



DEVELOPMENT OF AN ACACIA-BASED BIOREFINERY – AN ADVANCED SYSTEM FOR BIOMASS VALORIZATION AND ENVI- RONMENTAL SUSTAINABILITY

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Master in Energy and Bioenergy

DOCTORATE IN BIOENERGY

NOVA University Lisbon

April, 2023

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DOCTORATE IN BIOENERGY

NOVA University Lisbon
April, 2023

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To my beloved family

ACKNOWLEDGMENTS

The completion of this work would not have been possible without the presence and support from several people, whom I would like to thank.

I would like to thank my advisor Margarida Gonçalves, for being tireless in supporting the work carried out, and whose passion for research is inspiring and motivated me to start this journey. My special thanks also to my co-advisor José Carlos Quintela, for sharing knowledge and supporting during the realization of all the work.

I would like to thank the Portuguese funding agency, FCT - Fundação para a Ciência e a Tecnologia, for supporting this work through the PhD grant SFRH/BD/133300/2017. My thanks also to the company CMC Biomassa S.A., for facilitating access to some of the raw materials used in this work.

I would like to thank all my laboratory colleagues for their support, sharing, and good mood: Andrei Longo, Catarina Viegas, Liliana, Vasco, Mariana, João Pires, Leandro, Carolina, Ana Sofia, Ana Paula, and Luís Durão. My deep thanks. Thank you for existing. And to all the others that I have forgotten at this moment, my deep gratitude as well.

Catarina Nobre, I haven't forgotten you. Thank you so much for sharing, for helping in the lab, and for being a Google many times. Thank you so much.

However, my most special thanks go to my family. This effort was for you, my dear children Madalena, Matilde, and Henrique. I thank you from the bottom of my heart, Cristina, my beloved wife. Thank you for making this possible, for having to work extra hard, and for keeping the family together while I have been absent and unbearable for the last seven years. Thank you for everything.

My deep thanks also to the rest of my family, for all the support whenever it was needed. My dear mother, my dear father, my brothers Jorge and Carlos, and the whole family. Thank you for everything.

"What we know is a drop, what we don't know is an ocean." (Isaac Newton)

ABSTRACT

Acacia dealbata is an invasive species whose spreading must be controlled but with industrial applications as a source of concretes, tannins, wood materials and biofuels. Both removal operations and industrial applications generate biomass wastes that could be further valorized. The main goal of this thesis was to study the integral exploitation of *A. dealbata* biomass (flowers, leaves, twigs, wood, and bark) from a biorefinery perspective.

Extraction of terpenic and phenolic components from flowers collected at different locations, was evaluated using different solvents and extraction methods. Lipophilic extracts (absolutes) were characterized by SPME-GC-TOFMS showing the presence of organic acids, hydrocarbons and pentacyclic triterpenes. The phenolic fraction obtained from flowers had high levels of total phenolics and its characterization by HPLC-DAD and HPLC-DAD-MS/MS revealed the presence of flavonoid glycosides with relevant biological activities. Polar extracts obtained from leaves, twigs, wood, and bark showed high levels of total phenolics, flavonoids and proanthocyanidins. Leaves were also a source of lipophilic extracts rich in phytol, lupenone and lupeol. All phenolic extracts presented antioxidant properties (in DPPH and FRAP tests) and antimicrobial activity against gram-positive bacteria. The composition and properties of raw and spent biomass fractions were evaluated to validate their use as biofuels after extraction with solvents. Torrefaction and hydrothermal carbonization were used to convert these spent biomasses into biochars and hydrochars with improved fuel properties. Spent biomasses and corresponding chars were tested as adsorbents for cationic and anionic dyes, reaching removal efficiencies higher than 90%. The incorporation of the different *Acacia* biomass fractions in mortars to replace fillers was tested as a contribution to incorporate renewable resources in building materials.

The results obtained demonstrate multiple conversion pathways that can be used to valorise *Acacia* biomass producing energy and various marketable products, thus promoting bioeconomy and the sustainability of the forestry sector.

Keywords: *Acacia dealbata*, absolutes, phenolics, biomass, torrefaction, hydrothermal carbonization, biofuels, adsorbents, mortar

RESUMO

Acacia dealbata é uma espécie invasora cuja propagação deve ser controlada, mas com aplicações industriais como fonte de concretos, taninos, materiais lenhosos e biocombustíveis. Tanto a sua remoção como a exploração industrial geram resíduos de biomassa que poderiam ser mais valorizados. O principal objetivo desta tese foi estudar a exploração integral da biomassa de *A. dealbata* (flores, folhas, galhos, madeira e casca) numa perspetiva de biorrefinaria.

A extração de componentes terpénicos e fenólicos de flores recolhidas em diferentes localizações, foi avaliada usando diferentes solventes e métodos de extração. Os extratos lipofílicos (absolutos) foram caracterizados por SPME-GC-TOFMS mostrando a presença de ácidos orgânicos, hidrocarbonetos e tri-terpenos pentacíclicos. A fração fenólica das flores apresentou altos teores de fenólicos totais e sua caracterização por HPLC-DAD e HPLC-DAD-MS/MS revelou a presença de glicosídeos flavonoides com atividades biológicas relevantes. Extratos polares de folhas, galhos, madeira e casca apresentaram altos teores de fenólicos, flavonoides e proantocianidinas totais. Das folhas obtiveram-se extratos lipofílicos ricos em fitol, lupenona e lupeol. Todos os extratos fenólicos apresentaram propriedades antioxidantes (testes DPPH e FRAP) e atividade antimicrobiana contra bactérias gram-positivas. As biomassas brutas e extraídas foram caracterizadas para validar seu uso como biocombustíveis após extração com solventes. Torrefação e carbonização hidrotérmica foram utilizadas para produzir biocarvões e hidrocarvões com propriedades combustíveis melhoradas a partir das biomassas extraídas. As biomassas extraídas e os carvões foram testados como adsorventes para corantes catiónicos e aniónicos, alcançando eficiências de remoção superiores a 90%. Testou-se a incorporação das frações de biomassa de Acácia em argamassas como uma contribuição para a incorporação de recursos renováveis em materiais de construção.

Os resultados obtidos demonstram múltiplas vias de transformação que podem ser utilizados para valorizar biomassa de *Acacia* produzindo energia e diversos produtos comercializáveis, promovendo assim a bioeconomia e a sustentabilidade do setor florestal.

Palavras chave: *Acacia dealbata*, absolutos, fenólicos, biomassa, torrefação, carbonização hidrotérmica, biocombustíveis, adsorventes, argamassas

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ACRONYMS

A	Arrhenius pre-exponential factor
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate radical
ACE	Acetone
AI	Alkali index
ALC	Alcobaça
BAI	Bed agglomeration index
BHIA	Brain heart infusion agar
ButOH	Butanol
CatE	Catechin equivalent
ChIF	Chloroform
db	Dry basis
DCM	Dichloromethane
DiEtE	Diethyl ether
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
DTG	Derivative thermogravimetric
Ea	Activation energy
EtA	Ethyl acetate
EtOH	Ethanol

eV	Electron-volt
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
GC-TOFMS	Gas chromatography-time-of-flight mass spectrometry
HEX	Hexane
HHV	High heating value
HPLC-DAD	High-performance liquid chromatography with diode-array detection
ID	Inhibition diameter
m	Mass
MB	Methylene blue
MetOH	Methanol
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
MO	Methyl orange
PE	Petroleum ether
Pyc	Pycnogenol®
PycE	pycnogenol® equivalent
QE	Quercetin equivalent
q_t	Adsorption capacity
R	Universal gas constant
RE	Removal efficiency
RutE	Rutin equivalent
R_t	Retention time
SDA	Sabouraud dextrose agar
SEI	Slagging evaluation index
SI	Sintering index

T_b	Burnout temperature
TE	Trolox equivalent
TGA	Thermogravimetric analysis
T_i	Ignition temperature
TPC	Total phenolics content
TFC	Total flavonoids content
TPrAC	Total proanthocyanidins content
V	Volume
wb	Wet basis

GENERAL INTRODUCTION

1.1 Context and motivation

The genus *Acacia* comprises a large group of more than 1350 species, widely distributed throughout tropical and warm temperate areas of the world (Maslin, 2015). Most of the species are native to Australia but spread all over the world because of their wide variety of uses and economic importance such as for ornamental purposes, for sand and dune stabilization, as a fuel through the production of woodfuel and charcoal, as an important source of fodder, tannins for the leather industry, gums, and essences for perfumes (Breton et al., 2008; Kull et al., 2011; Seigler, 2003). In the Portuguese territory, its introduction was mainly for the stabilization of dunes and soil (Marchante et al., 2003), as well as for ornamental purposes (Nunes et al., 2019).

However, its worldwide cultivation led to its establishment and invasion in many regions of the world, with negative impacts on biodiversity and on ecosystem properties and functions (Miller et al., 2011). *Acacia* spp. are considered invasive in the Portuguese territory (*Ministério do Ambiente*, 2019), where the occupied area almost doubled from 1995 to 2010, passing from 2701 ha to 5351 ha, according to the last national forest inventory (ICNF, 2013). Amongst invasive *Acacia* spp, *A. cyclops*, *A. dealbata*, *A. longifolia*, *A. melanoxylon*, *A. saligna*, *A. retinodes*, *A. karroo*, *A. mearnsii* and *A. pycnantha* are the most representative, with *A. dealbata* being especially important once it forms the largest clusters and is the most-dispersed species (Nunes et al., 2019). *A. dealbata* is currently the invasive species that occupies the largest area in the national territory, having increased its area from about 2500 ha in the 70s to 8400 ha in 2015, as referred by Nunes et al. (Nunes et al., 2019). This species has indeed a large capacity for raw material supply, having been estimated that about 70 tons of biomass (whole tree) can

be obtained per hectare (Nunes et al., 2020). Figure 1.1 shows some areas invaded by *A. dealbata*.



Figure 1.1 — Examples of areas invaded by *A. dealbata* in (a) Alcobaça, (b) Caparica, and (c) Coruche.

The threat to protected areas, and the negative impacts on the ecosystems and the productivity of the forest sector have motivated expensive removal initiatives by local authorities in order to minimize its proliferation (Ferreira et al., 2011), generating high amounts of biomass which is usually burned for energy production or landfilled. Besides the valorization of some *Acacia* spp. for energy may have a positive contribution to the economic sustainability of the involved operations, especially if done locally, this option is often limited due to collection and transportation costs (Carneiro et al., 2014; Nunes et al., 2020). However, finding high-value applications for these species can contribute to overcoming these costs and make the process economically sustainable.

Acacia spp. fractions, namely bark, wood, leaves, flowers, pods, seeds or roots of *Acacia* spp. are rich sources of bioactive secondary metabolites (e.g. amines and alkaloids, cyanogenic glycosides, cyclitols, fatty acids and seed oils, gums, non-protein amino acids, terpenes, tannins and other flavonoids, and simple phenolics) (Jæger et al., 2019; Pereira et al., 1996; Seigler, 2003) that have been used in traditional medicine for a wide range of ailments, such as diabetes, worm infection, dysmenorrhea, eczema, malaria, gout, jaundice, abdominal pain, kidney problems, constipation, leprosy, piles, pneumonia, rheumatism, fever, and cancer (Subhan et al., 2018). These secondary metabolites can play an important role in reducing oxidative stress, by acting as antioxidants, and possess antimicrobial properties, which is important in the development of alternatives to antibiotics due to the increasing resistance to the conventional antimicrobial agents (Subhan et al., 2018). Besides, other important biological activities, such as anti-inflammatory, antiviral, anticancer, antidiabetic, immunomodulatory, hepatoprotective, cardioprotective, and anthelmintic were already reported for extracts obtained from *Acacia* spp. (Subhan et al., 2018). This way, the production of bioactive preparations of *Acacia* spp., either from well-established areas or from the biomass generated in the control operations, can be an option to bring an important economic return, overcoming expenses with collection

and transport, while simultaneously contributing to motivating forest cleaning and management actions, reducing the risk of fires, and improving the social–economic development of rural areas. Moreover, the valorization of its flowers, as an example, will have a positive contribution to the control of the proliferation by preventing the formation of seeds, thus minimizing the spread through the seed dispersal route (Lorenzo et al., 2019). Other well–studied applications of *Acacia* biomass fractions with the potential to contribute to the industrial exploitation of these species, and therefore to the control of their unregulated widespread, include its incorporation in polymers and composites, micro and nanomaterials, or as adsorbents.

This work intends to evaluate the obtention of bioactive extracts from several fractions of *A. dealbata* (flowers, leaves, twigs, bark, and wood), with potential for application in the lucrative nutraceutical, pharmaceutical, cosmetic, and food, and feed industries, as well as the valorization of the generated biomass residues for energy purposes, through its conversion in biochars, or in the development of other materials, such as adsorbents and its incorporation in mortars. Under the current transition towards a biobased economy, the cost–efficient use of biomass for the production of biobased products and energy is facilitated by biorefining systems (Jungmeier, 2015). Therefore, the knowledge of these options is essential to enable the valorization of this resource using a biorefinery approach, focused on the sustainable processing of biomass to yield a spectrum of marketable products and energy (Cherubini, 2010). It has been strongly recommended by specialists the search for these novel integrative and cost–effective solutions, involving the collaboration of society, politicians, and stakeholders, as necessary to achieve sustainable control of *Acacias* (Souza-Alonso et al., 2017).

1.2 Research goals

1.2.1 The problem

A. dealbata is an invasive species widely spread all over the Portuguese territory. Its phenotypic plasticity, conferring the ability to adapt to changing environments, the large seed production and accumulation of massive seed banks for long periods, the high capacity for vegetative reproduction from rhizomes after disturbances, such as fires and cuttings, and its allelopathic properties, inhibiting the growth of neighboring native species, are the most contributing factors for its success as an invader (Fuentes-Ramírez et al., 2011; Le Maitre et al., 2011; Lorenzo et al., 2010a).

As described, the uncontrolled proliferation of *A. dealbata*, as well as of other *Acacia* spp., has negative impacts on biodiversity and ecosystem properties and functions (Miller et al., 2011). Currently, this species is threatening protected areas and is harming the productivity of the forest sector in some regions, which has encouraged expensive removal initiatives by local authorities aiming its eradication or at least the minimization of its proliferation (Ferreira et al., 2011). To the best of our knowledge, the biomass residues generated in these operations are usually burned to produce energy or are landfilled. The first option does not bring enough economic return to overcome the expenses involved in collection and transportation and the second must be avoided due to environmental issues. Furthermore, given the magnitude of the invasion, the radicular system of these species, the dispersion of seeds in the natural environments, and the years necessary before soil nutrients and processes return to similar pre-invasion levels, their elimination by removal has been considered unfeasible from practical and economical perspectives (Souza-Alonso et al., 2017), and has the potential of by itself to cause significant environmental impacts, namely the destabilization of large volumes of soil.

1.2.2 Potential solutions

Acacia spp. fractions, including from *A. dealbata*, like bark, wood, leaves, and flowers, are reported to contain bioactive secondary metabolites (e.g. phenolics, flavonoids, proanthocyanidins, or terpenic compounds) that have been used in traditional medicine for a wide range of ailments, due to its antioxidant, antimicrobial, anti-inflammatory, antiviral, anticancer, anti-diabetic, immunomodulatory, hepatoprotective, cardioprotective, or anthelmintic properties, among others (Jæger et al., 2019; Pereira et al., 1996; Seigler, 2003; Subhan et al., 2018). Additionally, the lignocellulosic fraction of *A. dealbata* has been reported to have the potential for energy recovery, since possessing a high calorific power and low ash contents (Saldarriaga et al., 2015). Moreover, the use of *Acacia* spp. biomass or derived products in the production of materials has also been described, such as in nanomaterials (Khan et al., 2015; Martins et al., 2018; Missio et al., 2017), composites (Taflick et al., 2015), and adsorbents (Beckinghausen et al., 2020; da Silva et al., 2018; Taşkıran et al., 2017).

Therefore, the production of high-value bioactive extracts from *A. dealbata* biomass, together with its valorization for energy recovery and/or materials may have a fundamental contribution to the economic and environmental sustainability of its exploitation, either when using biomass residues from removal operations or when using biomass from zones where it is well-established, to control the proliferation. The use of flowers, as an example, will have a

positive contribution to the control of the proliferation by preventing the formation of seeds, thus minimizing the spread through the seed dispersal route (Lorenzo et al., 2019).

This approach of valuing the biomass of *A. dealbata* from a biorefinery perspective is an innovative concept on the national scene, which allows broadening the value generation throughout the entire processing chain. Combining energy recovery with obtaining products with high added value, enhances the economic viability of the global process and the most efficient and sustainable use of an endogenous resource, with positive consequences for the development of the forestry sector and rural areas.

1.2.3 General goal

This work intends to give a contribution to the exploitation of *A. dealbata* biomass (flowers, leaves, twigs, wood, and bark) from a biorefinery perspective, through the production of bioactive extracts, as well as solid biofuels and materials using the generated extraction residues.

1.2.4 Specific goals

- Evaluate the use of different solvents in obtaining bioactive preparations from the different fractions of *A. dealbata*, namely, aqueous solutions of acetone, ethanol, and methanol to retrieve more hydrophilic components, as well as petroleum ether to obtain components of more lipophilic character.
- Compare the use of a simple maceration process at room temperature with more energy-demanding processes requiring heat, such as Soxhlet or hot extractions at a temperature above room temperature.
- Evaluate the possibility of broadening the range of obtained products from the same amount of raw material, by performing sequential extraction with non-polar solvent (petroleum ether) and polar aqueous solutions of acetone, ethanol, and methanol.
- Evaluate the possibility of broadening the range of obtained products by performing a single extraction step with a mixture of non-polar and polar solvents.
- Perform the chemical characterization of the obtained extracts by colorimetric tests to determine their contents in some classes of compounds (e.g. phenolics, flavonoids, and proanthocyanidins), and chromatographic techniques (HPLC-DAD and GC-MS) to identify individual components.

- Characterize the residues obtained after extraction processes, to evaluate the potential for their application for energy purposes or the development of materials, such as adsorbents or their incorporation in mortars.
- Evaluate the production of biochars from the generated extraction residues by a conventional torrefaction method and by hydrothermal carbonization.
- Perform physical and chemical characterization of the produced solid biofuels to evaluate their potential for energy recovery or material valorization.

1.3 Document organization

- **Chapter 1:** General Introduction

Describes the context and motivation of this work, as well as the problem and the main goals to be achieved.

- **Chapter 2:** State-of-the-art

Presents information about the potential of the most representative *Acacia* spp. In the national territory to be explored from a biorefinery perspective.

The following review paper is based on this chapter:

Correia, R., Quintela, J.C., Duarte, M.P., Gonçalves, M., 2020. Insights for the valorization of biomass from Portuguese invasive *Acacia* spp. in a biorefinery perspective. *Forests* 11, 1–42. <https://doi.org/10.3390/f11121342>

- **Chapter 3:** Antioxidants and Antimicrobial Agents from the Flowers of Invasive *Acacia* spp.

Provides information on the obtention of antioxidant and antimicrobial extracts from the flowers of Portuguese invasive *Acacia dealbata* and *Acacia saligna*, with different geographical proveniences.

- **Chapter 4:** One-Pot Extraction for Simultaneous Obtention of Bioactive Compounds and Absolute Oils from Invasive *Acacia dealbata* Flowers

Presents a simple and innovative approach to obtain lipophilic and hydrophilic bioactive chemicals from *A. dealbata* flowers in a single extraction step.

- **Chapter 5:** Volatile Component Profile from Portuguese Mimosa (*A. dealbata*) Absolute Oils

Presents the first chemical profile of absolute oil produced from Portuguese *Acacia dealbata* flowers. Absolute oils produced by an innovative approach regarding obtaining hydrophilic components together with a lipophilic fraction were also characterized and compared.

- **Chapter 6:** Chemical and Functional Characterization of Extracts from Leaves and Twigs of *Acacia dealbata*

Describes the production of antioxidant and antimicrobial extracts from the leaves and twigs of *Acacia dealbata* from different locations, using different extraction methods, and different drying status of the biomass.

The following research paper is based on this chapter:

Correia, R., Duarte, M.P., Maurício, E.M., Brinco, J., Quintela, J.C., Silva, M.G. da, Gonçalves, M., 2022. Chemical and Functional Characterization of Extracts from Leaves and Twigs of *Acacia dealbata*. Processes 10, 2429. <https://doi.org/10.3390/PR10112429>

- **Chapter 7:** Chemical and Functional Characterization of Extracts from Wood and Bark of *Acacia dealbata*

Presents information on the production of antioxidant and antimicrobial extracts from the wood and bark of a Portuguese invasive species.

- **Chapter 8:** Characterization of *Acacia dealbata* Biomass for Energy and Material Application: Effect of the Previous Extraction of Bioactive Components

The remnants of each fraction were physically and chemically characterized to evaluate their potential for energetic or material applications.

- **Chapter 9:** Characterization of Biochars and Hydrochars Obtained from Different Fractions of *Acacia dealbata* Biomass for Energy or Material Applications

Torrefaction and hydrothermal carbonization were used to upgrade the physical and chemical properties of the spent biomass, for energetic or material application as adsorbents.

- **Chapter 10:** Effect of Different Fractions of *Acacia dealbata* Biomass on Fresh Gypsum Mortars for Eco Plasters

Presents a preliminary study on the effect of the incorporation of the spent *Acacia dealbata* fractions in the fresh-state behavior of gypsum mortars.

- **Chapter 11:** General Conclusions and Future Work

Includes the main conclusions that were drawn from this thesis work, as well as suggestions for future work.

STATE-OF-THE-ART

2.1 Introduction

The genus *Acacia* comprises a large group of more than 1350 species, widely distributed throughout tropical and warm temperate areas of the world (Maslin, 2015). Most of the species are native to Australia but are spread all over the world because of their wide variety of uses and economic importance such as for ornamental purposes, for sand and dune stabilization, as a fuel through the production of woodfuel and charcoal, as an important source of fodder, tannins for the leather industry, gums, and essences for perfumes (Breton et al., 2008; Kull et al., 2011; Seigler, 2003). In the Portuguese territory its introduction was mainly for stabilization of dunes and soil (Marchante et al., 2003), as well as for ornamental purposes (Nunes et al., 2019).

However, its worldwide cultivation has led to its establishment and invasion in many regions of the world, with negative impacts on biodiversity and on ecosystem properties and functions (Miller et al., 2011). Some key features contributing to the success of *Acacia* spp. as invaders include their phenotypic plasticity, which confers them with the ability to adapt to changing environments; their large seed production and accumulation of massive seed banks for long periods; their high capacity for vegetative reproduction from rhizomes after disturbances, such as fires and cuttings; and their allelopathic properties, inhibiting the growth of neighboring native species (Fuentes-Ramírez et al., 2011; Le Maitre et al., 2011; Lorenzo et al., 2010a).

Acacia spp. are considered invasive in the Portuguese territory (*Ministério do Ambiente*, 2019), where the occupied area almost doubled from 1995 to 2010, passing from 2701 ha to 5351 ha, according to the last national forest inventory (ICNF, 2013). Amongst invasive *Acacia*

spp., *A. cyclops* A. Cunn. ex G. Don fil., *A. dealbata* Link, *A. longifolia* (Andrews) Willd., *A. melanoxylon* R. Br., *A. saligna* (Labill.) H. L. Wendl., *A. retinodes* Schlecht., *A. karroo* Hayne, *A. mearnsii* De Wild. and *A. pycnantha* Bentham are the most representative. Figure 2.1 shows examples of invaded areas by *Acacia* spp. in the Portuguese territory.



Figure 2.1 — Examples of invaded areas by *Acacia* spp. in the Portuguese territory (elaborated from images taken from Plantas Invasoras em Portugal (<http://invasoras.pt>), accessed on 01/11/2020).

The threat to protected areas, and the negative impacts on the ecosystems and on the productivity of the forest sector have motivated expensive removal initiatives by local authorities in order to minimize its proliferation (Ferreira et al., 2011), generating high amounts of biomass which is usually burned for energy production or landfilled. The valorization of some *Acacia* spp. for energy may have a positive contribution to the economic sustainability of the involved operations (Carneiro et al., 2014), but this option is often limited due to collection and transportation costs. However, finding high-value applications for these species can contribute to overcome these costs and make the process economically sustainable. Furthermore, given the magnitude of the invasion, the radicular system of these species, the dispersion of seeds in the natural environments, and the years necessary before soil nutrients and processes return to similar pre-invasion levels, their elimination by removal has been considered unfeasible from practical and economical perspectives (Souza-Alonso et al., 2017), and has the potential of by itself to cause significant environmental impacts, namely the destabilization of large volumes of soil.

Acacia spp. fractions, namely bark, wood, leaves, flowers, pods, seeds or roots, are rich sources of bioactive secondary metabolites (e.g., amines and alkaloids, cyanogenic glycosides, cyclitols, fatty acids and seed oils, gums, non-protein amino acids, terpenes, tannins and other flavonoids and simple phenolics) (Jæger et al., 2019; Pereira et al., 1996; Seigler, 2003) that have been used in traditional medicine for a wide range of ailments, such as diabetes, worm infection, dysmenorrhea, eczema, malaria, gout, jaundice, abdominal pain, kidney problems, constipation, leprosy, piles, pneumonia, rheumatism, fever and cancer (Subhan et al., 2018). These secondary metabolites can play an important role in reducing oxidative stress, by acting as antioxidants, and possess antimicrobial properties, which are important in the development of alternatives to antibiotics due to the increasing resistance to the conventional antimicrobial agents (Subhan et al., 2018). Besides, other important biological activities, such as anti-inflammatory, antiviral, anticancer, antidiabetic, immunomodulatory, hepatoprotective, cardioprotective and anthelmintic, were already reported for extracts obtained from *Acacia* spp. (Subhan et al., 2018) In this way, the production of bioactive preparations of *Acacia* spp., either from well-established areas or from the biomass generated in the control operations, can be an option to bring an important economic return, overcoming expenses with collection and transport, while simultaneously contributing to motivating forest cleaning and management actions, reducing the risk of fires and improving the social-economic development of rural areas. Moreover, the valorization of its flowers, as an example, will have a positive contribution to the control of the proliferation by preventing the formation of seeds, thus minimizing the spread through the seed dispersal route (Lorenzo et al., 2019). Other well-studied applications of *Acacia* spp. biomass fractions with the potential to contribute to the industrial exploitation of these species, and therefore to the control of their unregulated widespread, include its incorporation in polymers and composites, micro and nanomaterials or as adsorbents.

This review presents various high-value applications of different biomass fractions from Portuguese invasive *Acacia* spp. (e.g. bark, wood, leaves, flowers, pods, seeds and roots) Besides the potential applications in the lucrative nutraceutical, pharmaceutical, cosmetic and food and feed industries, the production of innovative materials and biofuels will be also discussed. Under the current transition towards a biobased economy, the cost-efficient use of biomass for the production of biobased products and energy is facilitated by biorefining systems (Jungmeier, 2015). Therefore, the knowledge of these options is essential to enable the valorization of this resource using a biorefinery approach, focused on the sustainable processing of biomass to yield a spectrum of marketable products and energy (Cherubini, 2010). It has been strongly recommended by specialists the search for these novel integrative and

cost-effective solutions, involving the collaboration of society, politicians and stakeholders, as necessary to achieve sustainable control of *Acacias* (Souza-Alonso et al., 2017).

2.2 Bioactive extracts from *Acacia* spp. plant components

2.2.1 Bark

Overall, the bark is a by-product and is highly available from the adopted proliferation control operations or from the logging industries, whose disposal may cause environmental problems. For example, the bark of *A. dealbata* is frequently removed without felling the tree to reduce or prevent sprouting, and the bark of *A. melanoxylon* can come from industries that valorize the high-quality wood of this species (Neiva et al., 2020). Research has been conducted regarding the production of high-value products extracted from this biomass fraction, where it can be seen that *Acacia* spp. are one of a few 24 species that currently have commercialized products retrieved from their bark, especially for the leather and adhesive industries (Feng et al., 2013; Pásztor et al., 2016). Recent research has shown that the bark of *Acacia* spp. contains important chemicals with the potential to be applied in recently growing and profitable industries, such as nutraceutical, cosmetic, pharmaceutical or food industries (see Table 2.1).

Table 2.1 — Reported biological activities for extracts of *Acacia* spp. bark.

<i>Acacia</i> spp.	Extraction	Bioactivity/Application	Reference
<i>A. dealbata</i>	ACE (Soxhlet, previous defatting with PE)	Antioxidant	(Sowndhararajan et al., 2013),(Sowndhararajan et al., 2015)
	DCM (Soxhlet, 8h)	Pharmaceutics, nutraceutics, cosmetics (low cytotoxicity/safe use)	(C. S. D. Oliveira et al., 2020)
	ACE (Soxhlet, previous defatting with PE)	Anti-inflammatory	(Sowndhararajan et al., 2016)
	MetOH (room temperature, 24h)	Antimicrobial	(Yildiz et al., 2018)
	HEX, EtOH, H ₂ O (Soxhlet, 16–24h)	Antimicrobial, antioxidant	(Neiva et al., 2020)
<i>A. karroo</i>	H ₂ O (room temperature, 48h)	Anti-inflammatory	(Adedapo et al., 2008)
	PE, DCM, 80% EtOH, H ₂ O (sonication, 15 °C, 1h)	Antimicrobial, anti-HIV-1	(Mulaudzi et al., 2011)
	50% MetOH (sonication, 15 °C, 20 min) for anti-HIV-1 assay		
<i>A. longifolia</i> , <i>A. farnesiana</i> , <i>A. tortilis</i>	50% EtOH and 70% EtOH (room temperature)	Anti-inflammatory	(Gabr et al., 2018)
<i>A. mearnsii</i>	Commercial extract referred to as “hot water extract”	Antioxidant, antimicrobial, inhibition of digestive enzymes (lipase, α -amylase, α -glucosidase, maltase and sucrase), anti-obesity, antidiabetic, anti-inflammatory, etc.	(Ogawa et al., 2018),(Ogawa and Yazaki, 2018)
		Animal feed	(Denninger et al., 2020),(Orlandi et al., 2015),(Lima et al., 2019)
<i>A. melanoxylon</i>	HEX, EtOH, H ₂ O (Soxhlet, 16–24h)	Antimicrobial	(Neiva et al., 2020)

ACE: acetone; PE: petroleum ether; DCM: dichloromethane; MetOH: methanol; EtOH: ethanol; HEX: hexane; H₂O: water.

2.2.1.1 Commercial Tannin-Rich Extracts From Bark

The most known class of compounds present in the bark of *Acacia* spp. are the flavonoids, where tannins, and more specifically the proanthocyanidins, are included. After the discovery of the incredible superoxide scavenging activity of an aqueous extract obtained from black wattle (*A. mearnsii*), approximately 10 times higher than that of the pine bark supplements Pycnogenol® and Enzogenol®, clinical studies were made on the human safety use of this extract, which has been commercialized in Japan since 2007 with the label Acapolia® (Yazaki, 2015). The biological activities demonstrated by the proanthocyanidins derived from *A. mearnsii* bark, including antioxidant, antimicrobial, anti-obesity, antidiabetic, anti-inflammatory, among others, are extensively reviewed in the literature, the role of proanthocyanidin oligomers having 5-deoxyflavan-3-ol units and the presence of distinctive flavan-3-ols like such as robinetinidol and fisetinidol being highlighted (Ogawa et al., 2018; Ogawa and Yazaki, 2018; Yazaki, 2015). Another field of application of tannin bark extract from *Acacia* spp. is their inclusion in animal feed. Several studies used commercial tannin-rich extract obtained from *A. mearnsii* bark and highlighted its effect as a methane-mitigating agent for dairy cows (Denninger et al., 2020); in decreasing the urinary excretion of urea in sheep (Orlandi et al., 2020); in reducing the urinary nitrogen excretion and improving the amino acids supply in Holstein steers without significantly affecting the total organic matter digestibility (Orlandi et al., 2015); or as anthelmintic agents in sheep artificially infected with parasites *Trichostrongylus colubriformis* and *Haemonchus contortus* (Lima et al., 2019). However, precautions must be taken and further research must be developed, since reducing effects on *in vivo* nutrient digestibility and/or negative impacts on energy intake were observed in sheep and wethers (Gerlach et al., 2018; Kozloski et al., 2012).

2.2.1.2 Antioxidants, Anti-Inflammatory and Anti-Viral Agents from Bark

Sowndhararajan et al. (Sowndhararajan et al., 2013) compared acetone and methanol as extraction solvents of previously petroleum ether defatted bark of Indian *Acacias*, including *A. dealbata*, aiming to obtain antioxidants. The authors observed better outcomes using acetone, with remarkable results from free radical scavenging assays against DPPH, ABTS and OH free radicals, and from ferric-reducing antioxidant power, metal chelation, phosphomolybdenum reduction and peroxidation inhibition assays, which were comparable or in some cases higher than those obtained with the widely used antioxidants BHT (butylated hydroxytoluene) and α -tocopherol. The authors found strong positive correlations between these antioxidant activities

and the phenolic and flavonoid contents of the extracts. This acetone extract also demonstrated effective inhibition of H₂O₂-mediated oxidative stress in human hepatoma (HepG2) cells, in a concentration-dependent manner (25, 50 and 75 mg/mL), which gives it the potential to be explored as a therapeutic agent in preventing oxidative stress-mediated diseases (Sowndhararajan et al., 2015).

Treating inflammation has become the focus of global scientific research because of its implication in virtually all human and animal diseases, and because conventional drugs used to ameliorate this phenomenon are too expensive, toxic, and are not commonly available to the entire population of the world (Adedapo et al., 2008). Therefore, research has been done to find anti-inflammatory agents of natural origin, with *Acacia* spp. contributing in a significant manner to this purpose. Sowndhararajan et al. (Sowndhararajan et al., 2016) demonstrated that an acetone extract of *A. dealbata* bark effectively inhibited the production of nitric oxide and suppressed the expressions of cyclooxygenase (COX-2), inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF- α) in lipopolysaccharide (LPS) -stimulated RAW 264.7 macrophage cells, in a concentration-dependent manner (25, 50 and 75 mg/mL). These results suggest its potential use as a source of anti-inflammatory agents by suppressing the expression of pro-inflammatory mediators. Similarly, hydroalcoholic extracts from the bark of *A. longifolia*, *A. farnesiana* and *A. tortilis* showed anti-inflammatory activity by inhibition of COX-1 and COX-2 enzymes (Gabr et al., 2018). An *in vivo* study demonstrated the anti-inflammatory and also the analgesic activity of an aqueous extract from the stem bark of *A. karroo*, even when used by the oral route (Adedapo et al., 2008). Anti-inflammatory activity was accessed using the carrageenan-induced and histamine-induced rat paw oedema models, a strong reduction of the formation of oedema at 100 and 200 mg/kg doses for both models being observed. Analgesic activity was evaluated in mice using acetic acid-induced writhing response and the tail immersion test, with both 100 and 200 mg/kg doses showing a significant reduction in the number of writhes and increasing the reaction time to pain after 30 minutes of oral administration of the extract (Adedapo et al., 2008). The effects of the *A. karroo* extract were comparable to the observed with the reference drug indomethacin, administrated at a 10 mg/kg dose (Adedapo et al., 2008).

Extracts of the bark of *A. karroo* showed promise for the results as anti-HIV agents of natural origin. Mulaudzi et al. (Mulaudzi et al., 2011) demonstrated the potent HIV-1 reverse transcriptase inhibitory activity by hydromethanolic and aqueous extracts (over 70% of inhibition at 1 mg/mL based on COX assay), exhibiting IC₅₀ (i.e., the necessary concentration to attain 50% of inhibition) values of 30 μ g/mL and 100 μ g/mL, respectively (Mulaudzi et al., 2011). Since

medicines used to fight HIV are expensive and not available for the entire population, the use of drugs of natural origin can contribute to ameliorating this situation, and the extracts of *A. karroo* are good candidates to be further studied for that purpose (Mulaudzi et al., 2011).

2.2.1.3 Antimicrobial Agents from Bark

An important feature of extracts from plant origin is their antimicrobial activity, contributing to finding alternatives to synthetic antibiotics, due to the increasing pathogenic resistance to conventional antimicrobial agents (Ghannoum M. A. and Rice L. B., 1999). Microbial strains already studied with bark extracts of *Acacia* spp. are summarized in Table 2.2, where it can be seen that polar and apolar extracts from the bark of *A. dealbata*, *A. melanoxylon* and *A. karroo*, have shown antimicrobial activity against a variety of Gram-positive and Gram-negative bacteria and fungi. The reported values are comparable to the ones obtained with antibiotics ampicillin, gentamicin, tetracycline, cefotaxime and amphotericin B, which showed MIC values in the range 10–1250 µg/mL (Yildiz et al., 2018). Comparing them with other species, Neiva et al. (Neiva et al., 2020) found better antimicrobial activity against bacteria and fungi when using ethanolic and aqueous *A. dealbata* extract than when using the corresponding *Eucalyptus globulus* or *Picea abies* bark extracts. The extract of *A. karroo* exhibited a activity similar to those found with other species traditionally used to treat venereal diseases, such as *Aloe chabaudii*, *Adansonia digitata*, *Bolusanthus speciosus*, *Elephantorrhiza burkei*, *Ekebergia capensis*, *Grewia occidentalis*, *Osyris lanceolata*, *Peltophorum africanum*, *Pterocarpus angolensis*, *Pappea capensis* or *Ximenia caffra* (Mulaudzi et al., 2011)

Table 2.2 — Microbial strains inhibited by *Acacia* spp. bark extracts.

<i>Acacia</i> spp.	Extract	Microorganism strain	Antimicrobial activity	Reference
<i>A. dealbata</i>	Methanolic	GP: <i>Staphylococcus aureus</i> , <i>Listeria monocytogenes</i> GN: <i>Klebsiella pneumoniae</i> , <i>Pseudomonas aeruginosa</i> , <i>Proteus mirabilis</i> Fungi: <i>Candida albicans</i>	MIC: 156–625 µg/mL for bacteria MIC: 2500 µg/mL for fungi	(Yildiz et al., 2018)
<i>A. dealbata</i> , <i>A. melanoxylon</i>	Ethanollic; aqueous; hexanic	GP: <i>Staphylococcus aureus</i> , <i>Listeria monocytogenes</i> , <i>Bacillus cereus</i> , <i>Enterococcus faecalis</i> , GN: <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Pseudomonas aeruginosa</i> , <i>Acinetobacter baumannii</i> , <i>Salmonella enterica</i> serovar Typhimurium Fungi: <i>Candida albicans</i> , <i>Candida tropicalis</i>	MIC: 40–10000 µg/mL for bacteria MIC: 20–10000 µg/mL for fungi	(Neiva et al., 2020)
<i>A. karroo</i>	Hydroethanolic; aqueous; petroleum ether; dichloromethane	GP: <i>Staphylococcus aureus</i> , <i>Bacillus subtilis</i> GN: <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Neisseria gonorrhoeae</i> Fungi: <i>Candida albicans</i>	MIC: 195–3125 µg/mL for bacteria, except <i>N. gonorrhoeae</i> MIC: 3125–6250 µg/mL for fungi 44%–51% inhibition, compared to ciprofloxacin, for <i>N. gonorrhoeae</i>	(Mulaudzi et al., 2011)

GP: gram-positive bacteria; GN: gram-negative bacteria; MIC: minimum inhibitory concentration

2.2.1.4 Properties of the Lipophilic Fraction from Bark

The exploitation of lipophilic compounds from *Acacia* spp. bark has also been tried in the last few years. Freire et al. investigated bark of *A. dealbata*, *A. melanoxylon*, *A. longifolia* and *A. retinodes* for their sterol content using dichloromethane as an extraction solvent, having found free sterol contents between 213 mg/kg (*A. melanoxylon*) and 321 mg/kg (*A. longifolia*) relative to dried bark, with particularly high amounts of spinasterol (96–192 mg/kg of dry bark) and dihydrospinasterol (92–126 mg/kg of dry bark) (Freire et al., 2005). The corresponding unusual steryl glucosides, spinasteryl glucoside (88–186 mg/kg) and dihydrospinasteryl glucoside (58–166 mg/kg of dry bark) were also identified in significant amounts in the barks of *A. dealbata*, *A. longifolia* and *A. melanoxylon* (Freire et al., 2005). The biological activities of sterols are extensively reviewed in the literature (Kaur et al., 2011; Miras-Moreno et al., 2016), including important roles in reducing cholesterol levels in the blood, or as antidiabetic and anticancer agents. Specific bioactivity for spinasterol, dihydrospinasterol and respective glucosides were also referred, such as antitumorigenic potential and therapeutic potential in modulating the diabetic neuropathy evidenced by spinasterol, potent inhibitory effect on the Epstein-Barr virus early antigen attributed to dihydrospinasterol, and anticarcinogenic and cytotoxic potential exhibited by dihydrospinasterol glucoside (Freire et al., 2005). Recently, a broader characterization of the lipophilic fraction was performed for *A. dealbata* bark (C. S. D. Oliveira et al., 2020). Terpenic compounds (lupenone and lupenyl acetate) represent the major lipophilic family in *A. dealbata* bark (3451 mg/kg of dry bark), followed by long-chain aliphatic alcohols (1083 mg/kg of dry bark), fatty acids (1060 mg/kg of dry bark) (with more than 10% of these being unsaturated fatty acids), monoglycerides (692 mg/kg of dry bark), sterols (484 mg/kg of dry bark), aromatic compounds (29 mg/kg of dry bark) and others (744 mg/kg of dry bark), among which was α -tocopherol (a type of vitamin E) (46 mg/kg of dry bark) (C. S. D. Oliveira et al., 2020). Terpenic compounds, and more specifically lupenone, were reported to have good therapeutic potential in inflammation, virus infection, diabetes, cancer, and treatment of Chagas disease (Xu et al., 2018), and the positive effects of unsaturated fatty acids on the cardiovascular, respiratory, gastrointestinal, renal and immune systems are well known (Gill and Valivety, 1997). A cytotoxicity screening of the above-described lipophilic *A. dealbata* bark extract in several mammalian cell lines representing brain, immune system, skin, lung and liver cells, revealed encouraging results regarding its future incorporation in oral or topical pharmaceutical or nutraceutical formulations since there is a lack of toxicity in liver hepatocytes (HepG2), epidermis keratinocytes (HaCaT) and dermis fibroblasts (NIH/3T3) (C. S. D. Oliveira et al., 2020).

2.2.2 Wood

Wood from *Acacia* spp. can be an abundantly available resource from removal operations or as a residue from logging industries that use *Acacia* spp. as a raw material. As a rule, wood has a lower organic solvent and water-soluble extractives, including polyphenols, when compared to bark (Fradinho et al., 2002; Freire et al., 2005; Miranda et al., 2012; C. S. D. Oliveira et al., 2020). However, these extractives may contain bioactive secondary metabolites that can be exploited for incorporation in formulations of nutraceuticals, cosmetics, or other high-value applications. Extractives content for some *Acacia* spp. has been found in the range of 4.0–9.5% relative to dried wood (Domínguez et al., 2014; Ferreira et al., 2011; Lourenço et al., 2008). Table 2.3 resumes the reported biological activities of wood extracts from some species.

Table 2.3 — Reported biological activities for extracts of *Acacia* spp. wood.

<i>Acacia</i> spp.	Extraction	Bioactivity/Application	Reference
<i>A. dealbata</i>	MetOH (room temperature, 24 h)	Antimicrobial	(Yildiz et al., 2018)
<i>A. dealbata</i>	DCM (Soxhlet, 8 h)	Pharmaceutics, nutraceuticals, cosmetics (low cytotoxicity/safe use)	(C. S. D. Oliveira et al., 2020)
<i>A. karroo</i>	MetOH (room temperature, 48 h)	Antimicrobial	(Nielsen et al., 2012)

MetOH: methanol; DCM: dichloromethane.

2.2.2.1 Antimicrobial Agents from Wood

Yildiz et al. (Yildiz et al., 2018) observed that methanolic extracts from sapwood and heartwood of *A. dealbata* were revealed to be effective against *L. monocytogenes*, *S. aureus* and *K. pneumoniae*. MIC values ranged between 39 and 625 µg/mL (Yildiz et al., 2018). The extract was effective as the antibiotic ampicillin against *S. aureus*. A methanolic extract of *A. karroo* stem demonstrated efficiency as antimicrobial agent against several antibiotic-resistant bacteria (Methicillin-resistant *S. aureus* (MRSA), β -lactamase positive (β L⁺) *E. coli* (β L⁺ EC), Ampicillin-resistant *K. pneumoniae* (ARKP), Carbenicillin-resistant *P. aeruginosa* (CRPA) and Chloramphenicol-resistant *Citrobacter freundii* (CRCF)), fungi (*C. albicans* and *Microsporum audouinii*) and against the highly pathogenic *Mycobacterium tuberculosis* and non-pathogenic *Mycobacterium smegmatis* mycobacteria (Nielsen et al., 2012). MIC values varied in the range 78–313 µg/mL for bacteria (against 10–20 µg/mL showed by the antibiotic gentamicin), between 78 and 156 µg/mL for fungi (against 20 µg/mL for the antibiotic nystatin) and 1250–2500 µg/mL for mycobacteria (against values lower than 1 µg/mL for the antibiotics ciprofloxacin and isoniazid) (Nielsen et al., 2012). This antimicrobial activity of *A. karroo* wood extract was of the

same magnitude or higher than that observed with wood extracts of other medicinal plants, like *Acokanthera oppositifolia*, *Erythrophleum lasianthum* and *Ptaeroxylon obliquum* (Nielsen et al., 2012). These results encourage the possible use of wood extracts from *Acacia* spp. in the treatment of microbial infections, especially against bacteria.

2.2.2.2 Properties of The Lipophilic Fraction from Wood

The lipophilic extractives from *Acacia* spp. wood were also characterized, and the corresponding biological activities and potential applications were addressed in the literature. The main classes of compounds found in dichloromethane extracts of wood of *A. mearnsii* with ages between 4 and 13 years old, were fatty acids (27–95 mg/kg of dry wood), sterols (21–56 mg/kg of dry wood) and aromatic compounds (1–13 mg/kg of dry wood), followed by smaller amounts of long chain aliphatic alcohol (0–3 mg/kg of dry wood) and hydrocarbons (0–3 mg/kg of dry wood) (G. R. A. Oliveira et al., 2020). Sterols from the wood of *A. dealbata*, *A. melanoxylon*, *A. longifolia* and *A. retinodes* were found in amounts between 335 mg/kg (*A. longifolia*) and 652 mg/kg (*A. retinodes*) relative to dried wood, with particularly high amounts of spinasterol (128–359 mg/kg of dry bark), dihydrospinasterol (109–286 mg/kg of dry wood) and stigmasterol (only found in *A. longifolia*–61 mg/kg of dry wood) (Freire et al., 2005). The steryl glucosides spinasteryl glucoside (12–80 mg/kg of dry bark) and dihydrospinasteryl glucoside (20–79 mg/kg of dry bark) were also identified in significant amounts in the wood of all studied species, and campesteryl glucoside was identified only in *A. longifolia* wood (36 mg/kg of dry wood) (Freire et al., 2005). A more extensive characterization of the lipophilic fraction of *A. dealbata* wood revealed that sterols (spinasterol, sitostanol and dihydrospinasterol) represent the major lipophilic family in *A. dealbata* wood (total of 590 mg/kg of dry wood), followed by fatty acids (290 mg/kg of dry wood, with almost 41% of them being unsaturated fatty acids), long-chain aliphatic alcohols (44 mg/kg of dry wood), aromatic compounds (27 mg/kg of dry wood) and monoglycerides (18 mg/kg of dry wood) (C. S. D. Oliveira et al., 2020). This lipophilic fraction exhibited none or low cytotoxicity at the tested doses in different mammalian cell lines representing brain, immune system, skin, lung and liver cells, highlighting the potential to be further exploited for oral or topic nutraceutical or pharmaceutical applications, given the lack of toxicity observed in liver hepatocytes (HepG2), epidermis keratinocytes (HaCaT) and dermis fibroblasts (NIH/3T3) (C. S. D. Oliveira et al., 2020). The benefic biological activities of sterols (e.g., anti-cholesterol, antidiabetic, anticancer) are well-known (Kaur et al., 2011; Miras-Moreno et al., 2016), as well as the positive effects of unsaturated fatty acids in some human

systems (e.g., cardiovascular, respiratory, gastrointestinal, renal and immune systems) (Gill and Valivety, 1997).

2.2.3 Leaves

Leaves are a highly available component of *Acacia* spp., and is mainly responsible for the liberation of allelopathic compounds, which significantly contribute to their success as invaders. These compounds may be volatile or non-volatile compounds liberated by lixiviation, and the research into their identification and confirmation of allelopathic effects has contributed to interesting revelations about their potential applications as bioherbicides or even in pharmaceutical formulations. Besides the benefits that can be obtained from the allelopathic chemicals, the extraction of other bioactive compounds from *Acacia* spp. leaves has been attempted in the last few years using polar or non-polar solvents to recover a wide range of hydrophilic and lipophilic components, and interesting biological activities have been demonstrated (summarized in Table 2.4).

Table 2.4 — Reported biological activities for extracts of *Acacia* spp. leaves.

<i>Acacia</i> spp.	Extraction	Bioactivity/Application	Reference
<i>A. dealbata</i> ,	20% EtOH	Antimicrobial	(Silva et al., 2016)
	solid-liquid (20 °C, 1h), ultrasound (420W, room temperature, 1h), Soxhlet (16h), and microwave (1200 W, 2450 MHz), using H ₂ O, MetOH, EtOH, ACE, DCM and HEX	Antimicrobial, antioxidant	(Borges et al., 2020)
	DCM (Soxhlet, 8h)	Pharmaceutics, nutraceutics, cosmetics (low cytotoxicity/safe use)	(C. S. D. Oliveira et al., 2020)
<i>A. farnesiana</i>	H ₂ O (simulating natural conditions)	Herbicide	(Aguilera et al., 2015)
	PE, DCM, EtOH (Soxhlet, sequentially)	Antimicrobial, Antioxidant (low toxicity)	(Ramli et al., 2011)
<i>A. karroo</i>	EtA, ChlF	Antimicrobial	(Nyila et al., 2012)
	MetOH (room temperature, 48h)	Antimicrobial	(Nielsen et al., 2012)
	ChlF, MetOH, EtOH, EtA (sequentially)	Antimicrobial	(Priyanka et al., 2015)
	MetOH, H ₂ O (4 °C, 24h)	Antimicrobial	(Cock and van Vuuren, 2015)
<i>A. longifolia</i>	EtOH (room temperature, 1 week)	Herbicide, antimicrobial	(Lima et al., 2013)
<i>A. longifolia</i> , <i>A. farnesiana</i> , <i>A. tortilis</i>	50% EtOH and 70% EtOH (room temperature)	Anti-inflammatory	(Gabr et al., 2018)
<i>A. mearnsii</i>	80% MetOH + 70% ACE to obtain crude extract; further chromatographic fractionation	Anti-inflammatory	(Xiong et al., 2016)
<i>A. melanoxylon</i>	20% MetOH (Soxhlet, 49h minimum)	Herbicide	(Manikandan et al., 1998)
	H ₂ O (room temperature, 24h)	Herbicide	(Hussain et al., 2011)
	H ₂ O (room temperature, 48h)	Anthelmintic	(Payne et al., 2013)
<i>A. pycnantha</i>	EtOH, H ₂ O (room temperature, 72h)	Antimicrobial	(Edrah et al., 2018)
	ChlF, PE, MetOH, DiEtE, ACE (room temperature, 48h)	Antimicrobial	(Mahmoud et al., 2016)
<i>A. saligna</i>	H ₂ O (room temperature, 24h) for aqueous extracts; PE, EtA, MetOH (room temperature) (sequentially), and direct MetOH, for organic extracts	Herbicide	(El Ayeub et al., 2013)
	EtOH, MetOH, H ₂ O (room temperature, 15 days)	Antimicrobial	(Noreen et al., 2017)
	Aqueous EtOH (room temperature, 48h)	Antimicrobial	(Gumgumjee and Hajar, 2015)
	Defatting with DCM, followed by extraction with 70% MetOH (reflux, 10h) and further fractionation with EtA, MetOH and H ₂ O (Soxhlet)	Antimicrobial	(El-Toumy et al., 2010)
	Purified components from a crude 70% MetOH extract (room temperature)	Antioxidant, cytotoxicity	(Gedara and Galala, 2014)

EtOH: ethanol; H₂O: water; MetOH: methanol; ACE: acetone; DCM: dichloromethane; HEX: hexane; EtA:ethyl acetate; ChlF: chloroform; PE: petroleum ether; DiEtE: diethyl ether.

2.2.3.1 Potential Biopesticide Agents from Leaves

The continuous use of synthetic herbicides has resulted in the evolution of herbicide-resistant weeds and environmental pollution due to the toxicity associated with these products. The search for alternatives in the less toxic allelochemicals released by some plants has been a trend in the last few years, which will be a more sustainable and environmentally friendly approach to support the change to more sustainable agricultural practices (Jabran et al., 2015). In this view, invasive plants like *Acacia* spp. naturally came as candidates for this purpose, taking advantage of the allelochemicals these plants release to inhibit the growth of native species and their establishment. Souza-Alonso et al. (Souza-Alonso et al., 2014) demonstrated the allelopathic effect of volatile compounds from *A. dealbata* leaves on *Trifolium subterraneum*, *Lolium multiflorum* and *Medicago sativa* species, having identified phytol as the most abundant, representing almost half of the entire composition (44%) (Souza-Alonso et al., 2014). Phytol is a very interesting compound that has been pointed at as a candidate for a broad range of applications in the pharmaceutical and biotechnological industry, with recent investigations demonstrating its anxiolytic, metabolism-modulating, cytotoxic, antioxidant, autophagy and apoptosis-inducing, antinociceptive, anti-inflammatory, immune-modulating and antimicrobial effects (Islam et al., 2018). *A. longifolia* leaves proved their phytotoxicity against Portuguese native *L. multiflorum*, *T. subterraneum* and *Plantago lanceolata* species, having identified 2-heptanol (28%), nerol (19%) and geraniol (7%) as the most abundant (Islam et al., 2018). Nerol and geraniol are particularly important compounds due to their biological activities. Nerol evidenced significant inhibition of *L. monocytogenes* bacteria biofilms (Leonard et al., 2010) and exhibited better herbicidal effects than the commercial herbicide 2,4-D against the important weeds *Amaranthus retroflexus*, *Chenopodium album* and *Rumex crispus* (Kordali et al., 2007). Geraniol is a commercially important terpene alcohol in the flavour and fragrance industries, and presents several relevant biological activities, such as an insecticidal and insect repellent, a chemoprotective agent for cancer, antimicrobial, antioxidant and anti-inflammatory effects, among others reviewed by Chen and Viljoen (Chen and Viljoen, 2010).

Non-volatile compounds of *A. dealbata* showed allelopathic capacity against lettuce (*Lactuca sativa*), being identified resorcinol, benzophenone and the non-protein amino acid maculosin as the most representative chemicals in leaves (Aguilera et al., 2015). Resorcinol and resorcinol derivatives, as well as benzophenone, are referred to as possessing unique characteristics for developing drugs suitable for treating various human diseases due to their

antioxidant, anti-inflammatory, antimicrobial, anticancer, antiviral, antidiabetic and hepato and cardiovascular protection, among other important biological actions reviewed by Durairaj (Durairaj, 2005) and Wu et al. (Wu et al., 2014). In turn, maculosin was considered an ideal prototype for creating a safe and environmentally friendly anticknapweed herbicide (Bobylov et al., 1996).

Several polar leaf extracts of *Acacia* spp. exhibited promising properties for their application in biopesticides to control weeds and plant pathogens. An ethanolic extract from *A. longifolia* leaves showed allelopathic capacity against *L. sativa* by causing a small reduction on the germination percentage (4% reduction) and speed (5% reduction), but significantly decreasing the growth of hypocotyls by 41% (Lima et al., 2013). From *A. melanoxylon* leaves, an hydromethanolic extract and a water extract showed promising results having in view the development of bioherbicides, given the inhibitory effect on the growth of black gram (*Phaseolus mungo*) and *Cassia accidentalis* and *Cyperus rotundus* weeds (Manikandan et al., 1998), and on the growth of Galician native species *Dactylis glomerata*, *Lolium perenne* and *Rumex acetosa* (Hussain et al., 2011), respectively. Aqueous and organic extracts of leaves of *A. saligna* were also tested on the seeds of wheat (*Triticum aestivum*) and *L. sativa* crops, and on two weeds (*Peganum harmala* and *Silybum marianum*) by El Ayeb et al. (El Ayeb et al., 2013). Water extracts reduced seed germination of the crops to a small extent (5%–10% for *L. sativa*, and 7–11% for *T. aestivum*), but inhibition of weeds did not even reach 40% (28–35% against *P. harmala*, and 17%–38% for *S. marianum*). Organic extracts inhibited seed germination of crops by higher extents (9%–32% for *L. sativa*, and 5%–37% for *T. aestivum*), but were very efficient against weeds (maximum of 80% of inhibition for *P. harmala*, and 82% for *S. marianum*) (El Ayeb et al., 2013). These findings, together with the abovementioned allelopathic effects of volatile and non-volatile compounds identified in *Acacia* spp., make leaves an important raw material to be considered for the development of pesticides of natural origin.

2.2.3.2 Antimicrobial and Antioxidant Agents from Leaves

A. dealbata, *A. farnesiana*, *A. karroo*, *A. pycnantha* and *A. saligna* are some of the most studied *Acacia* spp. for the development of antibiotic agents, with several studies showing the antimicrobial effects of leaf extracts against pathogenic bacteria and fungi. A summary of the microorganism strains inhibited by leaf extracts is presented in Table 2.5.

Table 2.5 — Microbial strains inhibited by *Acacia* spp. leaf extracts.

<i>Acacia</i> spp.	Extract	Microorganism strain	Antimicrobial activity	Reference
<i>A. dealbata</i>	Ethanollic	GP: <i>Bacillus cereus</i>	ID: approximately 16 mm (at 10 mg/mL)	(Silva et al., 2016)
	Ethanollic; aqueous	GP: <i>Staphylococcus aureus</i> GN: <i>Escherichia coli</i>	ID: 9–10 mm (at 5 mg/mL)	(Borges et al., 2020)
<i>A. farnesiana</i>	Ethanollic	GP: <i>Bacillus subtilis</i> Fungi: <i>Saccharomyces cerevisiae</i>	MIC: 800 µg/mL for bacteria 2500 µg/mL for yeast	(Ramli et al., 2011)
<i>A. karroo</i>	Ethyl acetate; chloroform	GP: <i>Listeria monocytogenes</i>	MIC: 3100–6250 µg/mL	(Nyila et al., 2012)
	Methanollic	GP: Methicillin-resistant <i>S. aureus</i> (MRSA), <i>Mycobacterium smegmatis</i> GN: β -lactamase positive (β L ⁺) <i>E. coli</i> , Ampicillin-resistant <i>Klebsiella pneumoniae</i> (ARKP), Carbenicillin-resistant <i>Pseudomonas aeruginosa</i> (CRPA), Chloramphenicol-resistant <i>Citrobacter</i> (CRCF) Fungi: <i>Candida albicans</i> , <i>Microsporum audouinii</i>	MIC: 156–1250 µg/mL for bacteria MIC: 78–625 µg/mL for fungi	(Nielsen et al., 2012)
	Mathanollic; aqueous	GP: <i>B. subtilis</i> , <i>S. aureus</i> , <i>Staphylococcus epidermidis</i> GN: <i>Alicicigenes faecalis</i> , <i>Aeromonas hydrophilia</i> , <i>Citrobacter freundii</i> , <i>E. coli</i> , <i>K. pneumoniae</i> , <i>Proteus mirabilis</i> , <i>Proteus vulgaris</i> , <i>P. aeruginosa</i> , <i>Salmonella typhimurium</i> , <i>Serratia marcescens</i> , and <i>Shigella sonnei</i> Fungi: <i>Aspergillus niger</i>	MIC: 235–4836 µg/mL for bacteria MIC: 325–486 µg/mL for fungi	(Cock and van Vuuren, 2015)
	Methanollic; ethanollic; chloroform; ethyl acetate	GP: <i>S. aureus</i> , <i>B. subtilis</i> GN: <i>E. coli</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>P. vulgaris</i> , <i>Salmonella typhi</i>	ID: 10–20 mm (100 µL of extract)	(Priyanka et al., 2015)

GP: Gram-positive bacteria; GN: Gram-negative bacteria; MIC: minimum inhibitory concentration; ID: inhibition diameter;

Table 2.5 (cont.)— Microbial strains inhibited by *Acacia* spp. leaf extracts.

<i>Acacia</i> spp.	Extract	Microorganism strain	Antimicrobial activity	Reference
<i>A. longifolia</i>	Ethanollic	Fungi: <i>Fusarium oxysporum</i>	10% of reduction of mycelial growth (at 1 mg/mL)	(Lima et al., 2013)
<i>A. pycnantha</i>	Ethanollic, aqueous	GP: <i>S. aureus</i> , <i>S. epidermis</i> GN: <i>E. coli</i> , <i>K. pneumoniae</i>	ID: 8–15 mm (50 µL of extract)	(Edrah et al., 2018)
	Methanollic; acetonic; chloroform, diethyl ether, petroleum ether	GP: <i>S. aureus</i> GN: <i>K. pneumoniae</i> , <i>Klebsiella oxytoca</i> (all bacteria isolated from infected patients)	ID: 10–22 mm (at 500 mg/mL)	(Mahmoud et al., 2016)
<i>A. saligna</i>	Methanollic, ethanollic, aqueous	GP: <i>S. aureus</i> , <i>B. subtilis</i> GN: <i>E. coli</i> , <i>P. aeruginosa</i> , <i>Xanthomonas</i> , <i>Citrobacter</i>	ID: 7–10 mm (1–2 mg/disc)	(Noreen et al., 2017)
	Hydroethanollic	GP: <i>S. aureus</i> , Methicillin-resistant <i>S. aureus</i> (MRSA), <i>B. subtilis</i> , <i>Micrococcus luteus</i> GN: <i>E. coli</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> Fungi: <i>C. albicans</i> , <i>A. niger</i> , <i>Aspergillus fumigatus</i> , <i>Aspergillus flavus</i>	ID: 23–29 mm for bacteria (at 200 mg/mL) 20–26 mm for fungi (at 200 mg/mL)	(Gumgumjee and Hajar, 2015)
	Ethyl acetate	GP: <i>S. aureus</i> , <i>Streptococcus pyogenes</i> , <i>Bacillus cereus</i> , <i>B. subtilis</i> Fungi: <i>C. albicans</i>	MIC: < 1 µg/mL for bacteria MIC: 4 µg/mL for fungi	(El-Toumy et al., 2010)

GP: Gram-positive bacteria; GN: Gram-negative bacteria; MIC: minimum inhibitory concentration; ID: inhibition diameter.

A broad range of Gram-positive and Gram-negative bacteria, and fungi are sensitive to leaf extracts. The ethanolic extract of *A. dealbata* showed a higher inhibition against *B. cereus* than the antibiotic gentamicin (inhibition diameter between one and twofold the diameter exhibited by the antibiotic), possibly due to the presence of phenolic acids such as syringic, *p*-coumaric, ferulic and ellagic acids (Silva et al., 2016). Extracts of *A. dealbata* showed antibacterial activity of the same magnitude as that observed with *Eucalyptus nicholii* (Silva et al., 2016) and *Olea europaea* (Borges et al., 2020). Quercetin-galloylglucoside, rutin, quercetin-pentoside, diosmetin diglycoside and especially quercetin-deoxyhexoside, were found in the *A. farnesiana* extract and are suspected to be involved in the observed bioactivity (Ramli et al., 2011). Besides antimicrobial properties, the *A. farnesiana* extract was proved to have low toxicity and good antioxidant properties, accessed by the ability to chelate iron and reduce the power and scavenging capacity against nitric oxide and DPPH free radicals (Ramli et al., 2011). In *A. karroo*, multiple classes of compounds were already identified in their leaves that may be involved in its antimicrobial activity, such as flavonoids, phenols, phytosterols, proanthocyanidin, tannins or terpenes (Maroyi, 2017). The antimicrobial effects of *A. karroo* leaf extracts are probably due to the presence of epicatechin, β -sitosterol and epigallocatechin, some of the most abundant compounds present in the active extracts (Nyila et al., 2012). The antimicrobial activity against the microorganisms studied by Nielsen et al. (Nielsen et al., 2012) and Nyila et al. (Nyila et al., 2012) was lower than that observed with the drugs gentamicin, nystatin, ciprofloxacin, isoniazid and erythromycin, but were of the same magnitude as that observed with ampicillin, chloramphenicol and nyastin for the usually more resistant Gram-negative bacteria studied by Cock and van Vuuren (Cock and van Vuuren, 2015) (studied microorganisms showed in Table 5). The extracts of *A. karroo* exhibited better antimicrobial activity than many African medicinal plants already studied, such as *Eucomis autumnalis*, *Senecio inonartus*, *Drimia altissima*, *Aloe arborescens*, *Acokanthera oppositifolia*, *Bulbine latifolia*, *Curtisia dentata*, *Erythrophleum lasianthum*, *Ptaeroxylon obliquum*, *Searsia burchellii* and *Sansevieria hyacinthoides*, among others (Cock and van Vuuren, 2015; Nielsen et al., 2012; Nyila et al., 2012). *A. pycnantha* extract was found to contain alkaloids, sterols and triterpenes, tannins and phenols, saponins and flavonoids (Edrah et al., 2018), having been already identified as individual compounds of n-hexadecanoic acid, octadecanoic acid and phytol, which have recognized antimicrobial activity (Mahmoud et al., 2016). The activity of *A. pycnantha* extracts were comparable to the observed with tetracyclin and ciprofloxacin (16–22 mm of inhibition diameter) (Edrah et al., 2018) and cefoxitin (20–28 mm of inhibition diameter) (Mahmoud et al., 2016). The antimicrobial activity evidenced by *A. saligna* extracts may be attributed to phenolic

compounds, including complex flavonoids that were identified in the extracts, such as gallic, protocatioic, chlorogenic, *p*-hydroxybenzoic, *p*-coumaric, syringic, vanillic and salicylic acids (Mahmoud et al., 2016), and also catechin, 7-O-galloylcatechin, myricetin-3-*O*- α -L-arabinopyranoside, quercetin-3-*O*- β -D-glucopyranoside, quercetin-3-*O*- α -L-arabinopyranoside, apigenin-7-*O*- β -D-glucopyranoside and luteolin-7-*O*- β -D-glucopyranoside (El-Toumy et al., 2010). The aqueous and organic extracts of *A. saligna* evaluated by Noreen et al. (Noreen et al., 2017) revealed inhibition diameters approximately between 20 to 75% of the ones showed by azithromycin and ciprofloxacin, while the hydroethanolic extracts evaluated by Gumgumjee and Hajar (Gumgumjee and Hajar, 2015) were of the same magnitude of the obtained with streptomycin and ciprofloxacin with bacteria (19–27 mm, at 10 mg/mL), and with amphotericin and nystatin with fungi (25–32 mm, at 10 mg/mL).

These findings highlight that extracts from leaves of *Acacia* spp. and can be considered as a potential source of natural antimicrobials, which is of importance, since health hazards are frequently associated with the use of chemical antimicrobial agents or food preservatives.

2.2.3.3 Anthelmintic Agents from Leaves

In addition to antimicrobial effects, anthelmintic action was also observed with aqueous extracts of *A. melanoxylon* and *A. karroo*. Payne et al. (Payne et al., 2013) observed anthelmintic activity by a water extract from a mix of leaves and flowers of *A. melanoxylon* against cyathostomins parasites, which is very common in horses. The extract completely inhibited larval growth at a concentration of 1400 μ g/mL, encouraging its further study to possibly be included in parasite management programs for horses (Payne et al., 2013). In turn, Mølgaard et al. (Mølgaard et al., 2001) evaluated the anthelmintic effects of a water extract of *A. karroo* leaves against schistosomules of the trematode *Schistosoma mansoni* and cysticercoids of the cestode *Hymenolepis diminuta*. The extract killed the newly excysted cysticercoids of tapeworms within an hour when incubated in a culture medium, showing a lethal concentration of 3100 μ g/mL. This lethal concentration decreased to 800 μ g/mL when considering a period of 24 h. However, the activity against schistosomules was considered week, with the extract showing a lethal concentration of 103 mg/mL (Mølgaard et al., 2001). Anthelmintic effects on goats infected with *Haemonchus contortus* by the inclusion of *A. karroo* leaves in their diet was also reported (Kahiya et al., 2003; Marume et al., 2012).

2.2.3.4 Anti-Inflammatory, and Antitumoral Agents from Leaves

Extracts from leaves also have been proven to be good candidates for the production of plant-based anti-inflammatory agents, showing strong potential as a natural source of functional components related to human health. Xiong et al. (Xiong et al., 2016) found that crude *A. mearnsii* extract, but especially the purified proanthocyanidin-rich fraction, depressed reactive oxygen species in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells, and inhibited the release of nitric oxide, and significantly inhibited the mRNA expression levels of the anti-inflammatory interleukine IL-1 β , cyclooxygenase COX-2, inducible nitric oxide synthase (iNOS) and interleukin-6 (IL-6) in a dose-dependent manner (1–100 μ g/mL). In turn, Gabr et al. (Gabr et al., 2018) showed the anti-inflammatory activity of aqueous ethanolic extracts of *A. longifolia* leaves by inhibition of COX-1 and COX-2 enzymes. *A. longifolia* leaves were shown to contain relevant bioactive compounds, such as catechin, myricetin 3-*O*-rhamnoside, kaempferol 3-*O*-glucoside, quercitrin (quercetin 3-*O*-rhamnoside) and luteolin (Gabr et al., 2018).

Other relevant effects of a leaf extract were observed with *A. saligna* leaves by Gedara and Galala (Gedara and Galala, 2014). Authors isolated from a crude 70% aqueous methanol extract the spirostane saponine (25S)-5 β -spirostan-3 β -yl-3-*O*- β -D-xylopyranosyl(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl(1 $\rightarrow$ 4)- β -D-galactopyranoside, that showed potent cytotoxic activity against HEPG2 (liver cancer) cell line (IC₅₀ of 2.8 μ g/mL), the biflavonoid glycoside myricetin-3-*O*-rhamnoside (C7-*O*-C7) myricetin-3-*O*-rhamnoside, that showed potent free radical scavenging activity against ABTS free radicals, besides erythrodiol, 3 β -*O*-*trans*-*p*-coumaroyl-erythrodiol, quercetin-3-*O*- α -L-rhamnoside and myricetin-3-*O*- α -L-rhamnoside, that showed cytotoxicity and antioxidant activities, but to a lower extent.

2.2.3.5 Properties of the Lipophilic Fraction from Leaves

An extensive characterization of the lipophilic fraction of *A. dealbata* leaves revealed that terpenic compounds (14,635 mg/kg of dry leaf) represent the major lipophilic class (with lupenone representing almost half of the terpenics), followed by long-chain aliphatic alcohols (1892 mg/kg of dry leaf), fatty acids (1747 mg/kg of dry leaf, with almost 19% of them being unsaturated fatty acids), sterols (636 mg/kg of dry leaf), aromatic compounds (112 mg/kg of dry leaf), monoglycerides (49 mg/kg of dry leaf), and other compounds, including a remarkable quantity of α -tocopherol of 1936 mg/kg of dry leaf (Mahmoud et al., 2016). This lipophilic fraction showed good potential for the development of oral or topic nutraceutical or

pharmaceutical applications, since it was revealed to be non-toxic to different mammalian cell lines representing brain, immune system, skin, lung and liver cells (Mahmoud et al., 2016).

In turn, Borges et al. (Borges et al., 2020) found interesting free radical scavenging activities against DPPH (45–226 μM of trolox equivalents per gram of fresh leaf) and ABTS (393–539 μM of trolox equivalents per gram of fresh leaf) free radicals.

2.2.4 Flowers

Flowers of Portuguese invasive *Acacia* spp. can be of great economic importance, since they are important sources of essences for the cosmetic and perfume industries and are of valuable anthochlor pigments (chalcones), in addition to relevant biological activities that have been found in their extracts. Moreover, its exploitation can be of crucial importance to the control of the propagation of *Acacia* spp., once its collection during the flowering period prevents the formation of seeds, so hindering plant proliferation through the seed dispersal route (Lorenzo et al., 2019). The biological activities and uses of *Acacia* spp. flowers are summarized in Table 2.6.

Table 2.6 — Reported biological activities for extracts of *Acacia* spp. flowers.

<i>Acacia</i> spp.	Extraction	Bioactivity/Application	Reference
<i>A. dealbata</i> ,	Hexane to obtain concrete, solubilization with EtOH to obtain the absolute oil	Flavor and perfumes	(Perriot et al., 2010)
	97% aqueous MetOH (room temperature, 9 weeks) to obtain crude extract; further re-dissolution of dried extract in aqueous MetOH, fractionation with HEX and column chromatographic separation to obtain individual compounds	Herbicide	(Lorenzo et al., 2019)
	95% EtOH (boiling)	Pigments/dyes	(Imperato, 1982a),(Imperato, 1982b)
	96% EtOH (40 °C, 24h); HEX, DCM, EtA, BuOH (Soxhlet, 12h); green solvents (aqueous, acid and microwave hydrogravity extraction)	Antioxidant, cytotoxicity, hydrogels	(Casas et al., 2019)
	96% EtOH (50 °C, overnight)	Cosmetics	(Soto et al., 2018)
<i>A. melanoxylon</i>	H ₂ O (room temperature, 24h)	Herbicide	(Hussain et al., 2011)
<i>A. pycnantha</i>	EtOH, H ₂ O (room temperature, 72h)	Antimicrobial	(Edrah et al., 2018)
<i>A. saligna</i>	95% EtOH (boiling)	Pigments/dyes	(Imperato, 1977)
	MetOH (room temperature, 48h) for crude extract; re-dissolution in water, fractionation with ChlF and EtA and chromatographic separation to isolate individual compound	Pigments/dyes	(Ghouila et al., 2012),(Fajraoui et al., 2019)
	H ₂ O (room temperature, 24h) for aqueous extracts; PE, EtA, MetOH (room temperature) (sequentially), and direct MetOH, for organic extracts	Herbicide	(El Ayeb et al., 2013)
	70% MetOH (room temperature, 48h) for crude extract; fractionation with DCM, EtA, ButOH; Chromatographic separation of EtA fraction to obtain individual compounds	Antioxidant, anti-acetylcholinesterase	(Ghribia et al., 2014)
	H ₂ O (50 °C, 3h)	Antimicrobial	(Al-Huqail et al., 2019)

EtOH: ethanol; H₂O: water; MetOH: methanol; ACE: acetone; DCM: dichloromethane; HEX: hexane; EtA: ethyl acetate; ChlF: chloroform; PE: petroleum ether; DiEtE: diethyl ether.

2.2.4.1 Essences from Flowers

The intense and pleasant aroma of the flowers of *A. dealbata* is pronounced. The absolute oil, obtained from its flowers by hexane or petroleum ether extraction followed by fractionation with ethyl alcohol, is used in many perfumes, with a worldwide production estimated at five tons per year (Perriot et al., 2010). The chemical composition of the absolute oil was reported by Perriot et al. (Perriot et al., 2010) to know its constituents and to better understand its possible impact on both human health and the environment. Straight-chain compounds from C6 to C26 with different functional groups (hydro-carbons, esters, aldehydes, diethyl acetals, alcohols and ketones) were identified, with near 20% of volatiles (mainly alkanes, fatty acids (palmitic), and (Z)-heptadec-8-ene (6%)), 50% of semi-volatiles (triterpenic ketones (lupenone, 20%) and alcohols (lupeol, 8%)) and 30% of non-volatiles (aromatic and fatty acid triterpenic esters). Previous studies of *A. dealbata* absolute oil showed that it is neither an irritant, nor is it toxic by ingestion, and it does not show toxicity or phytotoxicity for microorganisms (Perriot et al., 2010).

2.2.4.2 Pigments and Dyes from Flowers

Currently, industries are returning to the use natural pigments or dyes to reduce the dangers caused by the synthetic materials (e.g., disruption of aquatic life through industrial discharges and adverse effects on human health, such as in the central nervous system, cancer of the thyroid and tumors of the adrenal glands as well as allergenic reactions), since natural molecules are mostly known to be eco-friendly, biodegradable, low toxic, less allergenic and non-carcinogenic (Fajraoui et al., 2019). Moreover, some natural pigment molecules, such as chalcones, are generally known for their antibacterial and antifungal activities (Ghouila et al., 2012). Chalcones are well-known anthochlor pigments and have been used for coloring textile materials for decades, and the flowers of some *Acacia* spp. can be an important source of this valuable material, which is responsible for its yellow colour. Moreover, (Imperato, 1977) isolated two chalcone glucosides from the flowers of *A. saligna*, isosalipurposide (4,2',4',6'-tetrahydroxychalcone 2'-glucoside) and 4,2',4',6'-tetrahydroxychalcone 4-glucoside, using preparative chromatographic techniques on a crude 95% ethanol extract. With the same procedure, the same author isolated the anthochlor chalcononaringenin 2'-[O-rhamnosyl-(1→4)-xyloside] (4,2',4',6'-tetrahydroxychalcone 2'-[O-rhamnosyl-(1→4)-xyloside]) (Imperato, 1982a), and later two more anthochlors were obtained: the chalcone glucoside 4,2',4',6'-tetrahydroxy-3-methoxychalcone 2'-O-β-D-glucoside and the aurone cernuoside (4, 6,3',4'-

tetrahydroxyaurone 4-O-fl-D-glucoside) (Imperato, 1982b). Amongst these chalcones, isosalipurposide was studied for its application as a dye on wool (Ghouila et al., 2012), and on the coloration of polylactic acid byopolymer, commonly used in food packaging (Fajraoui et al., 2019). Ghouila et al. (Ghouila et al., 2012) observed that isosalipurposide is more effective if used at a lower pH (pH 2) and lower temperatures (best result at 60 °C) to avoid its degradation. It provided bright hues with good colour fastness properties, which is improved if using zinc sulphate as a mordant (Ghouila et al., 2012). In turn, Fajraoui et al. (Fajraoui et al., 2019) studied several processing parameters to obtain a well finished and yellow coloured polylactic acid film, obtaining the best performance when using a dye concentration of 0.5% and blending at 50 rpm during 5 min at 90 °C, suggesting that these conditions allow the pigment to be able to disperse in the intermolecular chains of the amorphous byopolymer.

2.2.4.3 Potential Bioherbicide Agents from Flowers

As described for leaves, the search for natural herbicides is fundamental in the change for more sustainable agricultural practices. Along with leaves and leaf litter, flowers play an important role in releasing allelopathic compounds, and the allelopathic effects of *Acacia* spp. on native flora mostly occurs during the flowering period (Lorenzo et al., 2011, 2010b). Investigation of the allelopathic properties of flowers and the identification of individual volatile and non-volatile chemicals have been made and can bring important information for the development of bioherbicides. The allelopathic effects of *A. dealbata* and *A. longifolia* were observed in several Portuguese and Spanish native species, such as *Medicago sativa*, *Plantago lanceolata*, *Trifolium subterraneum* and especially on *Lolium multiflorum* (Souza-Alonso et al., 2018, 2014). Amongst the most abundant compounds identified in the volatiles, and probably responsible for the allelopathic effects, there are heptadecadiene (23% in *A. dealbata* and 7% in *A. longifolia*), *n*-nonadecane (19% in *A. dealbata* and 14% in *A. longifolia*), *n*-tricosane (11% in *A. dealbata* and 12% in *A. longifolia*), octadecene (9% in *A. dealbata*), nonanal (8% in *A. longifolia*) and kaurene (8% in *A. longifolia*) (Islam et al., 2018; Souza-Alonso et al., 2014).

The allelopathic effect of the non-volatile fraction of flowers was accessed for *A. dealbata*, which showed the allelopathic capacity against *L. sativa* (Aguilera et al., 2015). The most abundant compounds found in the non-volatile fraction were anisal, methyl *p*-hydroxycinnamate and *p*-anysil alcohol (Aguilera et al., 2015). Anisal is widely used in flavor and fragrance industries, methyl *p*-hydroxycinnamate previously showed anti-inflammatory activity (Vo et al., 2014), and nematocidal effects were associated with *p*-anysil alcohol (Stadler et al., 1994). Lorenzo et al. (Lorenzo et al., 2019) explored the potential herbicidal effect of two

compounds present in flowers of *A. dealbata*, methyl cinnamate and methyl anisate, against well-established plants, namely lettuce (*L. sativa*), the widely consumed wheat crop (*Triticum aestivum*), and against rigid ryegrass (*Lolium rigidum* Gaudin), a common weed in winter cereal and wheat crops. The authors found methyl cinnamate as the most active compound, which showed selective phytotoxicity by inhibiting early stem and radicle growth of dicotyledonous lettuce (60% and 89%, respectively) and monocotyledonous *L. rigidum* (76% and 87%, respectively), but not affecting wheat. These results encouraged the exploitation of *A. dealbata* flowers as a source of potential bioherbicides, namely to be applied in wheat crops infested by rigid ryegrass (Lorenzo et al., 2019). A water extract from *A. melanoxylon* leaves evidenced promising results as a potential bioherbicide agent, given the inhibitory effect on the growth of Galician native species (Hussain et al., 2011). The extract showed mean LC₅₀ values (i.e., the dose for 50% inhibition of seedling growth) of 43% against *Lolium perenne*, 40% against *Rumex acetosa* and 53% against *L. sativa* (doses referred to dilutions of the whole liquid crude extract) (Hussain et al., 2011). Water and organic extracts of flowers of *A. saligna* also exhibited potential for their application as bioherbicides (El Ayeb et al., 2013). These were tested on the seeds of wheat (*T. aestivum*) and lettuce (*L. sativa*) crops, and on two weeds (*Peganum harmala* and *S. marianum*). Water extracts reduced seed germination of the crops to a low extent (0%–2% for lettuce and 3%–13% for wheat), but were not remarkably efficient against weeds (maximum of 13% of inhibition for *S. marianum*) (El Ayeb et al., 2013). Organic extracts inhibited the seed germination of crops to higher extents (11%–40% for lettuce, and 0%–23% for wheat), but were very much efficient against weeds (maximum of 77% of inhibition for *P. harmala*, and 80% for *S. marianum*) (El Ayeb et al., 2013).

2.2.4.4 Antimicrobial Agents from Flowers

Flower extracts have demonstrated antimicrobial effects as well. Edrah et al. (Edrah et al., 2018) observed antibacterial activity of ethanol and water extracts of *A. pycnatha* leaves against *S. aureus*, *S. epidermis*, *K. pneumoniae* and *E. coli*, with inhibition diameters in the range 6–10 mm, against 16–22 mm showed by tetracyclin and ciprofloxacin. In turn, Al-Huqail et al. (Al-Huqail et al., 2019) evaluated the antibacterial activity of water extract of *A. saligna* flowers against phytopathogenic bacteria *Agrobacterium tumefaciens*, *Enterobacter cloacae*, *Erwinia amylovora* and *Pectobacterium carotovorum* subsp. *carotovorum*, causal agents of different infectious plant symptoms, such as blackleg, brown or soft rot on potato tuber and stems, and tumors on olive and other ornamental plants. The author tested the extract also against fungi *Fusarium culmorum*, *Rhizoctonia solani* and *Penicillium chrysogenum*, which cause root rot,

cankers and green fruit rot, respectively. These fungi can colonize and cause pigmentation and discoloration of different wood and wood-based products in humid conditions. All bacteria were sensitive to the extract, with MIC values ranging within 100–300 µg/mL, against 16–35 µg/mL obtained with the antibiotic tobramycin (Al-Huqail et al., 2019). For the fungi, the best performance was attained at a concentration of 3%, with inhibition percentages between 29% and 66% (Al-Huqail et al., 2019). The authors found benzoic acid, caffeine, *o*-coumaric acid, *p*-hydroxy benzoic acid and ellagic acid to be the most abundant phenolics (present in the extract in concentrations between 12 to 162 mg/100 g), and naringenin, quercetin and kaempferol as the most abundant flavonoids (44 to 145 mg/100 g) (Al-Huqail et al., 2019).

2.2.4.5 Other Applications of Flower Extracts

Other important characteristics were found for flower extracts that gave them potential to be applied in pharmaceuticals, cosmetics or in therapeutics against Alzheimer's disease.

Casas et al. (Casas et al., 2019) found a good free radical scavenging activity of crude ethanol extract against ABTS free radicals (850 mg of Trolox equivalents per gram of extract), which is probably attributed to the presence of phenolic compounds (270 mg of gallic acid equivalents per gram of extract). The authors found butanol, ethyl acetate, hexane and the crude ethanol extracts to be protective against neutrophils oxidative bursts, accessed in venous blood collected from healthy human volunteers, at concentrations in the range 63 to 500 µg/mL, and showed moderate cytotoxic activity against colon carcinoma HCT-116 and lung adenocarcinoma A549 cells (Casas et al., 2019). Moreover, encouraging results were obtained with the application of extracts as solvents in the preparation of hydrogels. Hydrogels with attractive mechanic properties were produced, thus opening a new possibility for the valorization of *A. dealbata* flowers through their inclusion in the preparation of these materials for personal care products, cosmetics or pharmaceuticals, taking advantage of their antiradical and anti-proliferative potential (Casas et al., 2019).

Soto et al. (Soto et al., 2018) evaluated the potential of the use of some vegetal raw materials, including *A. dealbata* flowers, in personal-care products (hand cream, body oil, shampoo, clay mask, body exfoliating cream and skin cleanser), with the evaluation being made by participants attributing a classification for some sensory attributes and overall acceptance. Between the four assayed vegetal extracts (grape pomace, *Pinus pinaster* wood chips, *A. dealbata* flowers and *Lentinus edodes*), the floral aroma and the yellow color provided by the *A. dealbata* flower extract to all produced cosmetic products, make the *Acacia*-based products preferred by all consumers, independent of gender or age (Soto et al., 2018). Besides, the *A.*

dealbata extract was the one with the highest antioxidant activity, given by the ferric-reducing antioxidant power assay (170 mg ascorbic acid equivalents per gram of extract) and the ABTS free radical scavenging assay (700 mg of trolox equivalents per gram of extract) (Soto et al., 2018).

Presently, amongst the treatments used against Alzheimer's disease are included the use of acetylcholinesterase inhibitors and antioxidants (Sharma, 2019). Ghribia et al. (Ghribia et al., 2014) evaluated the antioxidant and anti-acetylcholinesterase activities of extracts (dichloromethane, ethyl acetate, butanol and aqueous) and of the compounds isolated from flowers of *A. saligna* (isosalipurposide, quercetin and naringenin). In terms of IC_{50} values (i.e., the extract concentration providing 50% inhibition), quercetin ($IC_{50} = 5 \mu\text{g/mL}$) and ethyl acetate ($IC_{50} = 67 \mu\text{g/mL}$) showed the best antiradicalar activity against DPPH free radicals; dichloromethane extract displayed the best antiradicalar activity against ABTS free radical ($IC_{50} = 78 \mu\text{g/mL}$); and ethyl acetate extract was the one with the best reducing power. All extracts showed a dose-dependent acetylcholinesterase inhibition (IC_{50} between 16.03 and 52.04 $\mu\text{g/mL}$), with the butanolic extract being the most potent sample (Ghribia et al., 2014). The antioxidant and anti-acetylcholinesterase activity exhibited by these extracts encourage its further study for the development of alternative therapeutics to fight neurological disorders.

2.2.5 Pods and seeds

Collection of pods and seeds to obtain valuable products can be another way to promote the control of the proliferation of *Acacia* spp., by decreasing the amount of seeds in soils. The most reported biological activities are summarized in Table 2.7.

Table 2.7 — Reported biological activities for extracts of *Acacia* spp. pods and seeds.

<i>Acacia</i> spp.	Extraction	Bioactivity/Application	Reference
<i>A. dealbata</i> (pods and seeds)	50% ACE (25 °C) for crude extract; sequential extraction of the water fraction with EtA and ButOH	Herbicide	(Jelassi et al., 2016)
<i>A. farnesiana</i> (pods)	80% MetOH (room temperature, 24 h)	Antioxidant (animal feed)	(Delgadillo Puga et al., 2015)
<i>A. saligna</i> (seeds)	H ₂ O (25 °C, 48 h)	Immunomodulatory	(El Abbouyi et al., 2004)
<i>A. melanoxylon</i> (seeds)	70% EtOH (Soxhlet)	Antidiabetic	(Kumar, 2016)
<i>A. farnesiana</i> (seeds)	Defatting with HEX; extraction with NaCl 0.5M (room temperature, 3 h), centrifugation, dialyzation of soluble fraction against H ₂ O, centrifugation, and lyophilization to obtain protein	Antimicrobial	(Santi-Gadelha et al., 2012)

ACE: acetone; EtA: ethyl acetate; ButOH: butanol; MetOH: methanol; H₂O: water; EtOH: ethanol.

2.2.5.1 Bioactive Properties of Pods

Pods are formed after the flowering period. Studies about bioactive properties of pods from Portuguese invasive *Acacia* spp. are scarce. To the best of our knowledge, the focus has been mainly on the allelopathic effects of pods. Aguilera et al. (Aguilera et al., 2015) demonstrated the allelopathic capacity of a water extract from pods of *A. dealbata* against lettuce (*L. sativa*), identifying stigmasterol and D-alpha-tocopherol quinone as the most abundant chemicals in pods (Aguilera et al., 2015). Jelassi et al. (Jelassi et al., 2016) evaluated the allelopathic potential of *A. cyclops*, *A. mollissima* and *A. saligna* pods and seeds also on the germination of lettuce. The authors observed effects on the growth of roots and shoots, but found that the most pronounced effect was observed with an ethyl acetate extract from *A. saligna* seeds, that completely inhibited seed germination. The saponic triterpene glycoside 3-O-β-D-xylopyranosyl-(1→4)-α-L-rhampyranosyl-(1→6)-β-D-glucopyranoside, named mollisside B, isolated from the butanolic extract of *A. cyclops* pods, strongly inhibited lettuce root (88.21%) and shoot (63.68%) growth, being proposed as a lead compound for the development of new herbicides (Jelassi et al., 2016). In turn, *A. farnesiana* forms pods with good antioxidant properties, having been observed to protect against oxidative-induced damages in pig kidney cells LLC-PK1 and in *in vivo* studies with gerbils (Delgadillo Puga et al., 2015).

Considering the remarkable activities already demonstrated by pods of other *Acacia* species, more research must be conducted into the extraction of bioactive compounds from Portuguese invasive *Acacia* spp. Extracts from pods of *A. nilotica* showed good antimicrobial activity (Auwal et al., 2015; M. Abdallah, 2016), antidiabetic and hypolipidemic effects (Ahmad et al., 2008), antihyperglycemic properties (Omara et al., 2012), antihypertensive and antispasmodic actions (Gilani et al., 1999) and enhanced wound healing by alleviating oxidative stress and suppressing pro-inflammatory cytokines (Kankara, 2018). Tannins, saponins and flavonoids are known classes of compounds present in the pods of other species (Auwal et al., 2015), some of these having already been identified, such as catechin, catechin-7-O-gallate, catechin-4'-O-gallate, catechin-3'-O-gallate, gallic acid, quercetin and quercetin 3-O-glucoside (Omara et al., 2012).

2.2.5.2 Nutritional Potential of Seeds

The continuous increase in population has led to an increased demand for high-quality seed oils, and along with this, the necessity to diversify the sources of vegetable oil has emerged to meet that demand. Between the Portuguese invasive *Acacia* spp., the most studied for this

purpose is *A. saligna*, whose oil has already been widely characterized and very good properties have been reported (Jelassi et al., 2014; Youzbachi et al., 2012). Youzbachi et al. (2012) evaluated the composition of 12 *A. saligna* ecotypes, finding an oil content between 87 and 119 g/kg of dry seeds, with saturated fatty acid content in the range 12–13%, monounsaturated fatty acid content of 22%–25% and a high polyunsaturated fatty acid content between 62 and 66%. Smaller proportions of stearic (1%–2%), vaccenic (1%–2%) and palmitoleic (0%–1%) acids were also quantified. The most representative saturated fatty acid was palmitic acid (9%–10%) and oleic acid was the most abundant monounsaturated fatty acid (20%–23%) and a remarkable content of linoleic acid (ω 6) was found (61%–65%) (Youzbachi et al., 2012). The benefits of the inclusion of unsaturated fatty acids in the human diet are well known, such as reduction of serum cholesterol, atherosclerosis and on the modulation and/or prevention of cardiovascular diseases, type 2 diabetes, and several types of cancer (Jelassi et al., 2014; Kumar et al., 2019; Youzbachi et al., 2012). Along with such notable content of polyunsaturated fatty acids on its oil, *A. saligna* seeds also contain phenolics (1860–1970 mg of gallic acid equivalents per kg of dry seeds) and flavonoids (310–490 mg of rutin equivalents per kg of dry seeds), which confer to seeds important antioxidant properties, demonstrated by DPPH and ABTS free radical scavenging assays on methanolic extracts (430–660 μ M and 140–580 μ M of trolox equivalents, respectively (Youzbachi et al., 2012). A similar composition for seed oils was observed by Jelassi et al. (2014) with *A. cyclops*, *A. saligna* and *A. mollissima*, that found oil contents between 9 and 11%, saturated fatty acid contents ranging from 16%–17%, monounsaturated fatty acids in the range 21%–26% and polyunsaturated fatty acids between 57 and 62%. Besides, the authors determined also the phenolic composition of the seed oils, founding syringic (35%–39%) and ferulic (31%–34%) acids as the dominant phenolic compounds, and minor amounts of other phenolic acids, such as *o*-coumaric (1%–10%), *p*-coumaric (3%–7%) and protocatechuic (ca. 7%) (Jelassi et al., 2014). These studies confirm that *A. saligna* could be considered as a potential food source of ω 6 fats and antioxidants, showing that its seeds have the potential to replace conventional oil types such as soybean, olive, corn, coconut, peanut, cottonseed, palm, sunflower and rapeseed oils, which are more expensive and are not affordable to a considerable population in developing countries. The nutritional value of *A. retinodes* seeds was also investigated, having being found with a different composition profile when compared with *A. saligna* seeds. Shelat et al. (2019) observed *A. retinodes* seeds to contain a high protein (28%) and dietary fibre (34%) content and 16% of oil. The oil is characterized by 30% of saturated fatty acids, 53% of

monounsaturated fatty acids and 18% of polyunsaturated fatty acids, with palmitic acid (18%), oleic acid (50%) and linoleic acid (16%) the most representative fatty acids (Shelat et al., 2019).

Acacia seeds are characterized also by a mineral composition enriched in potassium (237–907 mg/100 g of dry seed), calcium and magnesium (61–509 mg/100 g of dry seed), phosphorous (20–85 mg/100 g of dry seed) (Hannachi et al., 2011; Youzbachi et al., 2012) and proteins (10–14 g/100 g of dry seed) (Hannachi et al., 2011). Hannachi et al. (Hannachi et al., 2011) investigated the chemical composition of seven *Acacia* species, including *A. saligna*, having found for this species 14% of seed storage protein, comprising 3% of albumin, 6% of globulin, 3% of prolamin and 2% of glutelin; potassium, phosphorous and calcium and magnesium contents of 493, 85 and 73 mg/100 g of dry seed, respectively; a phenolic content of 191 mg of gallic acid equivalents per 100 g of dry seed; flavonoids content of 29 mg of rutin equivalents per 100 g of dry seed; and a carotenoid content of 6 mg/kg of dry seed. In addition, the authors also found seeds to exhibit antioxidant activity, accessed by the DPPH (880 μ M of trolox equivalents) and ABTS (1290 μ M of trolox equivalents) free radical scavenging assays (Hannachi et al., 2011). These results makes seeds of *Acacia* spp., including those of the Portuguese invasive *A. saligna*, an interesting natural source of protein, minerals and antioxidants, with potential to be explored in the development of functional foods and dietary supplements.

2.2.5.3 Bioactive Properties of Seeds

In addition to the abovementioned properties, seeds extracts already demonstrated immunomodulatory, antidiabete, antimicrobial and antinematode effects. El Abbouyi et al. (El Abbouyi et al., 2004) investigated the effects of a water extract of *A. saligna* seeds on superoxide anions generation in rat pleural polymorphonuclear leukocytes, activated by opsonized zymosan, having observed a significant stimulatory effect in a dose-dependent manner. Moreover, the extract enhanced superoxide anions generation by polymorphonuclear leukocytes pre-treated with the anti-inflammatory diclofenac. The authors suggest that this stimulatory effect is probably due to the presence of polyphenols, such as tannins and/or lignins in the extract (El Abbouyi et al., 2004). Kumar (Kumar, 2016) studied the antidiabetic activity of a 70% ethanol extract of seed of *A. melanoxylon* in streptozotocin-induced diabetic rats. The extract, administered orally at 100, 200 and 400 mg/kg of body weight doses, exhibited significant hypoglycemic activities, significantly reducing blood glucose levels even at the lowest dosage (from 302 to 225 mg/dL) as compared with a diabetic control. Moreover, the administration of the extract avoided the decrease in body weight verified in the diabetic rats

without treatment (reduced from 228 to 212 g), and showed instead a continuous improvement in body weight from 208 to 211 g, when using the 100 mg/kg dosage. Lectin-like protein extracted from *A. farnesiana* seeds showed bacteriostatic effects against *Xanthomonas axonopodis* pv. *passiflorae* and, especially, against *Clavibacter michiganensis* subsp. *michiganensis*, inhibiting the growth of these phytopathogenic bacteria by 78% and 92%, respectively (Santi-Gadelha et al., 2012). Moreover, anti-nematode properties were also found against the root-knot nematode *Meloidogyne incognita*, involving both egg hatching and motility and reduction in larval mobility, suggesting its potential use as a component of integrated pest management programs (Santi-Gadelha et al., 2012).

2.2.6 Root

Within the Portuguese invasive *Acacia* spp., the most studied is what concerns the valorization of the roots is *A. karroo* (Table 2.8). The roots of *A. karroo* have been used in traditional medicine in some African regions for decades, as an aphrodisiac or in remedies for colic, convulsions, dizziness, general body pains, gonorrhea, malaria, sexually transmitted diseases, syphilis, urinary schistosomiasis, venereal diseases, diarrhea, etc. (Maroyi, 2017)

Table 2.8 — Reported biological activities for extracts of *A. karroo* roots.

Extraction	Bioactivity/Application	Reference
EtOH (room temperature, 72h)	Antimicrobial, antioxidant	(Tshikalange et al., 2016)
ChlF, MetOH, EtOH, EtA (sequentially)	Antimicrobial	(Priyanka et al., 2015)
70% EtOH (room temperature, 72h)	Antimicrobial, anti-inflammatory, anti-HIV-1	(Mamba et al., 2016)
H ₂ O (ultrasound, 30 min)	Anthelmintic	(Mølgaard et al., 2001)

EtOH: ethanol; ChlF: chloroform; MetOH: methanol; EtA: ethyl acetate; H₂O: water

2.2.6.1 Antimicrobial and Anthelmintic Agents from Roots

Extracts from *A. karroo* have demonstrated good antimicrobial activities. Priyanka et al. (Priyanka et al., 2015) verified good antibacterial activity of chloroform, ethanol, ethyl acetate and methanol extracts against *S. aureus*, *B. subtilis*, *E. coli*, *S. typhi*, *P. aeruginosa*, *K. pneumoniae* and *P. vulgaris* bacteria. The ethyl acetate extract caused the maximum zone of inhibition against *S. aureus* (33 mm) and the lowest against *E. coli* (9 mm). Tshikalange et al. (Tshikalange et al., 2016) and Mamba et al. (Mamba et al., 2016) investigated the antimicrobial activities of several plants traditionally used in the treatment of sexually transmitted diseases, including *A. karroo*. Tshikalange et al. (Tshikalange et al., 2016) found the antimicrobial effect of an ethanolic extract against *S. aureus*, *E. coli*, *K. oxytoca*, *K. pneumoniae* subsp. *Pneumoniae*, and *N. gonorrhoeae* bacteria and *C. albicans* fungi, with MIC values in the range 400–1600 µg/mL (Tshikalange et al., 2016). Mamba et al. (Mamba et al., 2016) accessed the efficacy of an hydroethanolic extract against *Gardnerella vaginalis*, *Neisseria gonorrhoeae* and *Oligella ureolytica* bacteria, and against *C. albicans* fungi, having observed the lowest MIC values against *N. gonorrhoeae* and *C. albicans* (800 µg/mL, against 10 µg/mL exhibited by the antibiotic ciprofloxacin), with *G. vaginalis* showing the highest resistance (6300 µg/mL). The *A. karroo* root extracts were among the most effective against most of the studied microorganisms, compared with other plants traditionally used to treat sexually transmitted diseases, such as *Abrus precatorius*, *Diospyros mespiliiformis*, *Ipomoea crassipes*, *Senna petersiana*, *Ximenia caffra*, *Elaeodendron croceum*, *Elaeodendron transvaalense*, *Hilliardiella nudicaulis*, *Jasminum fluminense*, *Peltophorum africanum*, *Schotia capitata*, among others (Mamba et al., 2016; Tshikalange et al., 2016).

Extracts of *A. karroo* root also exhibited anthelmintic effects against tapeworms, evaluated by Mølgaard et al. (Mølgaard et al., 2001) using newly excysted tapeworms (cestodes) of *Hymenolepis diminuta*. The water extract killed the newly excysted cysticercoids within an hour when incubated in a culture medium, showing a lethal concentration of 17 mg/mL. This lethal concentration decreased to 2 mg/mL when considering a period of 24 h. Besides verifying anthelmintic activity by the root extract, the authors observed that the extract obtained from the leaves proved to be even more effective (Mølgaard et al., 2001).

2.2.6.2 Antioxidants and Anti-Inflammatory Agents from Roots

In addition to the antimicrobial properties against microorganisms commonly associated with causing many sexually transmitted diseases and urinary tract infections, the ethanolic extracts

showed other important characteristics in this context, namely its safety, anti-inflammatory activity, and efficacy against HIV-1 virus (Mamba et al., 2016; Tshikalange et al., 2016). The ethanol extract of the *A. karroo* root showed remarkable antiradical activity given by the DPPH free radical scavenging assay, exhibiting an IC₅₀ value ((i.e., extract concentration providing 50% inhibition) of 830 µg/mL, lower than that of ascorbic acid used as control (1440 µg/mL) (Tshikalange et al., 2016). Moreover, the authors demonstrated the safety of its use by confirming its low cytotoxicity, assessed against Vero African monkey cells lines, having found an IC₅₀ value of 115 µg/mL (Tshikalange et al., 2016). In turn, Mamba et al. (Mamba et al., 2016) demonstrated the anti-inflammatory and anti-HIV-1 activity by a hydroethanolic extract of *A. karroo*. The anti-inflammatory activity was studied by measuring the inhibition of pro-inflammatory enzyme 15-lipoxygenase (15-LOX), with the extract showing an IC₅₀ value of 62 µg/mL (i.e., concentrations that resulted in 50% 15-LOX inhibition), comparable with that obtained with the positive control quercetin (IC₅₀ = 49 µg/mL). The antiviral activity against the HIV-1 virus was investigated measuring the inhibition of the HIV-1 reverse transcriptase enzyme, which is vital in the lifecycle of the HIV virus. The authors found that the *A. karroo* extract is amongst the ones with better inhibitory activity, showing an inhibitory percentage of 67%, demonstrating that it could be a good source of potent compounds for a therapeutic strategy against the HIV-1 reverse transcriptase enzyme (Mamba et al., 2016).

2.2.7 Exudates and gum

Exudates from *Acacia* spp. are of great economic importance, due to their numerous biological activities and wide range of applications and are one of the most traded products derived from these species. Gum from *A. senegal* or *A. seyal*, commercially known as gum arabic, is a global commodity generally harvested from stems and branches of these species in Africa and Western Asia (Patel and Goyal, 2015). It is a complex, branched heteropolysaccharide, either neutral or slightly acidic, and is composed of 1, 3-linked β-D-galactopyranosyl units, currently used in foods, pharmaceuticals and many other industries, taking advantage of its antioxidant properties, positive effects on renal function, reduction of blood glucose concentration, improvement of intestinal absorption, improvement of the lipid metabolism, improvement of the cardiovascular system, protection against dental erosion and hepatic protection, among others (Musa et al., 2018; Patel and Goyal, 2015). In the pharmaceutical industry, it is also used as a carrier of drugs; in the food industry it is used in confectionery, bakery, dairy, beverages, as a microencapsulating agent, as a stabilizer in frozen products like ice and ice cream, absorbing water and producing a finer texture and in the cosmetic industry its is used as smoothener in lotions and

protective creams and as an adhesive in facial masks or face powders (Musa et al., 2018). In food products, it received the number E414, being classified as an emulsifier, stabilizer, thickener or glazing agent.

Nevertheless, the search for alternatives to *A. senegal* or *A. seyal* gum is important to decrease the dependence on gum arabic, or to provide more affordable solutions to developing countries by using endogenous plants to achieve similar results. This has been the case for *A. karroo* and *A. mearnsii*. Gum of *A. karroo* has been used as an alternative to gum arabic in some African countries. It can be used in the commercial production of sweets and other confectioneries, and has been traditionally used in the treatment of several ailments, such as abscesses, diarrhoea, dysentery, haemorrhage, inflammation of eyes, mouth ulcers, oral thrush and osteomyelitis (Maroyi, 2017). Structural and physical chemical properties of *A. mearnsii* gum have been investigated and compared with commercial gum arabic, to better understand its behaviour and possible applications as an alternative to gum arabic (Grein-lankovski et al., 2018; Grein et al., 2013). Grein et al. (Grein et al., 2013) observed that *A. mearnsii* gum is more efficient in lowering the surface tension of water and saline solutions and is more efficient in emulsifying castor oil droplets, suggesting that polysaccharides from *A. mearnsii* are candidates as substitutes of currently commercialized arabic gums, even exhibiting improved properties, depending on their application.

An *A. pycnantha* exudate was characterized for its water-soluble prebiotic compounds (Vidanarachchi et al., 2009). It was found that the exudate contains 79% of water-soluble carbohydrates and 3% of protein. The carbohydrates are mainly constituted by galactose (78%) and arabinose (22%), while the dominant aminoacids are hydroxyproline (23%), aspartic acid (12%), leucine (11%) and proline (7%). Amongst the studied species by Vidanarachchi et al. (Vidanarachchi et al., 2009), the *A. pycnantha* exudate was the one with the highest percentages of hydroxyproline and proline. Proline plays an important role in protein synthesis and structure, metabolism (particularly the synthesis of arginine, polyamines and glutamate via pyrroline-5-carboxylate) and nutrition, as well as in wound healing, antioxidative reactions and immune responses, while hydroxyproline can participate in the synthesis of glycine (essential for chickens), pyruvate and glucose (important for ruminants), and may also scavenge oxidants and regulate the redox state of cells (Wu et al., 2011). These represent interesting compounds to be included in the development of dietary supplementation to benefit the health, growth and development of animals and humans (Wu et al., 2011). Vidanarachchi et al. (Vidanarachchi et al., 2009) studied the effect of some plant extracts, including an exudate of *A. pycnantha*, on growth performance, intestinal morphology, microflora composition and activity in broiler

chickens. The authors observed that the prebiotic-rich plant extracts had no negative effect on the performance of broilers, beneficially modulating the composition of the microflora in the ileum and caeca by increasing the number of lactobacilli, and reducing harmful bacteria, such as potential pathogenic coliforms and *Clostridium perfringens* bacteria (Vidanarachchi et al., 2009).

2.3 Material applications of *Acacia* spp. biomass

In addition to bioactive extracts and aromatic oil, with potential uses in the nutraceutical, pharmaceutical, cosmetic, perfume, food or feed industries, a diversity of other interesting materials can be obtained from *Acacia* spp. Some of these materials are already available on a commercial scale and will not be further discussed. These include the use of *A. mearnsii* for the production of tannins for the leather industry and for the manufacture of adhesives, which has been implemented since the sixties, as well the manufacture of coagulants used in the wastewater treatment, which were also commercialized about forty years ago (Chan et al., 2015; Ogawa and Yazaki, 2018; Yazaki, 2015). Besides these known applications, recent studies have shown that the potential of the *Acacia* spp. biomass or extracts obtained from it can have new applications with high added value, as in the polymer and composite industries, in the production of nanomaterials, in the manufacture of new adsorbents for the removal of pollutants as alternatives to conventional activated carbon, or wood and steel protection (Table 2.9).

Table 2.9 — Material applications from *Acacia* spp. components.

<i>Acacia</i> spp.	Component used	Product/Application	Reference
<i>A. dealbata</i>	Bark and wood	Wood protection	(Yildiz et al., 2018)
<i>A. karroo</i>	Charcoal (activated)	Adsorbent (heavy metals)	(Kumar, 2016)
<i>A. mearnsii</i>	Tannin extract from bark	Stabilizer for low-density polyethylene films	(Bridson et al., 2015)
		Superparamagnetic nanoparticles	(Khan et al., 2015)
		Nanocellulose films	(Missio et al., 2018)
		Nanofiber membranes	(Martins et al., 2018)
		Encapsulated nanoparticles	(dos Santos et al., 2017)
		Lactic- <i>co</i> -glycolic acid microparticles drug carriers	(Grasel et al., 2019)
	Spent bark from tannin production	Filler in polypropylene composites	(Taflick et al., 2015)
		Cellulose nanocrystals	(Taflick et al., 2017)
		Adsorbent (dyes)	(da Silva et al., 2018)
	Biochar from woodchips (activated)	Adsorbent (ammonium)	(Beckinghausen et al., 2020)
<i>A. melanoxylon</i>	Water extract from leaves	Colloidal silver nanoparticles	(Shashanka and Kumara Swamy, 2020)
<i>A. saligna</i>	Silver nanoparticles from water leaves extract	Adsorbent (dyes)	(Taşkıran et al., 2017)
	Water leaves extract	Corrosion inhibitor	(Avci and Keleş, 2011)

2.3.1 Polymers and composites from bark

The growing awareness of sustainability in the polymer industry has led to the development not only of biopolymers but also of bio-based additives, such as stabilisers, that are often required to extend the service life of the material. Bridson et al. (Bridson et al., 2015) investigated the use of a commercial tannin extract of *A. mearnsii* as a stabilizer of linear low density polyethylene films. The authors successfully increased the thermo-oxidative and UV stability of the films when using tannin extract compounded with co-polymers (ethylene vinyl alcohol, maleic anhydride modified polyethylene or ethylene acrylic ester maleic anhydride terpolymer) as additives. The tannin extract improved the strength and elongation retention of the polymer after the accelerated weathering, performed with UV light, elevated temperature and humidity to simulate in-service environmental conditions, probably due to radical scavenging reactions that reduces oxidation and limits the damage to the polymer (Bridson et al., 2015). The *A. mearnsii* extract is already produced at a commercial scale, so it can represent a cost-competitive additive for this purpose for industrial applications.

After the extraction of tannins from the bark of *A. mearnsii*, hundreds of tons of bark residue are produced, which represents an environmental problem if a proper strategy is not adopted. This residue is usually burned, but the alternative use of exhausted bark as a filler in polypropylene composites was investigated by Taflick et al. (Taflick et al., 2015). The authors studied the impact of the addition of bark in the mechanical and thermal properties of the produced materials, using maleic anhydride as a compatibilizer to improve the adhesion between the matrix and the bark particles. The incorporation of 10% of bark particles with sizes ranging from 106 to 425 nm produced composites with higher impact properties, higher crystallization and degradation temperatures and comparable tensile strength, compared with the polymer matrix without the addition of bark (Taflick et al., 2015). These composites were considered suitable for use in non-structural applications, such as in decoration, sound isolation and household goods, bringing the advantages of reducing the environmental impact of using synthetic polymers and adding value to an abundant waste formed after tannin production from *A. mearnsii* bark.

2.3.2 Micro and nanomaterials from leaves and bark

Shashanka and Kumara Swamy (Shashanka and Kumara Swamy, 2020) successfully synthesized colloidal silver nanoparticles using a water extract of leaves of *A. melanoxylon*, and studied their application as a dopamine and hydrogen peroxide sensor. Dopamine and hydrogen

peroxide are important intermediates in environmental and biological systems, whose determination has become important and has been given tremendous attention by neuroscientists and chemists in biomedical and bioanalytical research. The colloidal silver nanoparticles were prepared by treating the water *A. melanoxydon* extract with silver nitrate and were then used in the preparation of a carbon paste electrode, by mixing it with graphite powder and silicon oil (Shashanka and Kumara Swamy, 2020). The addition of silver nanoparticles prepared this way enhanced the sensitivity of the fabricated electrode towards the oxidation of both dopamine and hydrogen peroxide, which occurred at a physiological pH, by a diffusion-controlled process, and with a linear increase in peak current with different concentrations of both products (Shashanka and Kumara Swamy, 2020).

