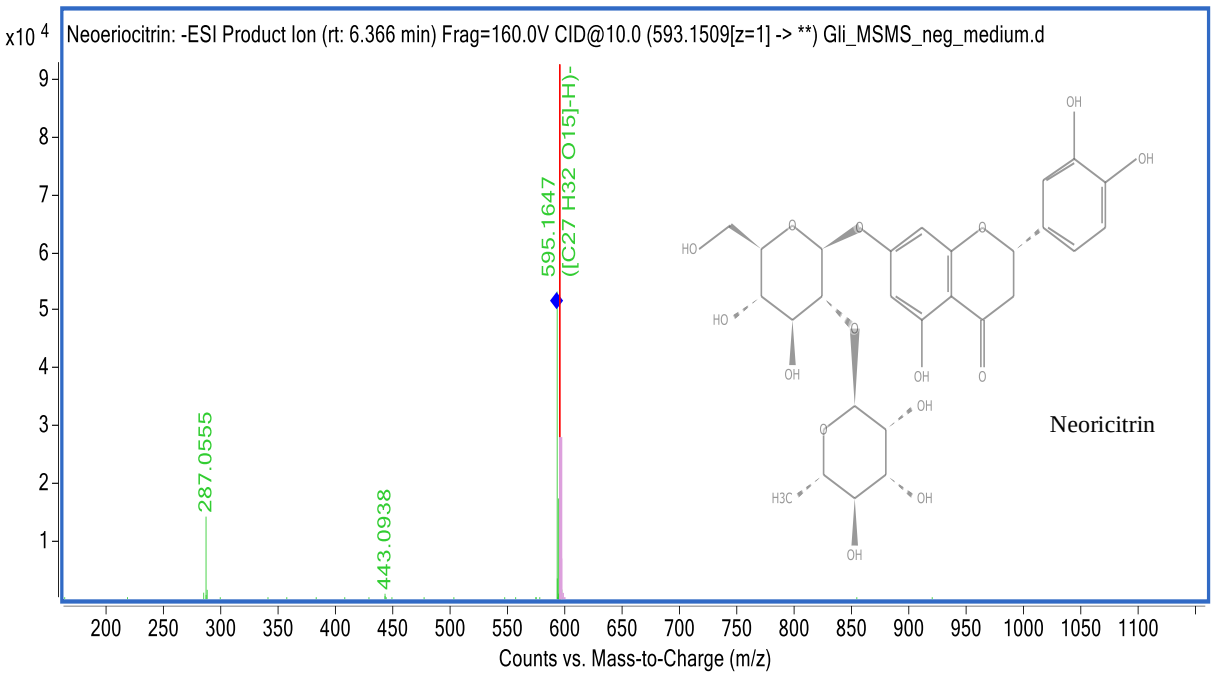
Appendix A- Supplementary information – Chapter 2

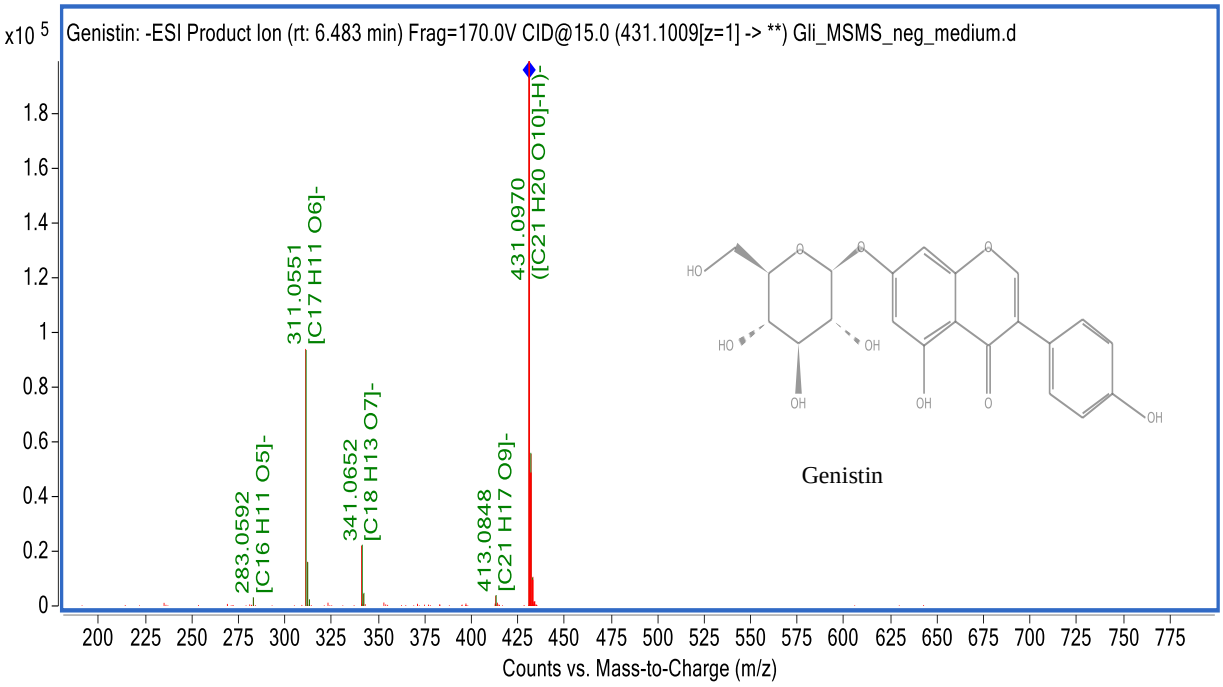
A.1- MS spectra of compounds tentatively identified from *C. latifolia*



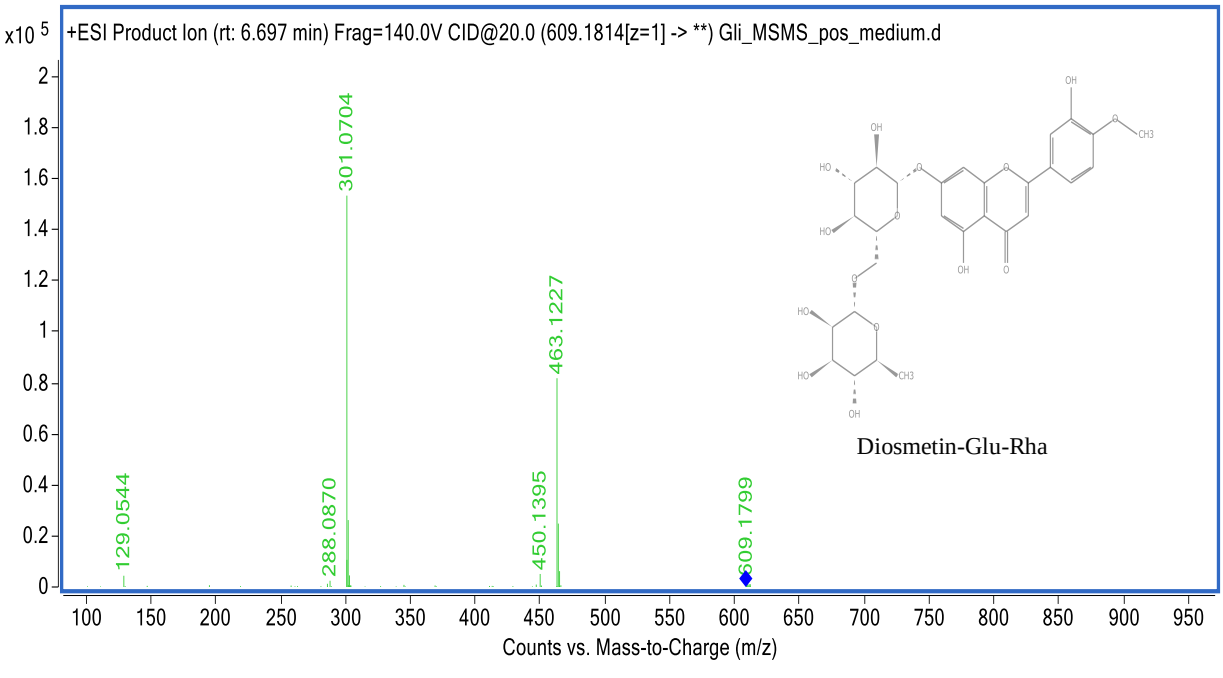



x10 5
Rutin: -ESI Product Ion (rt: 5.825, 5.965 min, 2 scans) (609.1443[z=1] -> **) Gli_MSMS_neg_medium.d
300.0264
609.1441
([C27 H30 O16]-H)-
151.0031
178.9980
255.0293
271.0239
343.0447
Rutin
Counts vs. Mass-to-Charge (m/z)

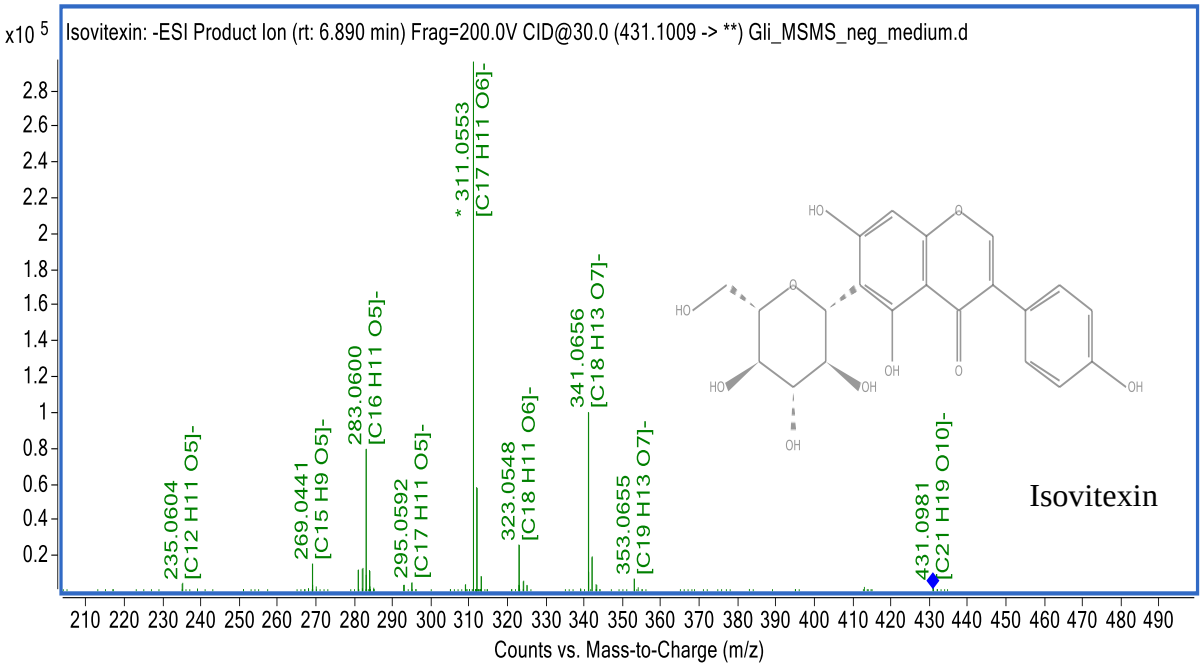

x10 4
Neoeriocitrin: -ESI Product Ion (rt: 6.366 min) Frag=160.0V CID@10.0 (593.1509[z=1] -> **) Gli_MSMS_neg_medium.d
595.1647
([C27 H32 O15]-H)-
287.0555
443.0938
Neoricitrin
Counts vs. Mass-to-Charge (m/z)

