UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA

TESIS DOCTORAL

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Supercritical technologies as sustainable tool for the concentration and co-encapsulation of Lavandula luisieri natural bioactives

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Directoras

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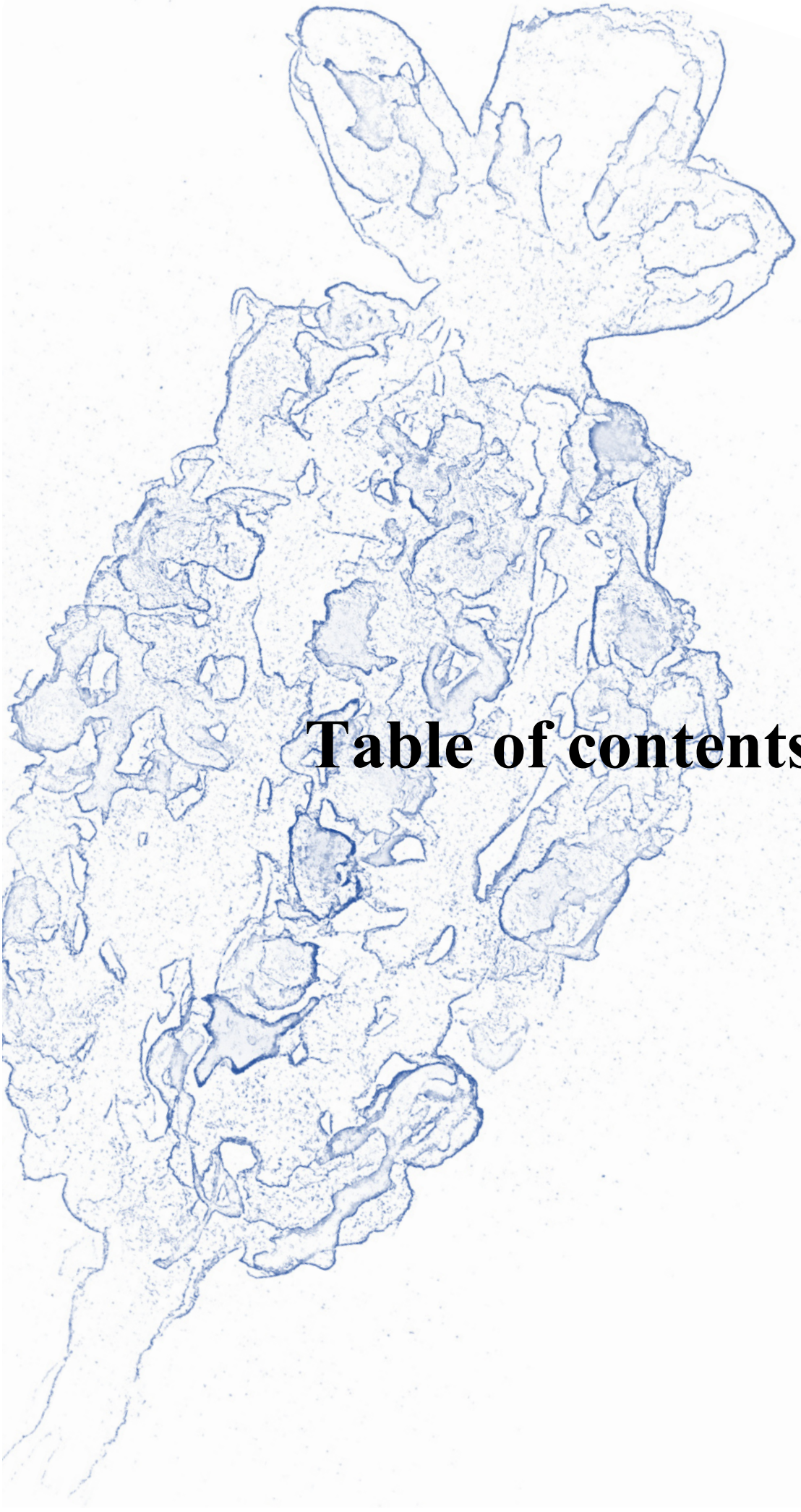
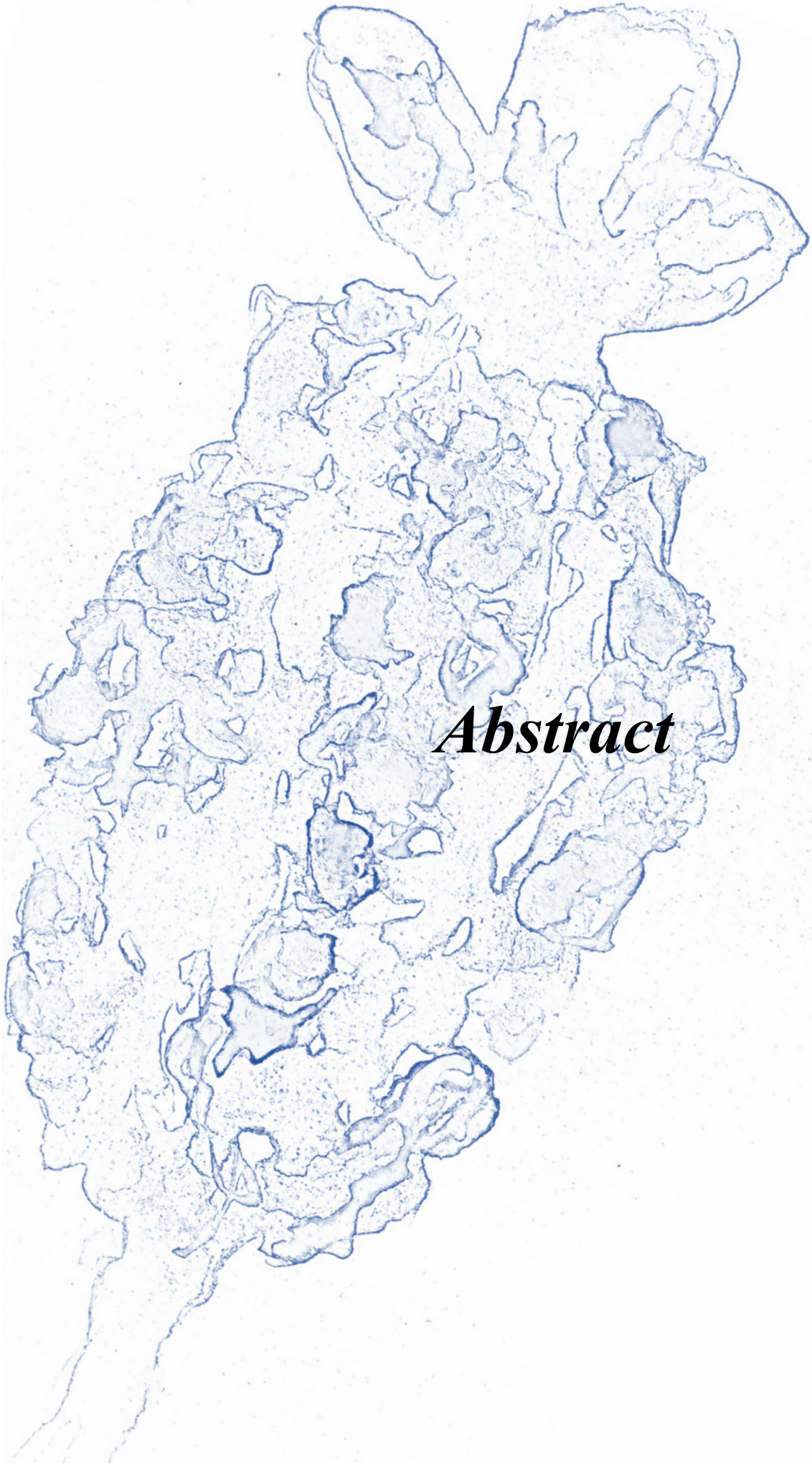