The concept of "active packaging" for food or pharmaceutical products arose a few years ago, with the intention of creating a packaging material to extend the shelf-life or the expiration date of products, which can be achieved by exerting antioxidant effects to protect against oxidation, UV radiation or moisture (Missio et al., 2018). The current trend is moving towards the use of green and sustainable resources, where nanocellulose and bio-based additives with antioxidant properties can play an important role. Missio et al. (Missio et al., 2018). prepared a nanocellulose film containing 190 mg/g of proanthocyanidin-rich tannin extract of *A. mearnsii*, which was incorporated into the matrix by mechanical fibrillation of cellulose pulp and tannin mixture. The addition of the tannin extract improved the surface hydrophobicity, which resulted in a 6-fold enhancement in their air-barrier properties, while the mechanic features of the cellulose matrix were not significantly affected (Missio et al., 2018). Moreover, good resistance to several organic solvents was verified, as well as a slow release of antioxidant components upon soaking (55% of the original tannins in one week), thus showing the potential use of tannin-enhanced biofilms as a valid green and non-toxic material for food, pharmaceutical or cosmetic products (Missio et al., 2018).

Nanofiber membranes are used for diverse objectives, from the removal of pollutants from wastewater to biomedical, tissue engineering and regenerative medicine applications (Ismail et al., 2019). Poly(ϵ -caprolactone) is one of the most important synthetic polymers used for developing nanofiber scaffolds, for repairing soft and hard tissues, due to its cytocompatibility, biodegradability and mechanical resistance, but it has also some limitations, such as high hydrophobicity and low water absorptivity (Martins et al., 2018). To overcome these shortcomings, Martins et al. (Martins et al., 2018) produced a novel poly(ϵ -caprolactone) electrospun membrane, by blending poly(ϵ -caprolactone) with a commercial amino-functionalized tannin extracted from *A. mearnsii*, to be used as a scaffold for wound healing and tissue repair. The

authors used different percentages of tannin incorporation (from 0% to 22%), which could be tuned to impart anchorage, adhesion and proliferation of human adipose-derived stem cells. Moreover, the incorporation of the tannin extract conferred bactericidal properties to the produced membrane against *Pseudomonas aeruginosa*, particularly with 22% of incorporation (Martins et al., 2018).

Dos Santos et al. (dos Santos et al., 2017) produced nanoparticles containing *A. mearnsii* tannin extract, encapsulated using sol-gel methods, and evaluated its application as antimicrobial agents. Amongst the sol-gel methods used, the acidic and alkaline routes affected the structure of the tannins during the synthesis and decreased the antibacterial activity of the material, while a non-hydrolytic route affected the final texture of the encapsulated materials, hindering the release of the extract. Only the silicate sol-gel option, performed by mixing a sodium silicate solution with dissolved extract in acidic medium, at 50 °C during 48–72 h, produced a material with a very small particle size (<1 nm), promoting a gradual release of tannins in aqueous medium and an improved interaction with microorganisms (moderate activity against *Staphylococcus aureus*, *Aspergillus niger* and *Candida* sp.).

Grasel et al. (Grasel et al., 2019) successfully incorporated *A. mearnsii* tannin extract on lactic- α -glycolic acid microparticles, having obtained 73% of encapsulation efficiency and a particle size distribution between 0.6 and 2.4 μm . The authors demonstrated *in vitro* the biocompatibility of the produced material with normal cell lines (Vero), with no effects on cell proliferation being observed after 24 h, using microparticle concentrations up to 200 $\mu\text{g/mL}$.

These findings for the utilization of *A. mearnsii* bark extract deserve further attention and research for the implementation at larger scales, not only due to the improved properties given by the tannin extract to silver nanoparticles, superparamagnetic nanoparticles, nanocellulose films and nanofiber membranes, but mainly because this extract can be delivered from well-established procedures at a commercial scale, and can be provided at a relatively low cost.

Another way to valorize the exhausted *A. mearnsii* bark produced after the extraction of tannins, avoiding an environmental problem, is its use in the production of cellulose nanocrystals, which are used usually for polymer reinforcement, as well as in transparent films for several applications (Taflick et al., 2017). The common procedure for isolating cellulose involves costly and time-consuming extraction steps to remove extractives, but Taflick et al. (Taflick et al., 2017) demonstrated, with spent *A. mearnsii* bark, that this step can be avoided if the purpose is producing cellulose nanocrystals. Only the common steps of delignification, bleaching and hydrolysis were applied, but without using chlorinated chemicals in the bleaching step. It was found that differences in the isolated cellulose, with the process without solvent extraction

showing a higher crystallinity index (86% versus 82%), larger and more agglomerated particles, and higher residual mass after thermal degradation; however, no significant difference was observed in the morphology and thermal stability of obtained nanocrystals. Such a result represents lower costs with solvents and a more eco-friendly process to produce cellulose nanocrystals, using a bark residue.

2.3.3 Alternative adsorbents from bark, wood, and leaves

The interest in the development of low-cost adsorbents for the removal of pollutants from wastewaters, such as dyes, heavy metals or organic compounds, has increased with the increased awareness to finding environmentally friendly and sustainable processes for that purpose, and preferentially at an affordable cost to make them usable in developing countries (De Gisi et al., 2016; Sulyman et al., 2017). Conventional activated carbon is still the most-used adsorbent, but its widespread use is frequently restricted due to its high cost, besides other issues related to its efficiency and the cost of its regeneration, and its disposal at the end of its life cycle (De Gisi et al., 2016). In this context, biomass from invasive species, such as *Acacia* spp., are good candidates due to their high abundance and availability, either in nature or as an industrial by-product from wood industries, charcoal production or tannin industries.

Silva et al. (da Silva et al., 2018) prepared an alternative adsorbent using *A. mearnsii* bark waste from a tannin industry. The bark was pretreated by steam explosion, the adsorbent was prepared by an acetosolv method (boiling for 24 h a mixture of steam-exploded bark, acetone and a small percentage of sulfuric acid) and is then used for crystal violet dye removal. Besides the small surface area determined for the adsorbent (near 5 m²/g), this procedure promotes a chemical modification of lignin and polysaccharides by the solvent and the acid, generating new functional groups like ketones, ethers, open aromatic rings and carbonyl groups, that can act as new sites for adsorption (da Silva et al., 2018). The authors observed removal percentages of 95% from an initial solution containing a 50 mg/L dye concentration, after two hours of contact, using an adsorbent dosage of 750 mg/L, at pH of 10. The maximum adsorption capacity was estimated at 280 mg of dye per gram of adsorbent (da Silva et al., 2018). These results illustrate a way to aggregate value to bark waste, by producing an effective dye adsorbent, and using a simple and low-cost method, without further activation steps.

Singh et al. . (Singh et al., 2013) tried the removal of heavy metals Ni(II) and Zn(II) from aqueous solutions using an industrially produced *A. karroo* charcoal. The authors performed a single activation process by boiling the charcoal in acidic medium (hydrochloric acid solution, 1 M) for 5 h and found removal percentages of Ni(II) and Zn(II) metals 90 and 83% at a dose

of 15 g/L. The maximum adsorption capacities for Ni(II) and Zn(II) metals were estimated in 9 and 8 mg of metal per gram of adsorbent, at an optimum pH of 6 and 4, respectively. These findings show that this material holds good potential for removing heavy metals from aqueous solutions and could be used for desalinating metal ions from industrial wastewater (Singh et al., 2013). Moreover, its production can be made using a discarded fraction from the charcoal production, which can be obtained at a regular rate and at a low cost.

Beckinghausen et al. (Beckinghausen et al., 2020) tried the removal of ammonium from artificial wastewater (1000 mg/L of ammonium solution) in a fixed-bed column, using activated biochar made from *A. mearnsii* woodchips. The woodchips were carbonized at 400 °C for two hours and then two activation processes were compared-steam activation at 850 °C and hydrogen peroxide activation by shaking the biochar in a 10% hydrogen peroxide solution. The steam activation proved to be the most suitable activation method, producing a material with a surface area of 340 m²/g, with an adsorption capacity of 1651 µg of ammonium per gram of adsorbent. Moreover, the steam activation decreased the desorption capacity of the ammonium-loaded adsorbent, making it suitable to use as a solid fertilizer, improving soil nitrogen content and plant growth (Beckinghausen et al., 2020). The process needs to be improved to increase the adsorption capacity for ammonium, but this approach has the advantage of producing a material that can remove ammonium from wastewater, and then be used as a soil fertilizer, returning a considerable amount of carbon into the earth (Beckinghausen et al., 2020).

Another alternative approach was performed by Taşkıran et al. (Taşkıran et al., 2017), through the use of silver nanoparticles produced with *A. saligna* leaves extract in a dye adsorption. The silver nanoparticles were produced by mixing the liquid water extract with silver nitrate, with the extract promoting the reduction of silver cations to its metallic form, and then used in the adsorption of the Basic Red 46 dye. The optimal conditions were found as pH 7, a temperature of 35 °C and an adsorbent dosage of 1 g/L, being estimated to have a maximum adsorption capacity of 126 mg of dye per gram of adsorbent. Moreover, the authors demonstrated that the reduction of dye concentration to a level below that required by legislation can be attained using four adsorption stages (i.e., four batches of adsorption), with the effluent of the first batch entering into the second batch, and so on (Taşkıran et al., 2017).

2.3.4 Wood and steel protection using bark and leaf extracts

The use of natural extracts to protect materials from degradation or corrosion, instead of using synthetic formulations, is of great importance when a change to more sustainable and environmentally friendly industrial practices is required.

Taking advantage of the antioxidant and antimicrobial properties of extracts of *A. dealbata*, Yildiz et al. (Yildiz et al., 2018) tried to use methanol extracts from this species as wood (Scots pine) protecting agents. The decay resistance of Scots pine wood against the brown-rot fungus (*Coniophora puteana*) was assessed comparing the impregnation of wood samples with water or methanol extracts from *A. dealbata* sapwood, heartwood and bark, using concentrations of 3% and 5% by weight. The weight loss caused by the fungus varied in the range of 7% to 24% for treated samples, against almost 40% of weight loss verified with untreated samples. Bark extracts were overall more effective than wood extracts, and methanol was the most appropriate solvent, being the highest protection observed when using a concentration of 5% of methanol bark extract (Yildiz et al., 2018). Although these experiments did not yet provide adequate protection to meet the requirements of European standards, these are encouraging results and deserve a further investigation to try higher concentrations or the mixing with other non-toxic antifungal materials (Yildiz et al., 2018).

The efficiency of *A. saligna* leaf extract as an environmentally friendly inhibitor for mild steel was investigated by Avci and Keleş (Avci and Keleş, 2011), using a sulphuric acid solution (1 M) as the corrosive agent. The authors concluded that water leaves extract adsorbs onto the mild steel surface and form a protective film against metal corrosion. The corrosion inhibition efficiencies determined by potentiodynamic polarization measurements and by electrochemical impedance spectroscopy techniques were in agreement, with the highest values observed when using an extract concentration of 20 g/L (93 and 95% for 1 h of immersion, respectively). The presence of the extract increased the activation energy for the corrosion process and slowed the corrosion rate down, even in long immersion experiments (up to 120 h of immersion time).

2.4 Energy from *Acacia* spp. and biorefinery approaches

The high availability of residues of *Acacia* spp. makes them an interesting resource to be studied, having in mind the production of solid, liquid or gaseous biofuels. This gains even more significance with a view to meeting targets for the incorporation of renewable energies according to the new directive on the promotion of the use of energy from renewable sources (Directive (EU) 2018/2001 of the European Parliament and of the Council, 2018). The solid biomass fuels, such as wood chips or pellets are now in the scope of the new directive. Its production to obtain heat or power by thermochemical processes can be an easy way to retrieve some value from *Acacia* spp. residues and mitigate eradication costs (Carneiro et al., 2014), instead

of being landfilled or left in place, but more investigation must be made to assess combustion, gasification or pyrolysis properties when using the Portuguese *Acacias*.

2.4.1 Combustion and pyrolysis

The overall chemical composition of the wood of some Portuguese *Acacia* spp. reported in the literature is summarized in Table 2.10. It is essentially a carbon-based material and its chemical composition does not significantly differ from other biomass sources (McKendry, 2002a). The reported lower heating values lay between 18,100–18,400 kJ/kg (Ferreira et al., 2014; Vicente et al., 2019).

Studies on combustion performance have been made using domestic boilers, where it was observed that CO and NO_x emissions are much higher than those observed with the combustion of pine, especially in the case of NO_x emissions, that are more than three times higher in the case of *A. dealbata* pellets (Ferreira et al., 2013). This can be improved if combustion takes place in a fluidized bed combustor, but CO and NO_x emissions from *Acacia* pellets are still considerably higher than those which occur with pine pellets (Ferreira et al., 2014). Concerning the NO_x emissions, the higher values observed with *Acacia* are due to the high nitrogen content found in these species (ca. 1% in *A. dealbata*, five times higher than in pine) (Ferreira et al., 2014). Furthermore, the particulate emissions are higher (two times higher) when combusting *Acacia* than when using pine, either in domestic or in fluidized bed boilers, according to experiments made with *A. longifolia* (whole tree, including bark, treetops and branches) (Ribeiro et al., 2017; Vicente et al., 2019).

Table 2.10 — Chemical composition of *Acacia* wood (values expressed as weight percentage, on a dry basis).

Chemical composition	Value	Reference
<i>Structural composition</i>		
Cellulose	36%–45%	(Acuña et al., 2017; Amutio et al., 2013a; Domínguez et al., 2014; Furtado et al., 2015; Yáñez et al., 2014)
Hemicelluloses	14%–28%	
Lignin	19%–47%	
Extractives	5%–9%	
<i>Proximate composition</i>		
Volatile matter	75%–88%	(Domínguez et al., 2014; Ferreira et al., 2014; Ribeiro et al., 2017; Saldarriaga et al., 2015; Vicente et al., 2019)
Fixed carbon	8%–19%	
Ashes	1%–3%	
<i>Elemental composition</i>		
C	47%–50%	(Amutio et al., 2013a; Ferreira et al., 2014; Ribeiro et al., 2017; Saldarriaga et al., 2015; Vicente et al., 2019)
H	5%–6%	
O	41%–47%	
N	ca. 1%	
S	<1%	

Further investigations on the impact of operational parameters or biomass pre-treatments in the performance of the selected thermochemical process are needed to better access the suitability of Portuguese invasive *Acacia* spp. for energy recovery purposes. For instance, torrefaction (Wilk et al., 2017) and hydrothermal carbonization (Wilk et al., 2020, 2019) of wood of other species of *Acacia* has been tried and successfully improved its calorific value, carbon content and mechanical properties. Even its co-gasification with plastic (polyethylene terephthalate) was already considered a possibility (Radenahmad et al., 2018).

The feasibility of the valorization by flash pyrolysis of forest shrub wastes, including of *A. dealbata*, was accessed by Amutio et al. (Amutio et al., 2013a), in a conical spouted bed reactor operating at 500 °C, with a continuous biomass feed and char removal. A high bio-oil yield of 72 wt.% was obtained, with a char yield of 23 wt.% and a low gas yield of 5 wt.%. The produced bio-oils are composed mainly of water (concentration of 40 wt.%), phenols (13 wt.%), ketones (9 wt.%), catechols (8%), acids (7 wt.%), and furans (5 wt.%), with lower contents of alkyl phenols (3 wt.%), aldehydes (2 wt.%), guaiacols (2 wt.%), saccharides (2 wt.%) and alcohols (1 wt.%). This process originated high bio-oil yields with a suitable composition for extracting individual components and further energetic or catalytic upgrading to produce hydrocarbons or syngas, thus

allowing the production of chemicals and energy (Amutio et al., 2013a). The produced char also evidenced a much higher calorific value of 27500 kJ/kg when compared with the original biomass (17100 kJ/kg), making it more suitable for energy recovery, besides other possible material applications such as in soil amendment or as adsorbents (Amutio et al., 2013a). Pyrolysis kinetics of these wastes were later studied in detail by the same authors (Amutio et al., 2013b).

Furtado et al. (Furtado et al., 2015) studied the composition of the pyrolysis liquor of *A. mearnsii* wood, in pyrolysis trials carried out in similar conditions to those used in the charcoal production. This liquor is usually released to the atmosphere in the traditional brick ovens, but its condensation can be easily performed, minimizing the amount of gas liberated and making possible the valorization of the condensables, along with the charcoal. This condensate contains important valuable chemicals derived from the thermal degradation of the lignocellulosic constituents, such as carboxylic acids, phenols, aldehydes, low molecular mass lignin fragments (e.g., guaiacol, syringol and eugenol), substituted aromatic compounds (e.g., vanillin, ethyl vanilli and 2-methoxy-4-propenyl-phenol), as well as polysaccharide derivatives from cellulose and hemicellulose depolymerization that occurs at higher temperatures (e.g., furan, 2-acetyl-furan, methyl-2-furoate and furfural) (Furtado et al., 2015). Amongst these chemicals, eugenol is worth highlighting and is used in dentistry, perfumes and food flavoring and protection; guaiacol, used in the pharmaceutical industry; syringol, used as a fuel additive and as a surfactant; 5-methylfurfural, which is an important flavor of balsamic vinegar; and 4-hydroxy-3-methoxyethylbenzaldehyde, which has a high commercial value for nutraceutical, pharmaceutical, cosmetics, agrochemical and dyeing purposes (Furtado et al., 2015).

2.4.2 Decomposition of biomass in the main constituents

More efficient use of biomass can be achieved by opting for a biorefinery approach, seeking for obtaining high-value chemical products and/or other materials and energy. For this purpose, the decomposition of biomass in its main constituents (cellulose, hemicelluloses and lignin) and the further valorization of each, has been suggested (Galbe and Wallberg, 2019; Kucharska et al., 2018; Narron et al., 2016). This can be achieved with the help of physical pretreatments (milling, microwaves, sonication, gamma radiation, pyrolysis), chemical pretreatments (acid hydrolysis, alkaline hydrolysis, ozonation, treatment with ionic liquids, deep-eutectic solvents or organic solvents (organosolv)), physico-chemical pretreatments (steam explosion, carbon dioxide explosion, ammonia fiber explosion (AFEX), hydrothermal treatment (autohydrolysis)), or even biological pretreatments (white rot, brown rot, soft rot, bacterial or

enzymatic treatments, pickling), which has already been reviewed in the literature (Galbe and Wallberg, 2019; Kucharska et al., 2018; Narron et al., 2016).

A common approach is the application of one or a combination of pretreatments to obtain a cellulose-enriched solid, by removing lignin or hemicelluloses, making it more susceptible to enzymatic hydrolysis and fermentation processes to produce liquid biofuels, while simultaneously obtaining valuable monosaccharides, sugar oligomers and other degradation products from hemicelluloses and lignin (such as furfural, 5-hydroxymethylfurfural, organic acids and phenolic compounds). A summary of the reported practices for invasive *Acacia* spp. is provided in Table 2.11.

Table 2.11 — Pretreatment approaches for the decomposition of *Acacia* biomass.

<i>Acacia</i> spp.	Component	Process	Goal	Reference
<i>A. dealbata</i>	wood	organosolv (glycerol-water mixtures)	delignification	(Domínguez et al., 2014)
<i>A. dealbata</i>	wood	ionic liquids (1-ethyl-3-methylimidazolium acetate)	delignification	(Yáñez et al., 2014)
<i>A. dealbata</i>	wood	autohydrolysis	solubilization of hemicelluloses	(Acuña et al., 2017; Yáñez et al., 2009)
<i>A. dealbata</i>	stalks, leaves	acid hydrolysis	solubilization of hemicelluloses	(Ferreira et al., 2011)

Delignification of *Acacia* wood was investigated by Domínguez et al. (Domínguez et al., 2014), who fractionated milled *A. dealbata* wood (250 µm to 1 mm) using an organosolv process with mixtures of glycerol-water (40%–80% glycerol) in a 600 mL stainless steel Parr reactor, with temperatures ranging from 200–230 °C, for 30 to 120 minutes, at a liquid to solid ratio of 6 and 10 g/g. This process promotes the delignification of the wood using a cheap industrial by-product, preserving cellulose in the solid phase, which can be later used as a substrate for enzymatic hydrolysis. The most favorable condition was using 80% glycerol at 230 °C for one hour, with a liquid to solid ratio of 6 g/g and introducing sodium hydroxide 1% in the washing steps, that led to the removal of 80% of the initially present lignin, allowing a solid yield of 51% (relative to the initial raw material) that preserved 93% and 23% of the initial cellulose and xylan contents, respectively (Domínguez et al., 2014). The enzymatic saccharification yielded glucose concentrations up to 85 g/L, with almost complete cellulose conversion into glucose, thus confirming the potential of glycerol as an environmentally friendly fractionation agent for biorefineries (Domínguez et al., 2014). Another way to promote delignification of biomass is by using pretreatment with acetate-based ionic liquids, which also promotes some loss of crystallinity

of cellulose (Doherty et al., 2010). Yáñez et al. (Yáñez et al., 2014) treated milled *A. dealbata* wood (1–250 μm) with the ionic liquid 1-ethyl-3-methylimidazolium acetate in flasks at temperatures between 90 and 150 $^{\circ}\text{C}$, for 30 to 1440 minutes, using a solid loading of 8 wt%, and testing water, methanol, ethanol and acetone-water (1:1 V/V) as anti-solvents to precipitate cellulose. The optimal condition (150 $^{\circ}\text{C}$ for 30 min, and water as anti-solvent) led to the removal of almost 20% of lignin and the preservation of 88% of cellulose and 66% of xylan in the solid fraction (82% of the initial raw material). Comparing with the untreated material, occurred an increase in cellulose and xylan conversion to fermentable sugars after enzymatic hydrolysis by 13-fold and 20-fold, respectively (Yáñez et al., 2014).

Solubilization of hemicelluloses was accessed by Yáñez et al. (Yáñez et al., 2009) by promoting the autohydrolysis of *A. dealbata* wood in a stainless steel Parr reactor at temperatures ranging within 170–240 $^{\circ}\text{C}$ and using a liquid to solid ratio of 8 kg of water per kg of oven-dried wood. In the selected condition (215 $^{\circ}\text{C}$), this procedure led to 70% of xylan conversion into xylooligosaccharides, whereas cellulose and lignin remained in the solid phase with little alteration. This method reduced the hemicelluloses content in the remaining solid phase from 16 to near 8%, and increased the cellulose and lignin contents from 42 to 65% and from 19 to 27%, respectively (Yáñez et al., 2009). The valorization of the solid phase (approximately 70% of the initial material) can be completed by subsequent processing, such as delignification and/or enzymatic hydrolysis of cellulose, enabling, for example, the production of bioethanol. The liquid phase contains saccharides (including monosaccharides and sugar oligomers suitable as ingredients of functional foods or as carbon sources for chemical or biotechnological processes), sugar-degradation compounds (furfural) and acetic acid (Yáñez et al., 2009). Acuña et al. (Acuña et al., 2017) investigated the potential of wood from *A. melanoxylon* and several *Eucalyptus* spp. for the production of bioethanol, considering also an autohydrolysis pretreatment (liquid to solid ratio of 6:1 (v/w), a heating rate of 3 $^{\circ}\text{C}/\text{min}$ and cooking at 194 $^{\circ}\text{C}$ for 1 min), followed by simultaneous saccharification and fermentation of the remaining cellulose- and lignin-enriched solid. *A. melanoxylon* outstands with a high average theoretical ethanol yield, after 48 months of plant growth, that reached 396 L of ethanol per ton of wood (Acuña et al., 2017). This estimation must be interpreted in the context of the referred work, that considered a plantation in marginal lands, with the sites presenting serious nutritional and hydric deficits, leading to low yields of wood production (Acuña et al., 2017).

Acid hydrolysis also favors the solubilization of the hemicelluloses fraction, as demonstrated by Ferreira et al. (Ferreira et al., 2011), studying the potential of stalks and leaves of *A. dealbata* for bioethanol production. The authors pretreated the biomass with diluted sulfuric

acid in various experimental conditions (up to approximately 4% of sulfuric acid (relative to dry biomass), 100–201 °C, 5–55 min and a liquid to solid ratio of 5:1), and then tried different methods for the production of ethanol from the obtained slurry, namely: the fermentation of the water-soluble fraction (72 h, 30 °C); the simultaneous saccharification and fermentation of the whole slurry (72 h, 30 °C); the simultaneous saccharification and fermentation (72 h, 30 °C), and the separate hydrolysis and fermentation (enzymatic saccharification for 72 h at 50 °C and fermentation for 24 hrs at 30 °C), in the case of the water-insoluble fraction (solid phase). Pre-treatment at 180 °C with 0.8% of sulfuric acid for 15 min led to an increase of cellulose and acid-insoluble lignin in the solid phase from 43 to 62% and from 21 to 44%, respectively, while completely removing xylan. This condition led to the production of the highest ethanol concentration (10 g ethanol/L) by using separate hydrolysis and fermentation of the water-insoluble fraction (Ferreira et al., 2011). With simultaneous saccharification and fermentation, results obtained for the water-insoluble fraction were better than those obtained with the whole slurry (6.8–7.5 g ethanol/L against ca. 1 g ethanol/L), which is due to the higher concentration of the fermentation inhibitors furfural and 5-hydroxymethylfurfural in the slurry, while the fermentation of the water-soluble fraction gave 1.9 to 2.6 g ethanol/L (Ferreira et al., 2011). The separate hydrolysis and fermentation led to the lowest presence of inhibitors due to the washing operations involved until the fermentation step. It is worth noting that the global ethanol concentration can be increased by running a parallel fermentation of the water-soluble fraction together with the separate hydrolysis and fermentation processes (Ferreira et al., 2011).

2.4.3 Integrated Scheme for the Valorization of Invasive *Acacia* spp.

Taking into consideration the valuable extracts that can be obtained from the various fractions of the invasive *Acacia* spp., and the abovementioned decomposition techniques to obtain valuable chemicals and biofuels, an integrated approach can be suggested for more efficient use of these biomass sources. Since the majority of the reported extraction processes involves mild conditions for obtaining extractives from biomass, there will be negligible impacts on its lignocellulosic structure, thus allowing the application of physical, chemical, physico-chemical or biological pre-treatments to the spent biomass to obtain more products, materials and solid or liquid biofuels.

The scheme in Figure 2.2 represents the possible options for the valorization of Portuguese invasive *Acacia* spp., based on what was already studied with these species. Instead of being directly burned for energy recovery, directly submitted to thermochemical processes or decomposed in its main constituents, an extraction step may be applied to recover high-value

extracts suitable to use in pharmaceutical, food and cosmetic industries, or in the growing nutraceutical industry. The spent biomass can then be submitted to thermochemical processes (route A in the diagram), such as combustion, gasification or pyrolysis. These options allow the production of heat and power, as well as important intermediates that can be further explored to obtain chemicals and fuels, such as the syngas obtained by gasification or the pyrolysis bio-oil, and also chars that can be the basis for the development of low-cost adsorbents or used in soil amendment (Akhtar et al., 2018; Benedetti et al., 2017). In another way, spent biomass can be submitted to the abovementioned decomposition pre-treatments to obtain biofuels (especially bioethanol) from the hydrolysis and fermentation of the celluloses and hemicelluloses fraction, and important chemicals from the decomposition of lignin and hemicelluloses and fermentation of the cellulose and hemicelluloses fraction, and important chemicals from the decomposition of lignin and hemicelluloses (Galbe and Wallberg, 2019; Kucharska et al., 2018; Narron et al., 2016). Ethanol, xylitol and other sugar alcohols, 2,3-butanediol, methyl ethyl ketone, 2-butanol, acetic acid, lactic acid, levulinic acid, fumaric acid, succinic acid, furfural and hydroxymethylfurfural are a few examples of industrially useful chemicals that can be derived from hemicelluloses (Ji et al., 2012; Kohli et al., 2019). From lignin, a broad range of chemicals can also be obtained, such as guaiacol, vanillin and a large variety of other phenols and phenolic acids, acetylene, ethylene and acetic acid (Kohli et al., 2019; Narron et al., 2016).

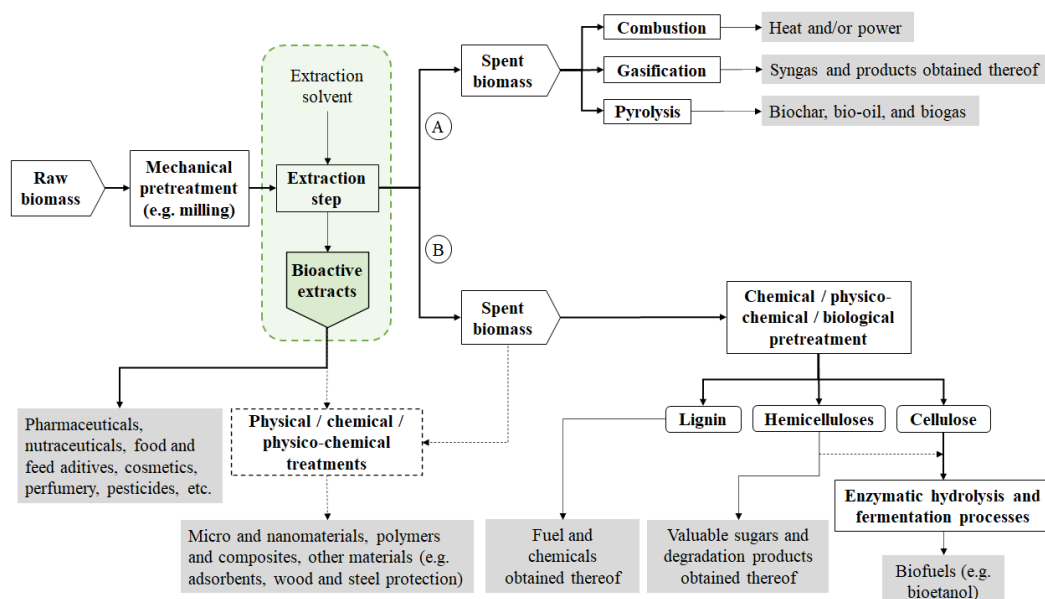


Figure 2.2 — Scheme for the valorization of biomass from Portuguese invasive *Acacia* spp., integrating an extraction step before application of advanced thermochemical processes or biomass decomposition pre-treatments

2.5 Conclusions

Acacia spp. are widespread all over the Portuguese territory, as well as across other territories around the world where they are considered invasive. Large amounts of residue have been generated from the proliferation control operations, whose valorization in an economic and environmentally sustainable manner is urgent. This review unified the results of the valorization of the various fractions of *Acacia* spp. present in the Portuguese territory, with a view to obtaining high-value extracts, chemicals, innovative materials and biofuels for energy recovery. The knowledge of these outputs is important regarding its sustainable valorization from a biorefinery perspective.

The general conclusion of researchers is that bark, wood, leaves, flowers, pods, seeds, roots and exudates can be exploited for their important biological activities, namely antioxidant, antimicrobial, anti-inflammatory, antitumoral, antidiabetic, antiviral, anthelmintic or pesticidal activities, and thus have the potential to be used by several profitable industries like pharmaceutical, nutraceutical, cosmetic or food and feed industries. Moreover, the incorporation of some fractions of the tree, or their extracts, in polymers and composites, nanomaterials and in the production of low-cost adsorbents as substitutes of commercial activated carbon, have been researched and highlighted by scientists.

Advanced decomposition processes to promote the fractionation of the biomass in its structural constituents (cellulose, hemicelluloses and lignin) have been studied in the last few years and are considered the basis for the valorization of these species in a biorefinery approach, taking advantage of the production of biofuels from the hydrolysis and fermentation of cellulose and hemicelluloses, as well as of the valuable chemicals that can be derived from the decomposition of hemicelluloses and lignin. Nevertheless, given the high-value products that can be extracted from *Acacia* spp., a scheme for the application of these decomposition procedures or of advanced thermochemical techniques (e.g., gasification or pyrolysis) to the spent biomass obtained from extraction processes is suggested, allowing us to obtain a broader range of products from the biomass.

ANTIOXIDANTS AND ANTIMICROBIAL AGENTS FROM THE FLOWERS OF INVASIVE *ACACIA* SPP.

Abstract: Antioxidant extracts rich in phenolic compounds were obtained from the flowers of Portuguese *Acacia* species grown in different locations (*A. dealbata* from Alcobaça, Caparica, and Coruche, and *A. saligna* from Caparica). Extracts were produced from fresh or spent flowers, by maceration in 70% aqueous organic solvents (acetone, ethanol, and methanol), at room temperature, for 24 h. The spent flowers were previously extracted with petroleum ether for the removal of lipophilic components (*Acacia* concrete). Aqueous acetone (70%) was selected as best extraction solvent, allowing high extraction yields for total phenolics (202.5 – 390.2 mg GAE/g extract), total flavonoids (97.3 – 136.3 mg CATE/g extract) and high values of antioxidant properties such as the scavenging activity against DPPH free radical (323.0 – 699.5 TE/g extract) and the reducing power given by FRAP assay (1.949 – 6.470 mmol Fe²⁺/g extract). Repeated maceration by adding fresh solvent to the extracted flowers increased the extract yield from 4.12 – 7.36% after a single extraction step to 7.5 – 12.7% after three sequential steps, without significantly affecting extract composition and antioxidant properties, exception made for fresh flowers of *A. dealbata* from Coruche. Significant and strong positive correlations were found between the total phenolic content, total flavonoid contents, and antioxidant activities given by DPPH and FRAP assays. Glycosides of p-coumaric acid, myricetin, quercetin, and naringenin, which have important biological activities widely referred in the literature, were tentatively identified in the extracts by HPLC-DAD, and LC-DAD-ESI-MS techniques. Results provide significant information to assess the potential of simultaneously producing *Acacia* concretes and high-value antioxidant extracts with possible applications in the nutraceutical or cosmetic

industries. The industrial exploitation of *Acacia* flowers can also contribute to the control of the propagation of this invasive species without requiring total removal of the plant, an operation with important costs and possible negative impacts.

3.1 Introduction

The genus *Acacia* encompasses over 1350 species (Seigler, 2003), most of them native to Australia but actually spread all over the world because of the wide variety of uses such as sand dunes stabilization, solid biofuel or charcoal production, source of tannins for the leather industry, and source of essences for perfume and cosmetic industry (Kull et al., 2011). However, its worldwide cultivation associated with the high capacity of *Acacia* spp. to colonize burned areas after forest fires led to its widespread and invasion in many regions of the world, with negative impacts on biodiversity. Presently 23 species of Australian *Acacias* are considered invasive outside Australia (Miller et al., 2011). For their success as invaders contribute factors such as its phenotypic plasticity, which confers to *Acacia* spp. the ability to adapt to changing environments; its large seed production and accumulation of massive seed banks for long periods; its high capacity for vegetative reproduction from rhizomes after disturbances, such as fires and cuttings; and its allelopathic properties, inhibiting the growth of neighboring native species (Fuentes-Ramírez et al., 2011; Le Maitre et al., 2011; Lorenzo et al., 2010a).

Acacia spp. are considered invasive in the Portuguese territory (Ministério do Ambiente, 2019), and the area occupied with *Acacia* spp. almost doubled from 1995 to 2010, reaching 5351 ha, according to the last national forest inventory (ICNF, 2013). The negative impacts on the ecosystems have motivated removal initiatives promoted by local authorities, generating high amounts of biomass which is usually used for pellet production, directly burned for energy production, or deposited in landfills. The valorization of the collected biomass for energy production may contribute to the economic sustainability of the removal operations but the dispersion of *Acacia* in the forest environments and the poor accessibility of some areas creates serious economic and technical limitations to selective removal as a propagation control method (Carneiro et al., 2014).

Another option to limit the dissemination of *Acacia* species is to promote the regular collection of its flowers, thus preventing seed formation and dispersion (Lorenzo et al., 2010a). In this context, flowers of *A. dealbata* and/or *A. saligna* have been studied as a source of herbicidal agents (El Ayeb et al., 2013; Lorenzo et al., 2019), yellow dyes (Fajraoui et al., 2019; Ghouila et al., 2012; Imperato, 1982b, 1982a), absolute oil (Perriot et al., 2010), cosmetic

additives (López-Hortas et al., 2020; Soto et al., 2018), and phenolic-rich extracts (Al-Huqail et al., 2019; Casas et al., 2019; Ghribia et al., 2014; Paula et al., 2022).

Casas et al. studied the extraction of *A. dealbata* flowers with ethanol, followed by fractionation of the solid residue with a sequence of organic solvents (hexane, dichloromethane, ethyl acetate, and n-butanol) and the microwave-assisted extraction with dilute acid solutions (Casas et al., 2019). The authors evaluated antioxidant, antibrowning, antilipogenic, anti-inflammatory properties of the extracts, and their cytotoxicity against tumoral cells. López-Hortas et al. evaluated the use of microwave extraction to extract bioactive components from *A. dealbata* flowers for replacement of commercial antioxidants used in sunscreen creams (López-Hortas et al., 2020). Soto et al. successfully incorporated an ethanolic extract of *A. dealbata* flowers with antioxidant properties in personal care products which had good acceptance by the tested consumers (Soto et al., 2018). Paula et al. found potential for the application of a hydroethanolic extract of *A. dealbata* flowers for the treatment of dementia, diabetes, as well as in antimicrobial treatments, due to the capacity of extracts to inhibit acetyl-cholinesterase, lipase, and α -glucosidase enzymes, and some Gram-negative and Gram-positive bacteria and yeasts (Paula et al., 2022). *A. saligna* flowers were also studied for obtaining antioxidant, antimicrobial, and anti-enzymatic agents. A hot-water extract of *A. saligna* flowers showed antibacterial, antifungal, and antioxidant properties (Al-Huqail et al., 2019), while the aqueous fraction of a hydromethanolic extract, and its fractions obtained after solvent fractionation showed antioxidant and anti-acetylcholinesterase activities (Ghribia et al., 2014).

In turn, flowers of *A. dealbata* are already used for decades for the production of absolute oil for the perfume industry, by fractionation of the *Acacia* flower concrete (Perriot et al., 2010). Once the worldwide production of *A. dealbata* absolute oil is estimated at five tons per year, with a yield of approximately 0.25% relative to the mass of flower (Perriot et al., 2010), we can estimate that around 2000 tons of flowers are used for this purpose, with the consequent production of a similar amount of spent flowers. The valorization of this spent material is of crucial importance for the sustainability of the exploration of *A. dealbata* flowers by this process.

Besides these previous studies, it is important to explore simple, easy-to-implement, and low-energy processes to retrieve bioactive components from these species. Moreover, the geographical origin of the plants determines the edaphoclimatic conditions of their production which can strongly influence the composition of lipophilic or phenolic extracts, therefore affecting their functional properties.

This study aims to investigate the production of bioactive extracts from flowers of *A. dealbata* and *A. saligna*, which are representative examples of Portuguese invasive *Acacia*

species, by a simple maceration process at room temperature with aqueous organic solvents. The influence of plant location in extract yield, composition, antioxidant, and antimicrobial activity was evaluated for *A. dealbata* species considering three regions of occurrence (Caparica, Coruche and Alcobaça). The extraction procedure was applied to fresh flowers and spent flowers after concrete removal to evaluate the possibility of obtaining two high-added-value products by sequential extraction of the same raw material. Furthermore, the extraction of concrete-free *Acacia* flowers allows to evaluate the influence of the concrete lipophilic components in the extraction of phenolic components with polar solvents. The bioactive extracts of both fresh and spent *A. dealbata* and *A. saligna* flowers were characterized for mass yield, total phenolics, total flavonoids, DPPH free radical scavenging assay, and the ferric reducing antioxidant power assay (FRAP). The antimicrobial activity of selected extracts against several bacteria was also determined to evaluate possible applications as natural biocides. Extract composition was characterized through HPLC-DAD profiles and LC-DAD-ESI-MS for tentative identification of individual components.

3.2 Materials and methods

3.2.1 Plant material

Fresh flowers of *A. dealbata* were collected in three different locations in the Center Region of Portugal: Alcobaça (39°36'36.5"N; 8°59'45.0"W, at 32 – 40 m of altitude), Caparica (38°37'22.6"N; 9°10'38.5"W, at 44 – 48 m of altitude), and Coruche (38°55'05.2"N; 8°27'31.6"W, at 56 – 58 m of altitude). *A. saligna* flowers were collected in Caparica (38°37'27.2"N; 9°12'03.8"W, at 76 – 78 m of altitude). The samples used for extraction are a result of the mixing of the flowers of at least ten different trees from each location.

3.2.2 Chemicals and reagents

Acetone, ethanol 96°, methanol, and petroleum ether (Carlo Erba Reagents, Val de Reuil, France) were used in the extraction processes. Total phenolics determination used Folin-Ciocalteu reagent (Panreac, Barcelona, Spain), sodium carbonate (Labkem, Barcelona, Spain), and gallic acid (Merck, Darmstadt, Germany). Sodium nitrite (NaNO₂), sodium hydroxide (NaOH), and aluminum chloride (AlCl₃) used in the determination of flavonoids were purchased from Merck (Darmstadt, Germany). The reagent 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, St. Louis, MO, USA) was used in the DPPH assay. The reagents 2,4,6-tris(2-pyridyl)-s-

triazine (TPTZ), acetate buffer 3.6, iron (III) chloride (FeCl_3), and iron (II) sulfate (FeSO_4) used in the ferric reducing antioxidant power (FRAP) assay were supplied by Merck (Darmstadt, Germany). Reagents for antimicrobial tests included ketoconazole, ofloxacin and vancomycin purchased from Sigma-Aldrich (St. Louis, MO, USA), Brain heart infusion agar (BHIA) purchased from Merck (Darmstadt, Germany) and Mueller-Hinton agar (MHA), Sabouraud dextrose agar (SDA) and Trypto casein-soy agar (TSA) purchased from Biokar diagnostics (Allonne, France).

3.2.3 Extraction procedure

Fresh flowers were collected before 10 a.m., rapidly transported to the laboratory, separated from twigs and leaves, roughly cut into smaller pieces (less than 0.5 cm), and immediately subject to the extraction procedures. The fresh flowers (100 g) were subject to maceration with 70% (v/v) aqueous solutions of acetone, ethanol and methanol (70% ACE, 70% ET, and 70% MET), at a flower:solvent ratio of 1:4. After a maceration time of 24h, the liquid extracts were separated by filtration, and the flower residues were re-extracted twice using the same maceration time but at a flower:solvent ratio of 1:3. Each collected extract was analyzed separately for mass yield, total phenolics, total flavonoids and antioxidant activity. The removal of the lipophilic components (*Acacia* concrete) from fresh flowers was performed by two sequential maceration cycles, using flower:solvent ratios of 1:3 and 1:2, and combining the obtained extracts. Concrete yield was determined for the combined petroleum ether extracts by solvent elimination at reduced pressure (Buchi rotavapor) and expressed as mass percentage, on a wet basis (wb). The spent flowers were spread in a tray and left to dry at room temperature for a period of 48 h, before subsequent extraction with aqueous organic solvents following the method applied to fresh flowers (Figure 3.1). Concrete samples and solvent extracts were stored in glass containers at $-20\text{ }^{\circ}\text{C}$, until further analysis. All extraction assays were performed in duplicate.

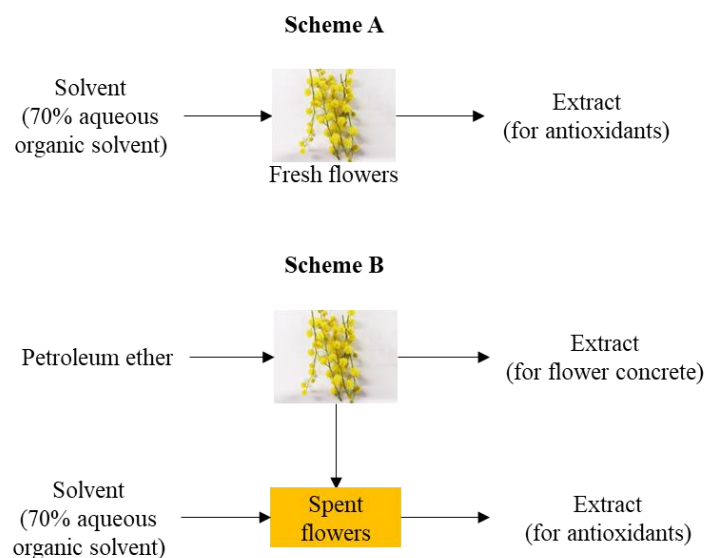


Figure 3.1 — Extraction scheme for the extraction of fresh and spent flowers.

3.2.4 Extracts characterization

Total phenolic content (TPC) of the extracts was measured by the Folin-Ciocalteu method, through an adaptation of the method described by Singleton et al. (Singleton et al., 1999). Briefly, 0.5 mL of extract, 2 mL of distilled water, and 0.5 mL of Folin-Ciocalteu reagent were added to a test tube and allowed to stand for 5 minutes. Then 2 mL of aqueous sodium bicarbonate (10% w/v) were added and the mixture was incubated for 1 h in the dark. Absorbance was measured at 760 nm and total phenolic contents (TPC) of the extracts were determined using a calibration curve constructed with gallic acid standards, and expressed as gallic acid equivalents (GAE), namely mg GAE/g of dry extract, and mg GAE/g of flowers, both in wet basis (wb) and dry basis (db).

Total flavonoid content (TFC) was determined as described by (Barros et al., 2010) and results were expressed in catechin equivalents (mg CatE/g of dry extract, and mg CatE/g of flower on both wet and dry basis). A calibration curve was also made using rutin standards in order to express total flavonoid content (TFC) in rutin equivalents (RutE), allowing comparison with results from the literature.

Antioxidant activity was evaluated by the DPPH free radical scavenging assay and by the ferric reducing antioxidant power assay. For the DPPH assay, 0.5 mL of extract were mixed with 4 mL of DPPH solution (2,2-diphenyl-1-picrylhydrazyl at 45 mg/L in methanol), incubated at room temperature for 30 min, and absorbance was measured at 517 nm. The DPPH scavenging activity was determined using a calibration curve constructed with Trolox standards and results

were expressed as Trolox equivalents by (mg TE/g of dry extract, and mg TE/g of flower on both wet and dry basis).

The FRAP assay was carried out using the procedure of Benzie and Strain with slight modifications (Benzie and Strain, 1996). The FRAP reagent was prepared from 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. This reagent was freshly prepared before analysis and warmed to 37 °C in a water bath prior to use. One hundred microliters of sample were added to 3 mL of the FRAP reagent and the mixture was kept at 37 °C for 30 minutes. After this period, absorbance was recorded at 593 nm. A standard curve was made using iron (II) sulfate solutions, and the results were expressed as mmol Fe(II)/g of dry extract, and as $\mu\text{mol Fe(II)/g}$ of flower on both wet and dry basis.

These characterization assays were performed in triplicate for each extract.

3.2.5 HPLC-DAD and HPLC-DAD-MS/MS analyses

High performance liquid chromatography was performed in a Thermo Spectra System, equipped with a binary pump (Spectra System P2000), autosampler (Spectra System AS1000), DAD detector (Spectra System UV6000LP), and UV controller (Spectra System SN4000). Chromatograms and UV spectra were acquired using the software XcaliburTM 2.0.6 (Thermo Fisher Scientific Corporation). The reverse phase chromatography separation was carried out with a Chromolith Performance RP-18e column (100 mm x 4.6 mm, Merck). The mobile phase consisted of (A) water (0.5% formic acid, v/v) and (B) acetonitrile (0.1% formic acid, v/v). The gradient profile of the mobile phase was optimized as follows: 100-95% A (0-5 min) at a flow rate of 1.0 mL/min, followed by 95-80% A (5-80 min), 80-10% A (80-90 min), 10% A (90-100 min), 10-100% A (100-105 min), and 100% A (105-110 min), at a flow rate of 0.7 mL/min. The absorption spectra were recorded in the range of 190-600 nm (increments of 1 nm), and at the specific wavelengths of 280, 320, and 360 nm. The injection volume of each sample was 20 μL .

In HPLC-DAD-MS/MS, some selected extracts were analyzed on an Alliance 2695 separation module HPLC system coupled to a 2996 Photodiode Array Detector and a Micromass[®] Quattro Micro triple quadrupole (TQ) (Waters, Ireland). The injection volume was 20 μL , and the chromatographic separation procedure was carried out using a Lichrocart[®] RP-18 column (250 x 4 mm, 5 μm , Merck) and a Manu-cart[®] RP-18 pre-column in a thermostated oven at 35°C. The mobile phase consisted of water with 0.5% formic acid as eluent A and acetonitrile with 0.5% formic acid as eluent B at a flow rate of 0.30 mL min⁻¹. The system was run with the

following gradient program: 0-5 min from 100 to 95% A; 5-120 min from 95 to 80% A; 120-140 min from 80 to 10% A; 140-160 min at 10% A; finally returning to the initial conditions for 20 minutes. PDA was used to scan wavelength absorption from 210 to 650 nm. Tandem mass spectrometry (MS/MS) detection was performed using an electrospray ionization (ESI) source operating at 120°C, applying a capillary voltage of 2.5 kV, cone voltage of 30 V, and collision energies of 10, 20, and 30 eV. Compounds were ionized in both negative and positive ion modes. High purity nitrogen (N₂) was used both as a drying gas and as a nebulizing gas. Ultra-high purity Argon (Ar) was used as the collision gas.

Before these chromatographic analyses, each analyzed sample (6 mL) was extracted with 3 mL of petroleum ether (twice) to remove lipids, and the volume was adjusted to 5 mL. The cleaned samples were diluted ten-fold in 0.5% of formic acid and filtered (0.22 µm) before injection.

3.2.6 Antimicrobial activity

The extracts used in the antimicrobial activity assays were all dissolved in 70% ethanol. When the original extraction solvent was 70% acetone or 70% methanol the organic solvent was removed under reduced pressure and replaced by ethanol, in the same concentration.

Antimicrobial activity was assayed against Gram-negative bacteria (*Escherichia coli* ATCC® 8739TM, and *Pseudomonas aeruginosa* ATCC® 9027TM, Gram-positive bacteria (*Staphylococcus aureus* ATCC® 6538TM, *Staphylococcus epidermidis* ATCC® 12228TM, methicillin resistant *Staphylococcus aureus* (MRSA) ATCC® 33591TM, *Enterococcus faecalis* ATCC® 29212TM, *Bacillus cereus* ATCC® 11778TM, *Streptococcus mutans* ATCC® 25175TM and *Streptococcus mitis* NCIMB® 13770), and yeast (*Candida albicans* ATCC® 10231TM). All microbial strains were kept at -70 °C in broth with glycerol (15% v/v). The antimicrobial activity was assessed by the well-diffusion assay, according to (P. Pereira et al., 2022). Briefly, microorganisms were subcultured on TSA (*E. coli*, *P. aeruginosa*, *S. aureus*, MRSA, *S. epidermidis*, *E. faecalis*, *S. mutans*, *S. mitis* and *B. cereus*), BHIA (*S. mutans* and *S. mitis*) or SDA (*C. albicans*) and incubated at 30 ± 2 °C (*C. albicans* and *B. cereus*) or 35 ± 2 °C (remaining bacteria). Isolated colonies were suspended in saline medium (NaCl, 0.85% w/v) and the turbidity was adjusted to 0.5 on the McFarland scale (approx. 1-2×10⁸ CFU/mL for bacteria and 1-5×10⁶ CFU/mL for yeasts) (DEN-1B McFarland Densimeter, Grant-bio). Subsequently, microbial suspensions were spread on BHIA (*S. mutans* and *S. mitis*) or MHA (remaining microorganisms) Petri dishes, wells (6 mm in diameter) were aseptically punched and 50 µL of extracts (1, 7, and 10 mg/mL) were poured

into the wells. The plates were incubated, in the dark, for 24 h at 30 ± 2 °C (*C. albicans* and *B. cereus*) or 35 ± 2 °C (remaining bacteria). Antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zone (in mm) around the well. Paper disks impregnated with vancomycin (Gram-positive bacteria), ofloxacin (Gram-negative bacteria), and ketoconazol (*C. albicans*) were used as positive controls. Ethanol (70% w/v) was used as a negative control. Data are presented as the mean $\pm$ standard deviation. All the determinations were performed in triplicates.

3.2.7 Statistical analysis

Statistical analysis of the results was performed using the SPSS software, version 23 (IBM Analytics, Armonk, NY, USA). Analysis of variance (ANOVA) was performed to evaluate significant differences between averages, and the Tukey post hoc test ($p < 0.05$) was used to perform multiple comparisons between averages.

3.3 Results and discussion

3.3.1 Extraction of lipophilic components from fresh flowers of *A. dealbata*

Acacia fresh flowers were extracted with petroleum ether to recover lipophilic components (*Acacia* concrete) and obtain spent flowers, to be used in the experiments of extraction of bioactive components with aqueous organic solvents. The concrete extraction yields are presented in (Figure 3.2) for *A. dealbata* flowers collected at Caparica, Coruche and Alcobaça and *A. saligna* flowers collected at Caparica.

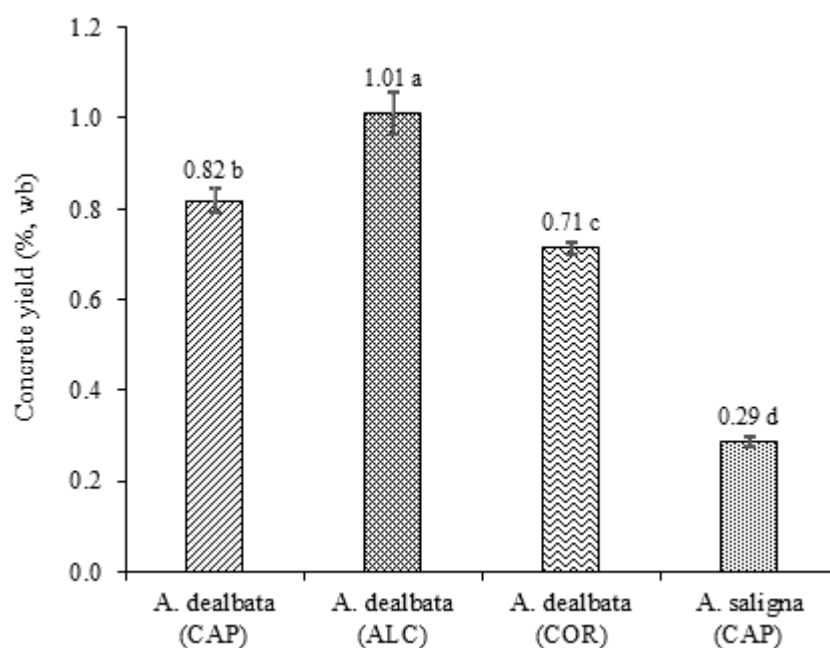


Figure 3.2 — Yields of concrete obtained with *A. dealbata* from Caparica (CAP), Alcobaça (ALC) and Coruche (COR), and with *A. saligna* from Caparica (CAP); different letters indicate significant differences of means flowers (one-way ANOVA, Tukey test at $p < 0.05$).

Both *A. saligna* and *A. dealbata* flowers yielded bright yellow concretes with an intense aroma. Concrete yields for *A. dealbata* flowers varied from 0.71 ± 0.01 % to 1.01 ± 0.05 %, with significative differences ($p < 0.05$) between concrete yields from flowers collected in the different locations. These results indicate that edaphoclimatic conditions influenced concrete yields, what is relevant for the the industrial application of this product. Concrete yields obtained for flowers of *A. saligna* were 2.5 to 3.5 times lower than those obtained from flowers of *A. dealbata*, which was expected since *A. dealbata* is the *Acacia* species traditionally exploited for production of concretes and absolutes for the perfume industry. Concrete values obtained for Portuguese *A. dealbata* flowers are comparable to those previously reported by Perriot et al. for *A. dealbata* flowers from Grasse (France) (Perriot et al., 2010). Casas et al. studied the extraction of *A. dealbata* flowers with supercritical CO_2 , at pressures of 10 – 35 MPa, and obtained lipophilic extracts with yields between 0.1 and 1.1% dry weight (Casas et al., 2021).

The characterization of the composition of flower concretes obtained in this work and of the corresponding absolute oils will be addressed in another report.

3.3.2 Extraction yield and composition of extracts from fresh and spent flowers

Fresh and spent flowers were extracted with different solvents (70% ACE, 70% ET, and 70% MET), to determine if the presence or absence of the concrete components in the flower matrix, might influence the subsequent removal of polar components by hydrophilic solvents. Extraction yields, TPC, and TFC for the extracts obtained with a single maceration of fresh and spent flowers are presented in Table 3.1.

Table 3.1 — Extraction yields (% wet basis), TPC (mg GAE/g extract) and TFC (mg CatE/g extract) of extracts obtained by a single maceration of fresh and spent flowers in different extraction solvents.

		Fresh flower			Spent flower		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
Yield	<i>A. dealbata</i> (ALC)	7.36 ± 0.24 ^{aA}	6.23 ± 0.05 ^{bA}	6.20 ± 0.14 ^{bA}	5.96 ± 0.22 ^{aA}	5.10 ± 0.21 ^{bB}	4.82 ± 0.21 ^{bB}
	<i>A. dealbata</i> (CAP)	6.46 ± 0.22 ^{aB}	5.09 ± 0.10 ^{bB}	5.14 ± 0.17 ^{bB}	5.38 ± 0.25 ^{aB}	5.08 ± 0.14 ^{aB}	4.27 ± 0.23 ^{bC}
	<i>A. dealbata</i> (COR)	6.35 ± 0.21 ^{aB}	5.02 ± 0.11 ^{bB}	4.89 ± 0.03 ^{bB}	5.95 ± 0.06 ^{aA}	5.62 ± 0.02 ^{bA}	5.47 ± 0.08 ^{cA}
	<i>A. saligna</i>	5.40 ± 0.18 ^{aC}	4.47 ± 0.13 ^{bC}	4.35 ± 0.03 ^{bC}	4.18 ± 0.19 ^{abC}	4.12 ± 0.05 ^{bC}	4.43 ± 0.07 ^{aBC}
TPC	<i>A. dealbata</i> (ALC)	261.6 ± 4.9 ^{aB}	219.4 ± 3.5 ^{bB}	209.3 ± 8.4 ^{cB}	257.1 ± 5.5 ^{aB}	200.4 ± 4.3 ^{bB}	189.0 ± 6.5 ^{cA}
	<i>A. dealbata</i> (CAP)	265.2 ± 4.3 ^{aB}	209.3 ± 1.8 ^{bC}	194.9 ± 7.8 ^{cC}	202.5 ± 5.9 ^{aC}	147.9 ± 6.1 ^{bC}	134.1 ± 5.7 ^{cC}
	<i>A. dealbata</i> (COR)	390.2 ± 1.9 ^{aA}	326.7 ± 3.6 ^{bA}	298.1 ± 6.7 ^{cA}	272.7 ± 3.9 ^{aA}	212.0 ± 2.2 ^{bA}	198.4 ± 7.9 ^{cA}
	<i>A. saligna</i>	209.0 ± 1.3 ^{aB}	156.3 ± 2.2 ^{bD}	133.5 ± 1.0 ^{cD}	203.7 ± 4.5 ^{aC}	148.2 ± 2.2 ^{bC}	145.0 ± 2.2 ^{bB}
TFC	<i>A. dealbata</i> (ALC)	97.3 ± 1.4 ^{aC}	84.3 ± 2.1 ^{bB}	79.9 ± 2.4 ^{cB}	130.0 ± 2.7 ^{aB}	100.3 ± 3.4 ^{bB}	94.2 ± 3.0 ^{cB}
	<i>A. dealbata</i> (CAP)	103.6 ± 4.9 ^{aB}	86.9 ± 4.3 ^{bB}	82.0 ± 2.0 ^{bB}	135.5 ± 2.5 ^{aA}	106.6 ± 2.1 ^{bA}	100.8 ± 3.5 ^{cA}
	<i>A. dealbata</i> (COR)	117.5 ± 3.8 ^{aA}	102.2 ± 4.1 ^{bA}	99.4 ± 2.3 ^{bA}	136.3 ± 0.8 ^{aA}	104.1 ± 3.4 ^{bAB}	90.8 ± 3.3 ^{cB}
	<i>A. saligna</i>	120.3 ± 2.6 ^{aA}	73.9 ± 3.8 ^{bC}	62.6 ± 3.4 ^{cC}	109.7 ± 2.6 ^{aC}	56.1 ± 1.3 ^{bC}	48.6 ± 1.4 ^{cC}

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 x 3); different letters (lower case a-c) indicate significant differences of means among solvents for each sample of fresh and spent flowers (one-way ANOVA, Tukey test at p<0.05); different letters (upper case A-D) indicate significant differences of means within flower samples of fresh and spent flowers, for each extraction solvent.

Extract yields ranged from 4.35 to 7.36% (wet basis) for fresh flowers (with moisture contents between 58.0 and 68.1%) and from 4.12 to 5.96% for air-dried spent flowers (with moisture contents in the range of 11.2 – 34.2%). Comparable mass yields (8%, wb and 7.45%, wb), were obtained from *A. nilotica* flowers extracted with aqueous methanol (El-Toumy et al., 2011) or from *A. saligna* flowers extracted with water (Al-Huqail et al., 2019). Other authors reported

lower mass yields for extracts of *Acacia* flowers with polar solvents, namely, 2.1% (db) for ethanolic extracts of *A. dealbata* flowers (López-Hortas et al., 2020), or 2% (db) for the methanolic extract of dried *A. saligna* flowers (Ghouila et al., 2012). Some authors obtained higher mass yields for extraction of *Acacia* dried flowers using different extraction conditions such as higher temperatures, higher solvent:flower ratios, longer extraction times or ultrasound assistance. Extracting *A. dealbata* flowers, Casas et al. obtained extracts with mass yields of 36.41% (db) (Casas et al., 2019), while Soto et al. report ethanolic extracts with mass yields of 16.9% (wb) (Soto et al., 2018). Also, aqueous and ethanolic extracts with mass yields of 21.2 – 26.3% were obtained from dried flowers of *A. confusa* (Tung et al., 2011).

The results obtained in the present work showed that the extraction mass yield was influenced by the nature of the sample (geographical origin, fresh or spent flowers) and by the type of extraction solvent. Aqueous acetone provided significantly higher extraction yields for all samples, when compared with the other tested solvents and the acetonetic extracts with higher mass yields were obtained from fresh flowers. Among fresh flowers, *A. dealbata* from Alcobaça gave the highest yields for each solvent. Among spent flowers, the highest yields were obtained from *A. dealbata* flowers from Coruche, regardless of the solvent, and from Alcobaça when using 70% ACE. *A. saligna* gave the lowest extraction yields. These results indicate that, as observed for concretes, both the *Acacia* species and their geographical origin affect the mass yields of polar extracts. In general, the extracts obtained from spent flowers had lower mass yields than those from fresh flowers, what may be the result of the co-extraction of some less polar components such as terpenic derivatives, during the extraction with hydrophilic solvents.

The polar extracts obtained with aqueous acetone, ethanol and methanol are expected to be rich in phenolic compounds, a major class of plant secondary metabolites that are associated with the antioxidant activity and other beneficial health effects of vegetal extracts (Miguel-Chávez, 2017; Minatel et al., 2017). The total phenolic content (TPC) was significantly higher for 70% ACE extracts of all fresh or spent flowers, when compared with the extracts obtained with aqueous ethanol or methanol, from the same samples. This result indicates that the higher yields of the acetonetic extracts result from a higher extraction of phenolic components and not to the co-extraction of other classes of metabolites. Comparing *A. dealbata* extracts from different locations, higher TPC values were generally obtained for the extracts of flowers collected at Coruche, regardless of their lower mass yield, indicating a more selective extraction of those phenolic components.

Extracts produced from *A. saligna* flowers showed the lowest TPC values, in accordance with their lower suitability for industrial valorization. The extracts obtained from spent flowers had TPC values slightly lower than comparable extracts obtained from fresh flowers, which suggests that phenolics with a more lipophilic character (like condensed polyphenols) may have been removed by the pre-extraction with petroleum ether. TPC in the extracts varied from 133.5 to 390.2 mg GAE/g and from 145.0 to 272.7 mg GAE/g for fresh and spent flowers, respectively. These values are comparable to TPC values of extracts obtained from flowers of *A. dealbata* by Casas et al. (38 – 279 mg GAE/g) (Casas et al., 2019) and Soto et al. (179 mg GAE/g) (Soto et al., 2018), or from *A. nilotica* flowers (101.17 – 342.03 mg GAE/g) (El-Toumy et al., 2011), and higher than the observed with flowers of *A. confusa* (28.6 – 105.1 mg GAE/g) (Tung et al., 2011). Therefore, the extracts obtained from spent flowers still contain valuable components that may be extracted and valorized. In order to facilitate comparison with literature results, TPC and TFC of the extracts was also expressed relative to the mass of flower and these results are presented in Appendix A1 (Table A1). Total phenolics yield varied from 5.8 to 24.8 mg GAE/g for fresh flowers and 5.7 – 16.2 mg GAE/g for spent flowers both on a wet basis (wb). Considering results in a dry basis (db) total phenolics varied from 18.2 – 71.3 mg GAE/g for fresh flowers and 6.9 – 24.7 mg GAE/g spent flowers, confirming the conclusion that some less polar phenolics were probably co-extracted with the terpenic components of *Acacia* concrete previously extracted with petroleum ether (full results in Appendix A1, Table A2). TPC of the extracts obtained in this work were higher than those reported by López-Hortas et al. for *A. dealbata* flowers (approximately 0.09 – 0.16 mg GAE/g flower (db)) (López-Hortas et al., 2020), or the TPC values reported for extracts of yellow tea (2.4 – 6.6 mg GAE/g of tea (wb)), green tea (1.1 – 3.6 mg GAE/g of tea (wb)), and black tea (0.6 – 4.2 mg GAE/g of tea (wb)) (Kopjar et al., 2015). Extracts with comparable values of TPC are reported for *Dendrobium catenatum* flowers (10.39 – 18.88 mg GAE/g of flower (db)) (Zhang et al., 2019), different edible flowers from Sri Lanka (1.1 – 57.4 mg GAE/g of flower (db)) (Janarney et al., 2021), raspberry fruits (10.4 – 20.6 mg GAE/g of fruit (wb)) or blackberry fruits (22.8 – 27.9 mg GAE/g of fruit (wb)) (Sariburun et al., 2010).

Flavonoids are a class of phenolics commonly found in flowers of *Acacia* spp. (Abdel-Farid et al., 2014; El-Toumy et al., 2011; Ghouila et al., 2012; Ghribia et al., 2014; Leela and Saraswathy, 2012; Tung et al., 2009; Wu et al., 2008). Flavonoids are well known for their wide spectrum of health-promoting effects and considered an indispensable component in a variety of nutraceutical, pharmaceutical, medicinal and cosmetic applications, because of their antioxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties (Panche et al.,

2016). Total flavonoid content (TFC) in the extracts of *Acacia* flowers ranged from 62.6 to 120.3 mg CatE/g extract and from 48.6 to 136.3 mg CatE/g extract for fresh and spent flowers, respectively. The highest TFC values were found in the 70% ACE extracts, when compared with the extracts obtained with 70% ET or 70% MET from the same samples. TFC was higher for *A. dealbata* extracts produced from spent flowers, and *A. saligna* extracts obtained from fresh flowers, which may indicate a different flavonoid profile in these two species. For this specific class of phenolic compounds, the pre-extraction with petroleum ether did not affect their concentration in the extracts since higher values of TFC were obtained for spent *A. dealbata* flowers than for fresh flowers from the same origin and extracted in the same conditions. This suggests that the flavonoid fraction is mainly composed by hydrophilic components, less prompt to be removed when pretreating with petroleum ether and becoming more accessible after removing the lipophilic extractives.

TFC ranged from 2.7 to 7.5 CatE/g of fresh flowers (wb) (8.5 – 21.5 CatE/g of fresh flowers (db)), and between 2.2 and 8.1 mg CatE/g of spent flowers (wb) (2.4 – 11.3 CatE/g of spent flower (db)), thus showing the high potential of *Acacia* flowers as a flavonoid source (Appendix A1, Tables A1 and A2). The TFC values found for the *Acacia* flowers extracts were higher than those observed in yellow (1.7 – 3.8 mg CatE/g (wb)), green (0.7 – 1.6 mg CatE/g (wb)) and black (0.2 – 0.3 mg CatE/g (wb)) teas (Kopjar et al., 2015), and in raspberry (0.2 – 0.4 mg CatE/g (wb)) and blackberry (0.3 – 0.8 mg CatE/g (wb)) red fruits (Sariburun et al., 2010). The TFC values of the *Acacia* flowers extracts expressed in rutin equivalents (RutE), for comparison with literature results, varied in the range of 192.1 to 368.8 mg RutE/g for fresh flowers and from 148.9 to 417.7 mg RutE/g for spent flowers (Appendix A1, Table A3). Those values were significantly higher than the reported for other *Acacia* spp., such as *A. confusa* (17.5 – 62.6 mg RutE/g) (Tung et al., 2011) or *A. nilotica* (11.83 – 30.33 mg RutE/g) (El-Toumy et al., 2011). Expressing per mass of flower (Appendix A1, Table A4), the results of this work (26.2 – 65.8 mg RutE/g of fresh flower (db) and 7.4 – 37.8 mg RutE/g of spent flower (db)) are in a higher range than the observed in *Dendrobium catenatum* flowers (7.24 – 18.56 mg RutE/g of flower (db)) (Zhang et al., 2019) and in Sri Lankan edible flowers (0.3 – 19.8 mg RutE/g of flower (db)) (Janarny et al., 2021), especially in the case of fresh flowers.

The results showed that the nature of the solvent greatly influenced not only the extract yield but also the removal of phenolic compounds. Varying the polarity of the solvents, the selectivity for more hydrophilic or more hydrophobic phenolic structures also changes, being the best results obtained when using 70% aqueous acetone as a solvent, regardless of the origin of samples or species. This is supported by other studies on phenolic removal from other

plants, such as whole wheat and bran (Abozed, 2014), stem bark of *Butea monosperma* (Lam.) Kuntze (Salar and Seasotiya, 2011), bark extracts of *A. leucophloea*, *A. ferruginea*, *A. dealbata* and *A. pennata* (Sowndhararajan et al., 2013), or flowers of safflower (*Carthamus tinctorius* L.), where also aqueous acetone solutions gave the best results for obtaining phenolics.

3.3.3 Antioxidant activity of extracts from fresh and spent flowers

The results for the antioxidant activity evaluated by the DPPH and FRAP assays are summarized in Table 3.2.

Table 3.2 — Antioxidant activity of extracts from fresh and spent flowers, given by DPPH (mg TE/g extract) and FRAP (mmol Fe²⁺/g extract), using different extraction solvents (results expressed per mass of raw material are presented in Appendix A1, Table A1).

		Fresh flower			Spent flower		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
DPPH	<i>A. dealbata</i> (ALC)	598.9 ± 10.6 ^{a,B}	487.4 ± 13.6 ^{c,B}	508.4 ± 13.1 ^{b,B}	447.2 ± 5.4 ^{a,A}	340.1 ± 6.7 ^{b,A}	318.5 ± 8.9 ^{c,A}
	<i>A. dealbata</i> (CAP)	577.1 ± 9.7 ^{a,C}	439.2 ± 12.0 ^{b,C}	399.1 ± 17.6 ^{c,C}	395.5 ± 4.1 ^{a,B}	183.7 ± 5.6 ^{b,C}	158.1 ± 8.1 ^{c,C}
	<i>A. dealbata</i> (COR)	699.5 ± 3.8 ^{a,A}	558.8 ± 7.3 ^{b,A}	554.2 ± 10.3 ^{b,A}	447.3 ± 8.1 ^{a,A}	262.8 ± 10.0 ^{b,B}	220.8 ± 6.2 ^{c,B}
	<i>A. saligna</i>	323.0 ± 4.8 ^{a,D}	188.8 ± 12.2 ^{b,D}	179.4 ± 10.3 ^{b,D}	243.3 ± 3.6 ^{a,C}	110.2 ± 1.3 ^{b,D}	103.5 ± 2.1 ^{c,D}
FRAP	<i>A. dealbata</i> (ALC)	5.355 ± 0.155 ^{a,B}	3.007 ± 0.183 ^{c,C}	3.760 ± 0.127 ^{b,A}	4.380 ± 0.181 ^{a,A}	2.966 ± 0.178 ^{b,A}	2.989 ± 0.106 ^{b,A}
	<i>A. dealbata</i> (CAP)	5.601 ± 0.133 ^{a,B}	3.360 ± 0.136 ^{b,B}	3.395 ± 0.192 ^{b,B}	3.715 ± 0.211 ^{a,B}	2.397 ± 0.121 ^{b,C}	2.086 ± 0.159 ^{c,C}
	<i>A. dealbata</i> (COR)	6.470 ± 0.199 ^{a,A}	4.894 ± 0.170 ^{b,A}	3.943 ± 0.202 ^{c,A}	4.581 ± 0.144 ^{a,A}	2.733 ± 0.146 ^{b,B}	2.664 ± 0.117 ^{b,B}
	<i>A. saligna</i>	3.004 ± 0.168 ^{a,C}	1.438 ± 0.111 ^{b,D}	1.302 ± 0.113 ^{b,C}	1.949 ± 0.160 ^{a,C}	1.127 ± 0.105 ^{b,D}	1.042 ± 0.097 ^{b,D}

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 × 3); different letters (lower case a-c) indicate significant differences of means among solvents for each sample of fresh and spent flowers (one-way ANOVA, Tukey test at p<0.05); different letters (upper case A-D) indicate significant differences of means within flower samples of fresh and extracted flowers, for each extraction solvent.

The DPPH assay is commonly performed to measure the ability of compounds to act as free radical scavengers or hydrogen donors, through the reduction of the odd electron of nitrogen atom in DPPH by hydrogen atoms received from the antioxidants (Kedare and Singh, 2011). The values for DPPH assay varied in the range 179.4 - 699.5 mg TE/g of dry extract for fresh flowers and from 103.5 - 447.3 mg TE/g of dry extract for spent flowers. No results were found in the literature for the DPPH assay with flowers of *Acacia* spp. expressed in Trolox equivalents per mass of flower or per mass of dry extract for a correct comparison of results. However, these were in a higher range than the observed with another known sources of antioxidants, such as pine bark extract (approximately between 50 and 350 mg TE/g of extract)

(Venkatesan et al., 2019) or an extract of a commercial polyherbal tea used in treatment of diabetes (Diabetea), which have a reported value of 75 mg TE/g of extract (Paddy et al., 2015). DPPH results expressed per mass of flower ranged from 7.8 to 44.4 mg TE/g and from 4.5 to 26.6 mg TE/g, for fresh and spent flowers, respectively. These values are much higher than the reported for yellow, green, and black teas (11.930 – 26.521 $\mu\text{mol TE}/100\text{g}$, which corresponds to 0.030 – 0.066 mg TE/g of tea leaves (wb)) (Kopjar et al., 2015). In the case of the 70% ACE extracts of all fresh and spent flowers from *A. dealbata*, the values were in the range of those reported for raspberry and blackberry fruits (64.14 – 177.11 $\mu\text{mol TE}/\text{g}$, corresponding to 16.1 – 44.3 mg TE/g of fruit (wb)) (Sariburun et al., 2010). The 70% ACE extracts demonstrated significantly higher DPPH scavenging activities, for both fresh and spent flowers. Significant differences were found between samples, with *A. dealbata* from Coruche showing the highest scavenging activity against DPPH free radical, amongst the fresh flowers, regardless of the solvent used. For spent flowers, the highest antioxidant activities were shown by 70% ACE extracts of Alcobaça and Coruche.

The FRAP assay is another common test used in the evaluation of the antioxidant activity of natural extracts, by accessing their total reducing power. It is a colorimetric method that evaluates the reduction of Fe^{3+} -the complex (Fe^{3+} -TPTZ) to a ferrous form (Fe^{2+} -TPTZ), by donation of electrons from the antioxidants, resulting in stopping the free radical production chain (Afsar et al., 2018; Sadiq et al., 2017; Santos-Sánchez et al., 2019). FRAP values ranged from 1.302 to 6.470 mmol Fe^{2+}/g extract for fresh flowers, and from 1.042 to 4.581 mmol Fe^{2+}/g extract for spent flowers. These values are higher than those found in leaves, pods, and seeds extracts of *A. leucophloea* (0.178 – 0.254 mmol Fe^{2+}/g extract) (Zia-UI-Haq et al., 2013), and in a similar range of the observed also in an *A. dealbata* flower extract (approximately 120 – 175 mg Fe^{2+}/g extract, equivalent to 2.148 – 3.133 mmol Fe^{2+}/g extract) (López-Hortas et al., 2020). Values of 152 mg Fe^{2+}/g extract (equivalent to 2.721 mmol Fe^{2+}/g extract) and 575 mg Fe^{2+}/g (equivalent to 10.296 mmol Fe^{2+}/g extract) were found for *Pinus radiata* bark extract and standardized *Pinus pinaster* bark extract Pycnogenol®, respectively (Astuya et al., 2016). For all samples of both fresh and spent flowers, significantly higher ferric reducing antioxidant power was found for 70% ACE extracts. Significant differences were also observed between samples. For the best extraction solvent, the highest reducing power was found in extracts from *A. dealbata* flowers from Coruche, and amongst spent flowers the best results were obtained with *A. dealbata* from Coruche and Alcobaça. *A. saligna* extracts also showed antioxidant activity by FRAP and DPPH assays, but to a lower extent than in any *A. dealbata* extract.

The extraction solvent significantly affected the antioxidant activity given by both methods applied, with the best results being obtained in 70% ACE extracts, regardless of the origin of samples or species. Higher antioxidant activities of extracts obtained with aqueous acetone solutions were also observed with stem bark extracts of *Butea monosperma* (Lam.) Kuntze (Salar and Seasotiya, 2011), bark extracts of *A. leucophloea*, *A. ferruginea*, *A. dealbata* and *A. pennata* (Sowndhararajan et al., 2013), and flower extracts of safflower (*Carthamus tinctorius* L.) (Salem et al., 2011).

3.3.4 Effect of increasing the number of extraction cycles on extraction yield

To further improve the extraction of antioxidants from fresh and spent flowers, a second and a third extraction step were performed on the same raw material, and its impact on extraction yield, composition, and antioxidant activities of extracts was assessed.

Figure 3.3 shows the effect of increasing the number of extraction cycles on the extract yield and concentration, for fresh and spent flowers of *A. dealbata* from Alcobaça and for *A. saligna*.

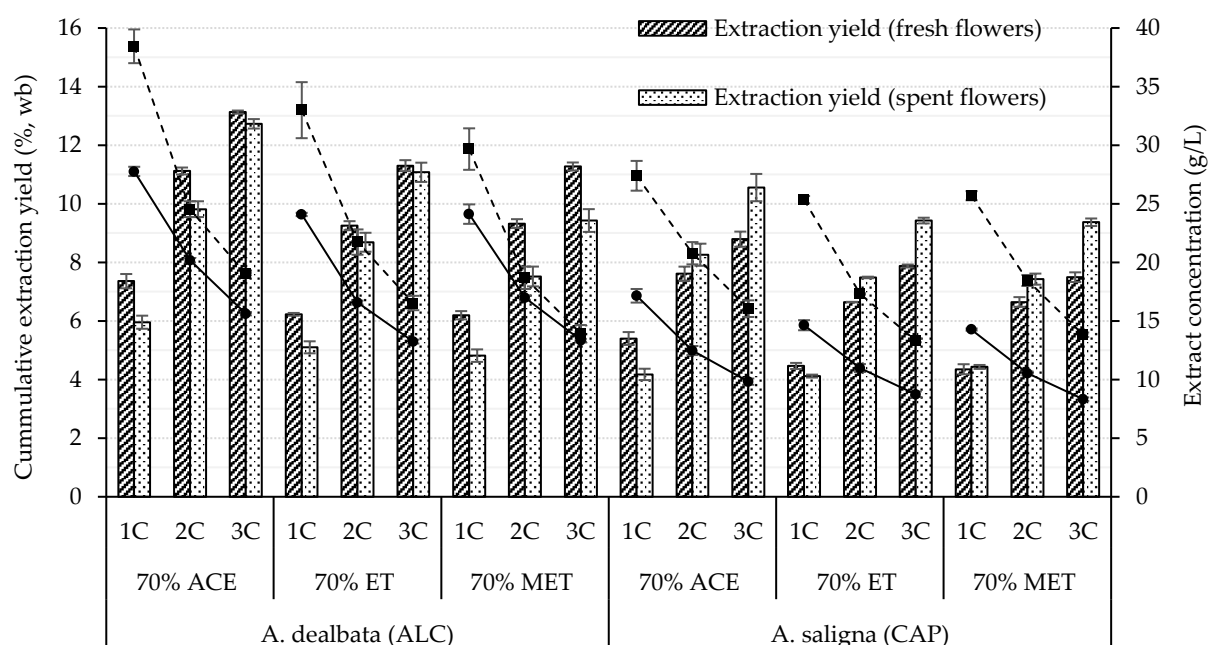


Figure 3.3 — Impact of number of extraction cycles on extraction yield and extract concentration, for 70% acetone, 70% ethanol and 70% methanol extracts of *A. dealbata* from Alcobaça and *A. saligna*. Bars represent the means and the error bars the standard deviation; 1C = one extraction cycle, 2C = accumulation of two extraction cycles, 3C = accumulation of three extraction cycles.

A notorious improvement in the extraction yield was observed as the number of extraction cycles increased. Yield increments of 41 – 85% and 49 – 98% were found with a second extraction cycle on fresh and spent flowers, respectively. When considering three extraction cycles, increments of 63 – 127% and 80 – 153% were found in fresh and spent flowers, respectively. Significantly higher extraction yields continued to be observed with 70% ACE compared with the other used extraction media, for each number of extraction cycles. For *A. dealbata* from Alcobaça (see Figure 3.3) and from Caparica (not shown; yields in the range 5.1 – 11.3% and 4.3 – 10.5% for fresh and spent flowers, respectively), extraction yields from fresh flowers were higher than those obtained from spent flowers, regardless the number of extraction cycles and extraction media used, while for *A. dealbata* from Coruche, this was verified only when using aqueous acetone as extraction solvent (not shown; yields in the range 4.9 – 14.4% for fresh flowers and 5.5 – 11.2% for spent flowers). For *A. saligna*, the extraction yield from spent flowers was higher than the obtained from fresh flowers, when more than one extraction cycle was performed (see Figure 3.3).

For large-scale extraction of these resources, economical considerations must be considered about the extraction process, such as the commercial value of the extract, solvent costs, extraction period, and energy consumption with solvent evaporation when the extract is commercialized as solid powder or in a more concentrated liquid or semi-solid form. As seen in Figure 3.3, the increment in the extract yield was accomplished by a significant dilution of the extract solution, which will increase processing time and energy costs with solvent evaporation if concentration or drying is needed. Moreover, expenses with solvent will also increase with increasing the number of extraction cycles and an efficient system of solvent recovery will be needed. Amongst other factors, these considerations will have to be considered and balanced with revenues from the increased quantity of the produced extract and format. Besides increments on extraction yields, it is also very important to evaluate the quality of the extract when collecting extract from additional extraction steps on the same flowers, which is shown in the following section.

3.3.5 Effect of increasing the number of extraction cycles on extract composition and antioxidant activity

The impact of increasing the number of extraction cycles on the extract composition and antioxidant activity is shown for 70% ACE extracts, which originated the best results in terms of phenolics content and antioxidant activities (Figure 3.4).

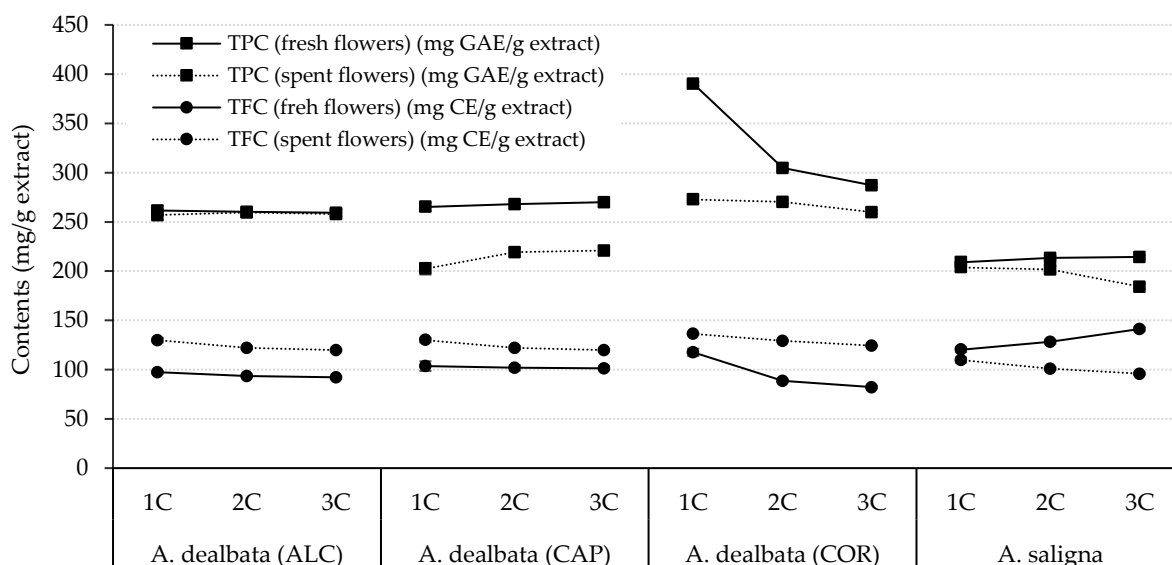


Figure 3.4 — Effect of increasing the number of extraction cycles on TPC and TFC, relative to extracts from all fresh and spent flowers obtained with 70% aqueous acetone.

Globally, TPC and TFC for fresh flowers varied in the range 209.0 – 390.2 mg GAE/g extract and 82.3 – 141.2 mg CatE/g extract, respectively. Figure 3.4 shows that increasing the number of extraction cycles did not substantially affect TPC and TFC of the extracts in the case of fresh flowers of *A. dealbata* from Alcobaça and Coruche, and of *A. saligna*, for which even a slight improvement was observed. The exception was the case of extracts from fresh flowers of *A. dealbata* from Coruche, that showed a marked decrease on TPC and TFC as more than one extraction step is performed. Besides the decay on TPC, this sample continued to be the one with the highest phenolic content after the three extraction cycles. For spent flowers, TPC and TFC varied between 184.3 and 272.7 mg GAE/g extract and between 95.9 and 136.3 mg CatE/g extract, respectively. The effect of increasing the number of extraction cycles was more identical between samples, being observed a global trend to a very slight decrease when collecting more than one extraction cycle. Exception made for TPC of *A. dealbata* from Caparica, that presented a slight improvement (from 202.5 to 220.9 mg GAE/g extract) with increasing the number of extraction cycles.

In what concerns the impact on antioxidant activity (Figure 3.5), a variation between 323.0 and 699.5 mg TE/g extract and between 217.9 to 447.3 mg TE/g extract was observed for the DPPH assay of fresh and spent flowers, respectively. For the FRAP assay, variations in the range 2962.9 – 6469.5 mmol Fe²⁺/g extract and 1949.2 – 4633.7 mmol Fe²⁺/g extract were observed

for fresh and spent flowers, respectively. The impact of collecting more than one extraction cycle in the DPPH and FRAP results followed a trend similar to that observed for TPC and TFC. Again, a negative impact was observed for the extracts of fresh flowers from *A. dealbata* from Coruche. For all other samples, either from fresh or spent flowers, it is worth mentioning even a slight increase in the FRAP values with increasing the number of extraction cycles.

Overall, with exception of extracts of fresh flowers of *A. dealbata* from Coruche, increasing the number of extraction steps with new solvent on the same raw material did not significantly affect neither the composition in terms of phenolic and flavonoid contents nor the antioxidant activity by DPPH and FRAP assays. Knowing the improvement of extraction yield and maintenance of the quality of an extract when increasing the number of extraction cycles is important to decide an approach to eventual large-scale production.

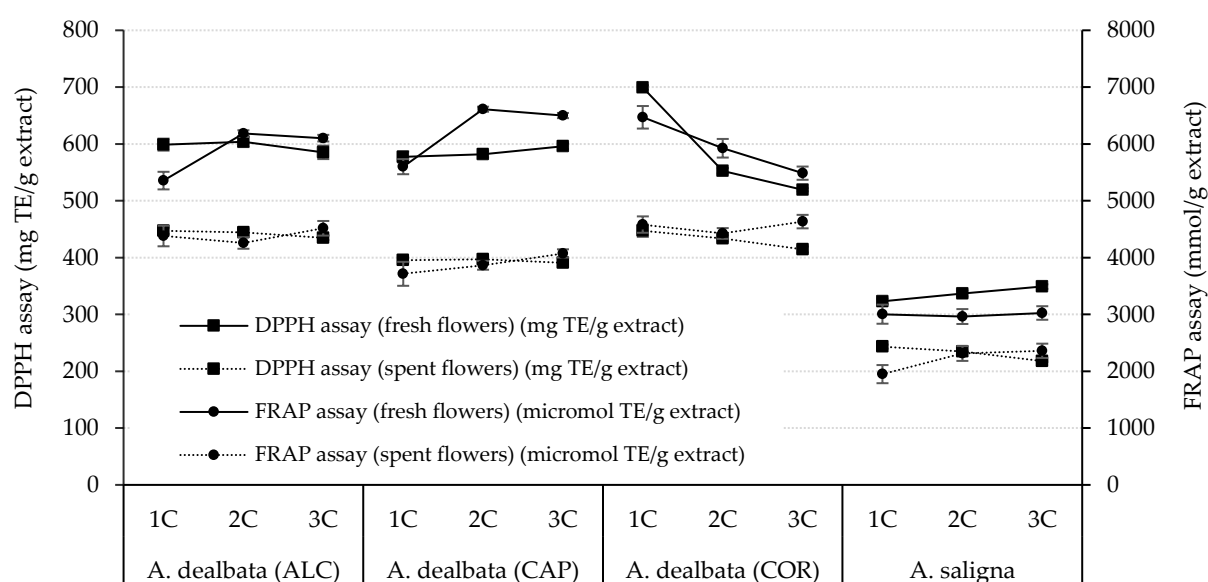


Figure 3.5 — Effect of increasing the number of extraction cycles on the antioxidant activity given by DPPH and FRAP assays, relative to extracts from all fresh and spent flowers obtained with 70% aqueous acetone.

3.3.6 Correlations between total phenolic and total flavonoid contents and antioxidant activities

The correlation analysis between TPC, TFC, and antioxidant activities by DPPH and FRAP assays for the extracts of fresh and spent flowers are given in Table 3.3.

Besides good correlations between variables, correlations must be statistically significant. All the correlations found in this work were statistically significant at $p < 0.01$, except for the

correlation between TPC and antioxidant activity by the DPPH assay for fresh flowers, which was significant at $p < 0.05$. The Pearson's correlation coefficients found indicate very strong correlations between total phenolics content and antioxidant activity given by DPPH and FRAP assays (Akoglu, 2018), for both fresh and spent flower extracts. In the case of flavonoids, for spent flowers, a strong correlation was found between this class of compounds and the antioxidant activity given by both assays, while for fresh flowers a moderate correlation was observed (Pearson's r of 0.621 and 0.718 for DPPH and FRAP, respectively). Yadav et al. (Yadav et al., 2018) also found strong correlations between TPC, TFC and DPPH values ($r = 0.771$ and $r = 0.815$), for extracts of *A. nilotica* leaves. However, (Abdel-Farid et al., 2014) found a non-significant correlation between TPC of extracts of different components (leaves, flowers, and pods) of three *Acacia* species (*A. nilotica*, *A. seyal*, and *A. laeta*) and DPPH results, having found a significant correlation between TFC and DPPH values instead. Significant and strong correlations between TPC and the DPPH and FRAP results were found in *Pinus densiflora* bark extract (Venkatesan et al., 2019) and in medicinal herbs and spices (Ulewicz-Magulska and Wesolowski, 2019).

Table 3.3 — Correlation analysis between total phenolics content, total flavonoid content and antioxidant activities, for fresh and spent flowers; significance of the correlations assessed at $p < 0.05$ and at $p < 0.01$ (two-tailed).

	TPC VS DPPH	TPC VS FRAP	TFC VS DPPH	TFC VS FRAP
Fresh flowers				
Pearson's r	0.883**	0.887**	0.621*	0.718**
p value	0.000	0.000	0.031	0.009
Spent flowers				
Pearson's r	0.891**	0.857**	0.845**	0.880**
p value	0.000	0.000	0.001	0.000

** correlation is significant at $p < 0.01$ (two-tailed); * correlation is significant at $p < 0.05$ (two-tailed)

These findings substantiate that phenolic compounds, including the flavonoids class, have an important role in the observed DPPH free radical scavenging activity and in the reducing power given by the FRAP assay. Moreover, the abundance of these compounds in the extracts obtained in this work suggests that collected fresh flowers, and even the spent flowers, have a strong potential to be explored for obtaining antioxidants for diverse applications, such as in nutraceutical or cosmetic industries.

3.3.7 Antimicrobial activity

Assessing the antimicrobial activity of plant extracts may contribute to finding alternatives to synthetic antibiotics, which is of crucial importance due to the increasing pathogenic resistance to conventional antimicrobial agents (Ghannoum M. A. and Rice L. B., 1999). A representative group of extract samples was selected to assess antimicrobial activity against bacteria and fungi, with results showing inhibition against the gram-positive bacteria (*S. aureus*, MRSA, *S. epidermis*, *E. faecalis*, *B. cereus*, *S. mutans* and *S. mitis*). *S. aureus* is one of the main pathogens of community- and hospital-acquired infections causing a wide variety of infectious diseases, including skin and soft tissue infections, endocarditis, osteomyelitis, and pleuropulmonary infections (Tong et al., 2015; Xiao et al., 2022), while the methicillin-resistant *S. aureus* is an emerging pathogen leading to more severe outcomes, being associated to higher mortality than the methicillin-sensitive *S. aureus* (Xiao et al., 2022). *S. epidermidis* is an opportunistic pathogen responsible for many healthcare-associated infections, and skin infections such as acne (Kumar et al., 2007; Lopes et al., 2021). *E. faecalis* is frequent in food and livestock, being associated to oral diseases and, in the last decades, have gained significance as leading opportunistic pathogen causing nosocomial infections (Anderson et al., 2016). *B. cereus* is an environmental contaminant present in soil, foods, insect larvae, and human skin, that induces local and systemic infections, being now considered a serious cause of nosocomial infections (Glasset et al., 2018). *S. mutans* and *S. mitis* are associated with caries and oral infections that can lead to tooth loss. Moreover, these two bacteria can cause endocarditis and other infections, particularly in patients with abnormal or damaged heart valves.

The inhibition zones against Gram-positive bacteria are presented in Table 3.4 and Table 3.5. Overall, extracts were more effective against *B. cereus* (inhibition zones between 8.0 and 13.0 mm) while *S. epidermis* (inhibition zones in the range 7.5 – 9.8 mm) and *S. mutans* (inhibition zones in the range 7.0 – 8.0 mm) were the most resistant bacteria, with acetone extracts showing the best performance. At 14 mg/mL, all 70% ACE extracts were effective against the tested Gram-positive bacteria, exception made for *A. saligna* extracts against *S. epidermis*, *E. faecalis*, *S. mutans* and *S. mitis*, and of spent flowers extracts from *A. dealbata* from Coruche against *S. epidermis*. At 7 mg/mL, no activity was observed against *S. epidermis* and *S. mutans*, and at 1 mg/mL only *B. cereus* revealed susceptibility (against acetone extracts of fresh flowers of *A. dealbata* from Coruche and Alcobaça). All tested samples revealed ineffective against the Gram-negative bacteria tested (*P. aeruginosa* and *E. coli*), as well as against *C. albicans*.

A. dealbata extracts were slightly more effective than *A. saligna* extracts. No significant differences were observed between fresh flowers and spent flowers extracts in the case of *A. dealbata*, however, slightly better results were found for fresh flowers in the case of *A. saligna*. Among plant locations, only marginal differences were found against *S. aureus*, MRSA and *S. mutans*, with 70% ACE extracts of flowers from Caparica and Alcobaça giving slightly better results than the corresponding extracts of flowers from Coruche.

Table 3.4 — Inhibition zones (mm) of selected extracts of *A. dealbata* and *A. saligna* against *S. aureus*, MRSA, *S. epidermis*, *E. faecalis*, and *Bacillus cereus*.

Samples	<i>S. aureus</i>			MRSA			<i>S. epidermis</i>			<i>E. faecalis</i>			<i>B. cereus</i>		
	1 mg/mL (50 mg)	7 mg/mL (350 mg)	14 mg/mL (700 mg)	1 mg/mL (50 mg)	7 mg/mL (350 mg)	14 mg/mL (700 mg)	1 mg/mL (50 mg)	7 mg/mL (350 mg)	14 mg/mL (700 mg)	1 mg/mL (50 mg)	7 mg/mL (350 mg)	14 mg/mL (700 mg)	1 mg/mL (50 mg)	7 mg/mL (350 mg)	14 mg/mL (700 mg)
Fresh flowers															
<i>A. dealbata</i> (COR)															
70% ACE	–	8.0 ± 0.0	11.5 ± 0.6	–	–	10.3 ± 0.5	–	–	9.8 ± 0.5	–	–	10.0 ± 0.0	8.0 ± 0.0	10.5 ± 0.6	12.3 ± 0.5
70% ET	–	–	10.3 ± 0.5	–	–	8.8 ± 0.5	–	–	–	–	–	8.0 ± 0.0	–	–	10.0 ± 0.0
70% MET	–	8.5 ± 0.6	10.3 ± 0.5	–	–	9.8 ± 0.5	–	–	7.5 ± 0.6	–	–	8.0 ± 0.0	–	10.0 ± 0.0	12.0 ± 0.0
<i>A. dealbata</i> (CAP)															
70% ACE	–	10.0 ± 0.0	12.8 ± 0.5	–	9.0 ± 0.0	11.8 ± 0.5	–	–	9.5 ± 0.6	–	8.3 ± 0.5	9.8 ± 0.5	–	10.5 ± 0.6	12.5 ± 0.6
<i>A. dealbata</i> (ALC)															
70% ACE	–	9.8 ± 0.5	12.0 ± 0.0	–	9.3 ± 0.5	12.0 ± 0.0	–	–	9.8 ± 0.5	–	8.3 ± 0.5	10.0 ± 0.0	8.0 ± 0.0	11.0 ± 0.0	13.0 ± 0.0
<i>A. saligna</i>															
70% ACE	–	8.0 ± 0.0	10.5 ± 0.6	–	8.0 ± 0.0	10.5 ± 0.6	–	–	–	–	–	–	–	8.8 ± 0.5	11.3 ± 0.5
70% ET	–	–	–	–	–	–	–	–	–	–	–	–	–	–	8.0 ± 0.0
70% MET	–	–	–	–	–	–	–	–	–	–	–	–	–	–	8.0 ± 0.0
Spent flowers															
<i>A. dealbata</i> (COR)															
70% ACE	–	9.5 ± 0.6	11.3 ± 0.5	–	8.8 ± 0.5	10.5 ± 0.6	–	–	–	–	–	9.8 ± 0.5	–	10.0 ± 0.0	12.0 ± 0.0
<i>A. saligna</i>															
70% ACE	–	–	8.0 ± 0.0	–	–	9.0 ± 0.8	–	–	–	–	–	–	–	7.0 ± 0.0	9.5 ± 0.6
Positive control															
Vancomycin (5.0 mg)		14.9 ± 0.7			13.9 ± 0.7			15.7 ± 1.0			12.5 ± 0.5			16.4 ± 1.1	

Table 3.5 — Inhibition zones (mm) of selected extracts of *A. dealbata* and *A. saligna* against *S. mutans*, *S. mitis* and *Bacillus cereus*.

Samples	<i>S. mutans</i>			<i>S. mitis</i>		
	1 mg/mL (50 mg)	7 mg/mL (350 mg)	14 mg/mL (700 mg)	1 mg/mL (50 mg)	7 mg/mL (350 mg)	14 mg/mL (700 mg)
Fresh flowers						
<i>A. dealbata</i> (COR)						
70% ACE	–	–	7.0 ± 0.0	–	8.0 ± 0.0	10.0 ± 0.0
70% ET	–	–	–	–	–	–
70% MET	–	–	–	–	–	9.0 ± 0.0
<i>A. dealbata</i> (CAP)						
70% ACE	–	–	8.0 ± 0.0	–	7.0 ± 0.0	10.0 ± 0.0
<i>A. dealbata</i> (ALC)						
70% ACE	–	–	8.0 ± 0.0	–	–	10.0 ± 0.0
<i>A. saligna</i>						
70% ACE	–	–	–	–	–	–
70% ET	–	–	–	–	–	–
70% MET	–	–	–	–	–	–
Spent flowers						
<i>A. dealbata</i> (COR)						
70% ACE	–	–	7.0 ± 0.0	–	8.0 ± 0.0	9.0 ± 0.0
<i>A. saligna</i>						
70% ACE	–	–	–	–	–	–
Positive control						
Vancomycin (50.0 mg)		19.3 ± 0.6			25.7 ± 0.6	

Studies including antimicrobial activity assessment with *A. dealbata* or *A. saligna* flower extracts are scarce in the literature. Only a recent study was found, showing antimicrobial capacity of 50% ethanol extracts of *A. dealbata* flowers against two strains of each *S. aureus* and *E. coli* bacteria, and *C. albicans* yeast (Paula et al., 2022). This activity against *E. coli* and *C. albicans*, which was not found in the present work, may be due to the different plant locations and/or extraction conditions and plant pretreatments, that may possibly affect the extract composition. A 60 mesh fraction of lyophilized and milled flowers was used in the extraction process by Paula et al., at a 1:10 flower-to-solvent ratio and under agitation for 15 hours (Paula et al., 2022), instead of the simpler approach assessed in this work, using maceration at a 1:4 flower to solvent ratio with roughly cut flowers. Regarding *A. saligna* flowers, Al-Huqail et al. studied the antifungal properties of a hot water extract against fungal wood pathogens (*Fusarium culmorum*, *Rhizoctonia solani*, and *Penicillium chrysogenum*) and phytopathogenic bacteria (*Agrobacterium tumefaciens*, *Enterobacter cloacae*, *Erwinia amylovora*, and *Pectobacterium carotovorum* subsp. *carotovorum*), having found promising results in the development of a natural wood preservative (Al-Huqail et al., 2019). No studies involving assessing antimicrobial activity against MRSA, *S. epidermis*, *E. faecalis*, *B. cereus*, *S. mutans* and *S. mitis* were found in the literature using flower extracts of *A. dealbata* and *A. saligna*. Inactivity against *E. coli* (Cock, 2012; Da Silva et al., 2022; Makeen et al., 2020), *P. aeruginosa* (Makeen et al., 2020), and *C. albicans* (Cock, 2012; Makeen et al., 2020) was also observed with extracts from flowers of other *Acacia* species, such as *A. ehrenbergiana*, *A. complanta*, and *A. mearnsii*.

The inhibition zones against *S. aureus* were lower than the 22 mm observed with ethanolic extracts of *A. ehrenbergiana* (Makeen et al., 2020), or of *A. salicina*, *A. laeta*, *A. hamulosa*, and *A. tortilis* (14.0 – 21.0 mm) (Alajmi et al., 2017), but in these examples, a much superior extract concentration was used in the antimicrobial assays, a crude extract in the first case and a 100 mg/mL extract concentration in the last case. The inhibition of *S. aureus* were equivalent to the ones found using more concentrated extracts of some medicinal flowers, such as *Pedaliium murex*, *Senna auriculata*, *Millingtonia hortensis*, and *Catharanthus roseus* (3.0 – 14.0 mm, at 25 – 100 mg/mL) (Abirami et al., 2021), *Tagetes patula* (12.0 mm, at 300 mg/mL) (Latifian et al., 2021), *Camellia japonica* (10.8 mm, at 20 mg/mL) (A. G. Pereira et al., 2022), or *Citrus aurantium* (7.8 – 13.8 mm, at 0 – 20 mg/mL) (Hashemi et al., 2016). Concerning *S. epidermis*, the inhibition zones were higher than the one found in the medicinal flower *Jasminum officinale* (7.0 mm, at 100 mg/mL) (Kumar et al., 2007), but lower than the one observed with *Camellia japonica* flowers (14.0 mm at 20 mg/mL) (A. G. Pereira et al., 2022). The inhibition zones against *E. faecalis* were lower than the one obtained with the crude extract of *A. ehrenbergiana* (18

mm) (Makeen et al., 2020), probably due to the reasons pointed out before, but were in the same magnitude as the ones found with more concentrated extracts of the medicinal flowers *Pedaliium murex*, *Senna auriculata*, *Millingtonia hortensis*, and *Catharanthus roseus* (2.0 – 11.0 mm, at 25 – 100 mg/mL) (Abirami et al., 2021), and *Tagetes patula* (11.5 mm, at 300 mg/mL) (Latifian et al., 2021). Against *B. cereus*, the inhibition zones were much higher than the one found with *Camellia japonica* flowers (5.0 mm at 20 mg/mL) (A. G. Pereira et al., 2022), and in a similar range to the observed with *Citrus aurantium* flowers (7.8 – 13.2 mm, at 0 – 20 mg/mL) (Hashemi et al., 2016).

3.3.8 Identification of compounds by HPLC-DAD and LC-MS

Extract samples were analyzed by HPLC-DAD and HPLC-DAD-MS/MS. HPLC-DAD chromatograms can be found in Appendix A2 (Figure A1 to A10). Figure 3.6 presents illustrative examples for acetone extracts of *A. dealbata* and *A. saligna*. The tentative identification of compounds was then performed by HPLC-DAD-ESI-MS/MS.

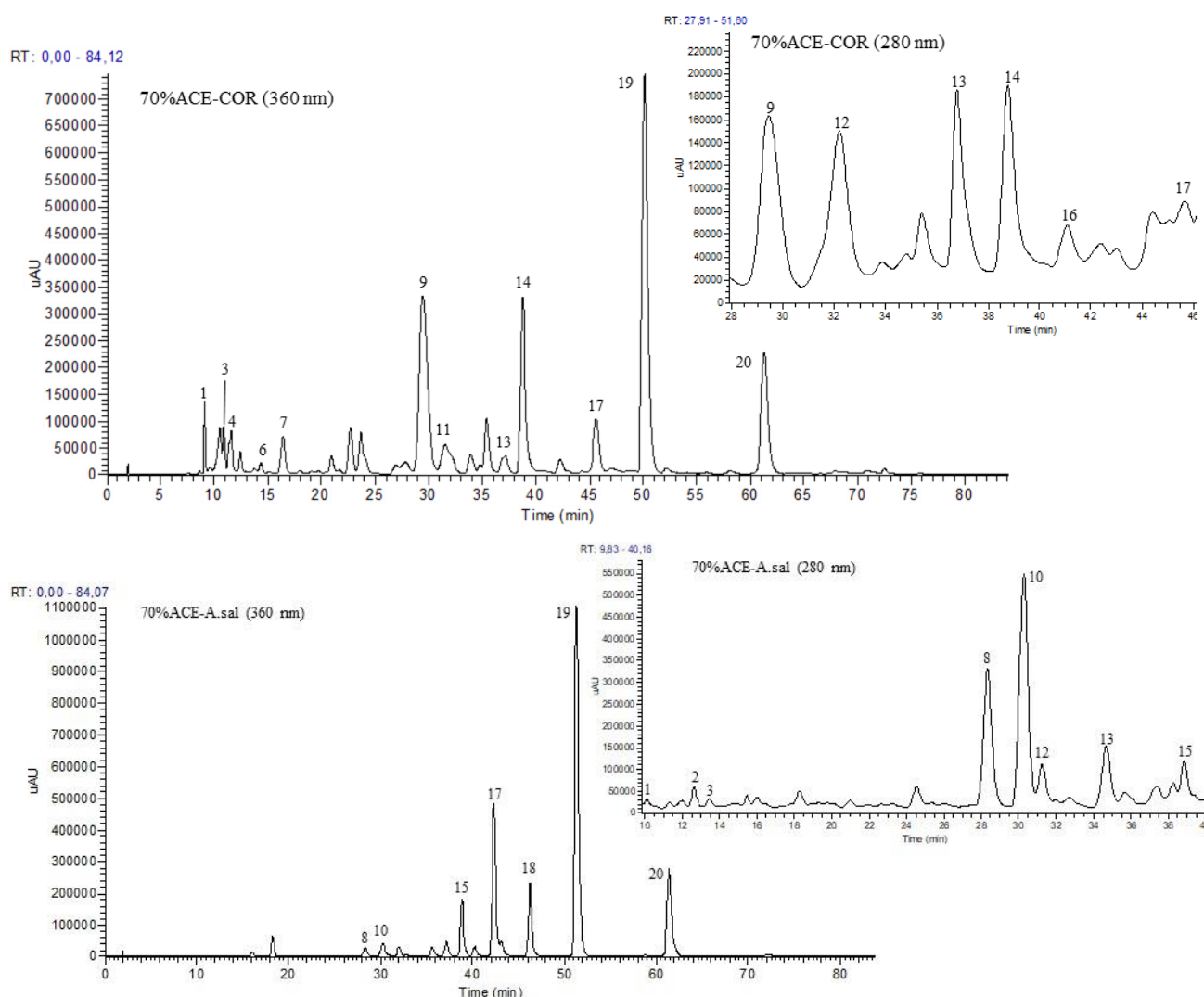


Figure 3.6 — Chromatographic profile at 360 nm for aqueous acetone extracts of *A. dealbata* from Coruche (top) and *A. saligna* (bottom); the inserts are parts of the chromatographic profile at 280 nm, to illustrate some peaks not easily visible at 360 nm.

Table 3.6 shows the list of 20 compounds tentatively identified by HPLC-DAD-MS/MS, along with their retention time (Rt), UV absorption maxima, monoisotopic mass (MM), precursor ion (in positive $[M+H]^+$ or negative $[M-H]^-$ ion mode), the MS/MS product ions and the bibliographic references to support the identification. The compounds were numbered by their elution order since most of them were not found in all extracts. The correspondence between HPLC-DAD and HPLC-DAD- MS/MS peaks was made according to UV spectra and elution order.

Table 3.6 — Compounds tentatively identified by LC-DAD-ESI-MS; MS/MS fragments presented in decreasing order of intensity.

No.	Rt (min)	λ_{max} (nm)	[M-H] ⁻ / [M+H] ⁺ (m/z)	MW	MS/MS fragments (m/z)	Compound	References
1	45.99	245, 298sh, 329	341 [M-H] ⁻	342	161	Cuminoid C	(Serag et al., 2019)
2	59.37	240, 280	291 [M+H] ⁺	290	139, 123, 147, 161, 119, 111	Catechin	(Li et al., 2015; Mandal and Chakraborty, 2013)
3	60.89	242, 314	325 [M-H] ⁻	326	145, 163, 119	p-coumaric acid O-hexoside I	(Bystrom et al., 2008; Radenkovs et al., 2020)
4	63.82	242, 314	325 [M-H] ⁻	326	145, 163, 119	p-coumaric acid O-hexoside II	(Bystrom et al., 2008; Radenkovs et al., 2020)
5	73.11	255, 267sh, 354	773 [M+H] ⁺	772	465, 303, 627	quercetin O-rhamnosylhexo- side-O-hexoside	(Lin et al., 2008)
6	77.88	238sh, 262	337 [M+H] ⁺	336	185, 175, 119, 127, 85	Unknown	
7	89.53	254, 267sh, 300sh, 355	627 [M+H] ⁺	626	319, 481	Myricetin O-rutinoside I	(Kelanne et al., 2020; Lin et al., 2008)
8	100.57	242, 291, 340sh	433 [M-H] ⁻	434	313, 343, 271	Naringenin C-hexoside I	(Lu et al., 2019; Righi et al., 2011; Vallverdú- Queralt et al., 2011)
9	101.47	258, 300sh, 355	627 [M+H] ⁺	626	319, 481	Myricetin O-rutinoside II	(Kelanne et al., 2020; Lin et al., 2008)
10	102.47	242, 291, 340sh	433 [M-H] ⁻	434	313, 343, 271	Naringenin C-hexoside II	(Lu et al., 2019; Righi et al., 2011; Vallverdú- Queralt et al., 2011)
11	103.92	255, 267sh, 295sh, 355	481 [M+H] ⁺	480	319	Myricetin O-hexoside	(Kelanne et al., 2020; Lin et al., 2008; Lin and Harnly, 2007)
12	105.66	241, 282	433 [M-H] ⁻	434	271, 151	Naringenin O-hexoside (I)	(Goufo et al., 2020; Kammerer et al., 2005; March et al., 2006; Pereira et al., 2013; Sánchez-Rabaneda et al., 2003)

Table 3.6 (cont.)— Compounds tentatively identified by LC-DAD-ESI-MS; MS/MS fragments presented in decreasing order of intensity.