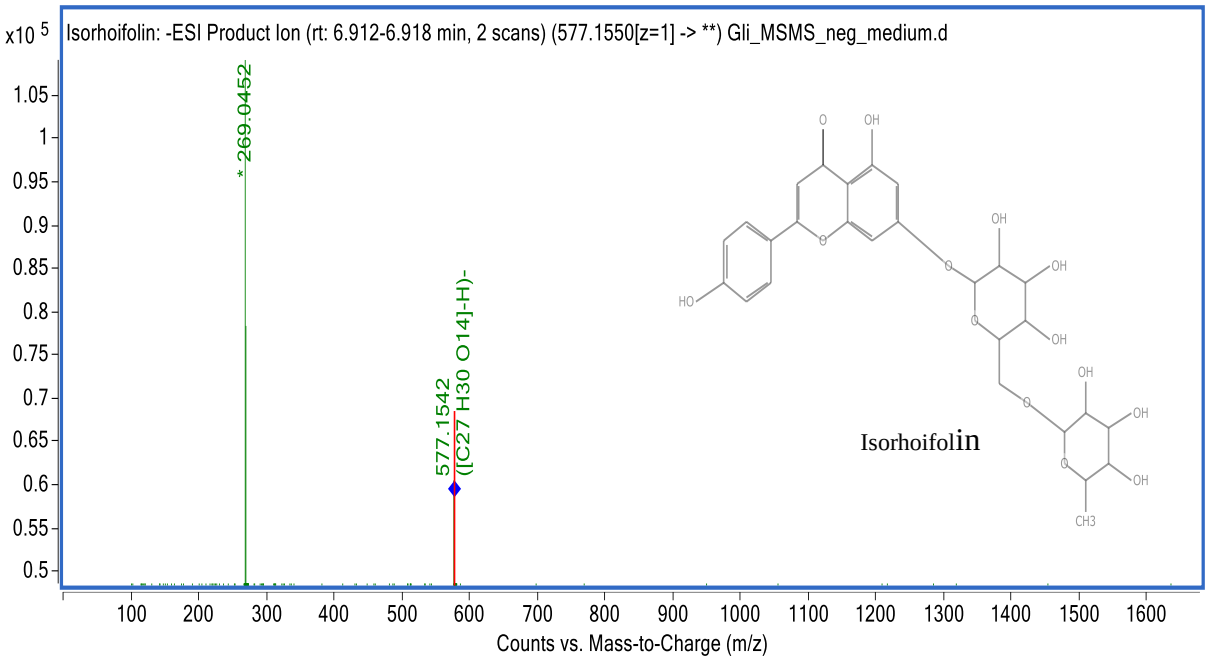


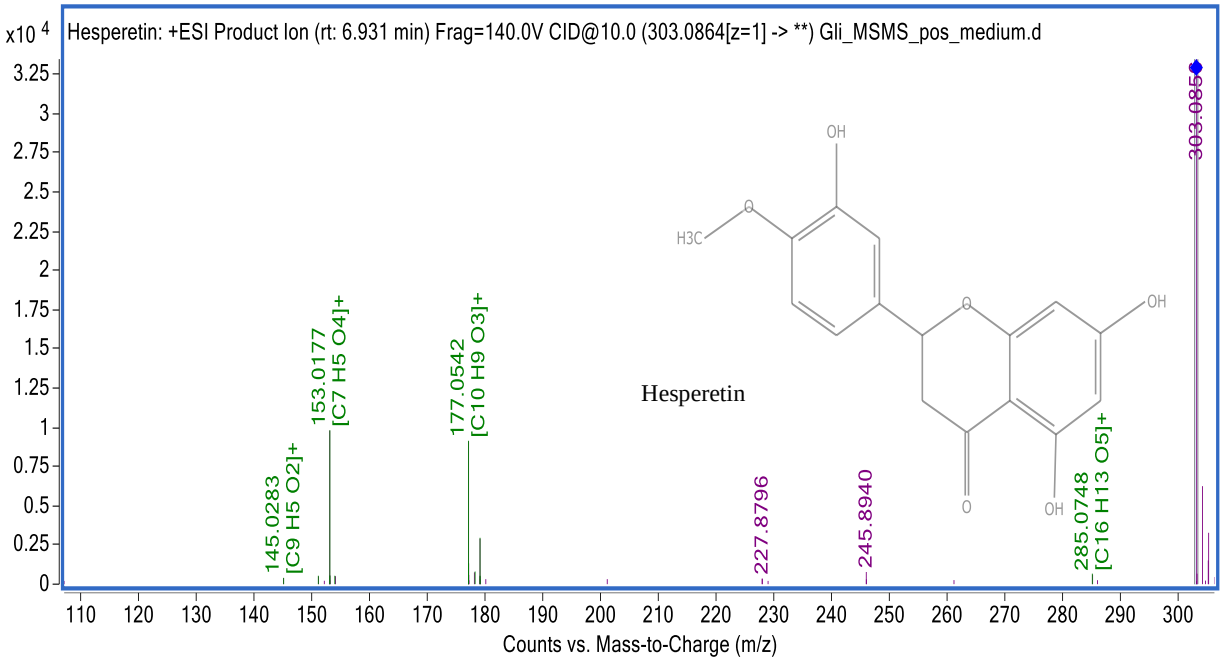
Genistin

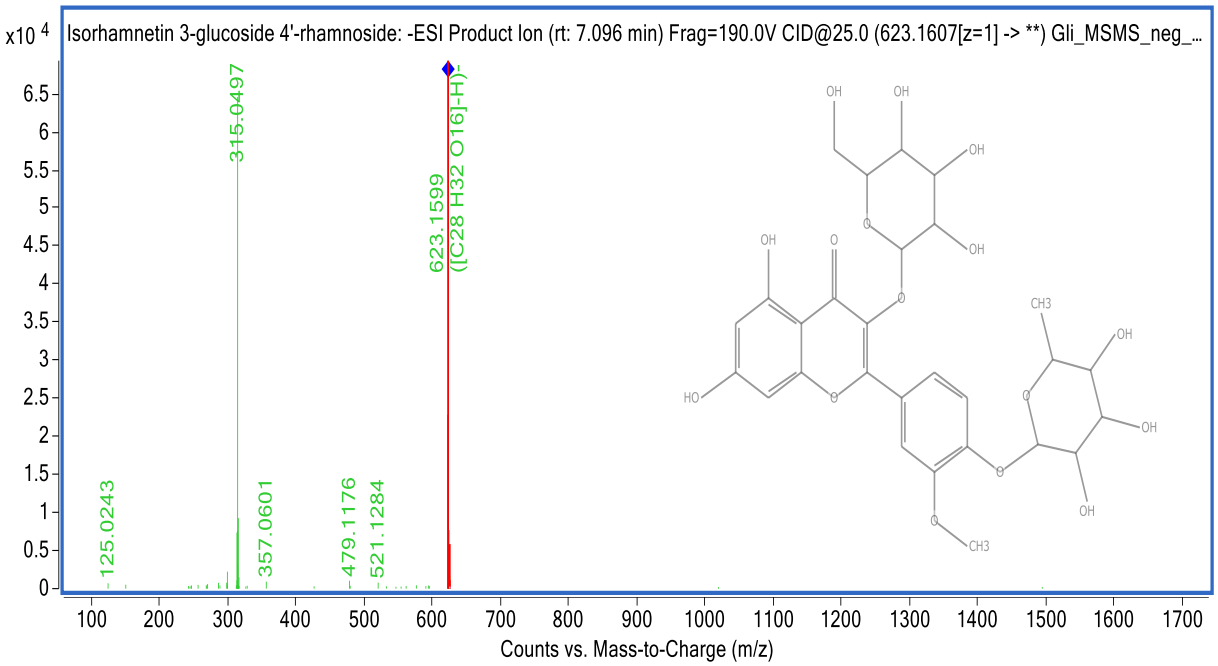


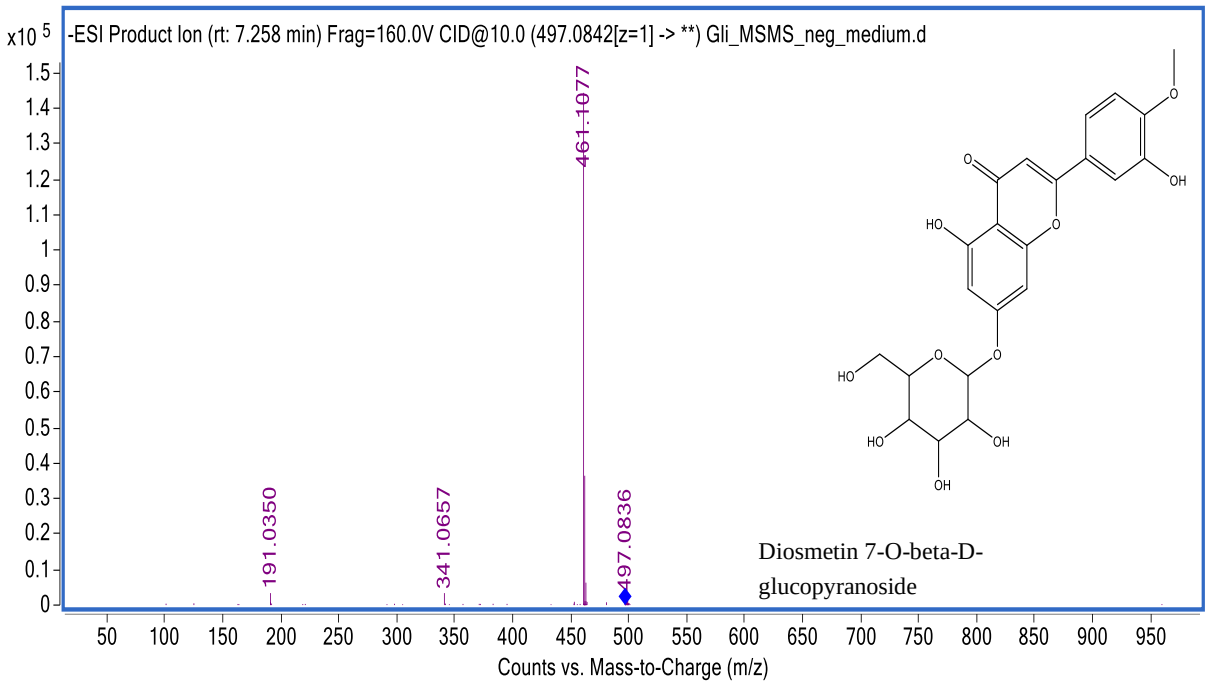
Diosmetin-Glu-Rha


x10 5
Isovitexin: -ESI Product Ion (rt: 6.890 min) Frag=200.0V CID@30.0 (431.1009 -> **) Gli_MSMS_neg_medium.d
235.0604 [C12 H11 O5]-
269.0441 [C15 H9 O5]-
283.0600 [C16 H11 O5]-
295.0592 [C17 H11 O5]-
* 311.0553 [C17 H11 O6]-
323.0548 [C18 H11 O6]-
341.0656 [C18 H13 O7]-
353.0655 [C19 H13 O7]-
431.0981 [C21 H19 O10]-
Isovitexin
Counts vs. Mass-to-Charge (m/z)

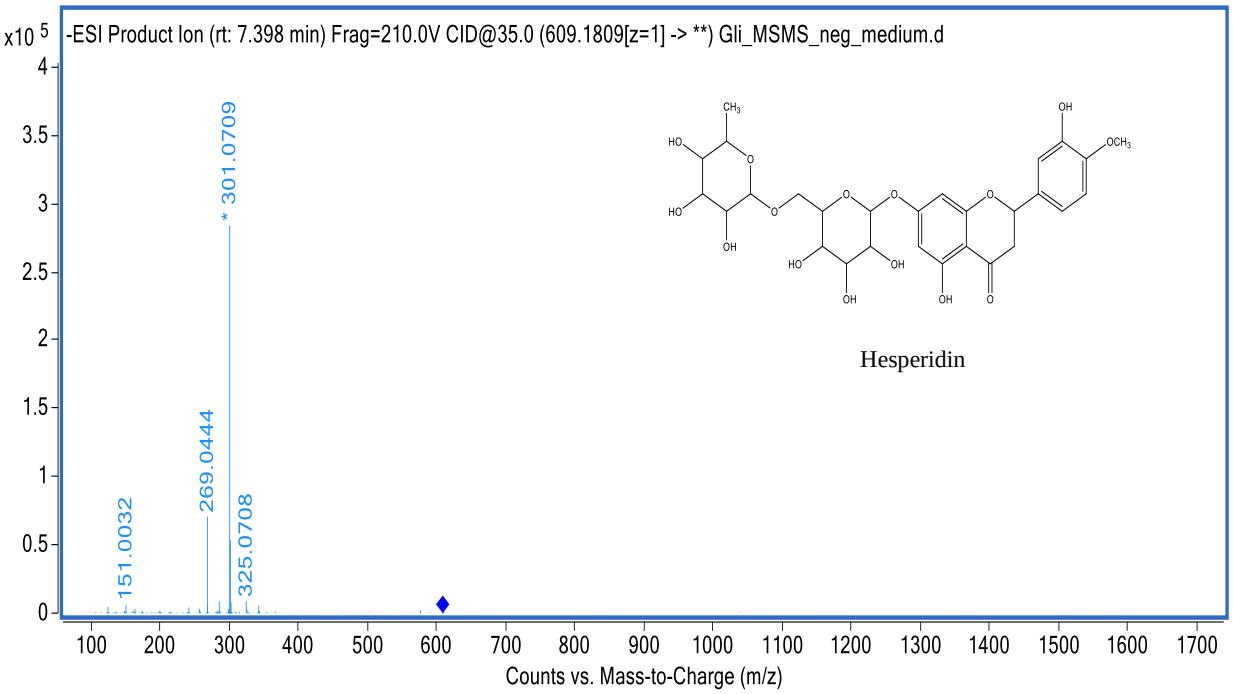

x10 5
Isorhoifolin: -ESI Product Ion (rt: 6.912-6.918 min, 2 scans) (577.1550[z=1] -> **) Gli_MSMS_neg_medium.d
* 269.0452
577.1542 ([C27 H30 O14]-H)-
Isorhoifolin
Counts vs. Mass-to-Charge (m/z)


x10 4
Hesperetin: +ESI Product Ion (rt: 6.931 min) Frag=140.0V CID@10.0 (303.0864[z=1] -> **) Gli_MSMS_pos_medium.d
145.0283 [C9 H5 O2]+
153.0177 [C7 H5 O4]+
177.0542 [C10 H9 O3]+
227.8796
245.8940
285.0748 [C16 H13 O5]+
303.0855
Hesperetin
Counts vs. Mass-to-Charge (m/z)

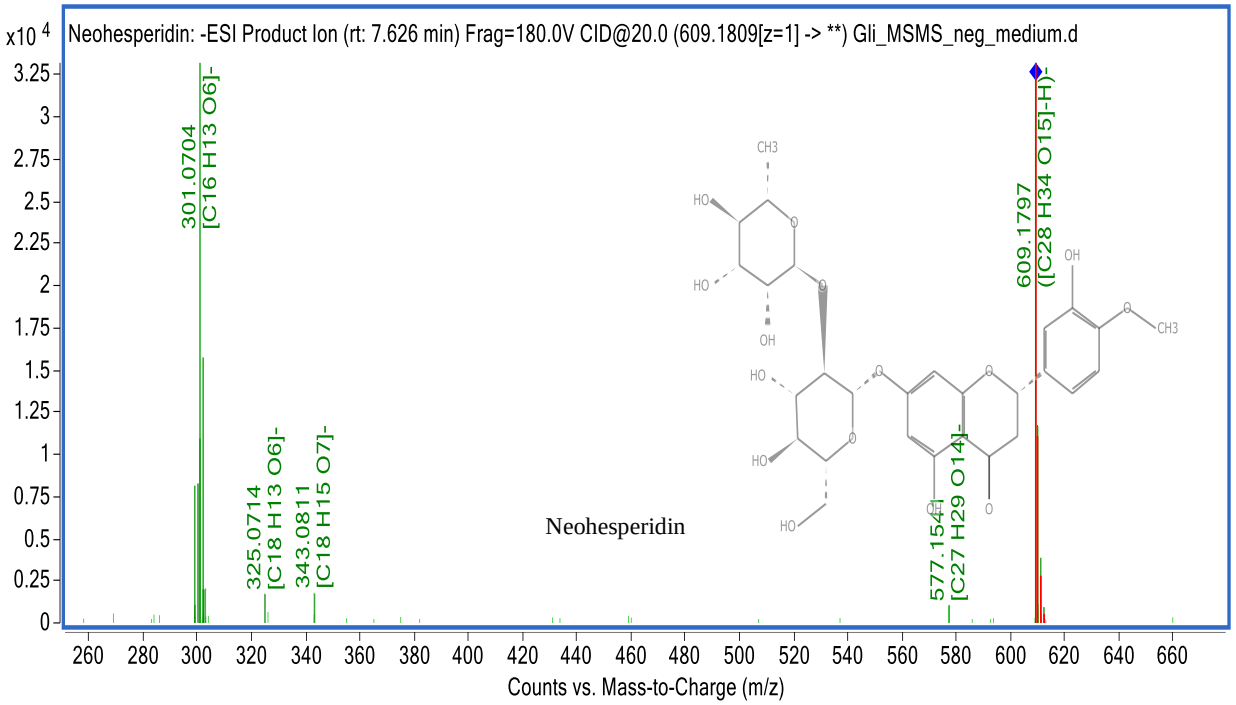

x10 4
Isorhamnetin 3-glucoside 4'-rhamnoside: -ESI Product Ion (rt: 7.096 min) Frag=190.0V CID@25.0 (623.1607[z=1] -> **) Gli_MSMS_neg_...
125.0243
315.0497
357.0601
479.1176
521.1284
623.1599 ([C28 H32 O16]-H)-
Counts vs. Mass-to-Charge (m/z)