Table of contents



Abstract

Traditionally, plants have been used for the treatment and prevention of diseases or food conservation. These diverse applications are a consequence of the production of secondary metabolites as defence against external damaging agents, presenting a varied activity with interest in many fields. In fact, the inclusion of plants as therapeutical tool in health systems is recommended by international organisations like the WHO. Nowadays, 52% of drugs are a natural compound or a derivate, and have a growing commercial interest because of the consumers' preference for natural products inclusion as part of natural habits to prevent chronic illnesses.

The species *Lavandula luisieri*, endemic in the south west of the Iberian Peninsula, has been traditionally used with medicinal purposes, and classified as species because of its essential oil content in compounds derived from necrodane with cyclic C5 structure. Although the study about other type of extract is limited, it has been reported its content on the polyphenol rosmarinic acid, antiinflamatory and antioxidant active, and the triterpenes oleanolic and ursolic acids with hepatoprotective, antiinflamatory, antiproliferative and antimicrobial properties.

The obtaining of secondary metabolites from plants has been traditionally performed with techniques that requires high temperatures or organic solvents, damaging for the environment or the extract itself. In the last decades, supercritical fluids, with physicochemical properties between liquids and gases, have emerged as a part of the trend to efficient production, residue reduction and noxious solvents avoidance, known as "Green Chemistry". Carbon dioxide is one of the most used compounds under supercritical conditions because of its abundance, innocuity and low critical temperature and pressure. These advantages allow the processing of natural compounds under mild enough conditions to avoid their degradation and the activity loss. The application of supercritical technologies to *L. luisieri* for the selective concentration and vehiculation of actives of interest such as rosmarinic, oleanolic and ursolic acids, presents a potential interest for their application in pharmaceutical, cosmetical or food products.

Because of all this, the aim of this thesis was the sustainable production of concentrated products from *Lavandula luisieri*, and the study of its bioactives encapsulation through supercritical technologies.

To achieve this goal, first the extraction and concentration of the three actives of interest; the polyphenol rosmarinic acid, and the triterpenes oleanolic and ursolic acids, was

performed through Supercritical Antisolvent Fractionation (SAF). Response Surface Methodology based on Central Composite Design was employed to statistically evaluate and optimise the experimental conditions of pressure and flow rate into the range 80-150 bar and 10-30 g/min CO_2 for a higher mass yield recovery in two different fractions and *L. luisieri* compound enrichment. Temperature and extract solution concentration and its flow rate were fixed at 40 ºC, 0.45 mL/min and 3% (wt/wt), respectively. Rosmarinic acid precipitated completely in the first fraction in the whole set of experiments, while ursolic and oleanolic acids were distributed among both of them. The concentration of the three actives was up to 1.8 times higher regarding their initial concentration in the ethanolic extract. According to the statistical optimization, the experimental conditions for a higher mass recovery and enrichment of the compounds were 130 bar and 30 g/min.

The antimicrobial and antioxidant activities of *L. luisieri* extracts and supercritical fractions obtained under optimized conditions were determined in this work. The assay was performed against 5 bacterial strains of interest in human health and food control, *Listeria monocytogenes, Enterococcus faecium, Staphylococcus aureus, Salmonella* Typhimurium and *Escherichia coli*. The activity of the pure rosmarinic and ursolic acids was evaluated parallelly. The minimum inhibitory and bactericidal concentrations were quantified in vitro with the dilution broth method. Gram positive bacteria were the most sensitive to the extracts and the studied fractions, being the precipitated fraction, the most active and *Listeria monocytogenes* was the most sensitive. According to the assays performed with the pure compounds, the increase of antimicrobial activity after the supercritical concentration was consequence of its enrichment in oleanolic and ursolic acids. The essential oil was studied too, and it showed also inhibitory and bactericidal properties but a lower concentration. Moreover, the antioxidant activity of the ethanolic extract and its fractions was tested against the free radical 2,2-diphenyl-1-picryl-hydrazil. Again, the precipitated fraction was more active than the initial extract, but in this case the increment of activity was a consequence of the enrichment in rosmarinic acid.