No.	Rt (min)	λ_{max} (nm)	[M-H] [−] / [M+H] ⁺ (m/z)	MW	MS/MS fragments (m/z)	Compound	References
13	111.59	241, 282	433 [M-H] [−]	434	271, 151	Naringenin O-hexoside (II)	(Goufo et al., 2020; Kammerer et al., 2005; March et al., 2006; Pereira et al., 2013; Sánchez-Rabameda et al., 2003)
14	117.35	256, 267sh, 290sh, 354	611 [M+H] ⁺	610	303	Rutin	(Hunyadi et al., 2012; Jang et al., 2018; Peres et al., 2013; Pollini et al., 2019)
15	121.91	256, 267sh, 290sh, 355	465 [M+H] ⁺	464	303	Quercetin O-hexoside	(Jang et al., 2018; Li et al., 2015; Lin et al., 2008; Zhang et al., 2018)
16	123.37	241, 283	403 [M-H] [−] 405 [M+H] ⁺	404	273, 153, 147 271, 151	Naringenin O-pentoside	(Bystrom et al., 2008)
17	126.36	246, 370	435 [M+H] ⁺	434	195, 219, 165, 285, 231, 249, 243, 351, 297	Unknown	
18	134.07	256, 267sh, 350	449 [M+H] ⁺ 447 [M-H] [−]	448	303, 129, 85, 71 300, 301	Quercetin O-rhamnoside	(Diaconeasa et al., 2014; Goufo et al., 2020; Jang et al., 2018; Lee et al., 2005; Lin et al., 2008)
19	135.86	247, 368	435 [M+H] ⁺ 433 [M-H] [−]	434	273, 153, 147 271, 151, 119	Isosalipurposide (chalcononaringenin 2'-glucoside)	(Kammerer et al., 2005; Li et al., 2009; Teppabut et al., 2018)
20	137.13	246, 368	405 [M+H] ⁺ 403 [M-H] [−]	404	273, 153, 147 271, 151, 119	Naringenin chalcone O-pentoside	

sh: shoulder

A terpene (peak 1, cuminoid C), a flavanol (peak 2, catechin), a hydroxycinnamic acid glycoside (peaks 3 and 4, *p*-coumaric acid *O*-hexoside isomers I and II), six flavone glycosides (peak 5, quercetin *O*-rhamnosylhexoside-*O*-hexoside; peaks 7 and 9, myricetin *O*-rutinoside isomers I and II; peak 11, myricetin *O*-hexoside; peak 14, rutin; peak 15, quercetin *O*-hexoside, and peak 18, quercetin *O*-rhamnoside), three flavanone glycosides (peaks 8 and 10, naringenin *C*-hexoside isomers I and II; peaks 12 and 13, naringenin *O*-hexoside isomers I and II; and peak 16, naringenin *O*-pentoside), and two chalcones (peak 19, isosalipurposide and peak 20, naringenin chalcone *O*-pentoside) were tentatively identified based on UV-spectra and MS/MS fragments, accordingly to the previously described in the literature. Peak 20, with molecular ion $[M+H]^+$ at m/z 405 in positive mode, and $[M-H]^-$ at m/z 403 in negative mode, was tentatively attributed to naringenin chalcone *O*-pentoside. The molecular ion gave origin to the main fragment ions at m/z 273 and m/z 271, respectively, corresponding to the loss of 132 Da, attributed to *O*-pentose unit. The following fragments in positive mode at m/z 153 and m/z 147 (Iijima et al., 2008), and in negative mode at m/z 151 and m/z 119 (Anton et al., 2017) were previously described for the naringenin chalcone aglycone. Moreover, the UV spectra was found identical to the observed for naringenin chalcone (Gurdon et al., 2019; Khan et al., 2011), which is referred to be identical to UV spectra of the respective glycosides (Gurdon et al., 2019).

Relative peak areas among samples can be found in Appendix A3 (Table A5). Table 3.7 shows the statistically significant correlations between peak areas and the observed antioxidant activities. Strong positive correlations were found for compounds tentatively identified as cuminoid C (peak 1), *p*-coumaric acid *O*-hexoside isomers (peaks 3 and 4), myricetin *O*-rutinoside isomers (peaks 7 and 9), myricetin *O*-hexoside (peak 11), rutin (peak 14), naringenin *O*-pentoside (peak 16), and for an unknown compound (peak 6). In turn, strong negative correlations were found for naringenin *C*-hexoside isomers (peaks 8 and 10), naringenin *O*-hexoside isomer (peak 13), quercetin *O*-hexoside (peak 15), quercetin *O*-rhamnoside (peak 18), and isosalipurposide (peak 19). These negative correlations do not necessarily mean that these compounds do not play a role in the observed antioxidant properties, because complex synergistic effects may be involved in the biological activities of plant extracts (Hubner et al., 2019; Qin et al., 2018; Xu et al., 2014).

Table 3.7 — Correlation analysis between antioxidant activities and relative areas of peaks; significance of the correlations assessed at $p < 0.05$ and at $p < 0.01$ (two-tailed).

	DPPH		FRAP	
	Pearson's r	p value	Pearson's r	p value
Peak 1	0.888**	0.001	0.881**	0.001
Peak 3	0.860**	0.001	0.735*	0.015
Peak 4	0.761*	0.011	0.673*	0.033
Peak 6	0.738*	0.015	0.667*	0.035
Peak 7	0.823**	0.003	0.732*	0.016
Peak 8	– 0.897**	0.000	– 0.886**	0.001
Peak 9	0.915**	0.000	0.820**	0.004
Peak 10	– 0.912**	0.000	– 0.899**	0.000
Peak 11	0.709*	0.022	0.684*	0.029
Peak 13	– 0.686*	0.029	– 0.656*	0.040
Peak 14	0.720*	0.019	0.688*	0.028
Peak 15	– 0.792**	0.006	– 0.759**	0.011
Peak 16	0.729*	0.017	---	---
Peak 18	– 0.920**	0.000	– 0.901**	0.000
Peak 19	– 0.785**	0.007	– 0.743*	0.014

** correlation is significant at $p < 0.01$ (two-tailed); * correlation is significant at $p < 0.05$ (two-tailed)

p-coumaric acid and quercetin were previously reported in *A. dealbata* and *A. saligna* flowers (Al-Huqail et al., 2019; Luís et al., 2012), and myricetin (Al-Huqail et al., 2019; Ghribia et al., 2014), naringenin (Al-Huqail et al., 2019; Ghribia et al., 2014), and isolalipurposide (Ghouila et al., 2012; Ghribia et al., 2014) were previously found in *A. saligna* flowers. Antioxidant activity of *p*-coumaric acid glycosides is reported in the literature (Deng et al., 2018; Strandås et al., 2008), as well as many other important biological effects of myricetin (Hopia and Heinonen, 1999; Riehle et al., 2013; Taheri et al., 2020; Toth et al., 2014), naringenin (Nouri et al., 2019; Venkateswara et al., 2017), quercetin (Batiha et al., 2020; Enogieru et al., 2018; Hopia and Heinonen, 1999; Pivec et al., 2019; Riehle et al., 2013; Toth et al., 2014), and respective glycosides. These include antioxidant, antidiabetic, anticancer, antiviral, antimicrobial, immunomodulatory, cardiovascular, analgesic, antihypertensive, anti-inflammatory, DNA protective, hepatoprotective, antiatherogenic, antidepressant, hypolipidaemic, peroxisome proliferator-

activated receptors activator, and memory improving (Batiha et al., 2020; Taheri et al., 2020; Venkateswara et al., 2017). Protection against neurodegenerative disorders, like Alzheimer, Parkinson, and Huntington diseases was particularly described for the quercetin glycoside rutin (Enogieru et al., 2018), myricetin (Taheri et al., 2020), and naringenin (Nouri et al., 2019). These flavonoids occur in plants mainly in the glycosylated form but are converted into aglycones by enterobacteria in the intestine before they are absorbed into enterocytes by the action of β -glycosidases enzymes (Batiha et al., 2020). In turn, the chalcone isosalipurposide was described to have antioxidant and anti-acetylcholinesterase effects (Ghribia et al., 2014), as well as cytoprotective effects against oxidative damage mediated through Nrf2 activation and induction of its target gene expression in hepatocytes, suggesting its potential to be used in therapeutics to prevent or treat oxidative stress-mediated liver disease (Han et al., 2015).

This way, *A. dealbata* and *A. saligna* flowers can be an important source of a complex mixture of phenolic compounds, particularly flavonoids. So important as their respective bioactivities are the synergistic effects amongst them, which are well described in the literature, and that also play an important role in the biological activities here referred, especially in the antioxidant activity (Hubner et al., 2019; Qin et al., 2018; Xu et al., 2014).

3.4 Conclusions

The results of the present investigation revealed demonstrated the possibility of producing phenolics rich extracts with antioxidant properties from flowers of Portuguese invasive *A. dealbata* and *A. saligna*, by a simple maceration process at room temperature for 24 h. Results revealed that 70% aqueous acetone extracts of *A. dealbata*, regardless its location, and of *A. saligna*, exhibited the highest phenolic contents and better antioxidant activities, either using fresh or spent flowers. Extracts of both species obtained from fresh flowers demonstrated higher total phenolic contents and higher antioxidant activities than those obtained from spent flowers. The results showed that the fresh flowers from these species, or even the spent flowers after their use in obtaining high-value concretes for the cosmetic or perfume industries, are important sources of phenolics with significant antioxidant activities, overall showing higher values than the reported for other well-known sources of antioxidants, such as teas and red fruits. Significant and strong positive correlations were found between the total phenolic content, total flavonoid contents and antioxidant activities given by DPPH and FRAP assays. *p*-coumaric acid, myricetin, quercetin, and naringenin glycosides were identified in the extracts, for which important biological activities are well described in the literature. The results also

demonstrated that a more exhaustive extraction, by repeating the addition of fresh extraction solvent, can be performed to increase the extraction yield without significantly affecting the composition and the antioxidant activity of the extracts, exception made for fresh flowers of *A. dealbata* from Coruche.

The present data provide significant information to ascertain the potential to produce high-value antioxidant extracts from an invasive species with possible applications in nutraceutical or cosmetic industries, simultaneously contributing for the control of their propagation by the collection of flowers and prevention of seed formation and dissemination. Moreover, besides the overall lower phenolic contents and antioxidant activity observed in spent flowers, the results revealed that these still a good source of antioxidants and their use may allow the recovery of two high-value materials, such as concretes for the perfume industry and antioxidants for nutraceuticals or cosmetic industries.

ONE-POT EXTRACTION FOR SIMULTANEOUS OBTENTION OF BIOACTIVE COMPOUNDS AND ABSOLUTE OILS FROM INVASIVE *ACA-* *CIA DEALBATA* FLOWERS

Abstract: Hydroalcoholic extraction of phenolic compounds from *Acacia* flowers is usually performed after removal of terpenic and hydrocarbon components (flower concrete), using a non-polar solvent. Nevertheless, this method requires pre-drying of the flowers to enable extraction with water-immiscible solvents such as petroleum ether, what may lead to some degradation of the phenolic fraction. In this work, the direct extraction of flower concrete and phenolic compounds was studied by maceration and Soxhlet extraction, using 100% acetone (ACE) and a mixture (1:1) of petroleum ether and acetone (PE/ACE), to evaluate the feasibility of obtaining non-polar and polar extracts, directly from fresh flowers, in a single extraction step. The complex extract can be fractionated into organic and aqueous phases in which are dissolved, respectively, the flower concrete and the polar phenolics. Extraction of the concrete flower was also evaluated by maceration and Soxhlet extraction, with 100% of petroleum ether for sake of comparison.

Concrete yields were higher for extractions with the mixture PE/ACE (1.26 – 1.28 %), followed by 100% PE (0.82 – 1.01%) and 100% ACE (0.53%). The PE/ACE solvent also had the highest yields of aqueous extracts (4.85-6.57 g/100g of flowers, wb), using maceration, and reached the highest aqueous extract concentrations (242 – 365.7 g/L), by Soxhlet extraction. The aqueous extracts obtained from extractions with PE/ACE or 100% ACE were characterized for total concentrations of phenolics (TPC), flavonoids (TFC), and proanthocyanidins (TPrAC).

Higher values of these parameters were obtained in the extracts obtained by maceration than those produced by Soxhlet extraction. Using 100% ACE favoured TFC and TFC values while the extracts obtained with PE/ACE showed higher TPrAC. Antioxidant activity was measured by DPPH and FRAP assays, and the highest values were obtained for the aqueous extracts of Alcobaça flowers macerated with 100% ACE. Strong correlations between TPC, TFC, and TPrAC and the antioxidant activity parameters showing the influence of the phenolic components in the functional properties of the aqueous extracts.

The crude aqueous extracts were effective against several Gram-positive bacteria (*S. aureus*, *MRSA*, *S. epidermis*, *E. faecalis*, *B. cereus*, *S. mutans* and *S. mitis*) but ineffective against Gram-negative bacteria *P. aeruginosa* and *E. coli*. Both extracts obtained by maceration of flowers from Alcobaça inhibited the growth of the yeast *Candida albicans*.

The main phenolic components of the aqueous extracts were evaluated by HPLC-DAD and HPLC-DAD-MS/MS leading to the identification of glycosides of *p*-coumaric acid, quercetin, myricetin, rutin, naringenin as well as isosalipurposide and naringenin chalcone O-pentoside.

The results obtained in this work demonstrate the feasibility of obtaining different types of high-value extracts from *Acacia* flowers, contributing to the exploitation of this biomass fraction, whose selective collection also constitutes a strategy to control the invasive spreading of this species.

4.1 Introduction

As referred in the previous chapter an option to prevent the dissemination of *A. dealbata* is through the exploitation of its flowers since its regular collection prevents seed formation and dispersion (Lorenzo et al., 2010a). The potential to produce value-added products by extraction processes from biomass of *Acacia* spp., including *A. dealbata* flowers, is already known, as extensively reviewed by Correia et al. (Correia et al., 2020). Besides its known use for the production of absolute oil for the perfume industry, by fractionation of the flower concrete (Perriot et al., 2010), the flowers of *A. dealbata* have been studied for obtaining herbicidal agents (Lorenzo et al., 2019), yellow dyes (Imperato, 1982a, 1982b), cosmetic additives (López-Hortas et al., 2020; Soto et al., 2018), as well as for obtaining phenolic-rich extracts with important biological activities (Casas et al., 2019; Paula et al., 2022).

Casas et al. performed a sequential extraction of *A. dealbata* flowers using organic solvents (ethanol, hexane, dichloromethane, ethyl-acetate, and n-butanol) and aqueous, acid, and

microwave-assisted extraction as an alternative, to evaluate antioxidant, antibrowning, antitumorigenic, anti-inflammatory, and cytotoxicity against tumoral cells by the obtained extracts (Casas et al., 2019). The authors highlight the potential for some selected extracts to be incorporated in the preparation of bioactive hydrogels. López-Hortas et al. incorporated an extract of *A. dealbata* flowers with antioxidant properties obtained by the microwave hydrodiffusion and gravity technique in the production of sunscreen creams (López-Hortas et al., 2020). Ethanolic extracts of *A. dealbata* flowers with antioxidant properties were also incorporated in personal care products, which showed pleasant sensory attributes and were well accepted by consumers (Soto et al., 2018). Paula et al. produced hydroethanolic extracts from *A. dealbata* flowers with antioxidant properties, inhibition of acetyl-cholinesterase, lipase and α -glucosidase enzymatic activities, and antimicrobial capacity against *Escherichia coli* and *Staphylococcus aureus* bacteria and antibiotic-resistant and sensitive strains of *Candida albicans* yeast (Paula et al., 2022). These attributes confer the potential for their application in the treatment of neurodegenerative disorders, diabetes, and microbial infections.

Besides these previous studies, it is important to explore simpler, easy-to-implement, and low-energy processes to obtain functional ingredients from this species to better understand the best options for the valorization of this resource. In this context, the present work aims to investigate an innovative way to retrieve a wider range of compounds from the same sample of flowers by applying simple extraction methods, thus using this raw material more efficiently. Two flower proveniences (Caparica and Alcobaça), two extraction methods (maceration at room temperature and Soxhlet extraction), and three extraction media (petroleum ether, acetone, and the mixture (1:1) of petroleum ether and acetone) were compared in order to obtain flower concretes and phenolics-rich aqueous phases. To the best of our knowledge, the use of pure acetone and the use of a mixture of petroleum ether and acetone were tried for the first time in the production of *A. dealbata* flower concretes and absolutes, as alternatives to the conventional use of petroleum ether or hexane. The purpose of using the mixture of petroleum ether and acetone was to obtain, in a single extraction step, polar compounds and a nonpolar fraction, simultaneously with the phase separation that occurs taking advantage of the vegetation water present in the fresh flowers. This work deals with the aqueous phases formed in the extraction processes when acetone is used, since the organic phases containing the flower concrete, used to produce absolutes after fractionation of the concrete with ethanol, will be studied in a separate work. The aqueous extracts were characterized for mass yield, total phenolic content, total flavonoid content, total proanthocyanidin content, and antioxidant activity through the DPPH free radical scavenging assay and the ferric reducing antioxidant power

assay (FRAP). Antimicrobial activity against several bacteria and fungi was also evaluated for selected extracts. Tentative identification of compounds was performed through HPLC-DAD and HPLC-DAD-MS/MS..

4.2 Materials and methods

4.2.1 Plant material

Fresh flowers of *A. dealbata* were collected in February of 2020 in two different locations in the Center region of Portugal: Alcobaça (ALC) (39°36'36.5"N; 8°59'45.0"W, at 32 – 40 m of altitude), and Caparica (CAP) (38°37'22.6"N; 9°10'38.5"W, at 44 – 48 m of altitude). The samples used for extraction are a result of the mixing of about 1 kg of flowers from at least ten different trees from each location.

4.2.2 Chemicals and reagents

Acetone and petroleum ether used in the extraction processes were purchased from CarloErba Reagents (Val de Reuil, France). Folin-Ciocalteu reagent (Panreac, Barcelona, Spain), sodium carbonate (Labkem, Barcelona, Spain), and gallic acid (Merck, Darmstadt, Germany) were used to determine phenolics content. The reagents sodium nitrite (NaNO_2), sodium hydroxide (NaOH), and aluminum chloride (AlCl_3) used in the determination of flavonoids were all purchased from Merck (Darmstadt, Germany). Butanol (Panreac, Barcelona, Spain), ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) (Panreac, Barcelona, Spain), hydrochloric acid (Chem-Lab NV, Zedelgem, Belgium), and Pycnognol® (generously provided by Horphag Research Ltd, Geneva, Switzerland) were used in the acid-butanol assay. The reagent 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, St. Louis, MO, USA) was used in the DPPH assay. The reagents 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), acetate buffer 3.6, iron (III) chloride (FeCl_3), and iron (II) sulfate (FeSO_4) used in the ferric reducing antioxidant power (FRAP) assay were also purchased from Merck (Darmstadt, Germany). Ketoconazole, ofloxacin and vancomycin were purchase from Sigma-Aldrich (St. Louis, MO, USA). Brain heart infusion agar (BHIA) was purchased from Merck (Darmstadt, Germany). Mueller-Hinton agar (MHA), Sabouraud dextrose agar (SDA) and Tryptone casein-soy agar (TSA) were purchase from Biokar diagnostics (Allonne, France).

4.2.3 Extraction procedure

Twigs with fresh flowers were separated from leaves and roughly cut into smaller pieces (about 0.5 cm), without mechanical grinding to avoid disaggregation and loss of compounds, and immediately used in the extraction processes. Maceration at room temperature and Soxhlet extraction were performed using a mixture (1:1) of petroleum ether and acetone (PE/ACE), and 100% of acetone (ACE). Extraction of concrete with 100% of petroleum ether (PE), was also made for sake of comparison.

Maceration was made during 24 h, at a flower:solvent ratio of 1:3 (weight:volume), followed by a second maceration step also with fresh solvent at flower:solvent ratio of 1:2, also during 24 h. The extracts were separated by filtration and combined.

The PE extracts (monophasic) were eliminated under reduced pressure to yield a semi-solid yellow extract (flower concrete).

The PE/ACE extracts were diphasic, because dissolution of the vegetation water in the mixed solvent, caused spontaneous separation into an organic phase (upper) rich in petroleum ether, and an aqueous phase (lower) rich in acetone and containing water. The upper phase was dried under sodium sulphate and the solvent was eliminated under reduced pressure to obtain a greenish semisolid concrete. The lower aqueous phase was concentrated under reduced pressure to eliminate acetone and stored at -20 °C until analysis.

The ACE extracts (monophasic) were concentrated under reduced pressure until half of their volume and fractionated by liquid-liquid extraction with equivalent volumes of petroleum ether and water. The upper organic and lower aqueous phases were treated like described for the phases obtained with PE/ACE to yield a greenish semisolid concrete and an aqueous extract that was also stored at -20 °C until analysis.

Soxhlet extraction was performed for 12 h, and the obtained PE, PE/ACE, and ACE extracts were processed as described for the maceration extracts.

The characterization of concretes and absolutes will be performed in a separate work.

4.2.4 Extracts characterization

Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity by DPPH and FRAP assays were performed as described in section 3.2.4.

The total proanthocyanidin content (TPrAC) was determined by the butanol-HCl assay, exactly as described by Škerget et al. (Škerget et al., 2005). Briefly, 5 mL of an acidic ferrous solution (77 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 500 mL HCl/BuOH (2/3)) was added to 0.5 mL of the extract.

The tubes were covered and put in a water bath at 95 °C for 15 min. The absorbance was read at 540 nm (Biochrom Libra S4). The same procedure was made using a proanthocyanidin commercial extract (Pycnogenol ®) to build a calibration curve and the results were expressed as Pycnogenol ® equivalents (mg PycE/g of dry extract, and mg PycE/g of fresh biomass).

4.2.5 HPLC-DAD and LC-DAD-ESI-MS analyses

High-performance liquid chromatography was performed in a Thermo Spectra System, equipped with a binary pump (Spectra System P2000), autosampler (Spectra System AS1000), DAD detector (Spectra System UV6000LP), and UV controller (Spectra System SN4000). Chromatograms and UV spectra were acquired using the software Xcalibur™ 2.0.6 (Thermo Fisher Scientific Corporation). The reverse phase chromatography separation was carried out with a Chromolith Performance RP-18e column (100 mm x 4.6 mm, Merck). The mobile phase consisted of (A) water (0.5% formic acid, v/v) and (B) acetonitrile (0.1% formic acid, v/v). The gradient profile of the mobile phase was optimized as follows: 100-95% A (0-5 min) at a flow rate of 1.0 mL/min, followed by 95-80% A (5-80 min), 80-10% A (80-90 min), 10% A (90-100 min), 10-100% A (100-105 min), and 100% A (105-110 min), at a flow rate of 0.7 mL/min. The absorption spectra were recorded in the range of 190-600 nm (increments of 1 nm), and at the specific wavelengths of 280, 320, and 360 nm. The injection volume of each sample was 20 µL.

In HPLC-DAD-ESI-MS/MS, the selected extracts were analyzed on an Alliance 2695 separation module HPLC system coupled to a 2996 Photodiode Array Detector and a Micromass® Quattro Micro triple quadrupole (TQ) (Waters, Ireland). The injection volume was 20 µL, and the chromatographic separation procedure was carried out using a Lichrocart® RP-18 column (250 x 4 mm, 5 µm, Merck) and a Manu-cart® RP-18 pre-column in a thermostated oven at 35°C. The mobile phase consisted of water with 0.5% formic acid as eluent A and acetonitrile with 0.5% formic acid as eluent B at a flow rate of 0.30 mL/min. The system was run with the following gradient program: 0-5 min from 100 to 95% A; 5-120 min from 95 to 80% A; 120-140 min from 80 to 10% A; 140-160 min at 10% A; finally returning to the initial conditions for 20 minutes. PDA was used to scan wavelength absorption from 210 to 650 nm. Tandem mass spectrometry (MS/MS) detection was performed using an electrospray ionization (ESI) source operating at 120°C, applying a capillary voltage of 2.5 kV, cone voltage of 30 V, and collision energies of 10, 20, and 30 eV. Compounds were ionized in both negative and positive ion modes. High purity nitrogen (N₂) was used both as drying gas and as a nebulizing gas. Ultra-high purity Argon (Ar) was used as the collision gas.

In all these chromatographic analyses, 6 mL of each analyzed sample was cleaned by liquid-liquid extraction with 3 mL of petroleum ether (twice) to remove lipids, and the final volume was adjusted to 5 mL. The cleaned samples were then ten-fold diluted in water (0.5% of formic acid) and filtered (0.22 mm) before injection.

4.2.6 Antimicrobial activity

The antimicrobial activity was determined in the aqueous extracts as described in section 3.2.6.

4.2.7 Statistical analysis

Statistical analysis of the results was performed using the SPSS software, version 23 (IBM Analytics, USA). Analysis of variance (ANOVA) was performed to evaluate significant differences between averages and the Tukey post hoc test ($p < 0.05$) was used to perform multiple comparisons between averages.

4.3 Results and discussion

4.3.1 Yields of concretes, absolutes, and aqueous phase extracts

The extraction procedures using the mixture (1:1) of petroleum ether and acetone (PE/ACE), and 100% acetone (ACE) originated organic phases rich in flower concrete components, and aqueous phases containing polar extractives (Figure 4.1). The mass yields of concrete and polar extractives relative to the fresh flowers, as well as the concentration of the aqueous phase extracts are presented in Table 4.1.

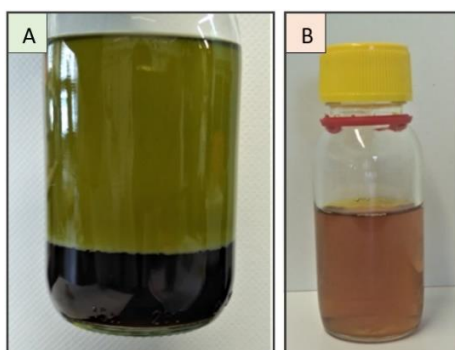


Figure 4.1 — Appearance of extracts obtained with PE/ACE and ACE: A) Diphasic extract obtained when using PE/ACE, with the upper organic phase containing flower concrete and the lower aqueous phase containing polar extractives; B) aqueous phase obtained with 100% ACE after se

Higher concrete yields were obtained for maceration with PE/ACE (1.26 – 1.28 %), relative to those obtained with 100% PE (0.82 – 1.01%) and 100% ACE (0.53%). Slightly lower concrete yields were obtained by Soxhlet extraction with the same solvents or with 100% PE (0.66%, wb) indicating that the extraction temperature was not a decisive parameter to improve extraction.

Table 4.1 — Yields of concretes and of aqueous phase extracts and respective concentration; extractions by maceration and Soxhlet made with the mixture (1:1) of petroleum ether and acetone (PE/ACE) and with 100% acetone (ACE), using flowers from Caparica (CAP) and Alcobaça (ALC); different letters indicate significant differences of means flowers (one-way ANOVA, Tukey test at $p < 0.05$).

Sample	Yield of concrete (g/100 g, wb)	Yield of aqueous extract (g/100 g, wb)	Concentration of aqueous extract (g/L)
Maceration			
PE/ACE-CAP	0.82 ± 0.03^c	4.85 ± 0.02^c	49.5 ± 1.2^e
PE/ACE-ALC	1.01 ± 0.05^b	6.57 ± 0.12^a	67.4 ± 0.4^d
ACE-CAP	1.26 ± 0.05^a	3.44 ± 0.04^e	28.9 ± 2.2^f
ACE-ALC	1.28 ± 0.02^a	4.40 ± 0.09^d	49.9 ± 1.6^e
Soxhlet			
PE/ACE-CAP	0.53 ± 0.03^e	5.66 ± 0.26^b	365.7 ± 63.3^a
PE/ACE-ALC	0.53 ± 0.03^e	5.51 ± 0.41^b	242.0 ± 66.7^b
ACE-CAP	0.66 ± 0.04^d	4.07 ± 0.27^d	70.3 ± 3.0^d
ACE-ALC	0.98 ± 0.06^b	5.37 ± 0.06^b	82.7 ± 1.5^c

The concrete obtained with PE presents the typical yellow color, while the concretes produced with PE/ACE or ACE, although obtained at a higher yield, show the presence of some chlorophyll pigments that should be eliminated during further processing of this extract. Concrete fractionation and composition will be addressed in chapter 5.

The extract yield in the aqueous phase was found slightly higher when using the mixture PE/ACE (4.85 – 6.57%) than with ACE (3.44 – 5.37%), especially for flowers from Alcobaça. It should be noted that the phase separation of organic and aqueous phases when using PE/ACE occurs spontaneously (see Figure 4.1 (A)), due to the presence of vegetable water in the fresh flowers, without the necessity of further fractionation steps besides decantation. This led to the production of a flower concrete and an aqueous extract in a single extraction step, that will only need to be dried or concentrated to further use. Moreover, by using this method were obtained polar extracts with significantly higher concentrations than those obtained with 100% ACE, that required further fractionation of the monophasic extract with petroleum ether and

water. This behavior may be relevant regarding the production of these extracts on a higher scale, since it reduces the number of unitary processing operations necessary to obtain dry extracts as well as the associated time and energy demand. The aqueous extract mass yields obtained by Soxhlet extraction were similar to those obtained maceration, but the concentrations of these Soxhlet extracts were much higher (70.3 to 365.7 g/L) than those obtained by maceration with equivalent solvents that showed concentration in the range of 28.9 to 67.4 g/L. This difference is a consequence of the extraction technique since Soxhlet extraction is meant to decrease the volume of solvent used in sequential extraction. In energetic terms, both processes will require some energy for heating, in the extraction step, for Soxhlet extraction and in the solvent evaporation step for maceration. Taking into account that the extract components will be exposed to lower temperatures and during a lower period of time, when the solvent is eliminated at reduced pressure than during Soxhlet extraction, this could be a factor in favor of the maceration process.

The extraction yields of the aqueous phases obtained in this work were higher than the ethanolic *A. dealbata* extract reported by López-Hortas et al. al (2.1% on a dry basis (db)) (López-Hortas et al., 2020), but lower than the ethanolic extracts obtained by Casas et al. (36.4%, relative to the dried flowers with 7.8% of moisture used in the extraction processes) (Casas et al., 2019) and Soto et al. (16.9% (wb)) (Soto et al., 2018), that used a much higher flower:solvent ratio of 1:20 (Casas et al., 2019) and/or high temperatures for a longer period under permanent agitation (Bobylev et al., 1996; Soto et al., 2018).

4.3.2 Chemical composition and antioxidant activity

Total phenolic (TPC), total flavonoid (TFC), and total proanthocyanidin (TPrAC) contents, as well as antioxidant activity given by DPPH and FRAP assays, are depicted in Table 4.2. It can be seen that, for each location and respective extraction solvent, the results of all evaluated parameters were better for maceration than for Soxhlet extraction. Since temperature generally increases solubility in a given solvent, this decrease in the concentrations of various phenolic components, suggests that partial degradation of the extracts may be occurring during Soxhlet extraction by the combined effects of temperature, exposure to atmospheric oxygen and light (Antony and Farid, 2022). The highest TPC and TFC contents were obtained by maceration or Soxhlet extraction using 100% ACE while TPrAC were better extracted by maceration or Soxhlet extraction with PE/ACE. This may result from the spontaneous phase separation that occurred during with this solvent mixture and that led to the separation

of non-polar and polar extractives creating a more polar aqueous phase during extraction. The flower source also affected their extraction behaviour and in particular, extract composition. The flowers collected at Caparica, produced extracts with higher values of DPPH and FRAP, when macerated with PE/ACE, while the flowers collected at Alcobaça, yielded extracts with higher antioxidant activity when macerated with 100% ACE.

Table 4.2 — Total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanidin content (TPrAC), and antioxidant activity given by DPPH and FRAP assays.

Sample	TPC (mg GAE/g dry extract)	TFC (mg CatE/g dry extract)	TPrAC (mg PycE/g dry extract)	DPPH (mg TE/g dry extract)	FRAP (μ mol Fe(II)/g dry extract)
Maceration					
PE/ACE-CAP	287.2 $\pm$ 6.2 ^c	139.4 $\pm$ 5.7 ^c	376.8 $\pm$ 5.6 ^b	795.1 $\pm$ 18.2 ^b	9484.1 $\pm$ 177.2 ^b
PE/ACE-ALC	259.5 $\pm$ 10.5 ^d	144.8 $\pm$ 7.0 ^{b,c}	406.8 $\pm$ 17.1 ^a	632.4 $\pm$ 24.4 ^d	7153.2 $\pm$ 275.1 ^d
ACE-CAP	344.7 $\pm$ 13.5 ^b	155.8 $\pm$ 7.9 ^{a,b}	314.7 $\pm$ 13.7 ^d	661.5 $\pm$ 22.7 ^d	8611.5 $\pm$ 387.2 ^c
ACE-ALC	546.8 $\pm$ 15.8 ^a	162.9 $\pm$ 8.3 ^a	403.9 $\pm$ 15.6 ^a	904.3 $\pm$ 34.9 ^a	10485.3 $\pm$ 409.7 ^a
Soxhlet					
PE/ACE-CAP	284.0 $\pm$ 7.1 ^c	127.9 $\pm$ 4.2 ^d	341.7 $\pm$ 13.2 ^c	492.5 $\pm$ 6.6 ^e	6920.6 $\pm$ 76.5 ^d
PE/ACE-ALC	235.6 $\pm$ 2.2 ^e	107.4 $\pm$ 3.0 ^e	100.2 $\pm$ 11.9 ^f	480.9 $\pm$ 6.4 ^e	5658.5 $\pm$ 78.5 ^f
ACE-CAP	177.1 $\pm$ 7.1 ^f	107.9 $\pm$ 4.9 ^e	199.3 $\pm$ 8.1 ^e	475.7 $\pm$ 18.9 ^e	6275.3 $\pm$ 244.3 ^e
ACE-ALC	359.6 $\pm$ 3.0 ^b	149.0 $\pm$ 5.9 ^{b,c}	346.1 $\pm$ 7.4 ^c	724.3 $\pm$ 12.6 ^c	8462.8 $\pm$ 113.7 ^c

Values (mean $\pm$ standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 x 3); different letters indicate significant differences of means (one-way ANOVA, Tukey test at p < 0.05)

Phenolics comprise a large group of metabolites associated with antioxidant activity and other important health benefits (Miguel-Chávez, 2017; Minatel et al., 2017). The TPC in the aqueous phases was in the range 177.1 – 546.8 mg GAE/g extract. The values are in a higher range than what was previously reported for *Acacia* flowers, such as *A. dealbata* (38 – 266 mg GAE/g extract) (Casas et al., 2019; Soto et al., 2018), *A. confusa* (28.6 – 263.2 mg GAE/g extract) (Tung et al., 2011; Wu et al., 2008), or *A. nilotica* (101.17 – 342.03 mg GAE/g extract) (El-Toumy et al., 2011), as well as for some medicinal flowers, such as elder flower (*Sambucus nigra* L.) (194.0 mg GAE/g extract) (Stoilova et al., 2007) or rose flower (*Rosa damascena* Mill.) (circa 35 – 276.02 mg GAE/g extract) (Özkan et al., 2004; Patil et al., 2015). Phenolics content in the extracts are comparable to the contents reported by Ferreira-Santos et al. (Ferreira-Santos et al., 2020) in another known source of phenolics, the *Pinus pinaster* bark (460 to 675 mg GAE/g

extract), or by Dudonné et al. (360.76 – 363.02 mg GAE/g extract) (Dudonné et al., 2009). It can be concluded that *A. dealbata* flowers are an important source of phenolics (7.2 – 24.0 mg GAE/g fresh flower (see Appendix B1, Table B1), higher than other well-known sources of these secondary metabolites, such as yellow tea (2.4 – 6.6 mg GAE/g of tea), green tea (1.1 – 3.6 mg GAE/g of tea) and black tea (0.6 – 4.2 mg GAE/g tea) (Kopjar et al., 2015), and of the same magnitude than what was found in raspberry (10.4 – 20.6 mg GAE/g of fruit) and blackberry (22.8 – 27.9 mg GAE/g of fruit) (Sariburun et al., 2010).

Flavonoids are a class of phenolics well known for their biological activities, being considered essential components in nutraceutical, pharmaceutical, and cosmetic applications, due to their antioxidant, anti-inflammatory, and anti-carcinogenic properties, among others (Panche et al., 2016). TFC in the aqueous extracts were found in the range 107.4 – 162.9 mg CatE/g extract (or 329.7 – 499.6 RE/g extract, expressed in rutin equivalents (RutE), see Appendix B1, Table B2). These values are considerably higher than the described for flower extracts of *A. confusa* (17.5 – 62.6 mg RutE/g extract) (Tung et al., 2011) and *A. nilotica* (11.83 – 30.33 mg RutE/g) (El-Toumy et al., 2011), as well as for rose flower extracts (circa 40 – 161 mg RutE/g extract) (Patil et al., 2015), and of the same magnitude of what is described for *P. pinaster* bark extracts (77 to 161 mg CatE/g extract) (Ferreira-Santos et al., 2020). *A. dealbata* flowers can be an important source of flavonoids (4.4 – 9.5 mg CatE/g fresh flower, see Appendix B1, Table B1), with contents in a much higher range than those observed in yellow tea (1.7 – 3.8 mg CatE/g), green tea (0.7 – 1.6 mg CatE/g), and black tea (0.2 – 0.3 mg CatE/g) (Kopjar et al., 2015), and in raspberry (0.2 – 0.4 mg CatE/g) and blackberry (0.3 – 0.8 mg CatE/g) red fruits (Sariburun et al., 2010).

Proanthocyanidins are oligomers or polymers of monomeric flavonoids (flavan-3-ols) with a known reputation for promoting health and wellbeing, due to their antioxidant, cardio-protective, neuroprotective, immunomodulatory, anti-obesity, antidiabetic, anticancer, or anti-microbial properties, among others (Beecher, 2004; Rauf et al., 2019). Pycnogenol® is a known proanthocyanidin commercial extract derived from *Pinus pinaster* bark, whose biological activities have been extensively reviewed (D'Andrea, 2010; Maimoona et al., 2011). The results of TPrAC were here expressed in Pycnogenol® equivalents. Values were in the range 100.2 – 406.8 mg PycE/g extract (5.5 – 26.7 mg PycE/g fresh flower, see Appendix B, Table B1). These are good results since the comparison is between a crude extract and Pycnogenol® is already a commercialized natural extract that is obtained after fractionation steps.

The DPPH assay evaluates the ability of extracts to act as free radical scavengers or hydrogen donors, by reducing the odd electron of the nitrogen atom in DPPH by hydrogen atoms

received from the antioxidant compounds present in the extract (Kedare and Singh, 2011). Highly reactive free radicals can be produced in biological systems and cause various degenerative disorders, like mutagenesis, carcinogenesis, cardiovascular disturbances, and aging, being of crucial importance to fight back free radicals, especially using antioxidants from natural origin (Singh and Singh, 2008). DPPH results revealed remarkable antioxidant activity, varying in the range 475.7 – 904.3 mg TE/g extract (19.3 – 41.6 mg TE/g fresh flower, see Appendix B1, Table B3). These values are much higher than the reported for known antioxidants, such as yellow, green, and black teas (11.930 – 26.521 $\mu\text{mol TE}/100\text{g}$, which corresponds to 0.030 – 0.066 mg TE/g of tea leaves) (Kopjar et al., 2015), and similar to those of red fruits raspberry and blackberry (64.14 – 177.11 $\mu\text{mol TE}/\text{g}$, corresponding to 16.1 – 44.3 mg TE/g of fruit) (Sariburun et al., 2010), suggesting *A. dealbata* to be an important source of antioxidants. These results are also considerably higher than the described for the commercial polyherbal tea extract used in the treatment of diabetes (Diabetea) (75 mg TE/g extract) (Paddy et al., 2015), or for *Pinus densiflora* bark extract (approximately between 50 and 350 mg TE/g extract) (Venkatesan et al., 2019).

Antioxidant activity was also evaluated by the FRAP assay. This assay assess the total reducing power of extracts, through the reduction of Fe^{3+} in the complex (Fe^{3+} -TPTZ) to a ferrous form (Fe^{2+} -TPTZ), by donation of electrons from the antioxidants present in the extract, with the subsequent stopping of the free radical production chain (Afsar et al., 2018; Sadiq et al., 2017; Santos-Sánchez et al., 2019). FRAP values were in the range 6275.3 – 10485.3 $\mu\text{mol Fe}^{2+}/\text{g}$ extract, which were much higher than the reported for leaves, pods, and seeds of *A. leucophloea* (178.14 – 254.42 $\mu\text{mol Fe}^{2+}/\text{g}$ extract) (Zia-UI-Haq et al., 2013), and for leaves, pods, and bark extracts of *A. nilotica* (2733 – 3355 $\mu\text{mol Fe}^{2+}/\text{g}$ extract) (Sadiq et al., 2017). The obtained values were also higher than what was found for *Pinus radiata* bark extract (152 mg Fe^{2+}/g extract, equivalent to 2721.2 $\mu\text{mol Fe}^{2+}/\text{g}$ extract), and of the same magnitude as the reported values for *P. pinaster* bark extracts (6.45 – 7.33 mmol Fe^{2+}/g extract) (Dudonné et al., 2009) and the commercial pine bark extract Pycnogenol® (575 mg Fe^{2+}/g extract, equivalent to 10296.4 $\mu\text{mol Fe}^{2+}/\text{g}$) (Astuya et al., 2016).

The correlation between TPC, TFC, TPrAC, and antioxidant activities determined by DPPH and FRAP assays were evaluated and are shown in Table 4.3. The correlations were found to be statistically significant, with the Pearson's correlation coefficients indicating strong correlations between TPC, TFC, and TPrAC and antioxidant activity (Pearson's r in the range 0.707 and 0.860) (Akoglu, 2018).

Table 4.3 — Correlation analysis between total phenolics content (TPC), total flavonoid content (TFV), total proanthocyanidins content (TPrAC) and antioxidant activities given by DPPH and FRAP assays, for aqueous extracts; significance of the correlations assessed at $p < 0.05$ and at $p < 0.01$ (two-tailed).

	TPC	TFC	TPrAC	DPPH	FRAP
TPC	1				
TFC	$r = 0.816^*$ $p = 0.014$	1			
TPrAC	not significant	$r = 0.828^*$ $p = 0.011$	1		
DPPH	$r = 0.826^*$ $p = 0.012$	$r = 0.841^{**}$ $p = 0.009$	$r = 0.707^*$ $p = 0.050$	1	
FRAP	$r = 0.833^*$ $p = 0.010$	$r = 0.860^{**}$ $p = 0.006$	$r = 0.736^*$ $p = 0.037$	$r = 0.963^{**}$ $p = 0.000$	1

** correlation is significant at $p < 0.01$ (two-tailed); * correlation is significant at $p < 0.05$ (two-tailed)

Similar observations were found in the literature. Yadav et al. (Yadav et al., 2018) found strong correlations between total phenolics and total flavonoids and antioxidant activity given by DPPH assay ($r = 0.771$ and $r = 0.815$, respectively), for extracts of *A. nilotica* leaves. However, Abdel-Farid et al. (Abdel-Farid et al., 2014) found significant correlations only between total flavonoids of extracts from leaves, flowers, and pods of three *Acacia* species, and the DPPH results, while non-significant correlations were found between total phenolics and DPPH. Significant and strong correlations between total phenolics and the DPPH and FRAP results were found also in *Pinus densiflora* bark extract (Venkatesan et al., 2019) and medicinal herbs and spices (Ulewicz-Magulska and Wesolowski, 2019).

4.3.3 Antimicrobial activity

The assessment of antimicrobial activity of extracts from plant origin has gained significance in the last years due to the increasing pathogenic resistance to conventional antimicrobial agents, thus contributing to finding alternatives to synthetic antibiotics (Ghannoum M. A. and Rice L. B., 1999). A representative group of extract samples was selected to evaluate the antimicrobial activity against different microorganisms frequently implicated in infectious and/or foodborne diseases, namely Gram-positive bacteria (*S. aureus*, MRSA, *S. epidermis*, *E. faecalis*, *S. mutans*, *S. mitis* and *B. cereus*), Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and fungi (*C. albicans*). The inhibition zones diameters are presented in Table 4.4.

Table 4.4 — Inhibition zones (mm) of selected extracts of *A. dealbata* and *A. saligna* against *S. aureus*, MRSA, *S. epidermis*, *E. faecalis*, *B. cereus*, *S. mutans*, *S. mitis* and *C. albicans*. Crude extracts concentrations are as follows (maceration: ACE-ALC (51 mg/ml), ACE-CAP (27 mg/mL), PE/ACE-ALC (190 mg/mL), PE/ACE-CAP (132 mg/mL); Soxhlet: ACE-ALC (84 mg/mL), PE/ACE-ALC (195 mg/mL).

Samples	<i>S. aureus</i>			MRSA			<i>S. epidermis</i>			<i>E. faecalis</i>		
	1 mg/mL (50 mg)	10 mg/mL (500 mg)	crude	1 mg/mL (50 mg)	10 mg/mL (500 mg)	crude	1 mg/mL (50 mg)	10 mg/mL (500 mg)	crude	1 mg/mL (50 mg)	10 mg/mL (500 mg)	crude
Maceration												
ACE-ALC	7.8 ± 0.5	12.0 ± 0.0	16.5 ± 0.6	nd	10.5 ± 0.6	16.3 ± 0.5	nd	10.3 ± 0.5	14.0 ± 0.0	nd	9.0 ± 0.0	14.0 ± 0.0
ACE-CAP	nd	9.8 ± 0.5	13.0 ± 0.0	nd	9.3 ± 0.5	13.0 ± 0.0	nd	8.0 ± 0.0	11.0 ± 0.0	nd	8.0 ± 0.0	10.5 ± 0.6
PE/ACE-ALC	nd	10.3 ± 0.5	18.3 ± 0.5	nd	9.8 ± 0.5	18.3 ± 0.5	nd	8.5 ± 0.6	17.0 ± 0.0	nd	9.5 ± 0.6	15.8 ± 0.5
PE/ACE-CAP	8.0 ± 0.0	11.3 ± 0.5	17.5 ± 0.6	nd	11.0 ± 0.0	17.8 ± 0.5	nd	9.3 ± 0.5	15.0 ± 0.5	nd	9.0 ± 0.0	15.5 ± 0.6
Soxhlet												
ACE-ALC	nd	10.5 ± 0.6	16.0 ± 0.0	nd	10.5 ± 0.6	16.5 ± 0.6	nd	8.5 ± 0.6	13.8 ± 0.5	nd	7.8 ± 0.5	13.8 ± 0.5
PE/ACE-ALC	nd	10.0 ± 0.0	15.8 ± 0.5	nd	8.8 ± 0.5	16.0 ± 0.0	nd	8.8 ± 0.5	14.3 ± 0.5	nd	7.8 ± 0.5	14.5 ± 0.6
Positive control												
Vancomycin (5.0 mg)		14.9 ± 0.7			13.9 ± 0.7			15.7 ± 1.0			12.5 ± 0.5	

Table 4.4 (cont.) — Inhibition zones (mm) of selected extracts of *A. dealbata* and *A. saligna* against *S. aureus*, MRSA, *S. epidermis*, *E. faecalis*, *B. cereus*, *S. mutans*, *S. mitis* and *C. albicans*. Crude extracts concentrations are as follows (maceration: ACE-ALC (51 mg/ml), ACE-CAP (27 mg/ml), PE/ACE-ALC (190 mg/ml), PE/ACE-CAP (132 mg/ml); Soxhlet: ACE-ALC (84 mg/ml), PE/ACE-ALC (195 mg/ml).

Samples	<i>B. cereus</i>			<i>S. mutans</i>			<i>S. mitis</i>			<i>C. albicans</i>		
	1 mg/mL (50 mg)	10 mg/mL (500 mg)	crude	1 mg/mL (50 mg)	10 mg/mL (500 mg)	crude	1 mg/mL (50 mg)	10 mg/mL (500 mg)	crude	1 mg/mL (50 mg)	10 mg/mL (500 mg)	crude
Maceration												
ACE-ALC	7.8 ± 0.5	12.5 ± 0.6	17.0 ± 0.0	nd	nd	10.0 ± 0.0	nd	7.00 ± 0.0	12.3 ± 0.6	nd	nd	9.5 ± 0.6
ACE-CAP	nd	11.0 ± 0.0	14.8 ± 0.5	nd	nd	9.7 ± 0.7	nd	7.00 ± 0.0	10.5 ± 0.7	nd	nd	nd
PE/ACE-ALC	7.0 ± 0.0	11.5 ± 0.5	20.0 ± 0.0	nd	nd	16.0 ± 0.0	nd	8.00 ± 0.0	16.0 ± 0.0	nd	nd	11.5 ± 0.6
PE/ACE-CAP	7.5 ± 0.6	12.3 ± 0.5	20.0 ± 0.0	nd	nd	13.3 ± 0.6	nd	8.00 ± 0.0	13.0 ± 1.0	nd	nd	10 ± 0.0
Soxhlet												
ACE-ALC	8.0 ± 0.8	12.5 ± 0.6	18.0 ± 0.0	nd	nd	10.3 ± 0.6	nd	7.00 ± 0.0	11.0 ± 0.0	nd	nd	nd
PE/ACE-ALC	nd	9.8 ± 0.5	17.0 ± 0.0	nd	nd	10.3 ± 0.6	nd	7.00 ± 0.0	12.3 ± 0.6	nd	nd	10.3 ± 0.5
Positive control												
Vancomycin (50.0 mg)		16.4 ± 1.1				19.3 ± 0.6		25.7 ± 0.6			na	
Ketoconazole (5.0 mg)		na				na		na			36.8 ± 0.5	

nd: not detected; na: not applied.

All crude extracts were effective against all Gram-positive bacteria, with zones of inhibition between 9.7 mm (*S. mutans*) and 20.0 mm (*B. cereus*). After standardization of concentrations at 10 mg/mL, all tested extract samples remained effective against Gram-positive bacteria, with the exception of *S. mutans*, although with lower inhibition zone diameters. Extracts obtained by maceration showed identical or higher inhibition zones than those obtained by Soxhlet. The highest inhibition zones were achieved against *B. cereus* while the most resistant bacteria was *S. mutans*. At a concentration of 1 mg/mL, the extracts showed activity only against *S. aureus* and *B. cereus*. All tested samples revealed ineffective against Gram-negative bacteria (*P. aeruginosa* and *E. coli*).

The antimicrobial activity of extracts from *A. dealbata* flowers is underevaluated. Only a recent study was found, showing antimicrobial capacity of 50% ethanol extracts of *A. dealbata* flowers against different strains of *S. aureus* and *E. coli* bacteria, and *C. albicans* yeast (Paula et al., 2022). The observed activity against *E. coli*, which was not found in the present work, may be due to a different extract composition resulting from the different plant locations and/or extraction conditions and plant pretreatments, once a 60 mesh fraction of lyophilized and milled flowers were used in an extraction process at a 1:10 flower to solvent ratio, under agitation for 15 hours, instead of the simpler approach assessed in this work. Inactivity against *E. coli* (Cock, 2012; Da Silva et al., 2022; Makeen et al., 2020) and *P. aeruginosa* (Makeen et al., 2020), was also observed with extracts from flowers of other *Acacia* species, such as *A. ehrenbergiana*, *A. complanta*, and *A. mearnsii*. No studies on antimicrobial activity against *S. epidermis*, MRSA, *E. faecalis*, *B. cereus*, *S. mutans* and *S. mitis* were found in the literature using flower extracts of *A. dealbata*.

The inhibition zones against *S. aureus* were in same range of those observed with ethanolic extracts of *A. salicina*, *A. laeta*, *A. hamulosa*, and *A. tortilis* (14.0 – 21.0 mm, at 100 mg/mL) (Alajmi et al., 2017), in the case of the crude extracts. The extracts with a concentration of 10 mg/mL gave similar zones of inhibition to the ones found in more concentrated extracts of some medicinal flowers, such as *Pedaliium murex*, *Senna auriculata*, *Millingtonia hortensis*, and *Catharanthus roseus* (3.0 – 14.0 mm, at 25 – 100 mg/mL) (Abirami et al., 2021), *Tagetes patula* (12.0 mm, at 300 mg/mL) (Latifian et al., 2021), *Camellia japonica* (10.8 mm, at 20 mg/mL) (A. G. Pereira et al., 2022), or *Citrus aurantium* (7.8 – 13.8 mm, at 0 – 20 mg/mL) (Hashemi et al., 2016).

S. epidermidis is a known pathogen responsible for many nosocomial infections, and skin infections such as acne (Kumar et al., 2007; Lopes et al., 2021). The inhibition zones against this bacteria were higher than the ones found in extracts of the medicinal flower *Jasminum*

officinale (7.0 mm, at 100 mg/mL), even at 10 mg/mL (Kumar et al., 2007), and of the same magnitude of the observed with extracts of *Camellia japonica* flowers (14.0 mm at 20 mg/mL) (A. G. Pereira et al., 2022), in the case of the crude extracts.

E. faecalis, frequently found in food and livestock and usually associated to oral diseases, has gained significance in the last decades as a leading opportunistic pathogen causing nosocomial infections (Anderson et al., 2016). The inhibition zones against *E. faecalis* by the extracts at a concentration of 10 mg/mL were similar to the ones found with more concentrated extracts of the medicinal flowers *Pedaliium murex*, *Senna auriculata*, *Millingtonia hortensis*, and *Catharanthus roseus* (2.0 – 11.0 mm, at 25 – 100 mg/mL) (Abirami et al., 2021), and *Tagetes patula* (11.5 mm, at 300 mg/mL) (Latifian et al., 2021), but lower than the one found in *A. ehrenbergiana* flower extract (18 mm) (Makeen et al., 2020), even in the case of the crude extracts.

B. cereus is an environmental contaminant present in soil, foods, insect larvae, and human skin, that induces local and systemic infections, being now considered a serious cause of nosocomial infections (Glasset et al., 2018). The inhibition zones against *B. cereus* were much higher than the one found with *Camellia japonica* flowers (5.0 mm at 20 mg/mL) (A. G. Pereira et al., 2022), and in a similar range to the observed with *Citrus aurantium* flowers (7.8 – 13.2 mm, at 0 – 20 mg/mL) (Hashemi et al., 2016).

Candida albicans yeast is a commensal of the human body, often found in gastrointestinal microbiota of healthy humans, and an opportunistic pathogen frequently responsible for nosocomial bloodstream infections (Bernard et al., 2020; Neville et al., 2015). Both extracts obtained by maceration of flowers from Alcobaça inhibited the growth of this microorganism. The zones of inhibition were lower than the ones obtained with *A. salicina*, *A. laeta*, *A. hamulosa*, and *A. tortilis* flower extracts (14.0 – 21.0 mm, at 100 mg/mL) (Alajmi et al., 2017). However, this is a good result since inactivity against *C. albicans* is often reported, such as in the case of extracts from flowers of *A. ehrenbergiana* (Makeen et al., 2020) and *A. complanta* (Cock, 2012).

4.3.4 Identification of constituents by HPLC-DAD and HPLC-DAD-MS/MS

Extract samples were analyzed by HPLC-DAD and HPLC-DAD-MS/MS. Figure 4.2 presents illustrative examples of the HPLC-DAD chromatograms obtained by maceration with ACE and PE/ACE (see all chromatograms in Appendix B2). The tentative identification of compounds was then performed by HPLC-DAD-ESI-MS/MS. The correspondence between HPLC-DAD and HPLC-DAD-MS/MS peaks was made according to UV spectra and elution order.

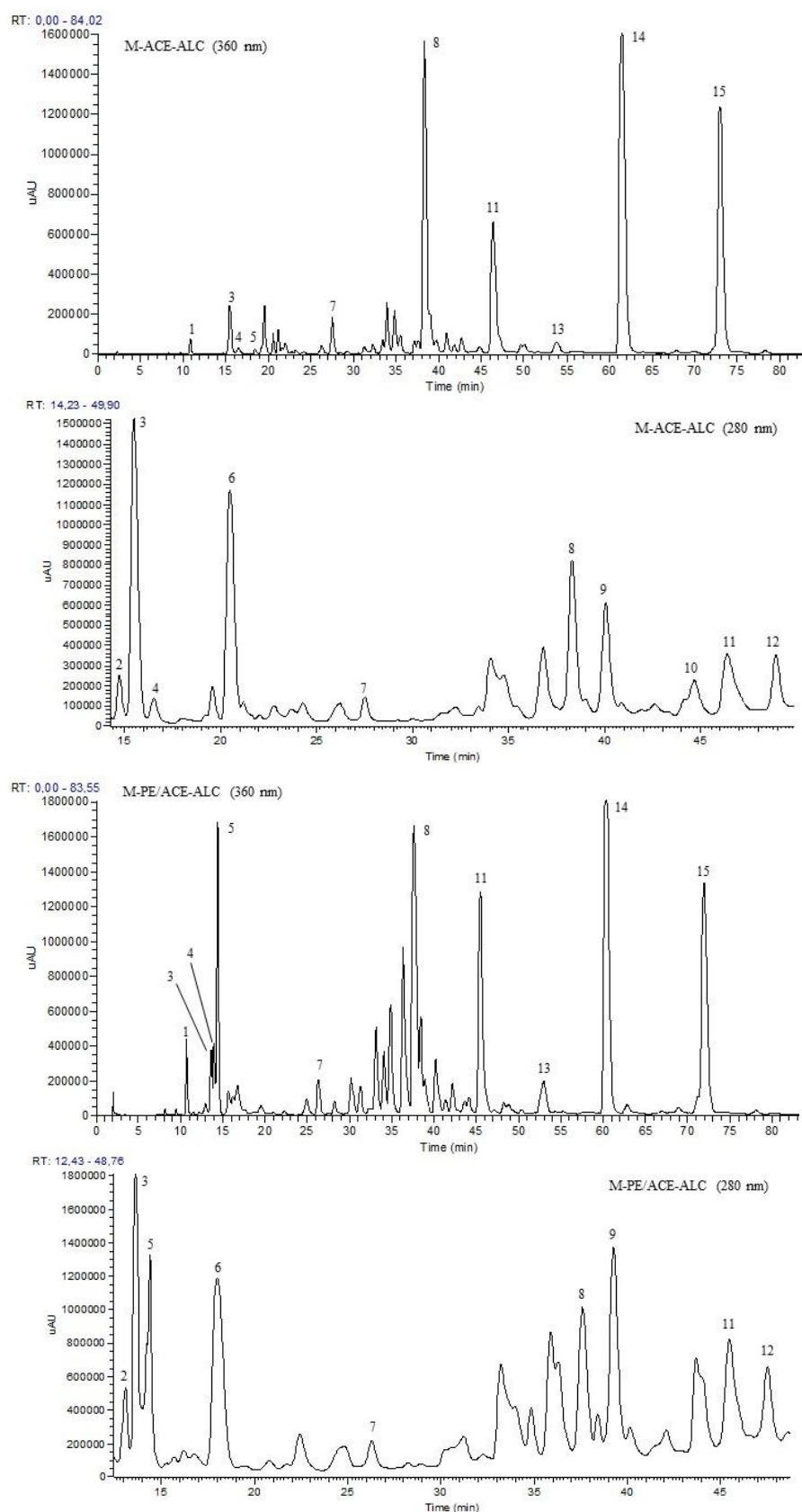


Figure 4.2 — Chromatographic profiles at 360 nm (and parts of the profile at 280 nm, to illustrate some peaks not easily visible at 360 nm) for ACE and PE/ACE extracts obtained by maceration with flowers from Alcobaça.

Table 4.5 shows a list of 15 compounds tentatively identified by HPLC-DAD-MS/MS, along with their retention time (Rt), UV absorption maxima, monoisotopic mass (MM), precursor ion (in positive $[M+H]^+$ or negative $[M-H]^-$ ion mode), the MS/MS product ions and the bibliographic references to support the identification.

A terpene (peak 1, cuminoid C), a flavanol (peak 2, catechin), a hydroxycinnamic acid glycoside (peaks 3 and 4, *p*-coumaric acid *O*-hexoside isomers I and II), four flavone glycosides (peak 5, quercetin *O*-rhamnosylhexoside-*O*-hexoside; peaks 7 and 8, myricetin *O*-rutinose isomers I and II; peak 11, rutin; and peak 13, quercetin *O*-rhamnoside), two flavanone glycosides (peaks 9 and 10, naringenin *O*-hexoside isomers I and II; and peak 12, naringenin *O*-pentoside), and two chalcones (peak 14, isosalipurposide and peak 15, naringenin chalcone *O*-pentoside) were tentatively identified based on UV-spectra and MS/MS fragments, accordingly to the previously described in the literature. Peak 15, with molecular ion $[M+H]^+$ at m/z 405 in positive mode, and $[M-H]^-$ at m/z 403 in negative mode, was tentatively attributed to naringenin chalcone *O*-pentoside, as described in the section 3.3.8.

Table 4.5 — Compounds tentatively identified by LC-DAD-ESI-MS; MS/MS fragments presented in decreasing order of intensity.

No.	Rt	λ_{max} (nm)	[M-H] ⁻ / [M+H] ⁺ (m/z)	MW	MS/MS fragments (m/z)	Compound	References
1	45.99	245, 298sh, 329	341 [M-H] ⁻	342	161	Cuminoid C	(Serag et al., 2019)
2	59.37	240, 280	291 [M+H] ⁺	290	139, 123, 147, 161, 119, 111	Catechin	(Li et al., 2015; Mandal and Chakraborty, 2013)
3	60.89	242, 314	325 [M-H] ⁻	326	145, 163, 119	p-coumaric acid O-hexose (I)	(Bystrom et al., 2008; Radenkovs et al., 2020)
4	63.82	242, 314	325 [M-H] ⁻	326	145, 163, 119	p-coumaric acid O-hexose (II)	(Bystrom et al., 2008; Radenkovs et al., 2020)
5	73.11	255, 267sh, 354	773 [M+H] ⁺	772	465, 303, 627	quercetin 3-O-rhamnosylhexoside-7-O-hexoside	(Lin et al., 2008)
6	77.88	260	337 [M+H] ⁺	336	185, 175, 119, 127, 85	Unknown	
7	89.53	254, 267sh, 355	627 [M+H] ⁺	626	319, 481	Myricetin O-rutinoside (I)	(Kelanne et al., 2020; Lin et al., 2008)
8	101.47	258, 300sh, 355	627 [M+H] ⁺	626	319, 481	Myricetin O-rutinoside (II)	(Kelanne et al., 2020; Lin et al., 2008)
9	105.66	241, 282	433 [M-H] ⁻	434	271, 151	Naringenin O-hexoside (I)	(Goufo et al., 2020; Kammerer et al., 2005; March et al., 2006; Pereira et al., 2013; Sánchez-Rabaneda et al., 2003)
10	111.59	241, 282	433 [M-H] ⁻	434	271, 151	Naringenin O-hexoside (II)	(Goufo et al., 2020; Kammerer et al., 2005; March et al., 2006; Pereira et al., 2013; Sánchez-Rabaneda et al., 2003)
11	117.35	256, 267sh, 290sh, 354	611 [M+H] ⁺	610	303	Rutin	(Hunyadi et al., 2012; Jang et al., 2018; Peres et al., 2013; Pollini et al., 2019)
12	123.37	241, 283	405 [M+H] ⁺ 403 [M-H] ⁻		273, 153, 147 271, 151	Naringenin O-pentoside	(Bystrom et al., 2008)
13	134.07	244sh, 264, 350	449 [M+H] ⁺ 447 [M-H] ⁻	448	303, 129, (103), 85, 71 300, 301	Quercetin O-rhamnoside	(Diaconeasa et al., 2014; Goufo et al., 2020; Jang et al., 2018; Lee et al., 2005; Lin et al., 2008)
14	135.86	247, 368	435 [M+H] ⁺ 433 [M-H] ⁻	434	273, 153, 147 271, 151, 119	Isosalipurposide (chalcononaringenin 2'-glucoside)	(Kammerer et al., 2005; Li et al., 2009; Teppabut et al., 2018)
15	137.13	246, 368	405 [M+H] ⁺ 403 [M-H] ⁻	404	273, 153, 147 271, 151, 119	Naringenin chalcone pentose	

p-coumaric acid and quercetin were previously reported in *A. dealbata* and *A. saligna* flowers (Al-Huqail et al., 2019; Luís et al., 2012), and myricetin (Al-Huqail et al., 2019; Ghribia et al., 2014), naringenin (Al-Huqail et al., 2019; Ghribia et al., 2014), and isosalipurposide (Ghouila et al., 2012; Ghribia et al., 2014) were previously found in *A. saligna* flowers.

The compounds *p*-coumaric acid, myricetin, naringenin, quercetin, and respective glycosides are associated with important biological activities, such as antioxidant, antidiabetic, anti-cancer, antiviral, antimicrobial, immunomodulatory, cardiovascular, analgesic, antihypertensive, anti-inflammatory, DNA protective, hepatoprotective, antiatherogenic, antidepressant, anti-arthritis, hypolipidemic, peroxisome proliferator-activated receptors (PPARs) activator, and memory improving (Batiha et al., 2020; Pei et al., 2016; Taheri et al., 2020; Venkateswara et al., 2017). Protection against neurodegenerative disorders, like Alzheimer, Parkinson, and Huntington diseases was already described for the quercetin glycoside rutin (Enogieru et al., 2018), myricetin (Taheri et al., 2020), and naringenin (Nouri et al., 2019). These flavonoids occur in plants mainly in the form of glycosides but are converted into the respective aglycones in the intestine by the action of β -glycosidases enzymes (Batiha et al., 2020). The chalcone isosalipurposide was described to have antioxidant and anti-acetylcholinesterase effects (Ghribia et al., 2014), as well as cytoprotective effects against oxidative damage mediated through Nrf2 activation and induction of its target gene expression in hepatocytes, what suggests its potential to be used in therapeutics to prevent or treat oxidative stress-mediated liver disease (Han et al., 2015).

Therefore, the results of this work show that *A. dealbata* flowers can be an important source of a complex mixture of phenolic compounds, particularly flavonoids. This variety is important since synergistic effects among them also play an important role in the biological activities here referred, especially in the antioxidant activity (Hubner et al., 2019; Qin et al., 2018; Xu et al., 2014).

4.4 Conclusions

The results obtained in this work showed the possibility of producing flavonoid-rich extracts with antioxidant and antimicrobial properties from the flowers of Portuguese invasive *A. dealbata*, using a simple maceration process at room temperature. Moreover, this investigation demonstrated the possibility of obtaining separately both nonpolar and polar extracts from the same sample of flowers, in a single extraction step, by using a mixture of petroleum ether and acetone.

Significant differences in TPC, TFC, TPrAC, and antioxidant activity by DPPH and FRAP were observed between the extraction methods, the extraction media, and the provenience of the flowers, with the best results being obtained by maceration with flowers from Alcobaça in the case of ACE extracts, and by maceration with flowers from Caparica in the case of PE/ACE extracts. Besides the best results obtained with ACE extracts, using the mixture PE/ACE allowed us to obtain extracts with a higher concentration, facilitating further processing from a practical and economic point of view, besides the advantage of obtaining separately a wider range of compounds from the same sample of raw material in a single extraction step.

Significant and strong positive correlations were found between TPC, TFC, TPrAC, and antioxidant activities given by DPPH and FRAP assays, having been identified *p*-coumaric acid, myricetin, quercetin, and naringenin glycosides in the extracts, for which important biological activities are well described in the literature.

This work contributes significant information to assess the potential to produce high-value antioxidant and antimicrobial extracts from the invasive *A. dealbata* plant, with possible applications in nutraceutical or cosmetic industries, simultaneously contributing to control its propagation by the collection of flowers and prevention of seed formation and dissemination.

VOLATILE COMPONENT PROFILE FROM PORTUGUESE MIMOSA (*A. DEALBATA*) ABSOLUTE OILS

Abstract: The purpose of this chapter was to produce and characterize absolute oils from Portuguese mimosa (*Acacia dealbata*) flowers. The conventional maceration process was compared with Soxhlet extraction, using flowers from different geographical proveniences. The traditional extraction with a non-polar solvent, such as petroleum ether, was compared for the first time with the use of a mixture of petroleum ether:acetone (1:1)) and pure acetone. The composition of the produced absolutes was assessed by GC-TOFMS, and principal component analysis (PCA) was performed to show eventual differences between extraction solvents, extraction methods, or the origin of the raw material. Absolutes with distinct but pleasant aromas were obtained with each extraction media. The chromatographic data revealed similar profile distribution of compounds regardless of the provenience of the flowers, extraction solvents, or extraction methods, but the PCA confirmed a distinct composition of absolutes determined by the extraction media. The most representative compounds in the produced absolutes were the organic acids *p*-anisic acid, palmitic acid, linoleic acid, and linolenic acid; the ester methyl *p*-anisate; the benzaldehyde anisaldehyde; the hydrocarbons nonadecane, heneicosane, heptadecane, and 8-heptadecene; the pentacyclic triterpenes α -amyirin, β -amyirin, lupenone (Lup-20(29)-en-3-one), and lupeol; and glycerol monostearate, which have particular importance due to their known applications and biological activities. The use of acetone, or the mixture of petroleum ether and acetone, allowed the production of distinct absolutes and simultaneously produces aqueous phases studied in the previous chapter. The results showed that the Portuguese *A. dealbata* is suitable for the production of absolutes in yields of the same magnitude

as the ones produced in the most typical region where they are commercially explored, such as in France, and with a similar profile of compounds.

5.1 Introduction

The flowers of *A. dealbata* are already used for decades to produce absolute oil for the perfume industry, which is obtained from flower concrete. The concrete is traditionally obtained from fresh flowers by extraction with hexane or petroleum ether, in yields of about 1%, while the absolute is extracted from the concrete after treatment with ethanol, corresponding to about 25% of the concrete weight (Perriot et al., 2010). Additionally, the flowers of *Acacia* spp. are reported to contain a variety of bioactive phenolic compounds with potential application as antioxidants in the pharmaceutical, nutraceutical, or cosmetic industries (Ghouila et al., 2012; Ghribia et al., 2014; Subhan et al., 2018). The successful removal of these valuable fractions from the flowers can bring an important economic return, overcoming expenses with biomass collection and transport, while simultaneously contributing to minimizing their proliferation by the spreading of seeds during the flowering season, once flower collection prevents seed formation (Lorenzo et al., 2010a).

The goal of this chapter is to investigate the production of concretes and absolutes from the flowers of *Acacia dealbata*, a representative example of Portuguese invasive *Acacia* species. Besides comparing two different geographical origins of this species (Alcobaça and Caparica) and two extraction methods (maceration at room temperature and Soxhlet), the use of pure acetone and the use of a mixture of petroleum ether and acetone was tried, to the best of our knowledge, for the first time. The purpose of using these alternative approaches is to obtain a wider range of compounds from the flowers in a single extraction step, instead of obtaining only essences for perfumes or cosmetics when using non-polar solvents, or only phenolic-rich extracts when using polar solvents. An aqueous phase is spontaneously produced when performing extraction with the mixture of petroleum ether and acetone, and after washing the pure acetone extract. These aqueous phases will be characterized in a separate work. Tentative identification of compounds was performed through GC-TOFMS analysis. Principal component analysis (PCA) was performed to show eventual differences between extraction solvents, extraction methods, or the origin of the raw material, as well as the compounds which have the main contribution to those eventual differences.

5.2 Materials and methods

5.2.1 Plant material

Fresh flowers of *A. dealbata* were collected in February of 2020 in two different locations in the Center region of Portugal: Alcobaça (ALC) (39°36'36.5"N; 8°59'45.0"W, at 32 – 40 m of altitude), and Caparica (CAP) (38°37'22.6"N; 9°10'38.5"W, at 44 – 48 m of altitude). The samples used for extraction are a result of the mixing of the flowers of at least ten different trees from each location.

5.2.2 Chemicals and reagents

Acetone, petroleum ether, and ethanol 96°, used in the extraction processes and in the production of the concretes, were purchased from CarloErba Reagents (France). The derivatizing agent N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and pyridine were purchased from Merck (Germany). Chloroform was purchased from Chem-Lab (Belgium).

5.2.3 Extraction procedure

The obtention of concretes and aqueous phases was performed as described in the previous chapter, section 4.2.3.

5.2.4 Preparation of absolutes from the concretes

About 500 mg to 1 g of concrete was weighed in a centrifuge tube. About 10 mL of ethanol were added and the mixture was manually stirred in a water bath at 40 °C for 5 min. Then the mixture was kept in a freezer at -20 °C for 30 min to promote the precipitation of waxes. After this period, the tube was immediately centrifuged at 5000 rpm for 2 min, and the supernatant was decanted into another centrifuge tube. The remaining spent concrete was extracted with about 5 mL of ethanol two more times, and the supernatants were collected with the previous one. The collected supernatants were kept in the freezer for 30 min and then immediately filtered into a distillation flask. Finally, the evaporation of ethanol was made under reduced pressure to obtain the absolute oil.

5.2.5 GC-TOFMS analysis

Absolutes (20 mL of a sample at 10 mg/L) were derivatized based on a procedure already described by Popova et al. (Popova et al., 2020). Briefly, 20 mL of each sample at 10 mg/L was diluted in 100 mL of pyridine and 100 mL of the derivatizing agent N,O-bis(trimethylsilyl)tri-fluoroacetamide (BSTFA), and incubated at 70 °C for 45 min. After derivatization, the samples were diluted with 150 mL of chloroform.

Analyses were performed with an Agilent 7890B (Palo Alto, California, USA) gas chromatograph equipped with a multi-mode inlet. An Agilent HP-5MS UI fused silica capillary column (30m × 0.25 mm i.d., 0.25 µm d f - film thickness) was used in all separations. For liquid samples: 1 µL of the derivatized sample was injected via a LECO L-PAL3 autosampler fitted with a 10 µL syringe. The injector was operated in solvent vent mode, with the split valve opened at 100 mL/min for 25 seconds, and the injector at 70 °C. Then the split valve was closed for 120 seconds, and the inlet temperature was raised to 290° C at 700 °C/min. Finally, the split valve was opened again at 20mL/min until the end of the run. The oven program was as follows: 50°C for 1 min, then 7 °C/min until 100 °C, then 3° C/min until 220 °C, and finally 10 °C/min until 295 °C, held for 8 minutes. The transfer line to the MS was kept at 300 °C. For Headspace-Solid Phase Microextraction (HS-SPME): samples were incubated at 40 °C and extracted for 30 minutes with a DVB/Carb/PDMS fiber, 50/30 µm df, supplied from Supelco (Bellefonte, PA, USA). The fiber was desorbed for 2 minutes with the injector at 250 °C. The oven program was: 40 °C for 1 minute, then 5 °C/min until 250 °C, and held for 5 minutes. The transferline was held at 250 °C. 99.9999% Helium was used at a constant flow rate of 1.2 mLmin⁻¹ in both analyses. Detection was performed with a LECO Pegasus BT Time-of-Flight mass spectrometer (Saint Joseph, Michigan, USA). The MS was operated with the ion source at 250 °C, electron ionization at 70 eV, acquisition from m/z 40 to 550, 10 spectra per second and an acquisition delay of 480 second for liquid samples. Data acquisition, system control and spectra deconvolution were performed using LECO ChromaTOF version 5.40. NIST MS Search Program Version 2.3 g was used for spectra matching (NIST, 2015). Linear retention index (LRIs) values for sample peaks were calculated by analyzing the commercial alkane standard solution C8 - C40, using the aforementioned chromatographic conditions (van Den Dool and Dec. Kratz, 1963).

5.2.6 Statistical analysis

For PCA analysis, the relative percent area of each peak was used, which assumes the sum of all peaks in a chromatogram to be 100%. Integration was performed on the m/z with the highest signal-to-noise ratio for each compound. Calculation and graphing were performed in Python (V. 3.9.5) using the following libraries: Scikit-learn (V. 0.24), namely the PCA class in the decomposition module (`sklearn.decomposition.PCA`); Pandas (V. 1.2.5); Seaborn (V. 0.11.1) and Matplotlib (V. 3.4.2).

5.3 Results and discussion

5.3.1 Yields of concretes and absolutes

The extraction procedures using 100% of petroleum ether (PE), the mixture (1:1) of petroleum ether and acetone (PE/ACE), and 100% acetone (ACE) originated concretes and, in the case of PE/ACE and ACE, also aqueous phases, as described in the previous chapter. The concretes were further processed to obtain absolutes, which are shown in Figure 5.1. The yields of concretes and absolutes are presented in Table 5.1.

The concrete yields obtained with PE are in agreement with about 1% expected for the traditional method (Perriot et al., 2010), although the values obtained with Caparica flowers were significantly lower than those obtained with Alcobaça flowers, either with extraction by maceration or with extraction by Soxhlet. For each extraction method and extraction solvent, the yields of concrete were found higher with flowers from Alcobaça, exception made for Soxhlet extraction with PE/ACE mixture. Comparing extraction methods, maceration at room temperature gave the highest concrete yields with PE and ACE, and yields of the same magnitude were found with PE/ACE.

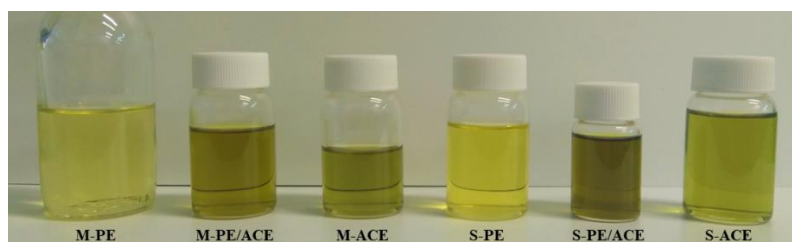


Figure 5.1 — Visual aspect of absolutes (10 g/L in ethanol) obtained by maceration with PE (M-PE), PE/ACE (M-PE/ACE) and ACE (M-ACE), and by Soxhlet also with PE (S-PE), PE/ACE (S-PE/ACE), and ACE (S-ACE).

Concerning the absolute yields, these were found also in agreement with the literature for the traditional method (PE), with slightly higher yields found from the concretes obtained by Soxhlet. The percentage of absolute removed from the concrete increased with the polarity of the extraction medium, which can be explained by the less amount of non-polar compounds (waxes) removed when extracting with PE/ACE or with ACE. This led to higher absolute yields when performing the fractionation with ethanol to obtain the absolute from the concrete.

Table 5.1 — Yields of concrete, absolute, and aqueous phase extracts; extractions made with 100% of petroleum ether (PE), with the mixture (1:1) of petroleum ether and acetone (PE/ACE), and with 100% acetone (ACE), using flowers from Caparica (CAP) and Alcobaça (ALC).

Sample	Yield of concrete (g/100 g, wb)	Yield of absolute (w/w percentage of concrete)
Maceration		
PE-CAP	0.82 ± 0.03	28.95 ± 0.67
PE-ALC	1.01 ± 0.05	23.77 ± 0.51
PE/ACE-CAP	1.26 ± 0.05	31.33 ± 0.73
PE/ACE-ALC	1.28 ± 0.02	30.72 ± 0.76
ACE-CAP	0.53 ± 0.03	40.25 ± 0.82
ACE-ALC	0.53 ± 0.03	39.44 ± 0.86
Soxhlet		
PE-CAP	0.66 ± 0.04	34.65 ± 0.71
PE-ALC	0.98 ± 0.06	32.97 ± 0.88
PE/ACE-CAP	1.36 ± 0.05	40.14 ± 1.00
PE/ACE-ALC	1.10 ± 0.02	37.08 ± 0.89
ACE-CAP	0.38 ± 0.03	74.16 ± 1.64
ACE-ALC	0.46 ± 0.03	73.36 ± 1.33

5.3.2 Composition of absolutes

5.3.2.1 GC-TOFMS and HS-SPME

The results from the GC-MS analysis of the chemical composition of the flower absolutes, after direct injection of derivatized samples, are presented in Figure 5.2 and Table 5.2.

Data revealed that the profile distribution of compounds is similar regardless of the provenience of flowers, extraction solvents, or extraction methods (see all chromatograms in Appendix C). However, it can be noticed that the peaks corresponding to the fatty acids palmitic acid (42), linoleic acid (45), α -Linolenic acid (46), and 11-cis-Octadecenoic acid (47) are higher in PE absolutes, compared with PE/ACE and ACE absolutes, and that these are higher when performing maceration instead of Soxhlet extraction.

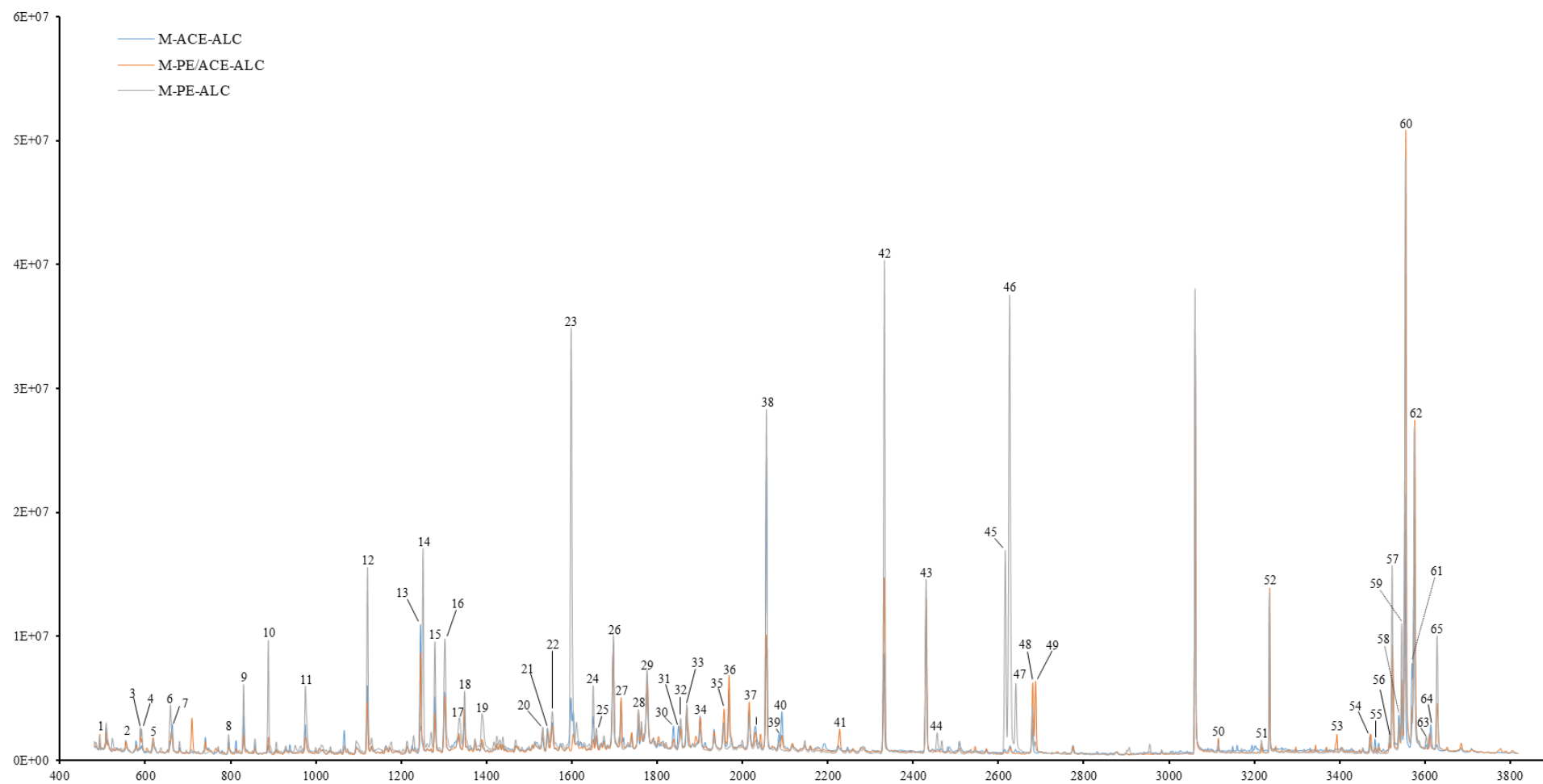


Figure 5.2 — Example of CG-MS chromatogram (TIC) of absolutes: macerations with 100% acetone (M-ACE-ALC), mixture (1:1) of petroleum ether and acetone (M-PE/ACE-ALC), and 100% petroleum ether (M-PE-ALC), with flowers from Alcobaça.

Table 5.2 — Absolute components tentatively Identified by GC-TOFMS by direct injection of derivatized samples; RT: retention time; RI: retention Index; ID: tentative identification based on RI and/or mass spectrum (MS).

No.	Compound (CAS, when available)	Formula (MW)	RT	RI	RI (lit.)	ID	Ref.
1	N-Butylamine 2TMS (18394-04-2)	C ₁₀ H ₂₇ NSi ₂ (217.5)	8.22	1116	1123	RI/MS	(Jiye et al., 2005)
2	Ethanediamide, N,N'-dibutyl- (14040-75-6)	C ₁₀ H ₂₀ N ₂ O ₂ (200.3)	9.23	1151		MS	
3	Unknown		9.80	1171			
4	Unknown		9.87	1173			
5	Unknown		10.28	1188			
6	2-Ketobutyric acid eo-TMS	C ₉ H ₁₉ NO ₃ Si (217.3)	10.98	1211		MS	(Lee et al., 2002)
7	Propanedioic acid, 2TMS (18457-04-0)	C ₉ H ₂₀ O ₄ Si ₂ (248.4)	11.05	1214	1218	RI/MS	
8	Unknown		13.25	1291			
9	N-Formylglycine, 2TMS (55517-31-2)	C ₉ H ₂₁ NO ³ Si ² (247.4)	13.83	1309	1371	RI/MS	(Asai et al., 2017)
10	N,O-Bis(tert-butyl dimethylsilyl)carbamate	C ₁₃ H ₃₁ NO ₂ Si ₂ (289.6)	14.80	1339		MS	
11	Methylmalonic monoamide, O,O'-bis(trimethylsilyl)-	C ₁₀ H ₂₃ NO ₃ Si ₂ (261.5)	16.25	1383		MS	(Isidorov et al., 2005)
12	Monoamidoethylmalonic acid, 3TMS	C ₁₄ H ₃₃ NO ₃ Si ₃ (347.7)	18.67	1452		MS	
13	Monoamidomalonic acid, tris(trimethylsilyl)	C ₁₂ H ₂₉ NO ₃ Si ₃ (319.6)	20.77	1511		MS	
14	p-Anisic acid, TMS (2078-14-0)	C ₁₁ H ₁₆ O ₃ Si (224.3)	20.85	1513	1526	RI/MS	
15	Unknown		21.32	1526			
16	N-(6-formamidoethyl)formamide (35161-65-0)	C ₈ H ₁₆ N ₂ O ₂ (172.2)	21.70	1538		MS	Zaikin (2008) ^a
17	Fumaric acid, isobutyl 3-methylbut-2-yl ester	C ₁₃ H ₂₂ O ₄ (242.3)	22.23	1553	1535	RI/MS	
18	Unknown		22.48	1558			
19	Unknown		23.15	1577			
20	Unknown		25.53	1644			
21	Alanylthreonine, 2TMS derivative (I)	C ₁₃ H ₃₀ N ₂ O ₄ Si ₂ (334.6)	25.73	1650	1723	RI/MS	(Tret'jakov, 2007)
22	Alanylthreonine, 2TMS derivative (II)	C ₁₃ H ₃₀ N ₂ O ₄ Si ₂ (334.6)	25.90	1655	1723	RI/MS	(Tret'jakov, 2007)
23	8-Heptadecene (54290-12-9)	C ₁₇ H ₃₄ (238.5)	26.63	1676	1677	RI/MS	(Qiming et al., 2006)
24	Heptadecane (629-78-7)	C ₁₇ H ₃₆ (240.5)	27.50	1700	1700	RI/MS	(Popova et al., 2020)
25	Unknown		27.62	1704			
26	L-Aspartic acid, 3TMS (55268-53-6)	C ₁₃ H ₃₁ NO ₄ Si ₃ (349.6)	28.28	1723		MS	(Isidorov et al., 2008)
27	2-(Ethylamino)ethanol, N,O-bis(trimethylsilyl)	C ₁₀ H ₂₇ NOSi ₂ (233.5)	28.57	1732		MS	
28	Unknown		29.27	1751			
29	3-Amino-5-methylisoxazole, N-tert-butyl dimethylsilyl (I)	C ₁₀ H ₂₀ N ₂ OSi (212.4)	29.60	1761		MS	
30	Unknown		30.63	1792			
31	Unknown		30.83	1797			(Isidorov et al., 2008)
32	Azelaic acid, 2TMS (17906-08-0)	C ₁₅ H ₃₂ O ₄ Si ₂ (332.6)	30.90	1799	1806	RI/MS	
33	3-Amino-5-methylisoxazole, N-tert-butyl dimethylsilyl (II)	C ₁₀ H ₂₀ N ₂ OSi (212.4)	31.15	1807		MS	
34	Citrulline, 4TMS	C ₁₈ H ₄₅ N ₃ O ₃ Si ₄ (463.9)	31.68	1822		MS	

Table 5.2 (cont.) — Absolute components tentatively Identified by GC-TOFMS by direct injection of derivatized samples; RT: retention time; RI: retention Index; ID: tentative identification based on RI and/or mass spectrum (MS).

No.	Compound (CAS, when available)	Formula (MW)	RT	RI	RI (lit.)	ID	Ref.
35	Myristic acid, TMS (18603-17-3)	C ₁₇ H ₃₆ O ₂ Si (300.6)	32.60	1850	1850	RI/MS	(Isidorov et al., 2005)
36	Leucylglycine, 2TMS	C ₁₄ H ₃₂ N ₂ O ₃ Si ₄ (332.6)	32.80	1856		MS	
37	Unknown		33.60	1880			
38	Nonadecane (629-92-5)	C ₁₉ H ₄₀ (268.5)	34.27	1900	1900	RI/MS	(Popova et al., 2020)
39	Heptadecanal (629-90-3)	C ₁₇ H ₃₄ O (254.5)	34.77	1916	1922	RI/MS	(Tkachev et al., 2000)
40	Tetrahydroactinidiolide (16778-27-1)	C ₁₁ H ₁₈ O ₂ (182.3)	34.88	1920		MS	
41	β-D-Glucopyranose, 5TMS (2775-90-8)	C ₂₁ H ₅₂ O ₆ Si ₅ (541.1)	37.13	1990	1971	RI/MS	(Tret'akov, 2011)
42	Palmitic acid, TMS (55520-89-3)	C ₁₉ H ₄₂ O ₆ Si (328.6)	38.85	2047	2050	RI/MS	(Kim, 2009)
43	Heneicosane (629-94-7)	C ₁₉ H ₄₂ (296.6)	40.50	2100	2100	RI/MS	(Popova et al., 2020)
44	10-Heptadecenoic acid, (Z)-, TMS	C ₂₀ H ₄₀ O ₂ Si (340.6)	40.93	2115	2126	RI/MS	(Tret'akov, 2007)
45	Linoleic acid, TMS (56259-07-5)	C ₂₁ H ₄₀ O ₂ Si (352.6)	43.60	2216	2209	RI/MS	(Tret'akov, 2007)
46	α-Linolenic acid, TMS (97844-13-8)	C ₂₁ H ₃₈ O ₂ Si (350.6)	43.78	2230	2219	RI/MS	(Tret'akov, 2007)
47	11-cis-Octadecenoic acid, TMS	C ₂₁ H ₄₂ O ₂ Si (354.7)	44.02	2251	2227	RI/MS	(Tret'akov, 2007)
48	Stearic acid, TMS (18748-91-9)	C ₂₁ H ₄₄ O ₂ Si (356.7)	44.65	2302	2279	RI/MS	(Karpe et al., 2015)
49	Tributyl acetylcitrate (77-90-7)	C ₂₀ H ₃₄ O ₈ (402.5)	44.78	2305	2269	RI/MS	(Yang et al., 2019)
50	1-Monopalmitin 2TMS (1188-74-5)	C ₂₅ H ₅₄ O ₄ Si ₂ (474.9)	51.92	2591	2600	RI/MS	(Frag et al., 2021)
51	2-Monostearin 2TMS (53336-13-3)	C ₂₇ H ₅₈ O ₄ Si ₂ (502.9)	53.60	2750	2772	RI/MS	(Isidorov et al., 2007)
52	Glycerol monostearate, 2TMS (1188-75-6)	C ₂₇ H ₅₈ O ₄ Si ₂ (502.9)	53.92	2782	2777	RI/MS	(Frag et al., 2021)
53	Glyceryl diacetate 1-oleate (55401-64-4)	C ₂₅ H ₄₄ O ₆ (440.6)	56.55	3100		MS	
54	Pseudotaraxasterol (=Urs-20-en-3-ol, (3b, 18a, 19a)-) (464-98-2)	C ₃₀ H ₅₀ O (426.7)	57.90	3241		MS	
55	Stigmasterol, TMS (14030-29-6)	C ₃₂ H ₅₆ O ₂ Si (484.9)	58.05	3259	3262	RI/MS	(Radulović and Dordević, 2011)
56	β-Amyrone (638-97-1)	C ₃₀ H ₄₈ O (424.7)	58.62	3310	3328	RI/MS	(Andriamaharavo, 2014)
57	Unknown		58.72	3317			
58	β-Amyrin, TMS (1721-67-1)	C ₃₃ H ₅₈ O ₂ Si (498.9)	58.98	3339	3348	RI/MS	(Todua, 2011)
59	Germanicol (465-02-1)	C ₃₀ H ₅₀ O (426.7)	59.08	3347	3282	RI/MS	(Ivanov et al., 2018)
60	Lup-20(29)-en-3-one (1617-70-5)	C ₃₀ H ₄₈ O (424.7)	59.23	3358	3384	RI/MS	(Andriamaharavo, 2014)
61	α-Amyrin, TMS	C ₃₃ H ₅₈ O ₂ Si (498.9)	59.48	3379	3382	RI/MS	(Todua, 2011)
62	Lupeol, TMS (I)(55804-02-9)	C ₃₃ H ₅₈ O ₂ Si (498.9)	59.57	3386	3435	RI/MS	(Andriamaharavo, 2014)
63	Moretenone (1812-63-1)	C ₃₀ H ₄₈ O (424.7)	60.17	3432		MS	
64	Unknown		60.22	3436		MS	
65	Lupeol, TMS (II) (55804-02-9)	C ₃₃ H ₅₈ O ₂ Si (498.9)	60.47	3456	3435	RI/MS	(Andriamaharavo, 2014)

^a Zaikin V. G., Personal Communication: Retention indices measured during the period 2007 to 2008, 2008. Available at: NIST Chemistry Webbook; <http://webbook.nist.gov> (last accessed 20th August 2022)

More accurate identification of volatiles was made by HS-SPME. The results of GC-MS chromatography after HS-SPME extraction are presented in Figure 5.3 and Table 5.3.

It can be observed that the most volatile chemicals present in the absolutes belong to the hydrocarbons, alcohols, and aldehydes families. As before, data revealed that the profile distribution of compounds did not significantly differ between the provenience of flowers, extraction solvents, or extraction methods (see all chromatograms in Appendix C, Figures C1 to C8). The main difference is that the compounds nonanal (8), benzeneethanol (9), and anisaldehyde (10) are only evident in the PE absolutes, regardless of the provenience or extraction method. Another visible difference was registered in PE and ACE extracts obtained by Soxhlet extraction from the flowers of Alcobaça, which showed a much higher peak corresponding to methyl *p*-anisate (15) when compared with the remaining absolutes.

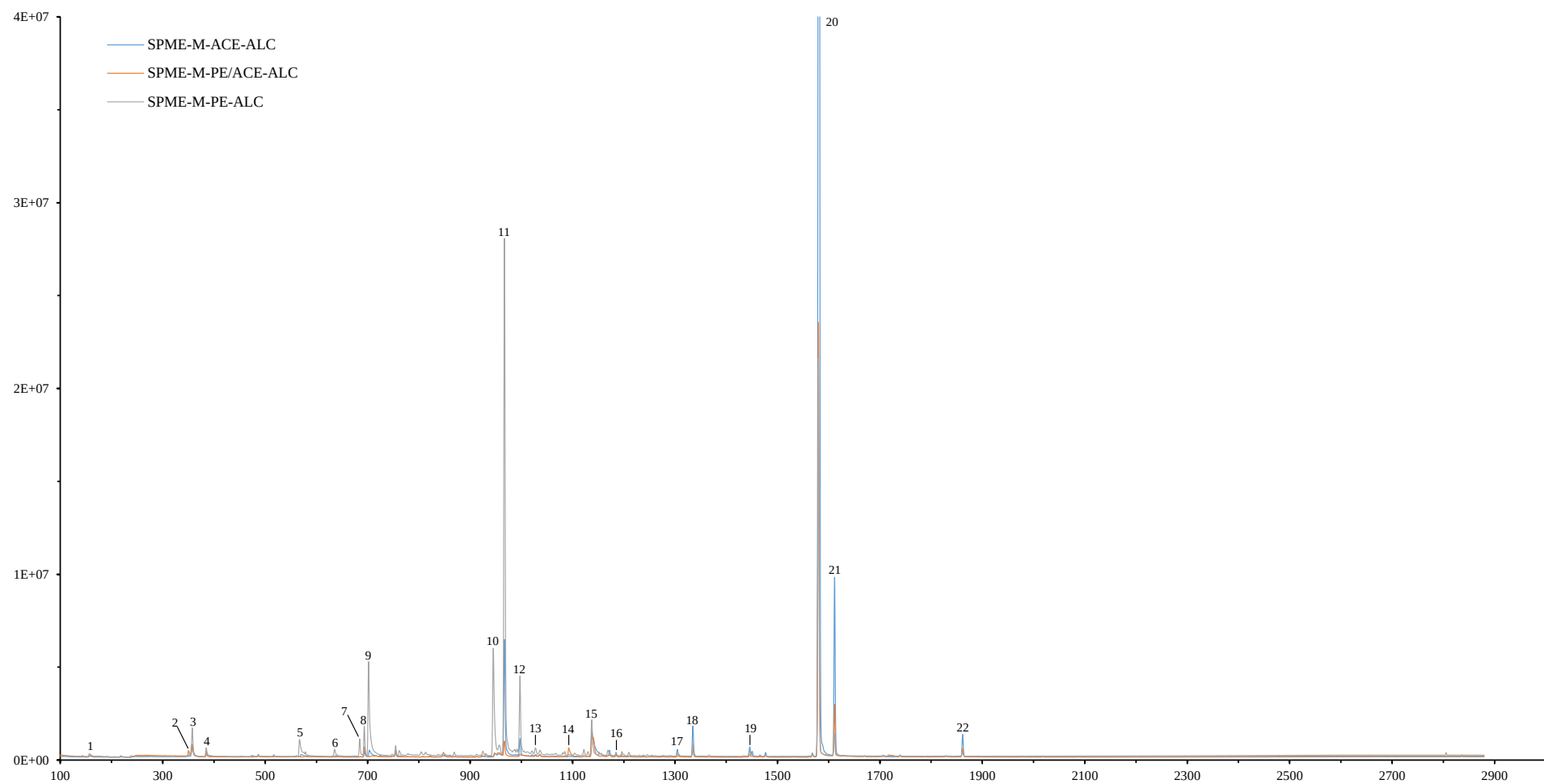


Figure 5.3 — Example of CG-MS chromatogram (TIC) after HS-SPME of absolutes: macerations with 100% acetone (M-ACE-ALC), mixture (1:1) of petroleum ether and acetone (M-PE/ACE-ALC), and 100% petroleum ether (M-PE-ALC), with flowers from Alcobaça.

Table 5.3 — Absolute components tentatively Identified by GC-TOFMS after HS-SPME; RT: retention time; RI: retention Index; ID: tentative Identification based on RI and/or mass spectrum (MS).

No.	Compound (CAS, when available)	Formula (MW)	RT	RI	RI (lit.)	ID	Ref.
1	2-Pentanol, 2-methyl- (590-36-3)	C ₆ H ₁₄ O (102.2)	2.62	798	784	RI/MS	(Begnaud et al., 2003)
2	Heptanal (111-71-7)	C ₇ H ₁₄ O (114.2)	5.83	908	901	RI/MS	(Asuming et al., 2005)
3	Carbonic acid, hexyl prop-1-en-2-yl ester	C ₁₀ H ₁₈ O ₃ (186.3)	5.95	912		MS	
4	2-Heptanone, 3-methyl- (2371-19-9)	C ₈ H ₁₆ O (128.2)	6.42	928		MS	
5	Benzenemethanol (100-51-6)	C ₇ H ₈ O (108.1)	9.45	1031	1024	RI/MS	(Van Durme et al., 2007)
6	1-Octanol (111-87-5)	C ₈ H ₁₈ O (130.2)	10.60	1070	1070	RI/MS	(Pino et al., 2005)
7	Linalool (1,6-Octadien-3-ol, 3,7-dimethyl-) (78-70-6)	C ₁₀ H ₁₈ O (154.3)	11.42	1098	1098	RI/MS	(Pino et al., 2005)
8	Nonanal (124-19-6)	C ₉ H ₁₈ O (142.2)	11.55	1103	1103	RI/MS	(Pino et al., 2005)
9	Benzeneethanol (60-12-8)	C ₈ H ₁₀ O (122.2)	11.72	1108	1114	RI/MS	(Pino et al., 2005)
10	p-anisaldehyde (123-11-5)	C ₈ H ₈ O ₂ (136.2)	15.75	1250	1251	RI/MS	(Pino et al., 2005)
11	Furan, tetrahydro-2,2-dimethyl-5-(1-methylethyl)- (97265-00-4)	C ₉ H ₁₈ O (142.2)	16.12	1263		MS	
12	Oxetane, 2-methyl-4-propyl- (7045-79-6)	C ₇ H ₁₄ O (114.2)	16.62	1281		MS	
13	Tridecane (629-50-5)	C ₁₃ H ₂₈ (184.4)	17.12	1299	1300	RI/MS	(Goodner, 2008)
14	Triacetin (102-76-1)	C ₉ H ₁₄ O ₆ (218.2)	18.22	1340	1344	RI/MS	(David et al., 2002)
15	Methyl p-anisate (121-98-2)	C ₉ H ₁₀ O ₃ (166.2)	18.97	1369	1373	RI/MS	(Bader et al., 2003)
16	Tetradecane (629-59-4)	C ₁₄ H ₃₀ (198.4)	19.75	1399	1400	RI/MS	(Goodner, 2008)
17	trans-7-pentadecene (15430-98-5)	C ₁₅ H ₃₀ (210.4)	21.73	1479	1485	RI/MS	(Arnold et al., 2019)
18	Pentadecane (629-62-9)	C ₁₅ H ₃₂ (212.4)	22.25	1499	1500	RI/MS	(Goodner, 2008)
19	3-Hexadecene, (Z)- (34303-81-6)	C ₁₆ H ₃₂ (224.4)	24.08	1577	1587	RI/MS	(Atalar and Türkan, 2018)
20	8-Heptadecene (2579-04-6)	C ₁₇ H ₃₄ (238.5)	26.33	1676	1687	RI/MS	(Arnold et al., 2019)
21	Heptadecane (629-78-7)	C ₁₇ H ₃₆ (240.5)	26.87	1699	1700	RI/MS	(Goodner, 2008)
22	Nonadecane (629-92-5)	C ₁₉ H ₄₀ (268.5)	31.02	1899	1900	RI/MS	(Goodner, 2008)

Amongst the most representative compounds in the produced absolutes, the organic acids *p*-anisic acid, palmitic acid, linoleic acid, and linolenic acid; the ester methyl *p*-anisate; the benzaldehyde anisaldehyde; the hydrocarbons nonadecane, heneicosane, heptadecane, and 8-heptadecene; the pentacyclic triterpenes a-amyrin, b-amyrin, lupenone (Lup-20(29)-en-3-one), and lupeol; and glycerol monostearate, have particular importance due to their known applications and biological activities. These compounds were already reported in *A. dealbata* absolute by Perriot et al. (Perriot et al., 2010). *p*-anisic acid has a mild fragrance and antioxidant, anti-inflammatory, anti-tumor, and antimicrobial properties, thus being common in cosmetic formulations (Lavery et al., 2015; Maksymiak et al., 2020). Palmitic acid is a saturated fatty acid that has antifungal properties (Altieri et al., 2007; Liu et al., 2008) and emollient properties,

being used to improve the textural properties of food and cosmetic preparations (Lima et al., 2011; Oliveira et al., 2016). Linolenic and linoleic acids are unsaturated fatty acids commonly used in cosmetic products as emollients or moisturizers (Ahmad and Ahsan, 2020; Muhamad et al., 2017), and present also antimicrobial and anti-inflammatory properties (Kusumah et al., 2020; Pauls et al., 2018). Methyl *p*-anisate and anisaldehyde are the main responsible compounds for a characteristic anise-like odor, being commonly used in food and fragrance industries (Kleofas et al., 2015), with the latter having reported antimicrobial activity (Adewunmi et al., 2020) and efficacy as an insect repellent (Showler and Harlien, 2021). Nonadecane is a long chain saturated aliphatic hydrocarbon used as a fragrance agent in cosmetics and perfumes, which was already found in other plants, such as rose (*Rosa damascena* Mill. and *Rosa moschata* Herrm.) and marine (*Heliotropium arborescens* L.) flowers, or sumach (*Rhus coriaria* L.) fruits (Bahar and Altug, 2009; Karami and Jandoust, 2016; Kays et al., 2005). Heneicosane and heptadecane are other saturated hydrocarbons, also used in fragrances (Kaur et al., 2018), with the former showing antimicrobial capacity (Vanitha et al., 2020), and the latter demonstrating anti-inflammatory properties (Kim et al., 2013). 8-heptadecene is an unsaturated hydrocarbon with antiparasitic capacity (Milani et al., 2004; Nazzi et al., 2002). Glycerol monostearate is used in the food industry as an emulsifier in some food products (Manley, 2011). The pentacyclic triterpenes α -amyrin, β -amyrin, lupeol, and lupenone (Lup-20(29)-en-3-one) have special importance due to their remarkable biological activities. α -amyrin and β -amyrin demonstrated antihyperglycemic, hypolipidemic, and anti-inflammatory properties (Pinto et al., 2008; Santos et al., 2012). Lupenone (Lup-20(29)-en-3-one) revealed good therapeutic potential in inflammation, virus infection, diabetes, cancer, and treatment of Chagas disease (Xu et al., 2018). Lupeol is known as well for its important health benefits, revealing anticancer, anti-inflammatory, antidiabetic, cardioprotective, skin protective, antiprotozoal, antimicrobial, or nephroprotective properties (Liu et al., 2021; Sharma et al., 2020). The compound furan, tetrahydro-2,2-dimethyl-5-(1-methylethyl)-, which showed a significant peak (peak 11 in the GC-MS chromatogram after HS-SPME), was already found in essential oil from the leaves of *Citrus aurantifolia* (Sarma et al., 2019).

5.3.2.2 Principal Component Analysis (PCA)

Principal Component Analysis (PCA) is a statistical technique very useful to interpret large datasets, as it allows reducing their dimensionality in an interpretable way, by preserving as much statistical information as possible (Jolliffe et al., 2016). PCA was used in this work to determine whether there were differences between the absolute samples produced by the different

extraction methods (maceration and Soxhlet), different extraction solvents (PE, PE/ACE and ACE), and using flowers from two locations (ALC and CAP), and which identified compounds were the most responsible for the observed variation between absolute samples.

All 65 compounds identified by GC-MS of the 12 derivatized absolute samples were analyzed by PCA based on the relative amount in each sample, as well as the 22 compounds identified after SPME extraction, resulting in datamatrices of 12 x 65 and 12 x 22, separately analyzed. In general, the maximum variation in information can be interpreted by the two first principal components, named PC1 and PC2, respectively. In the case of the derivatized samples, PC1 and PC2 explained 69.85 and 15.10% of the variation, respectively, meaning that 84.95% of the variation was explained by these two components. In the case of volatiles analyzed after SPME extraction, 56.48% of the variation was explained by PC1 and 28.68% by PC2, resulting in a total of 85.16% of variation explained by the first two components.

In PCA, the score plot places each sample in the bi-dimensional space, while the loading plot places the compounds (loadings). However, both scores and loading plots can be visualized and interpreted together in a single graphical display, denominated biplot. Biplots of the first two components for the derivatized samples data and HS-SPME are presented in the Figures 5.4 and 5.5, respectively.

Figure 5.4 shows a clear separation of absolute samples by PC1, with the formation of a group consisting of samples obtained with ACE, and a group formed with absolute samples obtained with the mixture PE/ACE on the opposite side. Nonadecane (38) is undoubtedly the one that is most responsible for this variation. ACE absolutes are characterized by higher contents of nonadecane, while PE/ACE absolutes have lower contents of this compound. In turn, by PC2 it is evident the group of PE samples on the positive side, characterized by higher contents of *p*-Anisic acid (14) and palmitic acid (42), and lower contents of leucylglycine (36) comparing with ACE and PE/ACE absolutes.

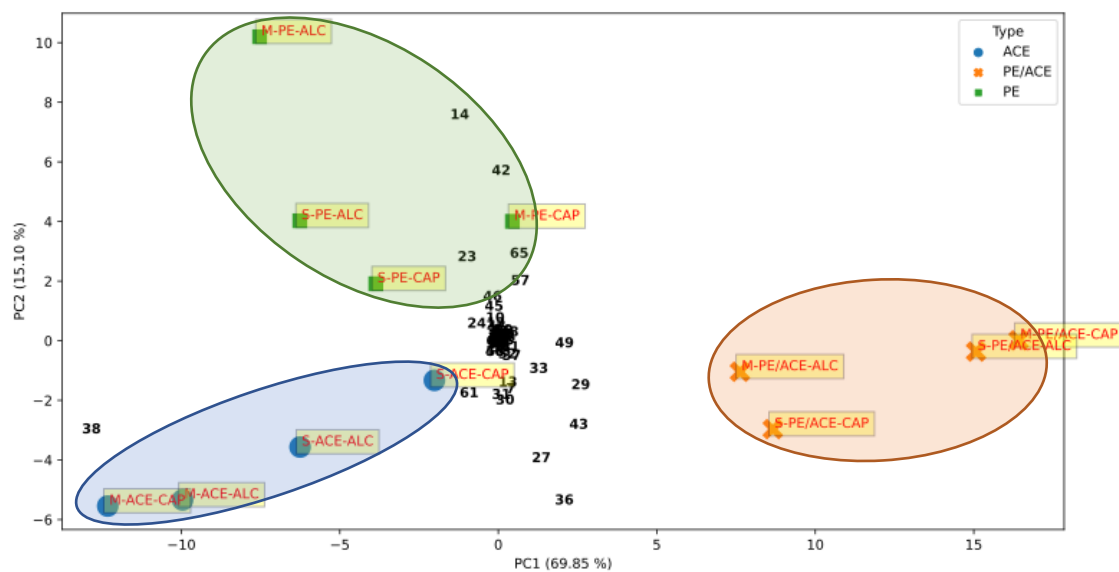


Figure 5.4 — Biplot of derivatized samples. The colored shapes represent samples, here cleanly separated by the extraction solvent used. The numbers represent the loadings, and refer to the peak numbers in table 5.2. Loadings were scaled to fit the biplot.

For the derivatized samples, PCA analysis didn't show substantial differences between maceration and Soxhlet methods, or between the flower provenience (Caparica and Alcobaça), indicating that the extraction solvent used was the factor that contributed the most to the differences observed in the composition of the absolutes.