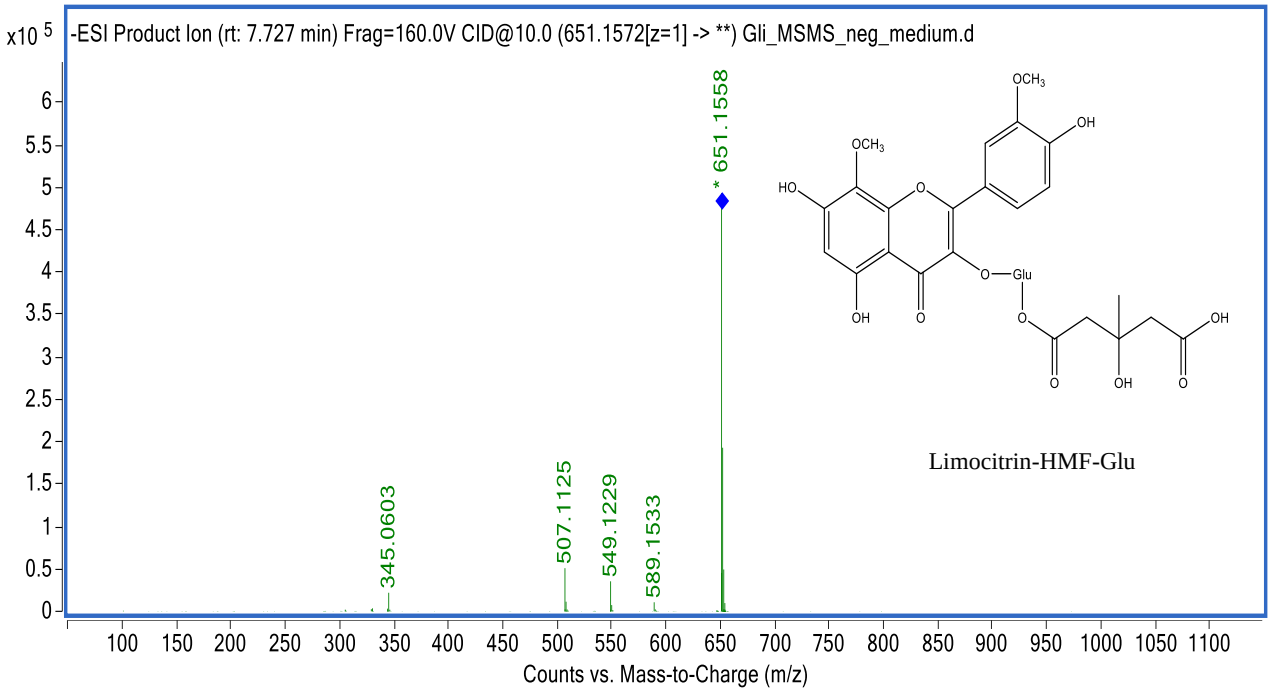


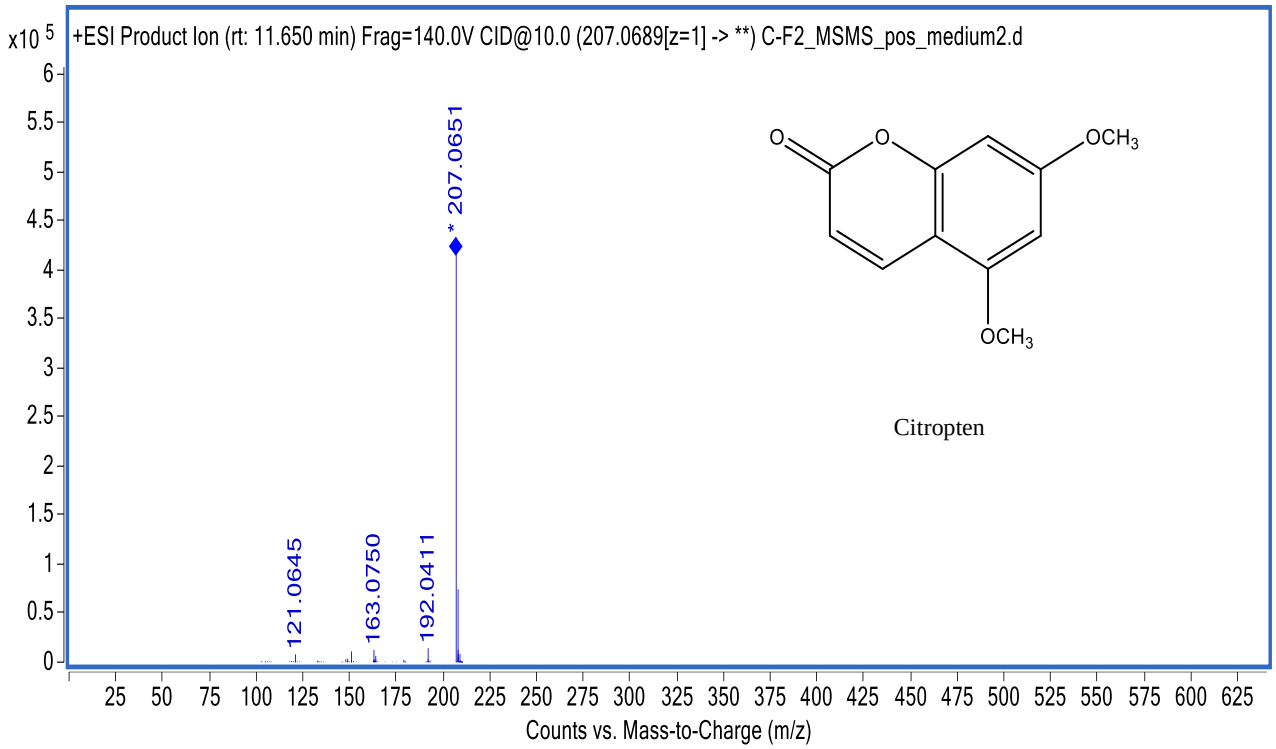
Diosmetin 7-O-beta-D-glucopyranoside



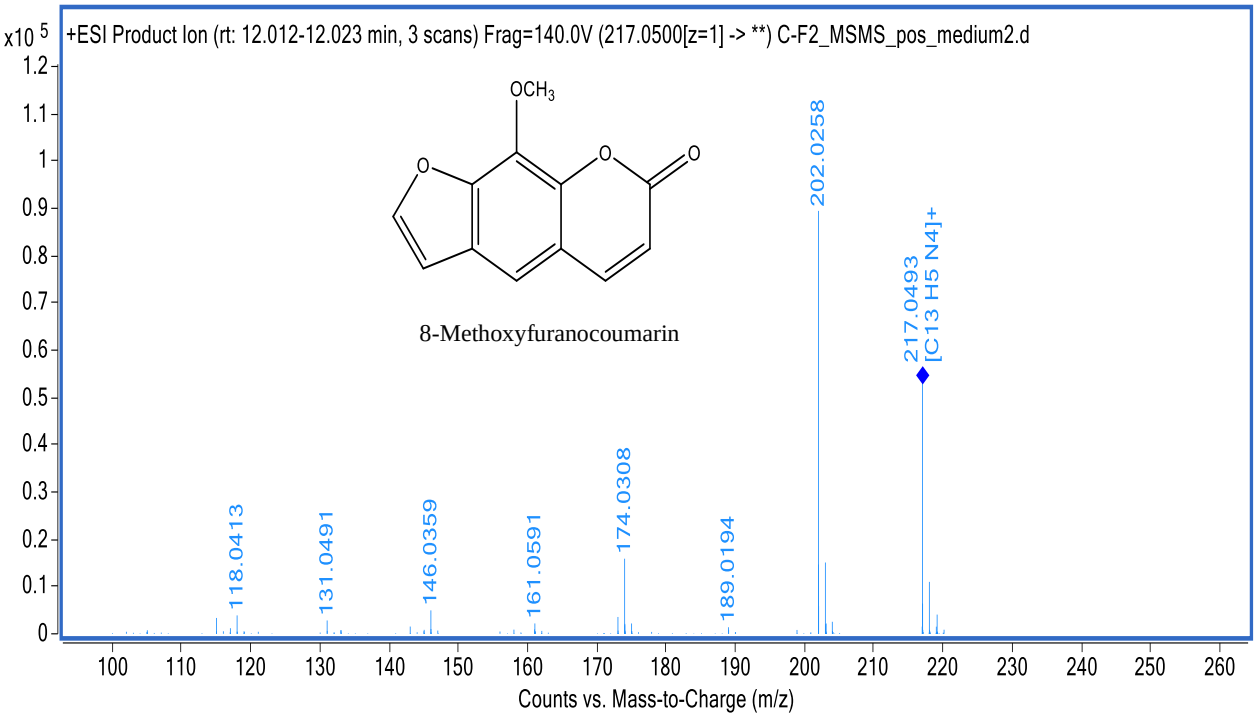
Hesperidin


x10 4
Neohesperidin: -ESI Product Ion (rt: 7.626 min) Frag=180.0V CID@20.0 (609.1809[z=1] -> **) Gli_MSMS_neg_medium.d
301.0704
[C16 H13 O6]-
325.0714
[C18 H13 O6]-
343.0811
[C18 H15 O7]-
577.1541
[C27 H29 O14]-
609.1797
([C28 H34 O15]-H)-
Neohesperidin
Counts vs. Mass-to-Charge (m/z)

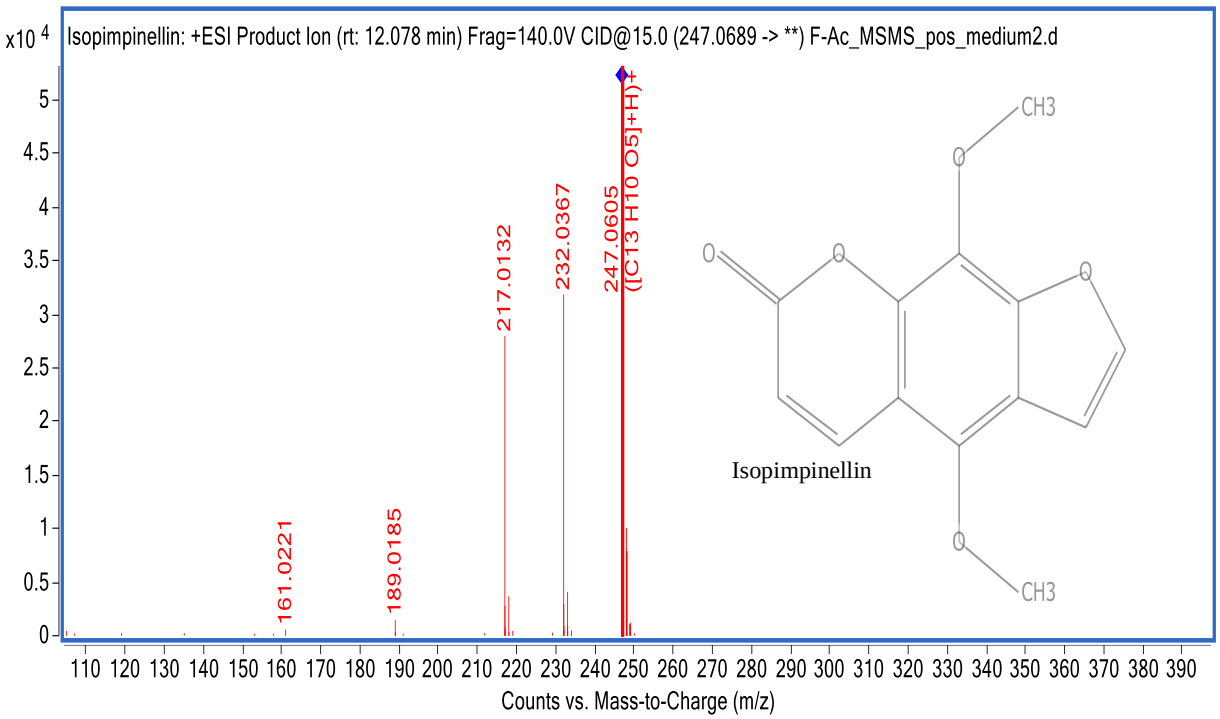

x10 5
-ESI Product Ion (rt: 7.727 min) Frag=160.0V CID@10.0 (651.1572[z=1] -> **) Gli_MSMS_neg_medium.d
* 651.1558
345.0603
507.1125
549.1229
589.1533
Limocitrin-HMF-Glu
Counts vs. Mass-to-Charge (m/z)