Finally, and because of natural compound lability to external agents, the encapsulation of rosmarinic acid, one of the more abundant and intense antioxidant activity compounds, as a model of *L. luisieri* supercritical fraction, was proposed. The encapsulation was performed using the technology Supercritical Emulsion Extraction (SEE) into the biopolymers polylactic and polylactic co-glycolic acids. To do so, the experimental conditions were fixed at 80 bar, 38 ºC and 1.4 kg/h of supercritical CO_2 and 0.14 kg/h of

emulsion solution. Different single and double emulsions were formulated in order to encapsulate the antioxidant rosmarinic acid, alone and with other natural antioxidants, β-carotene and α-tocopherol, widely used as preservatives. After every experiment, the emulsion droplets and capsules mean size and size distribution were determined and optical and electronic microscopy images obtained. Although different formulations were proposed, the encapsulation efficiency of rosmarinic acid was very low, probably due to its co-extraction with the organic solvent during the supercritical process. The encapsulation efficiency of rosmarinic acid was improved by its co-formulation with β-carotene and α-tocopherol, which showed a higher loading. The carriers mean size produced with this technique varied depending on the initial emulsion formulated, between 0.3 ±0.1 μm and 1.5 ±0.5 μm. The antioxidant activity of the actives was maintained after the encapsulation, besides, the protection of the actives inside the capsules prolonged their shelf life.

The obtained results showed that the techniques SAF and SEE using supercritical CO_2, allow the obtaining of two bioactive products with potential use in pharmacy, cosmetics and food.

Resumen

Tradicionalmente, las plantas han sido utilizadas para el tratamiento y prevención de enfermedades o conservación de alimentos. Su diversa aplicación deriva de la producción de metabolitos secundarios como defensa contra agentes externos dañinos, mostrando una variada actividad con interés en diferentes campos. De hecho, la inclusión de plantas como herramienta terapéutica en los sistemas de salud está recomendada por organismos internacionales como la OMS. Actualmente el 52% de los fármacos son o bien un compuesto natural o un derivado del mismo, con un interés comercial creciente debido a la preferencia que los consumidores muestran los productos de origen natural como parte de hábitos de vida saludables para la prevención de enfermedades crónicas.

La especie *Lavandula luisieri,* endémica del suroeste de la península ibérica, ha sido tradicionalmente utilizada con fines medicinales, y clasificada como especie por la riqueza de su aceite esencial en compuestos derivados del necrodano con estructura cíclica C5. Sin embargo, el conocimiento sobre otros extractos de esta planta es escaso, si bien ha sido descrito su contenido en fenoles como el ácido rosmarínico, antiinflamatorio y antioxidante, y en triterpenos hepatoprotectores, antinflamatorios, anticancerígenos y antimicrobianos como los ácidos ursólico y oleanólico.

Tradicionalmente la obtención de estos metabolitos secundarios se ha realizado con algunas técnicas que requieren altas temperaturas o solventes orgánicos, dañinos para el medioambiente o para el propio extracto. En las últimas décadas, y como parte de la tendencia hacia producción eficiente, reducción de residuos y solventes nocivos o "Química Verde", surgen los fluidos supercríticos, con propiedades fisicoquímicas intermedias entre líquidos y gases. Entre estos fluidos destaca el CO_2 por su abundancia, inocuidad y punto crítico muy accesible, ventajas que permiten el procesado de productos naturales bajo condiciones experimentales suaves que evitan la degradación de sus principios activos. La aplicación de tecnologías supercríticas a *L. luisieri* para el concentrado y vehiculizado selectivo de activos de interés como rosmarínico, oleanólico y ursólico, supone un potencial interés para su aplicación en formulaciones farmacéuticas, cosméticas o alimentarias.