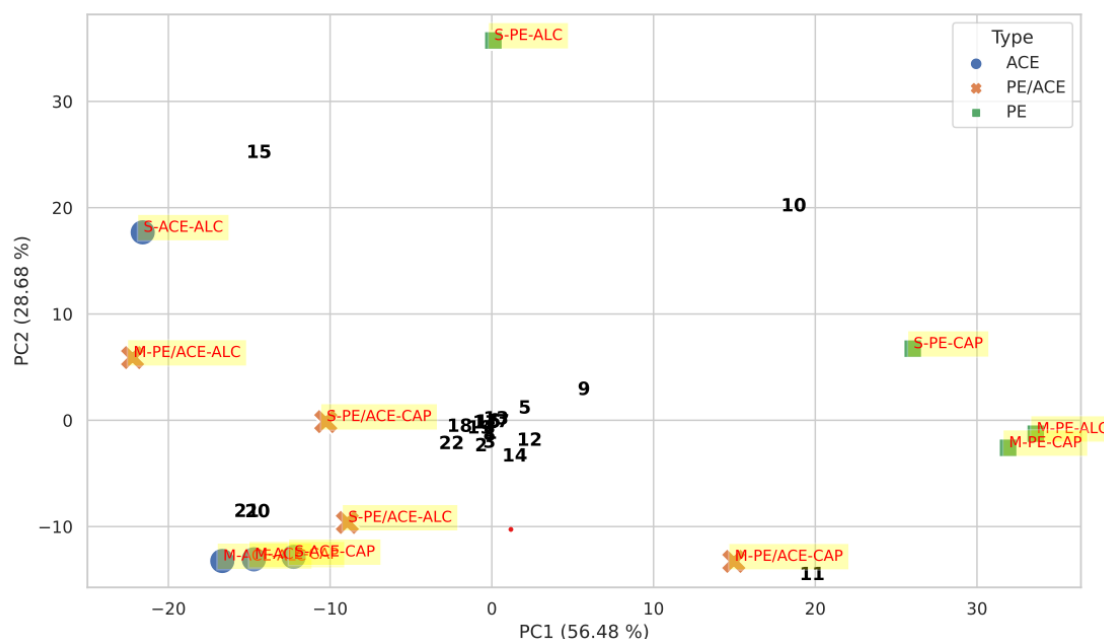


Figure 5.5 — Biplot of HS-SPME. The coloured shapes represent samples. The numbers represent the loadings, and refer to the peak numbers in table 5.3. Loadings were scaled to fit the biplot.

Figure 5.5 shows that no clear categorization based on flower provenience, the extraction solvents, or the extraction method was observed. The compounds that explain most of the variation are the compounds *p*-anisaldehyde (10), Furan, tetrahydro-2,2-dimethyl-5-(1-methylethyl)- (11), methyl *p*-anisate (15), 8-heptadecene (20), and heptadecane (21). There is a group formed by M-PE-CAP, M-PE-ALC, and S-PE-CAP absolutes clearly demarked on the positive side of PC1, showing high contents of *p*-anisaldehyde and furan, tetrahydro-2,2-dimethyl-5-(1-methylethyl)-, and lower contents of methyl *p*-anisate, 8-heptadecene, and heptadecane; a group involving M-ACE-ALC, M-ACE-CAP, S-ACE-CAP, and S-PE/ACE-ALC absolutes on the negative sides of PC1 and PC2, showing high contents of 8-heptadecene and heptadecane and lower contents of *p*-anisaldehyde; and a group formed by S-ACE-ALC and M-PE/ACE-ALC absolutes, characterized by high contents of methyl *p*-anisate and lower contents of furan, tetrahydro-2,2-dimethyl-5-(1-methylethyl)-. Isolated in the biplot there is S-PE-ALC absolute, with high levels of anisaldehyde and methyl *p*-anisate, and low levels of furan, tetrahydro-2,2-dimethyl-5-(1-methylethyl)-, 8-heptadecene, and heptadecane; M-PE/ACE-CAP absolute, with high levels of furan, tetrahydro-2,2-dimethyl-5-(1-methylethyl)- and relatively lower contents of *p*-anisaldehyde, methyl *p*-anisate, 8-heptadecene, and heptadecane; and the S-PE/ACE-CAP absolute, showing intermediate values of the referred compounds.

5.4 Conclusions

The chromatogram profile of the produced absolutes was similar, regardless of the provenience of the flowers, the extraction solvents, or the extraction methods. However, PCA analysis showed that the main differences were due to the extraction solvents used, in the case of data obtained after direct injection of derivatized samples. In what concerns the more volatile components, no clear categorization based on flower provenience, the extraction solvents, or the extraction method was observed.

The use of 100% of petroleum ether allowed extraction of some components that were extracted in much lower amounts when using 100% acetone or the mixture of petroleum ether and acetone, namely nonanal, benzeneethanol, and *p*-anisaldehyde, which may contribute to the different odor present in PE extracts. Nevertheless, the use of acetone, or the mixture of petroleum ether and acetone, allows the production of distinct absolutes and simultaneously produces aqueous phases that may be rich in phenolic compounds present in the flowers.

The results showed that the Portuguese *A. dealbata* is suitable for the production of absolutes in yields of the same magnitude as the ones produced in the most typical region where they are commercially explored, such as in France, and with a similar profile of compounds.

The exploitation of *A. dealbata* flowers may contribute to an economically sustainable manner of controlling its spread all over the territory, by preventing the seed dissemination.

CHEMICAL AND FUNCTIONAL CHARACTERIZATION OF EXTRACTS FROM LEAVES AND TWIGS OF *ACACIA DEALBATA*

Abstract: The recovery of bioactive extracts from *Acacia dealbata* leaves and twigs was evaluated, and their chemical composition and functional properties were determined. Fresh and air-dried samples were extracted by maceration at room temperature and by hot extraction at 60 °C using aqueous solutions of acetone, ethanol, and methanol. The highest extraction yields (14.8 and 12.0% for dried leaves and twigs, respectively) were obtained with 70% acetone, for both extraction procedures. Extracts were characterized for total phenolics content (TPC), total flavonoid content (TFC) and total proanthocyanidin content (TPrAC). Bioactive extracts with high TPC (526.4 mg GAE/g extract), TFC (198.4 mg CatE/g extract), and TPrAC (631.3 mg PycE/g extract) were obtained using maceration, a technically simple and low-energy process. The non-polar fraction of selected extracts was characterized using gas chromatography and time of flight mass spectrometry (GC-TOFMS). The main components detected were phytol, squalene, α -tocopherol, lupenone, and lupeol. The antioxidant activity of the extracts was characterized through DPPH and FRAP assays. Antimicrobial activity of the extracts against different bacteria was also determined. The highest DPPH and FRAP activities were obtained from dried twigs from Alcobaça (1068.3 mg TE/g extract and 9194.6 $\mu\text{mol Fe}^{2+}$ /g extract, respectively). Extracts from both leaves and twigs showed antimicrobial properties against *Staphylococcus aureus*, *Staphylococcus epidermidis*, methicillin resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis*, *Bacillus cereus*, *Streptococcus mutans*, and *Streptococcus mitis*. The results obtained demonstrate the feasibility of recovering valuable components from these biomass fractions that may be further valorized for energy production in a biorefinery concept.

6.1 Introduction

The genus *Acacia* comprises more than 1350 species distributed throughout tropical and warm temperate areas of the world (Maslin, 2015). Most of those species are native to Australia but spread all over the world due to a wide variety of useful applications such as sand and dunes stabilization, extraction of tannins, essences or gums, valorization as timber or fodder crop, and production of biofuels (Breton et al., 2008; Kull et al., 2011; Seigler, 2003). The ability to easily adapt to changing environments, the large seed production and accumulation of massive seed banks for long periods, the high capacity for reproduction after fires and cuttings, and the allelopathic properties are some of the characteristics that contribute to the success of *Acacia* spp. as invaders, leading to negative impacts on ecosystem biodiversity (Fuentes-Ramírez et al., 2011; Le Maitre et al., 2011; Lorenzo et al., 2010a).

A. dealbata is one of the most widespread *Acacia* species (Lorenzo et al., 2010a; Richardson and Rejmánek, 2011) and is classified as invasive in Portuguese territory (Ministério do Ambiente, 1999). Periodic removal operations to minimize its proliferation generate high amounts of biomass that is usually burned for energy production or landfilled, but these solutions are not economically sustainable due to the high costs of biomass collection and transportation. However, different fractions of *Acacia* spp. biomass have been evaluated as raw materials for the production of functional extracts that can be used in the nutraceutical, cosmetic, or food industries. In fact, it is widely documented that bark, wood, leaves, flowers, pods, seeds or roots of *Acacia* spp. are rich in bioactive secondary metabolites (e.g. amines and alkaloids, cyanogenic glycosides, cyclitols, fatty acids and seed oils, gums, non-protein amino acids, terpenes, tannins and other flavonoids, and simple phenolics) (Jæger et al., 2019; Seigler, 2003) and have been used in traditional medicine for a wide range of ailments, such as diabetes, worm infection, dysmenorrhea, eczema, malaria, gout, jaundice, abdominal pain, kidney problems, constipation, leprosy, piles, pneumonia, rheumatism, fever, and cancer (Subhan et al., 2018).

Plant extracts are known for their ability to act as antioxidants and reduce oxidative stress (Miguel-Chávez, 2017), a physiologic condition considered to play a key role in the pathogenesis of several degenerative diseases, such as cardiovascular diseases, diabetes neurodegeneration, or cancer (Santos-Sánchez et al., 2019). Additionally, has been reported that plant secondary metabolites also possess antimicrobial properties, which is important in the development of alternatives to antibiotics due to the increasing resistance to conventional antimicrobial agents (Ghannoum M. A. and Rice L. B., 1999). Therefore, the production of bioactive

extracts from *A. dealbata* biomass is an additional pathway for the valorization of these biomass materials, complementing energy applications and contributing to the sustainability of the forest cleaning and management actions, reducing the risk of fire and improving the social-economic development of rural areas.

Extraction of value-added components from *Acacia* spp. has been focused mainly on the bark, flower, wood, and leaves as reviewed by Correia et al. (Correia et al., 2020). Concerning the leaves fraction, antioxidant and/or antimicrobial activities have been determined for *A. farnesiana* (Ramli et al., 2011), *A. karroo* (Cock and van Vuuren, 2015; Nielsen et al., 2012; Nyila et al., 2012; Priyanka et al., 2015), *A. longifolia* (Lima et al., 2013), *A. pycnantha* (Edrah et al., 2018; Mahmoud et al., 2016), *A. saligna* (El-Toumy et al., 2010; Gedara and Galala, 2014; Gumgumjee and Hajar, 2015; Noreen et al., 2017). The antioxidant and antimicrobial activities of *A. dealbata* leaves were evaluated by Borges et al. (Borges et al., 2020), for acetonetic and ethanolic extracts of fresh *A. dealbata* leaves, obtained with different extraction methods. Ethanolic extracts of dried *A. dealbata* leaves were also found to have antimicrobial activity against the food poisoning agent *Bacillus cereus* (Silva et al., 2016).

Research on extraction of functional components from twigs of *Acacia* spp. is scarce. Extracts of *A. nilotica* twigs were characterized for antimicrobial activity against oral pathogens (Arshad et al., 2017; Kumari et al., 2020b, 2020a, 2019), while extracts of *A. pennata* twigs were described as having some potential application in the prevention of Alzheimer's disease (Lomarat et al., 2015).

The extraction methods explored so far included solid-liquid extraction for a pre-determined period under agitation at room temperature (1 h (Borges et al., 2020), 8 h (Arshad et al., 2017), or 2 days (Kumari et al., 2020a; Silva et al., 2016)) ultrasound-assisted extraction (Borges et al., 2020), Soxhlet (Borges et al., 2020; Lomarat et al., 2015), microwave-assisted extraction (Borges et al., 2020), cold percolation (Kumari et al., 2019), and supercritical fluid extraction (Kumari et al., 2020b; Rodrigues et al., 2021). Most of these methods are energy-consuming and/or involve high investment costs and may promote thermal degradation of the extract components (Azwanida, 2015).

This study aimed to investigate the production of bioactive extracts from leaves and twigs of *A. dealbata*, using maceration at room temperature, a method with low energy requirements and easy to implement on an industrial scale. Extraction with different aqueous solvents (acetone, ethanol, and methanol) was applied to fresh and dried biomass, collected in two different locations in order to evaluate the influence of solvent polarity, biomass water content, and geographic origin on the characteristics of the extracts, namely their yield, composition, and

properties. To the best of our knowledge, this is also the first study on the production and characterization of bioactive extracts from *A. dealbata* twigs. The extracts were characterized for mass yield, total phenolic content, total flavonoid content, total proanthocyanidins content, *in vitro* antioxidant activity tests, and antimicrobial activity against several bacteria and yeasts. To identify non-polar components that might contribute to the antioxidant or antimicrobial properties of the extracts, a representative set of leaves and twigs extracts was fractionated and characterized by GC-TOFMS.

6.2 Materials and methods

6.2.1 Plant material

Branches of at least ten different *A. dealbata* trees of Caparica (CAP) and Alcobaça (ALC) regions were collected. Samples of around 500g of branches were collected from each tree and combined to obtain a composite sample from that geographical origin. For each composite sample, leaves were separated from the twigs and both fractions were analyzed separately. Fresh and dried leaves and twigs were manually cut into small pieces about 1 cm in length before impregnation with the solvent.

6.2.2 Chemicals and reagents

Acetone, ethanol 96°, methanol, and petroleum ether used in the extraction processes were purchased from CarloErba Reagents (France). Folin-Ciocalteu reagent (Panreac, Spain), sodium carbonate (Labkem, Spain), and gallic acid (Merck, Germany) were used to determine phenolics content. The reagents sodium nitrite (NaNO_2), sodium hydroxide (NaOH), and aluminium chloride (AlCl_3) used in the determination of flavonoids were all purchased from Merck (Germany). Butanol (Panreac, Spain), ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) (Panreac, Spain), hydrochloric acid (Chem-Lab, Belgium), and Pycnognol® (generously provided by Horphag Research) were used in the acid-butanol assay. The reagent 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA) was used in the DPPH assay. The reagents 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), acetate buffer 3.6, iron (III) chloride (FeCl_3), and iron (II) sulfate (FeSO_4) used in the ferric reducing antioxidant power (FRAP) assay were also purchased from Merck (Germany). Ketoconazole, ofloxacin and vancomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brain heart infusion agar (BHIA) was purchased from Merck (Darmstadt,

Germany). Mueller-Hinton agar (MHA), Sabouraud dextrose agar (SDA) and Trypto casein-soy agar (TSA) were purchase from Biokar diagnostics (Allonne, France).

6.2.3 Extraction procedure

A portion of leaves and twigs was processed in a fresh state, during the first 24 h from harvest, while the remaining biomass was left to dry in air, at room temperature, until a constant weight was attained. The maceration at room temperature was performed for 48 h in the case of the leaves and 24 h for the twigs. Extraction times were selected for leaves and twigs as the maceration time necessary to achieve constant values of extract yield and extract composition, according to a previous study (Appendix D1; Table D1). Hot extraction was performed at 60 °C for 1 h, for both leaves and twigs, to evaluate the effect of temperature in extract yield and properties. Higher temperatures were not selected to avoid thermal degradation of the extracts. Maceration and hot extraction were performed in a single step, at a biomass:solvent ratio of 1:6 (w:v), using 70% of aqueous acetone (70% ACE), 70% aqueous ethanol (70% ET), and 70% of aqueous methanol (70% MET) as extraction solvents. After the extraction period, the liquid extract was separated from the biomass by filtration and stored at – 20 °C until analysis. An adequate aliquot was evaporated to dryness at reduced pressure, to determine the mass yield (expressed as percentage, on a wet basis). The extraction process was made in duplicate for each one of the four biomass samples of leaves and twigs (fresh and dried material, from Caparica and Alcobaça).

Extraction of spent raw material was also assessed, after the removal of lipophilic components by petroleum ether. Dried leaves and twigs were extracted with petroleum ether, by maceration at room temperature using a biomass:solvent ratio of 1:5 and the lipophilic extracts were stored for subsequent analysis, not included in this study. The spent biomass was left to dry in air at room temperature and then submitted to extraction of polar components by maceration at room temperature with 70% ACE, at the same conditions that were used for the raw leaves and twigs. Mass yields were determined as described above and the extracts were stored at -20 °C until analysis.

6.2.4 Extracts characterization

Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity by DPPH and FRAP assays were performed as described in section 3.2.4.

Total proanthocyanidin content (TPrAC) was determined by the butanol-HCl assay as described in section 4.2.4.

In all these characterization assays, two replicates of each sample were analyzed in triplicate.

6.2.5 Antimicrobial activity

The antimicrobial activity was determined as described in section 3.2.6.

6.2.6 GC-TOFMS analysis

The extracts selected for analysis of non-polar components were derivatized using a procedure adapted from Popova et al. (Popova et al., 2020). Briefly, 2 mL of each extract was evaporated to dryness and the residue was diluted in 100 mL of pyridine and 100 mL of the derivatizing agent N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA); the mixture was incubated at room temperature for 12h, diluted in petroleum ether (1.5 mL), dried with anhydrous sodium sulphate and kept at -5 °C until analysis. Analyses were performed with an Agilent 7890B (Palo Alto, California, USA) gas chromatograph equipped with a multi-mode inlet. An Agilent HP-5MS UI fused silica capillary column (30m × 0.25 mm i.d., 0.25 µm d f - film thickness) was used in all separations. An aliquot of the derivatized sample (1 µL) was injected via a LECO L-PAL3 autosampler fitted with a 10 µL syringe. The injector was operated in solvent vent mode, with the split valve opened at 100 mL/min for 25 seconds, and the injector at 70 °C. Then the split valve was closed for 120 seconds, and the inlet temperature was raised to 300 °C at 700 °C/min. Finally, the split valve was opened again at 20mL/min until the end of the run. The oven program was as follows: 50 °C for 1 min, then 7 °C/min until 100 °C, then 3 °C/min until 220 °C, and finally 10 °C/min until 295 °C, held for 8 minutes. The transfer line to the MS was kept at 300 °C. Detection was performed with a LECO Pegasus BT Time-of-Flight mass spectrometer (Saint Joseph, Michigan, USA). The MS was operated with the ion source at 250 °C, electron ionization at 70 eV, acquisition from m/z 40 to 550, 10 spectra per second and an acquisition delay of 480 second for liquid samples. Data acquisition, system control and spectra deconvolution were performed using LECO ChromaTOF version 5.40. NIST MS Search Program Version 2.3 g was used for spectra matching (NIST, 2015). Linear retention index (LRIs) values for sample peaks were calculated by analyzing the commercial alkane standard solution C8 - C40, using the aforementioned chromatographic conditions (van Den Dool and Dec. Kratz, 1963).

6.2.7 Statistical analysis

Statistical analysis of the results was performed using the SPSS software, version 23 (IBM Analytics, USA). Analysis of variance (ANOVA) was performed to evaluate significant differences between averages and the Tukey post hoc test ($p < 0.05$) was used to perform multiple comparisons between averages.

6.3 Results and discussion

6.3.1 Extract yield and composition

The recovery of bioactive extracts from *A. dealbata* leaves and twigs was evaluated for different extraction methods (maceration at room temperature and hot extraction), different solvents (70% ACE, 70% ET, and 70% MET), different geographical origins (Caparica and Alcobaça) and for different biomass water contents (fresh biomass and dried biomass).

Leaves and twigs were collected in Alcobaça (39°36'36.5"N; 8°59'45.0"W, at 32 – 40 m of altitude) and Caparica (38°37'22.6"N; 9°10'38.5"W, at 44 – 48 m of altitude). Both locations are classified as Csb (Warm-summer Mediterranean climate) according to Köppen-Geiger's classification system for climate, but with some differences between them. Monthly average daily temperatures are in similar ranges (10.6 – 20.4 °C for Alcobaça and 11.6 – 21.9 °C for Caparica), but Alcobaça is characterized by a higher level of annual rainfall (696 mm against 591 mm) and relative humidity (73 – 81% against 68 – 80%) ("Climate-Data.org. Clima Alcobaça,," n.d., "Climate-Data.org. Clima Almada,," n.d.). The fresh leaves and twigs, that were processed in the first 24 hours from the collection, had moisture contents in the ranges of 47.7 – 58.3% and 43.4 – 51.9% , respectively. After air-drying at room temperature, moisture contents were in the range of 8.1 – 9.3% for leaves and 8.8 – 11.2% for twigs.

Extraction yields and compositions (TPC, TFC, and TPrAC contents) for the extracts obtained leaves and twigs, at the different extraction conditions are presented in Table 6.1.

Making an overall analysis of this set of data, significantly higher extraction yields were obtained for dried raw material, and significantly higher TPC, TFC, and TPrAC contents were found in the extracts produced with 70% ACE (with the exception of hot extraction from twigs). The TPC, TFC, and TPrAC contents of twigs extracts with 70% ACE were also higher than those contents in the leaves extracts with the same solvent.

Table 6.1. — Extraction yields (% wet basis), TPC (mg GAE/g extract), TFC (mg CatE/g extract), and TPrAC (mg PycE/g extract) of extracts from fresh and dried leaves and twigs from Alcobaça (ALC) and Caparica (CAP), using different extraction solvents and extraction methods; TPC, TFC, and TPrAC expressed relative to the mass of biomass are presented in the Appendix D1, Table D2.

Samples		Maceration at room temperature			Hot extraction		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
Leaves							
Yield	Fresh (ALC)	12.2 ± 0.1 ^h	10.8 ± 0.1 ^j	11.6 ± 0.1 ⁱ	12.2 ± 0.1 ^h	11.0 ± 0.1 ^j	9.9 ± 0.1 ^l
	Fresh (CAP)	10.5 ± 0.2 ^k	10.0 ± 0.1 ^l	9.8 ± 0.1 ^l	11.7 ± 0.1 ⁱ	9.8 ± 0.1 ^l	9.9 ± 0.1 ^l
	Dried (ALC)	14.8 ± 0.1 ^c	14.4 ± 0.1 ^{de}	14.1 ± 0.1 ^{de}	15.8 ± 0.1 ^a	13.5 ± 0.1 ^f	13.0 ± 0.1 ^g
	Dried (CAP)	14.0 ± 0.1 ^e	14.3 ± 0.1 ^{de}	13.5 ± 0.1 ^f	15.6 ± 0.1 ^a	15.8 ± 0.1 ^a	15.2 ± 0.1 ^b
TPC	Fresh (ALC)	449.1 ± 8.0 ^b	338.9 ± 6.6 ^{ghi}	329.0 ± 7.6 ^{ijk}	428.4 ± 3.7 ^c	361.0 ± 5.9 ^{de}	336.7 ± 6.9 ^{hj}
	Fresh (CAP)	345.8 ± 4.0 ^{fgh}	295.8 ± 6.4 ^m	288.3 ± 3.4 ^{mn}	352.6 ± 5.8 ^{def}	324.5 ± 9.5 ^{kl}	314.7 ± 7.6 ^l
	Dried (ALC)	478.5 ± 4.5 ^a	324.9 ± 3.3 ^{ijkl}	336.1 ± 3.2 ^{hijk}	450.6 ± 3.7 ^b	362.2 ± 3.6 ^d	351.3 ± 5.4 ^{def}
	Dried (CAP)	350.2 ± 4.4 ^{efg}	292.3 ± 4.3 ^{mn}	255.2 ± 5.3 ^p	352.6 ± 5.4 ^{def}	272.4 ± 4.4 ^o	282.4 ± 4.7 ^{no}
TFC	Fresh (ALC)	98.7 ± 2.5 ^{cde}	81.4 ± 2.5 ^{kl}	75.3 ± 2.6 ^m	90.7 ± 3.1 ^{gh}	83.1 ± 3.4 ^{ijkl}	77.7 ± 3.3 ^{lm}
	Fresh (CAP)	94.9 ± 2.4 ^{efg}	97.7 ± 3.4 ^{cde}	89.3 ± 3.7 ^h	106.5 ± 2.8 ^b	96.6 ± 3.9 ^{def}	95.7 ± 2.8 ^{defg}
	Dried (ALC)	114.8 ± 2.0 ^a	82.7 ± 1.8 ^{ijkl}	81.7 ± 1.3 ^{ijkl}	103.2 ± 1.5 ^{bc}	88.2 ± 1.8 ^{hi}	87.0 ± 1.9 ^{hij}
	Dried (CAP)	105.4 ± 1.7 ^b	92.1 ± 1.8 ^{fgh}	78.9 ± 2.3 ^{klm}	101.2 ± 2.8 ^{bcd}	83.6 ± 2.3 ^{ijk}	82.1 ± 1.9 ^{jkl}
TPrAC	Fresh (ALC)	330.7 ± 7.9 ^b	192.4 ± 6.2 ^h	203.7 ± 9.3 ^{gh}	292.7 ± 9.4 ^c	191.0 ± 6.1 ^h	211.6 ± 8.4 ^g
	Fresh (CAP)	255.4 ± 5.5 ^{de}	140.2 ± 6.0 ^j	120.0 ± 5.1 ^k	263.6 ± 8.1 ^d	142.2 ± 8.3 ^j	146.4 ± 5.3 ^j
	Dried (ALC)	357.0 ± 8.0 ^a	201.5 ± 5.5 ^{gh}	189.4 ± 6.3 ^{hi}	331.5 ± 5.1 ^b	241.9 ± 8.8 ^{ef}	238.1 ± 7.8 ^f
	Dried (CAP)	279.3 ± 4.5 ^c	211.1 ± 4.9 ^g	133.5 ± 5.1 ^{jk}	280.0 ± 7.6 ^c	174.9 ± 8.5 ⁱ	198.2 ± 5.8 ^{gh}
Twigs							
Yield	Fresh (ALC)	7.9 ± 0.1 ^h	6.9 ± 0.1 ^{kl}	6.9 ± 0.1 ^{kl}	9.0 ± 0.1 ^e	8.3 ± 0.1 ^g	7.7 ± 0.1 ^{hi}
	Fresh (CAP)	7.3 ± 0.1 ^j	6.7 ± 0.1 ^l	7.0 ± 0.1 ^k	8.0 ± 0.2 ^g	7.6 ± 0.1 ⁱ	7.0 ± 0.1 ^k
	Dried (ALC)	12.0 ± 0.1 ^a	10.7 ± 0.1 ^b	10.6 ± 0.1 ^b	8.8 ± 0.1 ^{ef}	7.1 ± 0.1 ^k	10.1 ± 0.1 ^c
	Dried (CAP)	9.0 ± 0.1 ^e	8.6 ± 0.1 ^f	10.1 ± 0.1 ^c	8.8 ± 0.1 ^{ef}	8.3 ± 0.1 ^g	9.5 ± 0.1 ^d
TPC	Fresh (ALC)	526.5 ± 4.5 ^a	406.6 ± 4.3 ^g	348.3 ± 6.3 ^j	514.6 ± 2.2 ^b	420.9 ± 3.3 ^{ef}	419.1 ± 3.0 ^{ef}
	Fresh (CAP)	412.6 ± 6.7 ^{fg}	294.2 ± 4.9 ⁿ	273.7 ± 7.9 ^o	367.1 ± 3.8 ⁱ	344.8 ± 5.3 ^{jk}	324.0 ± 6.1 ^l
	Dried (ALC)	499.0 ± 3.9 ^c	389.5 ± 7.3 ^h	329.0 ± 8.3 ^l	485.6 ± 3.5 ^d	412.5 ± 5.5 ^{fg}	402.6 ± 6.2 ^g
	Dried (CAP)	419.1 ± 3.7 ^{ef}	311.0 ± 6.5 ^m	251.6 ± 3.4 ^p	425.1 ± 5.8 ^e	341.5 ± 6.1 ^{jk}	334.9 ± 3.3 ^{kl}
TFC	Fresh (ALC)	166.2 ± 4.3 ^{cd}	139.1 ± 4.0 ^{ijkl}	130.9 ± 2.1 ^m	166.3 ± 3.9 ^c	137.6 ± 3.3 ^{ijkl}	135.3 ± 2.6 ^{klm}
	Fresh (CAP)	178.7 ± 4.7 ^b	147.6 ± 2.3 ^{fgh}	130.0 ± 3.8 ^{mn}	153.0 ± 2.8 ^f	153.2 ± 2.4 ^{ef}	144.7 ± 4.5 ^{ghi}
	Dried (ALC)	166.3 ± 3.0 ^c	143.0 ± 3.4 ^{hij}	133.8 ± 2.0 ^{lm}	163.8 ± 1.7 ^{cd}	139.7 ± 2.8 ^{ijkl}	141.0 ± 2.9 ^{hijk}
	Dried (CAP)	198.4 ± 2.7 ^a	149.8 ± 3.3 ^{fg}	123.8 ± 2.4 ⁿ	201.0 ± 1.9 ^a	159.6 ± 1.4 ^{de}	151.5 ± 2.7 ^f
TprAC	Fresh (ALC)	585.8 ± 10.3 ^b	307.5 ± 7.1 ⁱ	286.4 ± 11.6 ^j	553.3 ± 7.0 ^c	375.2 ± 6.8 ^g	387.7 ± 9.0 ^g
	Fresh (CAP)	440.3 ± 9.1 ^e	94.2 ± 6.9 ^m	72.3 ± 6.3 ⁿ	388.3 ± 9.2 ^g	209.1 ± 7.8 ^k	142.1 ± 7.3 ^l
	Dried (ALC)	576.4 ± 5.5 ^b	340.3 ± 9.5 ^h	305.1 ± 6.9 ⁱ	516.9 ± 8.2 ^d	351.0 ± 8.1 ^h	384.6 ± 6.5 ^g
	Dried (CAP)	631.3 ± 7.9 ^a	379.9 ± 9.8 ^g	306.0 ± 7.2 ⁱ	614.3 ± 8.4 ^a	417.6 ± 6.1 ^f	432.4 ± 6.4 ^{ef}

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 × 3); different letters indicate significant differences of means within samples, for each determined parameter (one-way ANOVA, Tukey test at p<0.05).

The extraction yield obtained for leaves with 70% ACE (10.5 – 15.8%, on a wet basis) was higher than the ones reported by Borges et al. for *A. dalbata* leaves, extracted with different solvents (water, methanol, ethanol, acetone, dichloromethane, and hexane) and extraction methods (maceration under agitation, Soxhlet, and the use of ultrasound and microwaves), and solvent-to biomass ratio of 1:10, that were in the range of 2.8 – 12.0% of fresh weight (Borges et al., 2020). Extraction yields were also higher than those observed by Luís et al. (Luís et al., 2012) for ethanolic, hydroethanolic (1:1 in volume basis), methanolic, and acetonic extracts of aerial parts (wood, bark, and leaves) of *A. dealbata* (3.26 - 9.51% dry weight) using Soxhlet extraction, and in the same range of the described for *A. melanoxylon* aerial parts (11.88 – 15.41% dry weight) by the same authors. Concerning twigs, Lomarat et al. found an extraction yield of dried *A. pennata* twigs of 15.1%, obtained by Soxhlet extraction with methanol (Lomarat et al., 2015).

For the best extraction solvent (70% ACE), TPC in leaves and twigs extracts varied in the range 345.8 – 478.4 mg GAE/g extract and 367.1 – 526.5 mg GAE/g extract, respectively. These contents are higher than those found by Luís et al. (Luís et al., 2012) in extracts of aerial parts of *A. dealbata* (203.10 – 290.65 mg GAE/g extract) and *A. melanoxylon* (100.10 – 138.76 mg GAE/g extract), and higher than the reported for *A. farnesiana* leaves extracts (63.2 – 247.9 mg GAE/g extract) (Gabr et al., 2018), similar to the TPC contents found for *A. mearnsii* (163.9 – 646.6 mg GAE/ g extract) (Xiong et al., 2016), *A. tortilis* (260.7 – 512.4 mg GAE/g extract) (Gabr et al., 2018), *A. nilotica* (42.18 – 116.60 mg GAE/ g extract) (Zheleva-Dimitrova et al., 2021), and *A. ataxacantha* (63.26 – 115.57 mg GAE/ g extract) (Zheleva-Dimitrova et al., 2021) leaves extracts, but lower than the observed for *A. longifolia* leaves extracts (524.9 – 858.8 mg GAE/g extract) (Gabr et al., 2018). Leaves and twigs are important sources of phenolics, with contents of 36.2 – 71.2 mg GAE/g of fresh leaves and 30.1 – 59.9 mg GAE/g of fresh twigs, respectively (see full results in Appendix D1, Table D2). These are in a similar range as what was observed by Ferreira-Santos et al. (Ferreira-Santos et al., 2020) with *Pinus pinaster* bark, a known source of phenolic compounds, which gave 65.1 and 68.2 mg GAE/g of bark by extraction with 70% and 50% aqueous ethanol, respectively.

For 70% ACE, TFC in leaves and twigs extracts lied between 90.7 and 114.8 mg CatE/g extract and 153.0 to 198.4 mg CatE/g extract, respectively. A calibration curve was made using rutin as a standard for comparison purposes, giving 228.8 – 348.0 mg RutE/g extract for leaves, and 375.5 – 601.4 mg RutE/g extract (Appendix D1, Table D3). TFC in leaves extracts were in the range observed by Tung et al. (Tung et al., 2009) with leaves of *A. nilotica* (2.3 – 355.3 mg RutE/g extract), while TFC in twigs extracts were higher. The TFC values found in the extracts

of *A. dealbata* twigs with 70% ACE were higher than those observed in bark extracts of various *Acacia* spp. including *A. dealbata* (74.0 – 134.0 mg RutE/g extract) (Sowndhararajan et al., 2013), in leaves and bark extracts of *A. nilotica* and *A. ataxacantha* (0.34 – 49.90 mg RutE/g extract) (Zheleva-Dimitrova et al., 2021), or in *Pinus pinaster* bark extracts, a known source of flavonoids (77 – 161 mg CatE/g extract) (Ferreira-Santos et al., 2020). Relatively to the mass of raw material, TFC contents varied in the range 9.9 – 17.0 mg CatE/g of leaves (30.2 – 51.4 mg RutE/g of leaves), and 12.6 – 20.0 mg CatE/g of twig (38.2 – 60.5 mg RutE/g of twig) (see full results in Appendix D1, Table D4), which are much superior to the reported by Sowndhararajan et al. for the bark of *A. dealbata* and other *Acacia* spp. (2.4 – 15.5 mg RutE/ g of bark), and also other known sources of flavonoids such as yellow, green, and black teas (0.2 – 3.8 mg CatE/g of tea) (Kopjar et al., 2015), and red fruits (0.2 – 0.8 mg CatE/g of tea) (Sariburun et al., 2010).

Regarding TPrAC, the results were expressed relative to a standard source of proanthocyanidins, the commercial extract Pycnogenol®, a mixture of proanthocyanidins extracted from bark of Atlantic *Pinus* trees. As observed for TPC and TFC, the extracts with higher TPrAC contents were obtained with 70% ACE, with TPrAC values in the range of 255.4 – 357.0 mg PycE/g extract for leaves, and in the range of 388.3 – 631.3 mg PycE/g extract for twigs.

The results of this study showed that the leaves and twigs of *A. dealbata* from Caparica and from Alcobaça regions can be an abundant source of extracts rich in phenolics, especially flavonoids and proanthocyanidins, with average contents similar or higher than comparable extratcs described in the literature.

For a better interpretation of the impact of the extraction method (regardless of the drying status or the origin of the plant), the average of the measured variables for all extracts produced by maceration was compared with the average of all extracts produced by hot extraction, for each solvent. The same approach was followed to evaluate the average values of the measured variables for all extracts obtained from fresh and dried leaves (regardless of other extraction parameters). The average values of the measured variables for the extracts obtained from a specific origin (Caparica or Alcobaça) were also determined and compared. These results were then subject to analysis of variance (ANOVA) followed by Tukey's test at $p < 0.05$, and are shown in Appendix D1, Table D5).

For the extraction yield, statistically significant differences were only found between fresh and dried raw materials, as shown in the Figure 6.1. Yields in the range 13.9 - 15.0%, and 10.3 – 11.6% were found for dried and fresh leaves, respectively and yields in the range of 8.7 – 10.1%, and 7.2 – 8.1% were found for dried and fresh twigs, respectively.

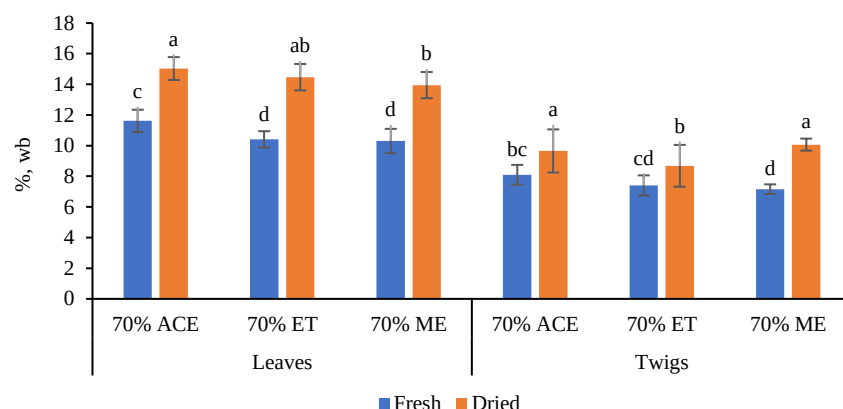


Figure 6.1 — Comparison of the average extraction yields for fresh and dried leaves and twigs (regardless the extraction method and the provenience of the raw material).

In what concerns the TPC, TFC, and TPrAC of the extracts, no significant differences were found between maceration and hot extraction, except for methanolic extracts that presented slightly higher contents for hot extraction (Appendix D, Table D5). Figure 6.2 highlights differences coming from the drying status and origin of the raw material, for all determined composition parameters.

Overall, it was confirmed that the higher average values for all the parameters considered were higher for the extracts obtained with 70% ACE. Regarding leaves, the extracts from Alcobaça region had higher average values of TPC and TPrAC, and higher average values of TFC (only for 70% ET and 70% ME extracts) than those from Caparica region. In the case of twigs, extracts from dried biomass had higher average values of TFC and TPrAC than those obtained from wet biomass with the same solvents. Extracts of twigs from Alcobaça region showed higher average values of TPC than those from Caparica region. Differences in the average values comparing fresh and dried plants were most noticed in the extraction yield, TFC, and TPrAC, with dried material originating better results. The differences of extraction yield or extract composition between fresh or dried biomass may be explained by the change of the extraction solvent characteristics, since the presence of biomass moisture corresponds to a reduction of the concentration of the organic component (acetone, ethanol or methanol). Differences coming from the plant origin can be explained by the fact that secondary metabolites are synthesized by plants to protect them in adverse conditions from pathogens and environmental stresses, being influenced by several biotic and abiotic factors, such as water, drought, salinity, temperature, radiation, or chemical stresses (Verma and Shukla, 2015). Thus, differences in

edaphoclimatic conditions between Alcobaça and Caparica can explain the differences observed.

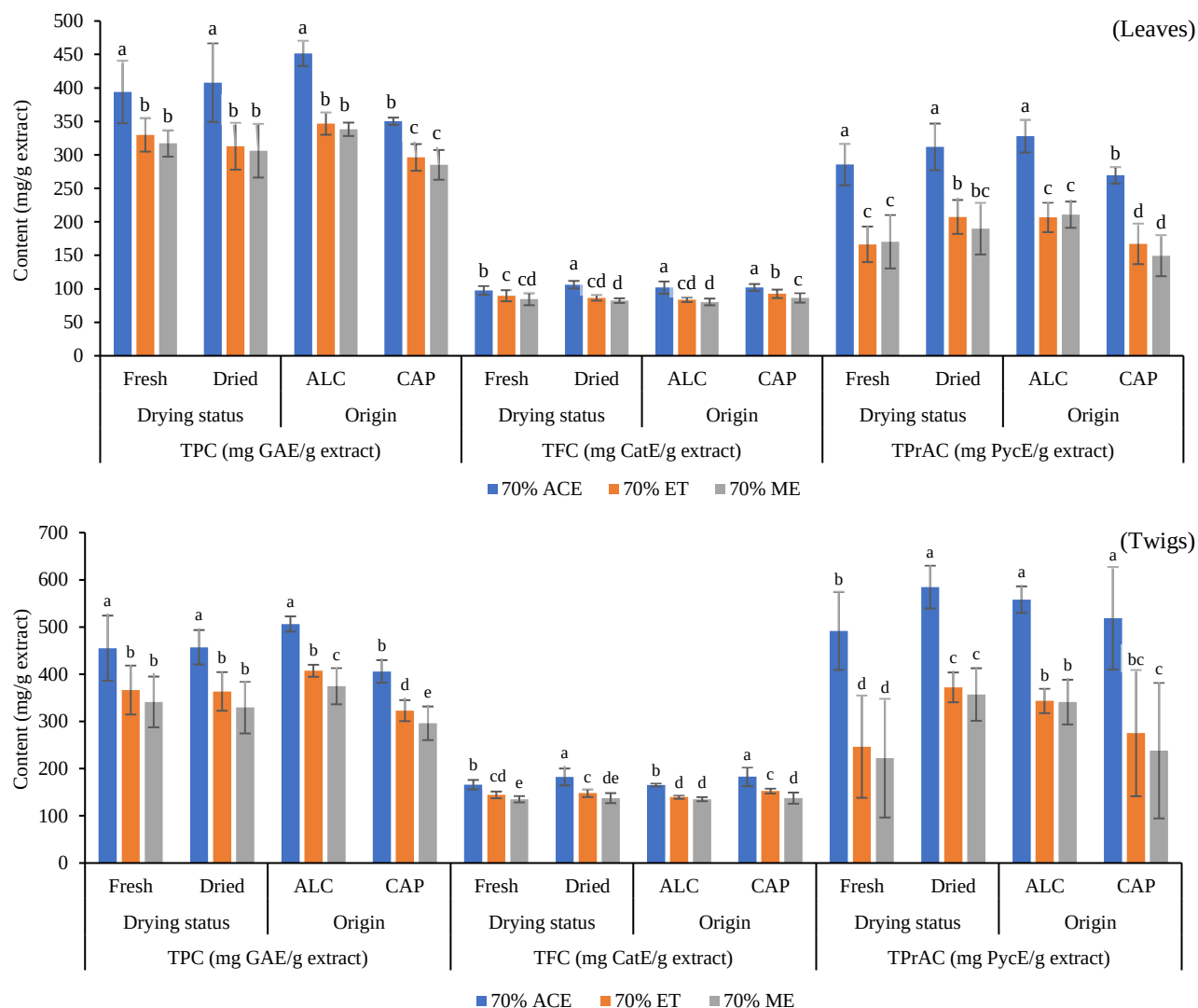


Figure 6.2 — Comparison of the average values of TPC (mg GAE/g extract), TFC (mg CatE/g extract), and TPrAC (mg PycE/g extract) for fresh or dried material (regardless the extraction method and the provenience of the raw material), and for plants from Alcobaça (ALC) and Caparica (CAP) (regardless the extraction method and the drying status).

6.3.2 Extraction of spent raw material

Leaves and twigs of *Acacia* spp. contain lipophilic compounds such as terpenes, long-chain alcohols, fatty acids, sterols, aromatic compounds, among others (C. S. D. Oliveira et al., 2020). These lipophilic components may be valorized in different application fields and their presence might limit the access of polar solvents to the matrix, affecting extraction of the bioactive components. To test this hypothesis the dried leaves and twigs were treated with petroleum ether,

to remove non-polar components and extraction with 70% ACE was performed on the spent leaves and twigs using equivalent conditions to those used with the raw biomass. The characterization of the lipophilic extracts will be addressed elsewhere.

The comparison of composition and antioxidant activity of extracts obtained from dried material macerated at room temperature with 70% ACE, and those of extracts obtained from spent material in similar conditions is shown in Table 6.4.

Table 6.2 — Extract yield, composition, and antioxidant activity of extracts obtained from raw and spent leaves and twigs, using maceration at room temperature with 70% ACE as extraction solvent.

Sample		Yield	TPC	TFC	TPrAC	DPPH	FRAP
Leaves	Alcobaça-spent	15.5 ± 0.1	436.4 ± 11.1	116.1 ± 2.8	441.0 ± 6.8	796.0 ± 9.9	8807.8 ± 225.1
	Alcobaça-raw	14.8 ± 0.1	478.5 ± 4.5	114.8 ± 2.0	357.0 ± 8.0	893.4 ± 10.8	8885.4 ± 93.1
	Caparica-spent	14.5 ± 0.1	350.5 ± 2.4	97.0 ± 1.8	356.9 ± 1.6	661.7 ± 3.5	6431.1 ± 85.8
	Caparica-raw	14.0 ± 0.1	350.2 ± 4.4	105.4 ± 1.7	279.3 ± 4.5	446.3 ± 7.0	5846.6 ± 90.7
Twigs	Alcobaça-spent	12.9 ± 0.1	448.3 ± 8.0	152.8 ± 3.6	610.1 ± 5.8	838.6 ± 8.6	8171.1 ± 153.0
	Alcobaça-raw	12.0 ± 0.1	499.0 ± 3.9	166.3 ± 3.0	576.4 ± 5.5	1068.3 ± 8.5	9194.6 ± 165.8
	Caparica-spent	10.2 ± 0.1	389.0 ± 5.3	147.6 ± 2.2	544.2 ± 4.7	644.8 ± 9.6	7151.6 ± 160.4
	Caparica-raw	9.0 ± 0.1	419.1 ± 3.7	198.4 ± 2.7	631.3 ± 7.9	731.3 ± 6.1	7754.4 ± 119.0

The extracts produced from spent leaves and twigs had a slightly higher yield, and slightly higher proanthocyanidin contents than those obtained from the raw dried materials. However, TPC and TFC, as well as the antioxidant activity were slightly lower when using spent raw material. The removal of lipophilic compounds from the plant matrix may facilitate the penetration of the solvent, thus allowing obtaining higher extract yields. The slight decrease in the antioxidant activity may be explained by the previous removal of lipophilic components with antioxidant activity, such as terpenes or sterols. Despite this, it should be noted that the values are quite close and that this option allows more efficient use of this resource by obtaining a wider range of products from the same amount of raw material.

6.3.3 Antioxidant activity

The antioxidant activity of the extracts obtained from *A. dealbata* leaves and twigs was assessed by DPPH and FRAP assays and the results are presented in Table 6.2.

The highest antioxidant activity of extracts from leaves or twigs was observed when using 70% ACE as extraction solvent, regardless of the extraction method, drying status, or the geographical origin of the plant. When using this solvent, the antioxidant activity of twigs extracts was higher than the one of corresponding leaves extracts.

Table 6.3 — Results from DPPH (mg TE/g extract) and FRAP ($\mu\text{mol Fe}^{2+}/\text{g extract}$) of extracts from fresh and dried leaves and twigs from Alcobaça (ALC) and Caparica (CAP), using different extraction solvents and extraction methods; DPPH and FRAP results expressed relative to the mass of biomass are presented in Appendix D1, Table D2.

Samples		Maceration at room temperature			Hot extraction		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
Leaves							
DPPH	Fresh (ALC)	740.8 $\pm$ 10.1 ^b	597.9 $\pm$ 8.3 ^e	535.8 $\pm$ 8.5 ⁱ	740.2 $\pm$ 6.1 ^b	636.7 $\pm$ 6.0 ^d	526.8 $\pm$ 5.3 ⁱ
	Fresh (CAP)	560.0 $\pm$ 7.2 ^h	348.5 $\pm$ 5.7 ⁿ	335.4 $\pm$ 5.9 ⁿ	540.0 $\pm$ 8.4 ^{gh}	428.4 $\pm$ 9.0 ^k	393.6 $\pm$ 7.2 ^m
	Dried (ALC)	893.4 $\pm$ 10.8 ^a	578.2 $\pm$ 6.5 ^{fg}	576.4 $\pm$ 8.2 ^{fg}	886.5 $\pm$ 7.5 ^a	634.3 $\pm$ 9.1 ^d	685.4 $\pm$ 5.5 ^c
	Dried (CAP)	446.3 $\pm$ 7.0 ^j	412.2 $\pm$ 5.6 ^l	337.0 $\pm$ 8.3 ⁿ	588.9 $\pm$ 5.5 ^{ef}	392.6 $\pm$ 7.3 ^m	406.6 $\pm$ 6.0 ^{lm}
FRAP	Fresh (ALC)	7664.1 $\pm$ 167.6 ^b	6285.3 $\pm$ 95.4 ^{ef}	5620.3 $\pm$ 118.5 ^{gh}	7702.8 $\pm$ 164.7 ^b	6501.3 $\pm$ 132.2 ^{de}	5423.2 $\pm$ 142.5 ^{hi}
	Fresh (CAP)	6612.1 $\pm$ 167.8 ^d	4386.9 $\pm$ 93.0 ^l	4403.9 $\pm$ 129.7 ^l	6015.2 $\pm$ 177.6 ^{def}	5072.4 $\pm$ 225.1 ^{jk}	4835.8 $\pm$ 178.6 ^k
	Dried (ALC)	8885.4 $\pm$ 93.1 ^a	6299.9 $\pm$ 71.9 ^{ef}	5334.0 $\pm$ 79.0 ^{ij}	6962.5 $\pm$ 63.4 ^c	6175.8 $\pm$ 138.3 ^f	3300.7 $\pm$ 98.7 ⁿ
	Dried (CAP)	5846.6 $\pm$ 90.7 ^g	4855.0 $\pm$ 58.6 ^k	3934.8 $\pm$ 103.5 ^m	5766.3 $\pm$ 81.2 ^g	4097.2 $\pm$ 124.8 ^m	4449.8 $\pm$ 82.2 ^l
Twigs							
DPPH	Fresh (ALC)	996.8 $\pm$ 6.7 ^b	757.2 $\pm$ 11.6 ^e	511.1 $\pm$ 11.3 ^j	994.7 $\pm$ 6.2 ^{bc}	778.8 $\pm$ 14.0 ^d	782.7 $\pm$ 8.1 ^d
	Fresh (CAP)	675.5 $\pm$ 11.0 ^h	254.2 $\pm$ 6.6 ^q	230.2 $\pm$ 9.2 ^r	596.3 $\pm$ 11.5 ⁱ	379.7 $\pm$ 11.8 ⁿ	358.4 $\pm$ 11.4 ^o
	Dried (ALC)	1068.3 $\pm$ 8.5 ^a	665.5 $\pm$ 11.2 ^h	514.2 $\pm$ 11.8 ^j	974.2 $\pm$ 6.4 ^c	791.2 $\pm$ 14.3 ^d	782.1 $\pm$ 7.1 ^d
	Dried (CAP)	731.3 $\pm$ 6.1 ^f	418.5 $\pm$ 7.9 ^m	321.3 $\pm$ 8.3 ^p	710.2 $\pm$ 5.2 ^g	474.5 $\pm$ 9.9 ^k	452.6 $\pm$ 8.5 ^l
FRAP	Fresh (ALC)	6852.4 $\pm$ 208.0 ^{de}	6546.5 $\pm$ 221.0 ^e	3989.4 $\pm$ 61.6 ^j	7829.9 $\pm$ 140.9 ^c	6851.8 $\pm$ 225.2 ^{de}	6554.3 $\pm$ 112.4 ^e
	Fresh (CAP)	6951.8 $\pm$ 146.0 ^d	3306.8 $\pm$ 109.3 ^k	3075.8 $\pm$ 75.0 ^k	6192.0 $\pm$ 53.1 ^f	4626.8 $\pm$ 255.7 ^{hi}	4461.1 $\pm$ 137.7 ⁱ
	Dried (ALC)	9194.6 $\pm$ 165.8 ^a	4770.3 $\pm$ 86.1 ^h	3783.0 $\pm$ 84.8 ^j	8804.2 $\pm$ 112.4 ^b	6987.7 $\pm$ 163.4 ^d	7098.5 $\pm$ 87.1 ^d
	Dried (CAP)	7754.4 $\pm$ 119.0 ^c	5354.9 $\pm$ 141.1 ^g	3969.0 $\pm$ 85.0 ^j	8003.8 $\pm$ 171.7 ^c	5585.2 $\pm$ 140.0 ^g	5429.1 $\pm$ 83.3 ^g

Values (mean $\pm$ standard deviation) are average of two replicates of each sample, analyzed individually in triplicate ($n = 2 \times 3$); different letters indicate significant differences of means within samples, for each antioxidant determination method (one-way ANOVA, Tukey test at $p < 0.05$).

The DPPH assay measures the ability of extract components to act as free radical scavengers or hydrogen donors antioxidants (Kedare and Singh, 2011). For 70% ACE extracts, the values lied between 446.3 – 893.4 mg TE/g extract (58.6 – 132.1 mg TE/g of leaves) for leaves extract, and between 596.3 – 1068.3 mg TE/g extract (49.0 – 128.3 mg TE/g of twigs). These values are higher than those reported by Zheleva-Dimitrova et al. for extracts from leaves (31.94 – 493.90 mg TE/g extract) and stem bark (8.51 – 349.17 mg TE/g extract) of *A. nilotica* and *A. ataxacantha* (Zheleva-Dimitrova et al., 2021). These results are also higher than the ones reported for known antioxidant sources such as yellow, green, and black teas (11.930 – 26.521

$\mu\text{mol TE}/100\text{g}$, which corresponds to 0.030 – 0.066 mg TE/g of tea leaves) (Kopjar et al., 2015), red fruits (64.14 – 177.11 $\mu\text{mol TE}/\text{g}$, corresponding to 16.1 – 44.3 mg TE/g of fruit) (Sariburun et al., 2010), or pine bark (approximately between 50 and 350 mg TE/g of extract) (Venkatesan et al., 2019).

The reducing power of the obtained extracts was measured through the FRAP assay, which evaluates the reduction of Fe^{3+} in the complex (Fe^{3+} – TPTZ) to a ferrous form (Fe^{2+} – TPTZ), by donation of electrons from the antioxidants, resulting in stopping the free radical production chain (Afsar et al., 2018; Sadiq et al., 2017; Santos-Sánchez et al., 2019). For 70% ACE the FRAP values were in the range 5766.3 – 8885.4 $\mu\text{mol Fe}^{2+}/\text{g}$ extract and 6192.0 – 9194.6 $\mu\text{mol Fe}^{2+}/\text{g}$ extract, for leaves and twigs extracts, respectively. These values higher than those found in leaves (233.17 $\mu\text{mol Fe}^{2+}/\text{g}$ extract), pods (254.42 $\mu\text{mol Fe}^{2+}/\text{g}$ extract), and seed (178.14 $\mu\text{mol Fe}^{2+}/\text{g}$ extract) extracts of *A. leucophloea* (Zia-Ul-Haq et al., 2013). Values of 152 mg Fe^{2+}/g (corresponding to 2721.2 $\mu\text{mol Fe}^{2+}/\text{g}$ extract) and 575 mg Fe^{2+}/g (corresponding to 10296.4 $\mu\text{mol Fe}^{2+}/\text{g}$ extract) were found for *Pinus radiata* bark extract and standardized *Pinus pinaster* bark extract Pycnogenol®, respectively (Astuya et al., 2016).

The extraction solvent significantly affected the antioxidant activity, with the best results being obtained when using 70% ACE, regardless the origin and the drying status of the raw material. Higher antioxidant activities of extracts obtained with aqueous acetone solutions were also observed with stem bark extracts of *Butea monosperma* (Lam.) Kuntze (Salar and Seasotiya, 2011), bark extracts of *A. leucophloea*, *A. ferruginea*, *A. dealbata* and *A. pennata* (Sowndhararajan et al., 2013), and flower extracts of safflower (*Carthamus tinctorius* L.) (Salem et al., 2011).

A better understanding of the influence of the extraction method, drying status, and provenience of the plant on the antioxidant activity can be derived from the analysis of variance applied using the same approach as for the impact of these factors on the composition and extract yield. The results are presented in Table 6.3. Besides the confirmation of 70% ACE as the best extraction solvent for the removal of antioxidant compounds, it can be seen that the geographical origin of the plant had the major impact on the antioxidant activity of the produced extracts, since the extracts of leaves and twigs from Alcobaça showed significantly higher antioxidant activity than the corresponding extracts from biomass collected in the Caparica region. The extraction method only gave significant differences on methanolic and ethanolic extracts of twigs, with hot extraction giving the highest antioxidant activity by both DPPH and FRAP assays. The effect of using fresh or dried material was most reflected in the

antioxidant activity of twigs given by the FRAP assay, with dried twigs extracted with 70% ACE originating a significantly higher value than the one obtained using fresh leaves.

Table 6.4 — Average values of DPPH (mg TE/g extract) and FRAP ($\mu\text{mol Fe}^{2+}/\text{g extract}$) assays for extracts obtained by maceration and hot extraction (regardless drying status and origin), from fresh and dried material (regardless extraction method and origin), and from plants from Alcobaça (ALC) and Caparica (CAP) (regardless extraction process and drying status).

Samples	Leaves			Twigs		
	70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
DPPH						
Maceration	660.1 $\pm$ 174.7 ^a	484.2 $\pm$ 109.0 ^b	446.1 $\pm$ 113.5 ^b	868.0 $\pm$ 171.5 ^a	523.8 $\pm$ 203.4 ^{bc}	394.2 $\pm$ 125.7 ^c
Hot extraction	696.2 $\pm$ 131.2 ^a	523.0 $\pm$ 115.9 ^b	503.1 $\pm$ 120.0 ^b	818.9 $\pm$ 174.4 ^a	606.0 $\pm$ 186.4 ^b	593.9 $\pm$ 195.7 ^b
Fresh	652.5 $\pm$ 90.2 ^a	502.9 $\pm$ 121.4 ^b	447.9 $\pm$ 88.0 ^b	815.8 $\pm$ 186.2 ^a	542.5 $\pm$ 235.1 ^b	470.6 $\pm$ 210.4 ^b
Dried	703.8 $\pm$ 197.2 ^a	504.3 $\pm$ 106.5 ^b	501.3 $\pm$ 140.5 ^b	871.0 $\pm$ 157.5 ^a	587.4 $\pm$ 152.6 ^b	517.5 $\pm$ 171.7 ^b
Alcobaça	815.2 $\pm$ 76.8 ^a	611.8 $\pm$ 26.2 ^b	581.1 $\pm$ 64.7 ^{bc}	1008.5 $\pm$ 37.0 ^a	748.2 $\pm$ 51.7 ^b	647.5 $\pm$ 138.1 ^c
Caparica	541.1 $\pm$ 57.3 ^c	395.4 $\pm$ 31.3 ^d	368.1 $\pm$ 33.6 ^d	678.3 $\pm$ 53.1 ^c	381.7 $\pm$ 83.2 ^d	340.6 $\pm$ 81.9 ^d
FRAP						
Maceration	7252.1 $\pm$ 1174.0 ^a	5456.8 $\pm$ 873.7 ^b	4823.3 $\pm$ 746.5 ^{bc}	7688.3 $\pm$ 969.5 ^a	4994.6 $\pm$ 1199.0 ^c	3704.3 $\pm$ 386.4 ^d
Hot extraction	6693.0 $\pm$ 746.5 ^a	5461.7 $\pm$ 981.1 ^b	4502.4 $\pm$ 801.6 ^c	7707.5 $\pm$ 976.6 ^a	6012.9 $\pm$ 1007.8 ^b	5885.8 $\pm$ 1046.0 ^b
Fresh	7079.9 $\pm$ 644.8 ^a	5561.5 $\pm$ 898.9 ^b	5070.8 $\pm$ 509.5 ^b	6956.5 $\pm$ 610.9 ^b	5333.0 $\pm$ 1491.8 ^{cd}	4520.2 $\pm$ 1306.5 ^d
Dried	6865.2 $\pm$ 1287.9 ^a	5357.0 $\pm$ 946.6 ^b	4254.8 $\pm$ 765.0 ^c	8439.3 $\pm$ 611.2 ^a	5674.5 $\pm$ 841.4 ^c	5069.9 $\pm$ 1364.5 ^{cd}
Alcobaça	7803.7 $\pm$ 715.8 ^a	6315.6 $\pm$ 159.6 ^b	4919.5 $\pm$ 966.2 ^c	8170.3 $\pm$ 940.4 ^a	6289.1 $\pm$ 926.3 ^c	5356.3 $\pm$ 1518.6 ^d
Caparica	6141.4 $\pm$ 380.2 ^b	4602.9 $\pm$ 412.3 ^{cd}	4406.1 $\pm$ 347.9 ^d	7225.5 $\pm$ 737.5 ^b	4718.4 $\pm$ 921.4 ^{de}	4233.7 $\pm$ 873.4 ^e

Different letters indicate significant differences of means within samples for each set of parameters (one-way ANOVA, Tukey test at $p < 0.05$).

6.3.4 Correlations between composition and antioxidant activity

The correlation analysis between TPC, TFC, TPrAC, and antioxidant activities by DPPH and FRAP assays for the extracts of fresh and spent flowers are given in Table 6.5.

All the correlations found in this work were statistically significant at $p < 0.01$, with the exception made for the correlation between TFC and antioxidant activity by the DPPH assay for leaves (non-significant) and twigs (significant at $p < 0.05$) and for the correlation between TFC and FRAP assay in the case of leaves (significant at $p < 0.05$). The Pearson's correlation coefficients found indicate very strong correlations between TPC and antioxidant activity given by DPPH and FRAP assays, and strong correlations between TPrAC and the same assays (Akoglu, 2018), for both leaves and twigs. Moderate correlation was found between TFC of leaves and twigs and both antioxidant assays. (Yadav et al., 2018) also found strong correlations between TPC, TFC and DPPH values ($r = 0.771$ and $r = 0.815$), for extracts of *A. nilotica* leaves.

However, (Abdel-Farid et al., 2014) found non-significant correlation between TPC of extracts of different components (leaves, flowers, and pods) of three *Acacia* species (*A. nilotica*, *A. seyal*, and *A. laeta*) and DPPH results, having found a significant correlation between TFC and DPPH values instead. Significant and strong correlations between TPC and the DPPH and FRAP results were found in *Pinus densiflora* bark extract (Venkatesan et al., 2019) and in medicinal herbs and spices (Ulewicz-Magulska and Wesolowski, 2019).

Table 6.5 — Correlation analysis between total phenolics content (TPC), total flavonoid content (TFC), total proanthocyanidins content (TPrAC) and antioxidant activities given by DPPH and FRAP assays, for leaves extracts; significance of the correlations assessed at $p < 0.05$ and at $p < 0.01$ (two-tailed).

		TPC	TFC	TPrAC	DPPH	FRAP
Leaves	TPC	1				
	TFC	$r = 0.563^{**}$ $p = 0.003$	1			
	TPrAC	$r = 0.801^{**}$ $p = 0.000$	$r = 0.553^{**}$ $p = 0.003$	1		
	DPPH	$r = 0.922^{**}$ $p = 0.000$	$r = 0.373$ $p = 0.061$	$r = 0.766^{**}$ $p = 0.000$	1	
	FRAP	$r = 0.864^{**}$ $p = 0.000$	$r = 0.490^{*}$ $p = 0.011$	$r = 0.742^{**}$ $p = 0.000$	$r = 0.778^{**}$ $p = 0.000$	1
Twigs	TPC	1				
	TFC	$r = 0.511^{**}$ $p = 0.008$	1			
	TPrAC	$r = 0.754^{**}$ $p = 0.000$	$r = 0.671^{**}$ $p = 0.000$	1		
	DPPH	$r = 0.967^{**}$ $p = 0.000$	$r = 0.396^{*}$ $p = 0.045$	$r = 0.765^{**}$ $p = 0.000$	1	
	FRAP	$r = 0.861^{**}$ $p = 0.000$	$r = 0.603^{**}$ $p = 0.011$	$r = 0.856^{**}$ $p = 0.000$	$r = 0.884^{**}$ $p = 0.000$	1

** correlation is significant at $p < 0.01$ (two-tailed); * correlation is significant at $p < 0.05$ (two-tailed)

These findings demonstrate that phenolic compounds have an important role in the observed antioxidant activity given by DPPH and FRAP assays. Moreover, the abundance of these compounds in the produced extracts suggests that leaves and twigs have a strong potential to be explored for obtaining antioxidants for diverse applications, such as in nutraceutical or cosmetic industries.

6.3.5 Antimicrobial activity

A representative group of extract samples was selected to assess antimicrobial activity against several microorganisms responsible for foodborne disease, skin infections, caries, and oral infections. Some of these microorganisms, namely *S. aureus*, *Enterococcus* spp, *P. aeruginosa*, *E. coli*, and *C. albicans*, are usually involved in hospital-acquired infections and have developed resistance to antibiotics (Jenkins, 2017; Zeng et al., 2018). Antibiotic resistance is emerging as a serious worldwide problem and natural extracts with antimicrobial activity have been suggested as a solution to this problem (Ganaie et al., 2017). Moreover, natural extracts with antimicrobial activity could be an less polluting and more safe alternative to synthetic antimicrobial substances applied in food industry and in oral, cosmetics and pharmacological formulations (P. Pereira et al., 2022).

Both leaves and twigs extracts were able to inhibit the growth of Gram-positive bacteria but were inactive against Gram-negative bacteria and *C. albicans*. The inhibition zones against bacteria *S. aureus*, MRSA, *S. epidermis*, *E. faecalis*, *B. cereus*, *S. mutans* and *S. mitis* are presented in Table 6.6. Overall, extracts were more effective against *B. cereus* (inhibition zones in the range 11.0 – 12.1 mm for leaves extracts and 9.3 – 13.0 mm for twigs extracts), while *E. faecalis* showed a sensitivity lower than the remaining microorganisms (inhibition zones in the range 8.3 – 8.8 mm for leaves extracts and 9.3 – 9.8 mm for twigs extracts).

Table 6.6 — Inhibition zones (mm) of selected extracts of *A. dealbata* leaves and twigs against *S. aureus*, MRSA, *S. epidermidis*, *E. faecalis*, *B. cereus*, *S. mutans* and *S. mitis*.

Samples (0.5 mg/well)	<i>S. aureus</i>	MRSA	<i>S. epidermidis</i>	<i>E. faecalis</i>	<i>B. cereus</i>	<i>S. mutans</i>	<i>S. mitis</i>
Leaves							
Dried-70%ACE-maceration (ALC)	10.3 ± 0.1	10.3 ± 0.0	9.8 ± 0.1	8.5 ± 0.1	12.1 ± 0.1	11.0 ± 0.0	11.3 ± 0.6
Dried-70%ET-maceration (ALC)	8.8 ± 0.0	9.5 ± 0.1	8.5 ± 0.1	8.0 ± 0.0	11.5 ± 0.0	10.0 ± 0.0	11.0 ± 0.0
Dried-70%MET-maceration (ALC)	8.5 ± 0.1	9.3 ± 0.1	8.0 ± 0.0	nd	11.0 ± 0.0	10.0 ± 0.0	nd
Dried-70%ACE-maceration (CAP)	9.3 ± 0.0	9.0 ± 0.0	8.5 ± 0.1	8.8 ± 0.0	11.0 ± 0.0	10.0 ± 0.0	9.0 ± 1.7
Dried-70%ACE-hot extraction (ALC)	9.5 ± 0.1	10.5 ± 0.1	9.5 ± 0.1	8.8 ± 0.1	12.0 ± 0.0	10.0 ± 0.0	nd
Fresh-70%ACE-maceration (ALC)	9.5 ± 0.1	9.8 ± 0.0	9.3 ± 0.1	8.3 ± 0.1	11.3 ± 0.0	10.0 ± 0.0	9.3 ± 0.6
Spent leaves-70%ACE-maceration (ALC)	10.5 ± 0.1	10.5 ± 0.1	9.3 ± 0.0	8.5 ± 0.1	11.8 ± 0.0	11.3 ± 0.0	nd
Twigs							
Dried-70%ACE-maceration (ALC)	10.8 ± 0.0	10.5 ± 0.0	10.3 ± 0.1	9.5 ± 0.1	12.5 ± 0.1	9.0 ± 0.0	12.0 ± 0.0
Dried-70%ET-maceration (ALC)	nd	7.8 ± 0.0	nd	nd	10.0 ± 0.0	9.0 ± 0.0	11.0 ± 0.0
Dried-70%MET-maceration (ALC)	nd	nd	nd	nd	9.3 ± 0.0	10.0 ± 0.0	10.0 ± 0.0
Dried-70%ACE-maceration (CAP)	11.0 ± 0.0	11.0 ± 0.0	10.0 ± 0.0	9.5 ± 0.1	13.0 ± 0.0	12.0 ± 0.0	10.0 ± 0.0
Dried-70%ACE-hot extraction (ALC)	10.0 ± 0.0	10.8 ± 0.1	9.8 ± 0.0	9.5 ± 0.1	13.0 ± 0.0	9.0 ± 0.0	11.0 ± 0.0
Fresh-70%ACE-maceration (ALC)	11.0 ± 0.0	10.7 ± 0.0	9.5 ± 0.1	9.3 ± 0.0	12.8 ± 0.1	11.0 ± 0.0	12.0 ± 0.0
Spent twigs-70%ACE-maceration (ALC)	11.0 ± 0.0	11.0 ± 0.0	9.5 ± 0.1	9.8 ± 0.0	12.1 ± 0.0	9.0 ± 0.0	10.0 ± 0.0
Positive control	12.5 ± 0.5 ^a	13.9 ± 0.7 ^a	15.7 ± 1.0 ^a	12.5 ± 0.5 ^a	16.4 ± 1.1 ^a	19.3 ± 0.6 ^b	25.7 ± 0.6 ^b

a) Vancomycin (5.0 mg); b) Vancomycin (50.0 mg); nd: not detected

Comparing leaves and twigs, twigs originated slightly higher inhibition diameters than leaves extracts, whenever acetone was used as extraction solvent. 70% ACE was the best extraction solvent regarding antimicrobial activity, for both leaves and twigs extracts, having produced significantly higher inhibition diameters than 70% ET or 70% MET extracts.

The origin of the raw material had a marked influence in the case of leaves extracts, with extracts obtained with leaves from Alcobaça having better results with all microorganisms, except for *E. faecalis*. In the case of twigs extracts, the differences were not so visible but extracts from Caparica twigs gave better results, except against *S. epidermidis*, *E. faecalis*, and *S. mitis*.

For both leaves and twigs extracts, the extraction process (maceration versus hot extraction), as well as the drying state of the raw material (dried versus fresh raw material), and the use of dried versus spent raw material did not significantly affect the antimicrobial activity, with extracts producing very similar inhibition zones against the microorganisms. Regarding the obtention of antibacterial extracts, these are important findings, once it supports the suggestion of using a more sustainable process (maceration at room temperature), with facilitated logistics due to not having the necessity of using fresh raw material. Moreover, the results show

the possibility of retrieving a broader range of products from the same amount of raw material, since the previous extraction with petroleum ether to get lipophilic compounds did not affect the antimicrobial activity of the extracts that were then obtained with 70% aqueous acetone.

Taking into account the concentration of 10 mg/mL used in the antimicrobial assays for comparison purposes, it can be observed that the leaves extracts obtained in this study produced similar effects on the microorganisms to the ones shown by other extracts from leaves of *Acacia* spp. reported in the literature. Antimicrobial effects of leaves extracts expressed by inhibition zones were already described against *S. aureus* with *A. dealbata* aqueous and ethanolic extract (10.0 – 10.2 mm of inhibition, at 5 mg/mL) (Borges et al., 2020), with *A. saligna* (15 mm by an ethyl acetate extract at 10 mg/mL (El-Toumy et al., 2010), and 24.7 mm by an ethanolic extract at 200 mg/mL (Gumgumjee and Hajar, 2015)), with extracts of *A. etbaica*, *A. laeta*, and *A. origena* (7.3 – 21.0 mm at 500 mg/mL) (Mahmoud et al., 2016), with *A. pycnantha* (10.2 – 21.0 mm at 500 mg/mL (Mahmoud et al., 2016), and 9 – 12 mm by aqueous and ethanolic extracts, no concentration reported (Edrah et al., 2018)), and with *A. rigidula* and *A. berlandieri* acetonic, methanolic, and acetic acid extracts (6 – 11.4 mm at 500 mg/mL); against MRSA with *A. saligna* ethanolic extract (22.7 mm at 200 mg/mL) (Gumgumjee and Hajar, 2015); against *S. epidermis* (6 – 10 mm by aqueous and ethanolic extracts, no concentration reported) (Edrah et al., 2018); against *E. faecalis* with *A. rigidula* and *A. berlandieri* acetonic, methanolic, and acetic acid extracts (6 – 12.1 mm at 500 mg/mL); and against *B. cereus* with *A. dealbata*, *A. melanoxylon*, *A. baileyana*, and *A. nicholli* aqueous, ethanolic, and methanolic extracts (6 – 19 mm at 10 mg/mL) (Silva et al., 2016), and with *A. saligna* ethyl acetate extract (inhibition zone of 16 mm at 10 mg/mL) (El-Toumy et al., 2010).

Concerning twigs in particular, just a few studies on antimicrobial effects involving *Acacia* spp. were found in the literature, most of them involving *A. nilotica* twigs (Arshad et al., 2017; Kumari et al., 2020b, 2020a, 2019). Antimicrobial activity of twigs extracts produced in this work was in a lower range than the reported by Kumari et al. for *A. nilotica* twigs against *S. aureus* (10.8 – 40.2 mm), *E. faecalis* (16.2 – 38.0 mm), and *C. albicans* (14.7 – 27.0 mm) (Kumari et al., 2019). However, these results were obtained by applying 2 mg of extract/well (Kumari et al., 2019), while in this work only 0.5 mg of extract/well was applied, which can at least partially explain the differences observed. To the best of our knowledge, no results of antimicrobial effects against MRSA, *S. epidermidis*, or *B. cereus* for twigs extracts of other *Acacia* spp. have been reported in the literature.

It is known that Gram-negative bacteria are more resistant to plant extracts than Gram-positive bacteria, due to the protection of the lipopolysaccharide layer in the outer membrane

(Ganaie et al., 2017). All tested samples revealed ineffective against Gram-negative *P. aeruginosa* and *E. coli*, which was equally reported by El-Toumy et al. (El-Toumy et al., 2010), as well as against *C. albicans* yeast. However, antibacterial effects against Gram-negative *E. coli* were already presented by some flower and leaves extracts of *Acacia* spp. (Borges et al., 2020; Edrah et al., 2018; Gumgumjee and Hajar, 2015; Noreen et al., 2017; Silva et al., 2016). In the same way, activity against *C. albicans* was previously reported (El-Toumy et al., 2010; Gumgumjee and Hajar, 2015; Kumari et al., 2019; Silva et al., 2016).

6.3.6 Characterization of the non-polar components of the extracts

To understand if the phenolic extracts also contained non-polar components with antioxidant or antimicrobial activities that might influence the properties of the extracts, a group of selected extracts were derivatized and fractionated to isolate these less polar components and analyzed by GC-TOFMS (see Appendix D2, Figures D1 to D3). A total of 32 compounds were tentatively identified using the NIST mass spectra library matching (Appendix D2, Table D6). Among them, phytol, squalene, α -tocopherol, lupeol, and lupenone (lup-20(29)-en-3-one) (Figure 6.3) are the most predominant, showing the highest peak areas. Squalene and lupenone have been previously identified in *A. dealbata* leaves (Rodrigues et al., 2021).

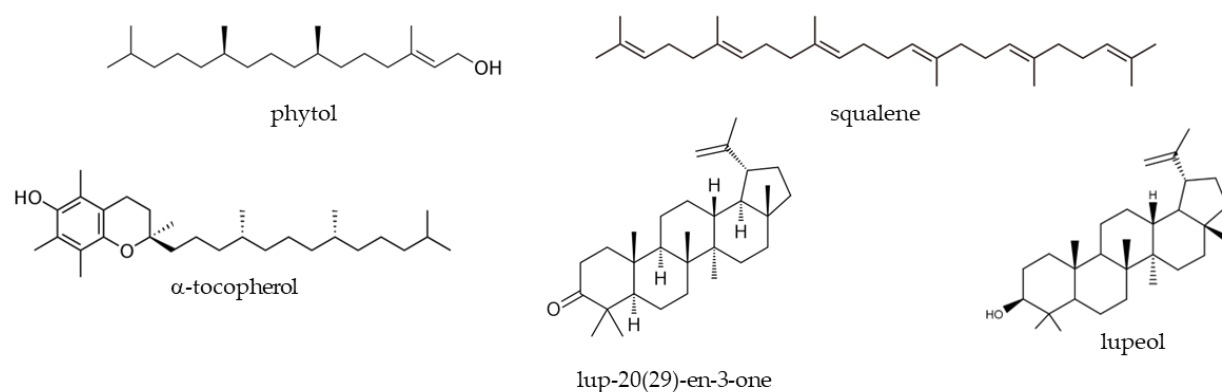


Figure 6.3 — Chemical structures of phytol, squalene, α -tocopherol, lupenone (lup-20(29)-en-3-one), and lupeol.

Phytol is a diterpene known for its wide range of biological activities, such as antimicrobial, antioxidant, cytotoxic, anxiolytic, anticonvulsant, immunomodulatory, antinociceptive, and anti-inflammatory activities, besides its role in the induction of apoptosis and protective autophagy, and in the treatment of metabolic disorders (Islam et al., 2018). Phytol is the most abundant compound in the analyzed extracts, especially in the leaves fraction, with the extracts

obtained from dried leaves showing higher content than the one obtained from fresh leaves (Figure 6.4).

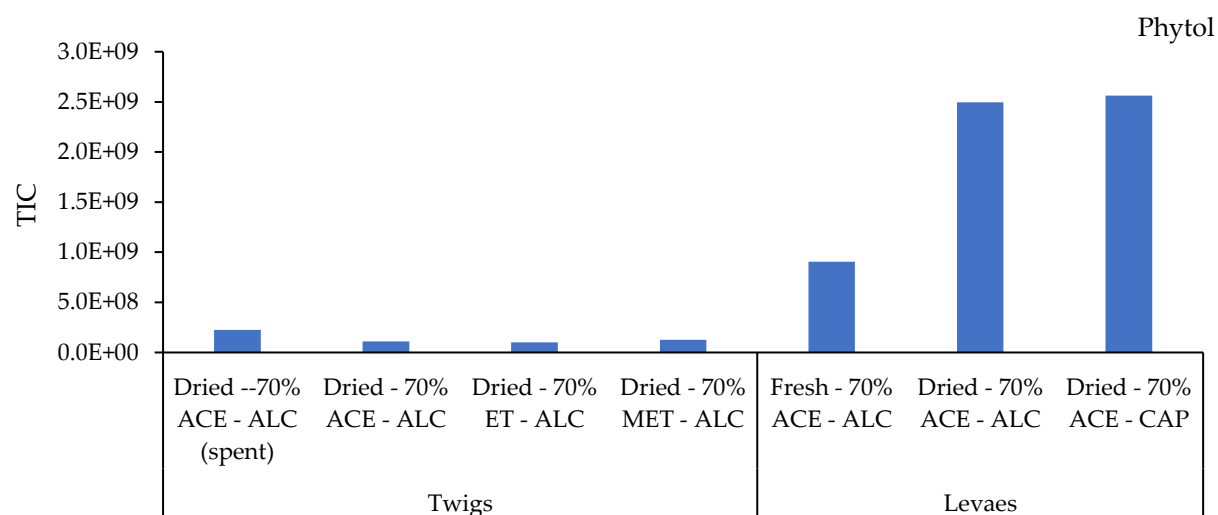


Figure 6.4 — Absolute peak areas of phytol in the analyzed extracts; TIC: Total Ion Current.

Figure 6.5 shows the absolute peak areas of squalene, α -tocopherol, lupenone, and lupeol for the selected extracts.

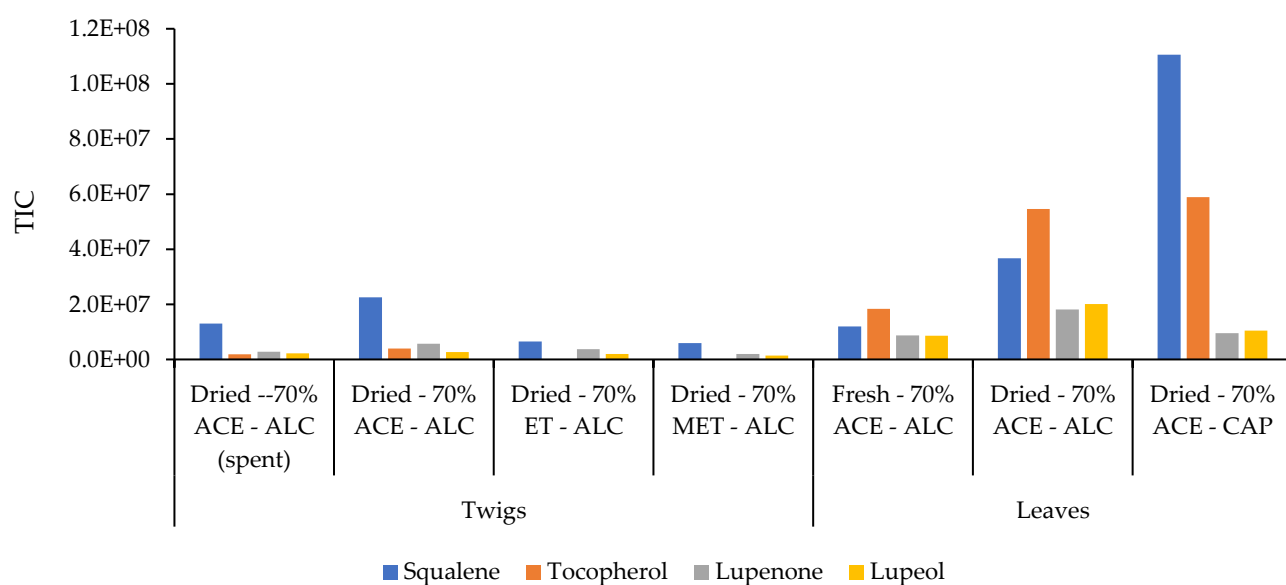


Figure 6.5 — Absolute peak areas of squalene, α -tocopherol, lupenone (lup-20(29)-en-3-one), and lupeol in the analyzed extracts; TIC: Total Ion Current.

Squalene is a triterpene with reported antioxidant, anti-inflammatory, and anti-atherosclerotic properties (Lou-Bonafonte et al., 2018), as well as presenting the potential for cosmetic dermatology applications due to its emollient and antioxidant properties, and for hydration and its antitumor activities (Huang et al., 2009). Squalene is more abundant in 70% ACE extracts than in 70% ET or 70% MET ones, with the highest amounts being observed in the dried leaves extracts.

α -Tocopherol is the main form of vitamin E, which has antioxidant, anti-inflammatory, and antitumor properties already described in the literature (Szewczyk et al., 2021). α -Tocopherol is not present in the 70% ET and 70% MET extracts, being particularly abundant in the dried leaves extracts.

Lupenone and lupeol are pentacyclic triterpenes showing a broad spectrum of biological activities. For the former, its importance as a therapeutic and chemopreventive agent for the treatment of inflammation, virus infection, diabetes, cancer, and Chagas disease was already described in the literature (Xu et al., 2018). For lupeol, antioxidant, antiprotozoal, anti-inflammatory, antitumor, and antimicrobial activities were previously reported, in addition to its cardio- and hepatoprotective effects and as a cancer chemopreventive agent (Wal et al., 2011). Both compounds are more abundant in leaves extracts than in twigs extracts. These results show the ability of acetone to extract both polar and non-polar compounds, which occurred to a lesser extent when using aqueous ethanol or methanol, given the higher polarity of these solvents. The use of dry material favored the extraction of this non-polar fraction as well. The previous extraction of the raw material with petroleum ether naturally decreased the amount of these non-polar compounds in the extract obtained from spent material. The presence of these bioactive compounds in a higher amount in the leaves extracts does not agree with the highest antioxidant and antimicrobial effects observed in the twigs extracts, which suggests that these effects are predominantly due to the action of the more polar compounds present in the extracts. In fact, it was observed a strong positive correlation between TPC and antioxidant activity.

6.4 Conclusions

This investigation demonstrated the possibility of producing extracts rich in phenolic compounds with antioxidant and antimicrobial properties using leaves and twigs of Portuguese *A. dealbata*, by a simple maceration process at room temperature. The highest values of extraction yield, TPC, TFC, and TPrAC as well as antioxidant activity measured by the DPPH and FRAP

assays were obtained with 70% ACE using leaves and twigs from Alcobaça. The drying status of the raw material also affected some characteristics of the extracts, improving extraction yield, TFC, and TPrAC for dried leaves and twigs.

As a rule, better results were observed in extracts from the twigs than in the corresponding extracts from the leaves, except for extraction yield. This observation shows the possibility of using the entire fraction of the branches, where the leaves represent by far the highest weight fraction, without the necessity of separating the twigs from the leaves, once they will add quality to the produced extracts.

Moreover, this work showed the possibility of obtaining a wider range of products from the same amount of raw material, by sequential extraction with petroleum ether to remove lipophilic compounds followed by the best extraction solvent to remove phenolic compounds, without significantly decreasing the quality of the extract.

Significant and strong correlations between TPC and TPrAC and antioxidant activity by DPPH and FRAP assays shows that those phenolic components are the main responsible for the antioxidant and antimicrobial properties of the extracts. Nevertheless, bioactive lipophilic components, such as phytol, squalene, α -tocopherol, lupenone, and lupeol were also detected, especially in the leaves extracts. Therefore, this work demonstrates the possibility of obtaining high-value extracts with antioxidant and antimicrobial properties from fractions of *A. dealbata* biomass that can be collected regularly to reduce widespread dissemination of this species.

CHEMICAL AND FUNCTIONAL CHARACTERIZATION OF EXTRACTS FROM WOOD AND BARK OF *ACACIA DEALBATA*

Abstract: The purpose of this work was to evaluate the recovery of bioactive extracts from *Acacia dealbata* wood and bark and to characterize their chemical composition and functional properties. Air-dried samples were extracted by maceration at room temperature and by hot extraction at 60 °C using aqueous solutions of acetone, ethanol, and methanol. The highest extraction yields (3.03 – 3.34 and 21.51 – 24.36% for wood and bark, respectively) were obtained with aqueous acetone and aqueous methanol in the case of wood, and with 70% acetone in the case of bark, for both extraction procedures. Extracts were characterized for total phenolics content (TPC), total flavonoid content (TFC), and total proanthocyanidin content (TPrAC), with the results showing that bark has a significantly higher content of phenolic compounds than the wood fraction. The highest TPC (308.8 – 311.0 mg GAE/g extract in the wood against 668.0 - 689.8 mg GAE/g extract in bark extracts), TFC (533.6 – 568.2 mg QE/g extract in the wood against 984.5 - 1015.9 mg QE/g extract in bark extracts), and TPrAC (638.4 - 639.5 mg PycE/g extract in the wood against 851.4 – 867.4 mg PycE/g extract in bark extracts) were obtained using aqueous acetone as extraction media, with no significant differences between maceration and the hot extraction methods. The antioxidant activity of the extracts was characterized through DPPH and FRAP assays, with better results obtained in bark extracts (1377.0 - 1405 mg TE/g extract and 10858.0 – 10979.8 mmol Fe²⁺/g extract, respectively), with no significant differences between both extraction methods. The antimicrobial activity of the extracts against different bacteria was also determined. Wood extracts showed antimicrobial properties against *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA),

Enterococcus faecalis, and *Bacillus cereus* bacteria, while bark extracts also inhibited *Staphylococcus epidermidis* bacteria and *Candida albicans* yeast besides the last ones. Overall, the extracts obtained from previously petroleum ether-extracted wood and bark showed increased phenolic contents and antioxidant activity, which allows the recovery of a broader range of chemicals from the same material, without compromising the quality of the obtained extracts. The results obtained demonstrate the feasibility of recovering valuable components from these biomass fractions that may be further valorized for energy production in a biorefinery concept.

7.1 Introduction

A. dealbata has a large capacity for raw material supply, having been estimated that about 70 tons of biomass (whole tree) can be obtained per hectare (Nunes et al., 2020), from which the bark and wood may represent a significant mass fraction. Research on the production of bio-active extracts from *A. dealbata* wood is scarce (Correia et al., 2020; López-Hortas et al., 2021). Yildiz et al. produced methanolic extracts from *A. dealbata* wood, which showed antioxidant activity and antimicrobial activity against *Listeria monocytogenes*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* bacteria (Yildiz et al., 2018). The phenolic and flavonoid contents in the heartwood were, respectively, ten and six times lower than the ones found in the bark, while the proanthocyanidin contents were similar in both fractions (Yildiz et al., 2018). Regarding bark, Yildiz et al. observed antioxidant and antimicrobial activity of methanolic extracts against *L. monocytogenes*, *S. aureus*, *Pseudomonas aeruginosa*, *K. pneumoniae*, and *Proteus mirabilis* bacteria, and *Candida albicans* yeast (Yildiz et al., 2018). In turn, acetonic extracts of previously petroleum ether-extracted bark were shown to possess antioxidant and anti-inflammatory properties (Sowndhararajan et al., 2016, 2015, 2013). The antioxidant activity of the acetonic extracts was well correlated with the extracts' phenolic and flavonoid contents (Sowndhararajan et al., 2013). Aqueous and ethanolic extracts of previously hexane-extracted bark also demonstrated antioxidant activity and antimicrobial activity against *Acinetobacter baumannii*, *Escherichia coli*, *K. pneumoniae*, *P. aeruginosa*, *Salmonella enterica* serovars, *Bacillus cereus*, *Enterococcus faecalis*, *L. monocytogenes*, and *S. aureus* bacteria, and *Candida albicans* and *Candida tropicalis* yeasts (Neiva et al., 2020). Antioxidant activity of aqueous ethanolic and aqueous methanolic extracts was already reported as well (Abilleira et al., 2021; Fernández-Agulló et al., 2012).

Despite the relevant outcomes from wood and bark reported so far, the use of a simple-to-implement and low-energy technique must be further studied and compared with more

energy-demanding techniques used so far, such as Soxhlet (Abilleira et al., 2021; Neiva et al., 2020; Sowndhararajan et al., 2016, 2015, 2013), agitation under heating (Abilleira et al., 2021; Fernández-Agulló et al., 2012), or just permanent agitation (Yildiz et al., 2018). Moreover, the comparison between using raw wood and bark or the spent material after the removal of lipophilic components with petroleum ether was not reported so far. In this context, this study aims to investigate the production of bioactive extracts from wood and bark of *A. dealbata* by a simple maceration process at room temperature and a hot extraction at 60 °C. The influence of the extraction solvent (70% acetone, 70% ethanol, and 70% methanol (V/V)) in extract yield, composition, antioxidant activity, and antimicrobial activity was evaluated. The extracts were characterized for mass yield, total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanidins content (TPrAC), for antioxidant activity through *in vitro* DPPH free radical scavenging assay and ferric reducing antioxidant power (FRAP) assay, and for antimicrobial activity against Gram-negative and Gram-positive bacteria and fungi.

7.2 Materials and methods

7.2.1 Plant material

Wood and bark of *A. dealbata* were collected in May of 2020 in the region of Alcobaça (Center region of Portugal: 39°36'36.5"N, 8°59'45.0"W, at 32 – 40 m of altitude). The samples used for extraction are a result of the mixing of at least ten different trees. Bark was separated from the wood and both fractions were analyzed separately. Biomass samples were left to dry in the air until a constant weight was attained (moisture content of 8.5 and 10.1% (wb) for wood and bark, respectively). The wood and bark were then cut into smaller pieces in an electric shredder (STILL GHE 140L, Hamburg, Germany), and then both bark and wood were grounded (Hukoer, Shanghai, China) and sieved to 5 mm before being processed in the extraction experiments. The visual aspect of the wood and bark used in this work is presented in Figure 7.1.



Figure 7.1 — Shredded wood (top, left) and bark (top, right); grounded wood (bottom, left) and bark (bottom, right).

7.2.2 Chemicals and reagents

Acetone, ethanol 96°, methanol, and petroleum ether used in the extraction processes were purchased from CarloErba Reagents (Val de Reuil, France). Folin-Ciocalteu reagent (Panreac, Barcelona, Spain), sodium carbonate (Labkem, Barcelona, Spain), and gallic acid (Merck, Darmstadt, Germany) were used to determine phenolics content. The reagents sodium nitrite (NaNO_2), sodium hydroxide (NaOH), and aluminium chloride (AlCl_3) used in the determination of flavonoids were all purchased from Merck (Darmstadt, Germany). Butanol (Panreac, Barcelona, Spain), ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) (Panreac, Barcelona, Spain), hydrochloric acid (Chem-Lab NV, Zedelgem, Belgium), and Pycnognol® (generously provided by Horphag Research Ltd, Geneva, Switzerland) were used in the acid-butanol assay. The reagent 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, St. Louis, MO, USA) was used in the DPPH assay. The reagents 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), acetate buffer 3.6, iron (III) chloride (FeCl_3), and iron (II) sulfate (FeSO_4) used in the ferric reducing antioxidant power (FRAP) assay were also purchased from Merck (Darmstadt, Germany). Ketoconazole, ofloxacin, and vancomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mueller-Hinton agar (MHA), Sabouraud dextrose agar (SDA), and Tryptone casein-soy agar (TSA) were purchased from Biokar diagnostics (Allonne, France). Brain heart infusion agar (BHIA) was purchased from Merck (Darmstadt, Germany).

7.2.3 Extraction procedure

Two extraction methods were compared with the fresh and dried raw material: maceration at room temperature for 24 h in the case of the wood and 48 h for the bark, and a hot extraction at 60 °C for 1 h (wood) or 2 h (bark). Higher temperatures were not selected to avoid thermal degradation of the extracts. Extraction times for bark and wood were selected as the time needed to achieve constant mass yield, TPC, and antioxidant activity given by the DPPH assay, according to a preliminary study (see Appendix E, Figures E1 to E10). Both approaches were performed in a single step using 20 g of biomass, at a biomass:solvent ratio of 1:6 (weight:volume), evaluating the use of 70% of aqueous acetone (70% ACE), 70% aqueous ethanol (70% ET), and 70% of aqueous methanol (70% MET) as extraction solvents. After the extraction period, the liquid extract was separated from the biomass by filtration and stored in a freezer (-20 °C) until used for analyses. An aliquot was evaporated to dryness in a rotavapor for determining the mass yield (expressed as a percentage, on a wet basis). The extraction process was made in duplicate.

Extraction of spent raw material was also assessed, after the removal of a lipidic fraction by extraction with petroleum ether. Wood and bark were extracted with petroleum ether by maceration at room temperature using a biomass:solvent ratio of 1:5, and the lipophilic extracts were stored for subsequent analysis and not included in this study. The biomass was left to dry in air at room temperature and then submitted to extraction by maceration at room temperature with 70% ACE. The mass yield was determined in the same manner and the extract samples were then stored at -20 °C until analysis.

7.2.4 Extracts characterization

Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity by DPPH and FRAP assays were performed as described in section 3.2.4. Total proanthocyanidin content (TPrAC) was determined by the butanol-HCl assay as described in section 4.2.4.

In all these characterization assays, two replicates of each sample were analyzed in triplicate.

7.2.5 Antimicrobial activity

The antimicrobial activity was determined as described in section 3.2.6.

7.2.6 Statistical analysis

Statistical analysis of the results was performed using the SPSS software, version 23 (IBM Analytics, Armonk, NY, USA). Analysis of variance (ANOVA) was performed to evaluate significant differences between averages, and the Tukey post hoc test ($p < 0.05$) was used to perform multiple comparisons between averages.

7.3 Results and discussion

7.3.1 Extract yield and composition

The use of different solvents (70% ACE, 70% ET, and 70% MET) and extraction methods (maceration at room temperature and hot extraction at 60 °C) was evaluated in obtaining bioactive extracts from wood and bark of *A. dealbata* collected in the region of Alcobaça (39°36'36.5"N; 8°59'45.0"W, at 32 – 40 m of altitude). The results of extraction yield, TPC, TFC, and TPrAC for each extraction solvent and extraction method, are presented in Table 7.1.

Extract yields varied in a short range for wood extracts (2.83 – 3.34% (wb)), with values much lower than the ones found for the bark extracts (17.50 – 24.36% (wb)). Expressing the extract yields in a dry basis (db), the values here obtained varied in the range 3.09 – 3.65% (db) and 19.47 – 27.10 % (db) for wood and bark respectively. Aqueous acetone was undoubtedly the extraction medium that gave the best yields for the bark extracts, while for the wood extracts, 70% ACE and 70% MET gave similar results. For each solvent, higher yields were obtained by maceration for both wood and bark extracts, with the exception made for the bark aqueous methanolic extracts. The extract yields obtained for the wood fraction are in accordance with the reported values for wood (3.1 – 5.85%) (López-Hortas et al., 2021). In the case of bark extracts, the obtained values were higher than the ones found in acetonic (2.6 – 11.6% (db)) and methanolic (3.6 – 8.7% (db)) bark of several *Acacia* species including *A. dealbata*, obtained by Soxhlet extraction (Sowndhararajan et al., 2013). The bark extract yields were in the range of the ones found in *A. dealbata* bark aqueous ethanolic (80% ethanol) extracts obtained by Soxhlet or hot extraction at 80 °C under stirring (11.9% - 29.5% (db)), and higher than the ones obtained using water in the same extraction conditions (5.2 – 10.1% (db)) (Abilleira et al., 2021). However, higher yields were reported by Neiva et al. using exhaustive Soxhlet extraction with ethanol (36.54 % (db)) or water (36.77% (db)) as extraction solvents (Neiva et al., 2020).

Table 7.1 — Extraction yields (EY), total phenolics content (TPC), total flavonoids content (TFC), and total proanthocyanidins content (TPrAC) of wood and bark extracts, using different extraction solvents and methods.

Solvent	EY (%, wb)	TPC (mg GAE/g extract, db)	TPC (mg GAE/g bi- omass, wb)	TFC (mg QE/g ex- tract, db)	TFC (mg QE/g bio- mass, wb)	TPrAC (mg PycE/g extract)	TPrAC (mg PycE/g biomass, db)
Wood							
Maceration							
70% ACE	3.23 ± 0.03 ^{ab}	308.8 ± 5.2 ^a	10.0 ± 0.1 ^a	533.6 ± 32.5 ^a	17.2 ± 0.9 ^a	638.4 ± 20.4 ^a	20.6 ± 0.7 ^a
70% ET	3.19 ± 0.03 ^{bc}	250.5 ± 5.5 ^b	8.0 ± 0.1 ^c	467.9 ± 15.5 ^{bc}	14.9 ± 0.3 ^{bc}	443.8 ± 13.7 ^c	14.2 ± 0.5 ^{bc}
70% MET	3.34 ± 0.03 ^a	239.6 ± 4.3 ^c	8.0 ± 0.1 ^c	452.6 ± 19.7 ^c	15.1 ± 0.5 ^b	441.3 ± 15.2 ^c	14.7 ± 0.4 ^b
Hot extraction							
70% ACE	3.09 ± 0.02 ^{cd}	311.0 ± 7.1 ^a	9.6 ± 0.1 ^b	568.2 ± 28.3 ^a	17.5 ± 0.7 ^a	639.5 ± 20.8 ^a	19.8 ± 0.7 ^a
70% ET	2.83 ± 0.03 ^e	257.4 ± 6.1 ^b	7.3 ± 0.1 ^e	494.7 ± 17.0 ^b	14.0 ± 0.3 ^c	488.1 ± 24.7 ^b	13.8 ± 0.6 ^{bc}
70% MET	3.03 ± 0.02 ^d	248.5 ± 2.1 ^{bc}	7.5 ± 0.1 ^d	483.4 ± 10.1 ^{bc}	14.7 ± 0.4 ^{bc}	447.4 ± 14.0 ^c	13.6 ± 0.5 ^c
Bark							
Maceration							
70% ACE	24.36 ± 0.44 ^a	668.0 ± 7.0 ^b	162.7 ± 1.8 ^a	984.5 ± 17.7 ^{ab}	239.7 ± 2.0 ^a	867.4 ± 26.9 ^a	211.2 ± 7.0 ^a
70% ET	19.85 ± 0.27 ^c	586.0 ± 4.2 ^e	116.4 ± 2.5 ^c	895.2 ± 32.7 ^e	177.7 ± 4.7 ^c	755.6 ± 18.0 ^c	150.1 ± 5.8 ^c
70% MET	17.50 ± 0.30 ^d	610.9 ± 6.3 ^d	106.9 ± 1.3 ^e	897.7 ± 26.1 ^{de}	157.1 ± 4.4 ^e	774.4 ± 17.8 ^{bc}	135.5 ± 4.1 ^d
Hot extraction							
70% ACE	21.23 ± 0.22 ^b	689.8 ± 9.8 ^a	148.4 ± 0.9 ^b	1015.9 ± 16.0 ^a	218.5 ± 3.3 ^b	851.4 ± 20.4 ^a	183.2 ± 4.1 ^b
70% ET	18.02 ± 0.06 ^d	627.0 ± 6.2 ^c	113.0 ± 1.2 ^d	969.5 ± 16.5 ^{bc}	174.7 ± 2.9 ^c	798.2 ± 26.4 ^b	143.8 ± 4.9 ^{cd}
70% MET	17.51 ± 0.24 ^d	569.7 ± 9.3 ^f	99.7 ± 0.8 ^f	937.3 ± 28.9 ^{cd}	164.0 ± 2.4 ^d	794.6 ± 29.7 ^{bc}	139.1 ± 3.9 ^d

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 x 3); different letters in each column indicate significant differences of means among extract samples (one-way ANOVA, Tukey test at p<0.05).

The TPC and TFC were significantly higher in 70% ACE extracts, for both fractions. Overall, no significant differences were found in TPC and TFC between extraction methods, meaning that we can produce phenolic and more specifically flavonoid-rich extracts by a simple and low-energy process such as maceration at room temperature. The bark is much richer in phenolics and flavonoids than wood and gave extracts with about twice the TPC and TFC contents.

The TPC in 70% ET and 70% MET wood extracts were of similar magnitude to the ones already observed with *A. dealbata* wood using similar extraction media (50% ethanol and 50% methanol) under heating (240.4 – 256.9 mg GAE/g extract) (Fernández-Agulló et al., 2012). The TPC in the 70% ACE wood extracts was in a higher range, showing that aqueous acetone is the best solvent to obtain phenolics from *A. dealbata* wood. Regarding bark, the TPC and TFC (in rutin equivalents (RutE), see Appendix E2, Table E1) obtained in the 70% ACE and 70% MET extracts by both methods were higher than the ones observed in acetonic (641 mg GAE/g

extract and 91.8 RE/g extract) and methanolic (465 mg GAE/g extract and 74.0 RE/g extract) *A. dealbata* bark extracts obtained by Soxhlet extraction (Sowndhararajan et al., 2013). Moreover, remarkably higher efficiency in the removal of the phenolics and flavonoids was observed in this work, with 99.7 – 162.7 mg GAE/g of bark and 157.1 – 239.7 RE/g of bark against 16.7 – 30.2 mg GAE/g of bark and 2.4 – 4.8 mg RutE/g of bark (Sowndhararajan et al., 2013). In fact, the use of mixtures of organic solvent and water are reported to be more efficient in removing phenolics, due to the facilitating role of water by increasing permeability of cell tissues and enabling mass transfer by molecular diffusion, which results in higher recovery of bioactive compounds (Boeing et al., 2014). The TPC observed in this work was comparable to the one found in *A. dealbata* bark extracts obtained by Soxhlet extraction with ethanol (704 mg GAE/g extract) or water (586 mg GAE/g extract), but the TFC was much higher (895.2 – 1015.9 mg QE/g extract against 3.9 – 15.9 QE/g extract) (Neiva et al., 2020). Abilleira et al. found a strong influence of the bark particle size in obtaining phenolics by Soxhlet and hot extraction at 80 °C under stirring, having found TPC values in the range of 232.5 – 661.1 mg GAE/g extract for particle sizes <0.5 mm or >1 mm, which are in the range of the TPC observed in this work, but significantly higher values when using particle sizes of 0.5 – 1 mm (775 mg GAE/g extract by hot extraction with 80% aqueous ethanol, or 820.2 mg GAE/g extract by Soxhlet extraction with same solvent) (Abilleira et al., 2021). The influence of the particle size was not evaluated in this work. An easy-to-implement approach was followed and particles <5 mm were used, avoiding eventual loss of biomass and operational issues that occurs when milling and sieving to much smaller sizes due to the tendency of wood and especially the bark fraction to form hair-like structures that may obstruct the sieves and feeding systems.

Regarding TPrAC, the results were expressed relative to a standard source of proanthocyanidins, the very well-known commercial extract Pycnogenol®. Both wood and bark fractions are good sources of proanthocyanidins, but these are more abundant in the bark fraction, following the trend observed for TPC and TFC. Again, aqueous acetone was the best extraction solvent, originating extracts with significantly higher TPrAC than 70% ET or 70% MET, with no significant differences between maceration and hot extraction being observed.

These results show that the different extraction solvents affect the quality of the produced extracts, while the different extraction methods had minor effects. It was demonstrated that it can be produced phenolic-rich extracts from the wood and bark of *A. dealbata* by an easy-to-implement and low-energy method such as maceration at room temperature, with 70% ACE as the best extraction media. However, the bark was found to be much richer in phenolics and produced extracts in a much higher yield, and with higher TPC, TFC, and TPrAC.

7.3.2 Antioxidant activity

The antioxidant activity of the wood and bark extracts obtained by maceration and by hot extraction was assessed by the DPPH and FRAP assays. The results are given in Table 7.2.

The DPPH assay measures the ability of compounds present in the extracts to act as free radical scavengers or hydrogen donors antioxidants (Kedare and Singh, 2011). In turn, the FRAP assay measures the reducing power of extracts, by evaluating the reduction of Fe^{3+} in the complex (Fe^{3+} – TPTZ) to a ferrous form (Fe^{2+} – TPTZ), by donation of electrons from the antioxidants present in the extracts, resulting in stopping the free radical production chain (Afsar et al., 2018; Sadiq et al., 2017; Santos-Sánchez et al., 2019).

Table 7.2 — Results from the DPPH and FRAP assays of the wood and bark extracts, using different extraction solvents and extraction methods.

Solvent	DPPH (mg TE/g ex- tract, db)	DPPH (mg TE/g bio- mass, wb)	FRAP ($\mu\text{mol Fe}^{2+}$ /g ex- tract, db)	FRAP ($\mu\text{mol Fe}^{2+}$ /g bi- omass, wb)
Wood				
Maceration				
70% ACE	536.3 $\pm$ 9.0 ^a	17.3 $\pm$ 0.1 ^a	4520.7 $\pm$ 75.0 ^a	145.8 $\pm$ 0.8 ^a
70% ET	304.7 $\pm$ 6.2 ^e	9.7 $\pm$ 0.1 ^f	3302.0 $\pm$ 45.0 ^c	105.5 $\pm$ 1.2 ^c
70% MET	303.2 $\pm$ 6.2 ^e	10.1 $\pm$ 0.1 ^e	3192.0 $\pm$ 83.8 ^c	106.6 $\pm$ 1.7 ^c
Hot extraction				
70% ACE	517.8 $\pm$ 6.0 ^b	16.0 $\pm$ 0.1 ^b	3801.2 $\pm$ 128.4 ^b	117.4 $\pm$ 3.1 ^b
70% ET	441.8 $\pm$ 2.9 ^c	12.5 $\pm$ 0.2 ^d	2977.6 $\pm$ 73.3 ^d	84.3 $\pm$ 2.4 ^e
70% MET	430.1 $\pm$ 2.8 ^d	13.0 $\pm$ 0.1 ^c	2920.9 $\pm$ 88.1 ^d	88.6 $\pm$ 2.0 ^d
Bark				
Maceration				
70% ACE	1377.0 $\pm$ 26.0 ^a	335.3 $\pm$ 1.1 ^a	10979.8 $\pm$ 194.0 ^a	2673.6 $\pm$ 39.7 ^a
70% ET	892.3 $\pm$ 15.2 ^b	177.2 $\pm$ 2.3 ^c	9468.6 $\pm$ 208.3 ^c	1880.4 $\pm$ 54.4 ^c
70% MET	886.4 $\pm$ 21.5 ^{bc}	155.1 $\pm$ 0.8 ^d	10461.7 $\pm$ 195.8 ^b	1830.7 $\pm$ 20.5 ^c
Hot extraction				
70% ACE	1405.0 $\pm$ 22.0 ^a	302.2 $\pm$ 2.9 ^b	10858.0 $\pm$ 103.0 ^a	2336.1 $\pm$ 41.5 ^b
70% ET	848.2 $\pm$ 22.9 ^c	152.8 $\pm$ 3.7 ^d	10444.9 $\pm$ 144.8 ^b	1882.1 $\pm$ 20.5 ^c
70% MET	802.1 $\pm$ 29.7 ^d	140.4 $\pm$ 3.0 ^e	8126.6 $\pm$ 170.5 ^d	1422.5 $\pm$ 16.1 ^d

Values (mean $\pm$ standard deviation) are average of two replicates of each sample, analyzed individually in triplicate ($n = 2 \times 3$); different letters indicate significant differences of means among solvents for each extraction process (one-way ANOVA, Tukey test at $p < 0.05$).

The DPPH and FRAP results were found higher when using 70% ACE as extraction media, for which no significant differences between the maceration at room temperature and the hot extraction processes were observed. Both assays indicated that the bark fraction is richer in antioxidant compounds than the wood, and originated extracts with much higher antioxidant contents.

The antiradicalar activity given by the DPPH assay in the 70% ET and 70% MET extracts of wood were of the same order as the ones found in 50% aqueous ethanol and 50% aqueous methanol extracts of *A. dealbata* wood (1.42 – 1.47 mmol TE/g extract, corresponding to 355.4 – 367.9 mg TE/g extract) (Fernández-Agulló et al., 2012). However, it was noticed again better results with the 70% ACE extract (517.8 – 536.3 mg TE/g extract), confirming aqueous acetone to be the most suitable solvent for obtaining antioxidant components from *A. dealbata* wood. Regarding bark, the DPPH values for the 70% ACE bark extract were found higher than the one obtained in an aqueous ethanolic extract (80% ethanol) of *A. mearnsii* bark (1220 mg TE/g extract) (Chen et al., 2018). In turn, the reducing power evaluated by the FRAP assay revealed that the antioxidant content in the wood was in the range of the observed in methanolic wood extract of *A. dealbata* (47.11 – 322.4 $\mu\text{mol Fe(II)/g bark}$) (Yildiz et al., 2018). The FRAP values of bark extracts were much higher than the ones observed in acetonic and methanolic bark extracts of several *Acacia* species, including *A. dealbata* (8126.6 – 10979.8 $\mu\text{mol Fe(II)/g bark}$ against 2177 – 2347 $\mu\text{mol Fe(II)/g bark}$) (Sowndhararajan et al., 2013).

The extraction solvent significantly affected the antioxidant activity given by both methods applied, with the best results being obtained in 70% ACE extracts. Higher antioxidant activities of extracts obtained with acetone or aqueous acetone solutions were also observed with stem bark extracts of *Butea monosperma* (Lam.) Kuntze (Salar and Seasotiya, 2011), bark extracts of *A. leucophloea*, *A. ferruginea*, *A. dealbata* and *A. pennata* (Sowndhararajan et al., 2013), and flower extracts of safflower (*Carthamus tinctorius* L.) (Salem et al., 2011).

7.3.3 Extraction of spent raw material

The wood and bark may have the potential for obtaining lipophilic compounds such as terpenes, long-chain alcohols, fatty acids, sterols, aromatic compounds, among others, (C. S. D. Oliveira et al., 2020). This way, to assess the possibility of enlarging the variety of products retrieved from the same amount of plant, and simultaneously evaluating the effect of the removal of lipids on the extraction of more hydrophilic components, an extraction experiment was performed on the wood and bark previously extracted with petroleum ether, using the

best extraction solvent (70% ACE). The characterization of the lipophilic fraction will be a subject of different work.

The comparison of the composition and antioxidant activity of extracts obtained from the raw material macerated at room temperature with 70% ACE, and from the spent material extracted in the same way with 70% ACE, is described in Table 7.3 and Table 7.4.

Table 7.3 — Comparison of the composition of the extracts obtained from raw and spent biomass, using maceration at room temperature with 70% ACE as extraction solvent.

Sample	EY (%, wb)	TPC (mg GAE/g extract, db)	TPC (mg GAE/g biomass, wb)	TFC (mg QE/g ex- tract, db)	TFC (mg QE/g bi- omass, wb)	TPrAC (mg PycE/g extract)	TPrAC (mg PycE/g biomass, db)
Wood							
spent	3.07 ± 0.08	324.5 ± 8.3	9.9 ± 0.0	620.2 ± 16.4	19.0 ± 0.3	577.9 ± 7.7	17.7 ± 0.6
raw	3.23 ± 0.03	308.8 ± 5.2	10.0 ± 0.1	533.6 ± 32.5	17.2 ± 0.9	638.4 ± 20.4	20.6 ± 0.7
Bark							
spent	17.95 ± 0.12	758.1 ± 8.6	136.1 ± 1.0	1190.1 ± 16.0	68.3 ± 1.4	1094.8 ± 26.1	196.6 ± 3.1
raw	24.36 ± 0.44	668.0 ± 7.0	162.7 ± 1.8	984.5 ± 17.7	239.7 ± 2.0	867.4 ± 26.9	211.2 ± 7.0

The extraction yields decreased when using wood or bark previously extracted with petroleum ether, suggesting that 70% ACE could have extracted lipophilic components in the raw biomass along with hydrophilic ones. However, it should be noted the increment in the TPC and TFC of both spent wood and bark extracts and also in the TPrAC of the spent bark extract.