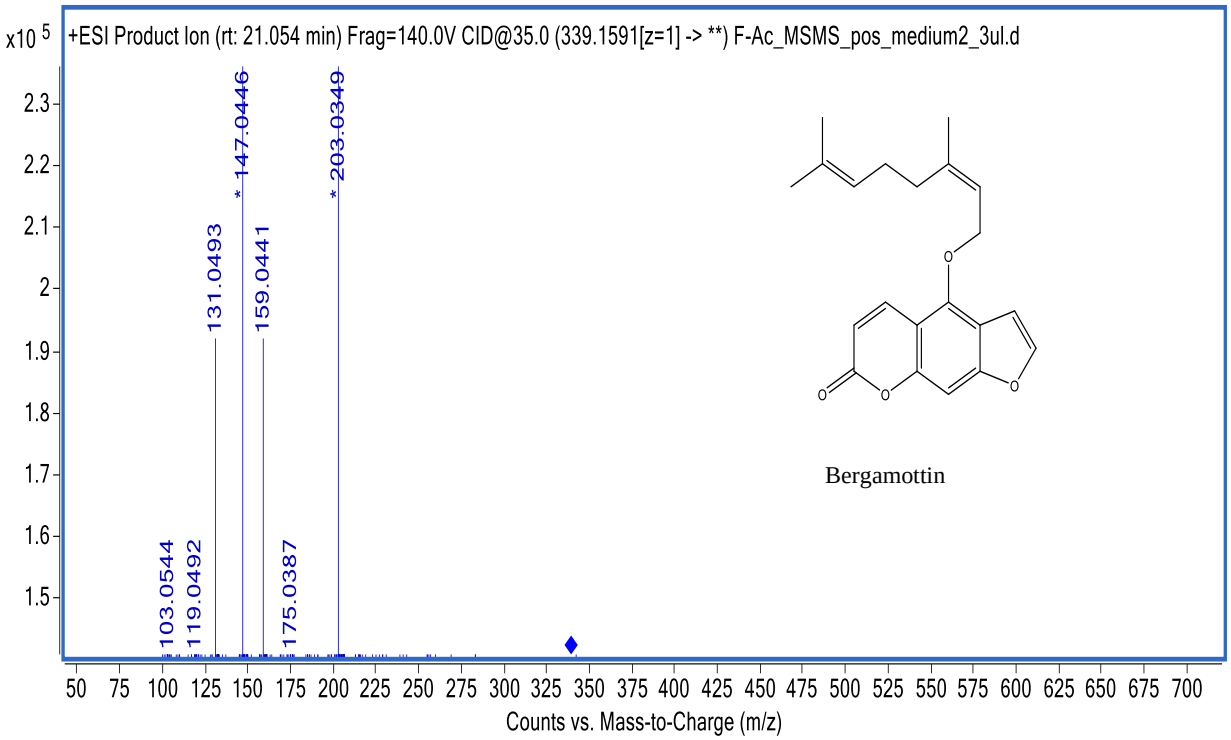


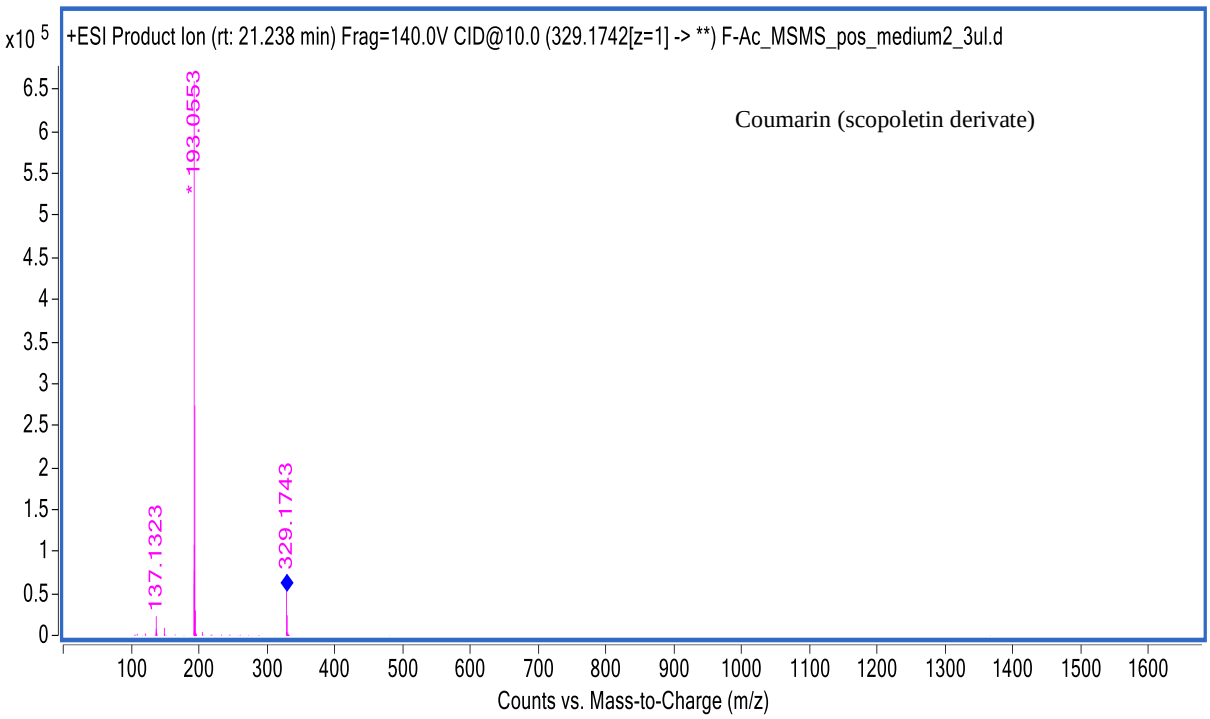
Citropten

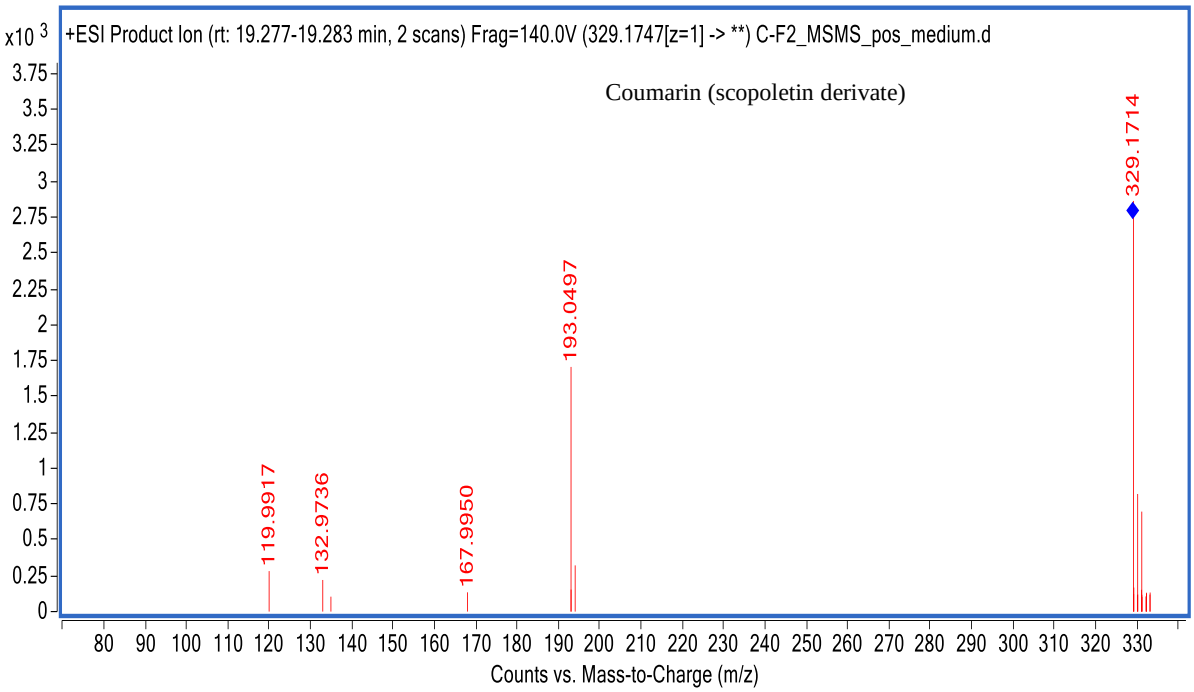


8-Methoxyfuranocoumarin


x10 4
Isopimpinellin: +ESI Product Ion (rt: 12.078 min) Frag=140.0V CID@15.0 (247.0689 -> **) F-Ac_MSMS_pos_medium2.d
161.0221
189.0185
217.0132
232.0367
247.0605
([C13 H10 O5]+H)+
CH3
CH3
Isopimpinellin
Counts vs. Mass-to-Charge (m/z)

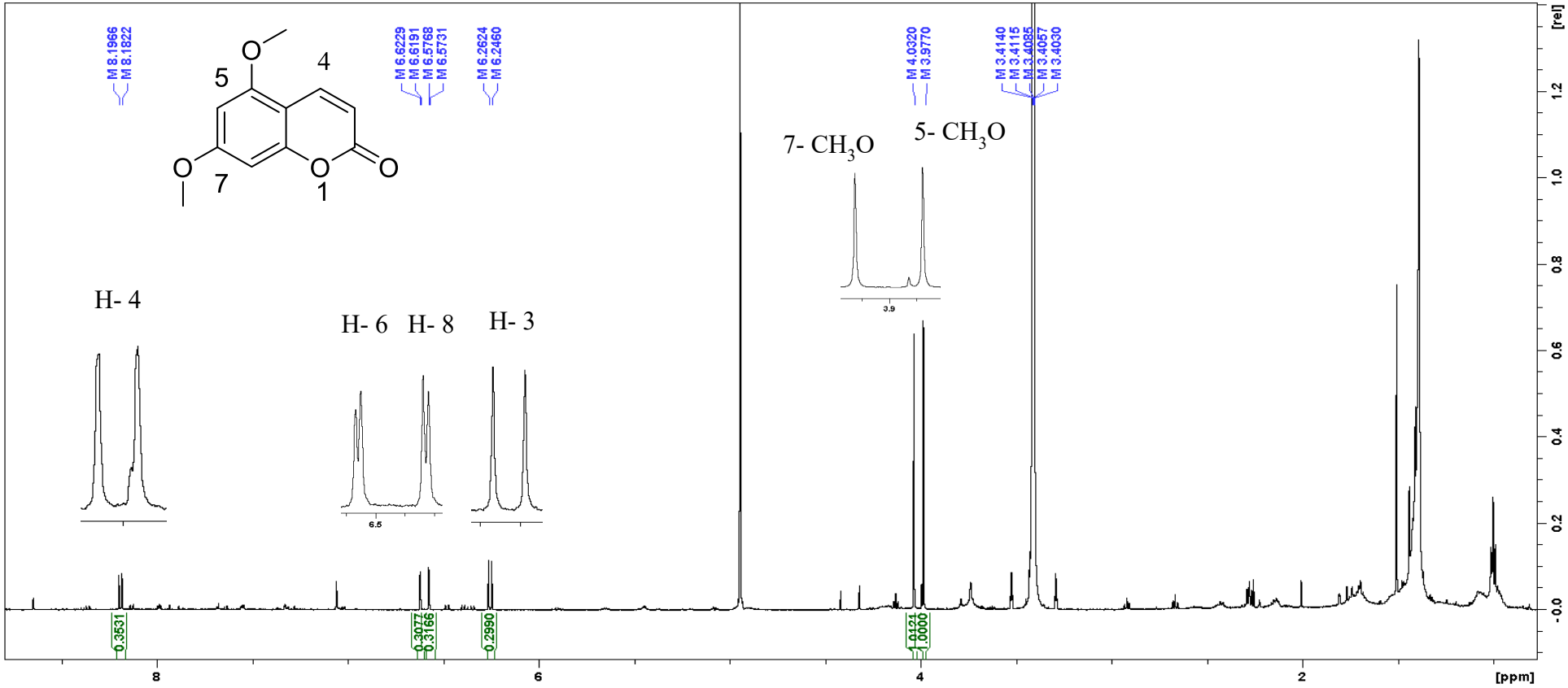

x10 5
+ESI Product Ion (rt: 21.054 min) Frag=140.0V CID@35.0 (339.1591[z=1] -> **) F-Ac_MSMS_pos_medium2_3ul.d
103.0544
119.0492
131.0493
147.0446
159.0441
175.0387
203.0349
Bergamottin
Counts vs. Mass-to-Charge (m/z)


x10 5
+ESI Product Ion (rt: 21.238 min) Frag=140.0V CID@10.0 (329.1742[z=1] -> **) F-Ac_MSMS_pos_medium2_3ul.d
Coumarin (scopoletin derivate)
* 193.0553
329.1743
137.1323
Counts vs. Mass-to-Charge (m/z)

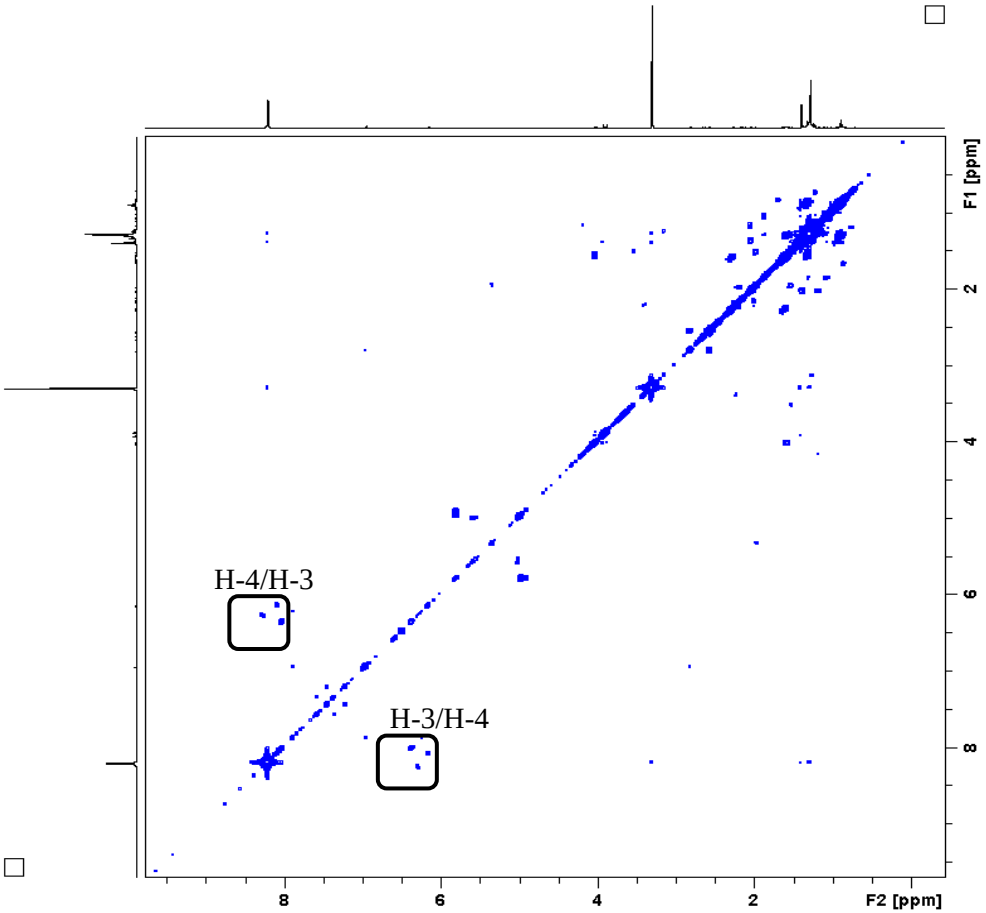

x10 3
+ESI Product Ion (rt: 19.277-19.283 min, 2 scans) Frag=140.0V (329.1747[z=1] -> **) C-F2_MSMS_pos_medium.d
Coumarin (scopoletin derivate)
329.1714
193.0497
119.9917
132.9736
167.9950
Counts vs. Mass-to-Charge (m/z)

A.2- 1D and 2D spectra of target compounds isolated from *C. latifolia.*

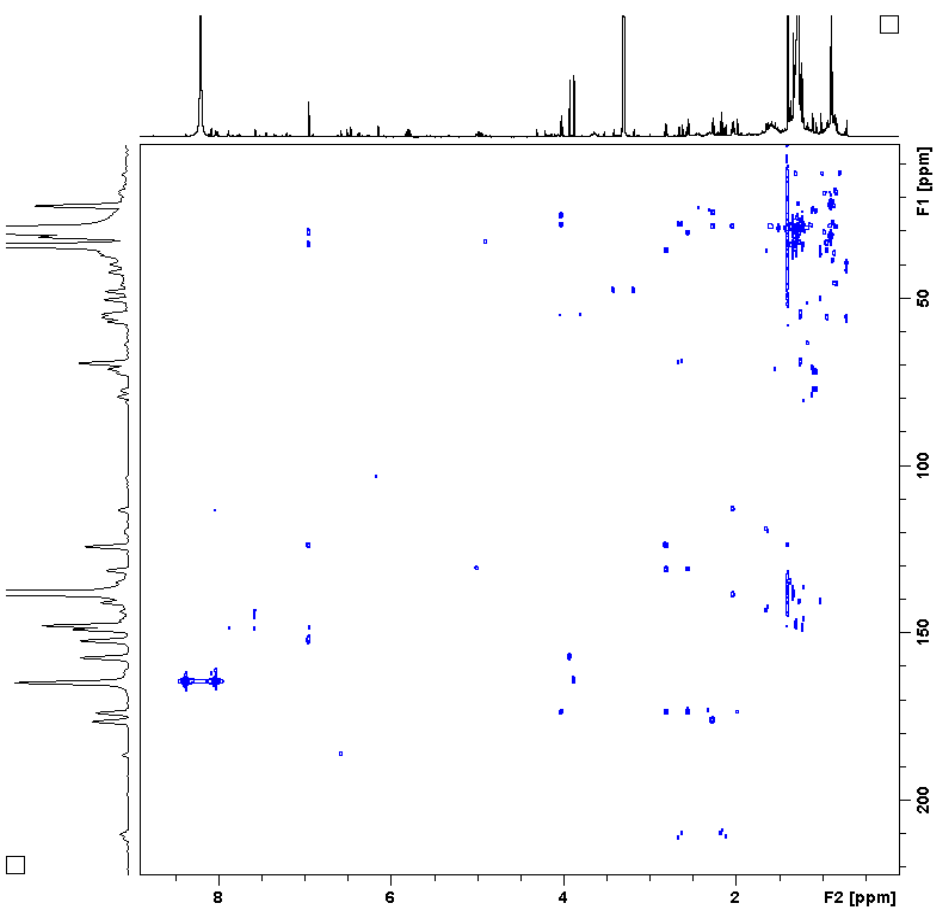
Compound 1: 5,7-dimethoxycoumarin



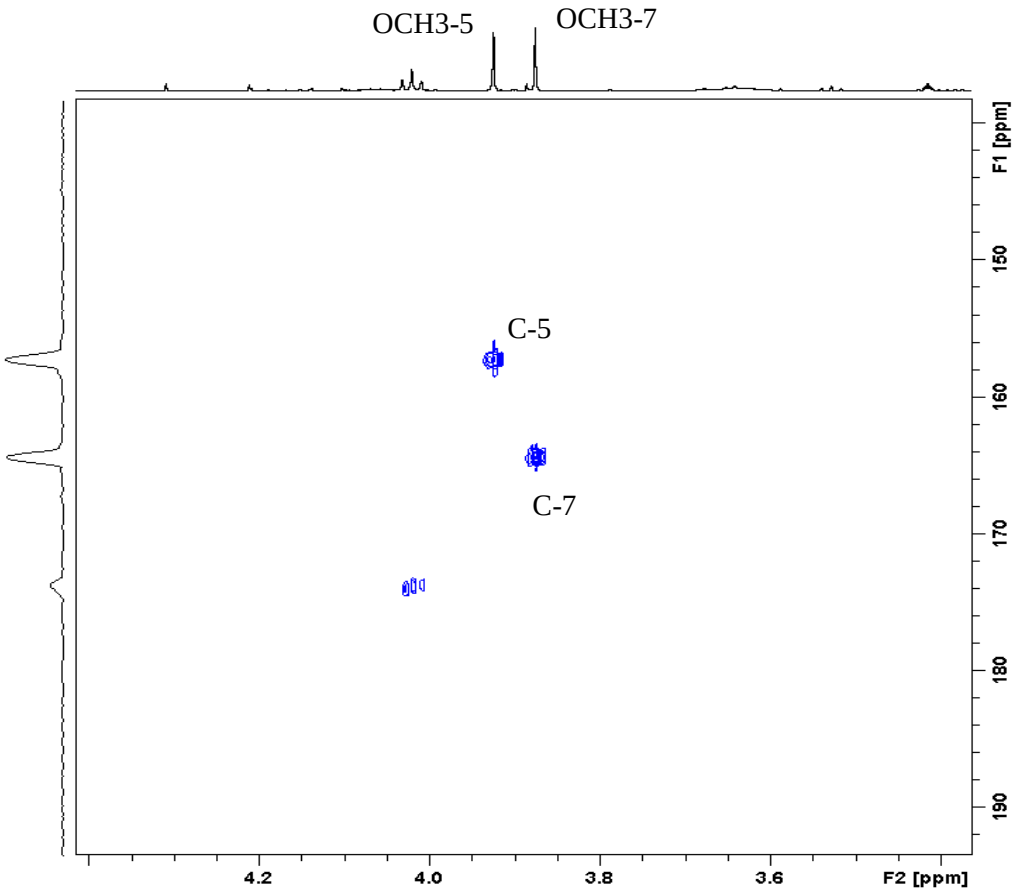
^{1}H NMR spectra of compound **01** (600 MHz in CH_3OH-d4).