Así, en este trabajo se plantea como objetivo general la obtención sostenible de productos concentrados de *Lavandula luisieri* y el estudio del encapsulado de sus bioactivos mediante tecnologías supercríticas.

Para alcanzar este objetivo, en primer lugar, se realizó la extracción y concentración de tres metabolitos secundarios de interés: el polifenol ácido rosmarínico y los triterpenos ácido oleanólico y ácido ursólico mediante Fraccionamiento Supercrítico Antidisolvente (SAF). Se utilizó una metodología de Superficie de Respuesta basada en un Diseño Central Compuesto para diseñar y, posteriormente, optimizar las condiciones experimentales de presión y caudal de CO_2 dentro de los rangos de 80-150 bar y 10-30 g/min, respectivamente. El resto de condiciones experimentales, temperatura, concentración y flujo de la solución etanólica a fraccionar, fueron fijados en 40 ^{o}C, 3% (*v/v*) y 0.45 ml/min. Independientemente de las condiciones de trabajo, el ácido rosmarínico quedó retenido en la cámara de precipitación de sólidos mientras que los ácidos oleanólico y ursólico se distribuyeron entre las dos fracciones obtenidas. Se consiguió concentrar los 3 activos de interés hasta 1.8 veces en una de las fracciones respecto de la disolución de entrada al separador. Estadísticamente las condiciones experimentales optimas para conseguir una mayor recuperación de masa alimentada y concentración de los activos, fueron 130 bar y 30 g/min.

A continuación, se evaluó la actividad antimicrobiana del extracto etanólico y de las fracciones del mismo obtenidas en las condiciones óptimas junto con la del aceite esencial, frente a 5 cepas bacterianas de importancia en salud humana y seguridad alimentaria: *Listeria monocytogenes*, *Enterococcus faecium*, *Staphylococcus aureus*, *Salmonella* Typhimurium y *Escherichia coli.* Paralelamente, se determinó la actividad antimicrobiana de los activos puros ácido rosmarínico y ursólico. Las concentraciones mínima inhibitoria y bactericida fueron cuantificadas mediante el método de dilución en caldo. Las bacterias gram positivas fueron las más sensibles a los extractos ensayados, siendo la primera fracción supercrítica la más activa y *L. monocytogenes* la cepa más sensible. De acuerdo al ensayo con los activos puros, el incremento de la actividad antimicrobiana tras el concentrado supercrítico, fue consecuencia del enriquecimiento en oleanólico y ursólico. El aceite esencial, también fue inhibitorio y bactericida, pero a concentraciones inferiores a los otros extractos estudiados. Además, la actividad antioxidante del extracto etanólico y fracciones supercríticas fue cuantificada frente al radical 2,2diphenil-1-picril-hydrazilo, y se determinó el IC50 de cada extracto y de los activos rosmarínico y ursólico. De nuevo, la primera fracción fue más activa que el extracto inicial, pero en este caso el incremento de actividad tras el procesado supercrítico se debe al enriquecimiento en ácido rosmarínico.

Finalmente, dada la labilidad de los compuestos naturales a agentes externos se propuso el encapsulado de ácido rosmarínico, uno de los activos más abundantes y con intensa actividad antioxidante, como modelo de la fracción supercrítica de *L. luisieri*. Dicho encapsulado se realizó utilizando la técnica de Extracción Supercrítica de Emulsiones (SEE) en los biopolímeros ácido poliláctico y ácido poliláctico co-glicólico. Las condiciones experimentales del proceso fueron fijadas a 80 bar, 1.4 kg/h de CO_2 0.14 kg/h de emulsión. Se formularon diferentes emulsiones simples y dobles para co-encapsular ácido rosmarínico con otros antioxidantes naturales β-caroteno y α-tocoferol, ampliamente utilizados como conservantes. La eficiencia de encapsulado del ácido rosmarínico fue muy baja a pesar de la formulación en diferentes emulsiones, probablemente debido a su co-extracción junto con la fase oleosa. Sin embargo, la eficiencia de encapsulado de este activo incrementó cuando se formuló junto a los antioxidantes β-caroteno y α-tocoferol, los cuales presentaron mayor eficiencia de encapsulado. El diámetro medio de las partículas producidas fue de 0.3 ±0.1 μm a 1.5 ±0.5 μm dependiendo de la emulsión formulada. La actividad antioxidante de los activos se mantuvo tras el encapsulado, además la protección de activos en el interior de las capsulas de polímero, prolongó su tiempo de vida media.