Table 7.4 — Comparison of the antioxidant activity of the extracts obtained from raw and spent biomass, using maceration at room temperature with 70% ACE as extraction solvent.

Sample	DPPH (mg TE/g ex- tract, db)	DPPH (mg TE/g bi- omass, wb)	FRAP (mmol Fe2+/g extract, db)	FRAP (mmol Fe2+/g biomass, wb)
Wood				
spent	558.3 ± 11.7	17.1 ± 0.4	4752.0 ± 128.3	145.6 ± 1.4
raw	536.3 ± 9.0	17.3 ± 0.1	4520.7 ± 75.0	145.8 ± 0.8
Bark				
spent	1410.1 ± 21.2	253.1 ± 2.4	12231.3 ± 200.9	2196.0 ± 51.7
raw	1377.0 ± 26.0	335.3 ± 1.1	10979.8 ± 194.0	2673.6 ± 39.7

Concerning antioxidant activity, very good results were obtained, once the removal of at least a part of the lipidic fraction from the wood or the bark increased the antioxidant activity

of the produced extracts. These findings are of crucial importance for the exploration of these resources from a biorefinery perspective because demonstrate the possibility of broadening the range of chemicals that can be obtained from the same amount of raw material, by performing sequential extraction with polar and non-polar solvents, such as petroleum ether followed by aqueous acetone.

7.3.4 Correlations between total phenolic, total flavonoid, and total proanthocyanidin contents and antioxidant activities

The antioxidant activity given by both methodologies followed the trend found for TPC, TFC, and TPrAC, which suggests the existence of some correlation between the TPC, TFC, TPrAC and the antioxidant activity. A correlation analysis using the Pearson correlation coefficient was performed and the results are presented in Table 7.5.

Besides good correlations between variables, correlations must be statistically significant. All the correlations found in this work were statistically significant at $p < 0.01$. The Pearson's correlation coefficients found indicate moderate ($0.700 < r < 0.800$) to very strong ($r > 0.800$) correlations (Akoglu, 2018) between TPC, TFC, TPrAC and antioxidant activity given by DPPH and FRAP assays, for both wood and bark extracts. Yadav et al. (Yadav et al., 2018) also found strong correlations between TPC, TFC, and DPPH values, for extracts of *A. nilotica* leaves. Significant and strong correlations between TPC and the DPPH and FRAP results were found in *Pinus densiflora* bark extract (Venkatesan et al., 2019) and in medicinal herbs and spices (Ulewicz-Magulska and Wesolowski, 2019).

Table 7.5 — Correlation analysis between total phenolics content (TPC), total flavonoid content (TFV), total proanthocyanidins content (TPrAC), and antioxidant activities given by DPPH and FRAP assays, for wood and bark extracts; significance of the correlations assessed at $p < 0.05$ and at $p < 0.01$ (two-tailed).

		TPC	TFC	TPrAC	DPPH	FRAP
Wood	TPC	1				
	TFC	$r = 0.933^{**}$ $p = 0.000$	1			
	TPrC	$r = 0.884^{**}$ $p = 0.000$	$r = 0.944^{**}$ $p = 0.000$	1		
	DPPH	$r = 0.884^{**}$ $p = 0.000$	$r = 0.727^{**}$ $p = 0.000$	$r = 0.708^{**}$ $p = 0.000$	1	
	FRAP	$r = 0.905^{**}$ $p = 0.000$	$r = 0.811^{**}$ $p = 0.000$	$r = 0.773^{**}$ $p = 0.000$	$r = 0.726^{**}$ $p = 0.000$	1
Bark	TPC	1				
	TFC	$r = 0.903^{**}$ $p = 0.000$	1			
	TPrC	$r = 0.904^{**}$ $p = 0.000$	$r = 0.753^{**}$ $p = 0.000$	1		
	DPPH	$r = 0.889^{**}$ $p = 0.000$	$r = 0.856^{**}$ $p = 0.000$	$r = 0.840^{**}$ $p = 0.000$	1	
	FRAP	$r = 0.902^{**}$ $p = 0.000$	$r = 0.726^{**}$ $p = 0.000$	$r = 0.722^{**}$ $p = 0.000$	$r = 0.758^{**}$ $p = 0.000$	1

$**$ correlation is significant at $p < 0.01$ (two-tailed).

These findings substantiate that phenolic compounds, including the flavonoids and proanthocyanidins class, have an important role in the observed DPPH free radical scavenging activity and the reducing power given by the FRAP assay. Moreover, the abundance of these compounds in the extracts obtained in this work, either from the raw material or from the spent material after the removal of the lipidic fraction, suggests that these *A. dealbata* fractions, especially the bark, have a strong potential to be explored for obtaining antioxidants for diverse applications, such as in nutraceutical, food or cosmetic industries.

7.3.5 Antimicrobial activity

The increasing pathogenic resistance to conventional antimicrobial agents has motivated research on finding alternatives to synthetic antibiotics by assessing the antimicrobial activity of extracts from plant origin (Ghannoum M. A. and Rice L. B., 1999). A representative group of extract samples was selected to assess antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi, including antibiotic resistant microorganisms. The tested microorganisms are frequently responsible for a wide variety of infectious diseases, including skin

and soft tissue infections, oral diseases and foodborne diseases (Tong et al., 2015; Xiao et al., 2022).

The inhibition zones (mm) of the tested extracts are presented in Table 7.6. Under the tested conditions, the negative control (70% ethanol) did not inhibit the growth of any of the microorganisms tested. None of the bark or wood extracts showed activity against Gram-negative bacteria. Bark extracts inhibited the growth of all Gram-positive bacteria and yeast tested. Concerning the wood extracts, no activity was observed against *S. epidermidis*, *S. mutans*, *S. mitis* and *C. albicans*, while for the remaining Gram-positive bacteria only the aqueous acetic extracts showed activity. Overall, extracts of both fractions were more effective against *B. cereus* (inhibition zones in the range 8.6 – 10.8 mm for wood extracts and 12.8 – 14.0 mm for bark extracts). The inhibition zones presented by bark extracts were always higher than the correspondent ones of the wood extracts. In agreement with the results obtained in the antioxidant activity assays, 70% ACE seems also to be the best solvent for extracting the antimicrobial compounds, since, generally, the extracts prepared in this solvent showed the highest inhibition diameters.

Table 7.6 — Inhibition zones (mm) of selected extracts of *A. dealbata* wood and bark against *S. aureus*, MRSA, *S. epidermis*, *E. faecalis*, *B. cereus*, *S. mutans*, *S. mitis*, and *C. albicans*.

Samples (10 mg/mL) (0.5 mg/well)	<i>S. aureus</i>	MRSA	<i>S. epidermis</i>	<i>E. faecalis</i>	<i>B. cereus</i>	<i>S. mutans</i>	<i>S. mitis</i>	<i>C. albicans</i>
Wood								
70%ACE-maceration	8.8 ± 0.0	8.3 ± 0.0	nd	7.1 ± 0.1	10.8 ± 0.1	nd	nd	nd
70%ET-maceration	nd	nd	nd	nd	8.8 ± 0.0	nd	nd	nd
70%MET-maceration	nd	nd	nd	nd	8.6 ± 0.0	nd	nd	nd
70%ACE-hot extraction	8.0 ± 0.0	7.8 ± 0.1	nd	7.0 ± 0.0	9.8 ± 0.0	nd	nd	nd
70%ACE-maceration – spent	7.8 ± 0.1	8.0 ± 0.1	nd	7.0 ± 0.0	10.0 ± 0.0	nd	nd	nd
Bark								
70%ACE-maceration	11.8 ± 0.0	11.5 ± 0.0	10.3 ± 0.0	10.0 ± 0.0	13.5 ± 0.0	11.0 ± 0.0	11.7 ± 0.6	10.0 ± 0.1
70%ET-maceration	11.0 ± 0.0	11.3 ± 0.0	10.0 ± 0.0	9.5 ± 0.1	12.8 ± 0.0	9.0 ± 1.0	11.0 ± 1.0	9.0 ± 0.0
70%MET-maceration	11.5 ± 0.0	11.8 ± 0.0	10.0 ± 0.1	10.0 ± 0.0	13.5 ± 0.0	9.3 ± 1.2	11.3 ± 1.5	8.0 ± 0.0
70%ACE-hot extraction	12.0 ± 0.0	12.0 ± 0.0	10.0 ± 0.0	10.3 ± 0.0	14.0 ± 0.0	9.7 ± 0.6	11.7 ± 1.2	8.3 ± 0.0
70%ACE-maceration – spent	12.6 ± 0.1	12.5 ± 0.1	10.3 ± 0.0	11.0 ± 0.0	13.5 ± 0.0	10.0 ± 1.7	11.7 ± 0.6	8.5 ± 0.1
Positive control								
Vancomycin (5.0 µg)	14.9 ± 0.7	13.9 ± 0.7	15.7 ± 1.0	12.5 ± 0.5	16.4 ± 1.1	na	na	na
Vancomycin (50.0 µg)	na	na	na	na	na	19.3 ± 0.6	25.7 ± 0.6	
ketoconazol (5.0 µg)	na	na	na	na,	na	na	na	36.8 ± 0.5

nd: not detected; na: not applied

All tested samples revealed ineffective against *P. aeruginosa* and *E. coli*. Inactivity against *P. aeruginosa* and *E. coli* bacteria and *Candida albicans* yeast was already observed with *A. dealbata* acetonic wood extracts (Yildiz et al., 2018). Using an eight times higher mass of aqueous or ethanolic bark extracts of *Eucalyptus globulus*, *Picea abies*, *Acacia melanoxylon*, and *A. dealbata*, Neiva et al. found inhibition zones of 11.7 – 21.8 mm against *S. aureus*, 9.9 – 17.8 mm against *E. faecalis*, 8.9 – 16.9 mm against *B. cereus*, and 10.2 – 12.9 mm against *C. albicans* (Neiva et al., 2020). Ferreira-Santos et al. found slightly lower inhibition zones against *S. aureus* (9.4 – 10.2 mm) and *B. cereus* (9.6 – 10.3 mm) using aqueous ethanolic extracts of *Pinus pinaster* bark, applying the same amount of extract in the antimicrobial assays (0.5 mg) (Ferreira-Santos et al., 2020). The authors did not find activity against *E. coli* and *C. albicans*. The produced bark extracts gave similar inhibition zones as the leaves and twigs extracts produced by the same procedure, with the exception made for *C. albicans* yeast, for which leaves and twigs extracts were ineffective (Correia et al., 2022).

These results show that it can be obtained antimicrobial extracts from *A. dealbata* wood and bark, with bark extracts showing activity against a wider range of microorganisms and higher inhibition zones. The extraction solvent influenced the antimicrobial activity only in the case of wood extracts. Extraction by maceration at room temperature proved to be a good option to obtain antimicrobial agents, even when using spent biomass.

7.4 Conclusions

This investigation demonstrated the possibility of producing phenolic-rich extracts with antioxidant and antimicrobial properties using wood and bark of Portuguese *A. dealbata* by a simple maceration process at room temperature. However, bark presented much higher extraction yields, TPC, TFC, TPrAC, and better results for antioxidant activity. Aqueous acetone proved to be the best extraction solvent, originating extracts with the highest TPC, TFC, and PrAC contents, and with the highest antioxidant activity as well. Moreover, this work showed the possibility of obtaining a wider range of products from the same amount of raw material via sequential extraction with petroleum ether to remove lipophilic compounds followed by the best extraction solvent to remove phenolic compounds, without compromising the antioxidant or antimicrobial activity of the extracts. The significant and strong correlations between TPC, TFC, TPrAC, and the antioxidant activity by DPPH and FRAP assays suggest that phenolic compounds, including the flavonoids and proanthocyanidins classes, may have an important role in the observed antiradical activity and reducing power.

Therefore, this work provides significant information on the possibility of obtaining high-value antioxidant and antimicrobial extracts from fractions of *A. dealbata* biomass that can be abundant from the removal operations, which is crucial to develop its exploitation from a bio-refinery perspective. Once the bark is much richer in phenolic compounds and presented significantly better bioactive properties, its separation from the wood may be considered.

CHARACTERIZATION OF *ACACIA DEALBATA* BIOMASS FOR ENERGY AND MATERIAL AP- PLICATION: EFFECT OF THE PREVIOUS EX- TRACTION OF BIOACTIVE COMPONENTS

Abstract: *Acacia dealbata* is an invasive species widespread in Portuguese territory, from which high amounts of biomass can be obtained, including flowers, leaves, twigs, wood, and bark. These fractions have been studied to obtain high-value chemicals through extraction procedures, thus making pertinent the characterization of the spent material for energetic and/or material applications, to allow exploring this resource from a biorefinery perspective. The purpose of this work was to evaluate the impact of extraction procedures on the thermochemical characteristics and adsorption ability of the different tree fractions. Proximate analysis revealed moisture, volatile, fixed carbon, and ash contents of 8.37 – 10.12 wt.%, 72.11 – 86.21 wt.%, 13.20 – 23.73 wt.%, and 0.58 – 5.86 wt.%, respectively, being observed an important reduction on ash content in spent flowers, leaves, twigs, and wood. Ultimate analysis showed minor differences between raw and spent material, with carbon, hydrogen, and oxygen contents of 46.67–52.91 wt.%, 5.94–7.07 wt.%, and 30.83–46.56 wt.% respectively, and lower values of sulfur and nitrogen. Higher heating values in the range of 18.45 – 23.47 MJ/kg, with no significant differences between raw and spent material, are of the same order as biomass traditionally used for energy purposes. FTIR analysis confirmed the heterogeneous character of the biomass with the presence of diverse functional groups, but with small differences among fractions. The mineral composition of leaves, twigs, wood, and bark ashes is reported to have a low tendency to provoke deposit problems, according to a recent predictive model developed for biomass. The

micro TGA revealed a decrease in burnout temperatures in the spent material, and the kinetic modeling allowed estimating that energies of activation decreased in the spent flowers, wood, and bark, compared with the respective raw materials. The macro TGA of spent material showed a typical biomass degradation behavior for all fractions, with the wood being the most reactive. Adsorption experiments showed that all fractions can adsorb the methylene blue dye in a much more extent than methyl orange dye, with removal efficiencies higher than 90% after 24 h of contact when using spent material. These results allowed us to conclude that biomass from *A. dealbata* has the potential to be explored from a biorefinery perspective, through the removal of high-value extractives and subsequent valorization of the remaining material for energy purposes or material application as adsorbents.

8.1 Introduction

As emphasized in the previous chapters, *Acacia dealbata* represent an abundant endogenous source of biomass with potential to be valorized from a biorefinery perspective. The possibility of recovering added-value functional extracts has been highlighted in this thesis. However, the evaluation of its fractions for energy purposes or material valorization is necessary to complete the biorefinery approach.

Energy applications of *Acacia* biomass are mostly restricted to the wood fraction but other parts of the tree have not been extensively characterized for this purpose. The thermochemical characterization of biomass through proximate and ultimate, mineral composition, and thermogravimetric analysis is a common practice to assess the viability of using a resource for energy production and has been made for diverse types of biomass (Ahmed et al., 2021a, 2021b; Charis et al., 2020; Ekinici et al., 2021; El may et al., 2012; Fraga et al., 2020; García et al., 2012; Okoroigwe et al., 2016; Şen et al., 2022; Shen et al., 2009). This characterization is fundamental to determine the fuel properties, including eventual environmental and operational equipment issues related to its use for energy purposes. Recently, Nunes et al. characterized the biomass of young and immature *A. dealbata* tree regarding the production of pellets. These authors concluded that *Acacia* biomass does not fit the certification standards for wood pellets due to high levels of nitrogen and chlorine (Nunes et al., 2021, 2020). Combustion experiments in a domestic pellet boiler evidenced higher CO and NO_x emissions when using *A. dealbata* pellets than when using *P. pinaster* (Ferreira et al., 2013). Regardless of these limitations for the production of certified *A. dealbata* pellets, the recovery of the residual biomass for domestic

applications as a fuel is an opportunity for the circularization of local resources associated with forest management operations (Nunes et al., 2021).

The potential of the different *Acacia* spp. fractions (flowers, leaves, wood, or bark) to be used in the production of materials (e.g. nanomaterials, filler in composites, or adsorbents) or added-value extracts is known, as described by Correia et al. (Correia et al., 2020). However, the subsequent valorization of the spent biomass as a fuel or as a feedstock for different materials, has not been extensively studied. The valorization of the spent bark from *Acacia mearnsii* after recovery of tannins, has been studied by Silva et al., that evaluated the performance of spent bark as an alternative adsorbent for crystal violet dye (da Silva et al., 2018). Carbonization of *Acacia* biomass to produce biochars with good adsorption properties has also been evaluated by several authors. Singh et al., used *Acacia karroo* activated biochar to remove the heavy metals Ni(II) and Zn(II) from aqueous solutions (Singh et al., 2013). Beckinghausen et al., assessed the removal of ammonium from artificial wastewater (1000 mg/L of ammonium solution) using activated biochar made from *A. mearnsii* woodchips (Beckinghausen et al., 2020).

The goal of this chapter is to evaluate the potential of the flowers, leaves, twigs, wood, and bark of the tree *A. dealbata* to be used as fuel or as a low-cost adsorbent of cationic and anionic dyes. A comparison of the properties and behavior of these raw biomass fractions and of the corresponding spent biomass fractions, after extraction of bioactive compounds, was made to evaluate the feasibility of a sequential valorization strategy according to a biorefinery concept. The biomass samples were characterized for their proximate, ultimate, and mineral composition, and their surface functional groups were described by Fourier-transform infrared spectroscopy (FTIR). The combustion behavior of the various fractions was studied by micro and macro thermogravimetric analysis (TGA). Batch adsorption experiments towards methylene blue (MB) and methyl orange (MO) dyes were performed to make a preliminary assessment of the potential of these biomass fractions to be used as low-cost adsorbents.

8.2 Materials and methods

8.2.1 Materials

Methylene blue and methyl orange used in this work were purchased from (Sigma-Aldrich, St. Louis, MO, USA).

8.2.2 Sample preparation

Flowers, leaves, twigs, bark, and wood of at least ten *A. dealbata* trees of the central region of Portugal (Alcobaça and Caparica) were used in this work. Samples of around 500 g of branches were collected from each tree and combined to obtain a composite sample, being labeled as raw flowers (Fl – raw), leaves (Le – raw), twigs (Tw – raw), wood (Wo – raw), and bark (Ba – raw). A sample of each fraction was used after an air-drying period at room temperature of two weeks. The samples were milled and sieved to 2 mm before characterization. A sample of each fraction was also studied after a sequential extraction step, with polar (70% aqueous acetone) and non-polar (petroleum ether) solvents (maceration at room temperature for 24 h), for removal of polar and non-polar extractives. These extraction steps originated spent flowers (Fl – spent), leaves (Le – spent), twigs (Tw – spent), wood (Wo – spent), and bark (Ba – spent). After the extraction, the milled samples were left to dry in the air for about one week and then processed as the original ones.

8.2.3 Physical and chemical analysis

Proximate analysis was conducted according to the European Standards: EN 14774-2 (2009) for moisture content, EN 14775 (2009), for ash content, and EN 15148 (2009) for volatile matter content. Fixed carbon was determined by difference, on a dry basis. Ultimate analysis (carbon, hydrogen, sulfur, and nitrogen contents) was performed using an elemental analyzer (Thermo Finnigan – CE Instruments Model Flash EA 112 CHNS series). Oxygen (O) was determined by difference on a dry basis, by subtracting the percentages of C, H, N, S, and ash from 100%. The mineral composition was evaluated through ICP-AES (Inductively Coupled Plasma – Atomic Emission Spectrometer Horiba Jobin-Yvon, Ultima).

Fourier transform infrared (FTIR) data were recorded using an Attenuated Total Reflectance (ATR) sampling accessory (Smart iTR) equipped with a single bounce diamond crystal on a Thermo Nicolet 6700 Spectrometer. The spectra were acquired with a 45° incident angle in the range of 4000–525 cm⁻¹ and with a 4 cm⁻¹ resolution.

Bulk density was determined by measuring the mass of material contained in a container of known volume. The biomass was dropped from a height of approximately 10 cm until the container was filled, without forcing compaction of the material. The biomass mass was determined by the difference between the full and empty container, and the loose bulk density was determined by dividing the determined mass by the container volume.

The higher heating value (HHV) of biomass was calculated according to the equation (8.1), proposed by Channiwala and Parikh (Channiwala and Parikh, 2002):

$$HHV = 0.3491 \times C + 1.1783 \times H + 0.1005 \times S - 0.1034 \times O - 0.0151 \times N - 0.0211 \times A \quad (8.1)$$

8.2.4 Ash slagging and fouling indices

The alkali index (AI), defined by the equation (8.2), expressing the quantity of alkali oxides in the fuel per unit of fuel energy (kg/GJ), is commonly used to predict fouling (Jenkins et al., 1998; Pena-Vergara et al., 2022; Vamvuka and Zografos, 2004; H. F. dos S. Viana et al., 2018):

$$AI = \frac{Y_f^a (Y_{K_2O}^a + Y_{Na_2O}^a)}{HHV} \quad (8.2)$$

where Y_f^a is the mass fraction of ash in the fuel, $Y_{K_2O}^a$ and $Y_{Na_2O}^a$ are the mass fractions of K_2O and Na_2O in the ash, and HHV is the high heating value (GJ/kg). Above 0.17 kg of alkali/GJ fouling is probable, and above 0.34 kg/GJ, fouling is virtually certain to occur.

A slagging evaluation index (SEI), was recently proposed and referred to as more suitable to be applied to biomass fuels than the commonly used base-to-acid index proposed for coal (Niu et al., 2016; Rico et al., 2020). The SEI is defined in defined by the following equation:

$$SEI = \frac{\%MgO + \%Al_2O_3 + \%Fe_2O_3}{\%CaO + \%P_2O_5} \quad (8.3)$$

in which the mass percentages of each component in the ashes are used. When the index value is less than 0.7, the softening temperature of ashes is less than 1000 °C and there is a high slagging risk; when it is above 1.7, the softening temperature is greater than 1200 °C and there is a low slagging risk; for values between 0.7 and 1.7 a medium slagging risk is considered (Niu et al., 2016; Rico et al., 2020).

A sintering index (SI), defined by the ratio between alkali earth and alkali metal fractions, is presented in equation (8.4) (Llorente and García, 2005; Viana et al., 2012):

$$SI = \frac{\%MgO + \%CaO}{\%K_2O + \%Na_2O} \quad (8.4)$$

where no sintering should be expected for $SI > 2$, while for $SI < 2$ the risk of sintering should be high (Llorente and García, 2005).

To predict bed agglomeration in the case of fluidized bed technologies, a bed agglomeration index (BAI) was previously defined as presented in equation (8.5) (Pena-Vergara et al., 2022; Vamvuka and Zografos, 2004; Viana et al., 2012):

$$BAI = \frac{\%Fe_2O_3}{\%K_2O + \%Na_2O} \quad (8.5)$$

Bed agglomeration occurs when this ratio between iron and alkali metals is lower than 0.15 (Pena-Vergara et al., 2022; Vamvuka and Zografos, 2004; Viana et al., 2012).

8.2.5 Thermogravimetric analysis

The thermal behavior of the biomass materials was studied under an oxidative atmosphere using a Netzsch STA 449 F3 Jupiter analyzer (Selb, Germany). TGA experiments were performed at a heating rate of 10 °C/min, from room temperature to 1100 °C, in an open Pt-Rh crucible. A low heating rate was chosen to promote a better heat transfer to the particles and to avoid thermal lag (Wnorowska et al., 2021).

The ignition temperature (T_i) was calculated using the intersection method and the burn-out temperature (T_b) using the conversion method at 99% conversion using thermogravimetric curves, as described by Lu and Chen (Lu and Chen, 2015). The temperature at maximum degradation rate (T_{peak}) and the maximum degradation rate value (R_{peak}) were obtained from the differential thermogravimetric (DTG) curves.

For the kinetic analysis, a first-order pseudocomponent fitting was applied, as described by Sen et al., which is here briefly described (Şen et al., 2022). A solid-state reaction dependent on the conversion (α) of the solid fraction and temperature (T) was considered, according to the Arrhenius equation (8.6):

$$\frac{d\alpha}{dt} = A \times e^{-\frac{E_a}{RT}} \times f(\alpha) \quad (8.6)$$

where A (s^{-1}) is the pre-exponential factor, E_a is the activation energy ($kJ\ mol^{-1}$), R is the universal gas constant ($8.314462\ J\ mol^{-1}\ K^{-1}$), and $f(\alpha)$ ($f(\alpha) = 1 - \alpha$) is the conversion function, with conversion α defined as the ratio between the actual mass at time t and the initial mass. In this

work, six pseudocomponents were considered, one assigned to water, two assigned to hemi-celluloses, one to cellulose, and two to lignin. Equation (8.6) thus changes to:

$$\frac{d\alpha}{dt} = \sum_{i=1}^6 c_i \times A_i \times e^{-\frac{Ea_i}{RT}} \times f(\alpha_i) \quad (8.7)$$

where biomass is considered as a sum of the six pseudocomponents. c_i is the fraction of the component i . The equation (8.7) was fitted using the nonlinear least-squares method. The fitting quality (FQ) was calculated as:

$$FQ(\%) = \left(1 - \left(\frac{N}{OF}\right)^{0.5}\right) \times 100 \quad (8.8)$$

where OF is the objective function after the least-squares fitting and N is the number of data points.

Concerning the macro TGA analysis, this was performed in a lab-scale reactor and following a procedure which are explained in detail in Silva et. al (Silva et al., 2022). A purpose-built macro TGA reactor was used in these tests composed of the following parts: an oven (200 mm in diameter and 350 mm in height) with capacity to be heated up to 1200 °C, a Nanodect Eurotherm controller connected to two type-K thermocouples, a stainless-steel sample basket connected by a 3 mm stainless-steel cable to a Shinko Denshi AJ-620CE scale to continuously measure the mass of the sample during the test. A TSI G4043 flow meter was used to control the airflow injected into the reactor. Both the scale and flow meter were connected to a PC with LabView software for data acquisition. A mass of sample of 10 g and an air-to-fuel ratio of 0.2 were used in the experiments. The macro TGA reactor is equipped with a sampling line that allows collection of the produced gases. The gases are cleaned from oils and particles by bubbling in two successive water scrubbers followed by a cellulose filter to retain small diameter particles and remove entrained moisture droplets. The cleaned gases were then sampled using a SKC 224-44MTX vacuum pump and measured using a a Rapidox 5100 Portable gas analyzer.

8.2.6 Adsorption experiments

Batch adsorption assays were performed to assess the capacity of these materials to act as low-cost adsorbents for MB and MO, that are respectively, cationic and anionic dyes. The biomass

samples used in the adsorption experiments were milled to a particle size lower than 500 μm . Experiments were performed using a 4 g/L adsorbent dose, by adding 0.200 g of the biomass sample to 50 mL of MB or MO solutions of known concentration (100 mg/L), and leaving this mixture in contact at room temperature, with manual agitation every 30 min until the first 4 h, and after 12 h of contact. The pH of the dye solutions was not adjusted and was 6.0 for the MB solution and 4.0 for the MO solution. After a preselected time (1, 4, and 24 h), a sample (2 – 3 mL) was taken from each flask and centrifuged (Hettich EBA 20) at 3000 rpm for 5 min to separate ensure full separation of the adsorbent particles from the dye solution. The dye concentration in the supernatants was determined, using a UV – vis spectrophotometer (Biochrom Libra S4 UV-Vis) at 664 nm for MB and 463 nm for MO, respectively. All assays were made in duplicate.

Adsorption capacity at time t , q_t (mg/g), and removal efficiency (RE (%)) were determined using equations (8.9) and (8.10), respectively:

$$q_t = V \times \frac{(C_0 - C_t)}{m} \quad (8.9)$$

$$RE (\%) = \frac{(C_0 - C_t)}{C_0} \times 100 \quad (8.10)$$

where C_0 and C_t represent initial and time t liquid-phase concentrations of MB (mg/L), respectively. V (L) is the volume of the MB solution and m (g) is the mass of the dry adsorbent used.

8.3 Results and discussion

The characteristics of the raw and spent *Acacia* biomass samples were evaluated by determination of their proximate, ultimate and mineral compositions. The bulk densities and estimated high heating values are also presented as relevant properties for fuel applications. The results are presented in Table 8.1.

Table 8.1 — Composition and properties of the raw and spent fractions of *A. dealbata*.

Parameters	Fl-raw	Fl-spent	Le-raw	Le-spent	Tw-raw	Tw-spent	Wo-raw	Wo-spent	Ba-raw	Ba-spent
Bulk density (kg/m ³) ^c	273.19 ± 4.26	204.30 ± 6.27	423.40 ± 14.3	366.01 ± 2.45	441.03 ± 13.0	330.76 ± 4.95	263.69 ± 3.40	221.27 ± 5.48	543.17 ± 2.02	327.72 ± 3.10
HHV (MJ/kg)	21.54 ± 0.06	22.49 ± 0.07	23.14 ± 0.07	23.47 ± 0.05	21.17 ± 0.25	21.01 ± 0.17	18.89 ± 0.18	18.45 ± 0.16	20.94 ± 0.20	19.98 ± 0.18
Proximate composition (wt.%)										
Moisture content ^a	8.90 ± 0.40	8.37 ± 0.12	9.30 ± 0.77	9.21 ± 0.11	9.98 ± 2.48	9.11 ± 0.17	8.53 ± 0.18	7.03 ± 0.24	10.12 ± 0.45	9.60 ± 0.13
Volatile matter ^b	76.90 ± 0.20	78.28 ± 0.83	78.26 ± 0.83	79.99 ± 0.63	75.39 ± 0.55	79.08 ± 0.45	84.83 ± 0.08	86.21 ± 0.45	72.11 ± 0.32	74.42 ± 0.47
Fixed carbon ^b	18.65 ± 0.21	17.72 ± 0.89	15.89 ± 0.77	14.60 ± 0.71	19.43 ± 0.54	16.74 ± 0.50	14.40 ± 0.08	13.20 ± 0.45	23.73 ± 0.33	20.59 ± 0.52
Ash ^b	4.45 ± 0.01	4.00 ± 0.06	5.86 ± 0.07	5.41 ± 0.16	5.18 ± 0.01	4.18 ± 0.05	0.77 ± 0.03	0.58 ± 0.01	4.15 ± 0.01	4.99 ± 0.07
Ultimate composition (wt.%) ^b										
Carbon (C)	50.27 ± 0.04	52.23 ± 0.11	52.73 ± 0.09	52.91 ± 0.06	50.02 ± 0.33	49.32 ± 0.02	47.00 ± 0.13	46.67 ± 0.02	50.86 ± 0.25	48.59 ± 0.23
Hydrogen (H)	6.66 ± 0.06	6.68 ± 0.01	6.89 ± 0.03	7.07 ± 0.01	6.45 ± 0.08	6.63 ± 0.13	6.15 ± 0.09	5.94 ± 0.13	6.08 ± 0.07	6.04 ± 0.06
Nitrogen (N)	2.19 ± 0.03	2.97 ± 0.01	3.16 ± 0.00	3.54 ± 0.03	1.92 ± 0.00	2.05 ± 0.01	0.25 ± 0.00	0.26 ± 0.01	1.55 ± 0.00	1.85 ± 0.04
Sulfur (S)	0.21 ± 0.03	0.19 ± 0.01	0.15 ± 0.01	0.24 ± 0.02	0.13 ± 0.01	0.07 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.01
Oxygen (O)	36.23 ± 0.02	33.93 ± 0.11	31.22 ± 0.11	30.83 ± 0.06	36.32 ± 0.42	37.75 ± 0.16	45.83 ± 0.22	46.56 ± 0.11	37.36 ± 0.32	38.45 ± 0.34

^a wet basis, ^b dry basis, ^c determined in air-dried material with the presented moisture contents.

8.3.1 Bulk density

The bulk density of biomass is an important property in terms of storage and transportation logistics, namely, for higher bulk densities the transportation costs are lower, and the storage capacity is improved (Oberberger and Thek, 2004).

Table 8.1 shows the bulk density of raw and spent fractions of *A. dealbata*. The bulk density of the raw material varied from 263.69 kg/m³ (Wo – raw) to 543.17 kg/m³ (Ba – raw), values that are in a higher range than those reported for *Eucalyptus* bark (156 – 192 kg m⁻³) (Miranda et al., 2013), *Pinus sylvestris* bark (approximately 185 – 245 kg m⁻³) (Miranda et al., 2012), spruce bark (approximately 195 – 305 kg m⁻³) (Miranda et al., 2012), *Acacia tortilis* barked wood (approximately 157 – 165 kg m⁻³) (Charis et al., 2020), *Pinus patula* wood (approximately 20 – 35 kg m⁻³) (Charis et al., 2020), *A. nilotica* wood (230.8 kg m⁻³) (Singh et al., 2020), or date palm biomass (199 – 316 kg m⁻³) (Saadaoui et al., 2013). Flowers, leaves and twigs presented higher bulk density than wood, therefore their incorporation in biomass pellets will not have a negative effect in this parameter. The removal of extractives from the *Acacia* biomass fractions (3.35 to 24.54% extraction yields) decreased the sample weights without significantly decreasing their volume, therefore bulk density of the spent biomass fractions was lower than the corresponding raw samples, varying between (204.30 kg m⁻³ (Fl – spent) and 366.01 kg m⁻³ (Le – spent). However, these are still high values of bulk density when compared with those of the above-mentioned biomasses from other species.

8.3.2 Composition and heating value

8.3.2.1 Proximate analysis

Proximate composition of the raw and spent biomass samples is given in Table 8.1. Moisture content affects the storage, handling, and transport of biomass, and should be below 10 wt.% for feedstocks of thermochemical conversion processes, such as pyrolysis, gasification, or combustion (Ahmed et al., 2021a; Williams et al., 2022). The process of air drying at room temperature could bring the moisture content of all samples to values lower or equal to 10%, in the range of other feedstocks for thermochemical processes, such as other biomass resources proposed for energy valorization (Álvarez-Álvarez et al., 2018; Amutio et al., 2013a; Kadri et al., 2015; Nunes et al., 2021).

The volatile matter is the fraction of the fuel that is released when it is heated at high temperature in the absence of air, consisting in a combustible part (gaseous C_xH_y, CO or H₂)

and a non-combustible fraction (CO_2 , SO_2 , NO_x , H_2O , and SO_3) (García et al., 2014). High volatile matter contents are associated with more reactive fuels since the produced gases can be easily oxidized. In Table 8.1 it can be observed that volatile matter of raw biomass samples varied from 72.11 to 84.83 wt.% (db), values identical to those described for date palm leaves and fronds (Sulaiman et al., 2016), *Eucalyptus* leaves (Liu and Balasubramanian, 2012), *Pinus pinaster* needles and branches (H. F. dos S. Viana et al., 2018), or *A. mangium* leaves and branches (Ahmed et al., 2021a). The volatile matter of the raw bark was 72.11 wt.% (db), a value inferior to those reported for *A. dealbata* bark (80.1 wt%, db) (Senelwa and Sims, 1999), eucalyptus bark (77.0 wt.%, db) (García et al., 2012), or *A. mangium* bark (81.3 wt.%, db) (Ahmed et al., 2021a), but higher than the value observed in *Pinus pinaster* bark (71.24 wt.% db) (Álvarez-Álvarez et al., 2018). Aside from the differences between species, *Acacia* bark represents a thin layer of external material that may be easily influenced by the edaphoclimatic conditions. Also, the physiological conditions of the *Acacia* trees are known to strongly influence the composition of its different biomass fractions (Lorenzo et al., 2011). Also the wood fraction presented a lower volatile matter content (84.83 wt.%, db) than several values reported for wood of other *Acacia* species, namely *A. dealbata* (87.87 – 91.5 wt.%, db) (Saldarriaga et al., 2015; Senelwa and Sims, 1999), *A. tortilis* (86.7 wt.%, db) (Nasser and Aref, 2014), *A. mearnsii* (88.09 wt.%, db) (Kosowska-Golachowska et al., 2018). On the other hand, lower volatile matter contents were reported for the woods of *A. mangium* (79.03 wt.%, db) (Ahmed et al., 2021a), or *A. longifolia* (79.9 wt.%, db) (Vicente et al., 2019). The volatile matter content found in this work for raw *Acacia* wood is in the range of the values found for wood of other fuel-relevant species, such as *Pinus pinaster* wood (85.7 wt.%, db) (H. F. dos S. Viana et al., 2018), and *Eucalyptus* wood (75.4 wt.%, db) (Channiwala and Parikh, 2002). The solvent extraction process caused a little increment in the volatile matter content of all the spent fractions relatively to the raw ones, maybe by solubilization and surface deposition of extractives that are less available for volatilization in the raw samples.

Ash is the inorganic residue remaining after complete oxidation in combustion conditions. Ash content decreases the fuel HHV, increases solid by-product formation both for gasification or combustion, and can lead to operational problems related to slagging and sintering depending on ash composition and the conversion technology chosen (García et al., 2014). Ash content varied from 0.77 wt.% (db) (for raw wood) to 5.86 wt.% (db) (for raw leaves) and according to the following order of increasing ash content values: wood < bark < flowers < twigs < leaves.

The low ash content of the wood fraction is characteristic from this type of lignocellulosic materials and similar to the ash contents reported for wood of different *Acacia* species. *A. dealbata* wood (1.4 – 2.50 wt.%, db) (Saldarriaga et al., 2015; Senelwa and Sims, 1999), *A. tortilis* (1.92 wt.%, db) (Nasser and Aref, 2014), *A. mangium* trunk (2.1 wt.%, db) (Ahmed et al., 2021a), *A. longifolia* (2.1 wt.%, db) (Vicente et al., 2019), or the mixture of *A. dealbata* wood, bark, and leaves (1.1 wt.%, db) (Amutio et al., 2013a). Relatively low values of ash content can also be found for woods of other species such as *Eucalyptus* (3.4 wt.%, db) (Channiwala and Parikh, 2002), date palm (3.9 wt.%, db) (Nasser et al., 2016), or *Pinus pinaster* (0.22 wt.%, db) (H. F. dos S. Viana et al., 2018).

The ash content of the bark fraction (4.15 wt.%, db) was similar to the observed for *A. dealbata* bark (Senelwa and Sims, 1999) or *Eucalyptus* bark (García et al., 2012). Lower ash content values were reported for the bark of other species such as *A. mangium* (1.57 wt.%, db) (Ahmed et al., 2021a), *A. auriculiformis* (2.46 wt.%, db) (Ahmed et al., 2021b), and *Pinus pinaster* (1.0 wt.%, db) (Álvarez-Álvarez et al., 2018).

The ash content of the twig fraction (5.18 wt.%, db) was higher than the values reported for the twigs of *A. mangium* (1.57 wt.%, db), *A. mangium* branches (2.50 wt.%, db), (Ahmed et al., 2021a) and *A. auriculiformis* branches (2.32 wt.%, db) (Ahmed et al., 2021b). Differences may be related to the species, tree age and local environmental conditions. The twig diameter that affects the proportion between peel and inner woody material, can also influence composition parameters such as the ash content.

Tree leaves tend to present high values of mineral components not only because of physiological reasons but also because of their high surface area enables them to act as passive samplers of atmospheric air particles. The value found for the leaves of *A. dealbata* (5.86 wt.%, db) is in the range of those found for leaves of other *Acacia* species, namely *A. mangium* (2.32 wt.%, db) (Ahmed et al., 2021a), *A. auriculiformis* (3.72 wt.%, db) (Ahmed et al., 2021b) or *A. dealbata* (7.9 wt.%, db) (García et al., 2012).

The solvent extraction step had in general a positive effect by slightly decreasing the ash content of the samples, probably by partial dissolution of the mineral components, in the 70% aqueous acetone solution. Therefore, and except in the case of bark, the spent samples presented lower ash contents than the corresponding raw samples.

The fixed carbon content is the mass remaining after the release of volatiles, excluding the ash and moisture contents (McKendry, 2002a). This parameter varied from 14.40 wt.%, (db), for the wood fraction to 23.73 wt.%, (db), for the bark fraction. The values found in wood were slightly higher than those reported for *A. dealbata* wood (7.2 – 9.64 wt.%, db) (Saldarriaga et

al., 2015; Senelwa and Sims, 1999), *A. tortilis* wood (11.4 wt.%, db) (Nasser and Aref, 2014), *A. mearnsii* wood (11.27 wt.%, db) (Kosowska-Golachowska et al., 2018), or the mixture of *A. dealbata* wood, bark, and leaves (8.9 wt.%, db) (Amutio et al., 2013a). Flowers and leaves presented fixed carbon contents lower than those found for leaves of *A. dealbata* (19.04 wt.%, db) (García et al., 2012), *A. auriculiformes* (21.56 wt.%, db) (Ahmed et al., 2021b) and *A. mangium* (20.13 wt.%, db) (Ahmed et al., 2021a) leaves. Twigs fixed carbon content was higher than the values reported for *A. mangium* twigs (16.13 wt.%, db) (Ahmed et al., 2021a), and similar to those observed with *A. auriculiformis* (Ahmed et al., 2021b). In turn, bark showed fixed carbon contents higher than the one found in *A. mangium* bark (17.12 wt.%, db), but lower than the ones found for *A. auriculiformis* (30.35 wt.%, db) (Ahmed et al., 2021b), *A. mearnsii* (26.82 wt.%, db)), and *Pinus pinaster* (28.58 wt.%, db)) (Álvarez-Álvarez et al., 2018) barks. The differences between species may be significant because fixed carbon content reflects the variations of both volatile matter and ash contents, that are strongly influenced by physiological conditions and environmental parameters (Stolarski et al., 2020).

The fixed carbon content was slightly lower in the spent materials than in the raw samples suggesting that some non-volatile organic components might be extracted by the solvent.

Overall, the proximate composition of all *A. dealbata* fractions were similar to those reported for other *Acacia* spp. as well as other species traditionally used for energy production, such as eucalyptus and pine. The proximate composition of all the *Acacia* fractions can be considered adequate for application in advanced thermochemical conversion processes, such as pyrolysis, gasification or combustion (Ahmed et al., 2021a; Singh et al., 2020). The process of solvent extraction did not have a negative influence in the fuel quality of these biomass fractions because it slightly decreased the fixed carbon content but also increased volatile matter and decreased ash content.

8.3.2.2 Ultimate analysis

The ultimate composition of the raw and spent biomass fractions are presented in Table 8.1.

Carbon content varied from 47.00 to 52.73 wt.%, db), for raw samples and from 46.67 to 52.91 wt.%, db), for spent samples. The extraction process led to an increase of carbon content for flowers and in a lesser degree for leaves, suggesting the removal of oxygenated components. Hydrogen content varied in the range of 5.94 – 7.07 wt.%, db), for raw and spent samples and the extraction process had minor effects in hydrogen concentrations, with variations lower than 0.5%. Carbon and hydrogen contents found for the raw samples were comparable to those found in *A. dealbata* wood (Saldarriaga et al., 2015), leaves and wood (García et al.,

2012), and for the mixture of wood, bark, and leaves of *A. mangium* or the mixture of leaves, trunk, bark, twigs, pods, and branches of *A. auriculiformis* (Ahmed et al., 2021a, 2021b), *A. longifolia* wood (Vicente et al., 2019). Similar values are also reported for biomass fractions of *Eucalyptus* species, namely leaves (Liu and Balasubramanian, 2012), bark (García et al., 2012), and wood (Channiwala and Parikh, 2002), and of *Pinus pinaster* wood, needles, branches, and bark (Álvarez-Álvarez et al., 2018; H. F. dos S. Viana et al., 2018).

Nitrogen content varied from 0.25 wt% (db), for raw wood to 3.54 wt% (db) for spent leaves and the extraction process caused a slight increase of nitrogen concentration (0.01 to 0.78 %) in the spent samples. Flowers and leaves presented the higher nitrogen concentrations which is consistent with the higher protein content found in plant flowers and leaves relatively to the ligneous components such as the wood or bark (Gbenga and Emmanuel, 2016; Polka et al., 2019). Similar nitrogen concentrations were reported for wood samples of *A. dealbata* (whole tree) (Nunes et al., 2020), *A. tortilis* (Nasser and Aref, 2014), *Eucalyptus* (Channiwala and Parikh, 2002), or *Pinus pinaster* (H. F. dos S. Viana et al., 2018). Nevertheless, slightly higher nitrogen values were found for woods of *A. dealbata* (1.14 wt.%, db) (Saldarriaga et al., 2015), *A. longifolia* (1.39 wt.%, db) (Vicente et al., 2019) and *A. mangium* (0.49%, db) (Ahmed et al., 2021a) reflecting different physiological conditions and genetic characteristics. Flowers and leaves had the highest nitrogen contents (> 2%) values that were higher than similar biomass fractions from *Acacia* species, namely *A. dealbata* leaves (0.75 wt.%, db) (García et al., 2012), or *A. mangium* leaves (1.72 wt.%, db). These *Acacia* biomass fractions with higher nitrogen contents could be combined with other biomass fractions, to minimize their contribution for NO_x emissions.

Sulfur was only detected in flowers, leaves, and twigs, and in very low percentage (0.07 – 0.24 wt.%, db), as found in *A. dealbata* (García et al., 2012) and *A. mangium* (Ahmed et al., 2021a) leaves, in *A. mangium* twigs and pods (Ahmed et al., 2021a), and in *Pinus pinaster* branches (0.03%, db) (H. F. dos S. Viana et al., 2018).

Oxygen content was lower in leaves (30.83 and 31.22 wt.%, db), followed by flowers, twigs, and bark (33.93 and 38.45 wt.%, db). The highest oxygen content values were found in wood (45.83 and 46.56 wt.%, db) as expected from the higher cellulose and hemicellulose content of this ligneous fraction. Similar oxygen contents were found in woods of *Acacia* species, namely *A. dealbata* (45.0 wt.%, db) (Saldarriaga et al., 2015) and *A. tortilis* (46.9 wt.%, db) (Nasser and Aref, 2014), and *A. mangium* trunk (48.3 wt.%, db).

The elemental composition of the raw and spent biomass fractions is represented in a Van Krevelen diagram and compared with lignite and with different biomass materials (Figure

8.1). The energy value of a fuel generally increases as the atomic O/C ratio decreases and the H/C ratio increases (McKendry, 2002a).

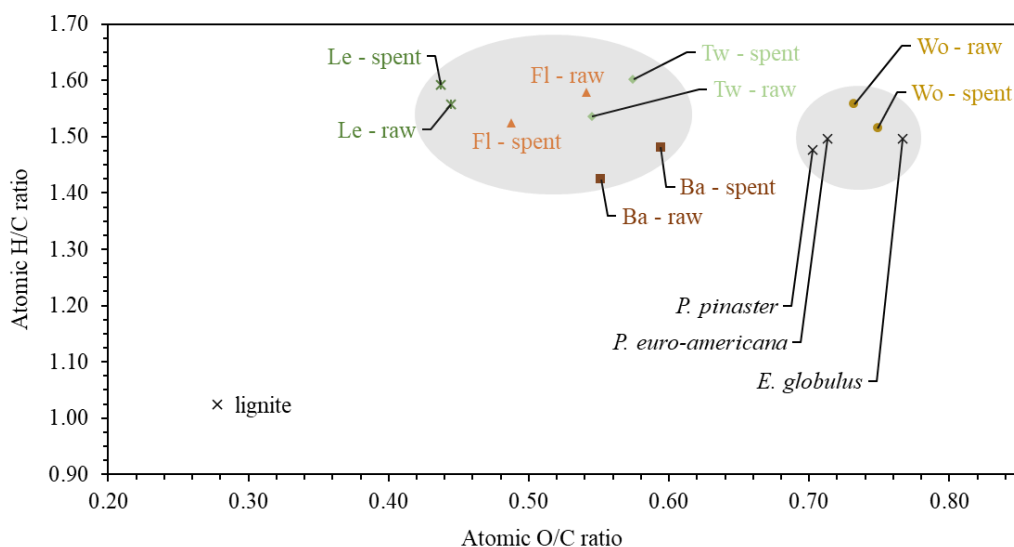


Figure 8.1 — Van Krevelen diagram, comparing the fossil fuel lignite (Vassilev et al., 2010) and wood of some common species used in the production of solid fuels, such as *Pinus pinaster*, *Populus euro-americana*, and *Eucalyptus globulus* (Telmo et al., 2010) with raw and spent fractions of *A. dealbata*.

As expected, the composition of the wood samples is comparable to the compositions of woods from other species commonly used as solid fuels, namely, *Pinus pinaster*, *Populus euro-americana*, and *Eucalyptus globulus*.

The leaves, flowers, twigs and bark samples present lower O/C values due to their lower contents in cellulose and hemicellulose. The bark samples present a slightly lower H/C content, probably due to a higher content of lignin, a polymer of aromatic phenolic acids. Nonetheless, the H/C ratio of all samples is much higher than that of lignite, therefore showing a lower degree of aromaticity than this fossil fuel. The flower fraction was the only whose composition showed a beneficial effect from the extraction with solvents since it decreased both the O/C and H/C ratios thus improved the relative carbon concentration in the spent flowers.

8.3.2.3 Higher heating value

Taking into account the elemental composition and ash content of the *Acacia* biomass samples their HHV was evaluated using an empirical equation (Channiwala and Parikh, 2002) and results are presented in Table 8.1. The estimated HHV varied from 18.89 to 23.14 MJ/kg (db) for raw samples, following the order of wood < bark < twigs < flowers < leaves. Solvent extraction

increased the HHV for flowers and leaves samples but decreased for the other biomass samples, with variations lower than 1 MJ/kg (db). These values indicate that the combination of the different *Acacia* biomass fractions would not have a negative effect in the HHV of the resulting solid biofuel, relatively to the use of only the wood fraction. Furthermore, the solvent extraction process did not have a significant impact in the HHV of the biomass fractions therefore validating the feasibility of their sequential valorization according to a biorefinery concept. The HHV estimated for the wood samples were comparable to those previously reported for *A. dealbata* (17.10 – 19.70 MJ/kg) (Saldarriaga et al., 2015; Senelwa and Sims, 1999), and slightly higher than those found for woods of other *Acacia* species namely, *A. auriculiformis* (17.85 MJ/kg) (Ahmed et al., 2021b), *A. mearnsii* (18.28 MJ/kg) (Kosowska-Golachowska et al., 2018) and *Robinia pseudoAcacia* (18.08 MJ/kg) (Wilk et al., 2020). These values are in the range of the HHV of woods from other species suitable for production of solid fuels such as eucalyptus (17.60 MJ/kg) (Telmo et al., 2010) or *Pinus pinaster* (19.09 – 21.60 MJ/kg) (Álvarez-Álvarez et al., 2018; H. Viana et al., 2018). Also the bark presented HHV values similar to those reported barks of other *Acacia* species such as for *A. dealbata* (Senelwa and Sims, 1999), *A. auriculiformis* (Ahmed et al., 2021b) and *A. mearnsii* (Kosowska-Golachowska et al., 2018). Slightly higher HHV were found for *A. mangium* bark (21.50 MJ/kg) but differences are within expected for different species and physiological conditions. The HHV estimated for the leaves fraction was higher than values reported for leaves of *A. dealbata* (19.44 MJ/kg) (García et al., 2012), *A. auriculiformis* (20.93 MJ/kg) (Ahmed et al., 2021b) and *A. mangium* (21.16 MJ/kg) (Ahmed et al., 2021a), but also in the studies the HHV of leaves was generally higher than the HHV of the wood fraction from the same species. This higher calorific value of the leaves relatively to other biomass fractions can also be observed in other forest species such as *Pinus pinaster* needles (21.61 MJ/kg) (H. F. dos S. Viana et al., 2018), and eucalyptus leaves (20.04 MJ/kg) (García et al., 2012), reflecting the presence of hydrocarbon-rich components such as essential oils or waxes and lower contents of cellulose or hemicellulose. HHV estimated for twigs were similar to the values found for *A. mangium* twigs (Ahmed et al., 2021a) and higher than those reported for *A. dealbata* (17.75 MJ/kg) (García et al., 2012) and *A. auriculiformis* (19.32 MJ/kg) (Ahmed et al., 2021b) branches.

8.3.2.4 Mineral composition

The mineral composition of the *Acacia* biomass samples is described in Table 8.2, expressed in terms of the corresponding oxides. Overall, it is clearly seen that the main mineral components of all raw and spent fractions are K, Ca, Mg, Na, and P, followed by Si, Al, Fe, and B, with Ba, Cr,

Cu, Mn, Sr, and Zn in very low concentrations. The predominance of the alkali elements K and Na, the alkali earth elements Ca and Mg, and P in the mineral composition of biomass ash is common for a large variety of biomass materials (Garcia-Maraver et al., 2017; Lachman et al., 2021; Nanda et al., 2013; Nunes et al., 2021; Telmo et al., 2010; Vassilev et al., 2010).

The effect of the previous solvent extraction step was notorious in the Ca and K metals, leading to a decrease of K_2O concentrations in the range of 5.2 to 15.9% for spent flowers, twigs, wood, and bark, compared to the respective raw materials. This removal of K_2O led to a concentration of the other main component CaO whose solubility in aqueous acetone was lower and therefore was not extracted. This may be a positive effect since high concentrations of alkali metals are usually associated with a lower ash melting point while high concentrations of alkali earth metals tend to increase the ash melting point (Lachman et al., 2021). Therefore reducing the concentration of K_2O and increasing the concentration of CaO and MgO could reduce ash melting related problems such as slagging, fouling, sintering, and bad agglomeration problems in thermal conversion equipments using spent *Acacia* biomass as a feedstock (Vassilev et al., 2013). The solvent extraction had a different effect on the mineral composition of leave fraction that suffered an increase of the K_2O concentration (+7.86%) and a decrease of the CaO and MgO concentrations by -2.48% and -4.92%, respectively. This may indicate a different molecular speciation for these mineral components in the leaves that influences their solubility in aqueous solvents differently from their correspondent forms in the other biomass fractions.

Table 8.2 — Composition and properties of the raw and spent fractions of *A. dealbata*.

Parameters	Fl-raw	Fl-spent	Le-raw	Le-spent	Tw-raw	Tw-spent	Wo-raw	Wo-spent	Ba-raw	Ba-spent
Mineral composition (% in ash)										
Al ₂ O ₃	0.53 ± 0.01	0.60 ± 0.01	0.91 ± 0.01	0.71 ± 0.01	0.88 ± 0.01	0.96 ± 0.01	0.81 ± 0.01	0.63 ± 0.01	2.84 ± 0.03	1.59 ± 0.02
B ₂ O ₃	0.15 ± 0.00	0.22 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.17 ± 0.00	0.25 ± 0.00	0.26 ± 0.00	0.17 ± 0.00	0.25 ± 0.00
BaO	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.05 ± 0.00
CaO	11.40 ± 0.25	20.77 ± 0.45	42.51 ± 0.92	40.03 ± 0.87	41.11 ± 0.89	49.91 ± 1.08	40.33 ± 0.87	47.65 ± 1.03	62.77 ± 1.36	72.89 ± 1.58
Cr ₂ O ₃	< 0.005	0.01 ± 0.00	0.01 ± 0.00	< 0.005	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
CuO	0.05 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.07 ± 0.00	0.05 ± 0.00	0.34 ± 0.01	0.26 ± 0.01	0.06 ± 0.00	0.06 ± 0.00
Fe ₂ O ₃	0.50 ± 0.01	0.68 ± 0.01	1.00 ± 0.02	0.68 ± 0.01	0.73 ± 0.01	0.97 ± 0.02	0.59 ± 0.01	0.45 ± 0.01	0.57 ± 0.01	0.48 ± 0.01
K ₂ O	60.15 ± 1.60	44.27 ± 1.18	7.90 ± 0.21	15.76 ± 0.42	20.30 ± 0.54	15.10 ± 0.40	30.74 ± 0.82	18.80 ± 0.50	11.67 ± 0.31	5.24 ± 0.14
MgO	18.25 ± 0.17	22.02 ± 0.20	38.67 ± 0.35	33.75 ± 0.31	26.54 ± 0.24	25.55 ± 0.23	17.94 ± 0.16	21.78 ± 0.20	15.42 ± 0.14	14.03 ± 0.13
MnO	0.12 ± 0.00	0.17 ± 0.00	0.24 ± 0.00	0.16 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.08 ± 0.00	0.10 ± 0.00	0.08 ± 0.00	0.09 ± 0.00
Na ₂ O	3.19 ± 0.04	3.52 ± 0.05	1.82 ± 0.02	2.08 ± 0.03	3.11 ± 0.04	2.06 ± 0.03	5.53 ± 0.07	6.81 ± 0.09	2.38 ± 0.03	1.86 ± 0.02
P ₂ O ₅	4.67 ± 0.10	6.17 ± 0.13	4.86 ± 0.10	5.26 ± 0.11	5.62 ± 0.12	4.18 ± 0.09	2.28 ± 0.05	2.28 ± 0.05	2.18 ± 0.04	1.96 ± 0.04
SiO ₂	0.89 ± 0.01	1.39 ± 0.01	1.72 ± 0.02	1.21 ± 0.01	1.20 ± 0.01	0.79 ± 0.01	0.79 ± 0.01	0.64 ± 0.01	1.56 ± 0.02	1.28 ± 0.01
SrO	0.01 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.09 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.14 ± 0.00	0.14 ± 0.00	0.13 ± 0.00
ZnO	0.07 ± 0.00	0.07 ± 0.00	0.08 ± 0.00	0.07 ± 0.00	0.10 ± 0.00	0.06 ± 0.00	0.15 ± 0.00	0.14 ± 0.00	0.09 ± 0.00	0.09 ± 0.00

8.3.3 Evaluation of ash-related problems

A major problem associated with biomass thermal conversion systems, such as pyrolysis, gasification, or combustion is related to ash deposit formation, which can significantly reduce heat transfer, cause erosion by channelizing gas flow, corrosion, and can accumulate in quantities unmanageable by the facilities (Baxter et al., 1998). The deposits are referred as slagging, when occurring in the high refractory sections of boilers where the radiative heat transfer is dominant, due to the presence of molten ashes, or as fouling, which forms in the convective heat transfer zones due to the formation of ash deposits while the gases cool down (Teixeira et al., 2012). In fluidized bed combustors, another issue may arise due to the ash melting behavior, which is the agglomeration of the bed material, usually sand. This agglomeration is enhanced with the sintering phenomenon, where loosely attached particles become denser due to adhesion on melted ash particles with low melting temperatures (Teixeira et al., 2012).

The tendency of biomass to produce ash deposits depends on its ash composition, with Si, K, Na, S, Cl, P, Ca, Mg, and Fe being involved in reactions leading to ash incrustations (Benavente and Fullana, 2015). Several empirical indexes based on ash composition have been developed based on the coal ash behavior and deposit formation and applied to predict ash-related problems when using biomass in thermal conversion facilities, despite knowing that biomass ash and coal ash composition can differ significantly (Garcia-Maraver et al., 2017). The alkali index (A), the slagging evaluation index (SE), the sintering index (S), and the bed agglomeration index (BA) were calculated and used to predict ash-related problems in boilers.

Table 8.3 shows the results of the calculation of the above-mentioned indexes, and associated fouling, slagging, sintering, and bad agglomeration risks.

Table 8.3 — Ash fouling, slagging, sintering, and bed agglomeration indexes and respective risks for all raw and spent fractions of *A. dealbata*.

	Alkali index (<i>A</i>)		Slagging evaluation index (<i>SE</i>)		Sintering index (<i>S</i>)		Bed agglomeration index (<i>BA</i>)	
	<i>A</i> /	Fouling risk	<i>SE</i> /	Slagging risk	<i>S</i> /	Sintering risk	<i>BA</i> /	Bed agglomeration
Fl-raw	1.31	Occur	1.20	Medium	0.47	High	0.01	Occur
Fl-spent	0.85	Occur	0.86	Medium	0.90	High	0.01	Occur
Le-raw	0.25	Probable	0.60	High	8.35	Low	0.10	Occur
Le-spent	0.41	Occur	0.45	High	4.14	Low	0.04	Occur
Tw-raw	0.57	Occur	0.29	High	2.89	Low	0.03	Occur
Tw-spent	0.34	Probable	0.86	Medium	4.40	Low	0.06	Occur
Wo-raw	0.15	Low	0.78	Medium	1.61	High	0.02	Occur
Wo-spent	0.08	Low	0.51	High	2.71	Low	0.02	Occur
Ba-raw	0.28	Probable	0.46	High	5.56	Low	0.04	Occur
Ba-spent	0.18	Probable	0.21	High	12.25	Low	0.07	Occur

The alkali index was below the 0.17 kg/GJ threshold for probable fouling only for both raw and spent wood. The higher alkali and ash contents in the other fractions increased this index to values indicating that fouling is probable or certain to occur, besides the improvement on the alkali index promoted by the previous extraction step in the case of flowers, twigs, and bark. In turn, the obtained values for the slagging evaluation index were lower than the 1.7 threshold for low slagging risk with all samples, meaning that slagging is probable to occur.

The sintering index values revealed low sintering risk for all fractions except for the raw and spent flowers, while for raw wood, the value is just slightly below the threshold of 2. It was observed that the previous extraction step improved the sintering index in flowers, twigs, wood, and bark. Regarding the bed agglomeration index, this was below the 0.15 limit in all samples, suggesting that bed agglomeration occurs.

Applying these indexes to biomass often gives this negative feedback, such as observed for the alkali and slagging evaluation indexes for vine prunings, gorse, and kiwi prunings (Rico et al., 2020); for the alkali and bed agglomeration indexes for eucalyptus and pine (Pena-Vergara et al., 2022), and olive kernel, olive tree wood, citrus tree wood and vine shoots (Vamvuka and Zografos, 2004); and for the sintering and bed agglomeration indexes for several woody shrub species (Viana et al., 2012). However, the application of these fouling, slagging and clustering predictive indexes has been recently reviewed and discussed in the literature by comparing the indexes information with experimental observations of ash fusion temperatures, and contradictory findings are common (Garcia-Maraver et al., 2017; Lachman et al., 2021).

Lachman et al. recently proposed a graphical approach consisting in a ternary diagram divided in three regions based on the initial deformation temperatures observed in a large variety of biomasses: region 1, referring to a high initial deformation temperature zone, thus meaning fuels less prone to cause ash related problems; region 2, which consist in a transition zone between the high and lower initial deformation temperature zones; and region 3, which refers to a low initial deformation zone, corresponding to fuels that must not be used without additives or co-firing with less problematic fuels (Lachman et al., 2021). In the ternary diagram axis the authors consider CaO and MgO together, once they are often found in high melting temperature ashes and show positive correlation with the ash fusion temperatures; SiO_2 is grouped with Al_2O_3 and Fe_2O_3 , which are often found in low quantities; and finally the alkali compounds K_2O and Na_2O are grouped with P_2O_5 once they were found to be the most abundant oxides in the low melting temperature ashes (Lachman et al., 2021). After the necessary calculations the ternary plot was built and is presented in Figure 8.2.

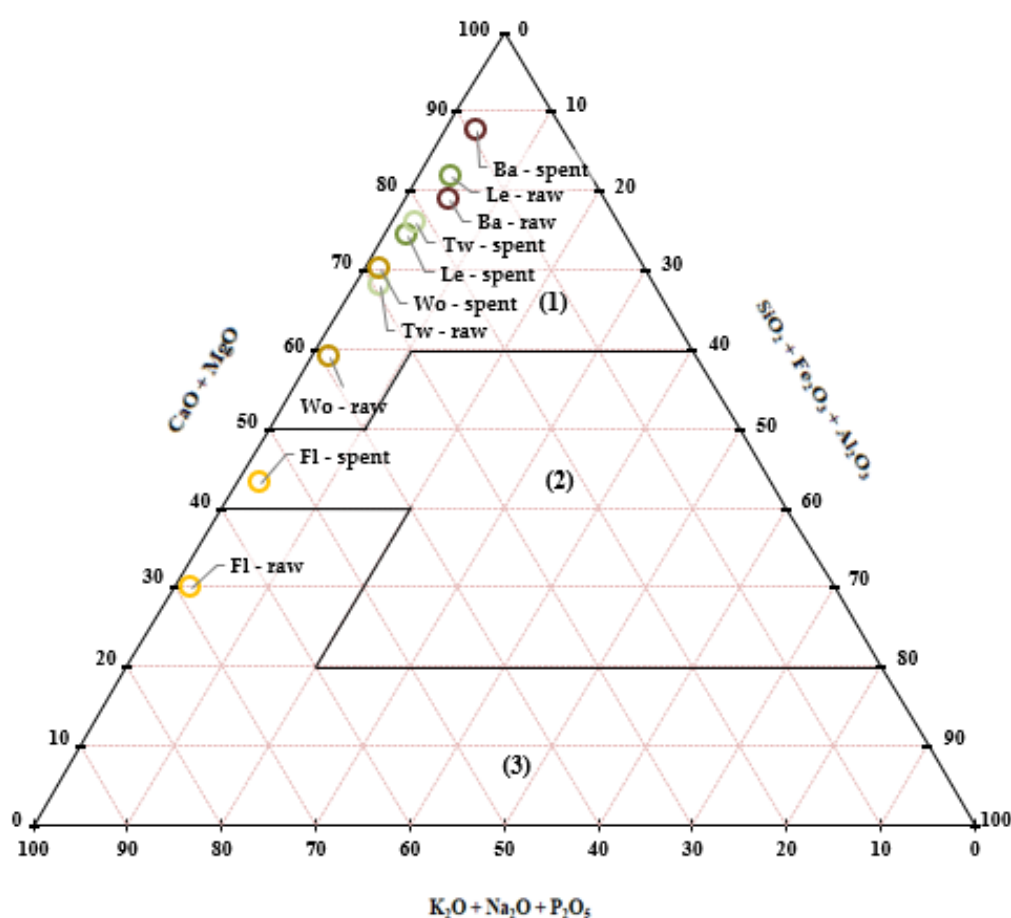


Figure 8.2 — Ternary diagram including all evaluated samples.

According to the diagram, all *A. dealbata* fractions except the flowers have a low tendency to cause ash-related problems when used in thermal conversion processes. Flowers must be used together with other less problematic fuels, taking advantage of their relatively high HHV. Furthermore, it should be noted that, in general, the prior solvent extraction of functional compounds improved the properties of the ash in terms of preventing deposit formation.

8.3.4 FTIR analysis

The surface properties of raw and spent fractions were analyzed by FTIR. The spectra are shown in Figure 8.3. Comparing the spectra, only slight differences can be found among the samples. Weak peaks between 719 and 895 cm^{-1} , assigned to aromatic C–H out-of-plane bend (Chupin et al., 2015; Coates, 2000) were evident for all samples. A strong peak at 1016 – 1026 cm^{-1} , assigned to C–O–C symmetric stretching in ethers and C–O stretching vibration in cellulose, hemicelluloses, and lignin (Saikia et al., 2015) was also observed in all samples. A weak peak at 1151 – 1161 cm^{-1} , related to alkyl substituted ether (O–CH₃ or O–CH₂) stretching (Abdullah et al., 2013) and O–C–O asymmetric stretching (glycosidic link) in polysaccharides (da Costa et al., 2022) was observed in all samples but were more pronounced in flowers and leaves. The peak at 1229 – 1232 cm^{-1} , associated with C–O–C stretching in phenol-ether bonds of lignin, C–O stretching in syringic rings, C–O–C symmetric stretching, and OH plane deformation (Lehto et al., 2018), and C–O vibrations of acetyl groups in hemicelluloses (da Costa et al., 2022) was most noted in wood, followed by twigs, while in bark it was only observed in the spent material. Weak peaks at 1311 – 1321 cm^{-1} , related with O–H bend in phenols and non-aromatic alcohols, C–N stretch in amines (Coates, 2000), and syringic monomer vibrations in lignin (da Costa et al., 2022), as well as at 1367 – 1369 cm^{-1} , associated with O–H bending in phenols and C–H bending in methyl groups (Chupin et al., 2015) in cellulose and hemicelluloses (Ahmed et al., 2021a) were more pronounced in wood, twigs, and leaves samples. A weak peak at 1423 cm^{-1} , assigned to C–O stretching or O–H deformation in carboxylic acids (Ahmad et al., 2007) and symmetric deformation of CH₂ in cellulose (Saikia et al., 2015), was only observed in wood samples. Peaks at 1441 – 1456 cm^{-1} , associated with C–H deformation and aromatic ring vibration (Abdullah et al., 2013), and aromatic C–C stretching and C–H deformation (Ahmed et al., 2021a), as well as the peaks at 1504 – 1516 cm^{-1} and at 1593 – 1612 cm^{-1} , assigned to C=C stretching in aromatic rings (Abdullah et al., 2013; Ahmad et al., 2007) in lignin (da Costa et al., 2022), were observed in all samples. In turn, the peak at 1635 cm^{-1} , attributed to C=O vibration in amides from proteins and the aromatic ring stretching (Nair et al., 2020), and and COO

vibrations in non-esterified carboxyl groups (da Costa et al., 2022), was only found in spent leaves. The peak at $1728 - 1734 \text{ cm}^{-1}$, assigned to C=O stretching in lactones (Ahmad et al., 2007), ketones, aldehydes, carboxylic acids (Islam et al., 2005) or esters (Coates, 2000), associated with hemicelluloses (da Costa et al., 2022), was found in all samples, as well as the peaks between 2850 and 2918 cm^{-1} , assigned to aromatic C–H out-of-plane bend (Chupin 2015, Coates 2000), and at $3280 - 3332 \text{ cm}^{-1}$, relative to O–H stretching in alcohols, phenols, or carboxylic acids (Chupin et al., 2015; Peydayesh and Rahbar-Kelishami, 2015), and N–H stretching in amines (Coates, 2000; Nair et al., 2020).

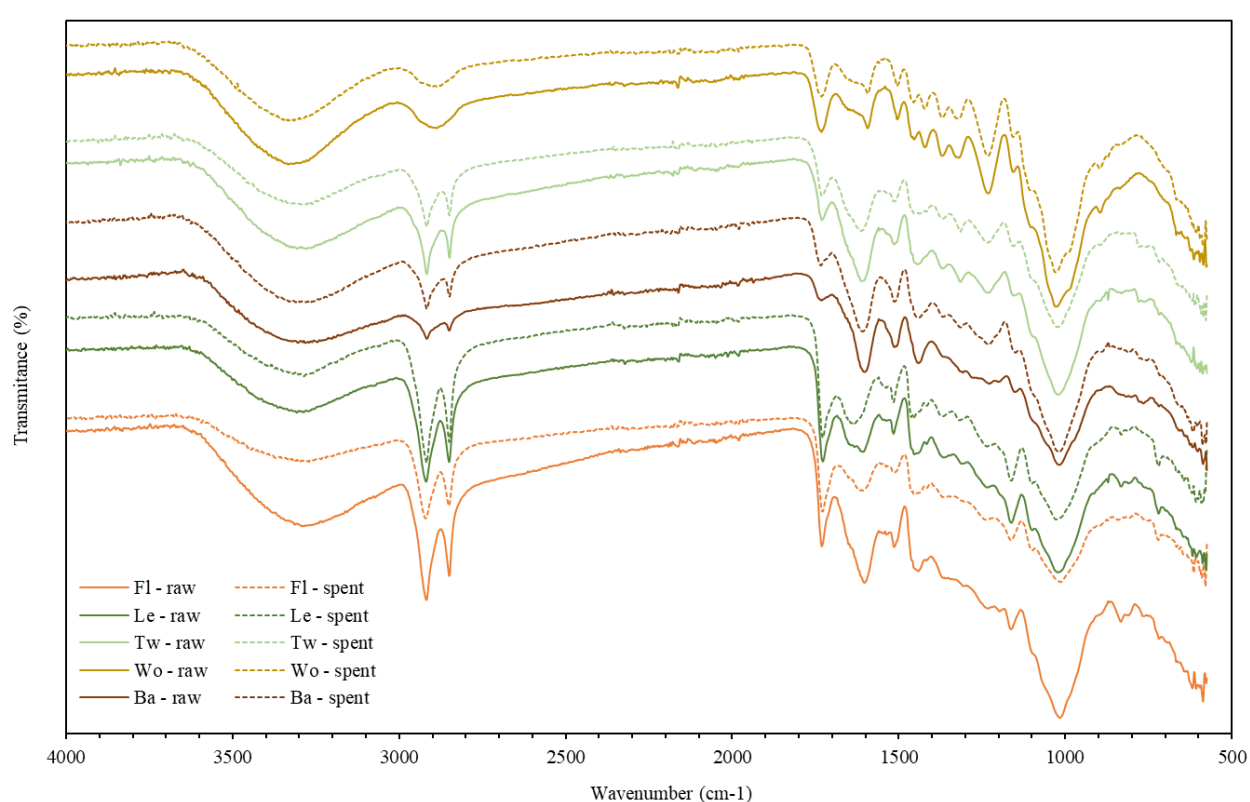


Figure 8.3 — FTIR spectra of raw and spent fractions of *A. dealbata*.

Differences between the FT-IR profile of raw and spent biomasses were more evident for the flower fraction, especially in the region below 1800 cm^{-1} , what may be explained by the removal of concrete and absolute components particularly abundant in this biomass fraction. The FTIR profile of the various fractions was similar to the ones reported for *A. dealbata* leaves and trunk (da Costa et al., 2022), *A. nilotica* wood (Singh et al., 2020), and *A. mangium* phylloides, trunk, twigs, pod, and branches (Ahmed et al., 2021a).

8.3.5 Thermal behavior under oxidative conditions

8.3.5.1 Micro TGA analysis

The combustion behavior of raw and spent biomasses was evaluated by thermogravimetric analysis under oxidative atmosphere (Figure 8.4).

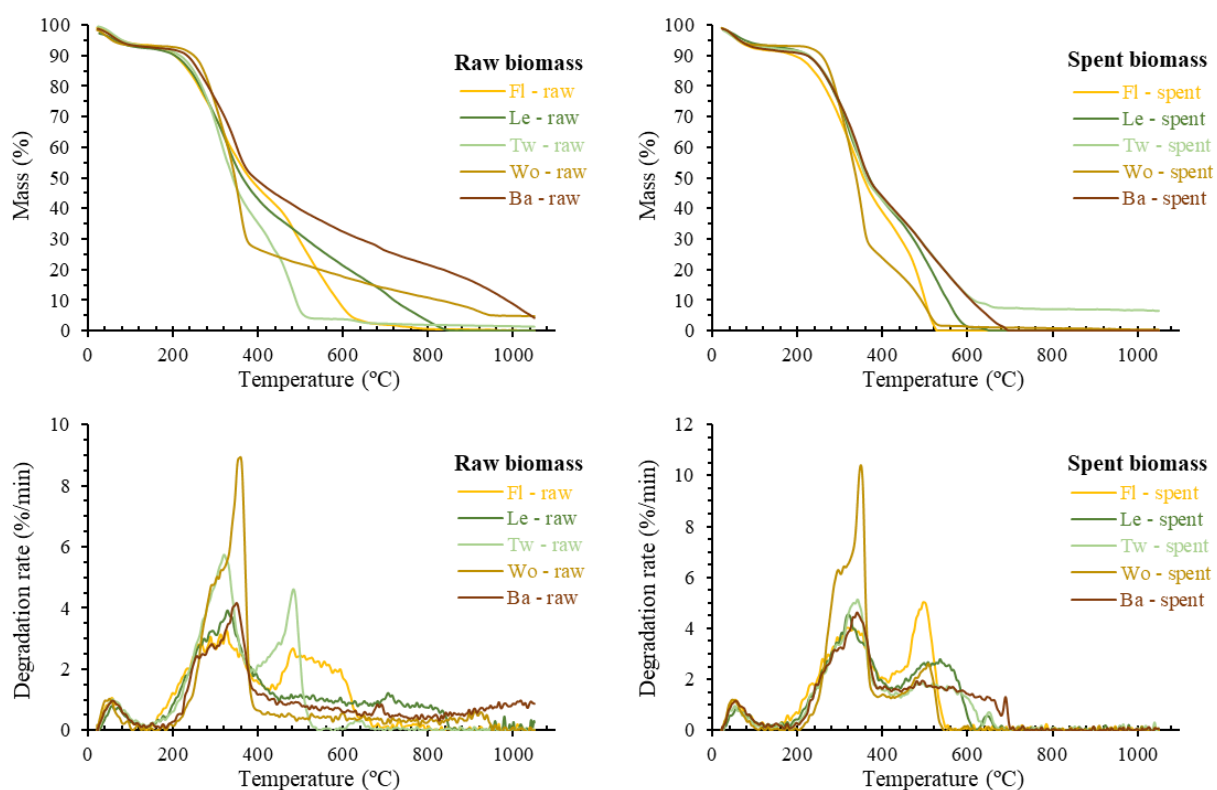


Figure 8.4 — TGA (top) and DTG (bottom) curves during the oxidation of samples under oxidative atmosphere.

The characteristic parameters of the thermal degradation of the biomass samples are described in Table 8.4. The mass weight loss profile follows three main steps: the first is associated with moisture evaporation, the second to devolatilization of organic components and the last corresponds to the oxidation of char.

The drying phase had mass loss rate at temperatures ranging from 47 °C (Wo – spent) to 58 °C (Tw – raw and Ba – raw).

The devolatilization stage occurred at temperatures between 138 °C and 458 °C (Table 8.4). The maximum decomposition rates occurred at temperatures between 317 °C (FI – spent) and 359 °C (Wo – raw). For the spent biomass it was noticed a decrease in this peak

temperature and an increase in the maximum decomposition rate, with the exception made for the twigs. The maximum decomposition rate was found for the wood fraction, with 8.9358 %/min for raw wood and 10.407 %/min for spent wood, which was expected due to the higher volatile content in this fraction.

Table 8.4 — Characteristic parameters of the thermal degradation of *A. dealbata* fractions under oxidative conditions.

Sample	Devolatilization zone			Char combustion zone			Ti (°C)	Tb (°C)
	Temperature range	R_{peak} (%/min)	T_{peak} (°C)	Temperature range	R_{peak} (%/min)	T_{peak} (°C)		
Fl – raw	138 – 417	3.2569	327	443 – 688	2.6759	482	220	766
Fl – spent	147 – 394	4.0256	317	414 – 557	5.0319	497	240	524
Le – raw	144 – 458	3.9187	328	-	-	-	230	831
Le – spent	184 – 415	4.5434	319	443 – 614	2.7927	536	250	622
Tw – raw	170 – 380	5.7428	320	406 – 540	4.6118	483	260	705
Tw – spent	200 – 420	5.1343	341	450 – 636	1.8639	488	270	684
Wo – raw	185 – 412	8.9358	359	-	-	-	300	930
Wo – spent	205 – 387	10.407	349	440 – 548	2.6152	508	300	602
Ba – raw	170 – 397	4.1634	350	-	-	-	260	1040
Ba – spent	182 – 385	4.6244	340	443 – 505	1.9489	494	270	687

In the char oxidation stage, some different behaviors were observed between samples. The maximum degradation rates were found at temperatures between 482 °C (Fl – raw) and 536 °C (Le – spent), with values between 1.8639 %/min (Tw – spent) and 5.0319 %/min (Fl – spent) (Table 8.4). However, for raw leaves, wood or bark, no evident peak was detected in the respective DTG curves, with the oxidation of the char occurring at an approximately constant rate until the end of the degradation. For these samples, combustion of the char occurred with an average mass loss rate of 0.9306 ± 0.1323 %/min, 0.3893 ± 0.0917 %/min, and 0.6806 ± 0.1945 %/min, respectively, measured from the end of devolatilization until burnout.

The ignition temperature (T_i) and the burnout temperature (T_b) are other important combustion parameters. T_i is the temperature at which major decomposition of the biomass samples began to take place (Okoroigwe et al., 2016). The values ranged from 220 °C (Le – raw) to 300 °C (Wo – raw and Wo – spent). Overall, the ignition temperature increases in the spent material, suggesting that the presence of extractives plays a facilitating role in this step of the combustion process. Ignition temperatures of flowers, leaves, twigs, and bark were similar to

the ones found in diverse types of biomass (El may et al., 2012; Lu and Chen, 2015; Plis et al., 2016; Wnorowska et al., 2021). In the case of wood samples, the values were identical to the ones reported for several wood species (Martínez-Cartas et al., 2022; Okoroigwe et al., 2016). T_b , which means the temperature at which the combustion ends, varied from 524 °C (Fl – spent) to 1040 °C (Ba – raw). An important feature is that despite a slight increase in the ignition temperature in spent biomass, the burnout temperature significantly decreased for the spent samples (524 – 687 °C for spent material versus 705 – 1040 °C for the raw material), indicating that the removal of at least a part of the extractives by the previous extraction of bioactive compounds increases the combustion efficiency by reducing the solid residence time (Sun et al., 2019; Yi et al., 2013). T_b temperatures of spent fractions are comparable to those found in diverse types of biomass (Kopczyński et al., 2015; Koprivica et al., 2022; Kosowska-Golachowska et al., 2018; Mahanta et al., 2020; Martínez-Cartas et al., 2022; Miranda et al., 2008; Okoroigwe et al., 2016; Plis et al., 2016; Vamvuka et al., 2019; Wattana and Kittayaruksakul, 2016; Wilk et al., 2020; Wnorowska et al., 2021).

The pseudo component model fitting results are presented in Table 8.5 and Figure 8.5. Very good fitting qualities were observed for all the samples. Activation energies ranged from 69.0 kJ/mol (Tw – raw) to 115.5 kJ/mol (Wo – raw). A smaller E_a means that combustion will be faster. It was observed that activation energy decreased in spent flowers, bark, and wood, remained in a similar value in spent leaves, and increased in spent twigs, compared with the respective raw materials. The E_a values obtained in this work are of a similar magnitude to the ones reported for different fractions of date palm (El may et al., 2012), and in a lower range than the reported for agriculture residues (shells and wood) (131.5 – 269.6 KJ/mol) (Okoroigwe et al., 2016), pine and processed wood wastes (145.7 – 190.0 kJ/mol) (Ekinci et al., 2021), or aspen, birch, oak, and pine woods (104 – 125 kJ/mol) (Okoroigwe et al., 2016).

Table 8.5 — First-order kinetic activation energies (E_a) and fitting qualities (FQ) obtained by first-order pseudo component fitting for the thermal degradation of *A. dealbata* raw and spent fractions.

Sample	E_a (kJ/mol)	FQ (%)	Sample	E_a (kJ/mol)	FQ (%)
Fl – raw	77.3	99.7	Fl – spent	74.2	99.5
Le – raw	74.7	99.6	Le – spent	75.6	99.8
Tw – raw	69.0	99.5	Tw – spent	75.3	99.5
Wo – raw	115.5	99.7	Wo – spent	99.4	99.5
Ba – raw	70.8	99.4	Ba – spent	68.5	99.7

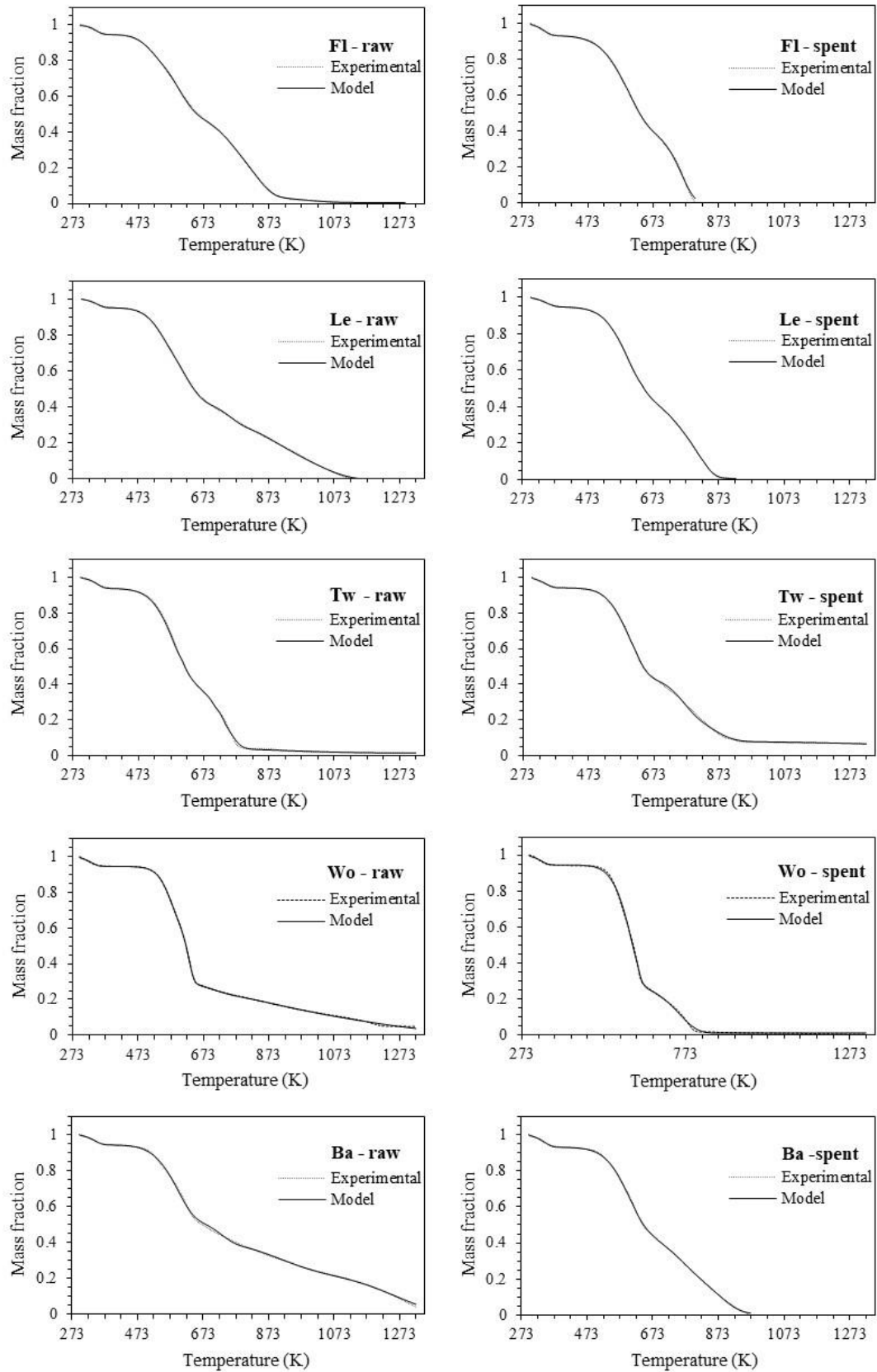


Figure 8.5 — Oxidation kinetic modelling of the different fractions of raw and spent *A. dealbata* fractions by pseudocomponent model fitting, showing the experimental and modeling results.

8.3.5.2 Macro TGA analysis

The thermal behavior of spent fractions was further investigated by macro TGA, using 10 g of sample, an oven temperature of 750 °C, and an air:fuel equivalence ratio of 0.2, therefore in conditions below the stoichiometric air-to-fuel ratio, corresponding to pyrolysis or gasification. The mass loss of samples over time given by the macro TGA is illustrated in Figure 8.6. The characteristic parameters of the devolatilization stage and char combustion are shown in Table 8.6. Two distinct zones can be identified: a first stage, characterized by a rapid mass loss, which includes the drying and devolatilization steps; and a second one, characterized by a much slower degradation rate until the end of the experiment, that corresponds to the char oxidation phase. A similar behavior was observed using eucalyptus woodchips (Silva et al., 2022). As can be observed, the spent wood had the faster decomposition rate (33.20%/min) when compared with the other samples with values in the range of 21.02 to 22.95 %/min. (Table 8.6). Also, in the end of the devolatilization phase, the spent wood sample presented the lower residual mass (16.99%) while the other biomass samples had residual masses in the range of 23.90 to 28.49%. These results are coherent with those found in micro-TGA and indicate that the wood sample presents a faster decomposition rate and at a higher extent, what may be due to its higher oxygen content that contributes to more oxidative conditions even at low air-to-fuel ratios.

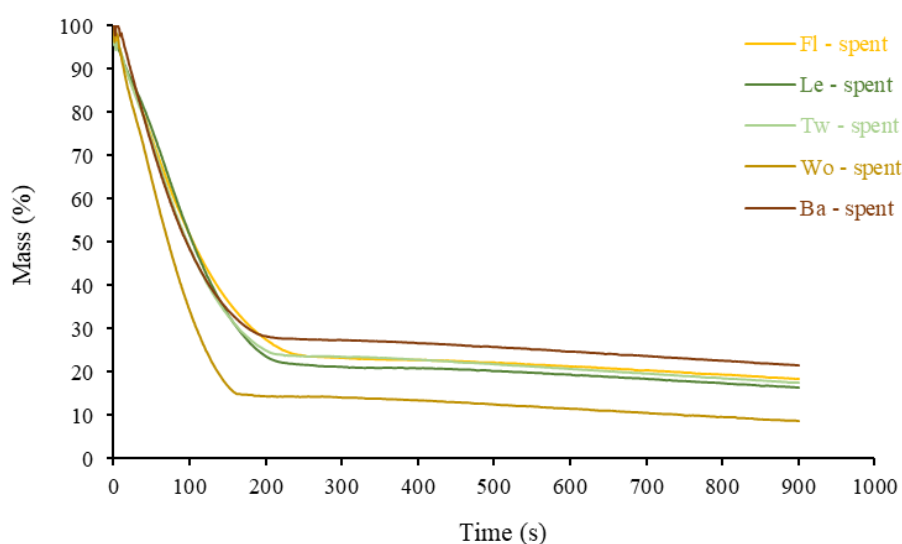


Figure 8.6 — Mass loss of the different spent fractions of *A. dealbata* given by the macro TGA.

Table 8.6 — Characteristics of the devolatilization and char oxidation stage on the thermochemical conversion of the various spent fractions of *A. dealbata* in the 900 s of the experiments.

Sample	Devolatilization			Char combustion	
	Time	Mass remaining (%)	Global rate (%/min)	Mass remaining (%)	Global rate (%/min)
Fl – spent	210	26.42	21.02	18.48	0.69
Le – spent	199	23.90	22.95	16.46	0.63
Tw – spent	197	25.48	22.70	17.67	0.55
Wo – spent	150	16.99	33.20	8.76	0.66
Ba – spent	194	28.49	22.12	21.43	0.60

The main gases released during the samples thermal decomposition was also analyzed and the results are presented in Figure 8.7. Given the equivalence ratio of 0.2 the experiments evaluated the gases produced by the sample decomposition under limited availability of oxidant agent therefore it was possible to detect H₂, CH₄ and CO, besides CO₂ that remains the major component of the permanent gas products. The most noticeable differences between samples were in CO₂ concentration, which varied between 2.46% when using spent leaves and 6.80% in the case of the bark. CO was found in a much lower concentration, which was similar when using spent flowers, twigs, and bark (0.31 – 0.49%), slightly higher with wood (0.89%), and in a very low concentration in the case of spent leaves (0.05%). No relevant differences were found between samples for H₂ and CH₄ concentration in the released gas.

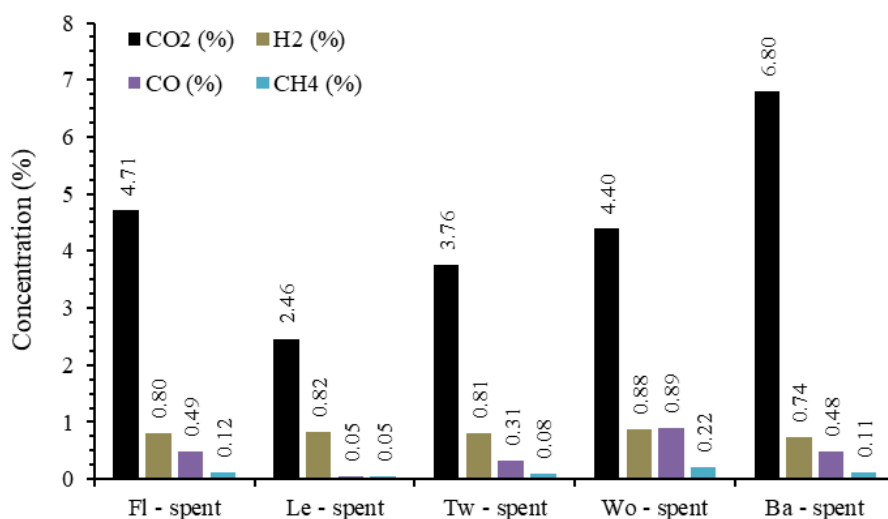


Figure 8.7 — Gas composition; each gas concentration determined by its amount in the total gas collected.

The concentration of each gas was also monitored over time and a clear peak was observed when devolatilization occurs, exception made for H₂, which remains approximately at a constant concentration during the experiments (See Appendix F1, Figure F1). Similar behavior was described by Silva et al. using eucalyptus woodchips (Silva et al., 2022).

8.3.6 Adsorption of cationic and anionic dyes

The adsorption ability of the different raw and spent fractions of *A. dealbata* towards the cationic MB and the anionic MO dyes was briefly studied. The adsorption capacity against MB increased with increasing the contact time, varying between 7.0 mg/g (Wo – spent, 1 h) and 24.6 mg/g (Ba – spent, 4 h and 24h), corresponding to removal efficiencies between 28.4 and 99.5% (see full results in Appendix F2, Figure F2). Until 4 h of contact time, better results were obtained with the raw material, with the exception made for bark after 4 h of contact time. After 24 h of contact time, MB removal efficiencies and adsorption capacity were found higher when using the spent material, with removal efficiencies higher than 90% with all spent samples. All the fractions were more effective against MB than against MO. The removal efficiencies were lower than 14% against MO, even after 24 h of contact time, with the exception made for spent leaves and twigs, which attained 58.6 and 58.3% of removal efficiency after 24 h, respectively. Adsorption capacity against MO varied between 0.2 mg/g (Le - spent, 1 h, and Wo – spent, 1 and 4h) and 14.6 mg/g (Le – spent, 24h), corresponding to removal efficiencies between 0.7 and 58.6% (Appendix F2, Figure F2).

Figure 8.8 presents the removal efficiencies and adsorption capacities obtained for the different contact times for leaves and bark, to illustrate the time dependence and some differences among samples. Raw and spent flowers, twigs, and wood followed a similar trend to that of leaves (Appendix, Figure F2). In the case of bark, only a small increment between 4 and 24 h of contact time was observed, once after 4 h the removal efficiency attained already 99.5%. The higher adsorption ability towards the cationic MB dye was evident for all fractions and contact times, with the exception made for spent leaves and twigs (Appendix, Figure F2) after 24 h of contact time, which attained almost 60% of MO removal. The highest affinity for the cationic dye suggests electrostatic attraction between negatively charged functional groups on the surface of adsorbents, like hydroxyls or carboxylic groups identified in the FTIR analysis, and the positively charged MB, which is less probable to occur with the negatively charged MO. This behavior was also observed by Zhou et al. in the adsorption of the same dyes on peach gum (Zhou et al., 2014).

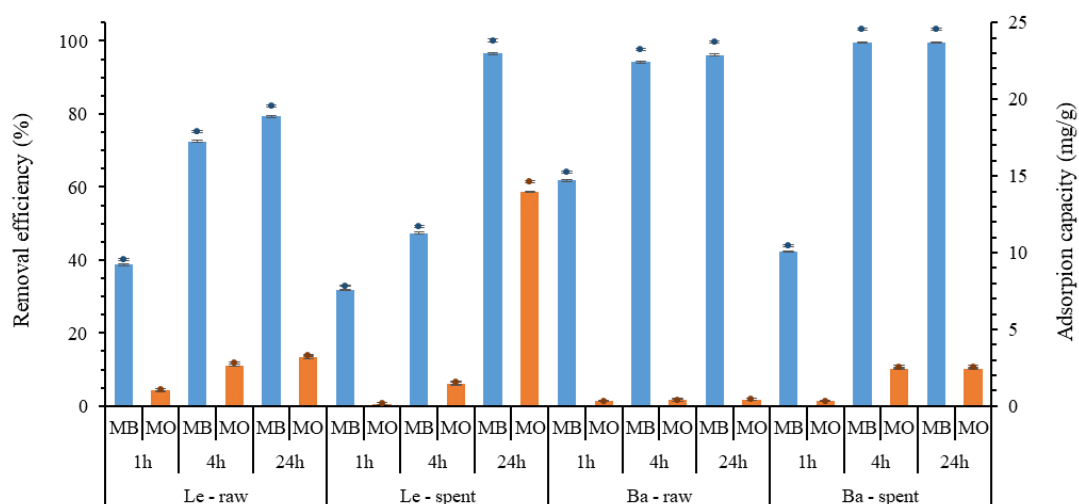


Figure 8.8 — Adsorption capacity (circles) and removal efficiency (bars) of MB and MO, for both raw and spent samples of leaves and bark, after 1 h, 4 h, and 24 h of contact time. Error bars represent the standard deviation of the mean.

The results here presented are, to the best of our knowledge, the first results on the adsorption of MB and MO using *A. dealbata* biomass. These are encouraging results regarding the exploitation of these residues from an invasive species, especially the ones from the spent material, after extraction of value-added chemicals. Adsorption ability must be further studied, evaluating different adsorption conditions (e.g. pH, agitation, adsorbent dose, particle size), as well as determining adsorption isotherms, adsorption kinetics, and performing activation procedures, to better understand the potential of these resource in the production of low-cost adsorbents. Nevertheless, results were better than the reported for beech sawdust (9.78 – 16.05 mg/g of maximum adsorption capacity towards MB) (Batzias and Sidiras, 2004) and comparable to those reported for oak and pitch-pine wood (29.94 and 27.78 mg/g of maximum adsorption capacity, respectively) (Ferrero, 2007). The raw and spent flowers, raw twigs, and raw bark showed similar removal efficiency as observed when using peach shell particles also from an aqueous 100 mg/L MB solution at a dosage of 4 g/L (Marković et al., 2015). The adsorption capacities and removal efficiencies obtained after 24 h were comparable to those found when using tea stem (Lee et al., 2019), although with a slower adsorption rate.

8.4 Conclusions

The thermochemical characterization of raw and spent flowers, leaves, twigs, wood, and bark of *A. dealbata* and their ability to be used as adsorbents of cationic and anionic dyes were

performed. Proximate and ultimate analysis showed that all fractions have a composition similar to that of other biomasses used for energy purposes, which was not significantly affected by the previous extraction step. The higher heating values were not affected by the removal of extractives as well and were in an attractive range for energetic applications. Moreover, according to a recent predictive model for ash-related problems in thermal conversion equipment, the mineral composition of the studied fractions is reported to have low tendency to cause ash deposit issues, with exception made for the raw and spent flowers, that must be used together with other less problematic fuels in energetic applications. It should be noted that, in general, the spent samples presented even higher CaO and lower K₂O concentrations in ashes, compared the respective raw materials, which favors the formation of ash with higher melting points and less prone to cause fouling or sintering.

The thermogravimetric analysis under oxidative conditions revealed ignition and burnout temperatures of the spent fractions comparable to the ones found in other fuels, being noticed a significant reduction of the burnout temperature with the removal of extractives in the previous extraction step, which means a more efficient conversion by reducing the solid residence time in the equipment. Moreover, a decrease in the activation energy and the consequent shortening of reaction time was observed in the case of spent flowers, wood, and bark, compared with the respective raw materials. The degradation rate in the macro TGA assay followed what is expected for biomass, with the wood fraction being clearly the most reactive.

The preliminary adsorption study revealed that all the raw and spent fractions can adsorb MB dye to a much higher extent than MO dye. The raw material showed better adsorption capacity for contact times of 1 and 4 h, but after 24 h the spent material had a better performance, being able to remove more than 90% of MB from an initial 100 mg/L MB solution. Further studies are needed to improve the adsorption ability of *A. dealbata* biomass, by applying agitation, evaluating the impact of changing pH, adsorbent dosage, the particle size, among other factors, or by performing some activation step.

Therefore, the results here presented provide significant information on the potential of using biomass from *A. dealbata* from a biorefinery perspective, through the removal of valuable extractives and subsequent valorization of the remaining material for energy purposes or material application as adsorbents.

CHARACTERIZATION OF BIOCHARS AND HYDROCHARS OBTAINED FROM DIFFERENT FRACTIONS OF *ACACIA DEALBATA* BIOMASS FOR ENERGY OR MATERIAL APPLICATIONS

Abstract: *Acacia dealbata* is an invasive species, whose widespread proliferation should be controlled but is also used in various industries for production of wood materials, biofuels, concretes and tannins. The biomass wastes produced by removal operations to limit dissemination or by the industrial transformation processes, could be further valorized in energy or material applications. In this work, different fractions of *Acacia* biomass (flowers, leaves, twigs, bark and wood) were sequentially extracted by non-polar and polar organic solvents to recover lipophilic and phenolic extractives and the spent biomasses were characterized for proximate, ultimate and mineral compositions, high heating value (HHV) and bulk density (BD). All biomass fractions had composition and properties adequate for valorization as solid biofuels, in particular the leaves fraction that presented the higher values of HHV and BD, 23.47 MJ/kg and 366.01 kg/m³, respectively. Torrefaction at 250 °C and hydrothermal carbonization at 200 °C and 250 °C were used to produce biochars and hydrochars, from each biomass fraction, with mass yields in the range of 46.4 to 89.5% and energy yields in the range of 61.8 to 94.5%. The produced biochars and hydrochars presented higher fixed carbon and ash contents, higher BD and HHV values, lower H/C and O/C ratios and lower K₂O and MgO concentrations than the corresponding spent biomasses. Hydrochars obtained at 200 °C and 250 °C also had higher CaO concentrations, leading to an increase of ash melting point. Evaluation of ash fouling, slagging, sintering, and bed agglomeration indexes as well as the representation of combined

mineral compositions in a ternary diagram led to identification of the flower fraction and related chars as the materials with higher risks of ash melting during combustion or gasification. FT-IR analysis of the spent biomasses and the corresponding biochars and hydrochars allowed the identification of significant modifications of the surface functional groups corresponding to the decomposition of the biomass polymers and extensive deoxygenation reactions. Analysis of the thermogravimetric profiles (TGA) and derivative curves (DTG) for the spent biomasses and of the respective biochars and hydrochars, obtained in and oxidative atmosphere, allowed to determine relevant combustion parameters such as ignition and burnout temperatures and maximum decomposition rates within the devolatilization and char oxidation regions. A model based on the Arrhenius equation was proposed to describe the TGA profiles of the *Acacia* biomass fractions and the corresponding biochars and hydrochars. The proposed equation was used to calculate first-order kinetic activation energies (E_a) for the thermal decomposition of each of these samples. The effluent of the hydrothermal process was characterized for pH, chemical oxygen demand (COD), total phenolics and for total, volatile and fixed solids, in order to discuss possible treatment or valorization options. High acidity and phenolic content were the limiting factors that may hinder the efficiency of bioremediation processes. The spent biomasses, biochars, hydrochars and corresponding activated chars produced by steam activation were evaluated as adsorbents for a cationic dye (methylene blue, MB) and an anionic dye (methyl orange, MO). The biochars and hydrochars obtained from wood or bark showed the higher adsorption capacity, especially after steam activation, reaching removal efficiencies higher than 90%. The results indicate that these biomass fractions can be valorized as biofuels, or used to produce biochars and hydrochars with improved fuel properties. Production of activated char by steam activation is another valorization pathway that contributes to the integral recovery of these biomass resources for energy or material applications.

9.1 Introdução

The growing concerns regarding the security of energy supply, energy sustainability, and the need to control the emission of greenhouse gases have motivated a global interest in the search for renewable energy sources, as an alternative to fossil fuels. Biomass plays here an important role, once it is highly available, is considered a carbon-neutral resource, and can be converted into solid, liquid or gaseous fuels (McKendry, 2002a, 2002b, 2002c). However, a significant fraction of biomass does not present the appropriate characteristics for energetic or material valorization, due to high moisture content and hydrophilic nature, low calorific value,

poor grindability, or low bulk density, which increases transportation and storage costs, limiting the storage time due to microbial degradation, and negatively affects combustion (Arias et al., 2007; Demirbas, 2004; Hellwig, 1985; Jenkins et al., 1998). In order to improve the physical and chemical characteristics of biomass for energy and material applications, torrefaction and hydrothermal carbonization (HTC) as been extensively studied in the last years, once they are low energy processes, when compared with slow pyrolysis for biochar, fast pyrolysis for bio-oil, or gasification technologies (Yan et al., 2017).

Torrefaction is a thermochemical process in which raw biomass is heated under atmospheric pressure, at a temperature range of 200–300 °C, in the absence of oxygen or under low oxygen levels (Rousset et al., 2011). It improves the calorific value of biomass, decreases its O/C ratio and moisture content, as well as confers to biomass a hydrophobic character, which improves its resistance to biological degradation during storage (Wilk et al., 2015). Besides, torrefaction also improves grindability, by breaking the bonds between lignocellulosic polymers and promoting the depolymerization of hemicellulose and cellulose (Bergman et al., 2005).

In turn, HTC arised as a promising and technically-attractive thermal process to improve the properties of biomass as a fuel. In this technique, biomass is submerged with water and typically heated to 180–260 °C in a confined system under autogenous pressures (2–6 MPa) for 5–240 min (Kambo and Dutta, 2015a). It has the obvious advantage over the traditional torrefaction process of not being affected by possible high moisture contents of the biomass feedstock, but the characterization of the generated process waters is needed once it may contain significant content of mineral parts and dissolved organic compounds (Wilk et al., 2020). Under these operational conditions, the water is present as a subcritical water, thus having properties not only as a solvent but also as a catalyst. It is reported that the presence of subcritical water allows decarboxylation, dehydration, decarbonylation, de-methoxylation, depolymerization, intermolecular derangement, condensation and aromatization reactions that occur during the thermal treatment to be initiated by hydrolysis, leading to lower biomass decomposition temperatures compared to traditional torrefaction (Kambo and Dutta, 2015b; Missaoui et al., 2017).

Both techniques have been applied to improve the fuel properties of diverse biomass. Torrefaction successfully improved the chemical composition and calorific value of oil palm fiber (Lu et al., 2012), eucalyptus wood (Arias et al., 2007; Lu et al., 2012), bamboo (Rousset et al., 2011), or *Acacia nilotica* wood (Singh et al., 2020), while HTC upgraded the fuel properties of miscanthus (Kambo and Dutta, 2015b, 2014), *Robinia pseudoAcacia* wood (Wilk et al., 2020),

coconut fiber and eucalyptus leaves (Liu and Balasubramanian, 2012), or eucalyptus bark (Gao et al., 2016). When comparing both techniques using the same raw material, higher improvements in the higher heating value, grindability or in hydrophobicity have been reported by using the HTC (Kambo and Dutta, 2015b; Wang et al., 2019; Yan et al., 2017).

To the best of our knowledge, no studies on hydrothermal carbonization of *A. dealbata* are reported in the literature, while just one work concerning the torrefaction of *A. dealbata* was found. Nunes et al. torrefied *A. dealbata* wood at 265 °C for 15 min, having observed a significant increment of the elemental carbon content (21.2% gain) along with a gain of 25% in the lower calorific value of the produced biochar (Nunes et al., 2018). Moreover, no studies on torrefaction and HTC have been found using spent biomass, after the previous extraction of added-value compounds in a biorefinery approach. This study aims to evaluate the potential of using spent flowers, leaves, twigs, wood, and bark of *A. dealbata* to produce biochars suitable for energetic applications as a solid fuel, or in material applications as low-cost adsorbents of cationic and anionic dyes from aqueous solutions.

A comparison of these spent fractions and the corresponding biochars was made to evaluate the feasibility of a sequential valorization strategy according to a biorefinery concept. The spent raw material and the produced biochars were characterized for their proximate, ultimate, and mineral composition, and their surface functional groups were described by Fourier-transform infrared spectroscopy (FTIR). The combustion behavior of the original material and respective biochars was studied by micro thermogravimetric analysis (TGA). Batch adsorption experiments towards methylene blue (MB) and methyl orange (MO) dyes were performed to make a preliminary assessment of the potential of these spent biomass fractions and biochars obtained thereof to be used as low-cost adsorbents.

9.2 Materials and methods

9.2.1 Biomass samples

The flowers, leaves, twigs, bark, and wood used in this work are the remnants of extraction procedures using non-polar (petroleum ether) and polar (70% aqueous acetone) solvents, sequentially, in order to obtain bioactive extracts that are being studied in separate works. The samples were grossly grinded before extraction procedures. After the extraction, the samples were left to dry in the air for about one week and then the particle size was uniformized by another grinding step and sieving to 2 mm.

9.2.2 Traditional torrefaction and hydrothermal carbonization procedures

The traditional torrefaction assays were performed under oxygen-limited conditions using a glass reactor heated in a chromatographic furnace equipped with a cooling trap for condensate recovery, as shown in Figure 9.1. The system was isolated to guarantee air-free conditions inside the reactor during the experiments. The initial residual air in the reactor is ejected during the initial devolatilization stage of the biomass.

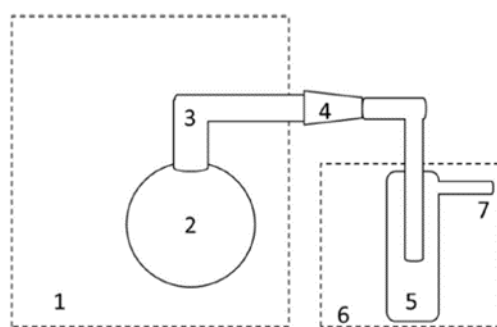


Figure 9.1 — Scheme representing the traditional torrefaction set-up (figure obtained from Şen et al. (Şen et al., 2020)). 1 – chromatographic furnace, 2 – glass reactor with biomass, 3 – connector, 4 – reducing adaptor, 5 – glass condensate collector, 6 – cooling ice bath, 7 – gas exit for sampling or vent.

A 250 mL glass reactor was filled with 40 to 80 g of biomass, depending on the bulk density of the fraction to be used, and heated up to the desired final torrefaction temperature using a heating rate of 12 °C/min. After reaching the desired temperature, the conditions were kept isothermal for 60 min, and then cooled to 35 °C with a cooling rate also of 12 °C/min. The produced biochars were left in atmospheric environment for five days before characterization. The mass of biochar was determined with an analytical balance (Mettler Toledo AB204-S) and the biochar yield determined as a mass percentage in a dry basis. The biochars of spent flowers, leaves, twigs, wood, and bark obtained by this torrefaction procedure were labelled as T-FI-250, T-Le-250, T-Tw-250, T-Wo-250, and T-Ba-250, respectively.

The HTC experiments were carried out using a 1 L stainless steel autoclave reactor (Parr Pressure Reactor) coupled with an electric heater and a controller (Parr 4848 Reactor controller), under autogenic pressure (Figure 9.2).



Figure 9.2 — Stainless steel autoclave reactor used in the HTC experiments.

For each experiment, 50 g of biomass sample was placed in the reactor and tap water was added at a solid-to-water ratio of 1:5 (m/m). After sealing and removing air with a vacuum pump, the reactor was heated to the desired carbonization temperature (200 and 250 °C), kept at that temperature for 60 min and cooled to room temperature using an internal refrigeration coil, fed with tap water. Any residual gaseous products were vented when the reactor reached room temperature. The hydrochars and liquid products were separated by filtration through a pre-weighted qualitative filter paper. The hydrochars were then air-dried overnight and oven-dried at 105 ± 2 °C for 12 h. The oven-dried hydrochars were then equilibrated to room temperature in a desiccator, weighted for yield determination and kept in atmospheric conditions for five days. The liquid product (process water) was stored at 4 °C until further analysis.

The flower, leaves, twigs, wood, and bark hydrochars obtained this way were labelled as H-Fl-200, H-Le-200, H-Tw-200, H-Wo-250, and H-Ba-250, or as H-Fl-250, H-Le-250, H-Tw-250, H-Wo-250, and H-Ba-250, respectively, depending on the used temperature.