^{1}H COSY spectra of compound **01 (**600 MHz in CH_3OH-d4)

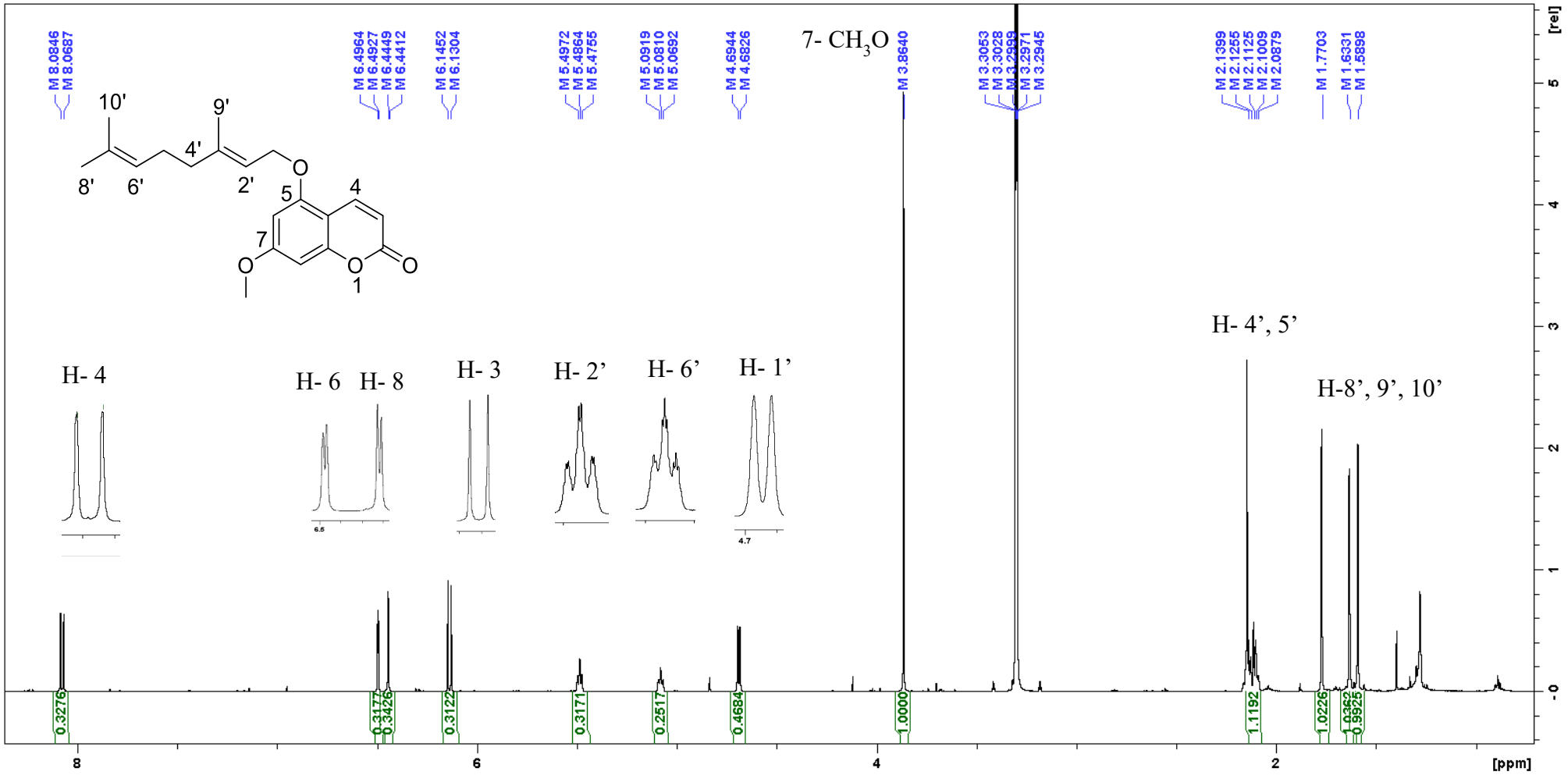


^{1}H, ^{13}C HMBC spectra of compound **01 (**600 MHz in CH_3OH-d4)

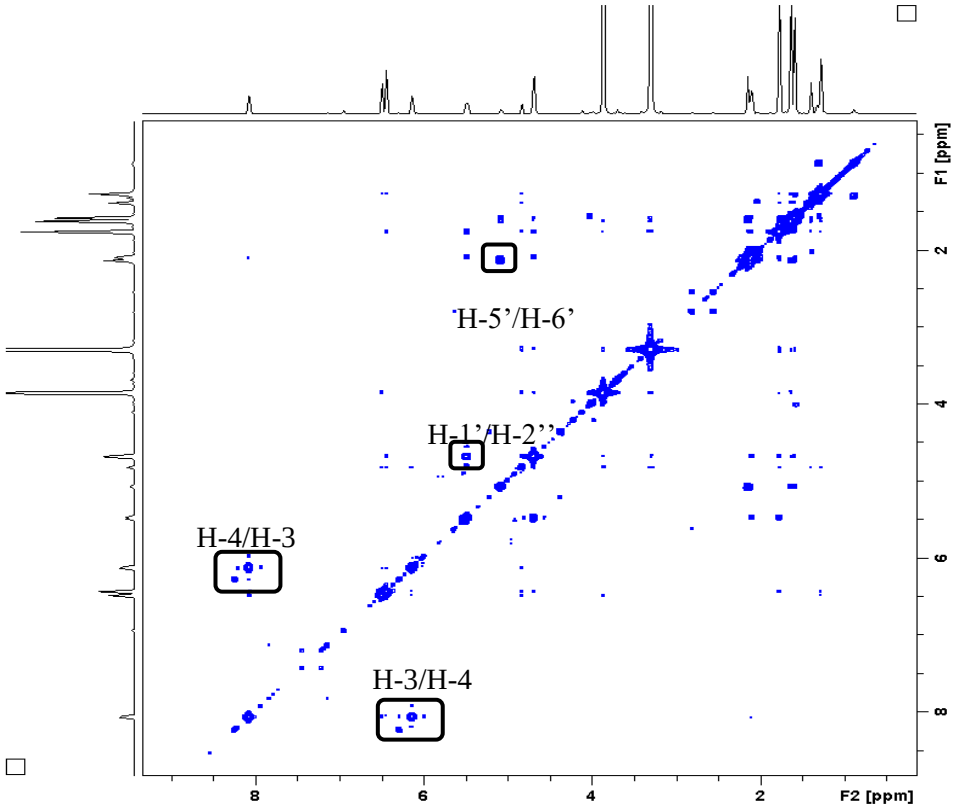


^{1}H, ^{13}C HMBC spectra of compound **01 (**600 MHz in CH_3OH-d4)

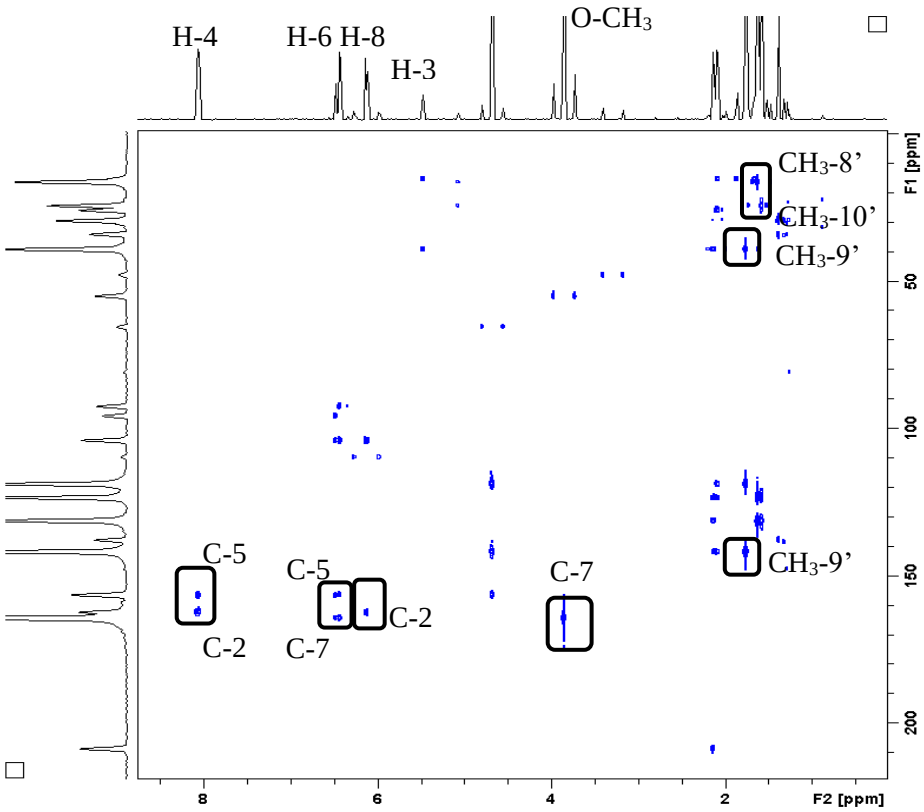
Compound 2: 5-geranyloxy-7-methoxycoumarin



[1]H NMR spectra of compound **02** (600 MHz in CH_3OH-d4).

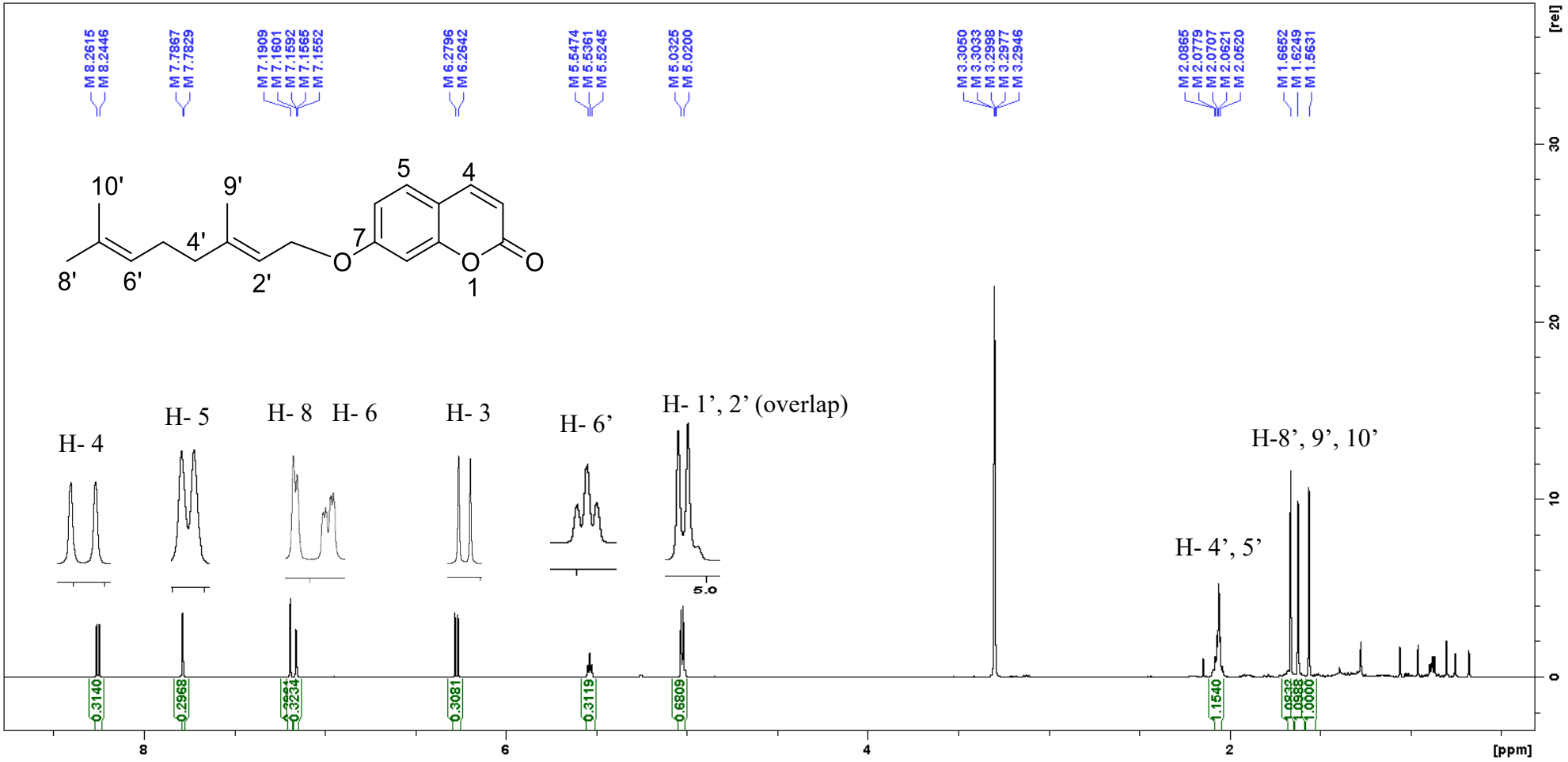


^{1}H COSY spectra of compound **02 (**600 MHz in CH_3OH-d4).

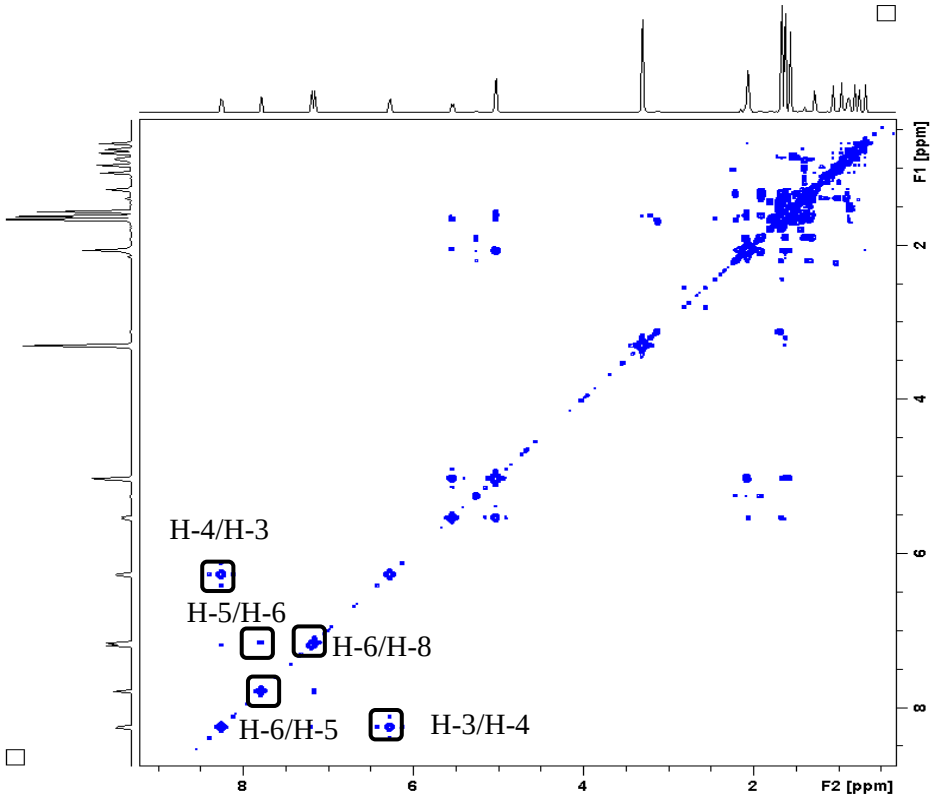


^{1}H, ^{13}C HMBC spectra of compound **02** (600 MHz in CH_3OH-d4).