The mass and energy yields of the produced biochars were calculated, in a dry basis, using equations (9.1) and (9.2), respectively:

$$\text{Mass yield (\%, db)} = \frac{m_{\text{char}}}{m_{\text{biomass}}} \times 100 \quad (9.1)$$

$$\text{Energy yield (\%, db)} = \text{Mass yield} \times \frac{HHV_{\text{char}}}{HHV_{\text{biomass}}} \quad (9.2)$$

where m_{char} and HHV_{char} are the oven-dried mass (kg) and high heating value (MJ/kg) of the biochars, and m_{biomass} and HHV_{biomass} are the oven-dried mass and high heating value of spent biomass used in the experiments.

9.2.3 Characterization of liquid phases from hydrothermal carbonization

The process waters from the HTC assays were characterized for their pH, chemical oxygen demand (COD), total solids (volatile and fixed solids), and total phenolic compounds.

The pH was determined with a pH meter (Crimson MicropH 2001 meter). The COD was determined through the high range dichromate method (Hanna Instruments test kits). Total phenolics were measured by an adaptation of the Folin-Ciocalteu method proposed by Singleton et al. (1998), exactly as described by Correia et al. (Correia et al., 2022). Evaluation of total solids was performed according to method 2540B from the Standard Methods for Water and Wastewater.

9.2.4 Physical and chemical analysis

The proximate and ultimate analyses, the bulk density, the FTIR data, and the HHV were obtained as described in the previous chapter (section 8.2.3).

9.2.5 Ash slagging and fouling indices

The alkali index (A_I), the slagging evaluation index (SEI), the sintering index (S_I), and the bed agglomeration index (BAI) were determined as described in the previous chapter (section 8.2.4).

9.2.6 Thermogravimetric analysis

The thermal behavior was studied by micro TGA, following the procedure described in section 8.2.5.

The first-order pseudocomponent model fitting was applied to fit experimental data and determine activation energy, as performed with raw and spent biomass in the previous chapter (section 8.2.5).

9.2.7 Adsorption

9.2.7.1 Adsorption experiments

The adsorption experiments were performed also as described in the previous chapter, with adsorption capacity and removal efficiency calculated using equations 8.9 and 8.10, respectively.

9.2.7.2 Steam activation of biochars and hydrochars

Steam activation of the biochars was carried out at a mass ratio 1:2 (w/w) for biochar and deionized water, respectively, under oxygen-lean conditions at 900 °C and 7-min reaction time. (Şen et al., 2020). Before this procedure the biochars and hydrochars were ground and water-soaked for 1h.

The steam activated biochars were then used in the adsorption experiments as described in the previous section.

9.3 Results and discussion

The different fractions of *Acacia* biomass (flowers, leaves, twigs, bark and wood) were converted to biochars at 250 °C, a temperature that is high enough to cause devolatilization of the hemicellulose and partial degradation and reorganization of the lignin and cellulose fractions. The same biomass fractions were also converted to hydrochars at 200 °C and 250 °C, using hydrothermal carbonization at a solid-to-water ratio of 1:5 (m/m).

9.3.1 Biochar yields and energy yields

The mass and energy yields for both carbonization processes are presented in Figure 9.3.

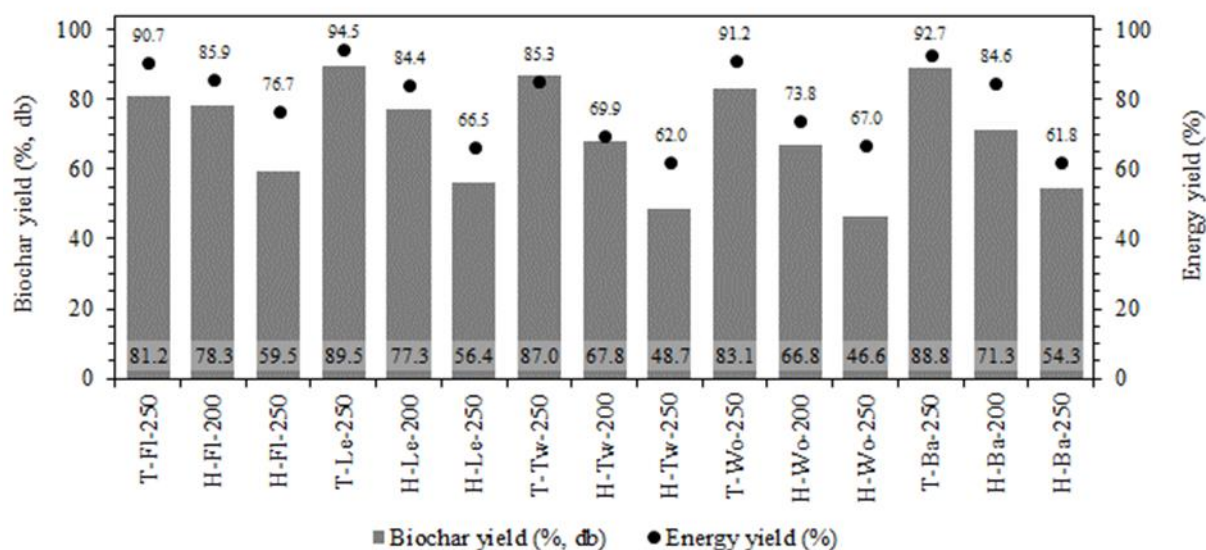


Figure 9.3 — Biochar yields and energy yield of traditional torrefaction and hydrothermal carbonization of spent fractions of *A. dealbata*.

As it can be seen in Figure 9.3, higher mass yields were obtained for torrefaction and varied from 81.2% for flowers to 89.5% for leaves. Bark, leaves and twigs were converted to biochar at 250 °C at a higher yield than the wood fraction, and in the case of leaves and bark at a higher energy yield. The mass yields of the hydrochars produced by hydrothermal carbonization at 200 °C were slightly lower than those obtained from torrefaction at 250 °C and varied from 66.8% for wood to 78.3% for flowers. These results indicate that at hydrothermal conditions was possible to convert the biomass fractions to hydrochars at a temperature 50 °C lower than that used in torrefaction, due to the oxidation effect of the water molecules. Moreover, the polymer degradation reactions that occur during the thermal treatments, such as decarboxylation, dehydration, decarbonylation, demethoxylation, intermolecular rearrangement, condensation and aromatization (Funke and Ziegler, 2010), are allowed to be initiated by hydrolysis with the presence of subcritical water, resulting in the cleavage of ether and ester bonds and in the reducing of activation energy of the biomass polymers being degraded (Kambo and Dutta, 2015b, 2014). When hydrothermal carbonization was performed at the same temperature as torrefaction both the mass and the energy yields were significantly lower than those of torrefaction due to the partial solubilization of carbonization products in the aqueous phase, while in torrefaction most of the liquid products remain trapped in the biochar pores (Kambo and Dutta, 2015b, 2014). Mass yields for hydrochars produced at 250 °C varied from 46.6% (for wood) to 59.5% (for flowers) while energy yields varied from 61.8% (for bark) to 76.7% (for flowers).

At all temperatures and for both types of conversion processes, the energy yield is higher than the mass yield indicating that there is some upgrading of the fuel quality of biochars and hydrochars relatively to the raw materials. Similar trends of the variation of the biochar and energy yields between torrefaction and hydrothermal carbonization, or for different hydrothermal carbonization temperatures were observed using miscanthus (Kambo and Dutta, 2015b, 2014) or moso bamboo (Yan et al., 2017).

9.3.2 Effect of thermal treatments on chemical composition and HHV

The proximate, ultimate and mineral composition of the spent fractions of *A. dealbata* and the respective biochars and hydrochars obtained by, respectively, traditional (T) and hydrothermal (H) carbonization are shown in Table 9.1.

Some properties relevant for the fuel properties of materials, namely bulk density (BD) and high heating value (HHV) were also determined in order to discuss possible effects of the carbonization processes.

Both torrefaction and hydrothermal carbonization increased fixed carbon and decreased moisture and volatile matter of the biochars or hydrochars relative to the corresponding raw materials. Ash content increased for the biochars produced at 250 °C but decreased for hydrochars produced from twigs and wood, indicating some degree of solubilization of ash components in the aqueous phase. In the case of the biochars produced by torrefaction at 250 °C, the increments of fixed carbon varied from 5.62% (for bark) to 8.3% (for flowers). At hydrothermal conditions the increase of fixed carbon was lower at 200 °C than at 250 °C, because elimination of volatile matter was higher at the higher temperature. At 200 °C the hydrochars presented fixed carbon increments of 3.02% (for twigs) to 7.18% (for wood) but at 250 °C the increase of fixed carbon varied from 11.81% (for leaves) to 32.08% for wood.

Table 9.1 — Physical and chemical characterization of spent fractions of *A. dealbata* and respective biochars obtained by torrefaction (T) and hydrothermal carbonization (H).

	BD (kg/m ³)	HHV (MJ/kg)	Proximate composition (wt. %)			FC ^b	Ultimate composition (wt. %) ^b				
			M ^a	VM ^b	Ash ^b		C	H	N	S	O
Fl	204.30 ± 6.27	22.49 ± 0.07	8.37 ± 0.12	78.28 ± 0.83	4.00 ± 0.06	17.72 ± 0.89	52.23 ± 0.11	6.68 ± 0.01	2.97 ± 0.01	0.19 ± 0.01	33.93 ± 0.11
T-Fl-250	218.63 ± 9.72	25.10 ± 0.21	1.26 ± 0.04	68.95 ± 0.63	5.03 ± 0.09	26.02 ± 0.54	58.82 ± 0.54	6.36 ± 0.02	2.80 ± 0.02	0.12 ± 0.01	26.89 ± 0.49
H-Fl-200	146.64 ± 5.15	24.67 ± 0.87	5.9 4 ± 0.00	72.43 ± 0.26	4.03 ± 0.03	23.54 ± 0.29	60.16 ± 0.58	5.64 ± 0.47	2.62 ± 0.02	nd	27.56 ± 1.07
H-Fl-250	212.64 ± 5.15	28.97 ± 0.17	3.2 8.06	64.94 ± 0.47	4.98 ± 0.04	30.08 ± 0.44	67.53 ± 0.54	6.35 ± 0.06	2.45 ± 0.09	nd	18.71 ± 0.57
Le	366.01 ± 2.45	23.47 ± 0.05	9.21 ± 0.11	79.99 ± 0.63	5.41 ± 0.16	14.60 ± 0.71	52.91 ± 0.06	7.07 ± 0.01	3.54 ± 0.03	0.24 ± 0.02	30.83 ± 0.06
T-Le-250	318.40 ± 1.98	24.80 ± 0.03	1.04 ± 0.05	71.84 ± 0.26	5.88 ± 0.04	22.28 ± 0.25	56.89 ± 0.05	6.68 ± 0.01	3.75 ± 0.01	0.12 ± 0.00	26.69 ± 0.07
H-Le-200	263.75 ± 4.42	25.64 ± 0.02	6.1 2 ± 0.10	74.79 ± 0.75	5.45 ± 0.04	19.76 ± 0.72	59.91 ± 0.14	6.37 ± 0.04	2.95 ± 0.03	nd	25.32 ± 0.13
H-Le-250	304.52 ± 6.49	27.68 ± 0.17	5.96 ± 0.07	67.07 ± 0.38	6.52 ± 0.12	26.41 ± 0.29	64.80 ± 0.28	6.18 ± 0.04	2.77 ± 0.01	nd	19.73 ± 0.30
Tw	330.76 ± 4.95	21.01 ± 0.17	9.11 ± 0.17	79.08 ± 0.45	4.18 ± 0.05	16.74 ± 0.50	49.32 ± 0.02	6.63 ± 0.13	2.05 ± 0.01	0.07 ± 0.00	37.75 ± 0.16
T-Tw-250	437.26 ± 1.64	20.59 ± 0.08	0.66 ± 0.03	72.31 ± 0.18	4.53 ± 0.13	23.16 ± 0.26	51.97 ± 0.18	5.38 ± 0.01	1.78 ± 0.06	nd	36.35 ± 0.24
H-Tw-200	345.03 ± 6.46	21.66 ± 0.04	4.5 4 ± 0.12	72.25 ± 0.47	4.00 ± 0.11	19.76 ± 0.72	54.36 ± 0.35	5.44 ± 0.08	1.13 ± 0.04	nd	35.08 ± 0.23
H-Tw-250	220.85 ± 6.05	26.75 ± 0.05	5.8 9 ± 0.17	58.76 ± 0.24	3.89 ± 0.05	37.35 ± 0.45	65.84 ± 0.11	5.34 ± 0.00	1.59 ± 0.02	nd	23.35 ± 0.13
Wo	221.27 ± 5.48	18.45 ± 0.16	7.03 ± 0.24	86.21 ± 0.45	0.58 ± 0.01	13.20 ± 0.17	46.67 ± 0.02	5.94 ± 0.13	0.26 ± 0.01	nd	46.56 ± 0.11
T-Wo-250	362.42 ± 4.10	20.25 ± 0.07	0.79 ± 0.04	78.84 ± 0.14	0.67 ± 0.03	20.48 ± 0.48	50.96 ± 0.08	5.82 ± 0.03	0.25 ± 0.00	nd	42.30 ± 0.11
H-Wo-200	504.86 ± 6.24	20.40 ± 0.18	7.32 ± 0.22	79.08 ± 0.47	0.54 ± 0.02	20.38 ± 0	52.26 ± 0.18	5.49 ± 0.07	0.18 ± 0.01	nd	41.53 ± 0.27
H-Wo-250	219.65 ± 4.64	26.52 ± 0.45	2.81 ± 0.13	54.24 ± 0.36	0.48 ± 0.02	45.28 ± 0.35	67.89 ± 0.78	4.75 ± 0.08	0.26 ± 0.00	nd	26.63 ± 0.86
Ba	327.72 ± 3.10	19.98 ± 0.18	9.60 ± 0.13	74.42 ± 0.47	4.99 ± 0.07	20.59 ± 0.52	48.59 ± 0.23	6.04 ± 0.06	1.85 ± 0.04	0.09 ± 0.01	38.45 ± 0.34
T-Ba-250	474.60 ± 1.09	20.86 ± 0.16	0.74 ± 0.01	68.64 ± 0.32	5.15 ± 0.07	26.21 ± 0.35	51.74 ± 0.16	5.61 ± 0.07	1.92 ± 0.00	nd	35.58 ± 0.23
H-Ba-200	368.47 ± 4.99	23.71 ± 1.03	5.36 ± 0.06	68.80 ± 0.51	4.96 ± 0.04	26.24 ± 0.47	56.52 ± 1.42	5.34 ± 0.30	1.65 ± 0.08	nd	30.16 ± 1.80
H-Ba-250	248.41 ± 4.48	22.77 ± 0.45	5.73 ± 0.06	56.50 ± 0.27	6.33 ± 0.07	37.17 ± 0.33	60.92 ± 1.15	4.63 ± 0.06	1.91 ± 0.02	nd	27.59 ± 1,12

^a wet basis; ^b dry basis; M: moisture; VM: volatile matter; FC: fixed carbon; BD: bulk density; HHV: higher heating value; nd: not detected

The moisture content of the biochars or hydrochars is in fact a measure of their hydrophobicity since both type of chars were produced at temperatures significantly higher than 100 °C therefore any water present in the raw materials would have been eliminated during the carbonization process. The moisture of biochars and hydrochars was determined as the water readsorbed from atmosphere when these dried materials were exposed to atmospheric air during several days, thus reflecting the presence of polar groups in the char surface, available for interaction with water molecules. The biochars produced at 250 °C by torrefaction showed the highest hydrophobicity with moisture contents that decreased -6.24% (wood) to -8.86% (bark) relative to the corresponding raw materials. In the case of the hydrochars produced at 200 °C the moisture contents increased 0.29% for wood derived hydrochar and decreased for all others. The hydrochars produced at 250 °C were all more hydrophobic than the corresponding raw biomasses, with moisture content reductions that varied from -3.22% (for twigs) to -5.09% (for flowers). The higher hydrophobicity of the biochars produced by traditional torrefaction at 250 °C may be related to the deposition of decomposition products in the char surface and pores, limiting the availability of polar surface groups to interact with water molecules.

The higher hydrophobicity of the hydrochars produced at 250°C relatively to the hydrochars produced at 200 °C reflects a more extensive rearrangement of their carbonaceous structure with further elimination of oxygenated groups and therefore lower capacity for water adsorption. The decrease of volatile matter was lower for hydrothermal carbonization at 200 °C (from -5.2% for leaves to -7.13% for wood) followed by torrefaction at 250 °C (from -5.78% for bark to -9.33% for flowers) and the higher devolatilization occurred during HTC at 250 °C with variations of volatile matter in the range of -12.92% (for leaves) to -31.97% (for wood). These results are in agreement with the previous results of mass yield and energy yield and reflect the extension of the sample decomposition.

The ultimate composition of the biochars and hydrochars showed a general tendency for increasing carbon content and decreasing oxygen and hydrogen contents, as a result of the deoxygenation reactions and aromatization of the carbonaceous structure, typical reactions of these thermochemical processes.

The carbon content of the biochars produced by torrefaction at 250 °C varied between 50.96% (for wood) to 58.82% (for flowers) and it was interesting to notice that the biochars produced from flowers or leaves presented a higher carbon content than those produced from wood. These results demonstrate that those *Acacia* fractions that are not usually valorized for energy purposes can be converted to biochars with high carbon concentration. The increments

of carbon content for all biochars ranged from 2.65% (for twigs) to 6.59% (for flowers), showing that torrefaction at a relatively moderate temperature could upgrade the fuel quality relative to the raw biomasses.

This upgrading effect was even more evident for the hydrochars produced at 200 °C, that showed carbon contents in the range of 52.26% (wood) to 60.16% (flowers), values higher than those obtained for the corresponding biochars produced at 250 °C by torrefaction. The increase of the carbon content was even more pronounced for the hydrochars produced at 250 °C, that presented carbon concentrations ranging from 60.92% (bark) to 67.53% (flowers), corresponding to a carbon content increase that varied from +11.89% for leaves to +21.22% for wood. An inverse trend was observed for the hydrogen content of the biochars and hydrochars that decreased leading to a carbonaceous structure more aromatic. The biochars produced by torrefaction at 250 °C decreased their hydrogen content from -0.12% (wood) to -1.25 (twigs) relative to the raw biomasses, while the hydrochars produced at 200 °C and 250 °C had hydrogen contents even lower. Therefore, although showing lower values of mass or energy yield the HTC process resulted in the production of hydrochars with higher carbon content and higher aromatic character that can be further valorized in energy or material applications.

The nitrogen content of the biochars and hydrochars was also generally lower than the corresponding raw materials. With some exceptions, HTC led to a higher decrease of the nitrogen content than torrefaction, but in both cases this decrease is beneficial since it contributes to lower the emissions of NO_x if these biochars or hydrochars are destined for energy applications (Ferreira et al., 2013).

The atomic ratios H/C and O/C from the spent *Acacia* biomass fractions and from the corresponding biochars and hydrochars are represented in a van Krevelen diagram, for comparison between themselves and with those of some typical fossil fuels (Figure 9.4).

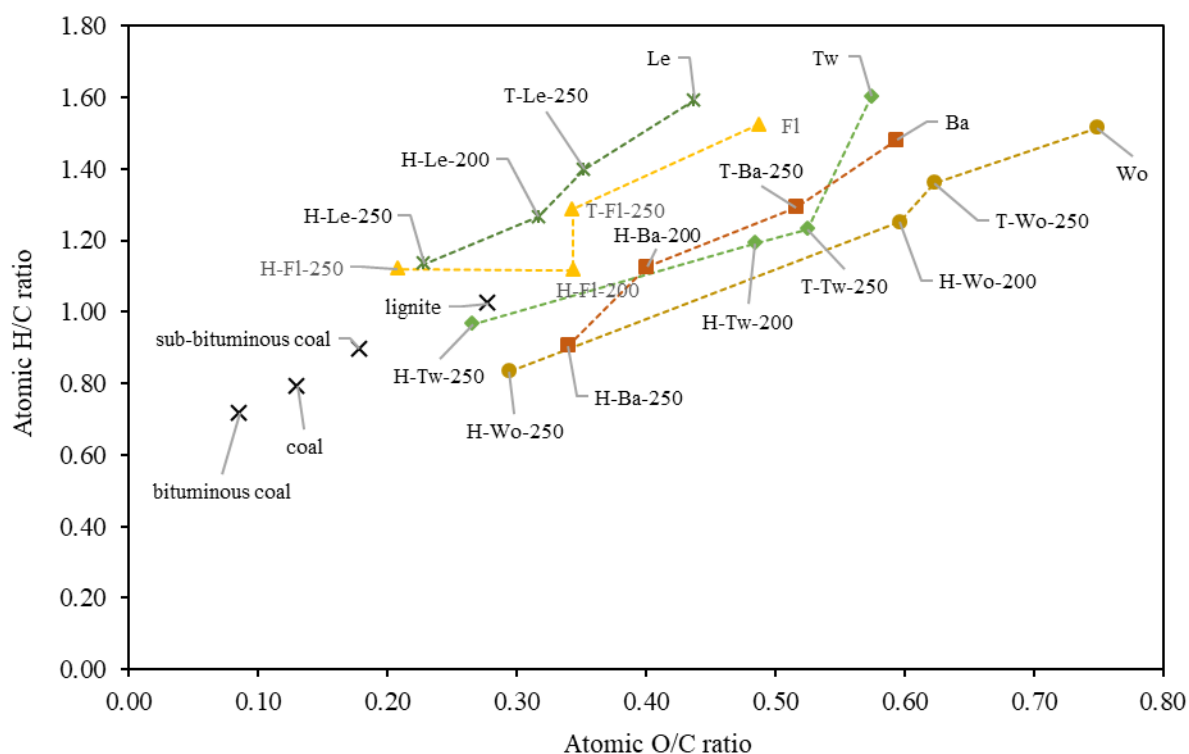


Figure 9.4 — Van Kravellen diagram for the original flowers (Fl), leaves (Le), twigs (Tw), wood (Wo), and bark (Ba) fractions, for the respective chars, and for fossil fuels (Vassilev et al., 2010) for comparison purposes.

As expected the torrefaction and carbonization processes introduced clear reductions in the O/C and in the H/C ratios of the hydrochars relatively to the corresponding raw materials (Nhuchhen and Afzal, 2017). For all samples, the decreasing order for O/C and H/C ratios were: raw biomass > biochars (T, 250 °C) > hydrochars (HTC, 200 °C) > hydrochars (HTC, 250 °C). Most hydrochars obtained at 250 °C presented O/C and H/C ratios similar or lower than those of lignite, showing that these conversion conditions increased the fuel quality of the hydrochars relatively to the raw materials (Wilk et al., 2020). Deoxygenation was less effective for wood or bark hydrochars, both at 200 °C and at 250 °C, but reduction of the H/C ratio was higher, suggesting that these biomass fractions suffered a higher degree of aromatization, therefore could have better properties for production of activated chars.

As a consequence of these changes in proximate and ultimate compositions the HHV of the biochars and hydrochars is expected to exhibit significant differences relative to the raw biomasses used in their production. Taking into account the main components that influence the calorific value the individual values of this parameter were estimated for the raw biomass

fractions and for the produced biochars and hydrochars using an empirical equation proposed by Channiwala and Parikh (Channiwala and Parikh, 2002).

The results obtained indicate that the overall modifications occurred during the carbonization processes increased the HHV of the chars of 0.65 MJ/kg (twigs, HTC at 200 °C) to 8.07 MJ/kg (wood, HTC, 250 °C). The hydrochars produced at 250 °C showed the highest upgrading of their estimated HHV with values that ranged from 22.77 MJ/kg (bark) to 28.97 (flowers). An exception to his behavior is the biochar produced from twigs at 250 °C, using TC, that presented an estimated HHV lower than the corresponding raw biomass (-0.42 MJ/kg), because the increase in ash content was not compensated by an equivalent deoxygenation rate.

The modifications in the carbonaceous structure and composition of the biochars and hydrochars relatively to the raw biomasses also had implications on the respective apparent densities, a parameter relevant for storage and transport operations as well as for the conception and optimization of the feeding systems of energy production units.

The biochars produced by torrefaction at 250 °C, from flowers, twigs, wood and bark presented higher apparent densities relatively to the raw materials with values in the range of 218.63 kg/m³ (for flowers) to 474.6 kg/m³ (for bark). The opposite was observed for most hydrochars produced by HTC at 200 °C or 250 °C with the exceptions of twigs, bark and wood hydrochars produced at 200 °C or flower hydrochar produced at 250 °C, that also presented higher apparent densities than the respective raw biomasses. The decrease of apparent density may be explained by the formation of a porous structure associated with the dissolution of liquid by-products in the aqueous phase that occurred mainly during HTC at 250 °C.

The mineral composition of the raw biomass fractions, and of the produced biochars and hydrochars was determined in order to understand the influence of the carbonization process on this composition parameter and to discuss the possible impact of the mineral composition in the choice of valorization pathways for these carbon materials, either in energy applications or as soil correctors to improve the organic carbon content. Results are presented in Table 9.2 and expressed as the predominant oxide forms corresponding to each mineral element detected.

Table 9.2 — Mineral composition of spent fractions of *A. dealbata* and respective biochars obtained by torrefaction (T) and hydrothermal carbonization (H).

	Al ₂ O ₃	B ₂ O ₃	BaO	CaO	Cr ₂ O ₃	CuO	Fe ₂ O ₃	K ₂ O	MgO	MnO	Na ₂ O	P ₂ O ₅	SiO ₂	SrO	ZnO
FI	0.60 ± 0.01	0.22 ± 0.00	0.03 ± 0.00	20.77 ± 0.45	0.01 ± 0.00	0.04 ± 0.00	0.68 ± 0.01	44.27 ± 1.18	22.02 ± 0.20	0.17 ± 0.00	3.52 ± 0.05	6.17 ± 0.13	1.39 ± 0.01	0.03 ± 0.00	0.07 ± 0.00
T-FI-250	0.76 ± 0.01	0.23 ± 0.00	0.03 ± 0.00	25.09 ± 0.54	0.00 ± 0.00	0.03 ± 0.00	0.58 ± 0.01	40.06 ± 1.07	19.66 ± 0.18	0.15 ± 0.00	4.36 ± 0.06	7.60 ± 0.16	1.30 ± 0.01	0.05 ± 0.00	0.09 ± 0.00
H-FI-200	1.03 ± 0.01	0.28 ± 0.00	0.03 ± 0.00	21.27 ± 0.46	0.03 ± 0.00	0.09 ± 0.00	1.05 ± 0.02	38.18 ± 1.02	25.22 ± 0.23	0.17 ± 0.00	4.22 ± 0.06	6.58 ± 0.13	1.76 ± 0.02	0.04 ± 0.00	0.06 ± 0.00
H-FI-250	1.22 ± 0.01	0.38 ± 0.01	0.03 ± 0.00	38.64 ± 0.84	0.03 ± 0.00	0.09 ± 0.00	1.18 ± 0.02	24.34 ± 0.65	17.82 ± 0.16	0.21 ± 0.00	3.69 ± 0.05	10.16 ± 0.21	1.99 ± 0.02	0.07 ± 0.00	0.14 ± 0.00
Le	0.71 ± 0.01	0.16 ± 0.00	0.03 ± 0.00	40.03 ± 0.87	0.00 ± 0.00	0.05 ± 0.00	0.68 ± 0.01	15.76 ± 0.42	33.75 ± 0.31	0.16 ± 0.00	2.03 ± 0.03	5.26 ± 0.11	1.21 ± 0.01	0.05 ± 0.00	0.07 ± 0.00
T-Le-250	1.05 ± 0.01	0.17 ± 0.00	0.03 ± 0.00	39.43 ± 0.85	0.01 ± 0.00	0.03 ± 0.00	0.82 ± 0.01	14.62 ± 0.39	33.67 ± 0.31	0.16 ± 0.00	2.22 ± 0.03	5.58 ± 0.11	2.08 ± 0.02	0.05 ± 0.00	0.08 ± 0.00
H-Le-200	3.28 ± 0.04	0.20 ± 0.00	0.03 ± 0.00	47.13 ± 1.02	0.02 ± 0.00	0.08 ± 0.00	1.33 ± 0.02	10.64 ± 0.28	22.99 ± 0.21	0.18 ± 0.00	3.10 ± 0.04	6.80 ± 0.14	4.01 ± 0.04	0.06 ± 0.00	0.15 ± 0.00
H-Le-250	1.52 ± 0.02	0.26 ± 0.00	0.03 ± 0.00	62.14 ± 1.34	0.02 ± 0.00	0.09 ± 0.00	1.58 ± 0.02	4.11 ± 0.11	15.81 ± 0.14	0.25 ± 0.00	2.09 ± 0.03	9.45 ± 0.19	2.41 ± 0.02	0.07 ± 0.00	0.16 ± 0.00
Tw	0.96 ± 0.01	0.17 ± 0.00	0.02 ± 0.00	49.91 ± 1.08	0.01 ± 0.00	0.05 ± 0.00	0.97 ± 0.02	15.10 ± 0.40	25.55 ± 0.23	0.06 ± 0.00	2.06 ± 0.03	4.18 ± 0.09	0.79 ± 0.01	0.10 ± 0.00	0.06 ± 0.00
T-Tw-250	0.52 ± 0.01	0.12 ± 0.00	0.02 ± 0.00	54.30 ± 1.17	0.00 ± 0.00	0.02 ± 0.00	0.32 ± 0.01	11.59 ± 0.31	25.75 ± 0.23	0.05 ± 0.00	1.87 ± 0.02	4.41 ± 0.09	0.86 ± 0.01	0.11 ± 0.00	0.05 ± 0.00
H-Tw-200	0.60 ± 0.01	0.14 ± 0.00	0.02 ± 0.00	80.04 ± 1.73	0.03 ± 0.00	0.07 ± 0.00	0.36 ± 0.01	2.28 ± 0.06	8.67 ± 0.08	0.05 ± 0.00	2.42 ± 0.03	3.45 ± 0.07	1.66 ± 0.03	0.13 ± 0.00	0.08 ± 0.00
H-Tw-250	1.18 ± 0.01	0.28 ± 0.00	0.02 ± 0.00	68.82 ± 1.49	0.08 ± 0.00	0.07 ± 0.00	0.79 ± 0.01	4.05 ± 0.11	12.48 ± 0.11	0.06 ± 0.00	2.37 ± 0.03	8.22 ± 0.17	1.27 ± 0.01	0.12 ± 0.00	0.20 ± 0.00
Wo	0.63 ± 0.01	0.26 ± 0.00	0.06 ± 0.00	47.65 ± 1.03	0.02 ± 0.00	0.26 ± 0.01	0.45 ± 0.01	18.80 ± 0.50	21.78 ± 0.20	0.10 ± 0.00	6.81 ± 0.09	2.28 ± 0.05	0.64 ± 0.01	0.14 ± 0.00	0.14 ± 0.00
T-Wo-250	0.34 ± 0.00	0.22 ± 0.00	0.05 ± 0.00	45.42 ± 0.98	0.01 ± 0.00	0.04 ± 0.00	0.19 ± 0.00	24.68 ± 0.66	20.05 ± 0.18	0.09 ± 0.00	5.89 ± 0.08	2.24 ± 0.05	0.57 ± 0.01	0.14 ± 0.00	0.08 ± 0.00
H-Wo-200	2.82 ± 0.03	0.34 ± 0.01	0.11 ± 0.00	57.06 ± 1.23	0.45 ± 0.02	0.31 ± 0.01	0.81 ± 0.01	4.88 ± 0.13	16.25 ± 0.15	0.08 ± 0.00	10.09 ± 0.13	2.92 ± 0.06	3.50 ± 0.03	0.18 ± 0.00	0.20 ± 0.00
H-Wo-250	3.66 ± 0.04	0.44 ± 0.01	0.05 ± 0.00	41.17 ± 0.89	0.39 ± 0.01	0.31 ± 0.01	2.15 ± 0.03	15.52 ± 0.41	20.21 ± 0.18	0.09 ± 0.00	9.64 ± 0.13	2.79 ± 0.06	3.34 ± 0.03	0.16 ± 0.00	0.09 ± 0.00
Ba	1.59 ± 0.02	0.25 ± 0.00	0.05 ± 0.00	72.89 ± 1.58	0.01 ± 0.00	0.06 ± 0.00	0.48 ± 0.01	5.24 ± 0.14	14.03 ± 0.13	0.09 ± 0.00	1.86 ± 0.02	1.96 ± 0.04	1.28 ± 0.01	0.13 ± 0.00	0.09 ± 0.00
T-Ba-250	0.88 ± 0.01	0.21 ± 0.00	0.05 ± 0.00	71.26 ± 1.54	0.00 ± 0.00	0.02 ± 0.00	0.39 ± 0.01	5.73 ± 0.15	16.52 ± 0.00	0.09 ± 0.02	1.57 ± 0.04	2.08 ± 0.00	1.00 ± 0.00	0.12 ± 0.00	0.07 ± 0.00
H-Ba-200	2.53 ± 0.03	0.18 ± 0.00	0.03 ± 0.00	84.11 ± 1.82	0.03 ± 0.00	0.06 ± 0.00	0.64 ± 0.01	0.06 ± 0.00	6.43 ± 0.06	0.06 ± 0.00	2.34 ± 0.03	1.27 ± 0.03	1.74 ± 0.02	0.13 ± 0.00	0.38 ± 0.00
H-Ba-250	1.17 ± 0.01	0.21 ± 0.00	0.02 ± 0.00	87.72 ± 1.90	0.03 ± 0.00	0.06 ± 0.00	0.50 ± 0.01	0.10 ± 0.00	4.58 ± 0.04	0.07 ± 0.00	1.75 ± 0.02	2.42 ± 0.05	0.88 ± 0.01	0.13 ± 0.00	0.34 ± 0.00

Calcium, potassium, and magnesium oxides were the main mineral components of all the biomass fractions as expectable for lignocellulosic biomass materials. Nevertheless, some differences were observed between samples, with distinct profiles being observed for bark and flowers relative to the other *Acacia* biomasses. Leaves, twigs and wood presented a similar composition with predominance of CaO with concentrations in the range of 40.03% to 49.91% followed by MgO at concentrations from 21.78% to 33.75 and finally K₂O at concentrations from 15.1% to 18.8%. Bark mineral composition followed the same relative order but with a higher prevalence of CaO (72.89%) relatively to the other main minerals MgO (14.03%) and K₂O (5.24%). In the case of the flower fraction, K₂O was the main mineral component with a concentration of 44.27%, while MgO and CaO had very similar concentrations, 22.02% and 20.77%, respectively. These three mineral components had different behaviors during carbonization. The K₂O and MgO contents decreased suggesting that the concentration effect related to devolatilization of organic components was compensated by dissolution of these minerals in the carbonization condensates or the hydrothermal aqueous phase. On the other hand, CaO concentrations generally increased, indicating that dissolution of this mineral in the process aqueous phases or condensates was residual, being concentrated in the biochars or hydrochars.

Other components with concentrations higher than 1% in the raw biomasses were Na₂O (from 1.86% for bark to 6.81% for wood), P₂O₅ (from 1.96 for bark to 6.17% for flowers) and SiO₂ (from 0.64% for wood to 1.39% for flowers). Both sodium and silicon oxides contribute to a decrease of the melting point of the ashes therefore low concentrations of those components reduce the probability of ash aggregation at high temperatures, a phenomenon that may occur in boilers or gasifiers. The relatively high concentrations of phosphorus oxide in the raw biomasses could suggest possible applications in soil amendment but this component was concentrated in the biochars and hydrochars indicating the phosphorus was present in a form not susceptible to be dissolved by water leaching therefore less available as a biofertilizer.

9.3.3 Evaluation of ash-related problems

The mineral composition data relative to the biomass fractions and the correspondent biochars and hydrochars was used to determine several indexes that relate ash composition with the probability of occurring ash melting and aggregation phenomena that hinder thermochemical conversion processes such as combustion or gasification (Teixeira et al., 2012).

These indexes take into account the concentrations of the mineral components Si, K, Na, S, Cl, P, Ca, Mg, and Fe because these are usually the main ash components and have different effects on the ash melting point (Benavente and Fullana, 2015; Garcia-Maraver et al., 2017). The alkali index (*AI*), the slagging evaluation index (*SEI*), the sintering index (*SI*), and the bed agglomeration index (*BAI*) were calculated and used to predict ash-related problems in boilers or gasifiers. Table 9.3 shows the results and the associated fouling, slagging, sintering, and bad agglomeration risks.

Table 9.3 — Ash fouling, slagging, sintering, and bed agglomeration indexes and respective risks for all raw and spent fractions of *A. dealbata*.

	Alkali index (<i>AI</i>)		Slagging evaluation index (<i>SEI</i>)		Sintering index (<i>SI</i>)		Bed agglomeration index (<i>BAI</i>)	
	<i>AI</i>	Fouling risk	<i>SEI</i>	Slagging risk	<i>SI</i>	Sintering risk	<i>BAI</i>	Bed agglomeration
FI	0.85	Occur	0.86	Medium	0.90	High	0.01	Occur
T-FI-250	0.89	Occur	0.64	High	1.01	High	0.01	Occur
H-FI-200	0.69	Occur	0.98	Medium	1.10	High	0.02	Occur
H-FI-250	0.48	Occur	0.41	High	2.01	Low	0.04	Occur
Le	0.41	Occur	0.45	High	4.14	Low	0.04	Occur
T-Le-250	0.40	Occur	0.79	Medium	4.34	Low	0.05	Occur
H-Le-200	0.29	Probable	0.51	High	5.10	Low	0.10	Occur
H-Le-250	0.15	Low	0.26	High	12.57	Low	0.26	No
Tw	0.34	Probable	0.86	Medium	4.40	Low	0.06	Occur
T-Tw-250	0.30	Probable	0.45	High	5.95	Low	0.02	Occur
H-Tw-200	0.09	Low	0.12	High	18.89	Low	0.08	Occur
H-Tw-250	0.09	Low	0.19	High	12.66	Low	0.12	Occur
Wo	0.08	Low	0.51	High	2.71	Low	0.02	Occur
T-Wo-250	0.10	Low	0.43	High	2.14	Low	0.01	Occur
H-Wo-200	0.04	Low	0.33	High	4.90	Low	0.05	Occur
H-Wo-250	0.05	Low	0.59	High	2.44	Low	0.09	Occur
Ba	0.18	Probable	0.21	High	12.25	Low	0.07	Occur
T-Ba-250	0.18	Probable	0.24	High	12.02	Low	0.05	Occur
H-Ba-200	0.05	Low	0.11	High	37.78	Low	0.27	No
H-Ba-250	0.05	Low	0.07	High	49.72	Low	0.27	No

The torrefaction process had a low impact on the ash fouling, slagging, sintering, and bed agglomeration indexes, therefore is not expected to produce significant changes in the associated risks.

Hydrothermal carbonization caused a decrease of the alkali index (lowering fouling risk), and an increase of the sintering index (lowering the sintering risk). These changes are mostly related with the solubilization of K_2O in the process water and the concentration of CaO in the hydrochars. The slagging risk was high for most hydrochars with SE /values typically lower than 0.7. The increase in the CaO and P_2O_5 concentrations in the hydrochars is responsible by the observed values. The sintering risk was low for most biomass fractions and the respective biochars and hydrochars except for the flowers. The high levels of K_2O and low levels of CaO of the flowers when compared with the remainder biomass fractions is responsible by this different probability of sintering occurrence, and changes during torrefaction or hydrothermal carbonization were not enough to change this tendency, except for the hydrochar produced at 250 °C, that reached a low sintering risk. The bed agglomeration index was lower than 0.15 for all biomass samples, suggesting that these should be mixed with other biomass sources to minimize this risk. Hydrothermal carbonization had the effect of increasing this index, due to the reduction of K_2O concentrations, but only for bark at 200 °C and leaves and bark at 250 °C reached BA /values corresponding to a low risk of bed agglomeration.

Applying these indexes to biomass often gives this negative feedback, such as observed for the alkali and slagging evaluation indexes for vine prunings, gorse, and kiwi prunings (Rico et al., 2020); for the alkali and bed agglomeration indexes for eucalyptus and pine (Pena-Vergara et al., 2022), and olive kernel, olive tree wood, citrus tree wood and vine shoots (Vamvuka and Zografos, 2004); and for the sintering and bed agglomeration indexes for several woody shrub species (Viana et al., 2012). However, the application of these fouling, slagging and clustering predictive indexes has been recently reviewed and discussed in the literature by comparing the indexes information with experimental observations of ash fusion temperatures, and contradictory findings are common (Garcia-Maraver et al., 2017; Lachman et al., 2021). Lachman et al. recently proposed a graphical approach consisting in a ternary diagram divided in three regions based on the initial deformation temperatures observed in a large variety of biomasses: region 1, referring to a high initial deformation temperature zone, thus meaning fuels less prone to cause ash related problems; region 2, which consist in a transition zone between the high and lower initial deformation temperature zones; and region 3, which refers to a low initial deformation zone, corresponding to fuels that must not be used without additives or co-firing with less problematic fuels (Lachman et al., 2021).

In the ternary diagram axis the authors consider CaO and MgO together, once they are often found in high melting temperature ashes and show positive correlation with the ash fusion temperatures; SiO_2 is grouped with Al_2O_3 and Fe_2O_3 , which are often found in low

quantities; and finally the alkali compounds K_2O and Na_2O are grouped with P_2O_5 once they were found to be the most abundant oxides in the low melting temperature ashes (Lachman et al., 2021). After the necessary calculations the ternary plot was built and is presented in Figure 9.5.

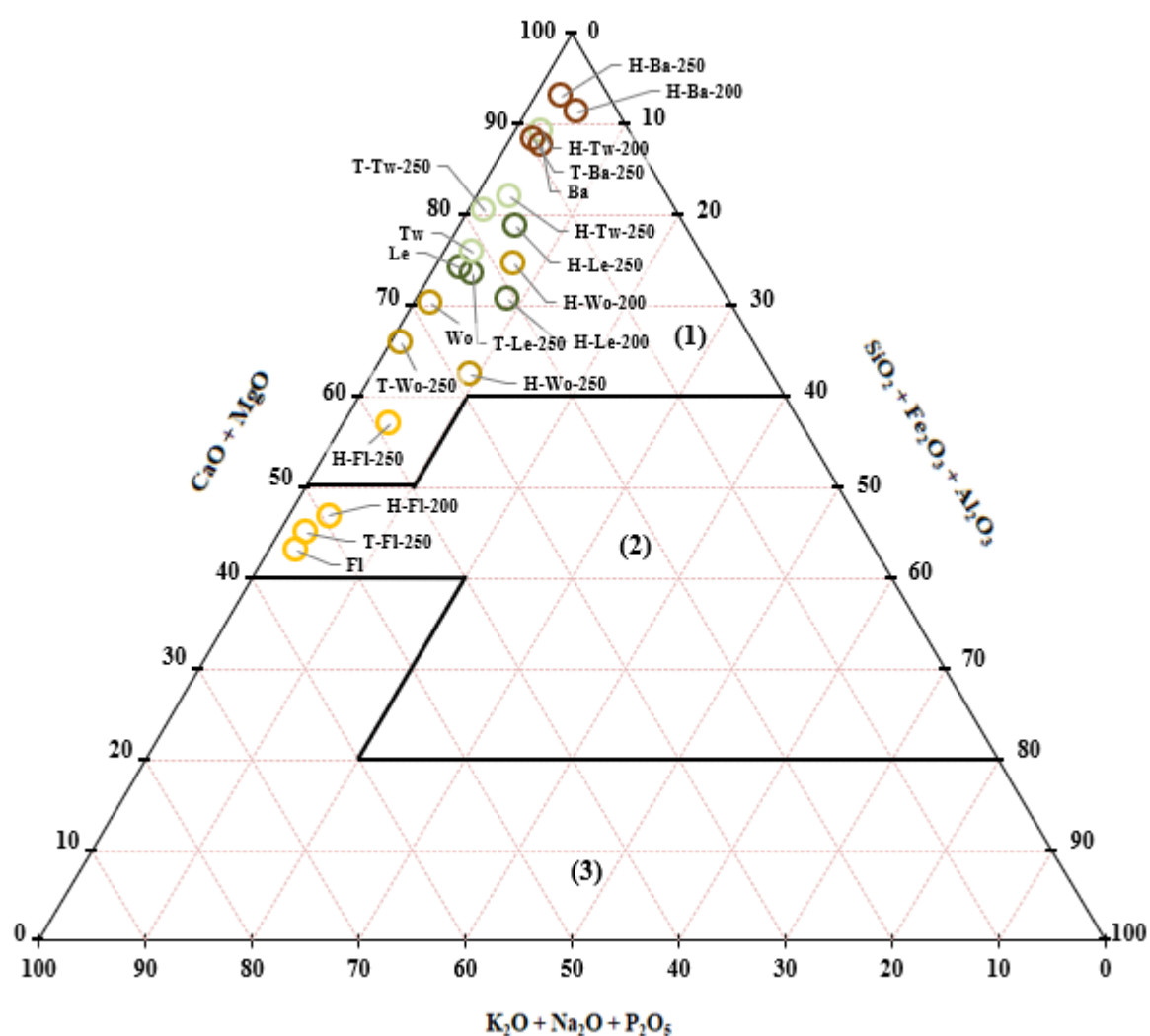


Figure 9.5 — Ternary diagram including all evaluated samples.

According to this criterion only the spent flowers, the corresponding biochars produced by torrefaction at 250 °C and the corresponding hydrochars produced at 200 °C present a mineral composition susceptible to cause problems related to the ash melting point, placing these samples in a transition zone between high and low deformation temperature. These more problematic products, but with relatively high HHV, can be used together with other fuels with different mineral composition. Except for the spent wood sample, the hydrochars

produced at 250 °C presented values of (CaO+MgO) higher than those of the corresponding to the raw biomasses, within the diagram region 1, indicating a positive effect of this process in the reduction of risks associated with ash melting and aggregation.

9.3.4 FTIR analysis

The surface properties of spent fractions and respective biochars were analyzed by FTIR. The spectra are shown in Figure 9.6. Comparing the spectra, the profile is very similar among samples but some peaks have different intensity.

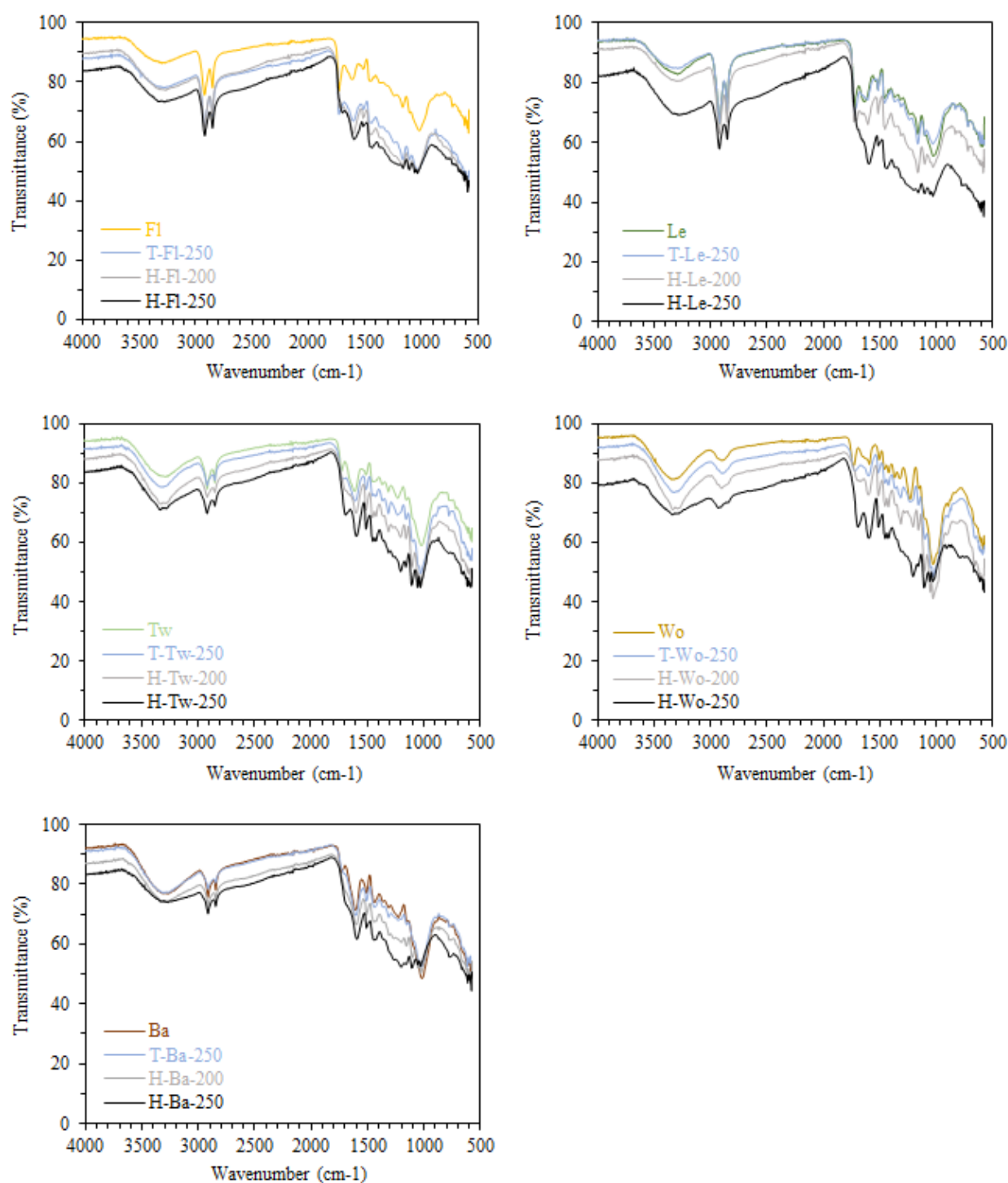


Figure 9.6 — FT-IR profiles of the spent biomass samples and of the biochars and hydrochars produced by torrefaction at 250 °C and hydrothermal carbonization at 200 °C and 250 °C.

Weak peaks between 719 and 895 cm^{-1} , assigned to aromatic C–H out-of-plane bend (Chupin et al., 2015; Coates, 2000) were evident for all samples, corresponding to the carbon backbone of the lignocellulosic polymers. A strong peak at 1014 – 1032 cm^{-1} , assigned to C–

O–C symmetric stretching in ethers and C–O stretching vibration in cellulose, hemicelluloses, and lignin (Saikia et al., 2015) was also observed in all samples, but significantly decreased in the hydrochars obtained at 250 °C, which is an indicator of the higher decomposition of these polymers with the hydrothermal treatment. A weak peak at 1151 – 1165 cm⁻¹, related to alkyl substituted ether (O–CH₃ or O–CH₂) stretching (Abdullah et al., 2013) and O–C–O asymmetric stretching (glycosidic link) in polysaccharides (da Costa et al., 2022) was observed in all samples but were more pronounced in flowers and leaves. The peaks at 1229 – 1232 cm⁻¹, associated with C–O–C stretching in phenol-ether bonds of lignin, C–O stretching in syringyl rings, C–O–C symmetric stretching, and OH plane deformation (Lehto et al., 2018), and C–O vibrations of acetyl groups in hemicelluloses (da Costa et al., 2022) is absent in both hydrochars from flowers and leaves, being evident mostly in the starting raw materials. In the hydrochars obtained from twigs, wood, and bark this peak apparently split in two small peaks at 1203 – 1207 cm⁻¹ and 1267 – 1279 cm⁻¹, which is an indicator of the thermal conversion of carbohydrates to hydroxymethylfurfural, a byproduct of cellulose thermal hydrolysis (Wilk et al., 2020). Weak peaks at 1311 – 1321 cm⁻¹, related with O–H bend in phenols and non-aromatic alcohols, C–N stretch in amines (Coates, 2000), and syringil monomer vibrations in lignin (da Costa et al., 2022), as well as at 1367 – 1369 cm⁻¹, associated with O–H bending in phenols and C–H bending in methyl groups (Chupin et al., 2015) in cellulose and hemicelluloses (Ahmed et al., 2021a) were more pronounced in the spent wood, twigs, and leaves samples, but almost disappeared in the hydrochars obtained at 250 °C, as a consequence of the thermal degradation. A weak peak at 1423 cm⁻¹, assigned to C–O stretching or O–H deformation in carboxylic acids (Ahmad et al., 2007) and symmetric deformation of CH₂ in cellulose (Saikia et al., 2015), was only observed in spent wood and respective chars, as well as in the biochars obtained from the spent twigs. Peaks at 1441 – 1456 cm⁻¹, associated with C–H deformation and aromatic ring vibration (Abdullah et al., 2013), and aromatic C–C stretching and C–H deformation (Ahmed et al., 2021a), as well as the peaks at 1504 – 1516 cm⁻¹ and at 1593 – 1612 cm⁻¹, assigned to C=C stretching in aromatic rings (Abdullah et al., 2013; Ahmad et al., 2007) in lignin (da Costa et al., 2022), were observed in all samples, but were more intense in the hydrochars obtained at 250 °C, indicating more exposed lignin as a consequence of the more intense degradation under this condition. In turn, the peak at 1635 cm⁻¹, attributed to C=O vibration in amides from proteins and the aromatic ring stretching (Nair et al., 2020), and COO vibrations in non-esterified carboxyl groups (da Costa et al., 2022), was only found in spent leaves. The peak at 1728 – 1734 cm⁻¹, assigned to C=O stretching in lactones (Ahmad et al., 2007), ketones, aldehydes, carboxylic acids (Islam et al., 2005) or esters (Coates, 2000), associated with hemicelluloses (da Costa

et al., 2022), was found in the starting material of all fractions. However, a shift of this peak to 1700 cm^{-1} was observed in the case of hydrochars obtained at $250\text{ }^{\circ}\text{C}$ from twigs, wood, and in a minor extent from the flowers, which is attributed to C=O stretching vibration and the formation, by crosslinking, of new quinones, carboxylates and aromatic compounds after this more severe thermal pretreatment (Wilk et al., 2020). The peaks between 2850 and 2918 cm^{-1} , assigned to aromatic C–H out-of-plane bend (Chupin 2015, Coates 2000) were observed in all samples, as well as the band at $3280 - 3332\text{ cm}^{-1}$, relative to O–H stretching in alcohols, phenols, or carboxylic acids (Chupin et al., 2015; Peydayesh and Rahbar-Kelishami, 2015), and N–H stretching in amines (Coates, 2000; Nair et al., 2020), besides some attenuation being observed in the case of the hydrochars from wood and bark obtained at $250\text{ }^{\circ}\text{C}$. Thus the changes observed in the FT-IR profiles confirm that the carbonization processes introduced substantive modifications in the surface groups of the raw materials, when these were converted to biochars and hydrochars, and the hydrothermal carbonization at $250\text{ }^{\circ}\text{C}$ was the process that resulted in a higher removal or rearrangement of oxygenated or nitrogenated functional groups. Furthermore the differences between the raw materials and the corresponding biochars or hydrochars were more evident for flowers and leaves than for the other biomass fractions, which is in agreement with the higher deoxygenation effect detected in the van Krevelen diagram for flowers and leaves hydrochars.

9.3.5 Thermogravimetric analysis

The thermogravimetric profile of the raw biomasses and of the corresponding biochars and hydrochars were determined as well as the respective derivatives to evaluate the thermal decomposition kinetics and combustion relevant parameters (Figures 9.7 and 9.8; Table 9.4).

As expected, the effect of the torrefaction and of the hydrothermal carbonization processes was to shift the mass loss profile to a higher temperature range, since the biochars and hydrochars have significantly less volatile matter than the original biomasses. This shift was particularly noticeable for hydrothermal carbonization at $250\text{ }^{\circ}\text{C}$ while torrefaction at $250\text{ }^{\circ}\text{C}$ and hydrothermal torrefaction at $200\text{ }^{\circ}\text{C}$ presented similar TGA profiles, especially for the twigs and the wood samples.

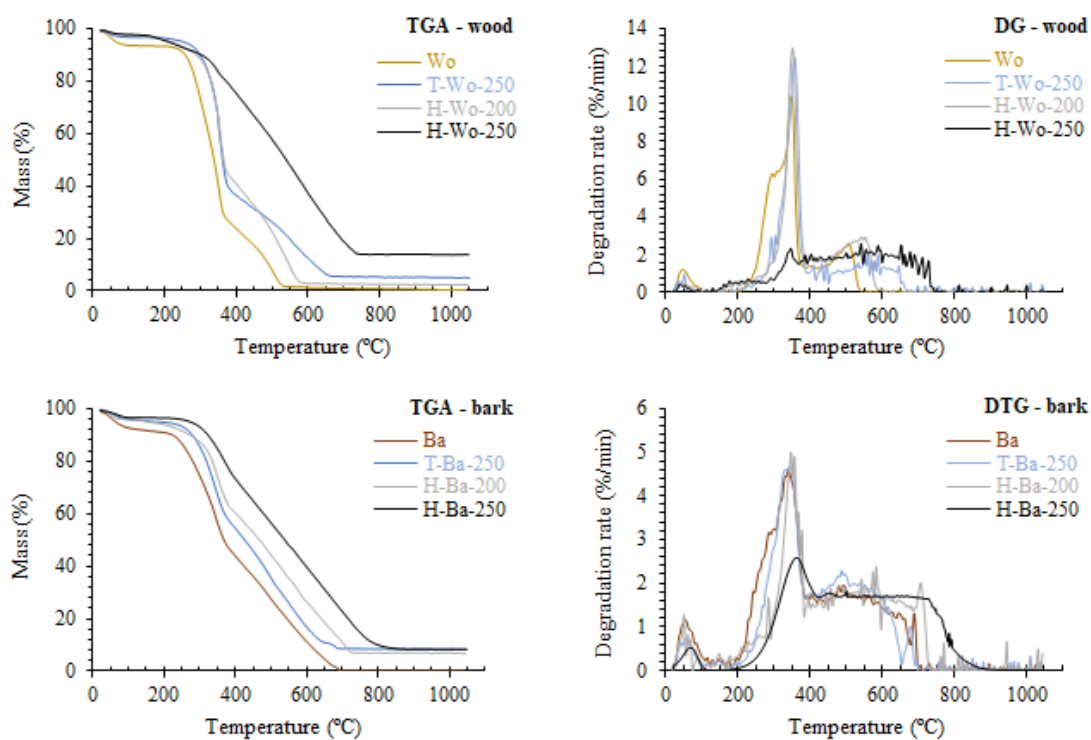


Figure 9.7 — TGA and DTG curves during the thermochemical decomposition of samples (wood and bark) under oxidative atmosphere.

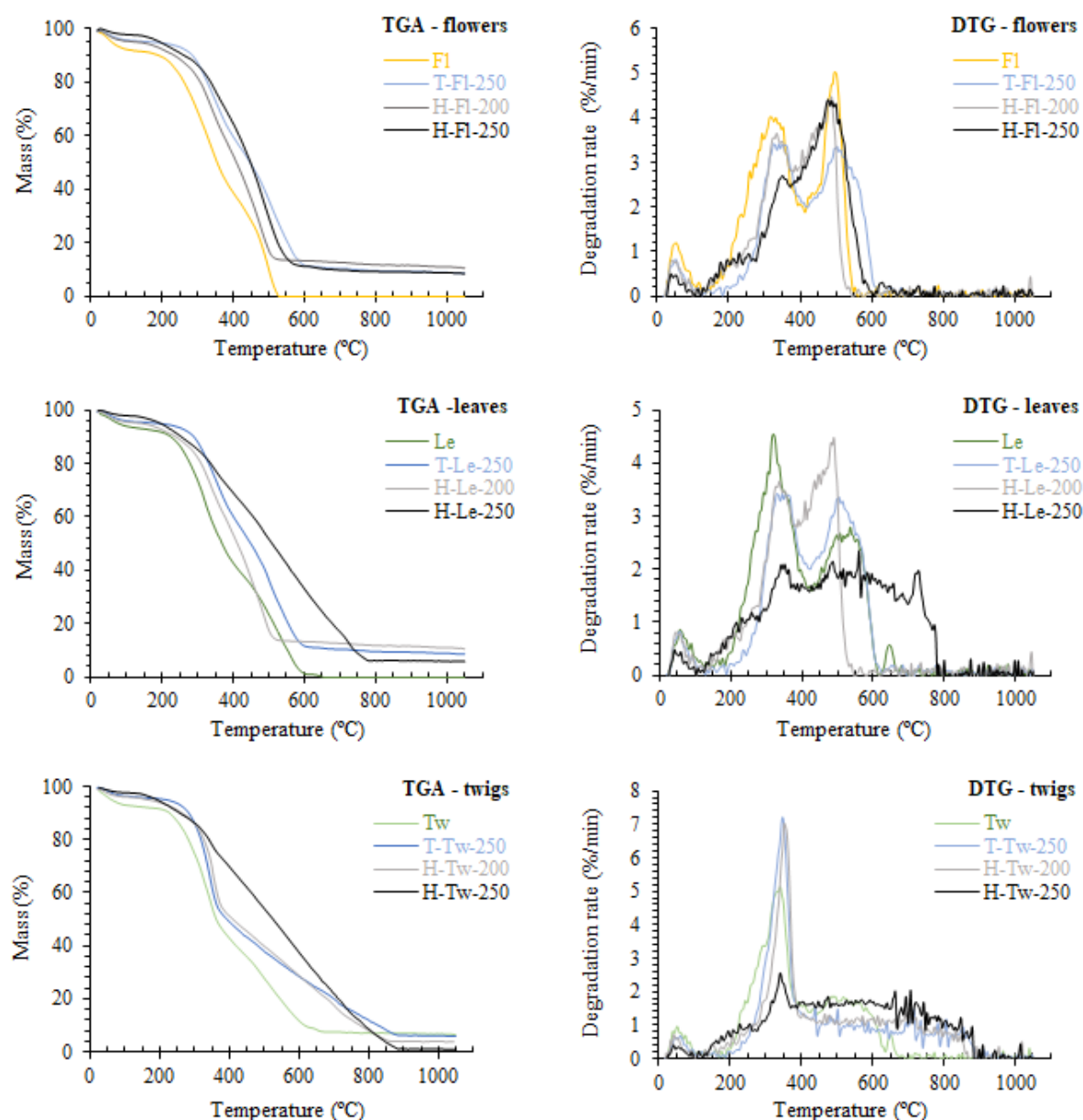


Figure 9.8 — TGA and DTG curves during the thermochemical decomposition of samples (flowers, leaves, and twigs) under oxidative atmosphere.

From the analysis of the TGA profiles of the biomasses and respective biochars and hydrochars were calculated combustion related parameters, namely the ignition temperature (T_i) and the burnout temperature (T_b). Also, for each combustion phase (devolatilization and char oxidation), the analysis of the DTG profiles allowed to determine thermal and kinetic parameters, including the temperature range of each stage, the maximum degradation rate (R_{peak}), and the temperature at maximum degradation rate (T_{peak}) (Lu and Chen, 2015; Rago et al., 2022).

Table 9.4 — Characteristic parameters of the thermochemical degradation of *A. dealbata* fractions and the corresponding biochars and hydrochars under oxidative conditions.

Sample	Devolatilization zone			Char combustion zone			T_i (°C)	T_b (°C)
	Temperature range	R_{peak} (%/min)	T_{peak} (°C)	Temperature range	R_{peak} (%/min)	T_{peak} (°C)		
Fl	147 - 394	4.0256	317	414 - 557	5.0319	497	240	524
T-Fl-250	220 - 390	3.4214	341	460 - 627	3.3619	503	280	809
H-Fl-200	225 - 370	3.6552	335	420 - 539	4.4818	487	270	920
H-Fl-250	280 - 398	2.7024	351	401 - 580	4.4090	478	260	747
Le	184 - 415	4.5434	319	443 - 614	2.7927	536	250	622
T-Le-250	239 - 399	4.4419	322	461 - 645	2.7647	532	270	679
H-Le-200	183 - 420	3.3952	336	---	1.4957*	---	260	771
H-Le-250	280 - 373	2.0869	342	452 - 502	2.1450	486	240	771
Tw	200 - 420	5.1343	341	450 - 636	1.8639	488	270	684
T-Tw-250	240 - 403	7.2129	341	---	0.5179*	---	290	870
H-Tw-200	275 - 400	7.0312	354	---	1.0411*	---	300	845
H-Tw-250	280 - 370	2.5560	340	---	1.4454*	---	250	871
Wo	205 - 387	10.407	349	440 - 548	2.6152	508	300	602
T-Wo-250	300 - 390	12.491	357	---	1.1934*	---	330	659
H-Wo-200	270 - 395	12.954	352	450 - 590	2.9450	550	320	581
H-Wo-250	302 - 370	2.3384	340	---	1.8388*	---	270	730
Ba	182 - 385	4.6244	340	443 - 505	1.9489	494	270	687
T-Ba-250	210 - 390	4.6733	345	430 - 518	2.2116	497	290	681
H-Ba-200	280 - 390	4.9994	348	---	1.6547*	---	300	721
H-Ba-250	220 - 420	2.5783	364	---	1.5100*	---	290	820

Values with * are averages of the decomposition rate in the temperature range between the end of the devolatilization phase and the burnout temperature.

Torrefaction at 250 °C and hydrothermal carbonization at 200 °C had the general effects of increasing T_i , T_b , as well as the temperature range and T_{peak} in the devolatilization zone. This is expectable taking into account the loss of volatile matter that occurs during the torrefaction and hydrothermal carbonization processes. Nonetheless, the hydrochars obtained at 250 °C, showed reductions of T_i relative to the hydrochars obtained at 200 °C, the biochars obtained by torrefaction and in some cases the raw biomasses. On the other hand, these hydrochars obtained at 250 °C, had a lower devolatilization rate than the raw biomasses and the other biochars and hydrochars, as a consequence of their lower contents of volatile matter. This behavior maybe the consequence of the catalytic effect of the mineral components concentrated in the hydrochar namely CaO and P₂O₅ that decreased the activation energy needed for the

decomposition of the carbon components of these hydrochars this initiating devolatilization at a lower temperature (Hu et al., 2023; Shi et al., 2019).

Using a model based on the Arrhenius equation, and for which the biomass, biochar or hydrochar samples were considered as a sum of the six pseudocomponents, was proposed an equation to describe the TGA profiles of the *Acacia* biomass fractions. This equation was fitted using the nonlinear least-squares method and the first-order kinetic activation energies (E_a) were calculated for the thermal decomposition of each of these samples (Table 9.5). The fitting between the experimental TGA profiles and the results of the model are presented in Figure 9.9.

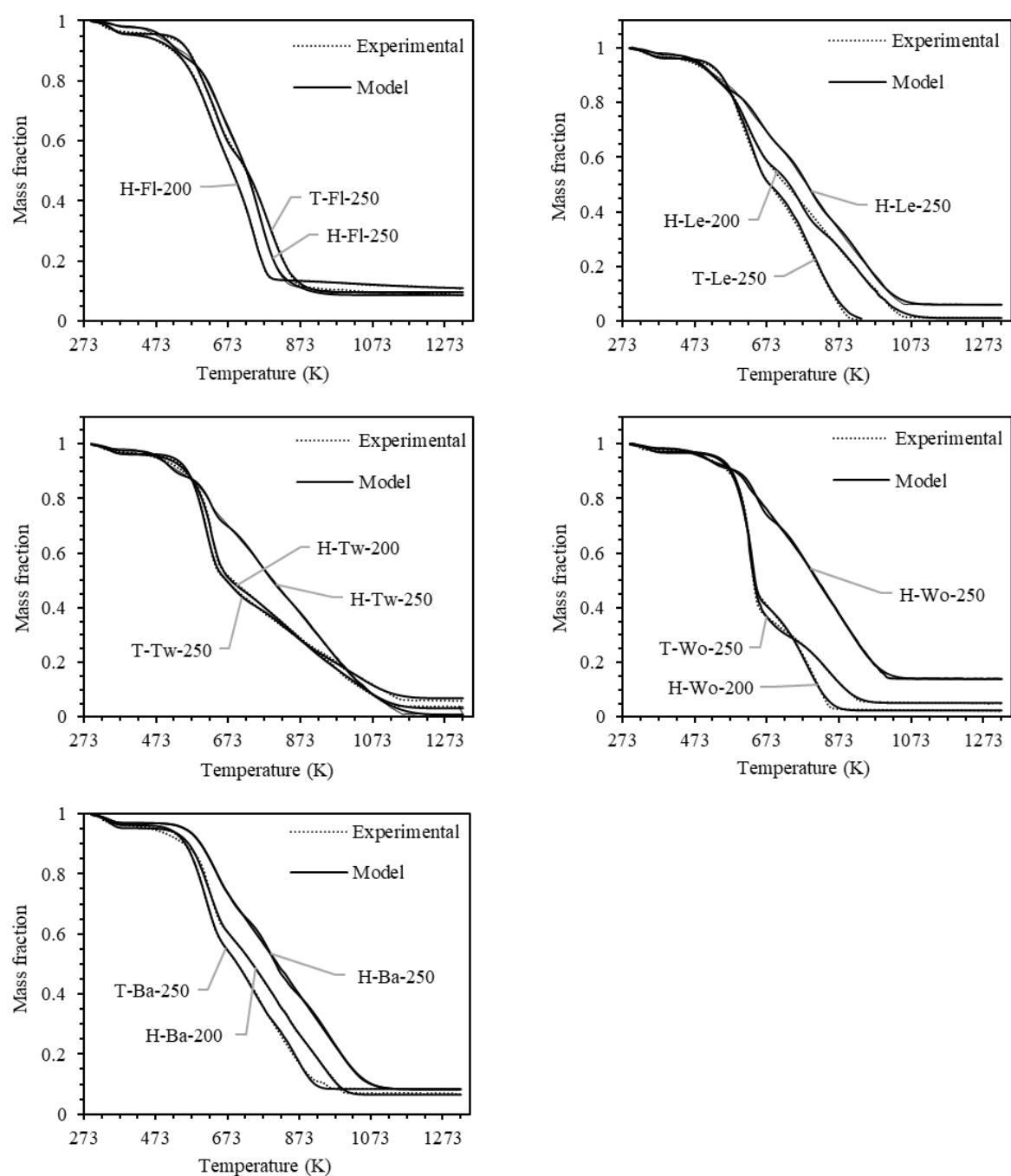


Figure 9.9 — Oxidation kinetic modeling of the different fractions of raw and spent *A. dealbata* fractions by pseudo-component model fitting, showing the experimental and modeling results.

The proposed model was able to accurately describe the thermogravimetric profiles of the spent biomass samples and of the corresponding biochars and hydrochars allowing to obtain fitting quality parameters that varied from 99.1 to 99.8 (Table 9.5).

Table 9.5 — First-order kinetic activation energies (E_a) and fitting qualities (FQ) obtained by first-order pseudo-component fitting for the thermal degradation of *A. dealbata* raw and spent fractions

Sample	E_a (kJ/mol)	FQ (%)	Sample	E_a (kJ/mol)	FQ (%)	Sample	E_a (kJ/mol)	FQ (%)	Sample	E_a (kJ/mol)	FQ (%)
Fl	74.2	99.5	T-Fl-250	80.6	99.4	H-Fl-200	77.5	99.6	H-Fl-250	76.1	99.3
Le	75.6	99.8	T-Le-250	79.0	99.4	H-Le-200	76.2	99.1	H-Le-250	75.4	99.3
Tw	75.3	99.5	T-Tw-250	80.7	99.2	H-Tw-200	80.9	99.2	H-Tw-250	75.7	99.2
Wo	99.4	99.5	T-Wo-250	105.0	99.4	H-Wo-200	102.3	99.1	H-Wo-250	73.5	99.3
Ba	68.5	99.7	T-Ba-250	81.4	99.3	H-Ba-200	82.4	99.2	H-Ba-250	82.0	99.4

The activation energy of *Acacia* wood was higher than all the other fractions suggesting that their co-combustion or co-gasification may have some advantages.

Traditional torrefaction at 250 °C and hydrothermal carbonization at 200 °C, increased E_a for all samples but especially at torrefaction conditions. This increase of E_a may be explained by the higher thermal stability of the carbon structure of biochars and hydrochars relative to the original lignocellulosic materials. As mentioned above, the lower E_a values of hydrochars obtained at 200 °C relatively to torrefaction biochars may be explained by the catalytic effect of CaO whose concentration increased after hydrothermal carbonization. This effect was even higher for the hydrochars obtained by HTC at 250 °C, leading to E_a values comparable to those of the spent raw biomasses and even lower for the leaves and wood fractions. Zang and co-workers reported a similar positive effect of CaO during gasification of biomass, namely by enhancing tar conversion, even at the lower gasification temperatures, and by reducing the activation energy for the generation of gas products, in particular, CH₄, H₂, CO₂, CO, C₂H₆, and C₃H₆ (Hu et al., 2023).

The activation energy showed to be correlated with the ignition temperature. The correlation analysis between ignition temperature values and the estimated activation energies revealed a strong and significative correlation (at $p < 0.01$ (two-tailed)), with Pearson correlation coefficient of 0.819. Finding a good correlation between Ignition temperature and activation energy could be interesting to predict activation energy for design purposes.

9.3.6 Characterization of process water from hydrothermal carbonization

Given the solid-to-water ratio of 1:5 (m/m) used in the hydrothermal carbonization experiments, it is useful to characterize this aqueous effluent in order to propose possible pathways for its treatment or valorization. The parameters evaluated were pH (because of its influence in most chemical or biological remediation processes), COD (as a generic evaluation of the

species requiring oxidation to be removed), total phenolics (because these specific organic components may have a negative effect in bioremediation processes due to antimicrobial activity) and total, volatile and fixed solids in order to evaluate the relative amounts of organic and inorganic contaminants (Table 9.6).

Table 9.6 — Characteristics of the aqueous phase collected after the hydrothermal carbonization of *A. dealbata* raw and spent fractions, at 200 °C and 250 °C.

Sample	pH	COD (g O ₂ /L)	Total phenolics (g GAE/L)	Total solids (mg/L)	Volatile solids (mg/L)	Fixed solids (mg/L)	Aqueous Phase Yield (%)
H-FI-200	nd	nd	nd	nd	nd	nd	83.6
H-FI-250	5.5	23.9	2.25	18,315	12,180	6,135	87.4
H-Le-200	4.6	42.1	3.65	40,105	35,040	5,065	82.6
H-Le-250	5.4	40.3	4.72	38,495	33,420	5,075	86.5
H-Tw-200	3.7	37.7	3.92	24,150	21,340	2,810	84.7
H-Tw-250	3.9	40.4	5.55	27,080	23,675	3,405	87.6
H-Wo-200	3.1	38.7	3.63	17,543	16,755	787.5	84.6
H-Wo-250	3.2	38.5	5.34	19,993	18,787	1,205	89.0
H-Ba-200	4.1	33.6	3.81	24,208	21,137	3,070	84.4
H-Ba-250	4.4	36.5	5.98	28,850	25,650	3,200	86.1

nd: not determined (not enough quantity of process water was collected to perform analyses)

The aqueous effluents collected after hydrothermal carbonization presented acid pH values (3.1 to 5.5) as a result of the dissolution of organic acids or other proton-donor species produced during the decomposition of the lignocellulosic polymers (Wilk et al., 2020). With the exception of the leaves hydrochars, the values of total, volatile and fixed solids, of COD and of total phenolics were higher for hydrothermal carbonization at 250 °C than at 200 °C, indicating that at a higher temperature, occurred a higher dissolution of both organic and inorganic components, in the aqueous phase (Wilk et al., 2020); (Kambo and Dutta, 2015b). The dissolution of mineral components in the aqueous phase was not proportional to the relative ash contents of the different biomass fractions but more related to the mineral composition of the different samples especially their content of K₂O and MgO and in a lesser extent Na₂O, Al₂O₃ and B₂O₃, since those were the minerals whose concentration decreased in the hydrochars relatively to the raw materials, for most of the biomass fractions. Given these characteristics, especially the moderate concentrations of total, volatile and fixed solids, these aqueous effluents could be

discharged in wastewater treatment units, but dilution or pre-treatment to decrease the total phenolics content might be advisable. Residual heat of the hydrochars could be used to partially distill this effluent, recovering water to reuse in the hydrothermal process.

9.3.7 Adsorption of cationic and anionic dye

Given the changes in the carbonaceous structure of the biochars and hydrochars relatively to the original biomasses some preliminary evaluation of their adsorption capacity towards cationic or anionic species was performed by determining the adsorption capacity relatively to methylene blue and methyl orange, two synthetic dyes, with contact times from 1h to 24h. Both the biochars produced at 250 °C and the hydrochars produced at 200 °C and 250 °C were subject to steam activation in order to remove condensates trapped in their pores and surfaces, thus increasing their porosity. Lignocellulosic materials are generally good adsorbents for cationic species because their surfaces are rich in negative dipoles associated to hydroxyl and carboxyl groups, able to have strong electrostatic interactions with positively charged species. As torrefaction or carbonization severity increases these groups are progressively eliminated by decarboxylation, decarbonylation and dehydration reactions, thus reducing the affinity of cationic species. But at the same time, as the process temperature increases, the progressive aromatization and reorganization of the carbonaceous structure also increases porosity, enhancing the adsorption of both cationic and anionic species, mainly by pore adsorption. This process of pore adsorption has some kinetic limitations to mass transfer, therefore usually requires higher contact times than surface adsorption due to electrostatic interactions (Correia et al., 2017; Wilk et al., 2015).

The adsorption capacities and dye removal efficiencies for torrefaction biochars, HTC hydrochars and the corresponding steam activated chars are compared in Figures 9.10 to 9.14 for the different biomass fractions.

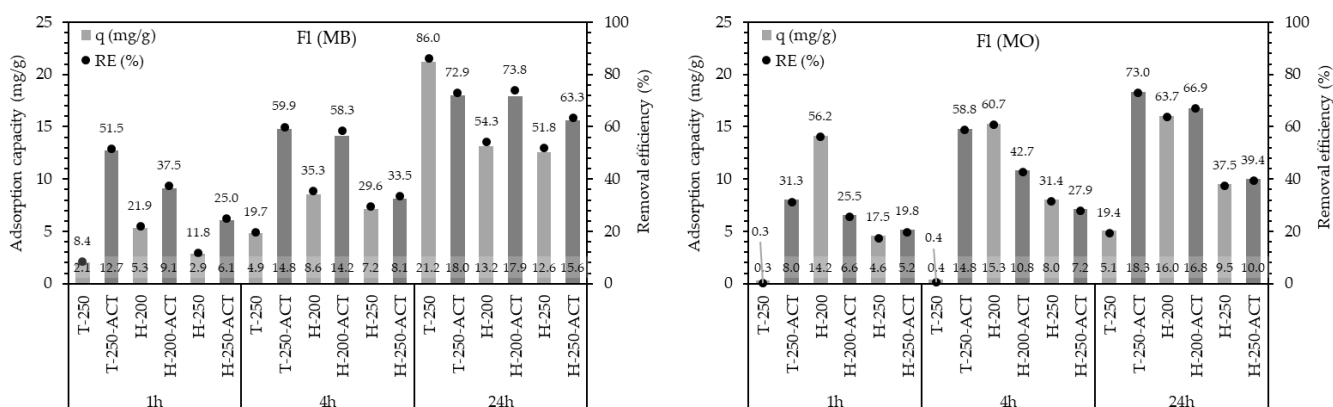


Figure 9.10 — Adsorption capacity (q) and removal efficiency (RutE) of methylene blue (MB) (left) and methyl orange (MO) (right) by activated (ACT) and non-activated biochars of flowers (Fl).

The higher values of adsorption capacity and removal efficiency of the flowers-derived chars were obtained for the torrefaction biochars and the hydrochars produced at 200 °C as well as for the corresponding activated chars, after 24h contact. MO removal efficiency reached values of 66.9 and 73% for the activated biochar and hydrochar produced at 200 °C, indicating some degree of pore adsorption. Removal rates for MB were higher than those of MO for T-250, H200-ACT and H250-ACT.

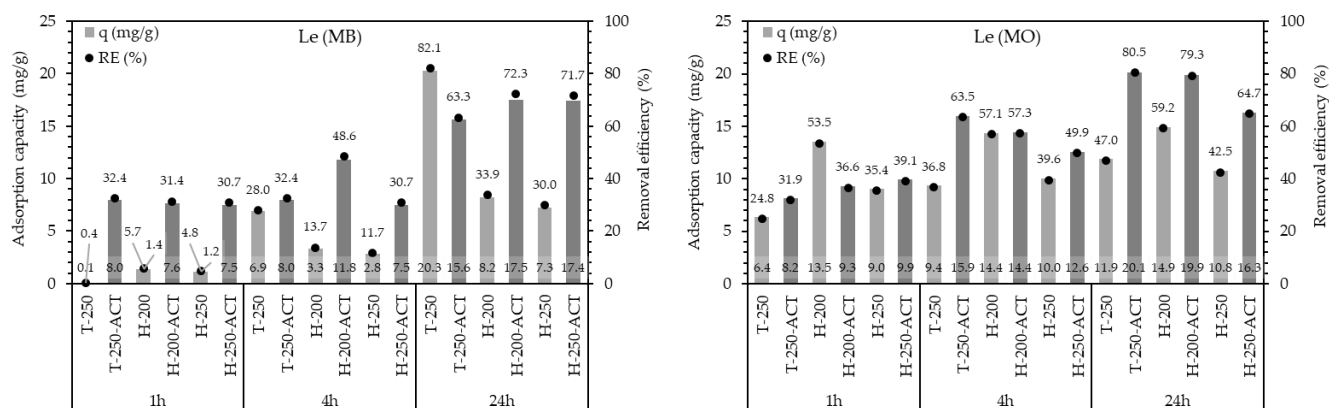


Figure 9.11 — Adsorption capacity (q) and removal efficiency (RutE) of methylene blue (MB) (left) and methyl orange (MO) (right) by activated (ACT) and non-activated biochars of leaves (Le).

Leaves biochar produced at 250 °C and all the activated chars showed removal rates in the range of 63.3 to 82.1, for both dyes, when the contact time was 24 h. In the case of MO, relatively high removal rates (> 50%) were also obtained for flowers and leaves activated

biochars after a contact time of 4 h, suggesting the development of a porous structure at these conversion conditions.

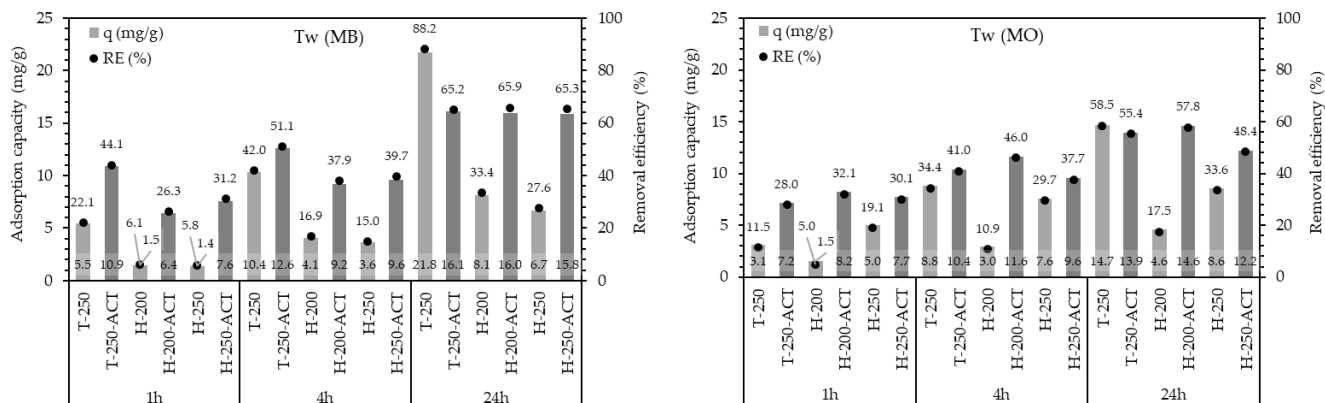


Figure 9.12 — Adsorption capacity (q) and removal efficiency (RutE) of methylene blue (MB) (left) and methyl orange (MO) (right) by activated (ACT) and non-activated biochars of twigs (Tw).

Twigs biochars showed a relatively good adsorption capacity for MB, especially for the activated chars but a lower removal efficiency for MO than the ones shown by the biochars and hydrochars obtained from flowers and leaves.

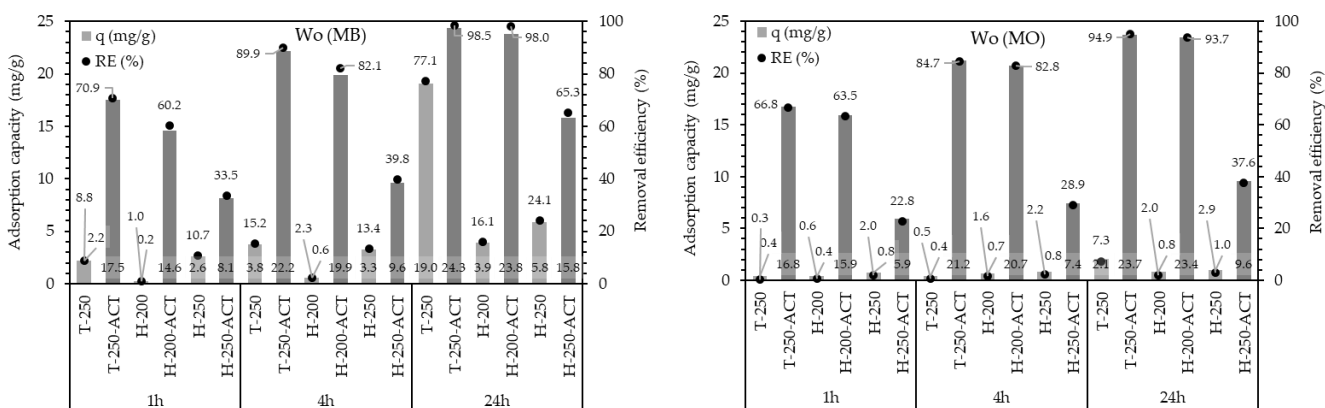


Figure 9.13 — Adsorption capacity (q) and removal efficiency (RutE) of methylene blue (MB) (left) and methyl orange (MO) (right) by activated (ACT) and non-activated biochars of wood (Wo).

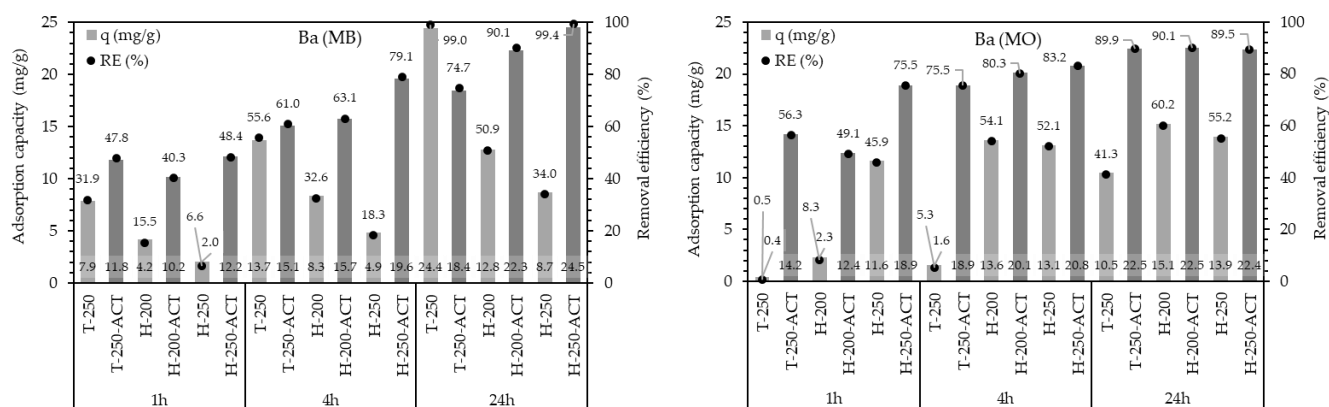


Figure 9.14 — Adsorption capacity (q) and removal efficiency (RutE) of methylene blue (MB) (left) and methyl orange (MO) (right) by activated (ACT) and non-activated biochars of bark (Ba).

Removal efficiencies higher than 90% were obtained for the activated biochars and hydrochars produced at 200 °C from wood and from bark, towards MO, after 24 h; even after a contact time of only 4 h, removal efficiencies of those activated chars reached values higher than 80%. In the case of MB, removal efficiencies > 90% were obtained for the T250-ACT and the H200-ACT produced from wood, and for T250, H-200-ACT and H250-ACT produced from bark.

These results indicate that the adsorption mechanisms must be complex, involving both electrostatic interactions and pore adsorption, as well as limited by mass transfer conditions, since the best adsorption results were not obtained for the hydrochars produced at 250 °C from wood or bark that presented the lowest H/C ratios (Figure 9.4), therefore should have the higher degree of aromaticity.

9.4 Conclusions

The different *Acacia* biomasses that are collected during operations of cutting for wood industry or removal to control invasive spreading have a potential to be valorized by themselves or after conversion by torrefaction or hydrothermal carbonization.

The raw biomasses (flowers, leaves, twigs, bark and wood) are already used as a source of terpenes or tannins for various applications but the spent biomass materials, still present sufficiently high values of bulk density and HHV that allow their valorization as biofuels. In particular the leave fraction that represents a significant amount of biomass in mature trees had the highest values of bulk density and HHV of 366.01 kg/m³ and 23.47 MJ/kg, respectively.

Torrefaction at 250 °C and hydrothermal carbonization at 200 °C and 250 °C yielded biochars and hydrochars with higher BD, HHV carbon content, and higher concentrations of CaO and P₂O₅, than the respective raw materials. On the other hand, the carbonization processes decreased H/C and O/C ratios, as well as K₂O and MgO concentrations yielding materials with composition more close to that of fossil fuels like lignite. Hydrochars obtained at 200 °C and 250 °C had higher CaO concentrations and lower K₂O concentration than the raw materials or the biochars obtained by torrefaction. This modification of the mineral composition had a positive effect in the combustion properties of these hydrochars, by reducing risks associated with ash melting. The presence of higher concentration of CaO was also related to a decrease of the activation energy for the devolatilization process.

Process water of the hydrothermal carbonization is an acidic effluent with relatively high concentrations of phenolic components although with moderate contents of total, volatile and fixed solids. Removal of the organic contaminants may allow the use of this effluent in irrigation due to the presence of various mineral components such as potassium and magnesium, besides minor amounts of zinc, copper, iron or manganese that have relevance as micro-nutrients for vegetal development. The biochars and hydrochars obtained from wood or bark showed the higher adsorption capacity towards the cationic and anionic dyes, MB and MO respectively, therefore are the fractions that could be used in more extensive studies of this possible valorization pathway.

EFFECT OF DIFFERENT FRACTIONS OF *ACA-* *CIA DEALBATA* BIOMASS ON FRESH GYPSUM MORTARS FOR ECO PLASTERS

Abstract: The use of renewable materials in the building construction is important to improve the sustainability of this sector. A possible approach is to incorporate biomass in the composition of structural elements or material formulations. In this work, different fractions of *Acacia* biomass (flowers, leaves, twigs, wood, and bark) were evaluated as additives for the preparation of mortars, at incorporation levels of 5% and 10% (vol.) in a premixed gypsum plaster product. The milled biomass fractions had been previously extracted with non-polar and polar solvents to recover terpenic and phenolic extractives and were dried at room conditions before characterization. The proximate and ultimate compositions of the biomass samples were determined. The biomass samples were characterized for physical properties, namely, loose bulk density (211.14 to 434.82 g/dm³), particle size distribution and average particle size diameters. The larger fractions of all biomasses corresponded to particles with diameters in the range of 100 to 1000 µm. Circularity index varied from 0.59 to 0.82 suggesting some variability of particle shapes, what may affect their mechanical behaviour during the mortar mixing and settling. Water holding capacity varied from 330.47 to 590.17 wt. %, and water vapour holding capacities from 20.42 to 23.08 wt. %, values that show a high capacity to interact with the added water during mortar preparation. Fresh mortars were characterized by the flow table consistency, drying shrinkage and fresh bulk density. Wood incorporation at 10% level resulted in a mortar with settling times very similar to the reference mortars but all other biomasses caused significant increases of both initial and final settling times. Flowers, leaves and twigs

had high increases of settling times and also a modified drying shrinkage behaviour that resulted in the formation of cracks during mortar drying. Flow table consistency of the mortars prepared with 5% and 10% biomass incorporation were below 150 mm diameter according to European standard (EN 13279-2:2014), but a second batch of mortars prepared with 10% biomass incorporation did not fulfil that standard. The results obtained suggest that *Acacia* wood and bark wastes have the characteristics more adequate for incorporation in mortars. To optimize the mortar final quality the biomass hygroscopicity should be taken into account when defining added water relative amount.

10.1 Introduction

In recent years, there has been growing pressure to promote sustainability policies in the buildings and construction sector, since they are responsible for a large consumption of energy (36% of global final energy use), as well as nearly 40% of energy-related carbon dioxide (CO₂) emissions (IEA and UNEP, 2018). Alongside the promotion of energy efficient construction systems, also the promotion of using renewable materials in the building construction is important to improve the sustainability of the sector, as required by the Energy Performance of Buildings Directive (Official Journal of the European Union, 2010).

Hence, due to the rising demand of low-embodied-energy and eco-friendly building materials, the inclusion of biomass wastes in the building sector has been widely promoted. A recent article (Cintura et al., 2021) shows that during the last decade several studies have been conducted on the incorporation of agro-wastes in the production of buildings materials, namely boards, concretes, bricks, and mortars (Akinyemi and Dai, 2020; Huang et al., 2019). In particular, mortars with incorporation of biomass wastes present an improvement of the moisture buffering capacity (Giosuè et al., 2017), a reduction of thermal conductivity (Maskell et al., 2017; Mazhoud et al., 2016; Rahim et al., 2015) and a mitigation of drying shrinkage (Abdul Shukor Lim et al., 2018; Lima and Faria, 2016).

The hygroscopicity of plasters and other indoor coatings can provide them the ability to act as passive moisture buffers. The sorption and desorption capacity of different plasters and plasterboard that can be applied to coat indoor rooms, has been compared by Lima et al. and Santos et al. (Lima et al., 2021; Santos et al., 2020). That comparison has shown a high hygroscopic capacity of clay plasters, but a low one of gypsum plasters.

Acacia dealbata is an invasive species native to Australia but widely spread in many regions of the world (Lorenzo et al., 2010a). The occupied area by *Acacia* spp. in the Portuguese

territory increased more than 400% since the 70s of the 20th century, currently occupying about 8400 ha (Nunes et al., 2021). *A. dealbata* is harming biodiversity, ecosystem properties and functions, and the productivity of the forest sector, in addition to threatening protected areas (Ferreira et al., 2011; Lorenzo et al., 2011, 2010a). This has motivated expensive removal operations by local authorities to control its proliferation, generating high amounts of biomass that is not currently valorized in a sustainable way. The remnants from removal operations are usually abandoned in the fields, increasing the risk of rural fires, landfilled, or directly burned to produce energy.

However, the potential of biomass from *Acacia* spp., including *A. dealbata*, to be used in the production of value-added products by extraction processes is known, as described by Correia et al. (Correia et al., 2020). A possible application for the spent biomass may be its incorporation in the construction sector, namely in the production of gypsum-based plastering mortars with improved hygroscopic capacity, thus promoting the efficient use of an endogenous resource, and contributing to the sustainability of the control of an invasive species and of the construction sector itself.

The aim of this work is to assess the effect that different fractions of *A. dealbata* (flowers, leaves, wood, twigs and barks) and two percentages of addition (5% and 10% in volume) can have on the fresh state performance of gypsum-based plastering mortars. The aim was to access the viability of this type of additions on the workability and vulnerability to cracking of these plasters, so that, later on, their effect could be accessed namely on hygroscopicity. Those further studies will be justified if the *A. dealbata* fractions allow to produce workable mortars with reduced drying shrinkage. The addition of the biomass fractions allows a reduced consumption of raw materials, replacing them by a renewable resource by the volume occupied by these wastes from an invasive species and, therefore, contribute to circular economy. different fractions of *Acacia* biomass (flowers, leaves, twigs, bark and wood) were converted to biochars at 250 °C, a temperature that is high enough to cause devolatilization of the hemicellulose and partial degradation and reorganization of the lignin and cellulose fractions. The same biomass fractions were also converted to hydrochars at 200 °C and 250 °C, using hydrothermal carbonization at a solid-to-water ratio of 1:5 (m/m).

10.2 Materials and methods

10.2.1 Materials

10.2.1.1 Premixed gypsum-based plaster

The premixed gypsum-based plaster used corresponded to the formulation defined by SIVAL for plaster tests.

10.2.1.2 Preparation of *Acacia dealbata* fractions

A. dealbata fractions used in this work were previously extracted with acetone to recover bio-active extractives. Each fraction (flowers (Fl), leaves (Le), twigs (Tw), wood (Wo), and bark (Ba)) was milled, sieved to 2 mm before extraction, macerated with acetone at 25 °C, and then air-dried at 25 °C for 48 h to eliminate solvent residues. Figure 10.1 shows the visual aspect of the various fractions used in this work.



Figure 10.1 — Visual aspect of the fractions of *A. dealbata*. From left to right: flowers, leaves, twigs, wood, and bark.

10.2.2 Methods

10.2.2.1 Biomass Characterization

Proximate and ultimate analysis were determined as described in section 8.2.3.

To carry out the particle size distribution, a sample of known mass (200 g) was placed on a vibrating sieve (Retsch) with an analytical sieve system (Retsch) with dimensions of 2000, 1000, 500, 200, 100 and 50 μm , making sieving for 10 min. After this period, the sample mass collected in each sieve and the fraction less than 50 μm were determined. A more detailed description of the size distribution and morphology, especially for the smaller particles (up to 1.8 μm) was obtained using a Morphologi G3 equipment from Malvern. Particle size distribution, as well as average particle size diameters of biomass samples, were determined using a Morphologi G3 equipment from Malvern. A typical analysis was performed by dispersing the

sample using the following conditions: sample volume 13 mm³, sample dispersion unit (SDU) settings (injection pressure: 4 bar, injection time: 40 ms, setting time: 120 s) selecting the optics 20x (detected range 1.8-100 µm) and 2.5x (detected range 13-1000 µm). The analyses were performed in duplicated from at least two different dispersions with 30000 particles counted. The instrument gave a detailed analysis by automatically capturing images of the sample scanned with microscopic optics. The size and shape of morphological descriptors were calculated using the Malvern Morphologi G3 software. Particle shapes were quantified in terms of the following parameters:

- Circularity (C), which measures how well the particle approximates a perfect circle, is given by equation (10.1):

$$C = \frac{4\pi A}{P^2} \quad (10.1)$$

where A is the particle area and P is the particle perimeter. The values can range between 0 (irregular object) and 1 (perfect circle).

- Convexity (C_x), which measures the edge roughness of a particle, is given by equation (10.2):

$$C_x = \frac{P_c}{P} \quad (10.2)$$

where P_c is the convex hull perimeter and P is the actual perimeter. Convexity ranges between 0 (rough shape) and 1 (smooth shape).

- Elongation Index (E_i), which measures the overall symmetry/asymmetry of the particle, and is calculated using equation (10.3):

$$E_i = 1 - \frac{width}{length} \quad (10.3)$$

where *width* and *length* are the shortest and longest object axes, respectively. Elongation indicates the symmetry (close to 0) or asymmetry (close to 1) of the particle in all directions.

Loose bulk density was determined by measuring the mass of material contained in a container of known volume (251.0 cm³). The biomass was dropped from a height of approximately 10 cm until the container was filled, without forcing compaction of the material. The biomass mass was determined by the difference between the full and empty container, and

the loose bulk density was determined by dividing the determined mass (g) by the container volume (dm³).

The water holding capacity was determined by adding 1 g of biomass sample in 100 ml of distilled water. The mixture was ultrasounded for 5 minutes and kept at rest for 24 h to extend contact. After this period, the samples were filtered and left for 2 h to drain. Then the wet biomasses were transferred to pre-weighed Petri dishes, weighed, and then left to oven dried for 12 h at 40°C. After this period, the biomasses were cooled in an excicator to room temperature, and weighed again to estimate the water holding capacity according to the equation (10.4):

$$\text{Water holding capacity (\%)} = \frac{m_2 - m_3}{m_3 - m_1} \times 100 \quad (10.4)$$

where m_1 is the mass of the Petri dish, m_2 is the mass drained sample (Petri dish + wet biomass), and m_3 is the mass of the oven-dried sample (Petri dish + oven-dried biomass).

The water vapor holding capacity was determined by keeping 10 g of biomass sample in open glasses inside a rounded metal container filled with warm water and covered with a plastic bag. The samples were kept for 5 days to promote water vapor absorption by the biomasses. After this period, the wet biomasses were transferred to pre-weighed Petri dishes, weighed, and then oven dried for 12 h at 40°C. Then the biomasses were cooled in an excicator to room temperature and weighed again to estimate the water vapor holding capacity using equation (10.5):

$$\text{Water vapor holding capacity (\%)} = \frac{m_2 - m_3}{m_3 - m_1} \times 100 \quad (10.5)$$

where m_1 is the mass of the Petri dish, m_2 is the mass drained sample (Petri dish + wet biomass), and m_3 is the mass of the oven-dried sample (Petri dish + oven-dried biomass).

10.2.2.2 Mortars Formulation and Production

Eleven mortars were formulated: a control mortar (REF) and mortars with additions of 5% and 10% (vol.) of each one of the five biomasses to the premixed gypsum plaster product. It was chosen to keep the added volume constant and not the mass as the biomasses present different loose bulk densities. By the loose bulk density of each material, the mass corresponding to each of the volumetric percentages were calculated and presented in Table 10.1.

Table 10.1 — Formulation of the test mortars indicating the amounts of water, premixed gypsum plaster product and biomass used in each test.

Mortar	H2O [ml]	PGPP [ml]	Biomass		
			type	[%]	[g]
REF	1000	3000	-	-	-
Fl5	1000	3000	flowers	5	32.6
Le5	1000	3000	leaves	5	62.2
Tw5	1000	3000	branchlet	5	56.7
Wo5	1000	3000	wood	5	31.7
Ba5	1000	3000	bark	5	65.2
Fl10	1000	3000	flowers	10	65.3
Le10	1000	3000	leaves	10	124.4
Tw10	1000	3000	branchlet	10	113.5
Wo10	1000	3000	wood	10	63.3
Ba10	1000	3000	bark	10	130.4

Notation: PGPP – premixed gypsum plaster product.

Except for the REF mortar, the biomass was manually homogenized with the gypsum premixed product. All the mortars were mixed mechanically, adding the dry components to the water of a mechanical mortar mixer. The same water content was used for all the mortars, as can be observed in Table 10.1. The mixing period had a duration of 1 min.

The same procedure was followed for a later production of a new batch of all the formulations with the addition of 10% (vol.) of the five biomasses together with the control mortar. Although the formulation of the second production was kept the same, the batch of premixed gypsum plaster product was different. Moreover, the production was run in a different laboratory to provide different curing conditions.

10.2.2.3 Fresh State Characterization and Samples Production

Fresh mortars were characterized by the flow table consistency, based on EN 1015-3 (1999), and density, based on EN 1015-6 (1999).

To assess the drying shrinkage prismatic 40 mm x 40 mm x 160 mm samples were prepared in metallic molds. The samples were visually observed after 2 days, when they were hard enough to be demolded.

The mortars of the second production were also casted in prismatic molds of 40 mm x 40 mm x 160 mm to assess drying shrinkage.

10.3 Results and discussion

10.3.1 Biomass fractions

10.3.1.1 Physical-Chemical characterization

The *A. dealbata* fractions used in this work have moisture contents between 7.52% (leaves) and 10.80% (bark). Chemical composition, proximate and ultimate composition, as well as bulk density, water holding capacity, and water vapor holding capacity of the different fractions, are shown in Table 10.2.

Table 10.2 — Physical and chemical characterization of the different fractions used in the production of mortars³⁰.

Parameters	Flowers	Leaves	Twigs	Wood	Bark
Proximate composition (wt.%)					
MCa	9.21 ± 0.04	7.52 ± 0.05	8.51 ± 0.11	10.05 ± 0.18	10.80 ± 0.26
VMb	79.88 ± 1.02	79.79 ± 1.81	78.83 ± 0.88	84.26 ± 0.19	72.93 ± 0.23
FCb	16.06 ± 1.19	14.26 ± 1.69	16.25 ± 0.92	15.00 ± 0.16	21.95 ± 0.15
Ashb	4.05 ± 0.17	5.94 ± 0.12	4.92 ± 0.08	0.74 ± 0.03	5.12 ± 0.13
Ultimate composition (wt.%) ^b					
C	48.22 ± 0.36	48.52 ± 0.12	44.61 ± 0.20	46.44 ± 0.24	49.75 ± 0.59
H	6.45 ± 0.13	6.42 ± 0.02	6.03 ± 0.06	6.09 ± 0.08	6.36 ± 0.28
N	2.06 ± 0.04	2.91 ± 0.02	1.44 ± 0.03	0.32 ± 0.01	1.89 ± 0.01
S	0.10 ± 0.01	0.15 ± 0.01	0.05 ± 0.01	nd	nd
O	39.12 ± 0.45	36.06 ± 0.14	42.95 ± 0.23	46.41 ± 0.31	36.88 ± 0.76
Bulk density (g/dm ³)	217.55 ± 4.43	414.75 ± 7.78	378.29 ± 9.20	211.14 ± 1.29	434.82 ± 7.18
Water Holding Capacity (wt.%)	590.17 ± 31.85	478.41 ± 28.50	423.51 ± 18.20	494.25 ± 24.01	330.47 ± 6.94
Water Vapor Holding Capacity (wt.%)	22.69 ± 1.48	20.07 ± 2.87	20.42 ± 0.87	21.64 ± 0.52	23.08 ± 0.29

^a on a wet basis; ^b on a dry basis, nd: not detected.

Proximate and ultimate composition is within the expected for biomass, despite the previous extraction process. The volatile matter of flowers (79.9%, db), leaves (79.8%, db), and twigs (78.8%, db) was very similar, and comparable to the contents found in date palm leaves (78.1%, db) and fronds (78.2 – 83.0%, db) (Sulaiman et al., 2016), eucalyptus leaves (79.2%, db) (Liu and Balasubramanian, 2012), Maritime Pine needles (79.4%, db) and branches (80.8%, db) (H. F. dos S. Viana et al., 2018), or *A. mangium* leaves (77.6%, db), pods (80.1%, db), and

branches (80.4%, db) (Ahmed et al., 2021a). The volatile matter was found lower in bark (72.9%, db), which is lower than what was found in *A. dealbata* bark by Senelwa and Sims (80.1%, db) (Senelwa and Sims, 1999), eucalyptus bark (77.0%, db) (García et al., 2012), or *A. mangium* bark (81.3%, db) (Ahmed et al., 2021a), and higher than the value of Maritime Pine bark (64.8%, db) (Álvarez-Álvarez et al., 2018). Wood presented the highest volatile matter content (84.3%, db), a slightly lower value than the ones found also in *A. dealbata* (87.9 – 91.5%, db) (Saldarriaga et al., 2015; Senelwa and Sims, 1999), *A. tortilis* wood (86.7%, db) (Nasser and Aref, 2014), and Maritime Pine wood (85.7%, db) (H. F. dos S. Viana et al., 2018), but higher than what was observed in eucalyptus wood (75.4%, db) (Channiwala and Parikh, 2002), *A. mangium* trunk (Ahmed et al., 2021a), or *A. longifolia* wood (Vicente et al., 2019).

The fixed carbon content was very similar for leaves (14.3%, db), wood (15.0%, db), flowers (16.1%, db), and twigs (16.3%, db), which were similar to what was found in oil palm fronds (15.2%, db) (Sulaiman et al., 2016), Maritime Pine wood (14.0%, db) (H. F. dos S. Viana et al., 2018), and *A. mangium* twigs (16.1%, db) (Ahmed et al., 2021a), higher than the values found in *A. dealbata* (7.2 – 9.6%, db) (Saldarriaga et al., 2015; Senelwa and Sims, 1999) and *A. tortilis* wood (11.4%, db) (Nasser and Aref, 2014), the mixture of *A. dealbata* wood, bark, and leaves (8.9%, db) (Amutio et al., 2013a), or eucalyptus leaves (10.3%, db) (Liu and Balasubramanian, 2012), and lower than the observed in *A. dealbata* (19.0%, db) (García et al., 2012) and *A. mangium* (20.1%, db) (Ahmed et al., 2021a) leaves, eucalyptus wood (21.3%, db) (Channiwala and Parikh, 2002), or *A. longifolia* wood (18.0%, db) (Vicente et al., 2019).

The ash contents were found lower in wood (0.74%, db), and varied between 4.05%, db (flowers) and 5.94%, db (leaves) for the other fractions studied. The ash content found in this work for the wood fraction was lower than the ones previously reported for *A. dealbata* wood (1.4 – 2.5%, db) (Saldarriaga et al., 2015; Senelwa and Sims, 1999), the mixture of *A. dealbata* wood, bark, and leaves (1.1%, db) (Amutio et al., 2013a), *A. tortilis* wood (1.92%, db) (Nasser and Aref, 2014), *A. mangium* trunk (2.1%, db) (Ahmed et al., 2021a), *A. longifolia* wood (2.1%, db) (Vicente et al., 2019), eucalyptus wood (3.4%, db) (Channiwala and Parikh, 2002), or date palm trunk (3.9%, db) (Nasser et al., 2016), but higher than the one found for Maritime Pine wood (0.22%, db) (H. F. dos S. Viana et al., 2018). For flowers, leaves, twigs, and bark, the values were in a similar range to those found for *A. dealbata* bark (3.9%, db) (Senelwa and Sims, 1999) or eucalyptus bark (6.2%, db) (García et al., 2012), lower than the ones found for *A. dealbata* (7.9%, db) (García et al., 2012) and eucalyptus (10.5%, db) (Liu and Balasubramanian, 2012) leaves, but higher than the ones found for *A. mangium* leaves (2.3%, db), bark (1.6%, db), twigs (1.7%, db), pods (2.9%, db), and branches (2.5%, db) (Ahmed et al., 2021a), Maritime Pine top

(0.24%, db), branches (1.0%, db), and needles (2.0%, db) (H. F. dos S. Viana et al., 2018), or Maritime Pine bark (1.0%, db) (Álvarez-Álvarez et al., 2018).

Carbon contents were found higher for flowers (48.2%, db), leaves (48.5%, db), and bark (50.0%, db) than for twigs (44.6%, db) and wood (46.4%, db). These values are in the same range as the ones found for *A. dealbata* leaves (45.8%, db) (García et al., 2012), wood (46.5%, db) (Saldarriaga et al., 2015), and the mixture of wood, bark, and leaves (46.5%, db), for *A. mangium* leaves (49.0%, db), trunk (45.5%, db), bark (47.7%, db), twigs (47.1%, db), pods (50.7%, db), and branches (48.1%, db) (Ahmed et al., 2021a), *A. longifolia* wood (49.6%, db) (Vicente et al., 2019), for other plants, such as eucalyptus leaves (47.0%, db) (Liu and Balasubramanian, 2012), bark (46.5%, db) (García et al., 2012), and wood (46.5%, db) (Channiwala and Parikh, 2002), and Maritime Pine wood (46.5%, db), needles (48.2%, db), branches (48.6%, db), and bark (50.4%, db) (Álvarez-Álvarez et al., 2018; H. F. dos S. Viana et al., 2018).

Hydrogen contents varied in a short range, from 6.0% (db) in twigs to 6.5% (db) in flowers, and are similar to the ones found in *A. dealbata* leaves (6.2%, db) (García et al., 2012), *A. tortilis* wood (6.0%, db) (Nasser et al., 2016), *A. mangium* leaves (6.0%, db), trunk (5.7%, db), bark (5.3%, db), twigs (5.4%, db), pods (5.4%, db), and branches (5.7%, db) (Ahmed et al., 2021a), *A. longifolia* wood (5.8%, db), eucalyptus leaves (6.2%, db) (Liu and Balasubramanian, 2012), bark (5.9%, db) (García et al., 2012), and wood (5.8%, db) (Channiwala and Parikh, 2002), or Maritime Pine wood (6.7%, db), branches (6.8%, db), and needles (6.9%, db) (H. F. dos S. Viana et al., 2018).

Nitrogen was found in lower amount in the wood fraction (0.32%, db), which agrees with the amount found in *A. tortilis* (0.34%, db) (Nasser and Aref, 2014), eucalyptus (0.30%, db) (Channiwala and Parikh, 2002), and Maritime Pine (0.13%, db) (H. F. dos S. Viana et al., 2018) wood, but lower than the one found by Saldarriaga et al. in *A. dealbata* wood (1.14%, db) (Saldarriaga et al., 2015), and in *A. longifolia* wood (1.39%, db) (Vicente et al., 2019) and *A. mangium* trunk (0.49%, db) (Ahmed et al., 2021a). Nitrogen amounts in flowers (2.06%, db), leaves (2.91%, db), twigs (1.44%, db), and bark (1.89%, db) are in a slightly higher range than the found in *A. dealbata* leaves (0.75%, db) (García et al., 2012), the mixture of *A. dealbata* wood, bark, and leaves (1.14%, db) (Amutio et al., 2013a), *A. mangium* leaves (1.72%, db), bark (0.94%, db), twigs (0.65%, db), pods (1.14%, db), and branches (0.48%, db) (Ahmed et al., 2021a), eucalyptus (1.69%, db) (García et al., 2012) and Maritime Pine (0.40%, db) (H. F. dos S. Viana et al., 2018) bark, eucalyptus leaves (1.23%, db) (Liu and Balasubramanian, 2012), and Maritime Pine branches (0.28%, db) and needles (0.74%, db) (H. F. dos S. Viana et al., 2018).

Sulfur was only detected in flowers (0.10%, db), leaves (0.15%, db), and twigs (0.05%, db), and in very low percentage, as expected for biomass. Similar amounts were found in *A. dealbata* (0.17%, db) (García et al., 2012) and *A. mangium* (0.20%, db) (Ahmed et al., 2021a) leaves, in *A. mangium* twigs (0.01%, db) and pods (0.16%, db) (Ahmed et al., 2021a), and in Maritime Pine branches (0.03%, db) (H. F. dos S. Viana et al., 2018). However, sulfur was detected in *A. dealbata* wood by Saldarriaga et al. (0.06%, db) (Saldarriaga et al., 2015), and in *A. mangium* trunk (0.06%, db) (Ahmed et al., 2021a) *A. longifolia* wood (0.09%, db) (Vicente et al., 2019), and Maritime Pine wood (0.03%, db) (H. F. dos S. Viana et al., 2018). Sulfur was found in higher amounts in eucalyptus bark (0.30%, db) (García et al., 2012), and in Maritime Pine needles (0.86%, db) (H. F. dos S. Viana et al., 2018) and bark (0.66%, db) (Álvarez-Álvarez et al., 2018).

Oxygen contents in flowers (39.1%, db), leaves (36.1%, db), and bark (36.9%, db) were lower than the ones found by Saldarriaga et al. in *A. dealbata* leaves (47.1%, db) (Saldarriaga et al., 2015), the mixture of *A. dealbata* wood, bark, and leaves (46.4%, db) (Amutio et al., 2013a), *A. mangium* leaves (43.1%, db) and bark (46.0%, db) (Ahmed et al., 2021a), eucalyptus (45.6%, db) (García et al., 2012) and Maritime Pine (42.7%, db) (H. F. dos S. Viana et al., 2018) bark, or Maritime Pine needles (42.1%, db) (H. F. dos S. Viana et al., 2018). Oxygen amount was higher in twigs (43.0%, db) and wood (46.4%, db), in agreement with the ones found in *A. dealbata* (45.0%, db) (Saldarriaga et al., 2015) and *A. tortilis* wood (46.9%, db) (Nasser and Aref, 2014), and *A. mangium* trunk (48.3%, db) and twigs (46.8%, db).

The particle size distribution is presented in Table 10.3. It can be seen that the main particle sizes lie between 200 and 1000 μm , with 51 wt.% of flowers, 73 wt.% of leaves, 62 wt.% of twigs, 77 wt.% of wood, and 75 wt.% of the bark lying within this range.

The Morphologi G3 analyzer allows the characterization of the particle shape by obtaining images and calculating morphological shape descriptors such as particle diameter (expressed as an equivalent circle diameter, based in the number of particles, denoted by *CE* diameter), circularity, convexity and elongation index. In Figure 10.2 it can be observed some heterogeneity between particles of the same sample, but it is evident the presence of more filamentous particles in flowers and wood samples, which is in accordance with the lower circularity values found for these fractions. The high convexity values found for the different fractions indicate a smooth particle surface, besides flowers and wood having shown slightly lower values, suggesting a slightly higher surface roughness in these samples. The flowers and wood samples were found to be also the more asymmetric particles, given the higher elongation values. The mean *CE* diameter varied between 4.42 μm (twigs) and 7.62 μm (wood).

The Morphologi G3 software allows obtaining the derived diameters (D), which are used to calculate the statistics of the particle size distributions and particle size expressed as $D[v, 0.1]$, $D[v, 0.5]$, and $D[v, 0.9]$ as standard percentile readings. As an example, the v in the expression $D[n, 0.1]$ refers to a volume distribution and, the 0.1 refers to the percentage, in this case, 10% of particles finer than the stated size. The derived diameters for all samples are described in Table 10.3. Bark was shown to be composed of the largest particles, with 10% of the volume being formed with particles smaller than 479.6 mm, 50% smaller than 924.1 mm, and 90% smaller than 1352 mm. The particle size distribution (volume distribution) is presented in Figure 10.3.

Table 10.3 — Particle size distribution and morphological shape descriptors, obtained from Morphologi G3 analysis.

Parameters	Flowers	Leaves	Twigs	Wood	Bark
Particle Size Distribution (wt.%)					
> 2000 μm	nd	nd	nd	nd	nd
1000 – 2000 μm	22.04	6.63	15.85	0.08	14.35
500 – 1000 μm	22.00	23.80	33.00	38.30	56.26
200 – 500 μm	29.43	48.72	28.95	39.03	18.51
100 – 200 μm	14.65	13.17	11.76	10.26	6.24
50 – 100 μm	5.22	3.62	5.33	5.30	2.69
< 50 μm	6.66	4.06	5.11	7.03	1.95
CE diameter (mm)	4.75 ± 0.28	5.29 ± 0.21	4.42 ± 0.04	7.62 ± 0.04	4.53 ± 0.10
Circularity	0.59 ± 0.02	0.77 ± 0.04	0.82 ± 0.00	0.63 ± 0.02	0.80 ± 0.02
Convexity	0.94 ± 0.00	0.97 ± 0.01	0.98 ± 0.00	0.95 ± 0.00	0.98 ± 0.00
Elongation index	0.34 ± 0.01	0.29 ± 0.01	0.30 ± 0.00	0.37 ± 0.00	0.29 ± 0.00
$D[v, 0.1]$	145.2 ± 18.67	247.3 ± 16.62	4.678 ± 2.61	115.8 ± 18.74	479.6 ± 15.02
$D[v, 0.5]$	455.7 ± 18.67	649.5 ± 16.62	8.234 ± 2.61	552.4 ± 18.74	924.1 ± 15.02
$D[v, 0.9]$	846.3 ± 18.67	1009 ± 16.62	11.91 ± 2.61	933.0 ± 18.74	1352 ± 15.02

^a on a wet basis; ^b on a dry basis, nd: not detected.

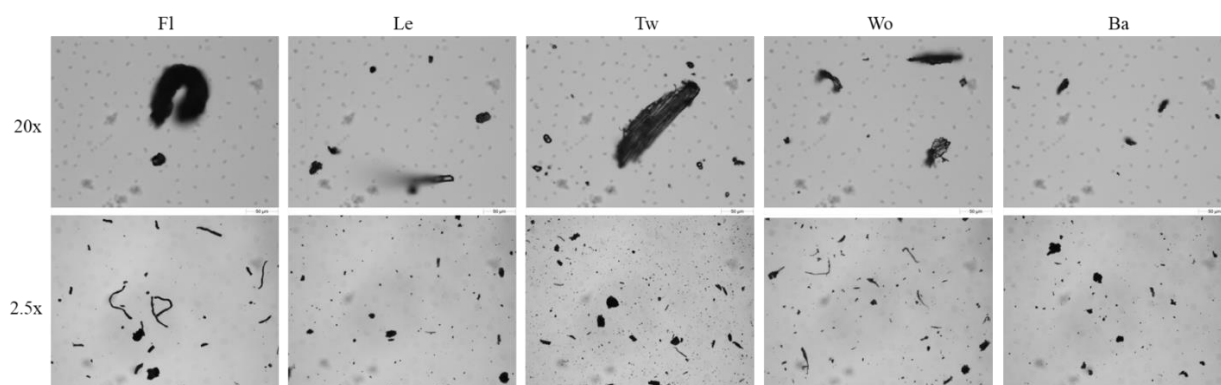


Figure 10.2 — Biomass particles dispersion images obtained by Morphologi G3 (20x and 2.5x magnification).

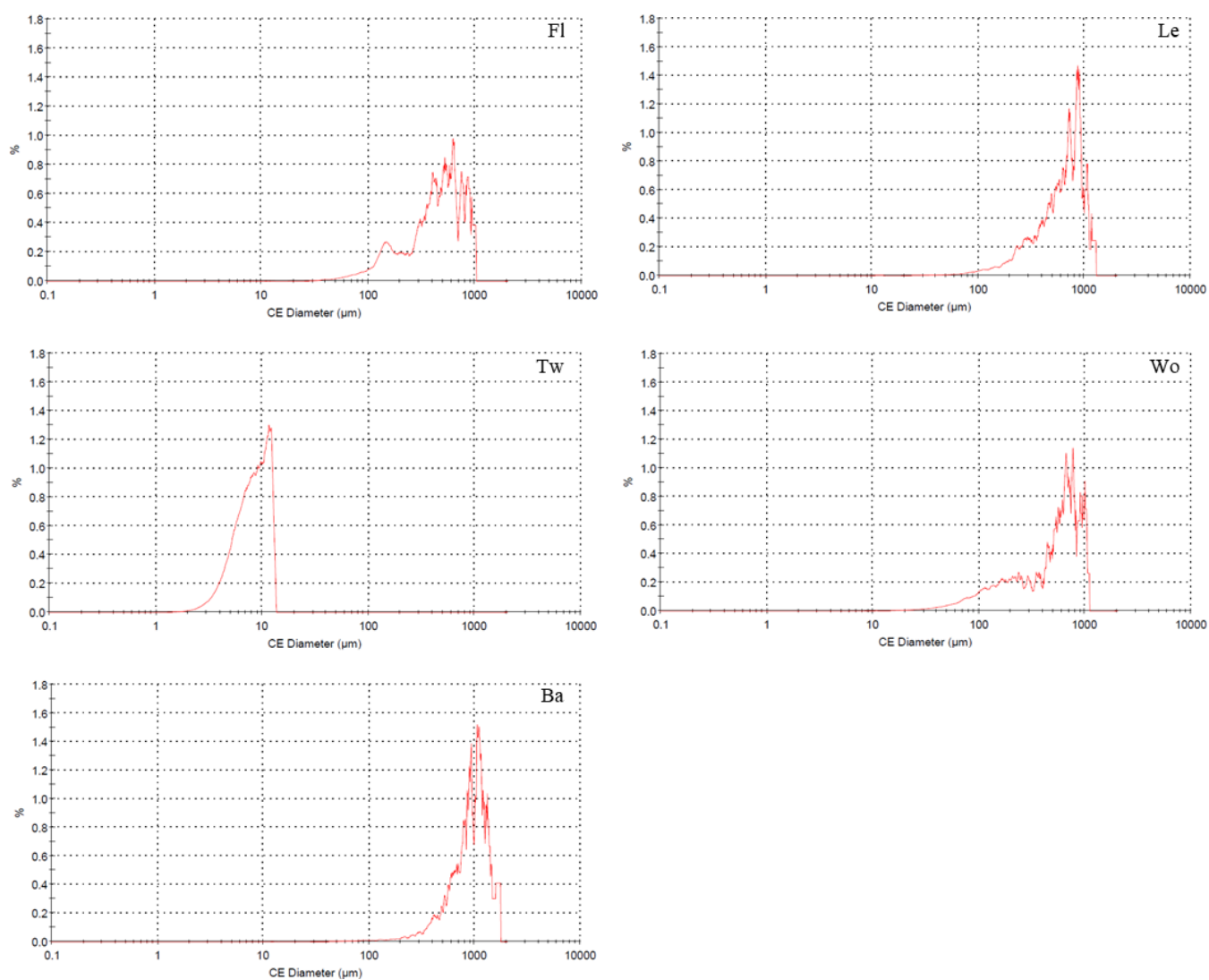


Figure 10.3 — Particle size distribution of the biomass samples obtained by Morphologi G3.

The bulk density varied in the range 211.14 (wood) – 434.82 (bark) kg/m³. Given these differences, it was decided to incorporate the biomass fractions in a volumetric percentage instead of a weight percentage, to have the same volume of biomass dispersed through the produced materials. The bulk density is expected to be affected by the particle size distribution, as shown in Table 10.4 for eucalyptus, *Pinus sylvestris*, or spruce barks, as well as for *Acacia tortilis* and *Pinus patula* wood. The values found for the materials used in this work were higher than the ones found in other biomasses used in the manufacture of mortars, such as hemp, barley, wheat, flax, miscanthus, reed, sunflower, rice husk, or maize bark, as well as of other forest residues, like eucalyptus bark, *Acacia tortilis* wood, and *Pinus patula* wood, and of the same order of the ones reported for pineapple crown, spruce bark, and *Pinus pinaster*, and *Pinus sylvestris* sawdust.

Table 10.4 — Bulk density of a variety of biomasses already used in the manufacture of mortars, and other forest residues comparable to *A. dealbata*.

Reference	Biomass	Particle size (mm)	Bulk density (kg/m ³)
(Braga et al., 2015)	Pineapple crown	0.074 – 0.104	421
(Laborel-Préneron et al., 2018)	Barley straw	2.3 - 7.6	57
(Laborel-Préneron et al., 2018)	Hemp shives	2.0 - 5.6	153
(Arufe et al., 2021)	Hemp shives	0.5 – 10	114
(Laborel-Préneron et al., 2018)	Corn cob	2.6 - 3.6	497
(Poongodi and Murthi, 2020)	Coconut shell	4.75 – 20	983
(Arufe et al., 2021)	Flax shiv	0.5 – 10	101
(Arufe et al., 2021)	Miscanthus	0.5 – 10	128
(Arufe et al., 2021)	Reed	0.5 – 10	81
(Arufe et al., 2021)	Rice husk	0.5 – 10	112
(Arufe et al., 2021)	Wheat	0.5 – 10	45
(Arufe et al., 2021)	Sunflower pith	0.5 – 10	31
(Lagouin et al., 2019)	Sunflower bark	< 0.95	120
(Arufe et al., 2021)	Sunflower bark	0.5 – 10	157
(Lagouin et al., 2019)	Maize bark	< 0.95	168
(Arufe et al., 2021)	Maize bark	0.5 – 10	67
(Miranda et al., 2013)	Eucalyptus bark	< 0.18	161
(Miranda et al., 2013)	Eucalyptus bark	0.18 - 0.25	156
(Miranda et al., 2013)	Eucalyptus bark	0.25 - 0.45	190
(Miranda et al., 2013)	Eucalyptus bark	0.45 - 0.85	192
(Miranda et al., 2013)	Eucalyptus bark	0.85 - 2.0	153
(Miranda et al., 2013)	Eucalyptus bark	> 2.0	181
(Miranda et al., 2012)	Pinus sylvestris bark	< 0.18	apr. 190
(Miranda et al., 2012)	Pinus sylvestris bark	0.18 - 0.25	apr. 185
(Miranda et al., 2012)	Pinus sylvestris bark	0.25 - 0.45	apr. 195
(Miranda et al., 2012)	Pinus sylvestris bark	0.85 – 1	apr. 235
(Miranda et al., 2012)	Pinus sylvestris bark	1 – 2	apr. 245
(Miranda et al., 2012)	Pinus sylvestris bark	> 2	apr. 175
(Miranda et al., 2012)	Spruce bark	< 0.18	apr. 275
(Miranda et al., 2012)	Spruce bark	0.18 - 0.25	apr. 245
(Miranda et al., 2012)	Spruce bark	0.25 - 0.45	apr. 275
(Miranda et al., 2012)	Spruce bark	0.85 – 1	apr. 300
(Miranda et al., 2012)	Spruce bark	1 – 2	apr. 305
(Miranda et al., 2012)	Spruce bark	> 2	apr. 195
(Mahdavi et al., 2010)	Date palm trunk	1.3 (mean)	82
(Charis et al., 2020)	Acacia tortilis barked wood	1.70 - 3.35	apr. 165
(Charis et al., 2020)	Acacia tortilis barked wood	3.35 - 5.0	apr. 157
(Charis et al., 2020)	Acacia tortilis barked wood	5.0 - 7.10	apr. 157
(Charis et al., 2020)	Pinus patula wood	1.70 - 3.35	apr. 35
(Charis et al., 2020)	Pinus patula wood	3.35 - 5.0	apr. 30
(Charis et al., 2020)	Pinus patula wood	5.0 - 7.10	apr. 20
(Singh et al., 2020)	Acacia nilotica wood	0.50 (mean)	230.8
(Herrero et al., 2011)	Pinus nigra sawdust	< 0.2	96 - 440
(Herrero et al., 2011)	Pinus pinaster sawdust	< 0.2	184 - 395
(Herrero et al., 2011)	Pinus sylvestris sawdust	< 0.2	200 - 356

The water holding capacity of the different fractions varied between 330.47% (bark) and 590.17% (flowers), which is higher than the reported for several other lignocellulosic materials used in this kind of application, such as date palm biomass (37 – 146%) (Almi et al., 2015), sisal (230%) and coconut (100%) fiber (Filho et al., 1999), maize fiber (214%) (Baranitharan and Mahesh, 2014), rice husk (210%) (Arufe et al., 2021), miscanthus (310%) (Arufe et al., 2021), reed (260%) (Arufe et al., 2021), sunflower bark (296 - 300%) (Arufe et al., 2021; Lagouin et al., 2019), corn cobs (90 – 150%) (Laborel-Préneron et al., 2018). The values found in this work are in the same order as the observed when using barley straw (400 – 600%) (Laborel-Préneron et al., 2018), wheat (490%) (Arufe et al., 2021), hemp shives (280 – 406%) (Arufe et al., 2021; Laborel-Préneron et al., 2018), flax shives (410%) (Arufe et al., 2021), and maize bark (254 – 580%) (Arufe et al., 2021; Lagouin et al., 2019).

The water vapor holding capacity was similar for all the plant fractions studied, varying between 20.1% (leaves) and 23.1% (bark).

10.3.2 Fresh state mortars

After mechanical mixing, all the mortar without and with biomass additions resulted in workable plastering mortars, although differences could be assessed. The effect of the biomass additions on manual workability was assessed for the second production and is presented in Table 5 together with starting and final setting times. Moreover, the occurrence of some cracks was observed on the surface of the layer applied on the brick before the setting was reached (Fig 10.4) and their assessment was included in Table 10.5.

Table 10.5 — Results of workability, setting times and cracks of the second production-mortars.

Mortar	Heavy	Sticky	IST	FST	Cracks
REF'	REF	REF	48'	1h20'	0
FI10'	+	REF	1h11'	2h24'	1
Le10'	+ +	–	1h16'	6h35'	2
Tw10'	–	+	2h03'	4h53'	2
Wo10'	–	–	48'	1h23'	0
Ba10'	REF	+	1h20'	2h43'	0

Notation: ST – initial setting time; FT – final setting time; REF – control mortar; + slightly higher than REF; + + higher than REF; – slightly lower than REF; 0 – no cracks; 1 – small cracks; 2 – cracks.

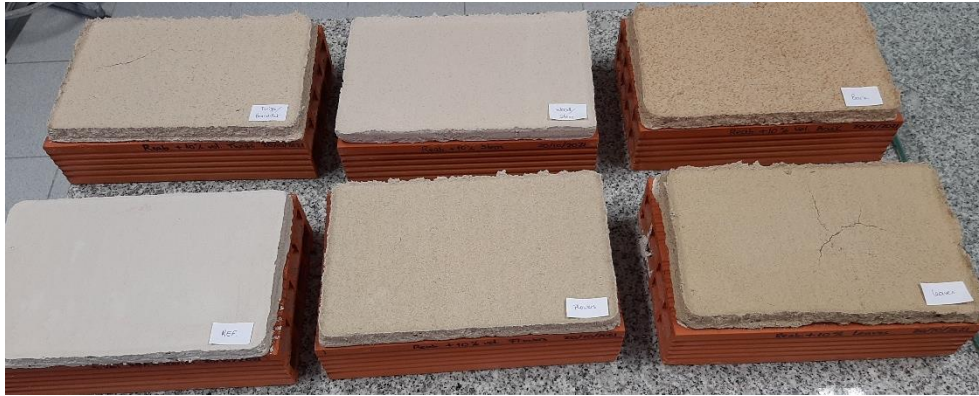


Figure 10.4 — Cracks observed for the layer applied on the brick of the six second production-mortars before the final setting was attained.

The Wo10' plastering mortar presented a better workability than the control one and a similar setting time, but the addition of all the other biomasses resulted in a worse workability and an increase of the setting time. Thus, the delayed loss of plasticity could have caused the cracks, except for the Ba10' plaster. The hygroscopicity of the biomass particles is much higher than that of sand particles therefore some water adsorption by the biomass particles would limit the availability of water to interact with the remainder mixture components. It is possible that the selected biomasses acted as a retarder, as it has been observed for the oil palm trunk by Selamat et al. (Selamat et al., 2019) and for the black tea by Huang et al. (Huang et al., 2019). The latter study referred that the addition of the black tea to the gypsum delayed the hydration process of the binder, creating a water film. The phenomenon is triggered by the addition of water to the hydrophilic groups contained in the black tea. Consequently, the same authors concluded that the addition of the black tea to gypsum mortar reduces water requirements. Moreover, Boquera et al. referred important cracking on samples with high percentage of lignin where the higher the percentage of lignin, the higher the water/powder ratio (Boquera et al., 2021). Results of flow table consistency and fresh bulk density of the first and second productions are presented in Figure 10.5.

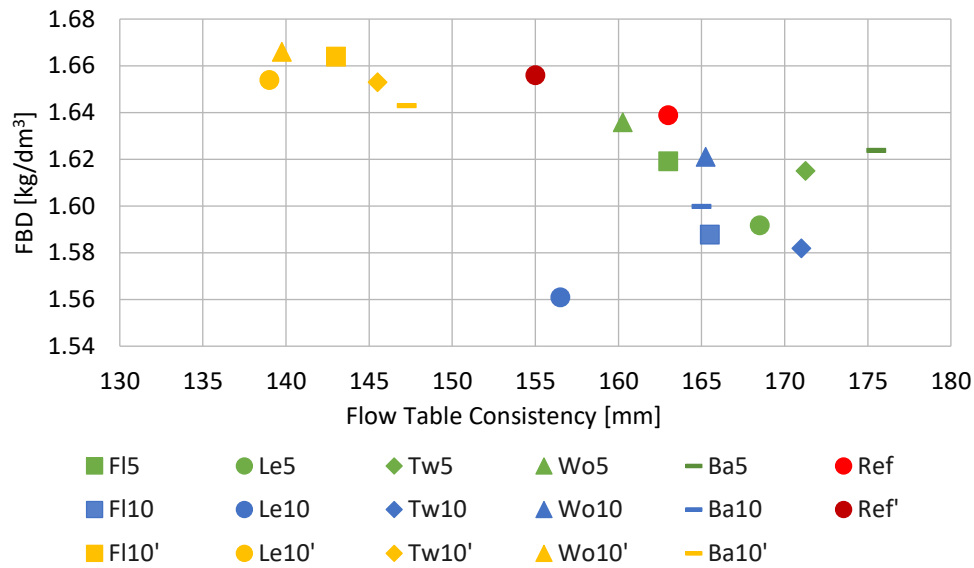


Figure 10.5 — Correlating Fresh Bulk density (*FBD*) with flow table consistency of mortars from the first and second (') production.

It can be observed that all the mortars from the second production show similar fresh bulk density and a flow table consistency, with the exception of the control mortar, below 150 mm diameter which is the lower limit prescribed by the European standard (EN 13279-2:2014). Similar results are reported by Boquera et al. (Boquera et al., 2021). The first production, instead, fulfilled the standard requirement of flow table consistency with values of fresh bulk density higher for the mortars with addition of 5% than 10% (vol.) of biomass, but still both lower than the control mortar.

10.4 Conclusions

Fresh mortars with incorporation of spent *A. dealbata* fractions were prepared and characterized by the flow table consistency, drying shrinkage and fresh bulk density.

Among the evaluated fractions, the wood was the one that gave mortars with more similar characteristics to the reference used, and with improved workability. Wood incorporation at 10% level resulted in a mortar with settling times very similar to the reference mortars but all other biomasses caused significant increases of both initial and final settling times. The flowers, leaves and twigs significantly increased the settling times and also modified drying shrinkage behaviour that resulted in the formation of cracks during mortar drying. Flow table consistency of the mortars prepared with 5% and 10% biomass incorporation were below 150

mm diameter according to European standard (EN 13279-2:2014), but a second batch of mortars prepared with 10% biomass incorporation did not fulfil that standard. The results obtained suggest that the wood and bark fractions, but especially the wood fraction, have the most promising characteristics for incorporation in mortars. To optimize the mortar final quality the biomass hygroscopicity should be considered when defining added water relative amount.

In future works mechanical-physical properties of the biomass plastering mortars must be tested, to assess the influence of the different types and percentages added, and the advantages and drawbacks that different fractions of *A. dealbata* can have on the performance of gypsum-based plasters.

GENERAL CONCLUSIONS AND FUTURE WORK

Acacia dealbata is currently the most aggressive invasive species in the national territory. The eradication attempts to control its proliferation have been costly and inefficient, generating high amounts of biomass that has not been economically and environmentally sustainably valorized. The main goal of the present thesis was to give a contribution to the exploitation of *A. dealbata* biomass (flowers, leaves, twigs, wood, and bark) from a biorefinery perspective, through the production of bioactive extracts, as well as solid biofuels and materials from the generated extraction residues.

The results demonstrated the possibility of producing added-value extracts with antioxidant and antimicrobial properties from each fraction, with the potential to be explored by nutraceutical, cosmetic, or food and feed industries. The simple-to-implement and low-energy technique of maceration at room temperature proved to be a suitable method to retrieve bioactive components from the biomass, instead of extraction at a higher temperature. Aqueous acetone proved to be the most suitable extraction media to remove bioactive components from the biomass, when compared with aqueous ethanol or aqueous methanol. Moreover, sequential extraction with polar petroleum ether followed by aqueous acetone proved to be feasible with all fractions, thus allowing obtaining a wider range of chemicals from the same amount of biomass.

Flowers from Coruche originated the extracts with the best composition in terms of bioactive phenolics compounds, as well as the highest antioxidant activity. An innovative extraction approach was successfully tried for the first time with flowers of *A. dealbata*, by using a mixture of petroleum ether and acetone (50% V/V) and pure acetone, that allowed the removal of lipophilic and hydrophilic components from the biomass in a single extraction step. Highly

concentrated phenolic-rich aqueous phases, with high antioxidant activity and antimicrobial capacity against several bacteria and fungi, were obtained by maceration at room temperature.

The polar extracts obtained with aqueous organic solvent or in the aqueous phases were found to be composed essentially by flavonoid glycosides of quercetin, naringenin, or myricetin, which have gained importance in nutraceutical, food and feed industries due to their relevant biological activities. Absolute oils with distinct aromas were produced and analyzed by GCTOF-MS, with results showing the organic acids *p*-anisic acid, palmitic acid, linoleic acid, and linolenic acid; the ester methyl *p*-anisate; the benzaldehyde anisaldehyde; the hydrocarbons nonadecane, heneicosane, heptadecane, and 8-heptadecene; the pentacyclic triterpenes α -amyrin, β -amyrin, lupenone, and lupeol; and glycerol monostearate as the most representative components. These compounds are particularly important due to their known applications and biological activities.

Leaves and twigs were demonstrated to be a good source of phenolics, having originated extracts with good antioxidant and antimicrobial activity, with twigs showing the best results. The extracts with the best properties in terms of composition and antioxidant activity were obtained with aqueous acetone as extraction solvent using leaves and twigs from Alcobaça. The use of air-dried material improved the extraction yield, the total flavonoid, and the total proanthocyanidin contents. Strong correlations between total phenolic and total proanthocyanidin contents and antioxidant activity were found. However, lipophilic components known for their relevant biological activities, such as phytol, squalene, α -tocopherol, lupenone, and lupeol were also detected, especially in the extracts from leaves.

Wood and bark also originated phenolic-rich extracts with antioxidant activity, especially the bark fraction which showed antioxidant activity higher than Trolox in the DPPH assay. Bark extracts showed also superior antimicrobial activity, even against *C. albicans* yeast. It is worth noting that in these fractions the extracts' composition and antioxidant activity were even improved by the previous extraction with petroleum ether. Once the bark is much richer in phenolic compounds and presented significantly better bioactive properties, its separation from the wood may be considered.

Regarding energy applications, raw and spent biomass revealed higher heating values in an attractive range for this purpose (18.45 MJ/kg in spent wood to 23.47 in spent leaves), which were significantly improved by the thermal treatments, especially the hydrothermal carbonization that raised the higher heating values to the range 22.77 MJ/kg (bark) - 28.97 MJ/kg (flowers).

All fractions showed proximate and ultimate composition similar to that of other biomasses used for energy purposes, which was not significantly affected by the previous extraction step. Torrefaction and hydrothermal carbonization, particularly hydrothermal carbonization at 250 °C, significantly decreased the volatile matter content and oxygen content and markedly increased the carbon content of the solid product, approaching their composition to the one of lignite. A higher conversion of biomass was achieved by hydrothermal carbonization, even when using a temperature 50 °C lower than that used in traditional torrefaction, due to the catalytic effect of subcritical water, that enhances the degradation reactions occurring in the biomass polymers during the thermal treatments.

The mineral composition of the studied fractions, as well as of the produced biochars and hydrochars, was found to have a low tendency to provoke ash deposits in the thermal conversion equipment, exception made for some flower samples which must be used together with other less problematic fuels in energetic applications.

The thermogravimetric analysis under oxidative conditions revealed ignition and burnout temperatures of the spent fractions comparable to the ones found in other fuels, being noticed a significant reduction of the burnout temperature with the previous removal of extractives. Moreover, a decrease in the activation energy and the consequent shortening of reaction time was observed in the case of spent flowers, wood, and bark, compared with the respective raw materials. The degradation rate in the macro TGA assay followed what is expected for biomass, with the wood fraction being clearly the most reactive.

Concerning the chars, the thermogravimetric data showed that hydrothermal carbonization gave products easier to ignite than traditional torrefaction at the same temperature. The activation energy was tendentially lower for hydrochars at 250 °C than for the torrefaction biochars, while the hydrochars obtained at 200 °C had similar activation energies than those of the torrefaction biochars obtained at a temperature 50 °C higher. A statistically significant correlation between ignition temperature and activation energy was found.

The preliminary adsorption study revealed that all the raw and spent fractions can adsorb methylene blue dye to a much higher extent than methyl orange dye, suggesting adsorption occurs by electrostatic interactions between negatively charged groups at the biomass surface and the positively charged methylene blue dye. The spent material had a better performance for the highest contact time, with methylene blue removal efficiencies higher than 90%. In the case of biochars and hydrochars this selectivity was not observed, suggesting that adsorption may occur by diffusion of the dye molecules through the porous structure formed during the thermal treatments, instead of electrostatic interactions. The removal efficiencies were lower in

the chars, however, these were significantly improved by steam activation, particularly in the case of the wood and bark fractions. The flowers, leaves, and twigs were the fractions that gave the products with less potential to be valorized as adsorbents. Nonetheless, their relatively higher contents in potassium and phosphorus may confer them the potential to be used in soil amendment.

Among the evaluated fractions, the spent wood and bark are the most suitable to be successfully incorporated in the formulation of gypsum mortars. The wood was the one that gave mortars with more similar characteristics to the reference used, and with improved workability.

Considering the importance of the biorefinery approach to contribute to the economic and environmental sustainability of the control of the proliferation of *A. dealbata*, some work must be continued. Suggestions on future work in this context are as follows:

- The validation of the produced extracts by its incorporation in cosmetic or nutraceutical formulations must be further studied.
- The valorization of the spent residues and biochars derived thereof as adsorbents must be further studied, once only a preliminary study was made in this thesis. The influence of operational parameters such as agitation, pH, particle size, or other activation procedures must be studied.
- The use of the spent residues and respective biochars and hydrochars in energy applications at pilot scale must be performed to validate some conclusions derived from this work, such as the ash-related problems, that are frequent when using biomass as a fuel.
- The mechanical-physical properties of the biomass plastering mortars must be tested, to assess the influence of the incorporation of wood and bark on the performance of gypsum-based plasters.
- The study of residual biomass of other species under this biorefinery concept must be performed, to maximize their valorization and sustainable exploitation.

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APPENDIX (CHAPTER 3)

A.1 Tables

Table A1 — TPC (mg GAE/g of flower, wb), TFC (mg CatE/g of flower, wb), DPPH (mg TE/g of flower, wb), and FRAP ($\mu\text{mol Fe}^{2+}$ /g flower, wb).

		Fresh flower			Spent flower		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
TPC	<i>A. dealbata</i> (ALC)	19.2 $\pm$ 0.6 ^{a,B}	13.7 $\pm$ 0.1 ^{b,B}	13.0 $\pm$ 0.3 ^{b,B}	15.3 $\pm$ 0.3 ^{a,B}	10.2 $\pm$ 0.3 ^{b,B}	9.1 $\pm$ 0.2 ^{c,B}
	<i>A. dealbata</i> (CAP)	17.1 $\pm$ 0.5 ^{a,C}	10.7 $\pm$ 0.3 ^{b,C}	10.0 $\pm$ 0.3 ^{b,C}	10.9 $\pm$ 0.3 ^{a,C}	7.5 $\pm$ 0.3 ^{b,C}	5.7 $\pm$ 0.1 ^{c,D}
	<i>A. dealbata</i> (COR)	24.8 $\pm$ 1.0 ^{a,A}	16.4 $\pm$ 0.3 ^{b,A}	14.6 $\pm$ 0.4 ^{c,A}	16.2 $\pm$ 0.4 ^{a,A}	11.9 $\pm$ 0.1 ^{b,A}	10.9 $\pm$ 0.4 ^{c,A}
	<i>A. saligna</i>	11.3 $\pm$ 0.4 ^{a,D}	7.0 $\pm$ 0.3 ^{b,D}	5.8 $\pm$ 0.1 ^{c,D}	8.5 $\pm$ 0.3 ^{a,D}	6.1 $\pm$ 0.2 ^{b,D}	6.4 $\pm$ 0.2 ^{b,C}
TFC	<i>A. dealbata</i> (ALC)	7.2 $\pm$ 0.2 ^{a,A}	5.2 $\pm$ 0.1 ^{b,A}	4.9 $\pm$ 0.1 ^{c,A}	7.7 $\pm$ 0.2 ^{a,B}	5.1 $\pm$ 0.1 ^{b,C}	4.5 $\pm$ 0.1 ^{c,B}
	<i>A. dealbata</i> (CAP)	6.7 $\pm$ 0.2 ^{a,B}	4.4 $\pm$ 0.2 ^{b,B}	4.2 $\pm$ 0.1 ^{b,B}	7.3 $\pm$ 0.3 ^{a,C}	5.4 $\pm$ 0.1 ^{b,B}	4.3 $\pm$ 0.1 ^{c,C}
	<i>A. dealbata</i> (COR)	7.5 $\pm$ 0.2 ^{a,A}	5.1 $\pm$ 0.1 ^{b,A}	4.9 $\pm$ 0.1 ^{c,A}	8.1 $\pm$ 0.1 ^{a,A}	5.9 $\pm$ 0.2 ^{b,A}	5.0 $\pm$ 0.2 ^{c,A}
	<i>A. saligna</i>	6.5 $\pm$ 0.3 ^{a,B}	3.3 $\pm$ 0.2 ^{b,C}	2.7 $\pm$ 0.1 ^{c,C}	4.6 $\pm$ 0.2 ^{a,D}	2.3 $\pm$ 0.1 ^{b,D}	2.2 $\pm$ 0.1 ^{b,D}
DPPH	<i>A. dealbata</i> (ALC)	44.1 $\pm$ 1.8 ^{a,A}	30.3 $\pm$ 0.6 ^{b,A}	31.5 $\pm$ 0.1 ^{a,A}	26.7 $\pm$ 1.2 ^{a,A}	17.3 $\pm$ 0.6 ^{b,A}	15.3 $\pm$ 0.5 ^{c,A}
	<i>A. dealbata</i> (CAP)	37.3 $\pm$ 0.9 ^{a,B}	22.3 $\pm$ 0.2 ^{b,C}	20.5 $\pm$ 0.6 ^{c,C}	21.3 $\pm$ 1.0 ^{a,B}	9.3 $\pm$ 0.5 ^{b,C}	6.7 $\pm$ 0.2 ^{c,C}
	<i>A. dealbata</i> (COR)	44.4 $\pm$ 1.8 ^{a,A}	28.0 $\pm$ 0.4 ^{b,B}	27.1 $\pm$ 0.7 ^{b,B}	26.6 $\pm$ 0.7 ^{a,A}	14.8 $\pm$ 0.6 ^{b,B}	12.1 $\pm$ 0.3 ^{c,B}
	<i>A. saligna</i>	17.5 $\pm$ 0.7 ^{a,C}	8.4 $\pm$ 0.3 ^{b,D}	7.8 $\pm$ 0.4 ^{b,D}	10.2 $\pm$ 0.5 ^{a,C}	4.5 $\pm$ 0.1 ^{b,D}	4.6 $\pm$ 0.2 ^{b,D}
FRAP	<i>A. dealbata</i> (ALC)	393.8 $\pm$ 5.3 ^{a,A}	187.2 $\pm$ 9.8 ^{c,B}	233.1 $\pm$ 13.0 ^{b,A}	104.5 $\pm$ 8.6 ^{a,A}	60.4 $\pm$ 1.1 ^{b,A}	57.5 $\pm$ 2.3 ^{b,A}
	<i>A. dealbata</i> (CAP)	361.5 $\pm$ 5.7 ^{a,B}	170.7 $\pm$ 3.8 ^{b,C}	174.3 $\pm$ 4.0 ^{b,C}	79.8 $\pm$ 1.2 ^{a,B}	48.7 $\pm$ 1.5 ^{b,B}	35.5 $\pm$ 1.4 ^{c,C}
	<i>A. dealbata</i> (COR)	411.3 $\pm$ 27.3 ^{a,A}	245.7 $\pm$ 14.1 ^{b,A}	192.7 $\pm$ 11.1 ^{c,B}	81.8 $\pm$ 2.1 ^{a,B}	46.1 $\pm$ 2.6 ^{b,B}	43.7 $\pm$ 1.4 ^{b,B}
	<i>A. saligna</i>	162.6 $\pm$ 14.8 ^{a,C}	64.1 $\pm$ 3.0 ^{b,C}	56.6 $\pm$ 4.5 ^{b,D}	32.5 $\pm$ 1.0 ^{a,C}	18.6 $\pm$ 2.0 ^{b,C}	18.5 $\pm$ 1.4 ^{b,D}

Values (mean $\pm$ standard deviation) are average of two replicates of each sample, analyzed individually in triplicate ($n = 2 \times 3$); different letters (lower case a-c) indicate significant differences of means among solvents for each sample of fresh and spent flowers (one-way ANOVA, Tukey test at $p < 0.05$); different letters (upper case A-C) indicate significant differences of means within flower samples of fresh and spent flowers, for each extraction solvent.

Table A2 — Extraction yield (% db), TPC (mg GAE/g flower, db), TFC (mg CatE/g flower, db), DPPH (mg TE/g flower, db), and FRAP (μmol Fe2+/g extract, db) (dry basis)

		Fresh flower			Spent flower		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
Yield	<i>A. dealbata</i> (ALC)	19.2 ± 0.6 ^{a,A}	16.2 ± 0.1 ^{b,A}	16.1 ± 0.4 ^{b,A}	7.08 ± 0.27 ^{a,C}	6.06 ± 0.24 ^{b,C}	5.72 ± 0.25 ^{b,C}
	<i>A. dealbata</i> (CAP)	16.7 ± 0.6 ^{a,C}	13.1 ± 0.3 ^{b,C}	13.3 ± 0.4 ^{b,C}	8.16 ± 0.38 ^{a,B}	7.70 ± 0.22 ^{a,B}	6.47 ± 0.34 ^{b,B}
	<i>A. dealbata</i> (COR)	18.3 ± 0.6 ^{a,AB}	14.4 ± 0.3 ^{b,B}	14.1 ± 0.1 ^{b,B}	9.04 ± 0.08 ^{a,A}	8.55 ± 0.02 ^{b,A}	8.31 ± 0.12 ^{c,A}
	<i>A. saligna</i>	16.9 ± 0.6 ^{a,BC}	14.0 ± 0.14 ^{b,B}	13.7 ± 0.1 ^{b,BC}	4.70 ± 0.22 ^{ab,D}	4.64 ± 0.06 ^{b,D}	4.99 ± 0.08 ^{a,D}
TPC	<i>A. dealbata</i> (ALC)	50.1 ± 1.6 ^{a,B}	35.6 ± 0.4 ^{b,B}	33.7 ± 0.7 ^{c,B}	18.2 ± 0.4 ^{a,B}	12.1 ± 0.3 ^{b,B}	10.8 ± 0.2 ^{c,B}
	<i>A. dealbata</i> (CAP)	44.2 ± 1.3 ^{a,C}	27.5 ± 0.8 ^{b,C}	25.8 ± 0.8 ^{c,C}	16.5 ± 0.4 ^{a,C}	11.4 ± 0.5 ^{b,C}	8.7 ± 0.1 ^{c,C}
	<i>A. dealbata</i> (COR)	71.3 ± 2.8 ^{a,A}	47.1 ± 0.8 ^{b,A}	41.9 ± 1.2 ^{c,A}	24.7 ± 0.6 ^{a,A}	18.1 ± 0.2 ^{b,A}	16.5 ± 0.8 ^{c,A}
	<i>A. saligna</i>	35.4 ± 1.4 ^{a,D}	21.9 ± 0.8 ^{b,D}	18.2 ± 0.1 ^{c,D}	9.6 ± 0.3 ^{a,C}	6.9 ± 0.2 ^{b,D}	7.2 ± 0.2 ^{b,D}
TFC	<i>A. dealbata</i> (ALC)	18.6 ± 0.6 ^{a,C}	13.7 ± 0.3 ^{b,B}	12.9 ± 0.2 ^{c,B}	9.2 ± 0.2 ^{a,C}	6.1 ± 0.1 ^{b,C}	5.4 ± 0.1 ^{c,C}
	<i>A. dealbata</i> (CAP)	17.2 ± 0.5 ^{a,D}	11.4 ± 0.6 ^{b,C}	10.9 ± 0.2 ^{b,C}	11.0 ± 0.4 ^{a,B}	8.2 ± 0.1 ^{b,B}	6.5 ± 0.2 ^{c,B}
	<i>A. dealbata</i> (COR)	21.5 ± 0.5 ^{a,A}	14.7 ± 0.3 ^{b,A}	14.0 ± 0.3 ^{c,A}	12.3 ± 0.2 ^{a,A}	8.9 ± 0.3 ^{b,A}	7.5 ± 0.3 ^{c,A}
	<i>A. saligna</i>	20.4 ± 0.8 ^{a,B}	10.3 ± 0.6 ^{b,D}	8.5 ± 0.5 ^{c,D}	5.2 ± 0.2 ^{a,D}	2.6 ± 0.1 ^{b,D}	2.4 ± 0.1 ^{b,D}
DPPH	<i>A. dealbata</i> (ALC)	114.8 ± 4.8 ^{a,B}	79.0 ± 1.5 ^{b,A}	82.0 ± 0.3 ^{b,A}	31.7 ± 1.5 ^{a,C}	20.6 ± 0.7 ^{b,B}	18.2 ± 0.5 ^{c,A}
	<i>A. dealbata</i> (CAP)	96.1 ± 2.4 ^{a,C}	57.6 ± 0.4 ^{b,B}	52.9 ± 1.5 ^{c,C}	32.3 ± 1.5 ^{a,B}	14.2 ± 0.8 ^{b,C}	10.2 ± 0.4 ^{c,B}
	<i>A. dealbata</i> (COR)	127.8 ± 5.1 ^{a,A}	80.6 ± 1.0 ^{b,A}	77.9 ± 1.9 ^{b,B}	40.5 ± 1.1 ^{a,A}	22.5 ± 0.9 ^{b,A}	18.3 ± 0.2 ^{c,A}
	<i>A. saligna</i>	54.7 ± 2.1 ^{a,D}	26.4 ± 0.9 ^{b,C}	24.5 ± 1.3 ^{b,D}	11.4 ± 0.5 ^{a,C}	5.1 ± 0.1 ^{b,D}	5.2 ± 0.2 ^{b,C}
FRAP	<i>A. dealbata</i> (ALC)	1025.4 ± 13.79 ^{a,B}	487.37 ± 25.63 ^{c,B}	607.02 ± 33.74 ^{b,A}	310.27 ± 21.24 ^{a,B}	179.70 ± 11.61 ^{b,B}	171.08 ± 11.59 ^{b,B}
	<i>A. dealbata</i> (CAP)	932.27 ± 14.32 ^{a,C}	440.33 ± 9.91 ^{b,C}	449.62 ± 10.28 ^{b,C}	302.93 ± 17.83 ^{a,B}	184.45 ± 5.66 ^{b,B}	134.63 ± 5.46 ^{c,C}
	<i>A. dealbata</i> (COR)	1182.9 ± 78.59 ^{a,A}	706.62 ± 40.44 ^{b,A}	554.35 ± 31.98 ^{c,B}	414.35 ± 14.50 ^{a,A}	233.57 ± 12.88 ^{b,A}	221.15 ± 7.20 ^{b,A}
	<i>A. saligna</i>	509.93 ± 46.52 ^{a,D}	200.95 ± 9.30 ^{b,D}	177.70 ± 14.23 ^{b,D}	91.37 ± 2.94 ^{a,C}	52.28 ± 5.18 ^{b,C}	51.97 ± 4.51 ^{b,D}

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 x 3); different letters (lower case a-c) indicate significant differences of means among solvents for each sample of fresh and spent flowers (one-way ANOVA, Tukey test at p<0.05); different letters (upper case A-C) indicate significant differences of means within flower samples of fresh and extracted flowers, for each extraction solvent.

Table A3 — TFC expressed in rutin equivalents (mg RutE/g of dry extract), of extracts from fresh and spent flowers using different extraction solvents.

		Fresh flower			Spent flower		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
TFC	<i>A. dealbata</i> (ALC)	298.2 ± 4.4 ^{a,C}	258.5 ± 6.3 ^{b,B}	244.8 ± 7.4 ^{c,B}	398.5 ± 8.2 ^{a,B}	307.5 ± 10.5 ^{b,B}	288.7 ± 9.3 ^{c,B}
	<i>A. dealbata</i> (CAP)	317.4 ± 15.1 ^{a,B}	266.6 ± 13.1 ^{b,B}	251.5 ± 6.1 ^{b,B}	415.1 ± 7.8 ^{a,A}	326.8.4 ± 6.4 ^{b,A}	309.0 ± 10.8 ^{c,A}
	<i>A. dealbata</i> (COR)	360.2 ± 11.6 ^{a,A}	313.4 ± 14.5 ^{b,A}	304.8 ± 7.1 ^{b,A}	417.7 ± 2.5 ^{a,A}	319.2 ± 10.3 ^{b,AB}	278.3 ± 10.1 ^{c,B}
	<i>A. saligna</i>	368.8 ± 8.0 ^{a,A}	226.6 ± 11.6 ^{b,C}	192.1 ± 10.5 ^{c,C}	336.2 ± 8.0 ^{a,C}	171.8 ± 3.9 ^{b,C}	148.9 ± 4.2 ^{c,C}

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 x 3); different letters (lower case a-c) indicate significant differences of means among solvents for each sample of fresh and spent flowers (one-way ANOVA, Tukey test at p<0.05); different letters (upper case A-C) indicate significant differences of means within flower samples of fresh and extracted flowers, for each extraction solvent.

Table A4 — TFC expressed in rutin equivalents (mg RutE/g of biomass), of extracts from fresh and spent flowers using different extraction solvents.

		Fresh flower			Spent flower		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
TFC	<i>A. dealbata</i> (ALC)	57.1 ± 0.8 ^{a,C}	41.9 ± 1.0 ^{b,B}	39.5 ± 1.2 ^{c,B}	28.2 ± 0.5 ^{a,C}	18.6 ± 0.6 ^{b,C}	16.5 ± 0.5 ^{c,C}
	<i>A. dealbata</i> (CAP)	52.9 ± 2.5 ^{a,D}	35.0 ± 0.5 ^{b,C}	33.4 ± 0.8 ^{b,C}	33.9 ± 0.6 ^{a,B}	25.2 ± 0.5 ^{b,B}	20.0 ± 0.7 ^{c,B}
	<i>A. dealbata</i> (COR)	65.8 ± 2.1 ^{a,A}	45.2 ± 2.1 ^{b,A}	42.8 ± 1.0 ^{c,A}	37.8 ± 0.2 ^{a,A}	27.3 ± 0.2 ^{b,A}	23.1 ± 0.8 ^{c,A}
	<i>A. saligna</i>	62.5 ± 1.4 ^{a,B}	31.7 ± 1.6 ^{b,D}	26.2 ± 1.4 ^{c,D}	15.8 ± 0.4 ^{a,D}	8.0 ± 0.2 ^{b,D}	7.4 ± 0.2 ^{b,D}

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 x 3); different letters (lower case a-c) indicate significant differences of means among solvents for each sample of fresh and spent flowers (one-way ANOVA, Tukey test at p<0.05); different letters (upper case A-C) indicate significant differences of means within flower samples of fresh and extracted flowers, for each extraction solvent.

A.2 Chromatograms

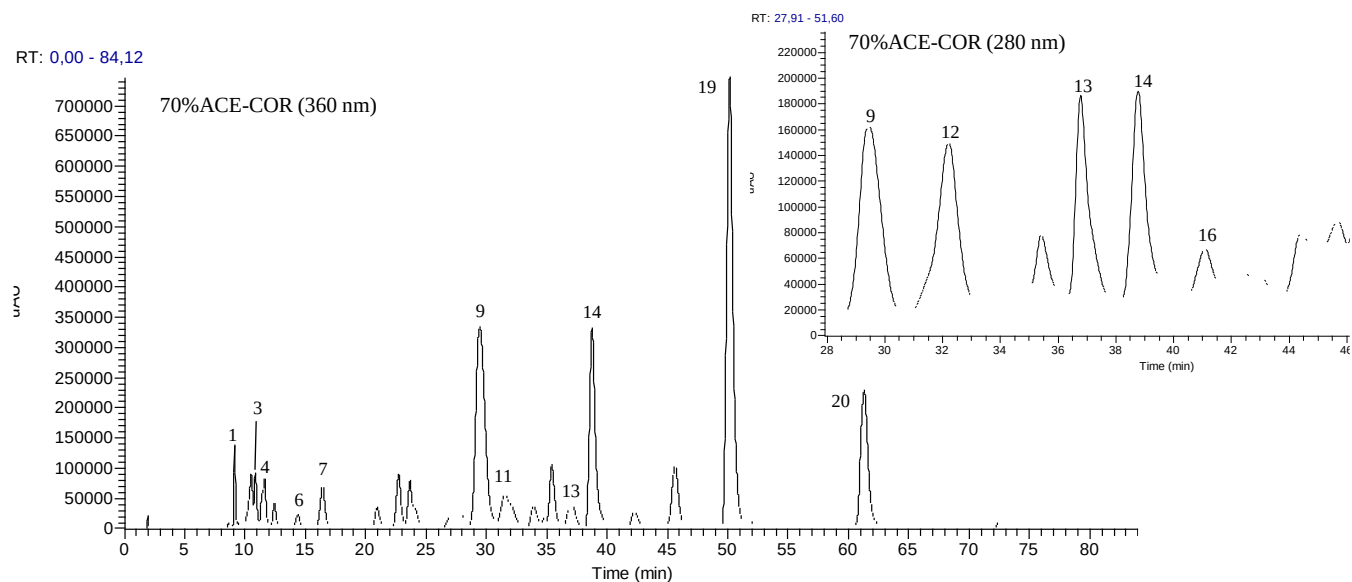


Figure A1 — Chromatographic profile at 360 nm for 70% ACE extract of fresh flowers of *A. dealbata* from Coruche at 360 nm; the insert is a part of the chromatographic profile at 280 nm, to illustrate some peaks not easily visible at 360 nm.

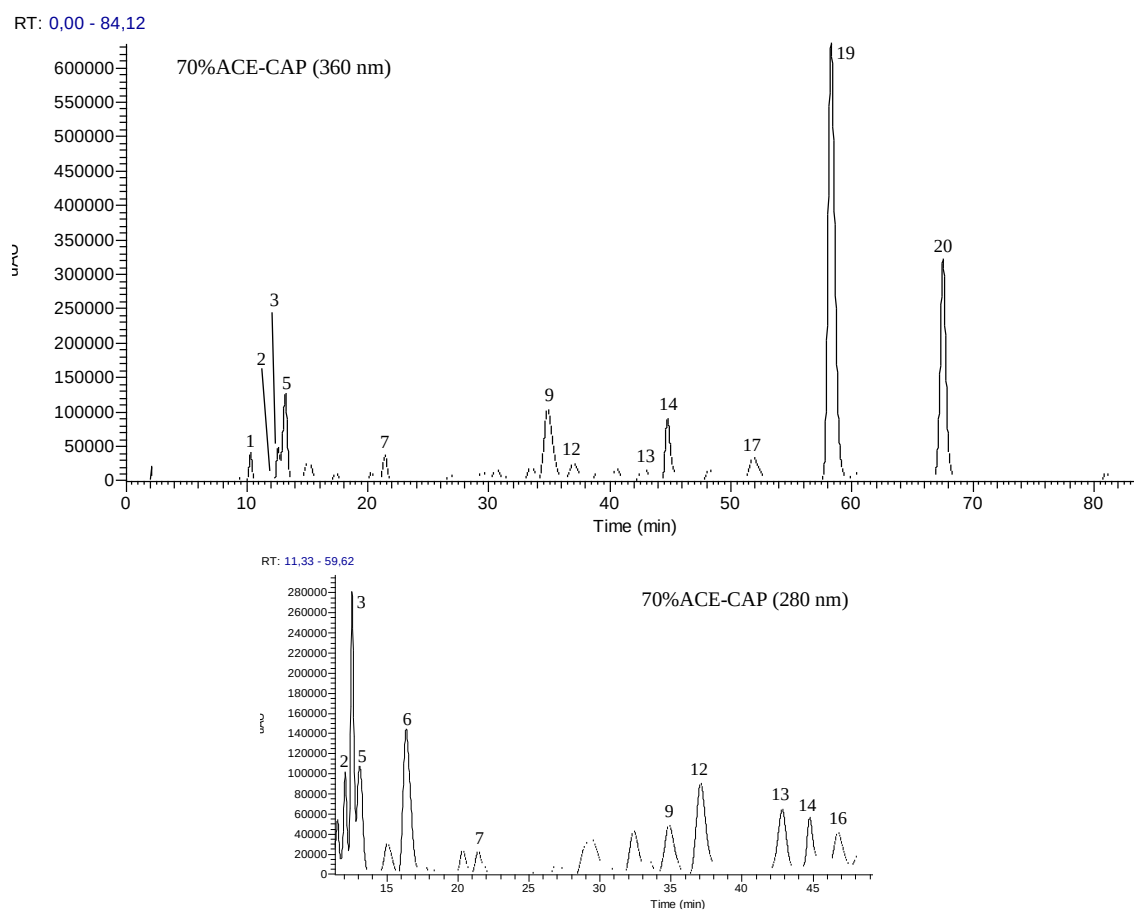


Figure A2 — Chromatographic profile at 360 nm for 70% ACE extract of fresh flowers of *A. dealbata* from Caparica at 360 nm (top); a part of the chromatographic profile at 280 nm, to illustrate some peaks not easily visible at 360 nm (bottom).

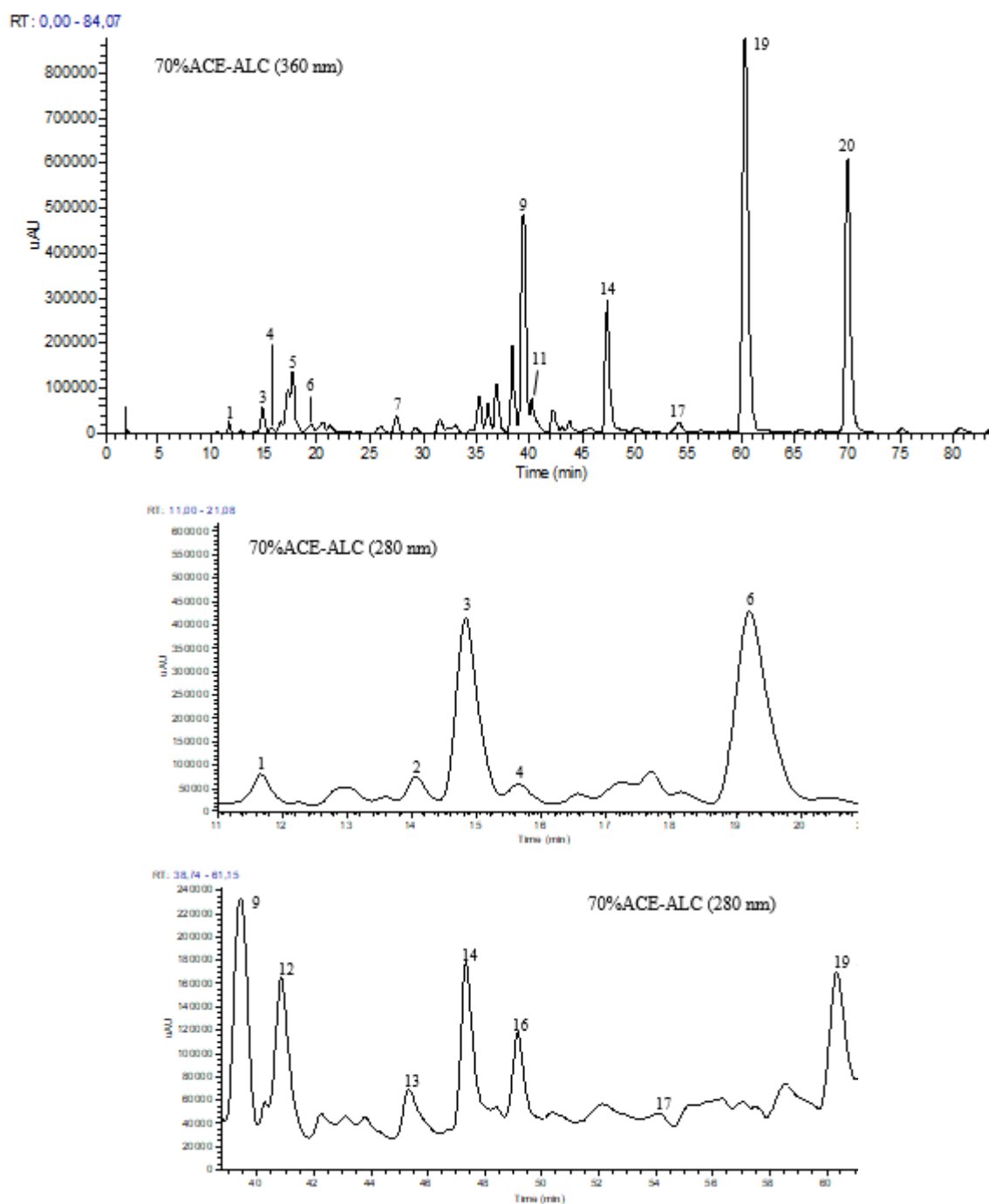


Figure A3 — Chromatographic profile at 360 nm for 70% ACE extract of fresh flowers of *A. dealbata* from Alco-baça at 360 nm(top); a part of the chromatographic profile at 280 nm, to illustrate some peaks not easily visible at 360 nm (middle and bottom).

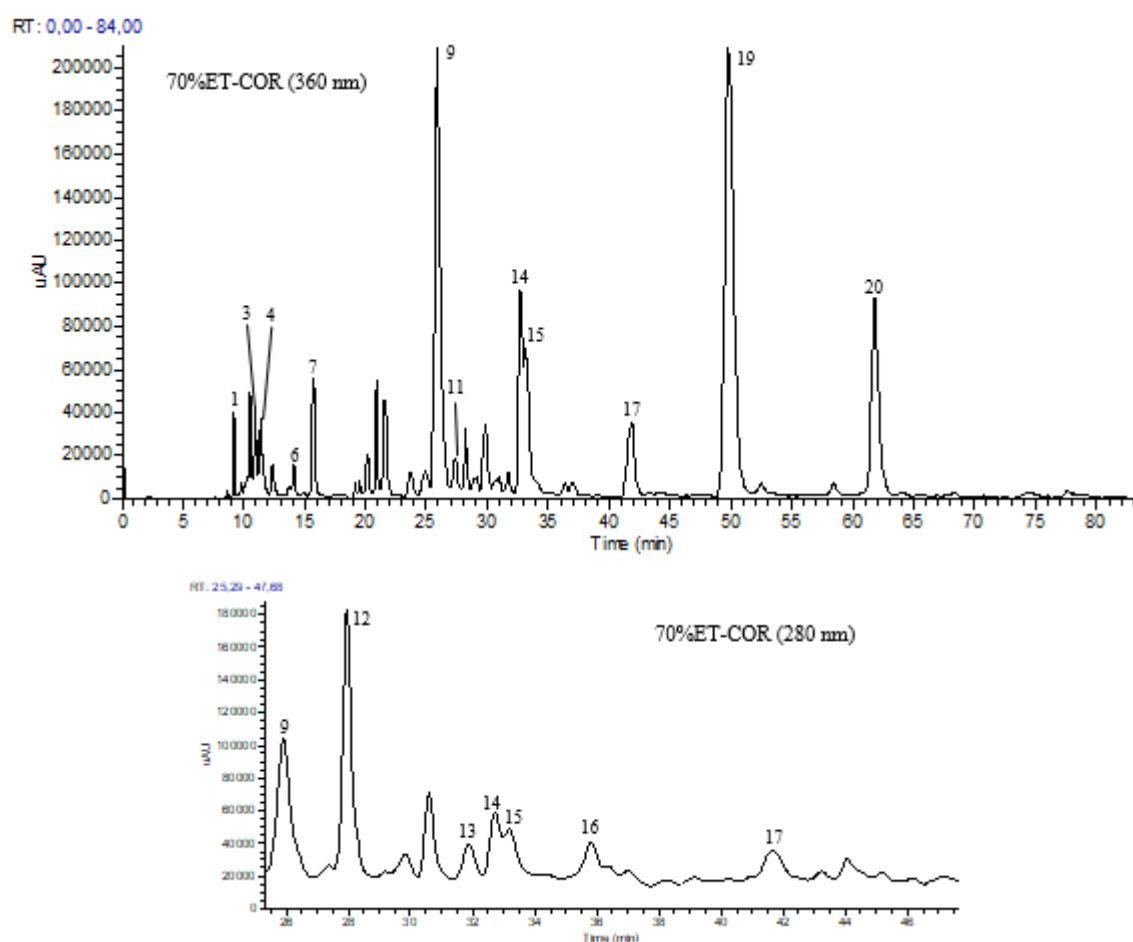


Figure A4 — Chromatographic profile at 360 nm for 70% ET extract of fresh flowers of *A. dealbata* from Coruche at 360 nm(top); a part of the chromatographic profile at 280 nm, to illustrate some peaks not easily visible at 360 nm (bottom).

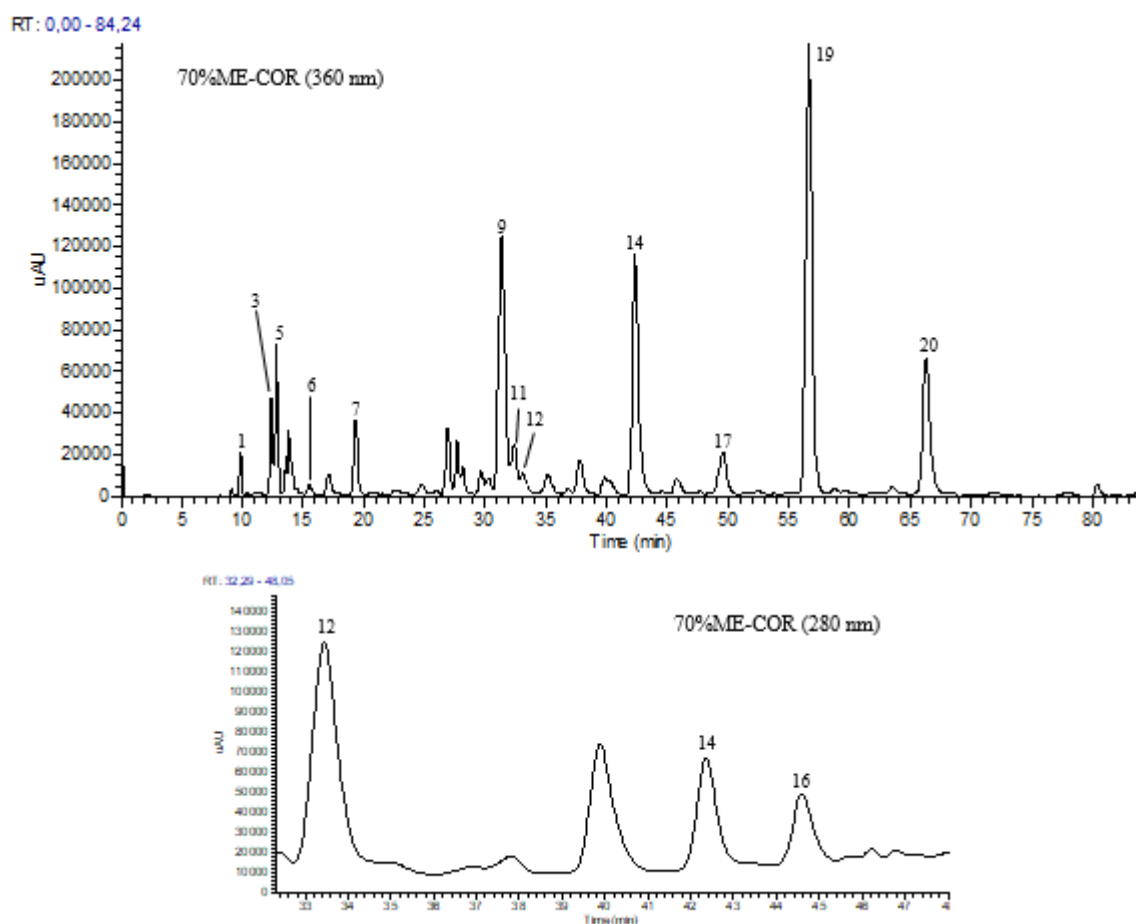


Figure A5 — Chromatographic profile at 360 nm for 70% MET extract of fresh flowers of *A. dealbata* from Coruche at 360 nm(top); a part of the chromatographic profile at 280 nm, to illustrate some peaks not easily visible at 360 nm (bottom).

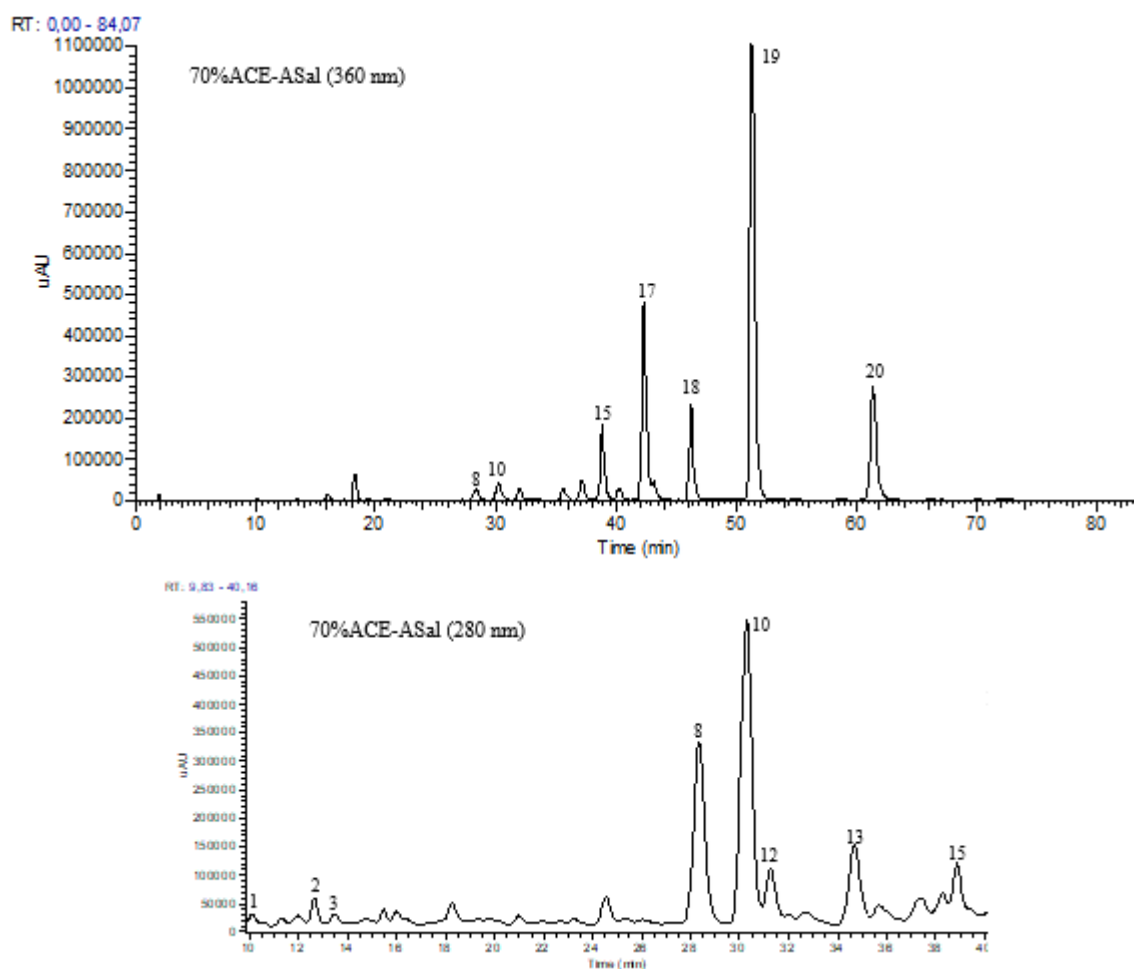


Figure A6 — Chromatographic profile at 360 nm for 70% ACE extract of fresh flowers of *A. saligna* at 360 nm(top); a part of the chromatographic profile at 280 nm, to illustrate some peaks not easily visible at 360 nm (bottom).

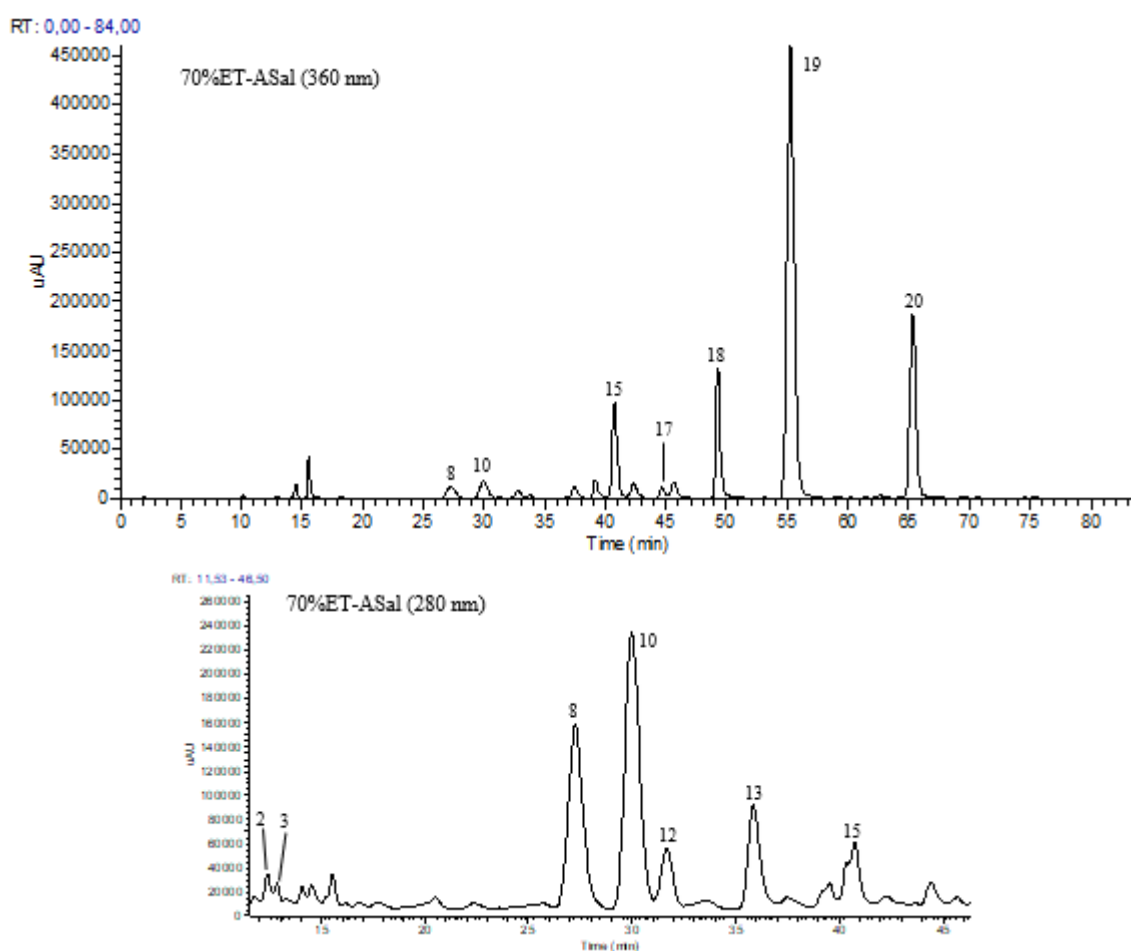


Figure A7 — Chromatographic profile at 360 nm for 70% ET extract of fresh flowers of *A. saligna* at 360 nm(top); a part of the chromatographic profile at 280 nm, to illustrate some peaks not easily visible at 360 nm (bottom).

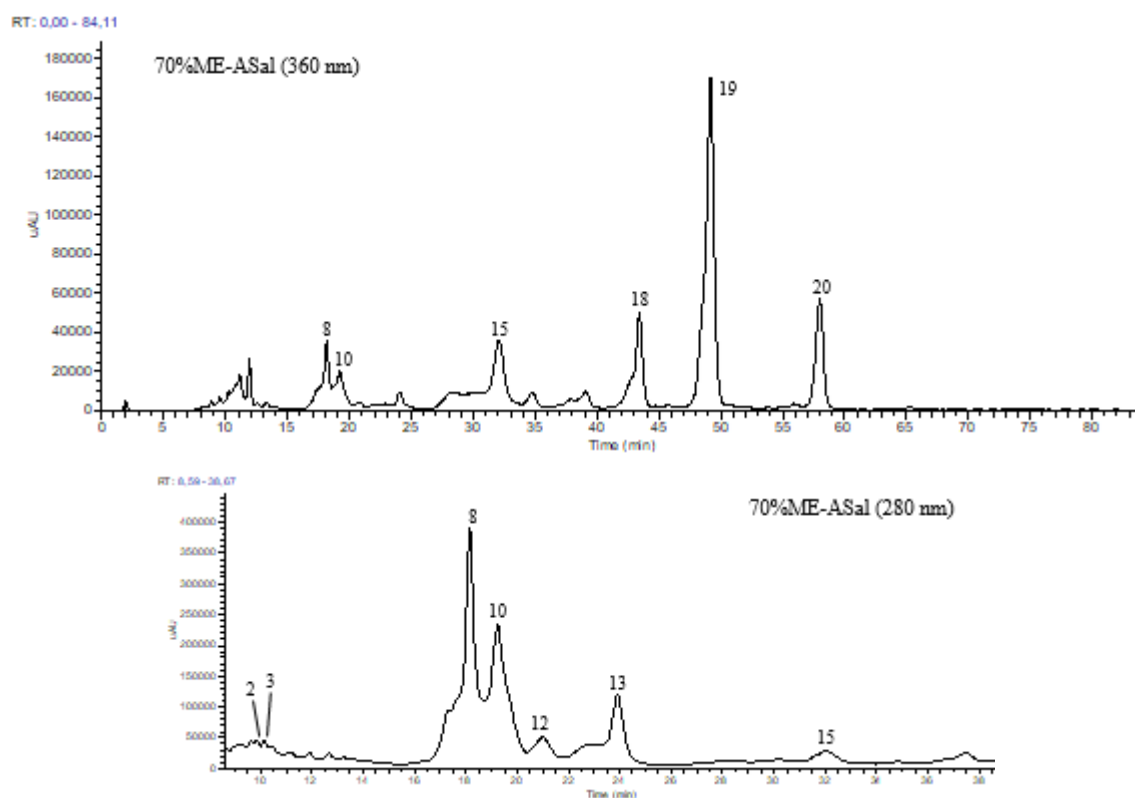


Figure A8 — Chromatographic profile at 360 nm for 70% MET extract of fresh flowers of *A. saligna* at 360 nm(top); a part of the chromatographic profile at 280 nm, to illustrate some peaks not easily visible at 360 nm (bottom).

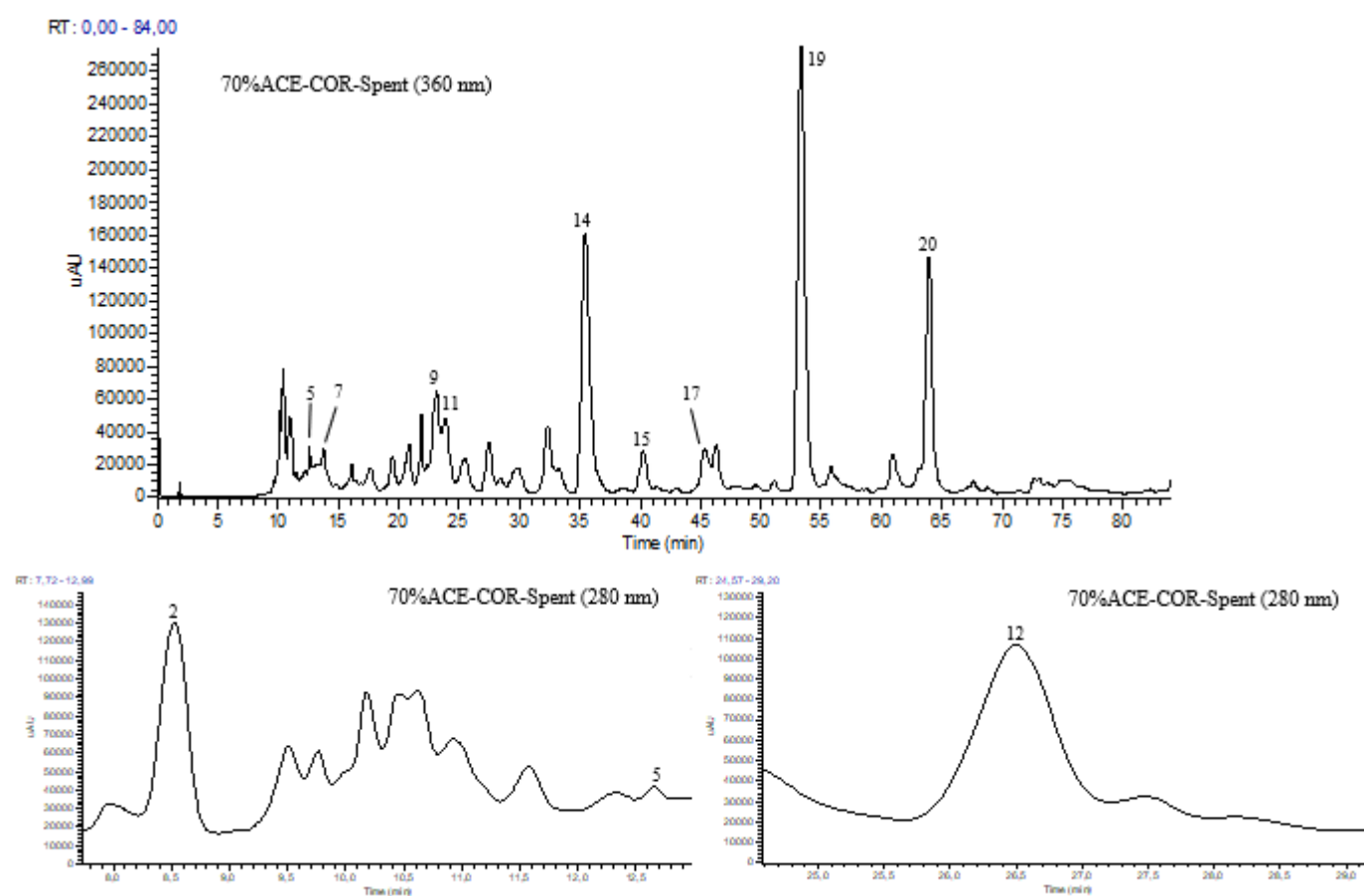


Figure A9 — Chromatographic profile at 360 nm for 70% ACE extract of spent flowers of *A. dealbata* from Coruche at 360 nm; the insert is a part of the chromatographic profile at 280 nm, to illustrate some peaks not easily visible at 360 nm.

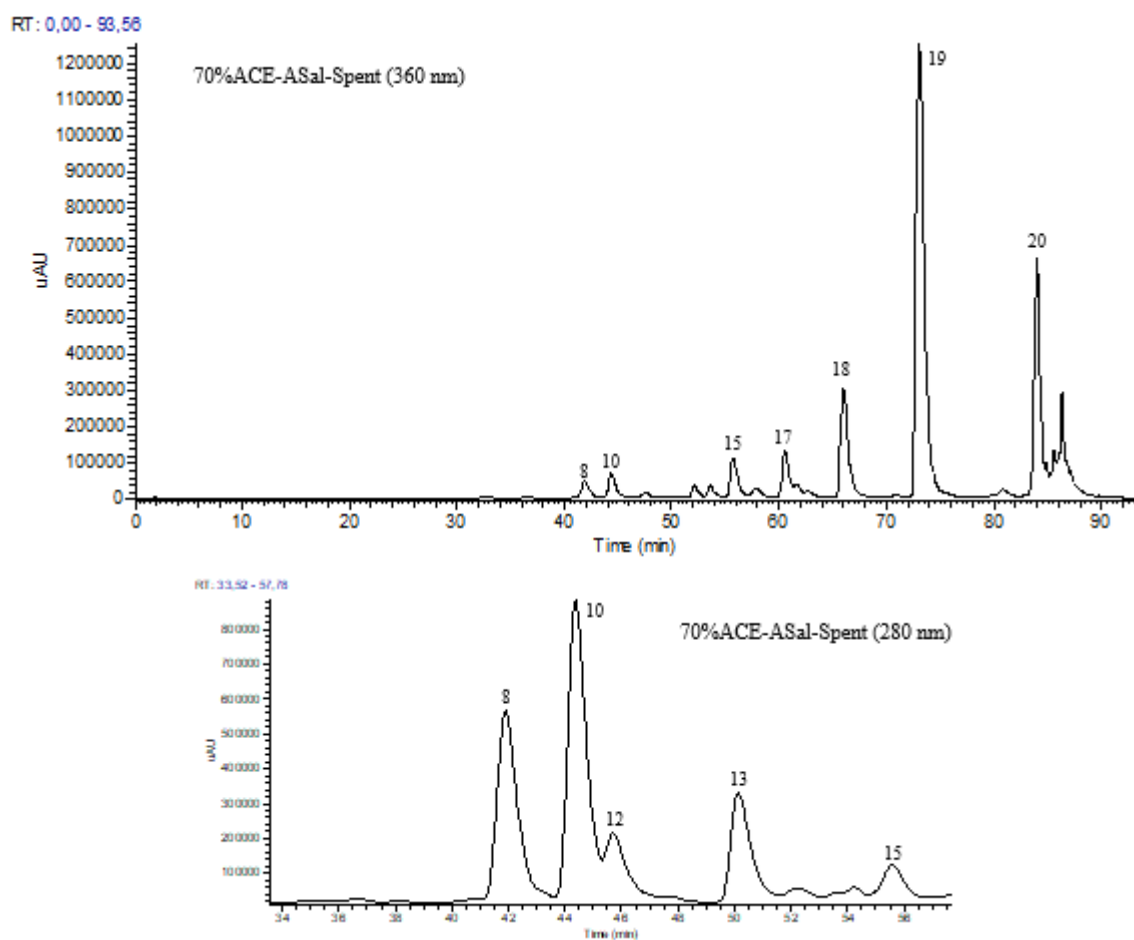


Figure A10 — Chromatographic profile at 360 nm for 70% ACE extract of spent flowers of *A. saligna* at 360 nm; the insert is a part of the chromatographic profile at 280 nm, to illustrate some peaks not easily visible at 360 nm.

A.3 Peak areas

Table A5 — Individual peak areas of the identified compounds in the HPLC-DAD chromatograms The wavelength considered for each compound was the one that gave the highest peak area.

No.	Class	Compound	λ (nm)	Sample									
				<i>A. dealbata</i>					<i>A. saligna</i>				
				fresh		spent			fresh		spent		
				COR-70% ACE	COR-70% ET	COR-70% MET	ALC-70% ACE	CAP-70% ACE	COR-70% ACE	70% ACE	70% ET	70% MET	70% ACE
1	Terpene	Cuminoid C	320	3.72	2.48	1.87	1.19	3.36	0.92	0.57	nd	nd	nd
2	Flavanol	Catechin	280	nd	nd	1.30	0.81	1.46	nd	0.80	1.25	0.84	nd
3	Hydroxycinnamic acid glycoside	p-coumaric acid O-hexoside I	320	19.81	21.03	22.84	20.28	15.61	nd	1.52	1.92	1.76	nd
4	Hydroxycinnamic acid glycoside	p-coumaric acid O-hexoside II	320	3.62	3.65	5.13	1.62	6.06	nd	nd	nd	nd	nd
5	Flavone glycoside	quercetin O-rhamnosylhexo- side-O-hexoside	360	nd	nd	0.68	2.12	5.21	0.89	nd	nd	nd	nd
6		Unknown	280	3.01	3.99	4.31	9.43	4.84	nd	nd	nd	nd	nd
7	Flavone glycoside	Myricetin O-rutinoside I	360	1.88	2.34	2.63	0.87	1.48	1.45	nd	nd	nd	nd
8	Flavanone glyco- side	Naringenin C-hexoside I	280	nd	nd	nd	nd	nd	nd	8.21	17.01	30.23	11.43
9	Flavone glycoside	Myricetin O-rutinoside II	360	17.53	17.43	14.98	14.24	8.43	4.44	nd	nd	nd	nd
10	Flavanone glyco- side	Naringenin C-hexoside II	280	nd	nd	nd	nd	nd	nd	13.90	24.94	20.18	16.27
11	Flavone glycoside	Myricetin O-hexoside	360	3.52	1.06	2.20	1.98	nd	2.88	nd	nd	nd	nd

Table A3 (cont) — Individual peak areas of the identified compounds in the HPLC-DAD chromatograms The wavelength considered for each compound was the one that gave the highest peak area.

No.	Class	Compound	λ (nm)	Sample									
				<i>A. dealbata</i>					<i>A. saligna</i>				
				fresh		spent			fresh		spent		
				COR-70% ACE	COR-70% ET	COR-70% MET	ALC-70% ACE	CAP-70% ACE	COR-70% ACE	70% ACE	70% ET	70% MET	70% ACE
12	Flavanone glycoside	Naringenin O-hexoside I	280	4.48	7.88	10.93	3.08	4.25	4.10	2.07	4.20	2.24	3.31
13	Flavanone glycoside	Naringenin O-hexoside II	280	3.37	1.31	nd	1.01	2.37	nd	3.64	7.85	11.61	6.58
14	Flavone glycoside	Rutin	360	10.83	5.88	12.96	7.64	4.62	15.18	nd	nd	nd	nd
15	Flavone glycoside	Quercetin O-hexoside (glucoside)	360	nd	5.48	nd	nd	nd	2.05	5.66	7.25	9.26	3.47
16	Flavanone glycoside	Naringenin O-pentoside	280	0.89	1.84	2.62	1.64	1.26	nd	nd	nd	nd	nd
17		Unknown	360	3.79	3.90	2.88	0.88	2.75	2.33	16.14	0.80	nd	3.76
18	Flavone glycoside	Quercetin O-rhamnoside	360	nd	nd	nd	nd	nd	nd	7.41	8.63	10.07	9.89
19	Chalcone	Isosalipurposide (chalcononaringenin 2'-glucoside)	360	27.04	27.94	25.25	30.16	40.61	22.81	44.20	50.68	37.41	44.88
20	Chalcone	Naringenin chalcone O-pentoside	360	9.72	9.90	9.38	18.41	17.34	9.92	13.05	16.78	11.32	16.85

APPENDIX (CHAPTER 4)

B.1 Tables

Table B1 — Total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanidin content (TPrAC), expressed relative to the weight of dry extract and to the weight of fresh flower.

Sample	TPC (mg GAE/g dry extract)	TPC (mg GAE/g fresh flower)	TFC (mg CatE/g dry extract)	TFC (mg CatE/g fresh flower)	TPrAC (mg PycE/g dry extract)	TPrAC (mg PycE/g fresh flower)
Maceration						
PE/ACE-CAP	287.2 ± 6.2 ^c	13.9 ± 0.3 ^e	139.4 ± 5.7 ^c	6.8 ± 0.3 ^c	376.8 ± 5.6 ^b	18.3 ± 0.2 ^{b,c}
PE/ACE-ALC	259.5 ± 10.5 ^d	17.1 ± 0.5 ^c	144.8 ± 7.0 ^{b,c}	9.5 ± 0.4 ^a	406.8 ± 17.1 ^a	26.7 ± 0.8 ^a
ACE-CAP	344.7 ± 13.5 ^b	11.9 ± 0.4 ^g	155.8 ± 7.9 ^{a,b}	5.4 ± 0.2 ^d	314.7 ± 13.7 ^d	10.8 ± 0.4 ^d
ACE-ALC	546.8 ± 15.8 ^a	24.0 ± 0.3 ^a	162.9 ± 8.3 ^a	7.2 ± 0.4 ^c	403.9 ± 15.6 ^a	17.8 ± 0.4 ^c
Soxhlet						
PE/ACE-CAP	284.0 ± 7.1 ^c	16.0 ± 0.6 ^d	127.9 ± 4.2 ^d	7.2 ± 0.2 ^c	341.7 ± 13.2 ^c	19.3 ± 0.6 ^b
PE/ACE-ALC	235.6 ± 2.2 ^e	13.0 ± 0.6 ^f	107.4 ± 3.0 ^e	5.9 ± 0.5 ^d	100.2 ± 11.9 ^f	5.5 ± 0.8 ^f
ACE-CAP	177.1 ± 7.1 ^f	7.2 ± 0.5 ^h	107.9 ± 4.9 ^e	4.4 ± 0.3 ^e	199.3 ± 8.1 ^e	8.1 ± 0.5 ^e
ACE-ALC	359.6 ± 3.0 ^b	19.3 ± 0.3 ^b	149.0 ± 5.9 ^{b,c}	8.0 ± 0.4 ^b	346.1 ± 7.4 ^c	18.6 ± 0.5 ^{b,c}

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 x 3); different letters indicate significant differences of means (one-way ANOVA, Tukey test at p<0.05)

Table B2 — Total flavonoid content (TFC), expressed as mg of rutin equivalents (RutE), expressed relative to the weight of dry extract and to the weight of fresh flower.

Sample	TFC (mg RutE/g dry ex- tract)	TFC (mg RutE/g fresh flower)
Maceration		
PE/ACE-CAP	427.5 ± 17.6 ^c	20.7 ± 0.8 ^e
PE/ACE-ALC	443.9 ± 21.6 ^d	29.2 ± 1.1 ^c
ACE-CAP	478.1 ± 24.2 ^b	24.2 ± 0.4 ^g
ACE-ALC	499.6 ± 25.3 ^a	22.0 ± 1.2 ^a
Soxhlet		
PE/ACE-CAP	392.1 ± 12.8 ^c	22.1 ± 0.7 ^d
PE/ACE-ALC	329.7 ± 9.4 ^e	18.2 ± 1.5 ^f
ACE-CAP	331.1 ± 15.0 ^f	13.5 ± 0.9 ^h
ACE-ALC	457.0 ± 18.1 ^b	24.5 ± 1.1 ^b

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 x 3); different letters indicate significant differences of means (one-way ANOVA, Tukey test at p<0.05)

Table B3 — Antioxidant activity given by DPPH and FRAP assays, expressed relative to the weight of dry extract and to the weight of fresh flower.

Sample	DPPH (mg TE/g dry extract)	DPPH (mg TE/g fresh flower)	FRAP (μmol Fe(II)/g dry extract)	FRAP (μmol Fe(II)/g fresh flower)
Maceration				
PE/ACE-CAP	795.1 ± 18.2 ^b	38.6 ± 0.8 ^c	9484.1 ± 177.2 ^b	460.1 ± 10.3 ^a
PE/ACE-ALC	632.4 ± 24.4 ^d	41.6 ± 1.1 ^a	7153.2 ± 275.1 ^d	470.0 ± 11.9 ^a
ACE-CAP	661.5 ± 22.7 ^d	22.8 ± 0.6 ^e	8611.5 ± 387.2 ^c	296.5 ± 11.1 ^c
ACE-ALC	904.3 ± 34.9 ^a	39.8 ± 0.9 ^{a,b}	10485.3 ± 409.7 ^a	461.1 ± 10.7 ^a
Soxhlet				
PE/ACE-CAP	492.5 ± 6.6 ^e	27.8 ± 0.2 ^d	6920.6 ± 76.5 ^d	390.6 ± 1.8 ^b
PE/ACE-ALC	480.9 ± 6.4 ^e	26.5 ± 1.9 ^d	5658.5 ± 78.5 ^f	312.2 ± 21.9 ^c
ACE-CAP	475.7 ± 18.9 ^e	19.3 ± 1.2 ^f	6275.3 ± 244.3 ^e	255.1 ± 16.1 ^d
ACE-ALC	724.3 ± 12.6 ^c	38.9 ± 0.4 ^c	8462.8 ± 113.7 ^c	454.3 ± 10.0 ^a

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 x 3); different letters indicate significant differences of means (one-way ANOVA, Tukey test at p<0.05)

B.2 Chromatograms

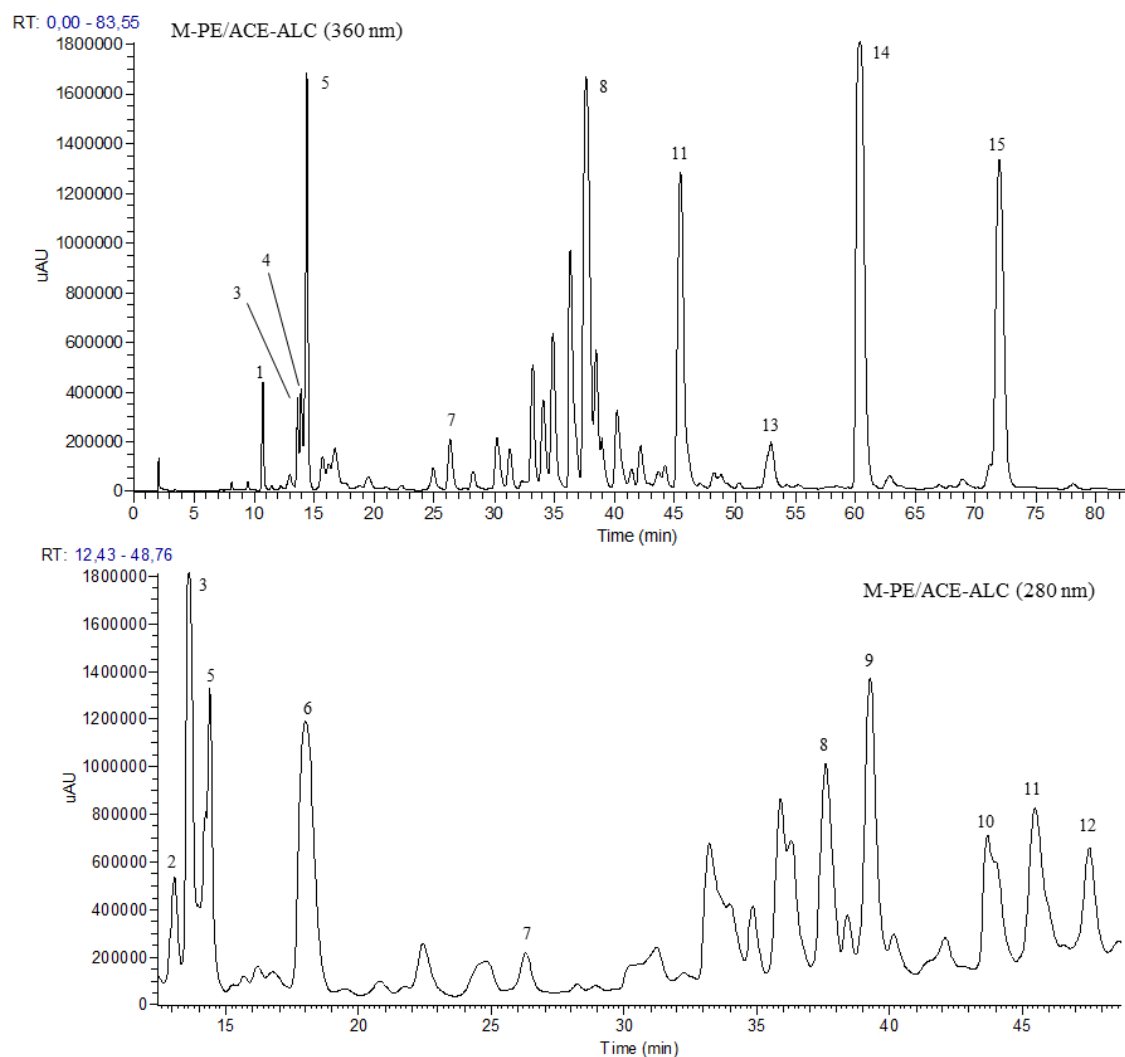
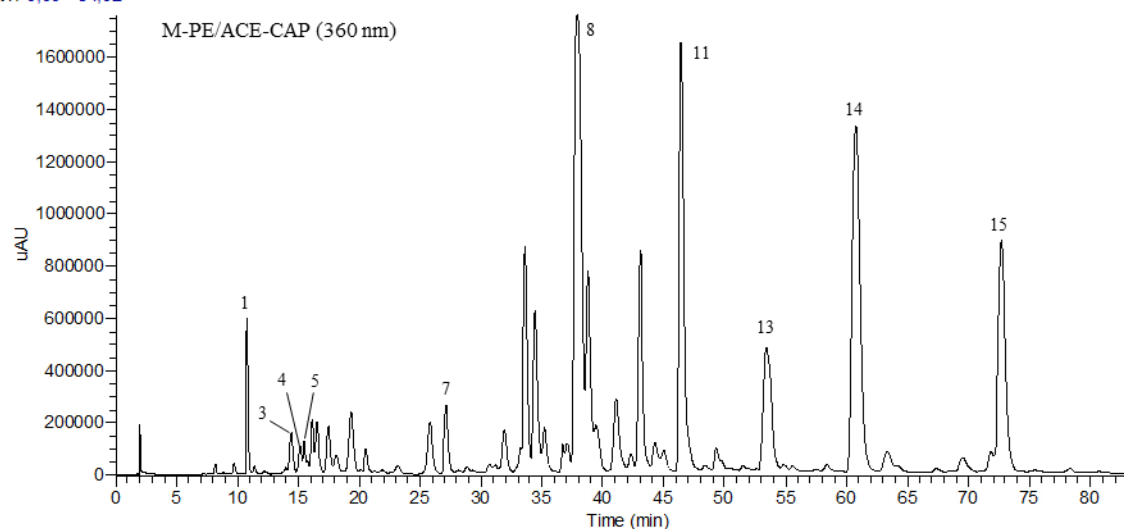


Figure B1 — Chromatographic profiles at 360 nm (and parts of the profile at 280 nm, to illustrate some peaks not easily visible at 360 nm) for PE/ACE extracts obtained by maceration with flowers from Alcobaça

RT: 0.00 - 84.02



RT: 12.64 - 49.69

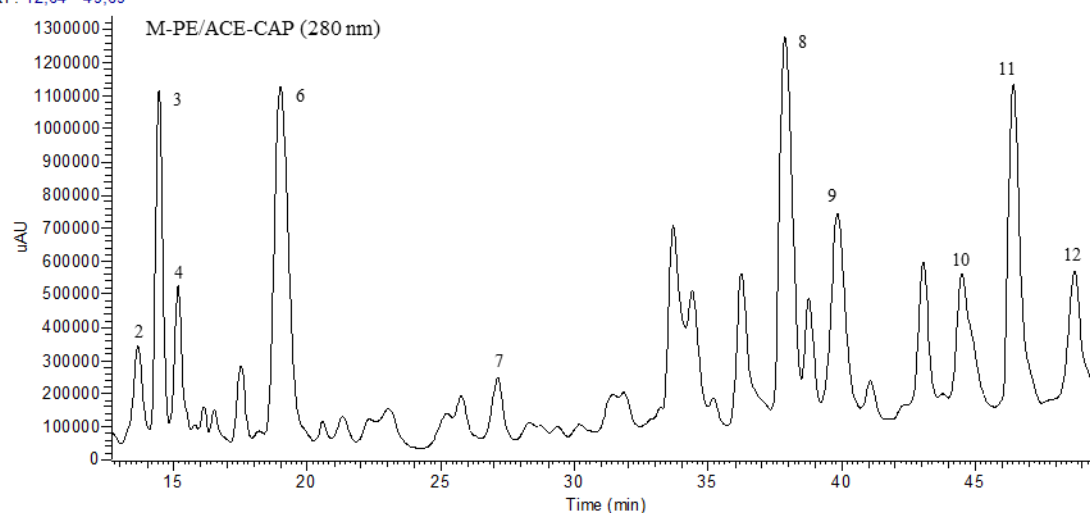
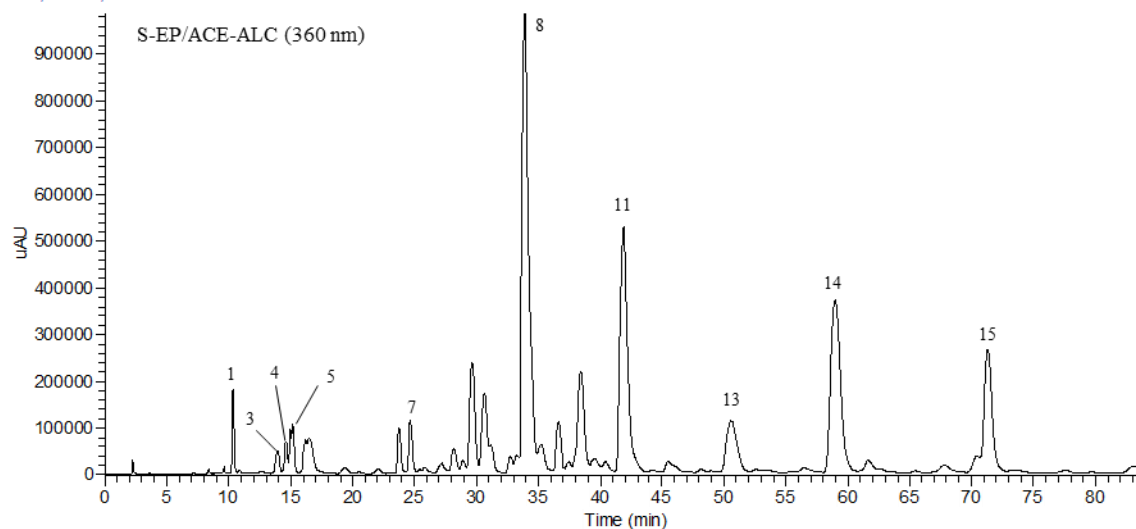


Figure B2 — Chromatographic profiles at 360 nm (and parts of the profile at 280 nm, to illustrate some peaks not easily visible at 360 nm) for PE/ACE extracts obtained by maceration with flowers from *Caparica*

RT: 0,00 - 84,19



RT: 12,14 - 46,05

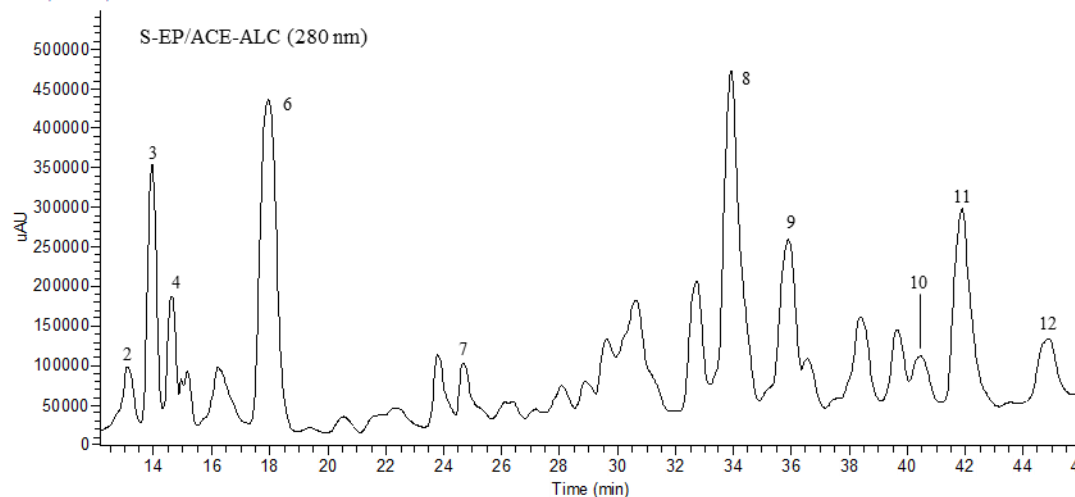
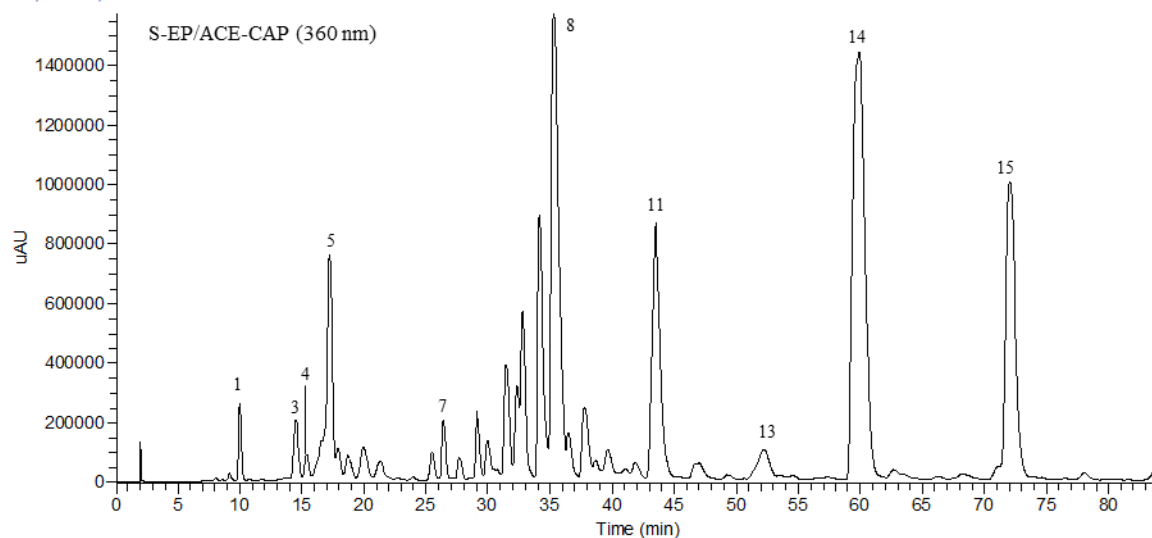


Figure B3 — Chromatographic profiles at 360 nm (and parts of the profile at 280 nm, to illustrate some peaks not easily visible at 360 nm) for PE/ACE extracts obtained by Soxhlet with flowers from Alcobaça

RT: 0.00 - 84.05



RT: 12.15 - 48.00

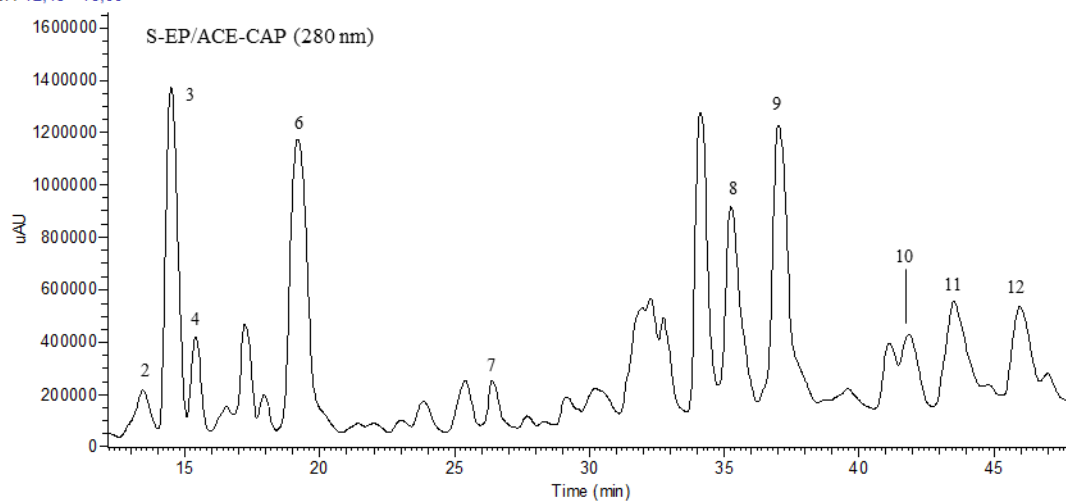


Figure B4 — Chromatographic profiles at 360 nm (and parts of the profile at 280 nm, to illustrate some peaks not easily visible at 360 nm) for PE/ACE extracts obtained by Soxhlet with flowers from Caparica