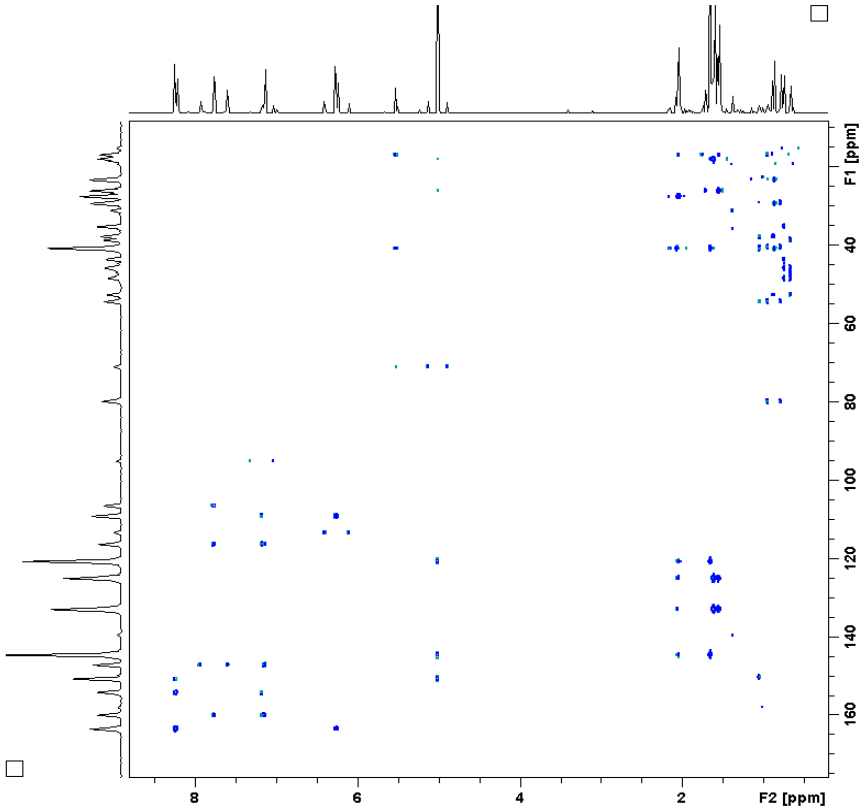
Compound 3: 7-geranyloxy-coumarin



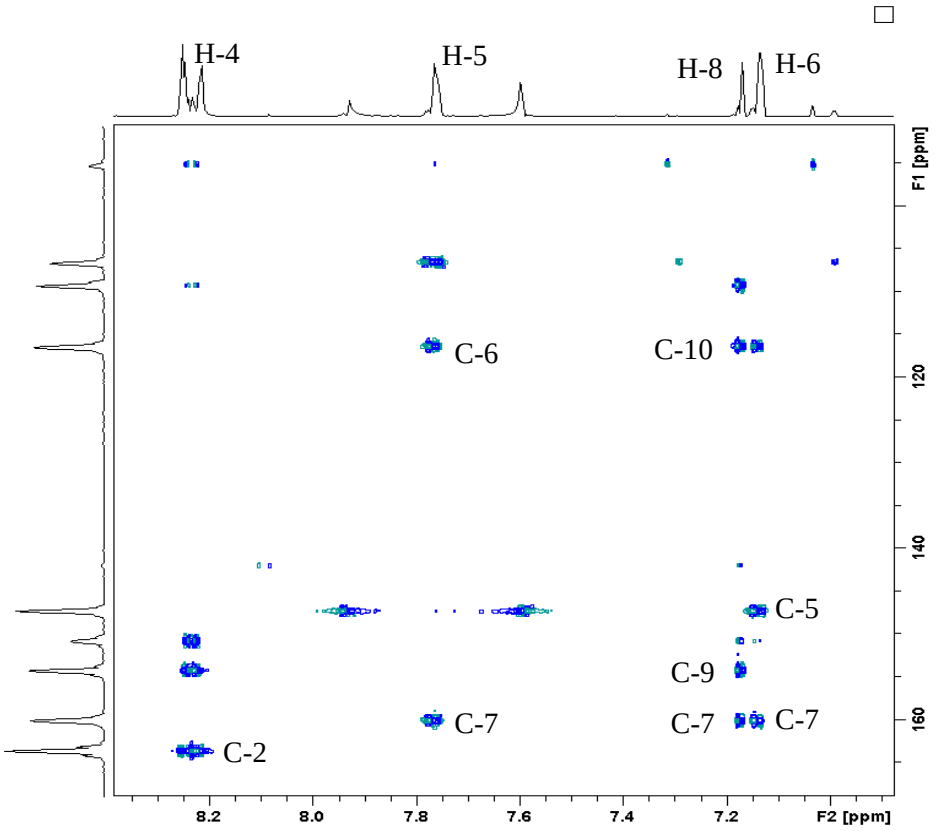
^{1}H NMR spectra of compound **03 (**600 MHz in CH_3OH-d4).



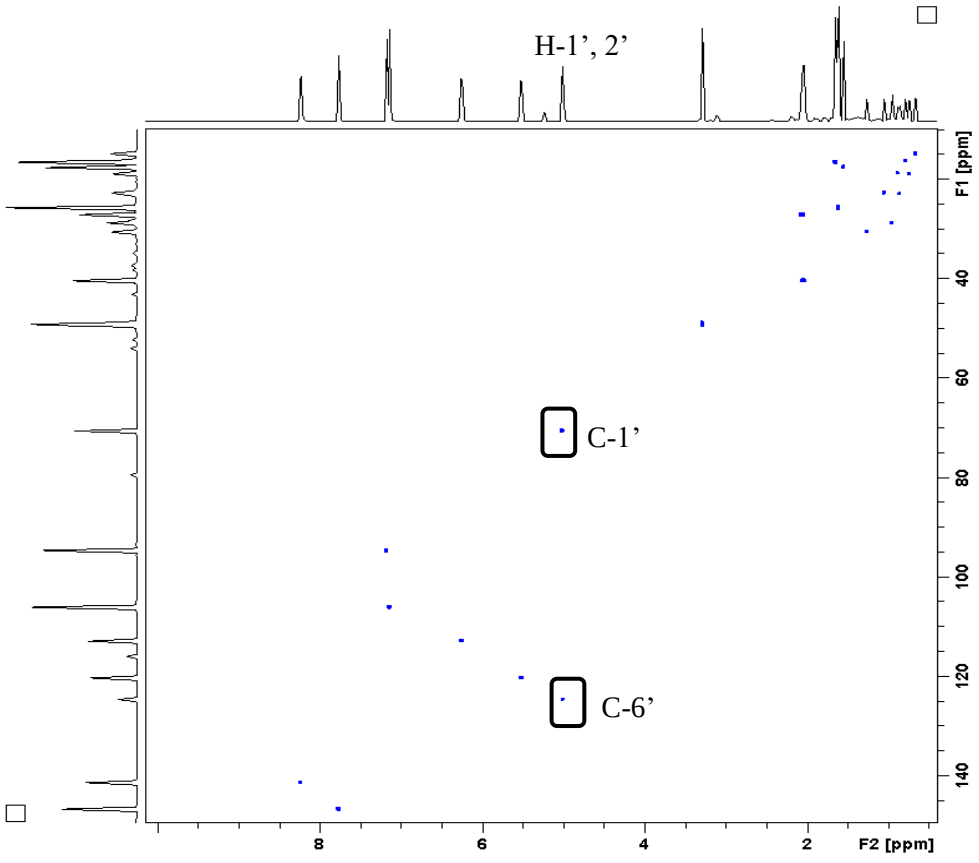
^{1}H COSY spectra of compound **03 (**600 MHz in CH_3OH-d4).



^{1}H, ^{13}C HMBC spectra of compound **03** (600 MHz in CH_3OH-d4).

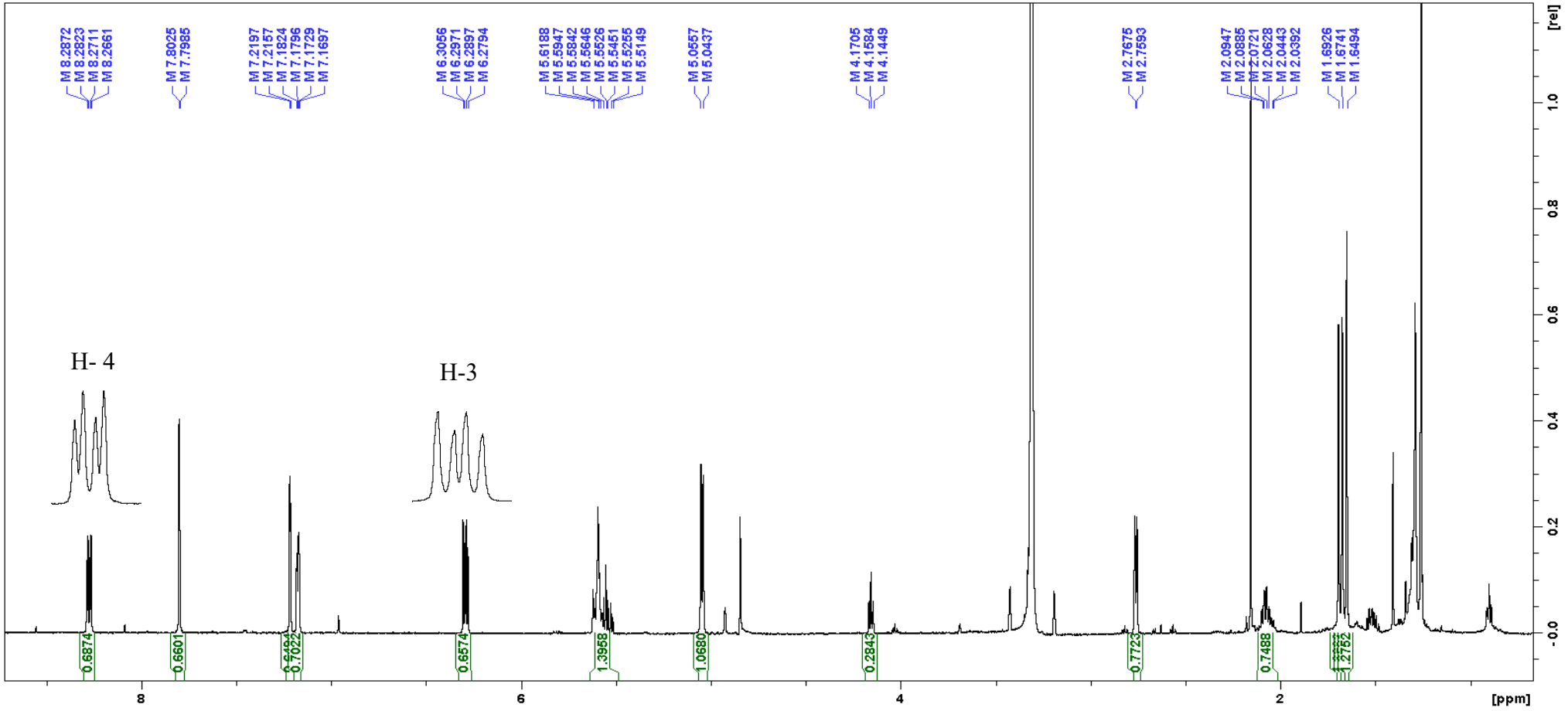


^{1}H, ^{13}C HMBC spectra of compound **03** (600 MHz in CH_3OH-d4).



^{1}H, ^{13}C HSQC spectra of compound **03** (600 MHz in CH_3OH)

Compound 4: Non elucidated



^{1}H NMR spectra of compound **04 (**600 MHz in CH_3OH-d4).

Appendix B- Supplementary information- Chapter 4

B.1- Putative compounds from *Phyllosticta citricarpa*, annotated by GNPS library

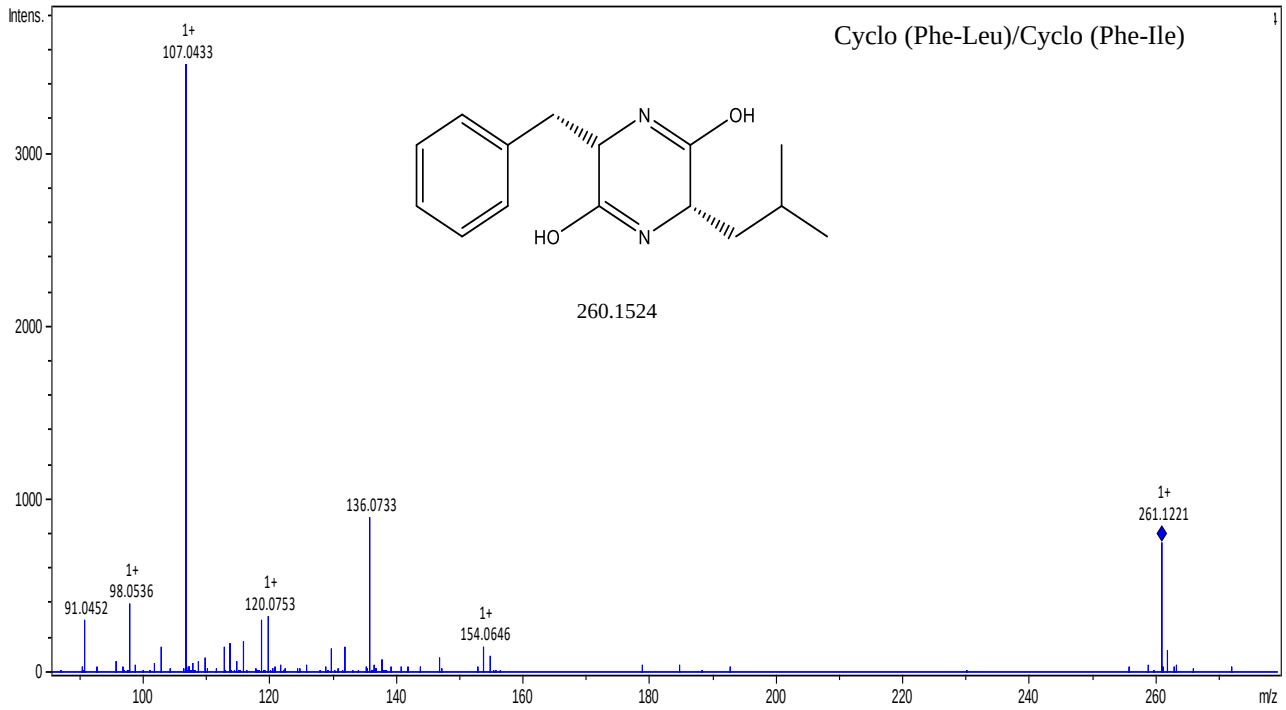
	Compound Name	**Instrument**	**precursor mass**	**Adduct**	**MQ Score**	**Compound class**
1	Cis, cis-9,12-Octadecadien-1-ol	Ion trap	277.223	M+H	0.75	N/A
2	Naringin	LC-ESI-QTOF	611.207	[M+H] +	0.90	Flavonoids
3	13-Docosenamide	QqQ	310.317	M+H	0.72	N/A
4	1,10a-dihydroxy-4,4,7,11b-tetramethyl-1,2,3,4a,5,6,6a,7,11,11a-decahydronaphtho[2,1-f][1] benzofuran-9-one	ESI-QFT	304.109	[M-H]-	0.75	N/A
5	Phe-Leu	Q-TOF	321.184	M+H	0.90	N/A
6	9-hydroxy-1,4a-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-1-carboxylic acid	ESI-QFT	331.254	[M+H] +	0.70	Prenol lipids
7	(3,4-dimethyl-2-oxochromen-7-yl) oxy-N-[2-(1H-indol-3-yl) ethyl] propanamide	Q-TOF	424.148	M+H	0.84	N/A
8	Bestatin	Ion trap	291.175	M+H	0.75	N/A
9	Cevane-3,4,7,14,15,16,20-heptol, 4,9-epoxy-, (3beta,4alpha,7alpha,15alpha,16beta)	Q-TOF	476.331	M+H	0.70	Steroids and steroid derivatives
10	Tyr-Pro	QqQ	279.141	M+H	0.84	N/A
11	Spectral Match to Phe-Pro from NIST14	QqQ	265.158	M+H	0.78	N/A
12	FruLeuIle	Q-TOF	404.247	M+H	0.72	Carboxylic acids and derivatives
13	Dicyclopenta[a,d]cycloocten-3(3aH)-one	Q-TOF	383.258	M+H	0.745	Prenol lipids
14	Genistein	LC-ESI-QTOF	271.069	[M+H] +	0.74	Isoflavonoids

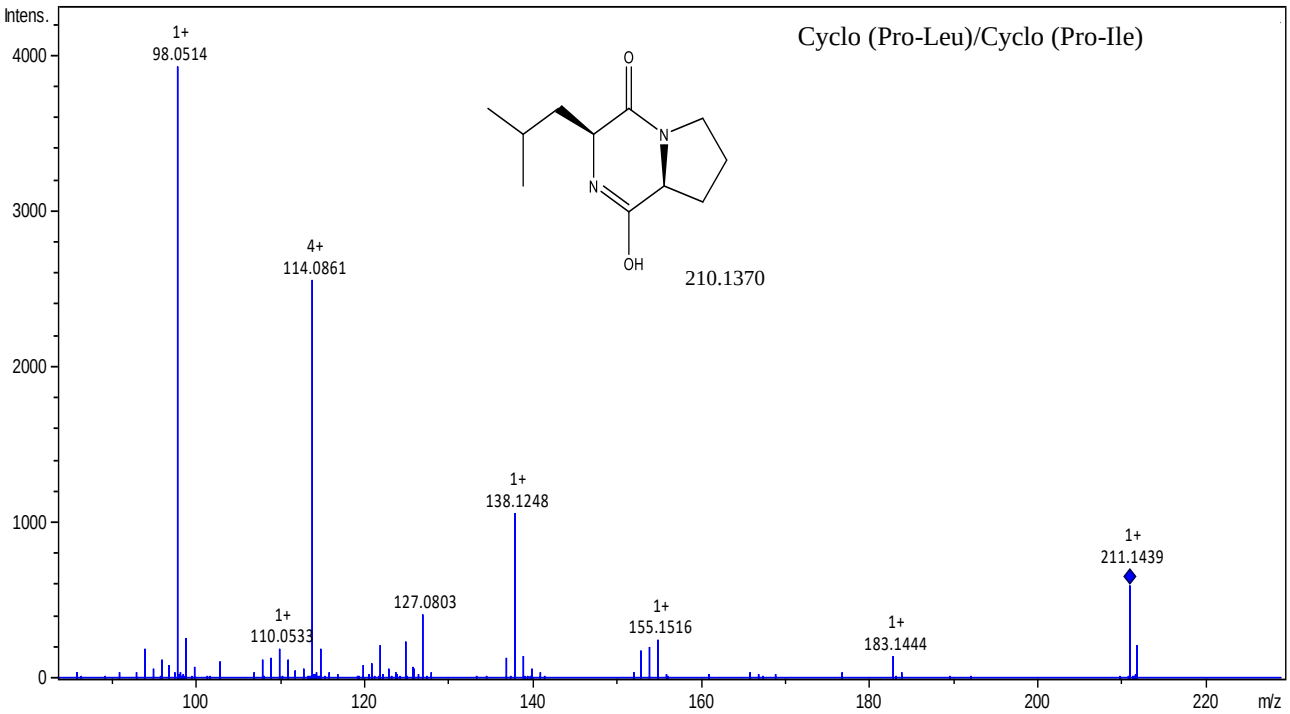
15	[5-[[4-[5-[acetyl(hydroxy)amino] pentylamino] -4-oxobutanoyl] -hydroxyamino]pentylamino]-4-oxobutanoic acid	Q-TOF	466.302	$M+NH_4$	0.85	Fatty Acyls
16	Phe-Val	Q-TOF	307.195	M+H	0.86	N/A
17	1-(hexadecanoyloxy)-3-hydroxypropan-2-yl-octadec-9-enoate	Q-TOF	596.46	$M+NH_4$	0.73	N/A
18	3',4',5',6'-tetradehydrogeissospermine	Q-TOF	663.34	M+	0.73	N/A
19	Phe-Val	Q-TOF	276.145	M+H	0.82	N/A
20	Acebutolol	HCD	356.15	M+H	0.80	N/A
21	(2R)-5,8-dihydroxy-2-(2-hydroxyphenyl)-7-methoxy-2,3-dihydrochromen-4-one	ESI-QFT	285.086	[M-H]-	0.71	Flavonoids
22	[5-(Benzoyloxy)-3-chloro-4,6-dihydroxy-1-cyclohexen-1-yl] methyl benzoate	Q-TOF	417.304	M+H	0.70	Benzene and substituted derivatives
23	Gestodene	Q-TOF	351.248	M+H	0.70	N/A
24	(2E,6E,10Z)-12-hydroxy-10-(hydroxymethyl)-6-methyl-2-(4-methylpent-3-enyl) dodeca-2,6,10-trienoic acid	ESI-QFT	369.28	[M+Na] +	0.78	Prenol lipids
25	Tyr-Pro	Q-TOF	316.195	M+H	0.75	N/A
26	2-hydroxy-3-(2-hydroxyacetoxy) propyl palmitate	Q-TOF	454.301	M+H	0.76	N/A
27	NCGCevane-3,4,7,14,15,16,20-heptol, 4,9-epoxy-, (3beta,4alpha,7alpha,15alpha,16beta) -	Q-TOF	492.327	M+H	0.73	Steroids and steroid derivatives
28	sphinganine	Q-TOF	312.297	M+H	0.87	N/A
29	C17_Sphingosine	Q-TOF	314.28	M+H	0.79	Organonitrogen compounds
30	(3-hydroxyhexadecanoyl) lysine	Q-TOF	401.342	M+H	0.89	N/A
31	4-[2-[(1R,4aS,5R,6R,8aS)-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylidene-3,4,4a,6,7,8-hexahydro-1H-naphthalen-1-yl]-1-hydroxyethyl]-2H-furan-5-one	ESI-QFT	386.219	[M-H]-	0.786	Prenol lipids

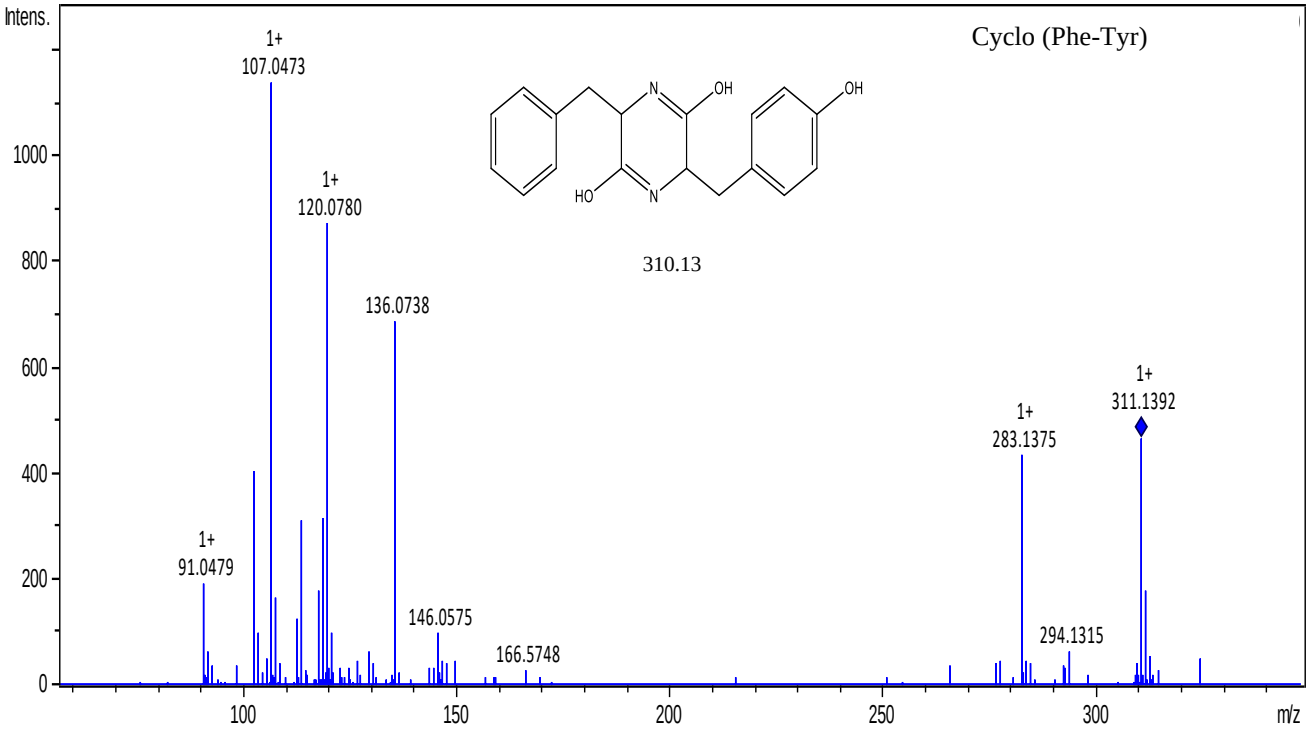
32	Rubranoside B	Q-TOF	478.23	M-H	0.82	Diarylheptanoids
33	Daidzein	LC-ESI-QTOF	255.07	[M+H] +	0.836	Isoflavonoids
34	(3-hydroxypentadecanoyl) lysine	Q-TOF	359.3	M+H	0.817	N/A
35	9-hydroxy-1,4a-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-1-carboxylic acid	ESI-QFT	313.163	$[M+NH_4]$ +	0.711	Prenol lipids
36	N-Acetylphenylalanylvaline	Q-TOF	310.132	M+H	0.809	Carboxylic acids and derivatives
37	isomaltulose	ESI-QFT	360.153	$[M+NH_4]$ +	0.802	Organooxygen compounds
38	Coproporphyrin I	HCD	655.283	M+H	0.86	N/A
39	Salmeterol	Q-TOF	458.324	[M+H]	0.75	Benzene and substituted derivatives
40	[(3S,5S,8R,10S,13R,14S,17R)-3-[4,5-dihydroxy-6-(hydroxymethyl)-3-[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxyoxan-2-yl] oxy-14-hydroxy-10,13-dimethyl-1,2,3,4,5,6,7,8,9,11,12,15,16,17-tetradecahydrocyclopenta[a]phenanthren-17-yl]-2H-furan-5-one	Q-TOF	698.449	$M+NH_4$	0.735	Steroids and steroid derivatives
41	Phytosphingosine	ion trap	334.303	M+H	0.753	N/A
42	Cholic acid from	QqQ	352.23	$M+H-2H_2O$	0.701	N/A
43	9-hydroxy-1,4a-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-1-carboxylic acid	ESI-QFT	353.28	[M+NH4] +	0.702	Prenol lipids
44	L-Tyrosine	HCD	182.088	M+H	0.974	N/A
45	5'-Methylthioadenosine	Q-TOF	282.123	M+H	0.922	5'-deoxyribonucleosides
46	His-Ile	Q-TOF	311.175	M+H	0.864	N/A
47	Tyr-Phe	Q-TOF	295.164	M+H	0.772	N/A

48	Cevane-3,4,7,14,15,16,20-heptol, 4,9-epoxy-, (3beta,4alpha,7alpha,15alpha,16 beta) -	Q-TOF	474.32	M+H	0.766	Steroids and steroid derivatives
49	1-Kestose from NIST14	ion trap	543.145	M+Na	0.756	N/A
50	Egtazic acid from NIST14	HCD	387.123	M+Na	0.766	N/A
51	Glycocholic Acid	ESI-QFT	488.297	[M+K] +	0.777	Steroids and steroid derivatives
52	[4a,5-bis(hydroxymethyl)-1,2-dimethyl-2,3,4,7,8,8a-hexahydronaphthalen-1-yl]-3-methylpentanoic acid	Q-TOF	330.269	M+NH4	0.753	Phenol lipids/ diterpenoids
53	(2E,6E,10Z)-12-hydroxy-10-(hydroxymethyl)-6-methyl-2-(4-methylpent-3-enyl) dodeca-2,6,10-trienoic acid	ESI-QFT	321.251	[M+Na] +	0.836	Phenol lipids/ diterpenoids

B.2- MS spectra of compounds identified from *Phyllosticta citricarpa*.






Intens.
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136.0738
1+
91.0479
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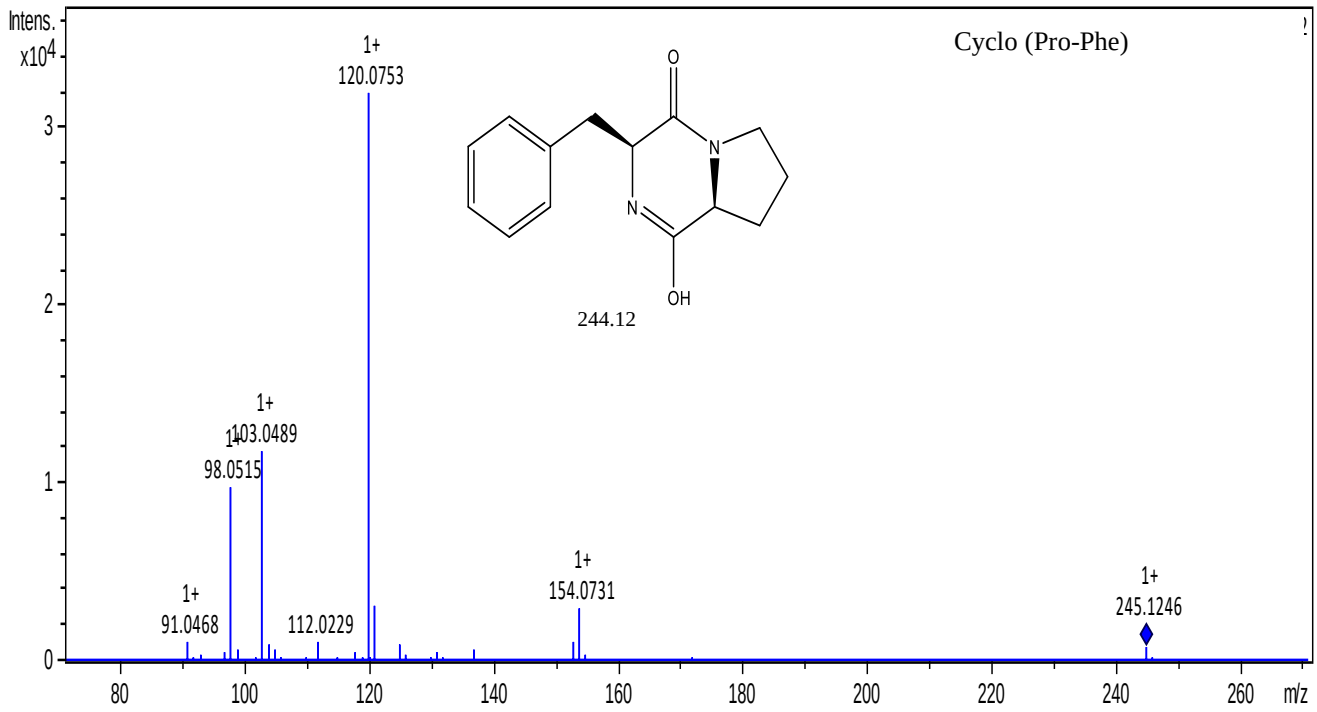
166.5748
1+
283.1375
1+
311.1392
294.1315
310.13
1000
800
600
400
200
0
100
150
200
250
300
m/z