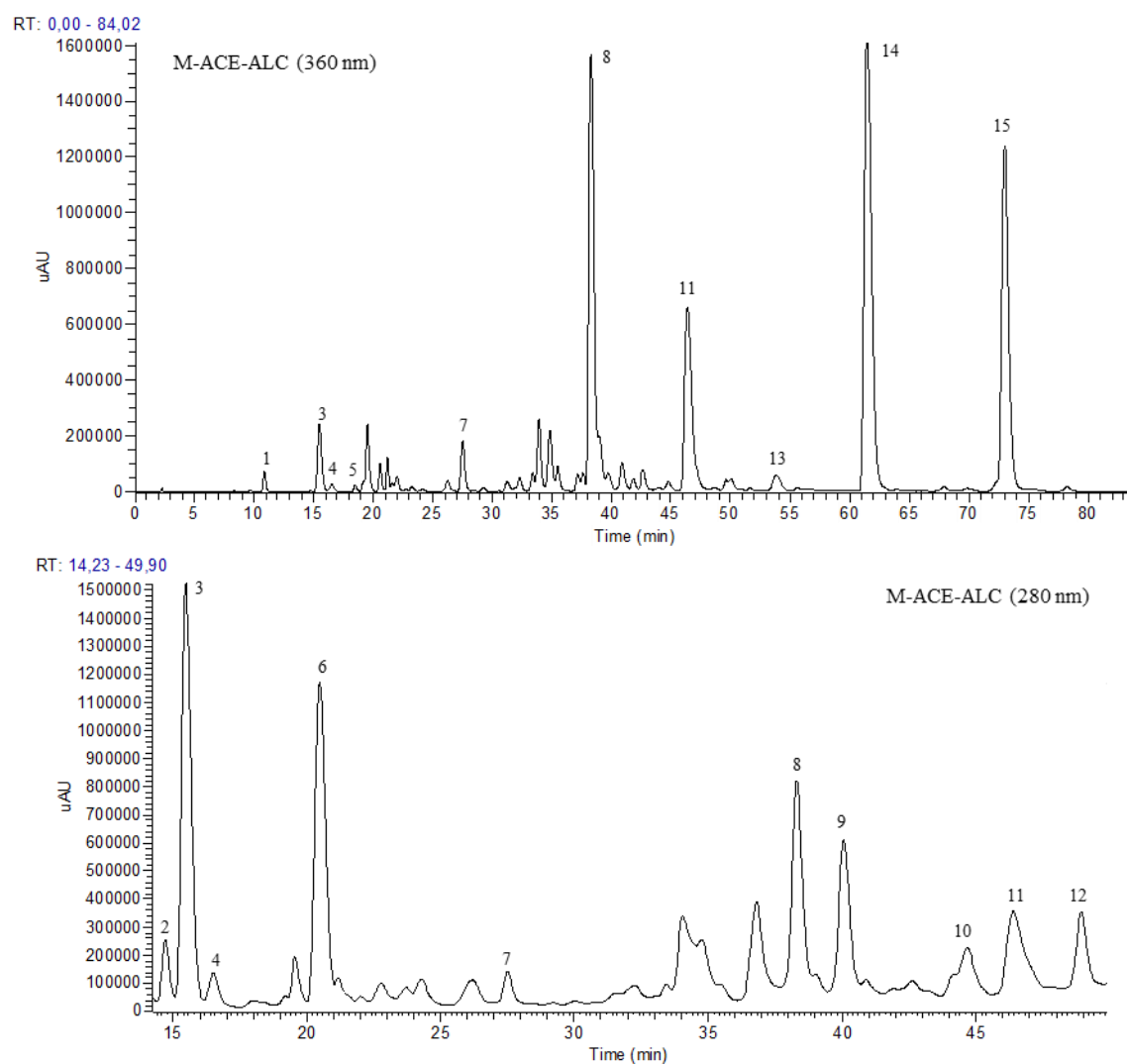
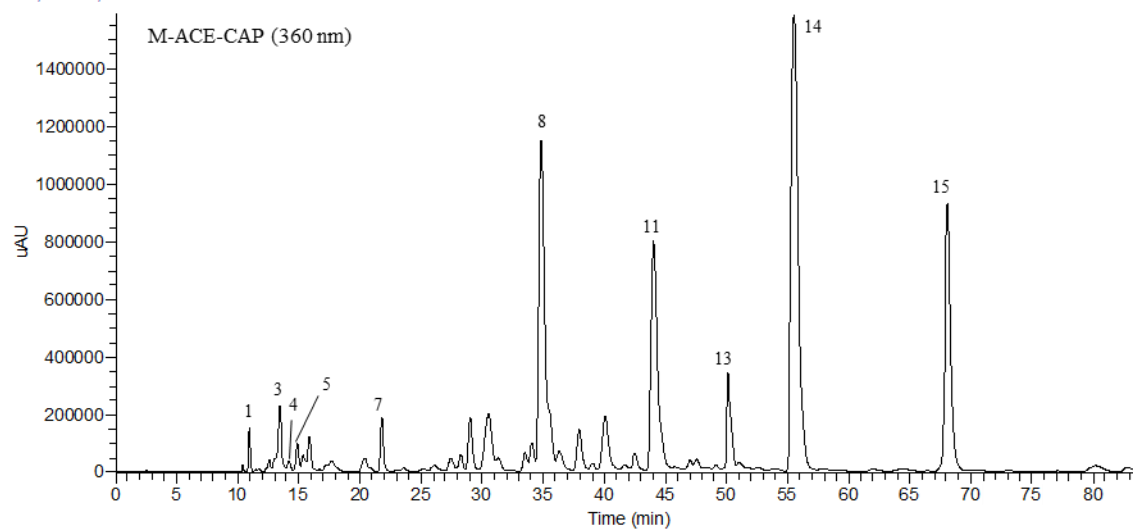


Figure B5 — Chromatographic profiles at 360 nm (and parts of the profile at 280 nm, to illustrate some peaks not easily visible at 360 nm) for ACE extracts obtained by maceration with flowers from Alcobaça

RT: 0,00 - 83,95



RT: 11,97 - 47,66

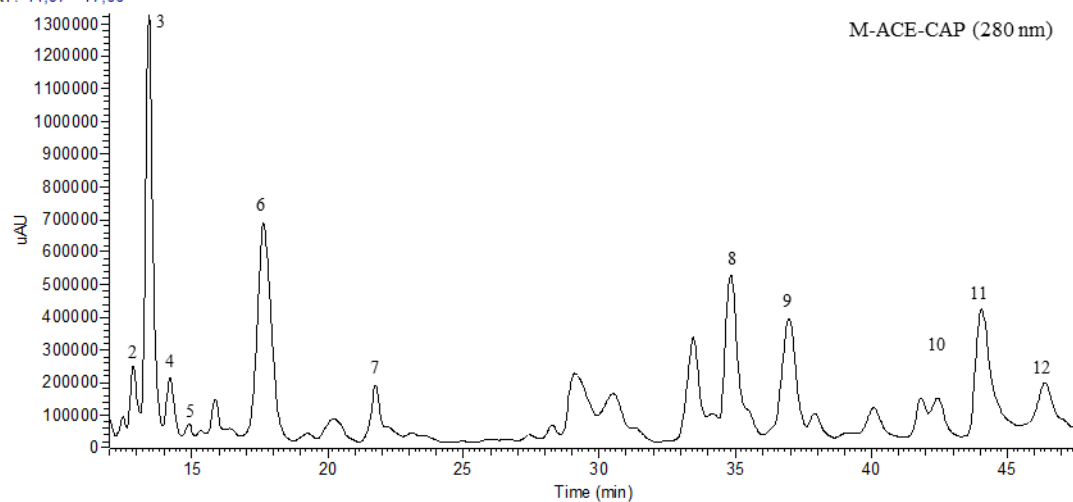


Figure B6 — Chromatographic profiles at 360 nm (and parts of the profile at 280 nm, to illustrate some peaks not easily visible at 360 nm) for ACE extracts obtained by maceration with flowers from Caparica

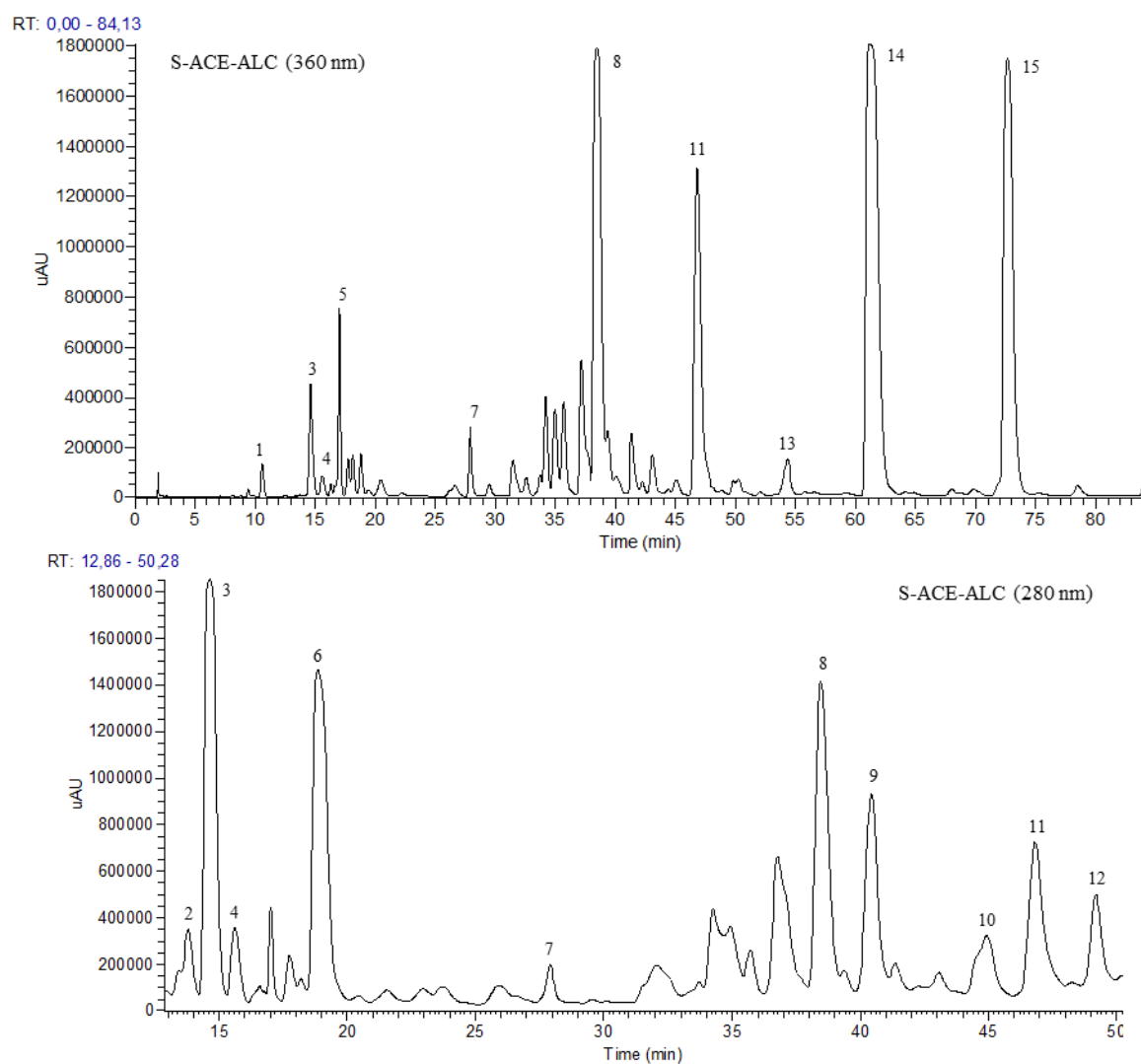
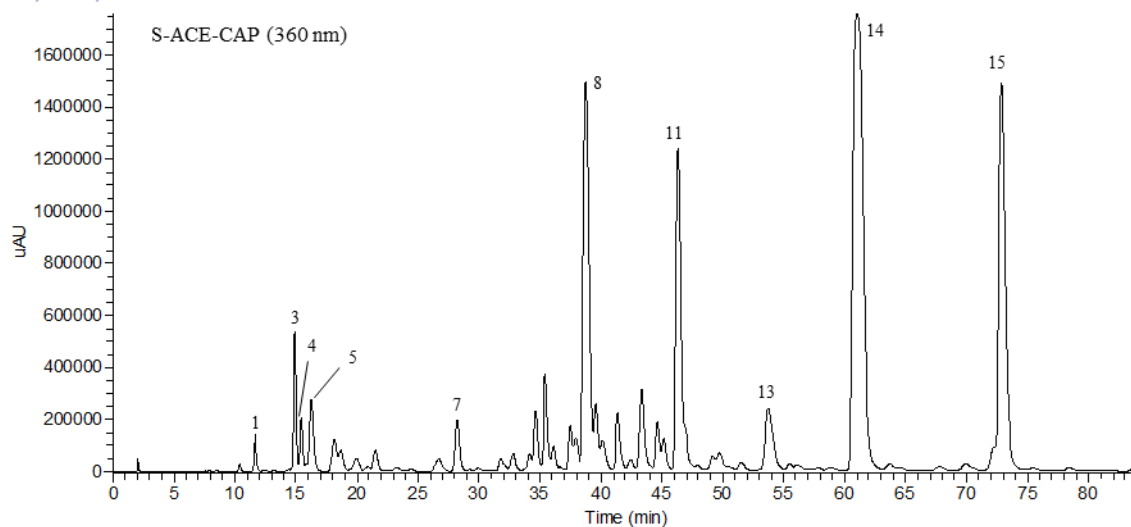


Figure B7 — Chromatographic profiles at 360 nm (and parts of the profile at 280 nm, to illustrate some peaks not easily visible at 360 nm) for ACE extracts obtained by Soxhlet with flowers from Alcobaça

RT: 0.00 - 84.13



RT: 13.76 - 49.56

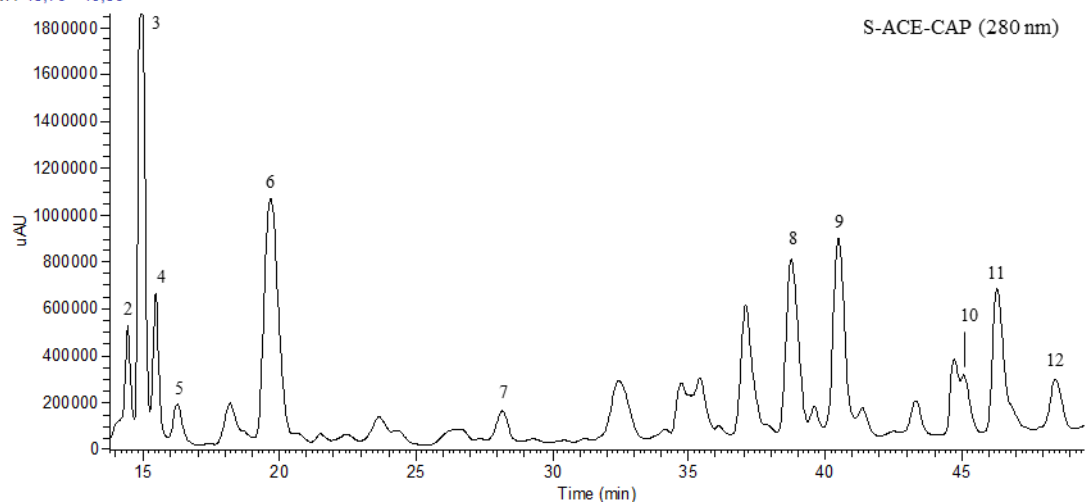


Figure B8 — Chromatographic profiles at 360 nm (and parts of the profile at 280 nm, to illustrate some peaks not easily visible at 360 nm) for ACE extracts obtained by Soxhlet with flowers from *Caparica*

| c

APPENDIX (CHAPTER 5)

C.1 Chromatograms GC-TOFMS

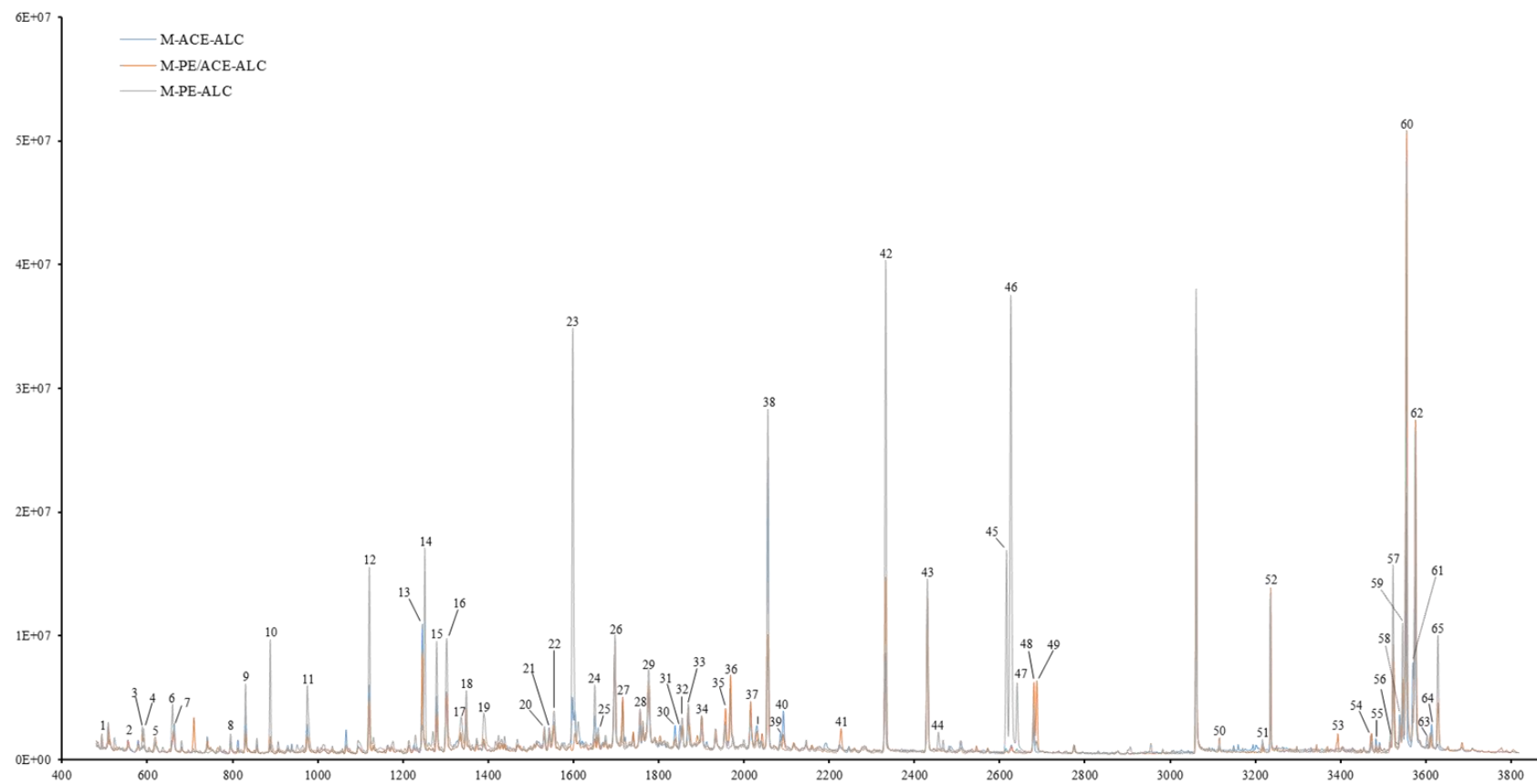


Figure C1 — CG-TOFMS chromatograms (TIC) of absolutes: macerations with 100% acetone (M-ACE-ALC), mixture (1:1) of petroleum ether and acetone (M-PE/ACE-ALC), and 100% petroleum ether (M-PE-ALC), with flowers from Alcobaça.

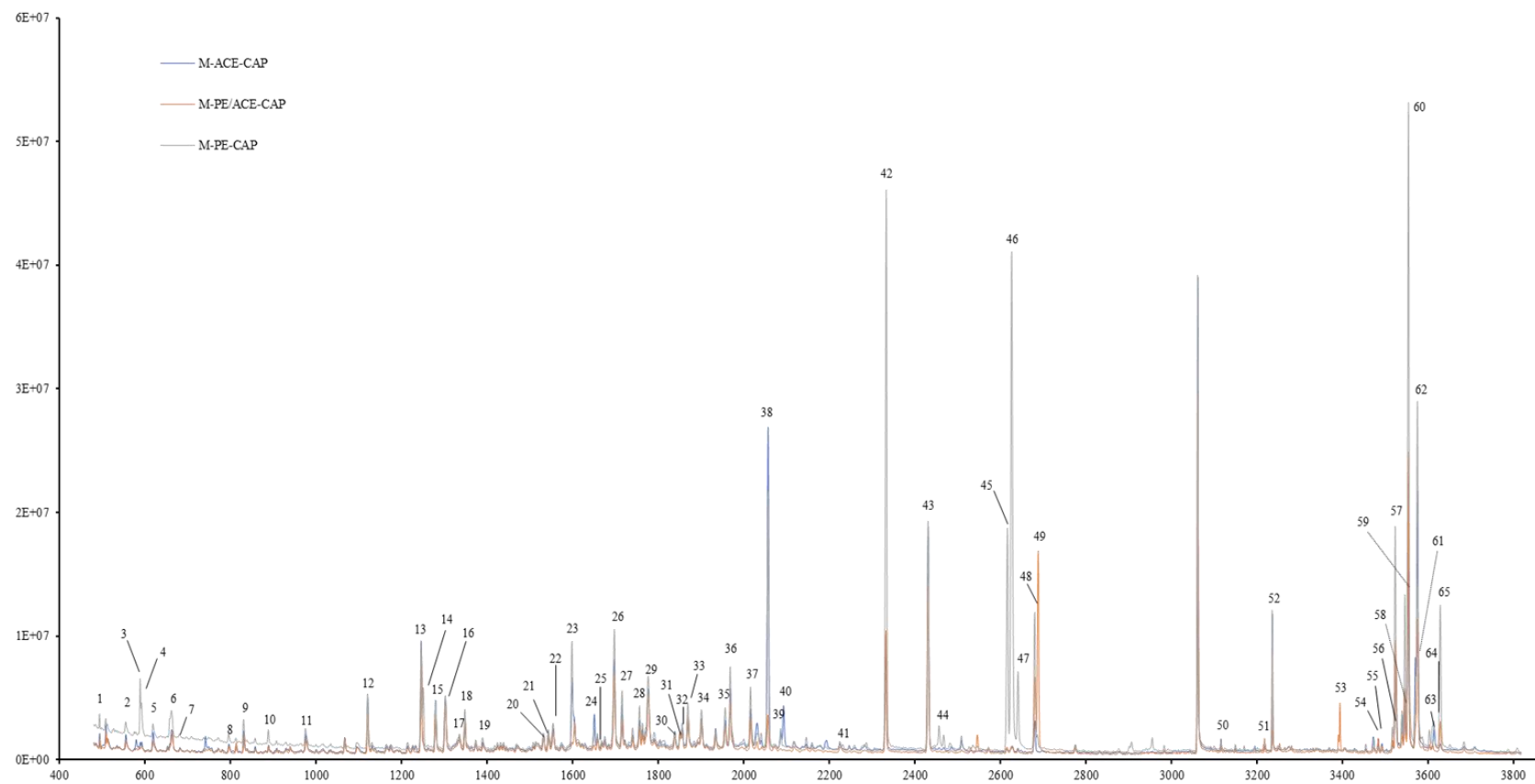


Figure C2 — CG-TOFMS chromatograms (TIC) of absolutes: macerations with 100% acetone (M-ACE-CAP), mixture (1:1) of petroleum ether and acetone (M-PE/ACE-CAP), and 100% petroleum ether (M-PE-CAP), with flowers from Caparica.

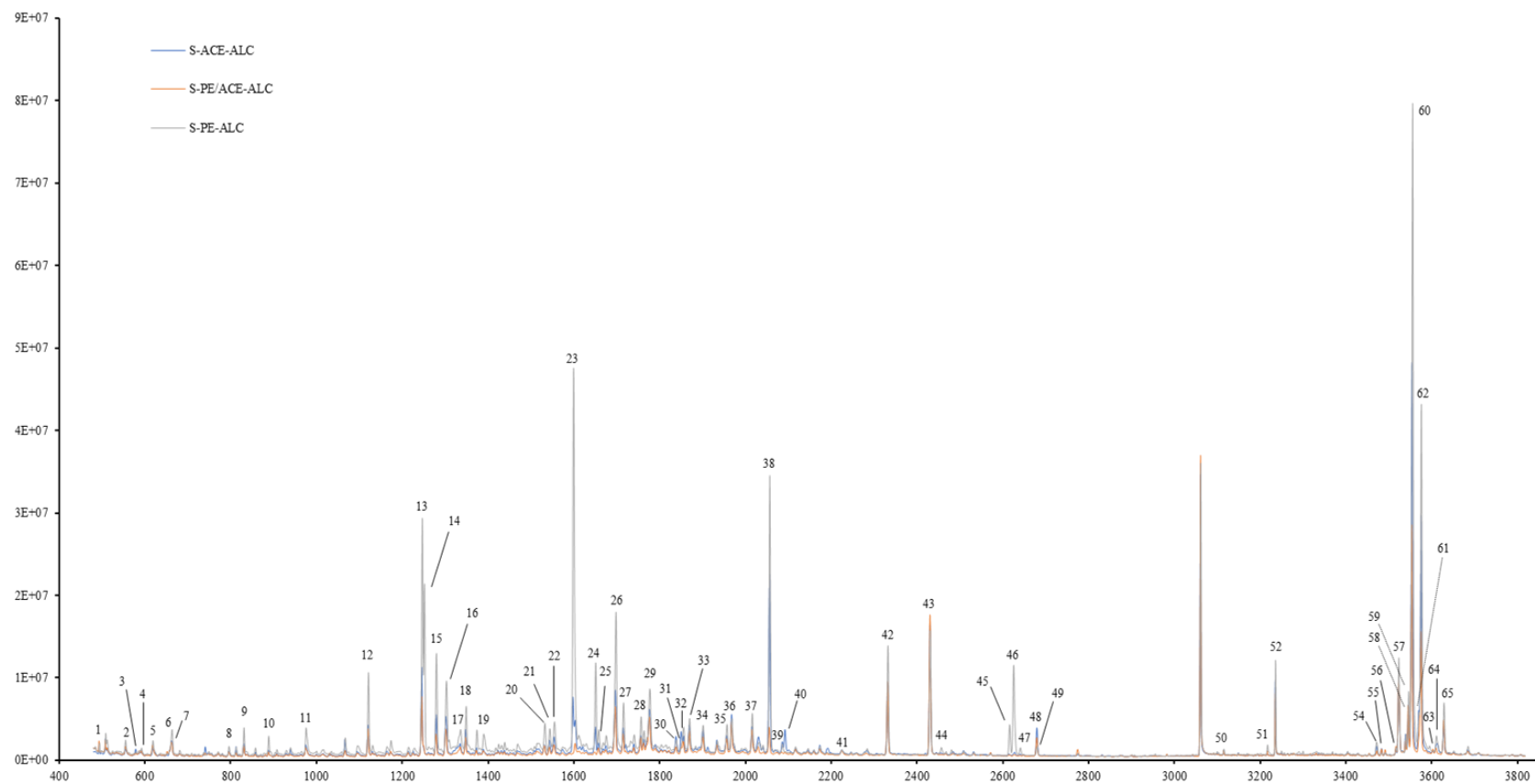


Figure C3 — CG-TOFMS chromatograms (TIC) of absolutes: Soxhlet with 100% acetone (S-ACE-ALC), mixture (1:1) of petroleum ether and acetone (S-PE/ACE-ALC), and 100% petroleum ether (S-PE-ALC), with flowers from Alcobaça.

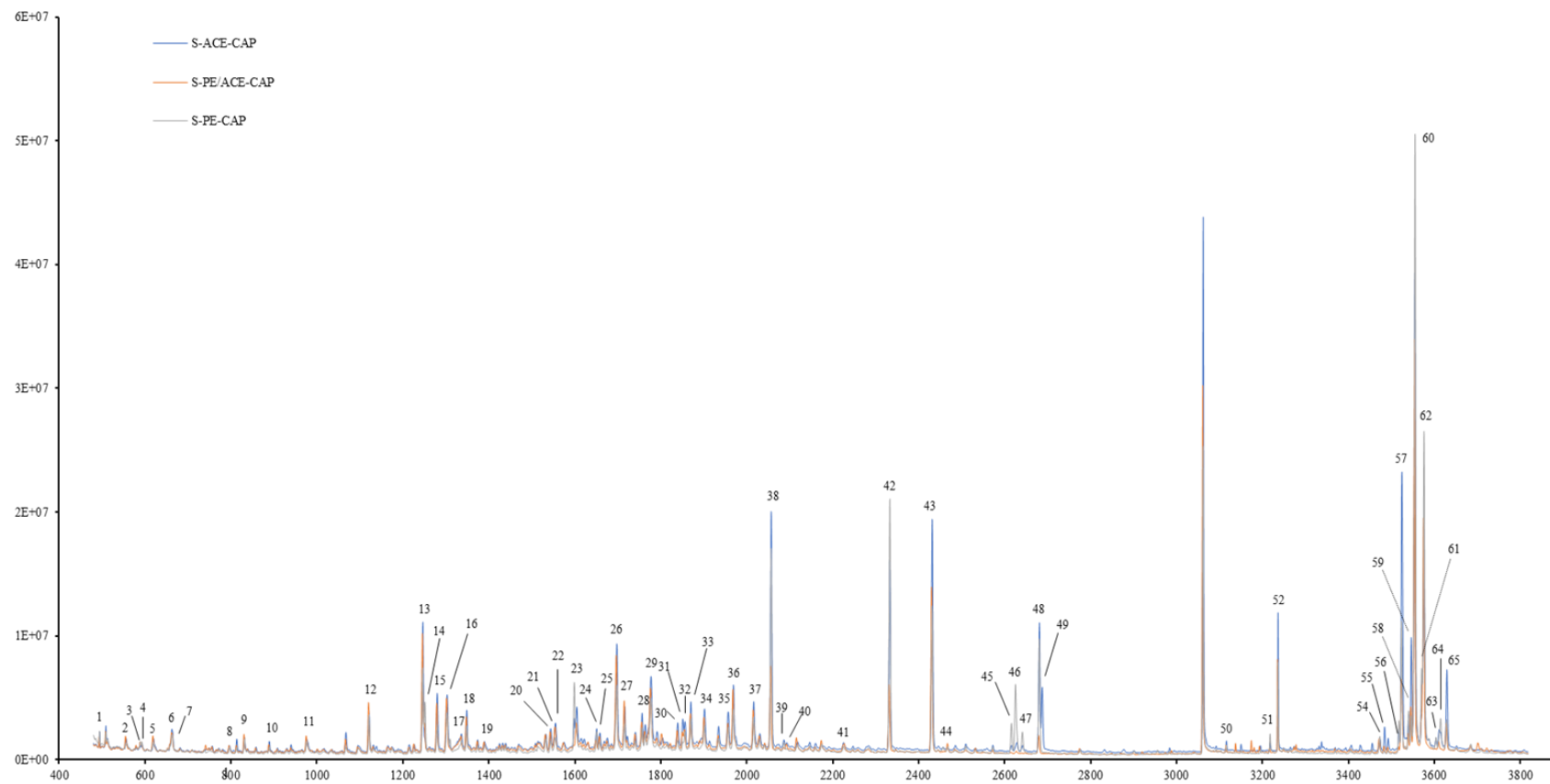


Figure C4 — CG-TOFMS chromatograms (TIC) of absolutes: Soxhlet with 100% acetone (S-ACE-CAP), mixture (1:1) of petroleum ether and acetone (S-PE/ACE-CAP), and 100% petroleum ether (S-PE-CAP), with flowers from Caparica.

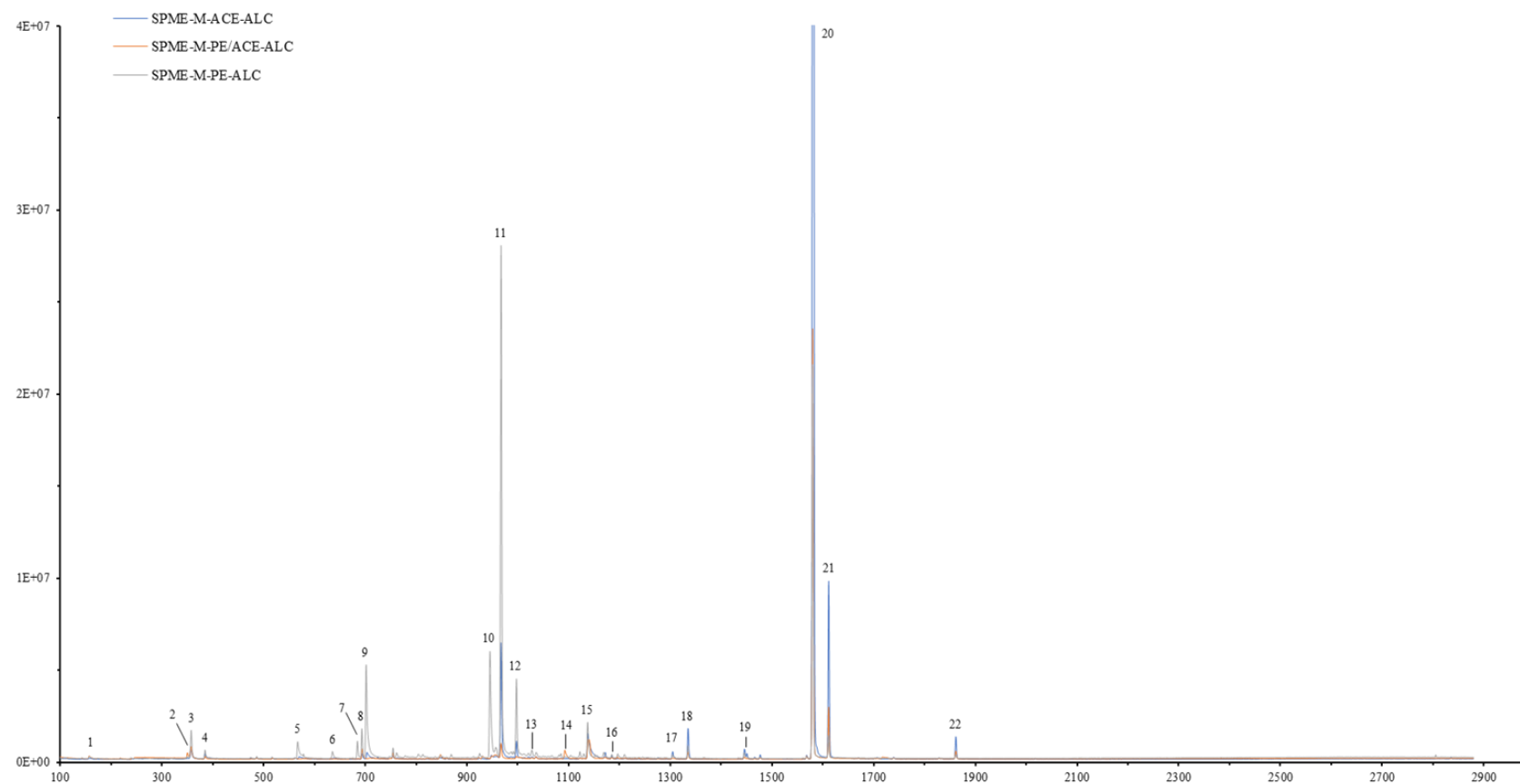


Figure C5 — CG-TOFMS chromatograms (TIC) of absolutes after SPME adsorption: maceration with 100% acetone (SPME-M-ACE-ALC), mixture (1:1) of petroleum ether and acetone (SPME-M-PE/ACE-ALC), and 100% petroleum ether (SPME-M-PE-ALC), with flowers from Alcobaça.

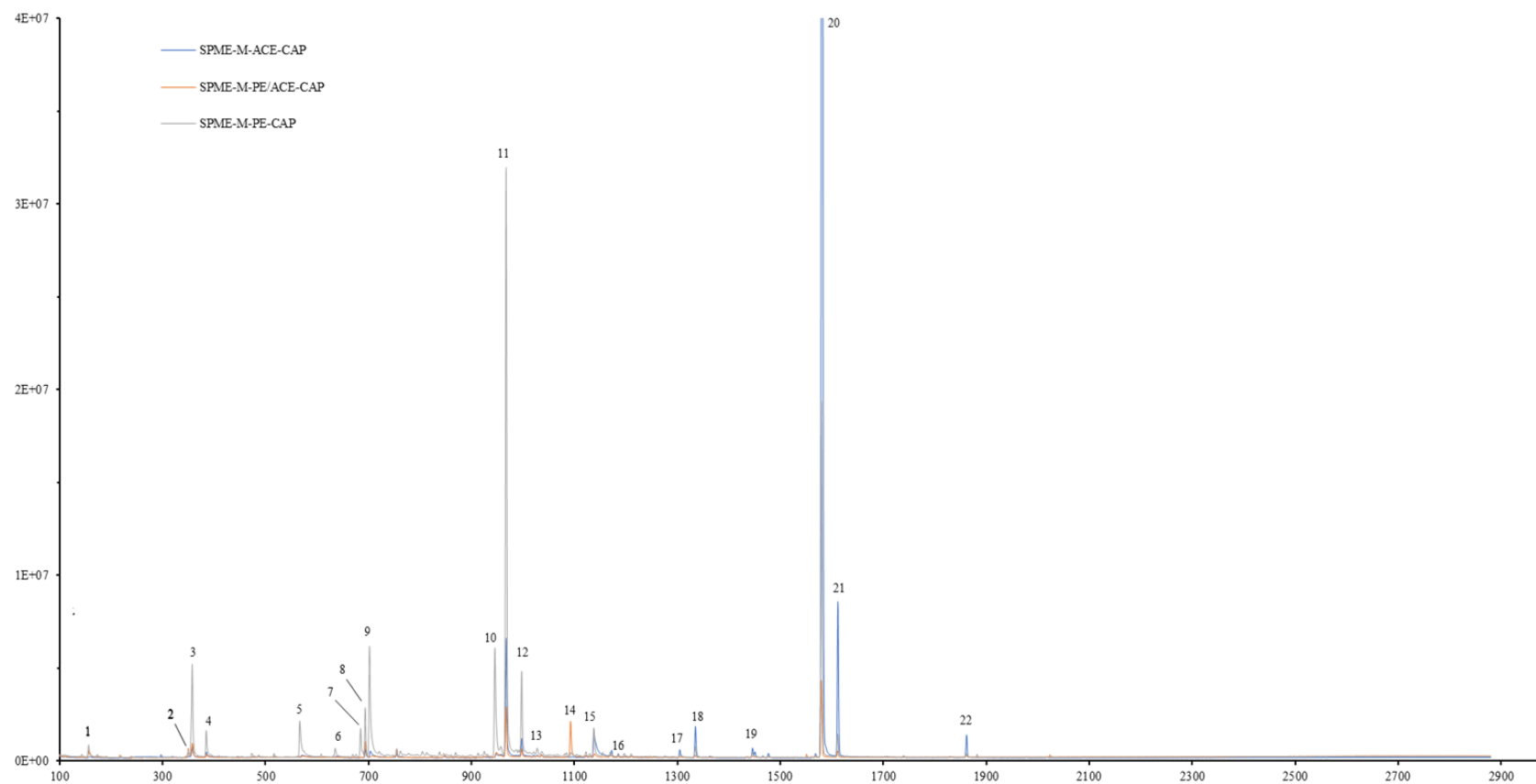


Figure C6 — CG-TOFMS chromatograms (TIC) of absolutes after SPME adsorption: maceration with 100% acetone (SPME-M-ACE-ALC), mixture (1:1) of petroleum ether and acetone (SPME-M-PE/ACE-ALC), and 100% petroleum ether (SPME-M-PE-ALC), with flowers from Caparica.

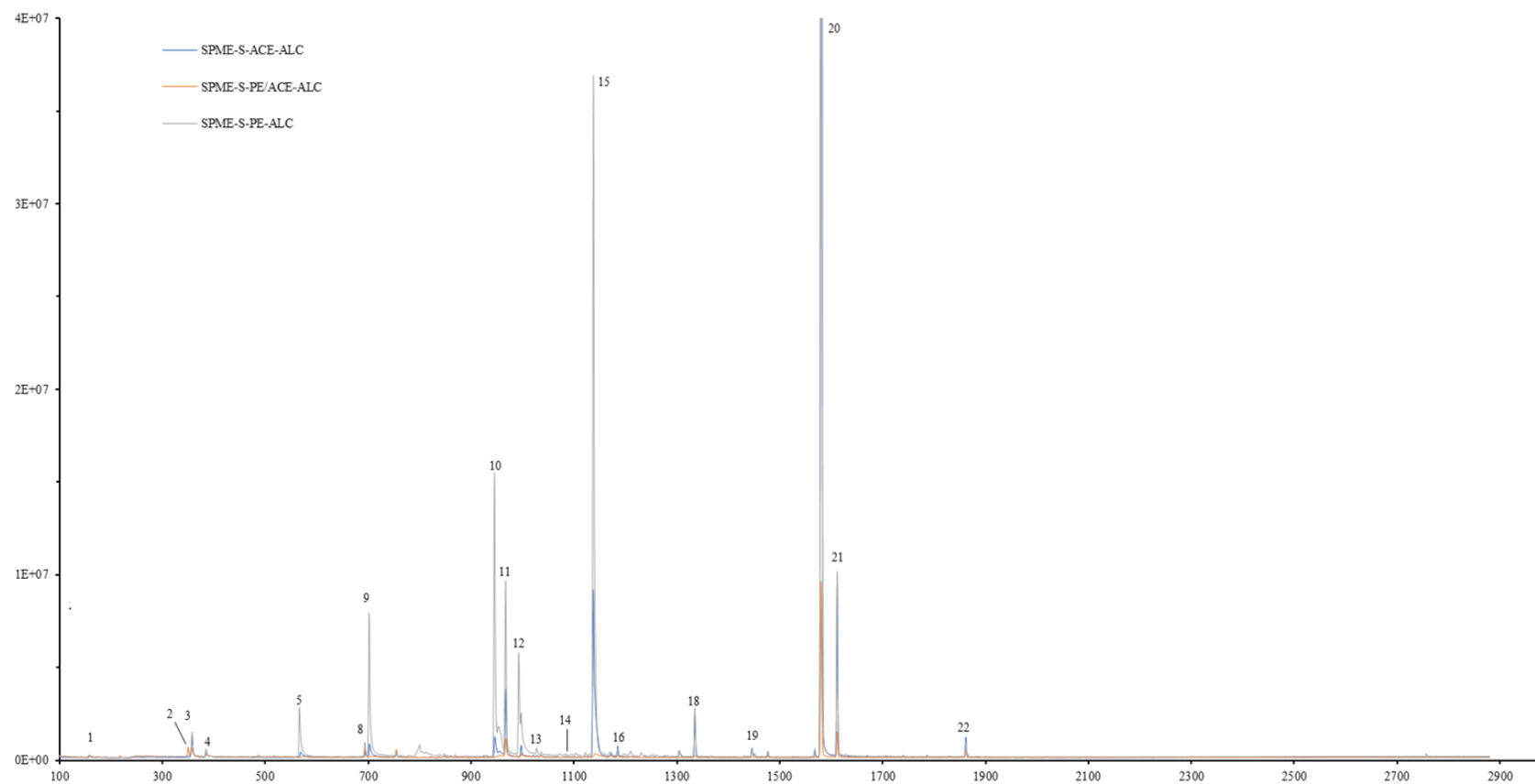


Figure C7 — CG-TOFMS chromatograms (TIC) of absolutes after SPME adsorption: Soxhlet with 100% acetone (SPME-M-ACE-ALC), mixture (1:1) of petroleum ether and acetone (SPME-M-PE/ACE-ALC), and 100% petroleum ether (SPME-M-PE-ALC), with flowers from Alcobaça.

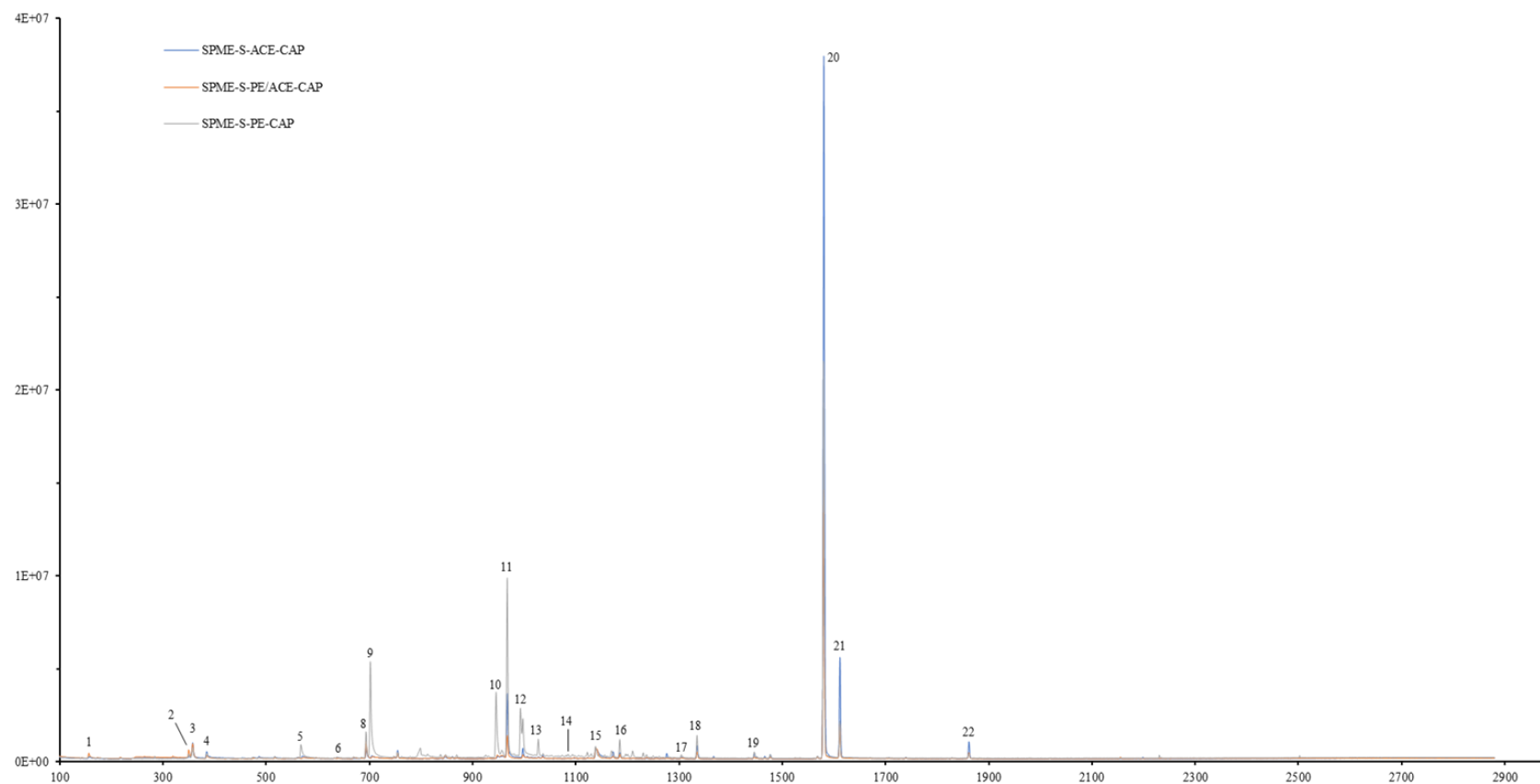


Figure C8 — CG-TOFMS chromatograms (TIC) of absolutes after SPME adsorption: Soxhlet with 100% acetone (SPME-M-ACE-ALC), mixture (1:1) of petroleum ether and acetone (SPME-M-PE/ACE-ALC), and 100% petroleum ether (SPME-M-PE-ALC), with flowers from Caparica.

APPENDIX (CHAPTER 6)

D.1 Tables (colorimetric tests)

Table D1 — Extraction yield, TPC, and DPPH values for different times of maceration at room temperature, of plants collected in Caparica using 70% ACE as extraction solvent.

		Yield (%, wet basis)	TPC (mg GAE/g ex- tract)	TPC (mg GAE/g of bio- mass)	DPPH (mg TE/g ex- tract)	DPPH (mg TE/g of bio- mass)
Leaves						
TFC	12 h	9.2 ± 0.2	333.7 ± 4.3	30.5 ± 0.6	537.5 ± 8.1	49.2 ± 0.7
	24 h	9.9 ± 0.1	340.1 ± 1.8	33.5 ± 0.2	544.2 ± 8.5	53.6 ± 1.0
	48 h	10.5 ± 0.2	345.8 ± 4.0	36.2 ± 1.1	560.0 ± 7.2	58.6 ± 0.6
	96 h	10.6 ± 0.2	344.3 ± 4.8	36.6 ± 0.8	561.8 ± 10.1	59.7 ± 0.6
Twigs						
TFC	12 h	6.8 ± 0.1	369.1 ± 4.0	24.9 ± 0.5	598.1 ± 18.5	40.4 ± 1.6
	24 h	7.3 ± 0.1	412.6 ± 6.7	30.1 ± 0.8	675.5 ± 11.0	49.3 ± 1.1
	48 h	7.5 ± 0.1	395.4 ± 3.6	29.6 ± 0.6	674.4 ± 7.6	50.4 ± 1.2
	96 h	7.5 ± 0.1	385.9 ± 4.7	28.8 ± 0.4	638.9 ± 18.6	47.7 ± 1.4

Table D2 — TPC (mg GAE/g of biomass, wet basis), TFC (mg CatE/g of biomass, wet basis), and antioxidant activity by DPPH (mg TE/g of biomass) and FRAP ($\mu\text{mol Fe}^{2+}$ /g of biomass) of extracts from fresh and dried leaves and twigs from Alcobaça and Caparica, using different extraction solvents and extraction methods.

Samples		Maceration at room temperature			Hot extraction		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
Leaves							
TPC	Fresh (ALC)	54.6 $\pm$ 0.7	36.6 $\pm$ 0.7	38.2 $\pm$ 0.5	52.2 $\pm$ 0.9	39.8 $\pm$ 0.9	33.3 $\pm$ 1.0
	Fresh (CAP)	36.2 $\pm$ 1.1	29.7 $\pm$ 0.6	28.2 $\pm$ 0.7	41.1 $\pm$ 0.4	31.8 $\pm$ 0.6	31.1 $\pm$ 1.5
	Dried (ALC)	70.7 $\pm$ 0.2	46.7 $\pm$ 0.8	47.4 $\pm$ 0.7	71.2 $\pm$ 1.1	48.8 $\pm$ 0.5	45.5 $\pm$ 0.3
	Dried (CAP)	48.9 $\pm$ 0.4	41.7 $\pm$ 0.5	34.5 $\pm$ 0.5	54.9 $\pm$ 0.5	43.0 $\pm$ 0.5	42.9 $\pm$ 0.5
TFC	Fresh (ALC)	12.0 $\pm$ 0.3	8.8 $\pm$ 0.2	8.7 $\pm$ 0.3	11.1 $\pm$ 0.3	9.2 $\pm$ 0.3	7.7 $\pm$ 0.3
	Fresh (CAP)	9.9 $\pm$ 0.4	9.8 $\pm$ 0.2	8.7 $\pm$ 0.3	12.4 $\pm$ 0.2	9.5 $\pm$ 0.3	9.5 $\pm$ 0.2
	Dried (ALC)	17.0 $\pm$ 0.2	11.9 $\pm$ 0.3	11.5 $\pm$ 0.2	16.3 $\pm$ 0.3	11.9 $\pm$ 0.2	11.3 $\pm$ 0.3
	Dried (CAP)	14.7 $\pm$ 0.3	13.1 $\pm$ 0.2	10.7 $\pm$ 0.2	15.8 $\pm$ 0.3	13.2 $\pm$ 0.3	12.5 $\pm$ 0.2
TPrAC	Fresh (ALC)	40.2 $\pm$ 0.7	20.8 $\pm$ 0.6	23.7 $\pm$ 0.9	35.7 $\pm$ 0.9	21.1 $\pm$ 0.8	20.9 $\pm$ 1.0
	Fresh (CAP)	26.7 $\pm$ 1.0	14.1 $\pm$ 0.6	11.7 $\pm$ 0.6	30.7 $\pm$ 0.7	13.9 $\pm$ 0.6	14.5 $\pm$ 0.7
	Dried (ALC)	52.8 $\pm$ 0.8	28.9 $\pm$ 0.7	26.7 $\pm$ 1.1	52.4 $\pm$ 0.9	32.6 $\pm$ 1.2	30.9 $\pm$ 0.7
	Dried (CAP)	39.0 $\pm$ 0.9	30.1 $\pm$ 0.9	18.1 $\pm$ 0.6	43.6 $\pm$ 1.1	27.6 $\pm$ 1.2	30.1 $\pm$ 1.0
DPPH	Fresh (ALC)	90.0 $\pm$ 0.8	64.5 $\pm$ 0.5	62.3 $\pm$ 0.7	90.3 $\pm$ 0.9	70.2 $\pm$ 1.1	52.1 $\pm$ 1.0
	Fresh (CAP)	58.6 $\pm$ 0.6	34.9 $\pm$ 0.8	32.9 $\pm$ 1.0	66.3 $\pm$ 0.5	42.0 $\pm$ 0.5	38.9 $\pm$ 0.5
	Dried (ALC)	132.1 $\pm$ 0.8	83.1 $\pm$ 1.3	81.4 $\pm$ 0.6	140.1 $\pm$ 1.8	85.5 $\pm$ 1.8	88.9 $\pm$ 1.1
	Dried (CAP)	62.4 $\pm$ 0.7	58.8 $\pm$ 1.1	45.6 $\pm$ 0.8	91.7 $\pm$ 0.8	61.9 $\pm$ 0.8	61.8 $\pm$ 0.8
FRAP	Fresh (ALC)	931.2 $\pm$ 15.2	678.1 $\pm$ 16.2	653.2 $\pm$ 10.1	939.1 $\pm$ 14.8	716.8 $\pm$ 19.4	536.7 $\pm$ 18.4
	Fresh (CAP)	691.6 $\pm$ 7.5	440.0 $\pm$ 15.6	431.6 $\pm$ 19.5	738.8 $\pm$ 17.1	497.5 $\pm$ 15.8	478.0 $\pm$ 13.7
	Dried (ALC)	1313.5 $\pm$ 21.8	904.9 $\pm$ 10.5	753.0 $\pm$ 11.4	1100.6 $\pm$ 17.4	831.9 $\pm$ 12.8	427.9 $\pm$ 10.0
	Dried (CAP)	817.0 $\pm$ 11.7	692.6 $\pm$ 11.6	532.1 $\pm$ 11.7	897.9 $\pm$ 17.1	646.4 $\pm$ 16.6	676.0 $\pm$ 13.0
Twigs							
TPC	Fresh (ALC)	41.4 $\pm$ 0.8	28.1 $\pm$ 0.6	24.1 $\pm$ 0.7	46.4 $\pm$ 0.6	35.1 $\pm$ 0.6	32.2 $\pm$ 0.4
	Fresh (CAP)	30.1 $\pm$ 0.8	19.8 $\pm$ 0.7	19.3 $\pm$ 0.5	30.2 $\pm$ 0.7	26.3 $\pm$ 0.4	22.7 $\pm$ 0.3
	Dried (ALC)	59.9 $\pm$ 0.9	41.8 $\pm$ 0.5	34.7 $\pm$ 0.6	42.9 $\pm$ 0.3	29.1 $\pm$ 0.3	40.7 $\pm$ 1.1
	Dried (CAP)	37.6 $\pm$ 0.7	26.9 $\pm$ 0.5	25.4 $\pm$ 0.6	37.4 $\pm$ 0.2	28.3 $\pm$ 0.3	31.8 $\pm$ 0.3
TFC	Fresh (ALC)	13.1 $\pm$ 0.3	9.6 $\pm$ 0.4	9.1 $\pm$ 0.2	15.0 $\pm$ 0.2	11.5 $\pm$ 0.2	10.4 $\pm$ 0.3
	Fresh (CAP)	13.0 $\pm$ 0.2	9.9 $\pm$ 0.3	9.2 $\pm$ 0.2	12.6 $\pm$ 0.3	11.7 $\pm$ 0.3	10.1 $\pm$ 0.2
	Dried (ALC)	20.0 $\pm$ 0.3	15.3 $\pm$ 0.2	14.1 $\pm$ 0.2	14.5 $\pm$ 0.2	9.9 $\pm$ 0.2	14.2 $\pm$ 0.3
	Dried (CAP)	17.8 $\pm$ 0.4	13.0 $\pm$ 0.2	12.5 $\pm$ 0.2	17.7 $\pm$ 0.2	13.2 $\pm$ 0.2	14.4 $\pm$ 0.4
TprAC	Fresh (ALC)	46.0 $\pm$ 0.4	21.3 $\pm$ 0.4	19.9 $\pm$ 0.8	49.9 $\pm$ 0.7	31.3 $\pm$ 0.7	29.7 $\pm$ 0.9
	Fresh (CAP)	32.1 $\pm$ 0.3	6.4 $\pm$ 0.5	5.1 $\pm$ 0.5	31.9 $\pm$ 0.6	16.0 $\pm$ 0.8	9.9 $\pm$ 0.6
	Dried (ALC)	69.2 $\pm$ 0.9	36.5 $\pm$ 0.7	32.2 $\pm$ 0.7	45.7 $\pm$ 0.7	24.8 $\pm$ 0.5	38.8 $\pm$ 0.8
	Dried (CAP)	56.6 $\pm$ 0.9	32.9 $\pm$ 0.7	30.9 $\pm$ 0.8	54.1 $\pm$ 0.9	34.7 $\pm$ 0.6	41.1 $\pm$ 0.7
DPPH	Fresh (ALC)	78.3 $\pm$ 1.2	52.3 $\pm$ 0.6	35.4 $\pm$ 1.2	89.6 $\pm$ 1.3	64.9 $\pm$ 0.5	60.0 $\pm$ 1.1
	Fresh (CAP)	49.3 $\pm$ 1.1	17.1 $\pm$ 0.7	16.2 $\pm$ 0.8	49.0 $\pm$ 1.5	29.0 $\pm$ 0.6	25.1 $\pm$ 0.5
	Dried (ALC)	128.3 $\pm$ 1.3	71.4 $\pm$ 1.0	54.3 $\pm$ 0.8	86.1 $\pm$ 0.6	55.8 $\pm$ 0.7	79.0 $\pm$ 0.6
	Dried (CAP)	65.6 $\pm$ 1.0	36.2 $\pm$ 1.1	32.5 $\pm$ 1.2	62.5 $\pm$ 0.6	39.4 $\pm$ 0.5	43.0 $\pm$ 0.4
FRAP	Fresh (ALC)	538.3 $\pm$ 11.4	452.4 $\pm$ 14.3	276.5 $\pm$ 7.0	705.5 $\pm$ 6.5	570.9 $\pm$ 16.6	502.7 $\pm$ 4.9
	Fresh (CAP)	507.0 $\pm$ 5.2	223.0 $\pm$ 10.7	216.7 $\pm$ 8.1	509.1 $\pm$ 8.9	352.7 $\pm$ 15.1	312.0 $\pm$ 5.6
	Dried (ALC)	1103.9 $\pm$ 9.6	511.9 $\pm$ 6.6	399.4 $\pm$ 8.2	777.8 $\pm$ 5.7	493.1 $\pm$ 6.7	716.9 $\pm$ 9.2
	Dried (CAP)	695.7 $\pm$ 18.4	463.2 $\pm$ 16.9	400.9 $\pm$ 11.1	704.2 $\pm$ 10.6	463.5 $\pm$ 9.4	515.7 $\pm$ 7.0

Values (mean $\pm$ standard deviation) are average of two replicates of each sample, analyzed individually in triplicate ($n = 2 \times 3$).

Table D3 — TFC (mg RutE/g extract) of extracts from fresh and dried leaves and twigs from Alcobaça and Caparica, using different extraction solvents and extraction methods.

Samples		Maceration at room temperature			Hot extraction		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
Leaves							
TFC	Fresh (ALC)	299.7 ± 7.6	247.2 ± 7.7	228.8 ± 7.7	275.3 ± 9.3	252.5 ± 10.3	236.3 ± 9.9
	Fresh (CAP)	288.3 ± 7.2	296.8 ± 10.2	271.3 ± 11.1	323.2 ± 8.4	293.6 ± 11.7	290.9 ± 8.6
	Dried (ALC)	348.0 ± 6.1	250.9 ± 5.5	247.7 ± 3.8	312.9 ± 4.6	267.6 ± 5.4	264.0 ± 5.7
	Dried (CAP)	319.5 ± 5.3	279.3 ± 5.5	239.3 ± 6.8	306.7 ± 8.5	253.5 ± 6.8	249.1 ± 5.9
Twigs							
TFC	Fresh (ALC)	504.4 ± 12.9	422.3 ± 12.0	397.6 ± 6.53	504.5 ± 11.7	417.7 ± 10.1	410.9 ± 7.9
	Fresh (CAP)	542.3 ± 14.1	447.8 ± 7.1	394.9 ± 11.6	464.3 ± 8.6	465.2 ± 7.4	439.7 ± 13.5
	Dried (ALC)	504.1 ± 9.2	433.9 ± 10.4	406.0 ± 6.1	496.8 ± 5.2	424.2 ± 8.4	427.6 ± 8.8
	Dried (CAP)	601.4 ± 8.3	454.5 ± 9.9	375.5 ± 7.3	609.5 ± 5.6	484.2 ± 4.1	459.6 ± 8.2

Table D4 — TFC (mg RutE/g of raw material, wet basis) of extracts from fresh and dried leaves and twigs from Alcobaça and Caparica, using different extraction solvents and extraction methods.

Samples		Maceration at room temperature			Hot extraction		
		70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
Leaves							
TFC	Fresh (ALC)	36.4 ± 0.7	26.7 ± 0.7	26.6 ± 0.8	33.6 ± 1.0	27.8 ± 1.0	23.34 ± 0.8
	Fresh (CAP)	30.2 ± 1.1	29.7 ± 0.6	26.6 ± 0.8	37.7 ± 0.7	28.8 ± 0.9	28.8 ± 0.5
	Dried (ALC)	51.4 ± 0.7	36.0 ± 1.0	35.0 ± 0.7	49.5 ± 0.8	36.0 ± 0.6	34.2 ± 2.9
	Dried (CAP)	44.6 ± 1.0	39.8 ± 1.0	32.3 ± 0.7	47.8 ± 1.0	40.0 ± 0.9	37.8 ± 0.7
Twigs							
TFC	Fresh (ALC)	39.6 ± 1.0	29.2 ± 1.7	27.6 ± 0.7	45.5 ± 0.7	34.8 ± 0.7	31.5 ± 0.8
	Fresh (CAP)	39.5 ± 0.6	30.2 ± 0.8	27.8 ± 0.5	38.2 ± 0.9	35.5 ± 0.8	30.8 ± 0.5
	Dried (ALC)	60.5 ± 0.9	46.6 ± 0.7	42.9 ± 0.7	43.9 ± 0.6	29.9 ± 0.6	43.2 ± 1.0
	Dried (CAP)	54.0 ± 1.3	39.3 ± 0.6	37.9 ± 0.6	53.6 ± 0.6	40.2 ± 0.6	43.7 ± 1.1

Table D5 — Average values of extraction yield (% wet basis), TPC (mg GAE/g extract), TFC (mg CatE/g extract) and TPrAC (mg PycE/g extract) for extracts obtained by maceration and hot extraction (regardless of drying status and origin), from fresh and dried material (regardless extraction method and origin), and from plants from Alcobaça and Caparica (regardless extraction process and drying status).

Samples	Leaves			Twigs		
	70% ACE	70% ET	70% MET	70% ACE	70% ET	70% MET
Yield						
Maceration	12.8 ± 1.7 ^a	12.4 ± 2.0 ^a	12.3 ± 1.8 ^a	9.0 ± 1.9 ^a	8.3 ± 1.7 ^a	8.7 ± 1.7 ^a
Hot extraction	13.8 ± 2.0 ^a	12.5 ± 2.4 ^a	12.0 ± 2.3 ^a	8.7 ± 0.3 ^a	7.8 ± 0.5 ^a	8.6 ± 1.3 ^a
Fresh	11.6 ± 0.7 ^c	10.4 ± 0.5 ^d	10.3 ± 0.8 ^d	8.1 ± 0.6 ^{bc}	7.4 ± 0.7 ^{cd}	7.2 ± 0.3 ^d
Dried	15.0 ± 0.7 ^a	14.5 ± 0.9 ^{ab}	13.9 ± 0.9 ^b	9.7 ± 1.4 ^a	8.7 ± 1.4 ^b	10.1 ± 0.4 ^a
Alcobaça	13.7 ± 1.7 ^a	12.4 ± 1.6 ^a	12.2 ± 1.6 ^a	9.4 ± 1.6 ^a	8.3 ± 1.6 ^{ab}	8.8 ± 1.6 ^{ab}
Caparica	12.9 ± 2.1 ^a	12.5 ± 2.7 ^a	12.1 ± 2.4 ^a	8.3 ± 0.7 ^{ab}	7.8 ± 0.8 ^b	8.4 ± 1.5 ^{ab}
TPC						
Maceration	405.8 ± 60.3 ^a	312.95 ± 20.7 ^b	302.1 ± 33.7 ^b	464.3 ± 50.7 ^a	350.3 ± 49.8 ^b	300.6 ± 40.7 ^c
Hot extraction	396.1 ± 45.3 ^a	330.1 ± 37.8 ^b	321.3 ± 27.6 ^b	448.1 ± 58.2 ^a	379.9 ± 38.0 ^b	370.2 ± 42.5 ^b
Fresh	394.0 ± 46.7 ^a	330.1 ± 25.1 ^b	317.2 ± 19.8 ^b	455.2 ± 69.0 ^a	366.6 ± 51.9 ^b	341.3 ± 53.8 ^b
Dried	408.0 ± 58.8 ^a	313.0 ± 35.0 ^b	306.2 ± 40.1 ^b	457.2 ± 36.5 ^a	363.6 ± 41.0 ^b	329.5 ± 54.9 ^b
Alcobaça	451.6 ± 18.8 ^a	346.8 ± 16.7 ^b	338.3 ± 10.0 ^b	506.4 ± 16.2 ^a	407.4 ± 12.8 ^b	374.8 ± 38.5 ^c
Caparica	350.3 ± 5.5 ^b	296.2 ± 20.0 ^c	285.1 ± 22.2 ^c	406.0 ± 23.9 ^b	322.9 ± 22.3 ^d	296.0 ± 35.6 ^e
TFC						
Maceration	103.5 ± 8.0 ^a	88.5 ± 7.3 ^b	81.3 ± 5.8 ^c	177.4 ± 13.9 ^a	144.8 ± 5.2 ^b	129.6 ± 4.5 ^c
Hot extraction	100.4 ± 6.5 ^a	87.9 ± 6.2 ^b	85.7 ± 7.2 ^{bc}	171.0 ± 18.6 ^a	147.5 ± 9.7 ^b	143.1 ± 6.6 ^b
Fresh	97.7 ± 6.5 ^b	89.7 ± 8.3 ^c	84.5 ± 9.0 ^{cd}	166.0 ± 10.0 ^b	144.3 ± 7.1 ^{cd}	135.2 ± 6.8 ^e
Dried	106.1 ± 5.7 ^a	86.7 ± 4.3 ^{cd}	82.4 ± 3.5 ^d	182.4 ± 17.9 ^a	148.0 ± 8.2 ^c	137.5 ± 10.6 ^{de}
Alcobaça	101.9 ± 9.2 ^a	83.9 ± 3.5 ^{cd}	80.4 ± 5.0 ^d	165.6 ± 3.3 ^b	139.9 ± 3.8 ^d	135.2 ± 4.3 ^d
Caparica	102.0 ± 5.2 ^a	92.5 ± 6.3 ^b	86.5 ± 7.2 ^c	182.8 ± 19.9 ^a	152.5 ± 5.2 ^c	137.5 ± 11.8 ^d
TprAC						
Maceration	305.6 ± 41.6 ^a	186.3 ± 28.5 ^{bc}	161.6 ± 36.9 ^c	558.5 ± 73.3 ^a	280.5 ± 113.2 ^{bc}	242.5 ± 101.0 ^c
Hot extraction	291.9 ± 26.6 ^a	187.5 ± 37.5 ^{bc}	198.6 ± 34.7 ^b	518.2 ± 84.8 ^a	338.2 ± 80.2 ^b	336.7 ± 116.6 ^b
Fresh	285.6 ± 31.0 ^a	166.4 ± 26.6 ^c	170.4 ± 39.9 ^c	491.9 ± 82.7 ^b	246.5 ± 108.4 ^d	222.1 ± 125.8 ^d
Dried	311.9 ± 34.8 ^a	207.4 ± 25.3 ^b	189.8 ± 38.6 ^{bc}	584.7 ± 45.4 ^a	372.2 ± 31.6 ^c	357.0 ± 55.7 ^c
Alcobaça	328.0 ± 24.5 ^a	206.7 ± 22.1 ^c	210.7 ± 19.6 ^c	558.1 ± 28.1 ^a	343.5 ± 25.9 ^b	340.9 ± 47.4 ^b
Caparica	269.6 ± 12.3 ^b	167.1 ± 30.3 ^d	149.5 ± 30.7 ^d	518.6 ± 108.6 ^a	275.2 ± 133.7 ^{bc}	238.2 ± 143.8 ^c

Different letters indicate significant differences of means within samples for each set of parameters (one-way ANOVA, Tukey test at $p < 0.05$).

D.2 GC-TOFMS Data

Table D6 — Chromatographic peak areas of non-polar components co-extracted with phenolic compounds for a group of selected extracts obtained by maceration at room temperature.

Extraction Solvent		70% ACE	70% ACE	70% MET	70% ET	70% ACE	70% ACE	70% ACE
Status		Dried	Dried	Dried	Dried	Fresh	Dried	Dried
Biomass / Geographical Origin		Twigs Al- cobaça	Twigs Al- cobaça	Twigs Al- cobaça	Twigs Al- cobaça	Leaves Al- cobaça	Leaves Al- cobaça	Leaves Caparica
No.	Compound Name							
1	Neophytadiene	3870564	2506597	2890708	2618226	18259744	27923663	41468761
2	6,10-dimethyl-2-Undecanone	8158101	2564086	4494088	3725733	7331571	10800282	8370053
3	Tetramethyl-2-hexadecen-1-ol	1201880	5812381	1066436	11693842	-	7938807	11693842
4	1-Hexadecanol	87416039	25671111	66275268	-	24259269	100999797	88173650
5	6-Dodecanone	1727458	5261270	7875074	10994683	916754	18323890	4763041
6	Methyl hexadecanoate	4148491	6833789	3.63E+08	4951273	6613855	19613167	8733044
7	1-Hexadecanol (TMS derivative)	85956786	48164147	70724059	47115507	42892333	42989469	35478974
8	Ethyl Hexadecanoate	-	-	-	4.58E+08	-	2544219	4242189
9	Palmitic Acid (TMS derivative)	27290494	2012970	2886685	6886565	4467915	7193467	-
10	1-Octadecene	64559734	21311256	37927976	36479409	19383671	68066659	55719274
11	Methyl-9,12-Octadecadienoate	905917	-	1.21E+08	5901530	-	2904050	534514
12	Methyl 9,12,15-Octadecatrienoate	-	-	1.21E+08	-	-	5506842	2753922
13	Methyl 9-Octadecenoate	--	-	35381563	-	-	-	-
14	Phytol	1.05E+08	39975742	54131526	54207934	3.65E+08	1.563E+09	1.905E+09
15	Diallyl acetal palmitaldehyde		1073505	1370100	1933436	8332209	22549852	27671761
16	1-Octadecanol, (TMS derivative)	78279383	42840515	58752712	41575022	35633240	48195737	26179740
17	Methyl 9,12-Octadecadienoate	-	-	1653183	93285165	-	-	1005962
18	Tri-n-butyl aconitate	4614209	-	2782973	-	15034848	40758680	112844093
19	Ethyl Oleate	-	-	-	39360244	-	-	--
20	Phytol (TMS derivative)	1.19E+08	70571077	71045757	47618417	5.4E+08	934008233	656783661
21	Ethyl octadecanoate		-	-	15659060	-	-	145600
22	Tributyl acetylcitrate	1003654	-	-		67819179	-	83457851
23	Eicosane	2559784	1536828	1314349	877072	889677	4193106	1910845
24	Squalene	13056697	22489185	5888245	6495633	12029161	36719498	110601809
25	dl- α -Tocopherol	1817566	3912929	-	-	18389246	54634933	58847032
26	Stigmasterol	3126933	2201904	-	1318946	1772095	4185504	3225958
27	Olean-18-ene	-	164102	-	123966	2483215	3993612	4295146
28	Lupeol isomer	-	173770	-	-	3062321	5721073	5158152
29	Lup-20(29)-en-3-one	2780597	5672836	2008534	3682556	8761884	18184112	9564442
30	Lupeol	2181198	2712110	1382327	1969921	8633173	20098838	10493844
31	A'-Neogammacer-22(29)-en-3-one	-	1132595	-	1393234	536941	2072609	1198093
32	Lupeol isomer	-	917528	382291	548299	1038714	1757054	5495892

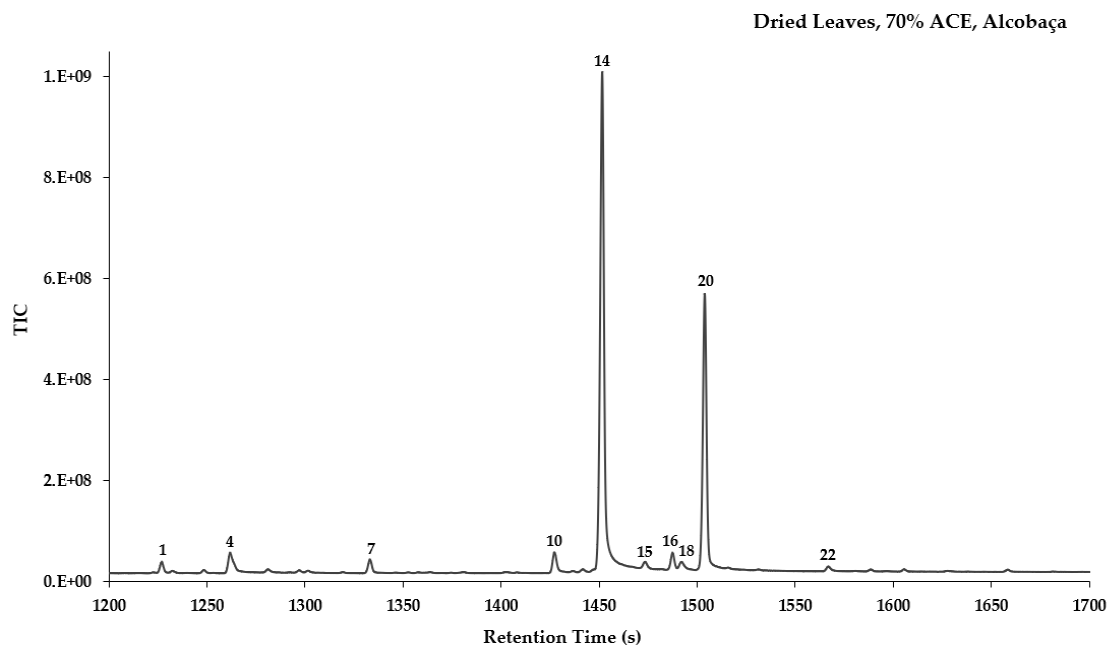


Figure D1 — Chromatographic profile of the non-polar fraction of the extract obtained with 70% acetone by maceration of dried *Acacia* leaves collected in Alcobaça region. Part 1 – Low retention time components: 1. Neophytadiene; 4. 1-Hexadecanol; 7. 1-Hexadecanol (TMS derivative); 10. 1-Octadecene; 14. Phytol; 15. Diallyl acetal palmitaldehyde; 16. 1-Octadecanol, (TMS derivative) 17. Methyl 9,12-Octadecadienoate; 18. Tri-*n*-butyl aconitate; 20. Phytol (TMS derivative); 22. Tributyl acetylcitrate.

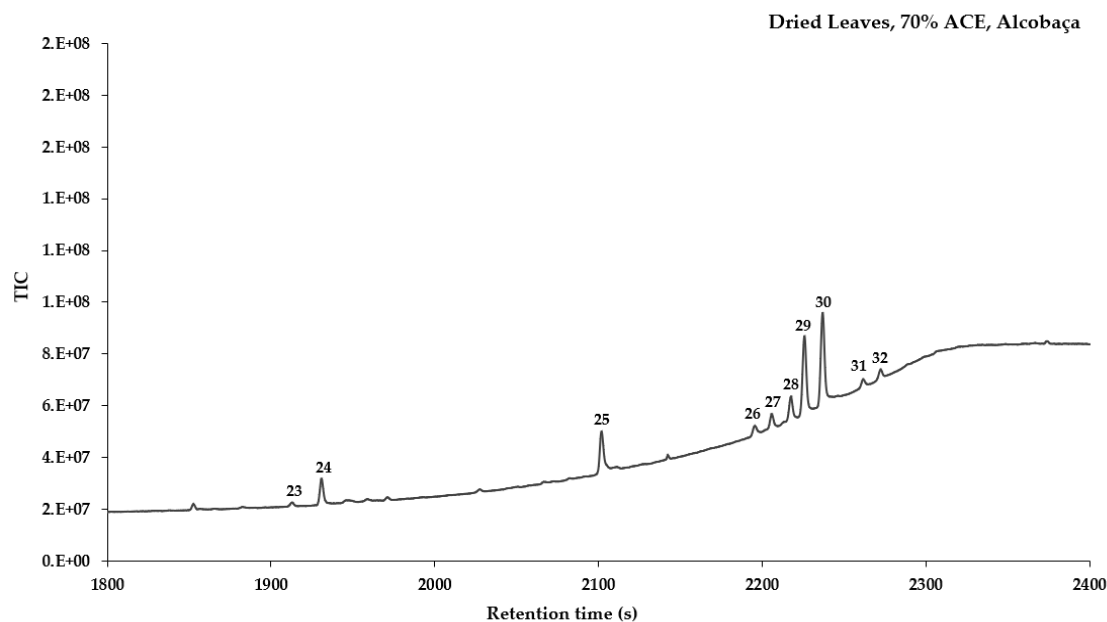


Figure D2 — Chromatographic profile of the non-polar fraction of the extract obtained with 70% acetone by maceration of dried *Acacia* leaves collected in Alcobaça region. Part 2 – High retention time components: 23. Eicosane; 24. Squalene; 25. dl- α -Tocopherol; 26. Stigmasterol; 27. Olean-18-ene; 28. Lupeol isomer; 29. Lup-20(29)-en-3-one; 30. Lupeol; 31. A'-Neogammacer-22(29)-en-3-one; 32. Lupeol isomer.

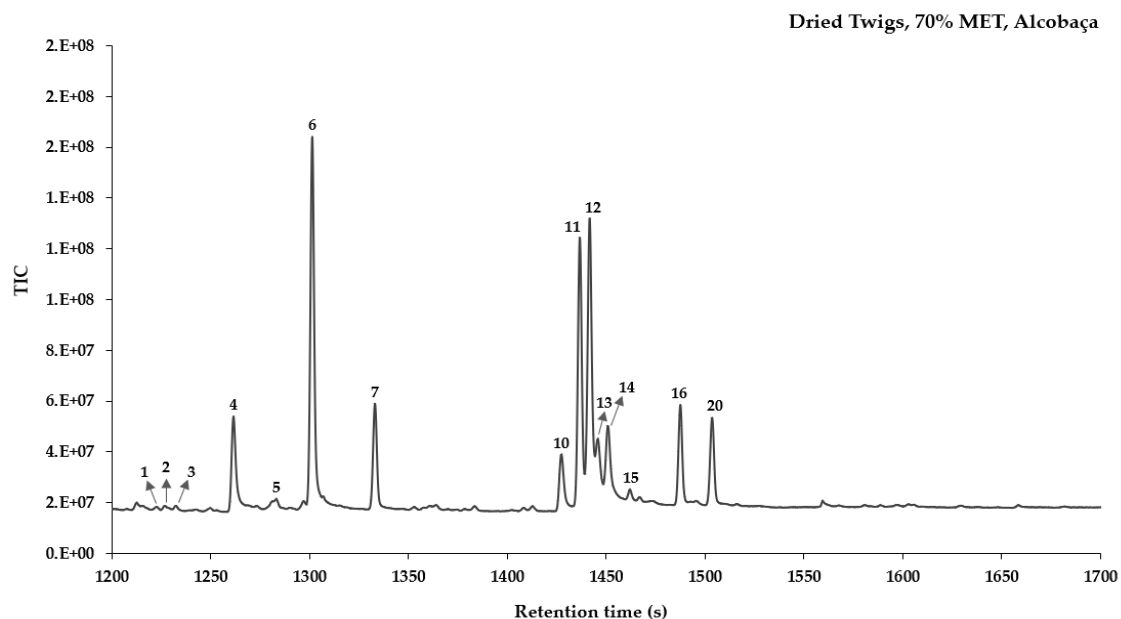


Figure D3 — Chromatographic profile of the non-polar fraction of the extract obtained with 70% methanol by maceration of dried *Acacia* twigs collected in Alcobaça region. Part 1 – Low retention time components: 1. Neophytadiene; 2. 6,10-dimethyl-2-Undecanone; 3. Tetramethyl-2-hexadecen-1-ol; 4. 1-Hexadecanol; 5. 6-Dodecanone; 6. Methyl hexadecanoate; 7. 1-Hexadecanol (TMS derivative); 10. 1-Octadecene; 11. Methyl-9,12-Octadecadienoate; 12. Methyl 9,12,15-Octadecatrienoate; 13. Methyl 9-Octadecenoate; 14. Phytol; 15. Diallyl acetal palmitaldehyde; 16. 1-Octadecanol, (TMS derivative); 20. Phytol (TMS derivative).

APPENDIX (CHAPTER 7)

E.1 Optimization of extraction time

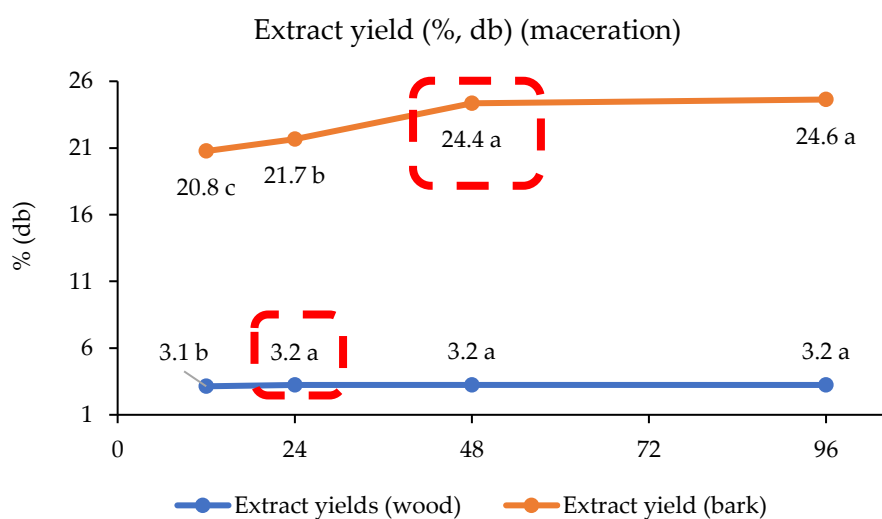


Figure E1 — Extraction yield for the different maceration times using aqueous acetone as extraction solvent. Different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

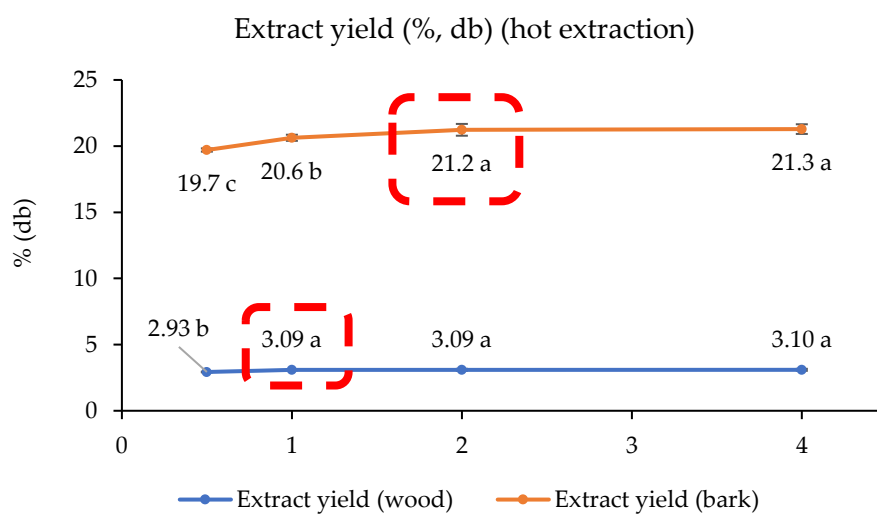


Figure E2 — Extraction yield for the different hot extraction times using aqueous acetone as extraction solvent. Different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

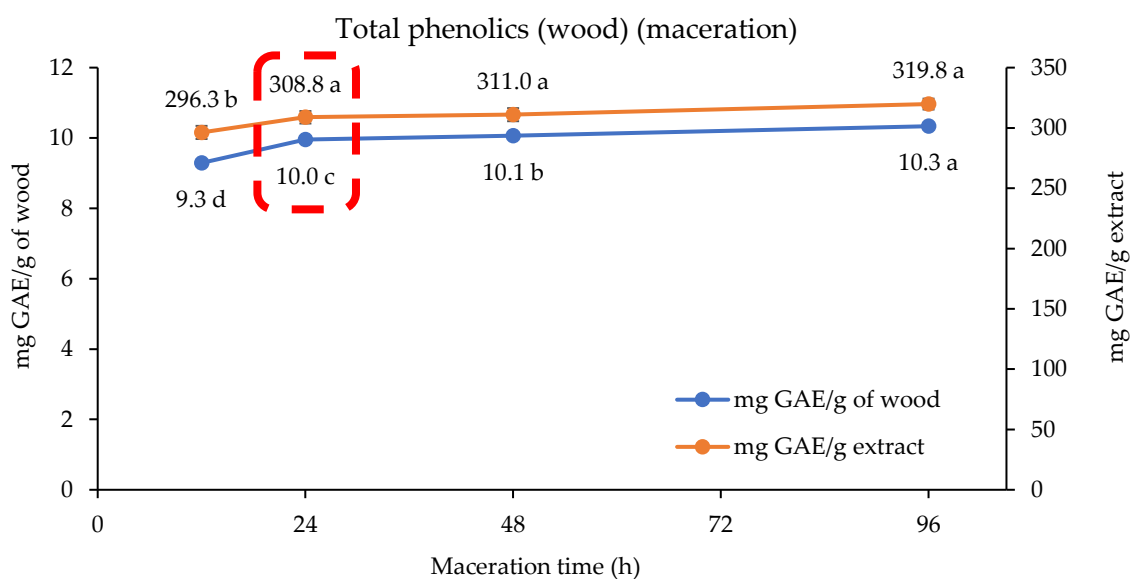


Figure E3 — Total phenolic content of aqueous acetonetic wood extracts for the different maceration times. Different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

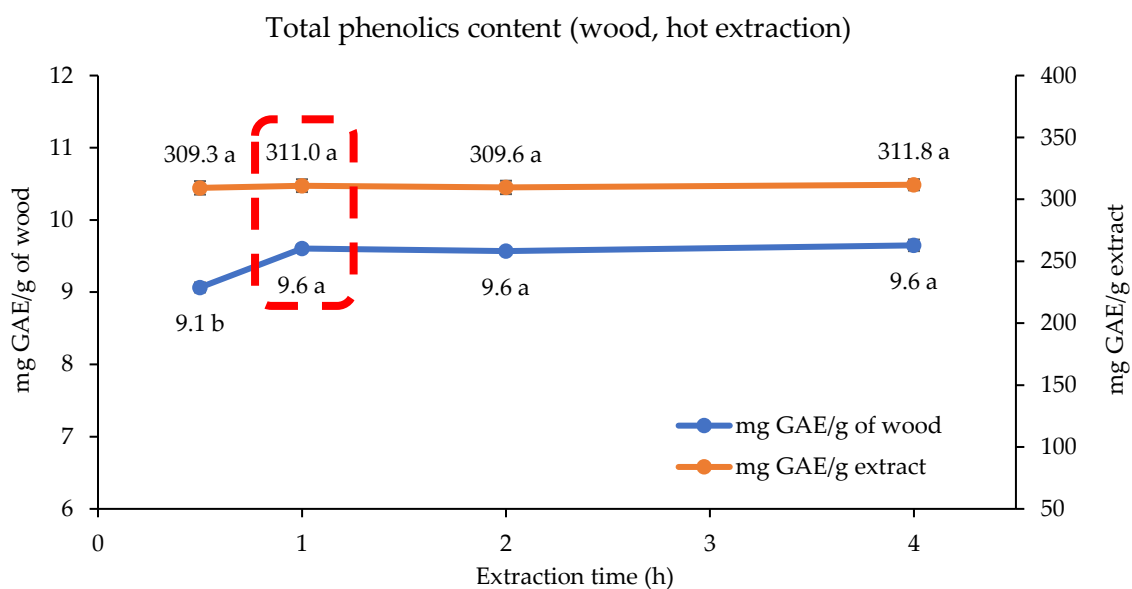


Figure E4 — Total phenolic content of aqueous acetonetic wood extracts for the different hot extraction times. Different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

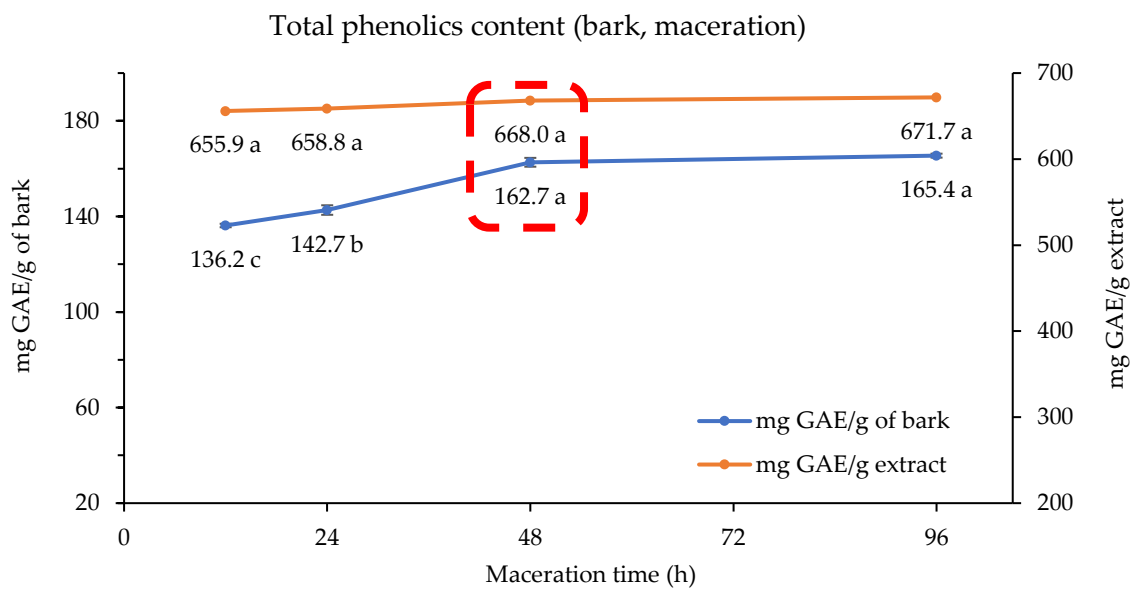


Figure E5 — Total phenolic content of aqueous acetonetic bark extracts for the different maceration times. Different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

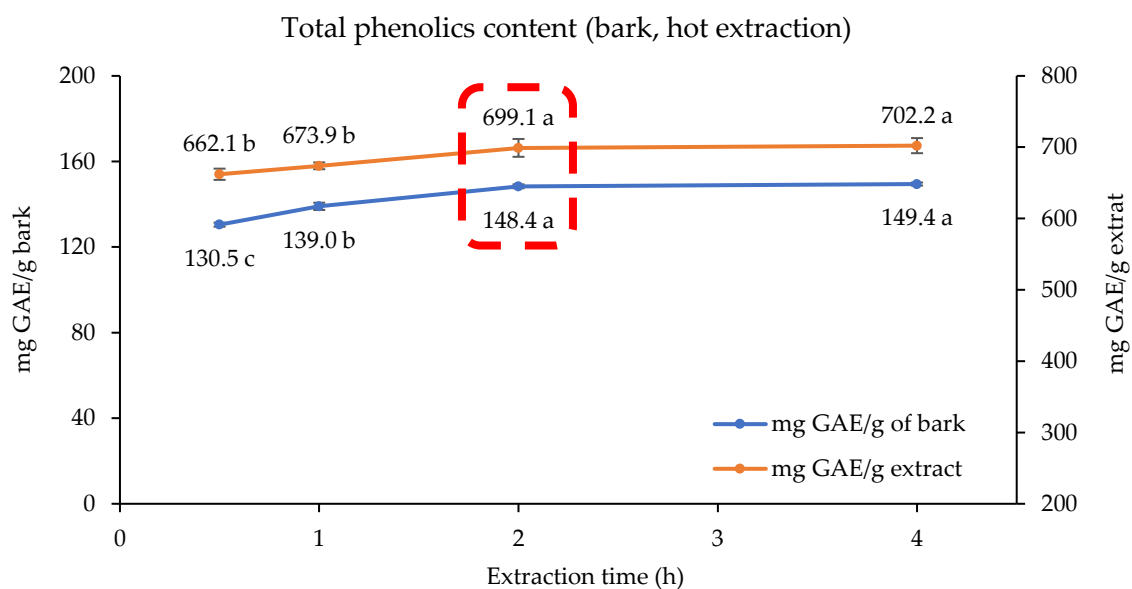


Figure E6 — Total phenolic content of aqueous acetonetic bark extracts for the different hot extraction times. Different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

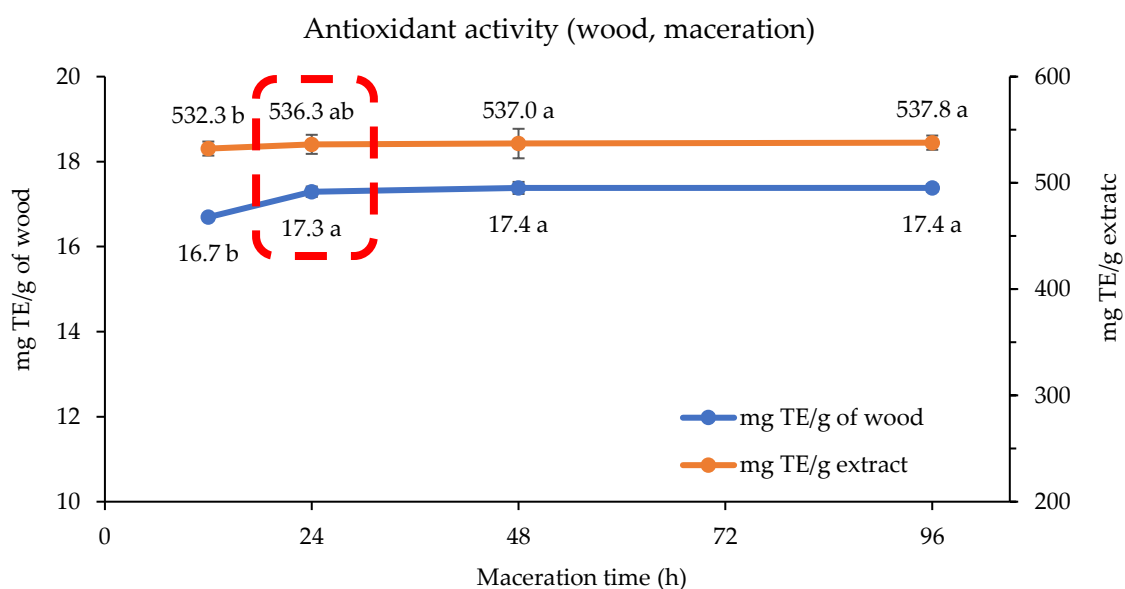


Figure E7 — Antioxidant activity by DPPH assay of aqueous acetonetic wood extracts, for the different maceration times. Different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

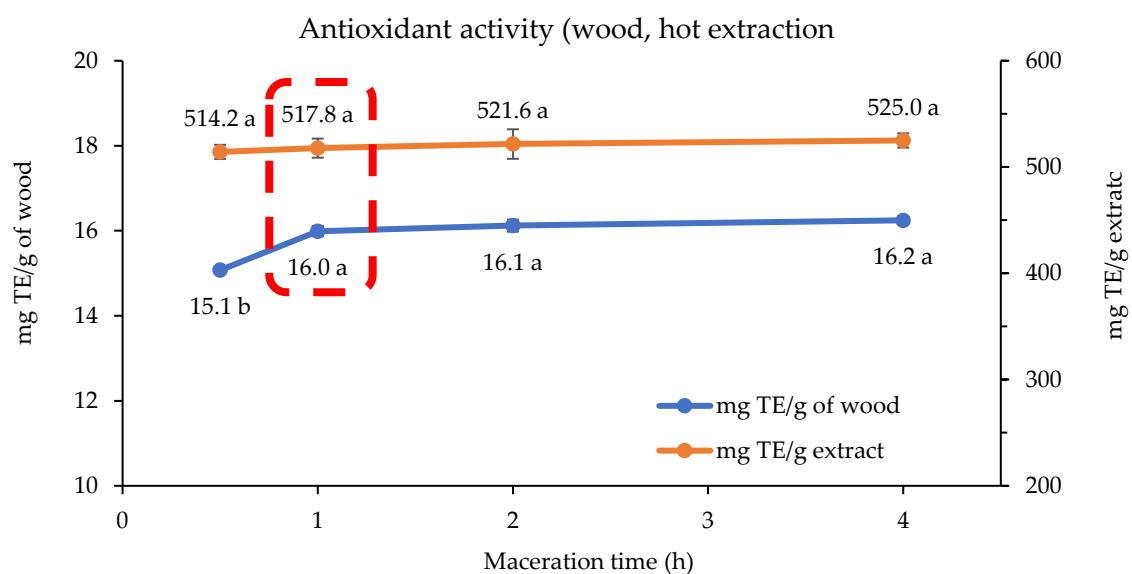


Figure E8 — Antioxidant activity by DPPH assay of aqueous acetonetic wood extracts, for the different hot extraction times. Different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

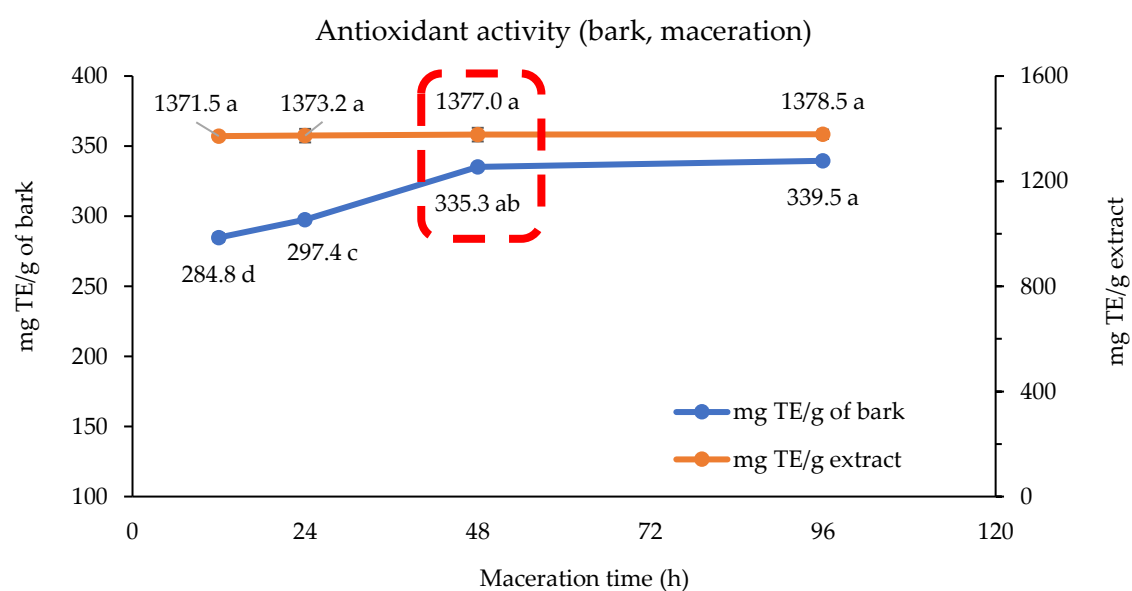


Figure E9 — Antioxidant activity by DPPH assay of aqueous acetonetic bark extracts, for the different maceration times. Different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

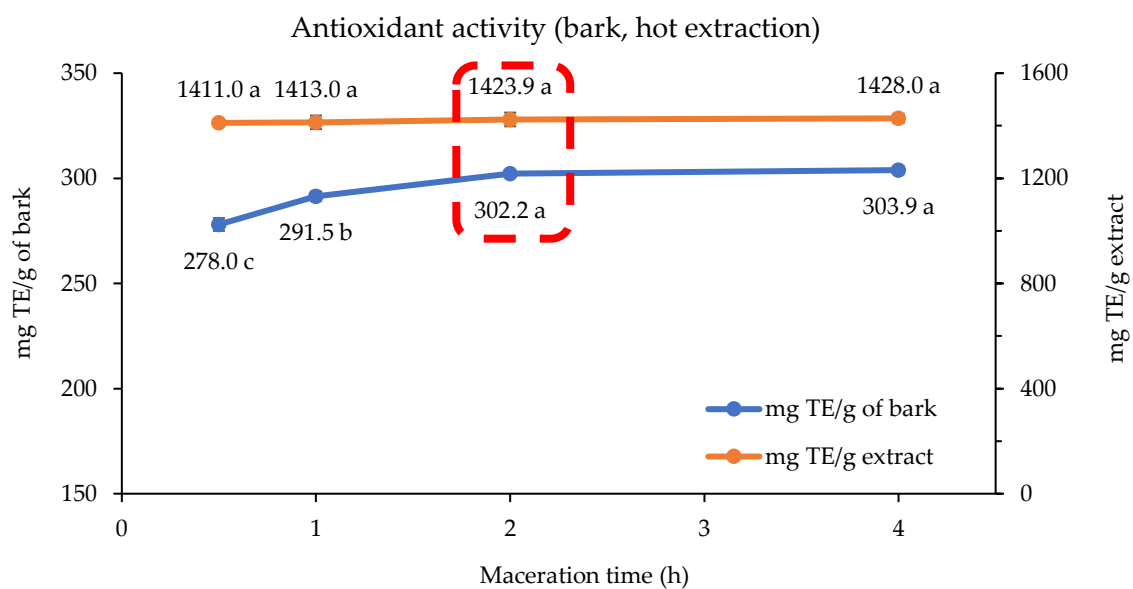


Figure E10 — Antioxidant activity by DPPH assay of aqueous acetonc bark extracts, for the different hot extraction times. Different letters indicate significant differences of means (one-way ANOVA, Tukey test at $p < 0.05$).

E.2 Total flavonoids content using different standards

Table E1 — Total flavonoid content (TFC) of wood and bark extracts, expressed in rutin equivalents (RutE) and in catechin equivalents (CatE)

Sample	catechin equivalents (CatE)			
	TFC (mg RutE/g extract, db)	TFC (mg RutE/g bio- mass, wb)	TFC (mg CatE/g extract, db)	TFC (mg CatE/g bio- mass, wb)
Wood				
Maceration				
70% ACE	516.5 ± 31.8 ^a	16.6 ± 0.9 ^a	169.9 ± 7.6 ^a	5.5 ± 0.2 ^a
70% ET	451.6 ± 15.1 ^{bc}	14.4 ± 0.3 ^{bc}	148.4 ± 5.0 ^{bc}	4.7 ± 0.1 ^{bc}
70% MET	436.8 ± 19.3 ^c	14.6 ± 0.5 ^b	143.6 ± 6.4 ^c	4.8 ± 0.2 ^b
Hot extraction				
70% ACE	550.1 ± 27.8 ^a	17.0 ± 0.7 ^a	181.0 ± 5.6 ^a	5.6 ± 0.1 ^a
70% ET	477.0 ± 16.5 ^b	13.5 ± 0.3 ^c	156.8 ± 5.5 ^b	4.4 ± 0.1 ^c
70% MET	466.5 ± 9.9 ^{bc}	14.1 ± 0.4 ^{bc}	153.4 ± 3.3 ^{bc}	4.7 ± 0.1 ^{bc}
Bark				
Maceration				
70% ACE	956.4 ± 17.2 ^{ab}	232.9 ± 2.0 ^a	315.0 ± 5.7 ^{ab}	76.7 ± 0.7 ^a
70% ET	866.6 ± 32.0 ^e	172.0 ± 4.7 ^c	285.1 ± 10.6 ^e	56.6 ± 1.5 ^c
70% MET	868.2 ± 25.5 ^{de}	151.9 ± 4.3 ^e	285.6 ± 8.4 ^{de}	50.0 ± 1.4 ^e
Hot extraction				
70% ACE	986.7 ± 15.7 ^a	212.3 ± 3.2 ^b	324.9 ± 5.2 ^a	69.9 ± 1.5 ^b
70% ET	938.9 ± 16.2 ^{bc}	169.2 ± 2.9 ^c	309.0 ± 5.3 ^{bc}	55.7 ± 1.7 ^c
70% MET	906.7 ± 28.2 ^{cd}	158.7 ± 2.4 ^d	298.2 ± 9.3 ^{cd}	52.2 ± 0.8 ^d

Values (mean ± standard deviation) are average of two replicates of each sample, analyzed individually in triplicate (n = 2 x 3); different letters in each column indicate significant differences of means among extract samples (one-way ANOVA, Tukey test at p<0.05).

APPENDIX (CHAPTER 8)

F.1 Gas composition

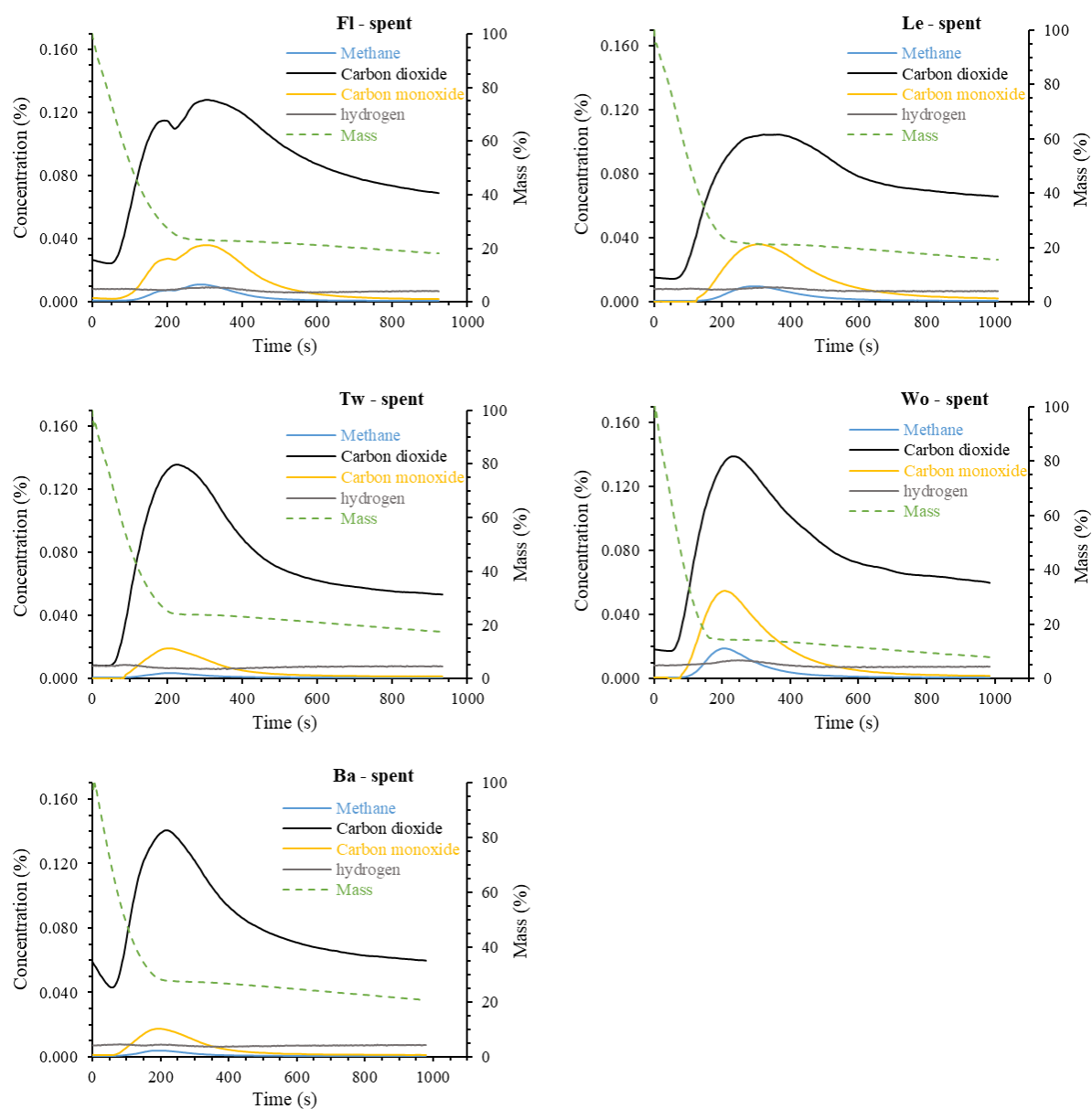


Figure F1 — Gas emissions during the macro TGA experiment with spent flowers (FI), leaves (Le), twigs (Tw), wood (Wo), and bark (Ba)..

F.2 Adsorption full data

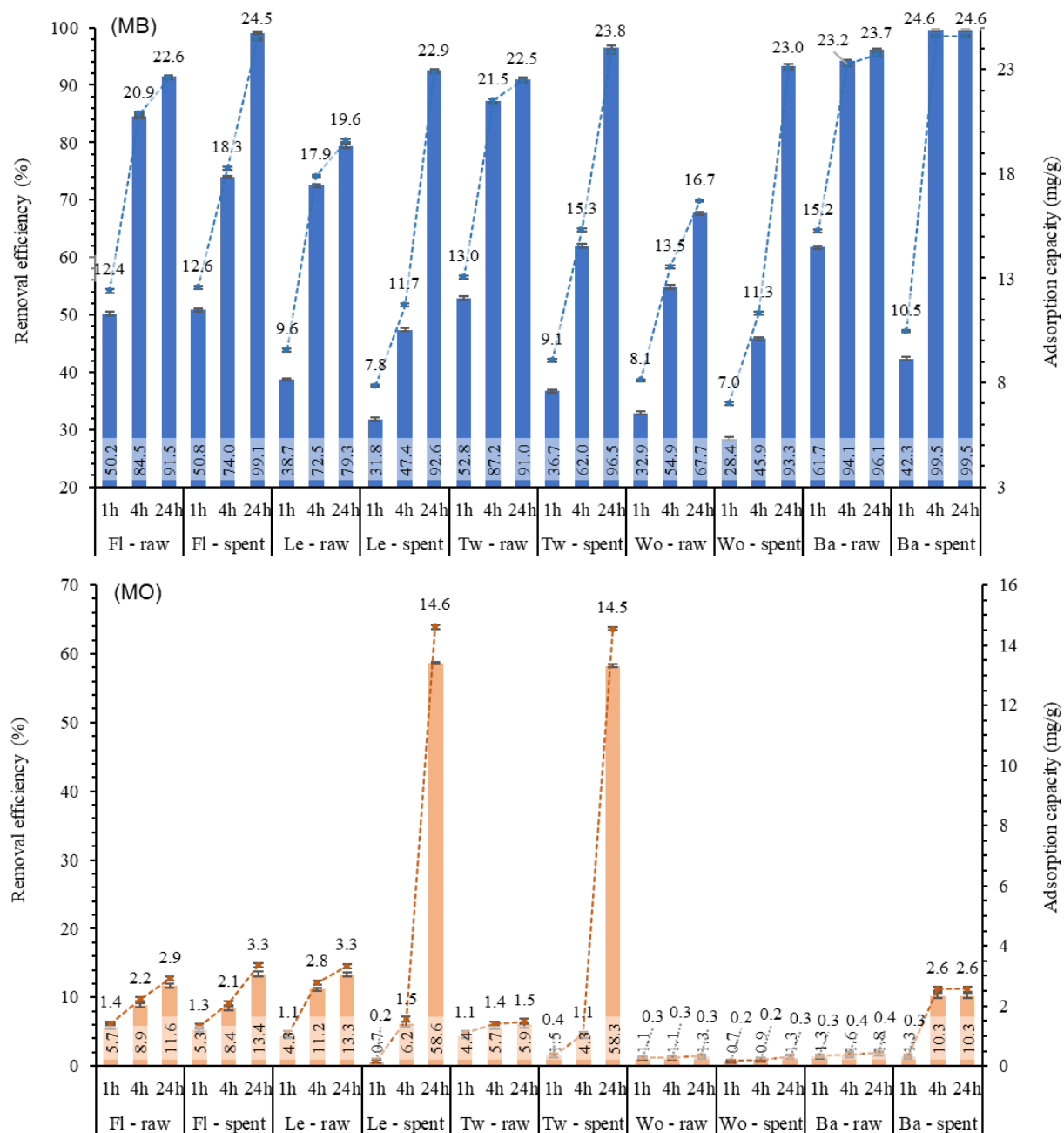


Figure F2. Removal efficiency (bars) and adsorption capacity (circles) towards MB (top) and MO (bottom) dyes, for raw and spent fractions at different contact times.