Intens.
x10^4
Cyclo (Pro-Phe)
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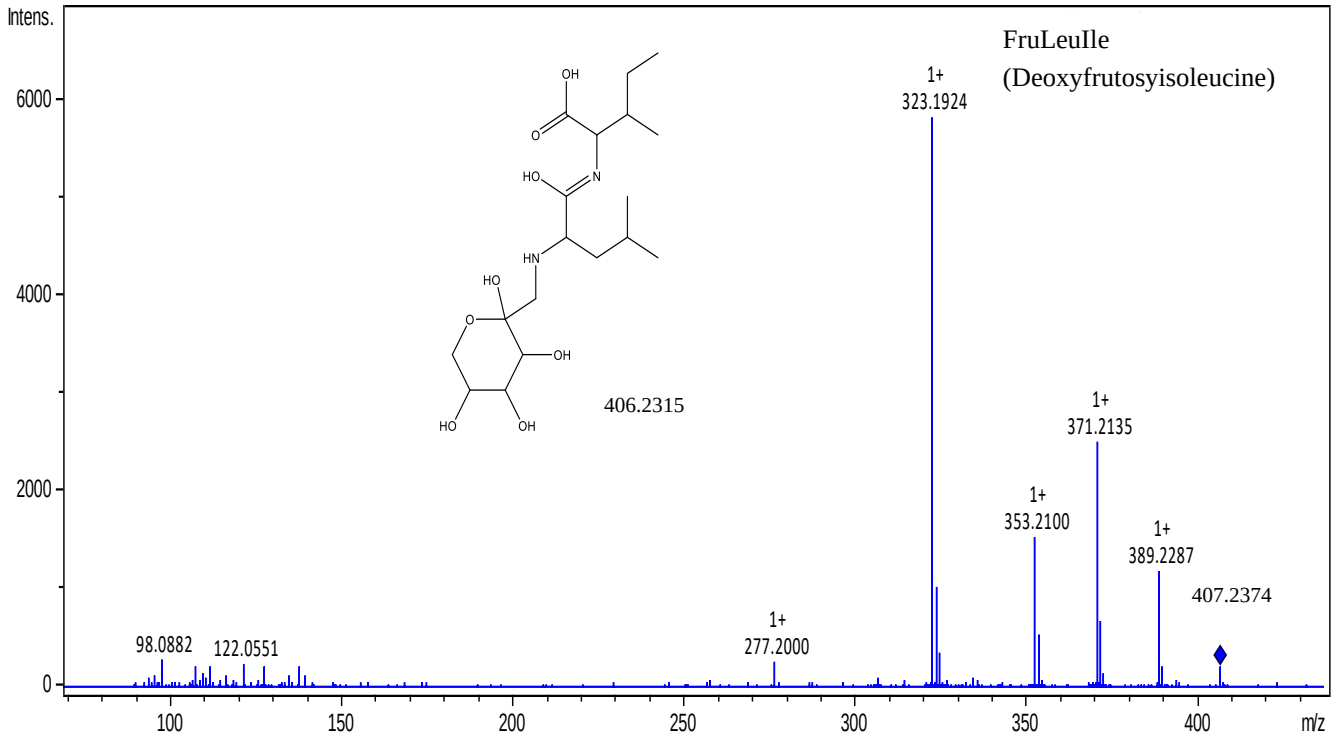
120.0753
1+
103.0489
98.0515
1+
91.0468
112.0229
1+
154.0731
1+
245.1246
244.12
3
2
1
0
80
100
120
140
160
180
200
220
240
260
m/z


Intens.
6000
4000
2000
0
FruLeuIle
(Deoxyfrutosyisoleucine)
1+
323.1924
406.2315
1+
371.2135
1+
353.2100
1+
389.2287
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98.0882
122.0551
1+
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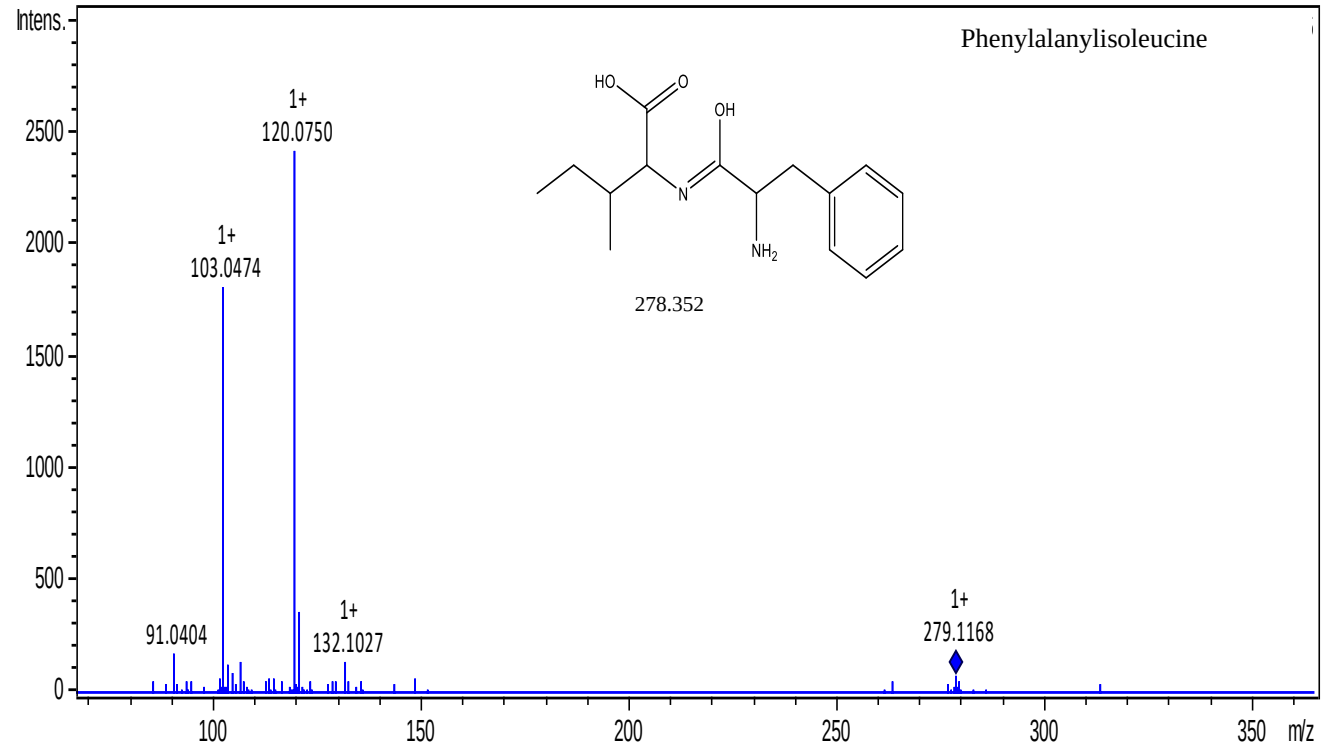
100
150
200
250
300
350
400
m/z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1500
1000
500
0
Phenylalanylisoleucine
1+
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1+
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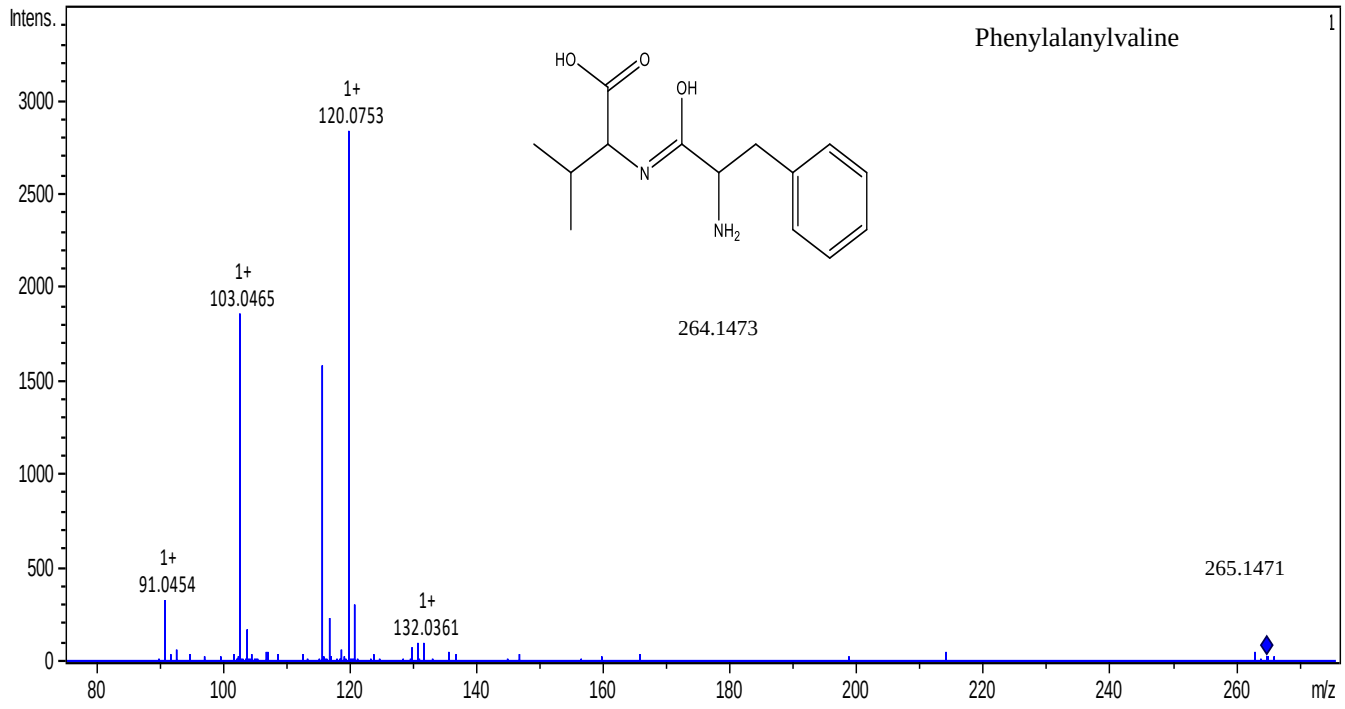
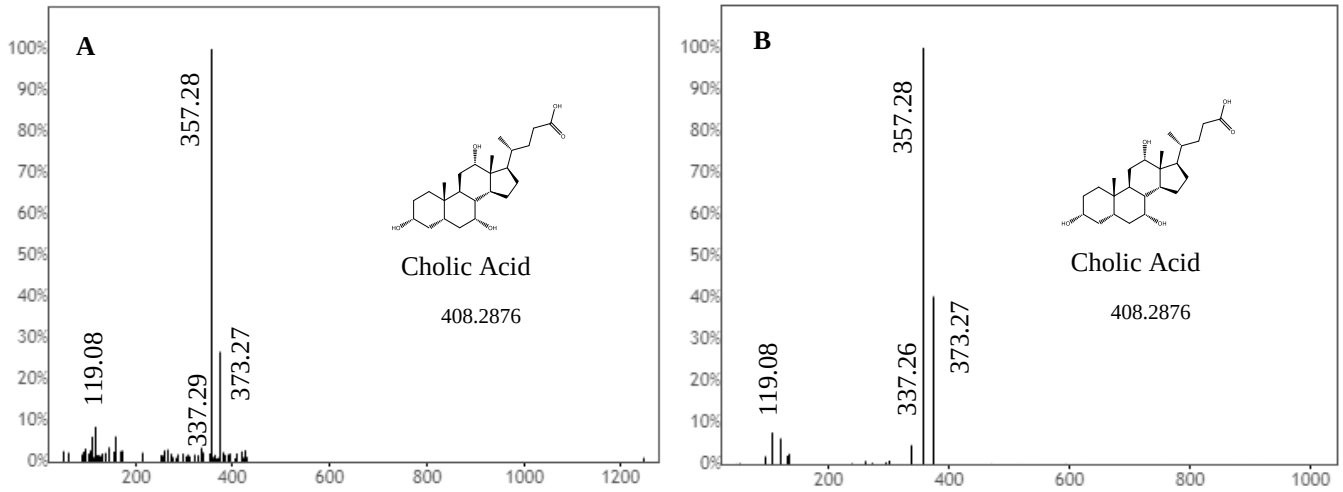
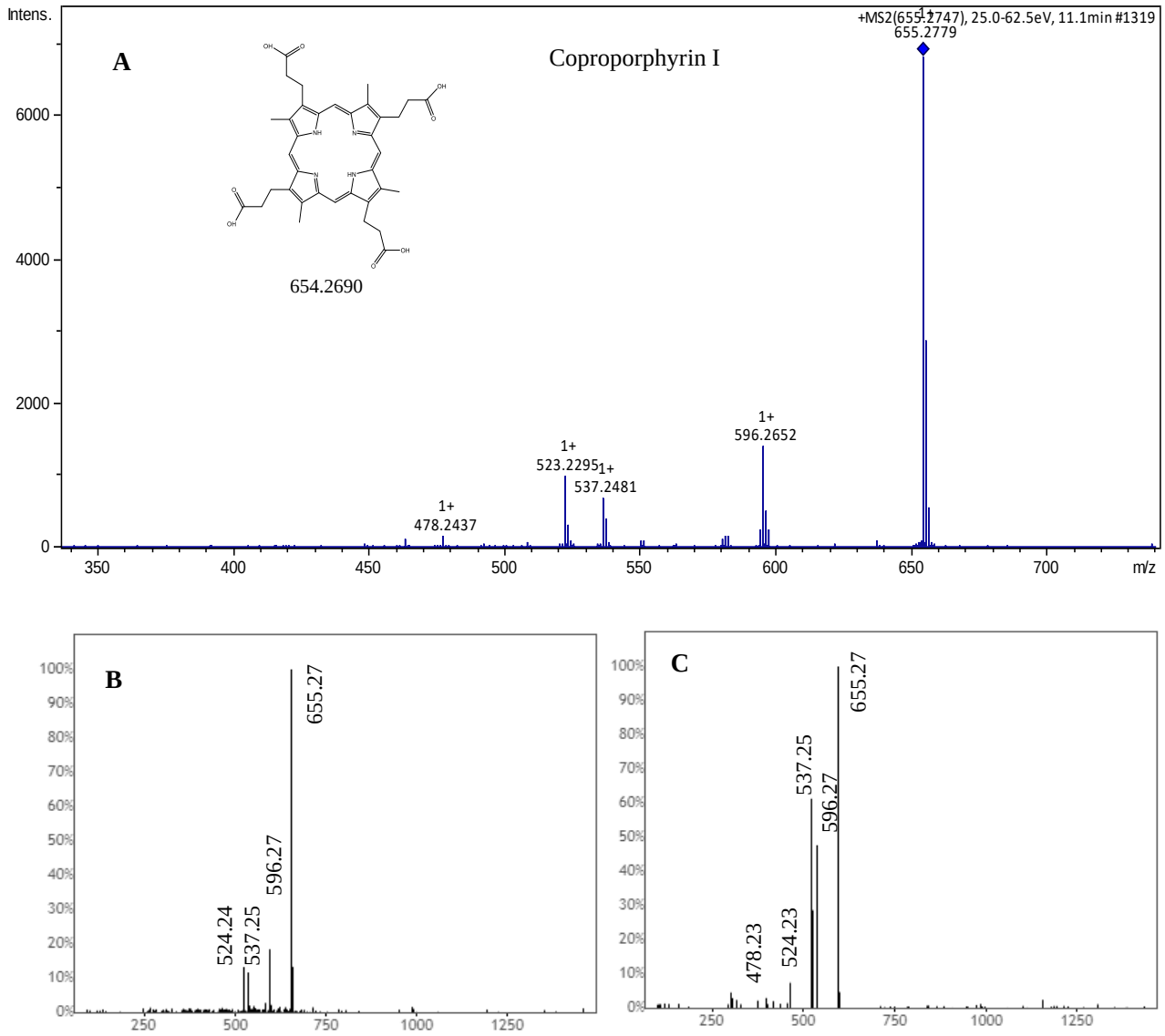
1+
279.1168
100
150
200
250
300
350
m/z





Cholic Acid (**A**) MS/MS parent ion fragmentation for this study (**B**) MS/MS parent ion fragmentation for the molecular networking standard (m/z 373.27 + [M+H-2H_2O]).



Coproporphyrin I (A) MS/MS from extract YMA culture media (B) MS/MS parent ion fragmentation for this study (C) MS/MS parent ion fragmentation for the molecular networking standard.