Los resultados alcanzados muestran que el CO_2 supercrítico en las técnicas SAF y SEE permite la obtención de dos productos bioactivos con potencial uso en farmacia, cosmética o alimentación.

List of figures

Figure IV.3. Pictures of the solid precipitated in PV fraction after experiment 9 (p = 115 bar and Q_{CO_2} = 20 g/min). a) Precipitation vessel injector, b) Precipitation vessel inside view once opened, c) and d) Powder recovered from PV.

Figure IV.4. SEM Picture of the powder obtained in the precipitation vessel of experiment 10 (p = 90 bar and Q_{CO_2} = 27 g/min). A) 5.00 K X, B) 10.00 K X, C) 20.00 K X, and D) 40.00 K X. particle diameters measured 82.66 nm and 71.16 nm.

Figure IV.4 (continued). Image from the powder obtained in the precipitation vessel of experiment 10 (p = 90 bar and Q_{CO_2} = 27 g/min). A) 5.00 K X, B) 10.00 K X, C) 20.00 K X, and D) 40.00 K X. particle diameters measured 82.66 nm and 71.16 nm.

Figure IV.5. SEM Picture of the powder obtained in the precipitation vessel of experiment 10 (p = 90 bar and Q_{CO_2} = 27 g/min) (5.00 K X). Non-spherical, cuboid shape.

Figure IV.6. SEM Picture of the powder obtained in the precipitation vessel of experiment 11 (p = 115 bar and Q_{CO_2} = 10 g/min) (20.00 K X). Particle diameter measured 52.67 nm.

Figure IV.7. SEM Picture of the powder obtained in the precipitation vessel of experiment 11 (p = 115 bar and Q_{CO_2} = 10 g/min) (10.00 K X). Polyhedral structure.

Figure IV.8. Chromatogram obtained after an injection of the 3 standards used. 1st peak RA (1.6 min), 2nd peak OA (7.5 min), and 3rd peak UA (7.8 min).

Figure IV.9. Overlaid chromatograms of PV (blue), ME (red) and DV (black) of experiment 2 (p = 140 bar and Q_{CO_2} = 13 g/min)

Figure IV.10. Response surface plots of the SAF fraction yields: A) Y_{PV} (%); B) Y_{DV} (%); C) Y_{SAF} (%) as a function of XP and XQ_{CO_2}

Figure IV.11. Response surface plots of $E_{TC\ PV/FS}$ as a function of XP and XQ_{CO_2}

Figure IV.12. Optimal combined conditions for a maximum mass recovery in PV, DV and SAF and maximum $E_{TC\ PV/FS}$

Figure IV.13. Macrodilution antimicrobial test of *L. luisieri* essential oil solutions against *Enterococcus faecium.* C+, Positive control, C-, Negative control *Satureja montana* essential oil, and the tested concentrations of *L. luisieri* essential oil 0.5, 1, 2, 3, 5, 7.5, 15 and 30 μL/mL.

Figure IV.14. Macrodilution antimicrobial test of *L. luisieri* essential oil solutions against *Salmonella* Typhimurium. C+, Positive control, C-, Negative control of *Satureja montana* essential oil, and the tested concentrations of *L. luisieri* essential oil 0.5, 1, 2, 3, 5, 7.5, 15 and 30 μL/mL.

List of tables

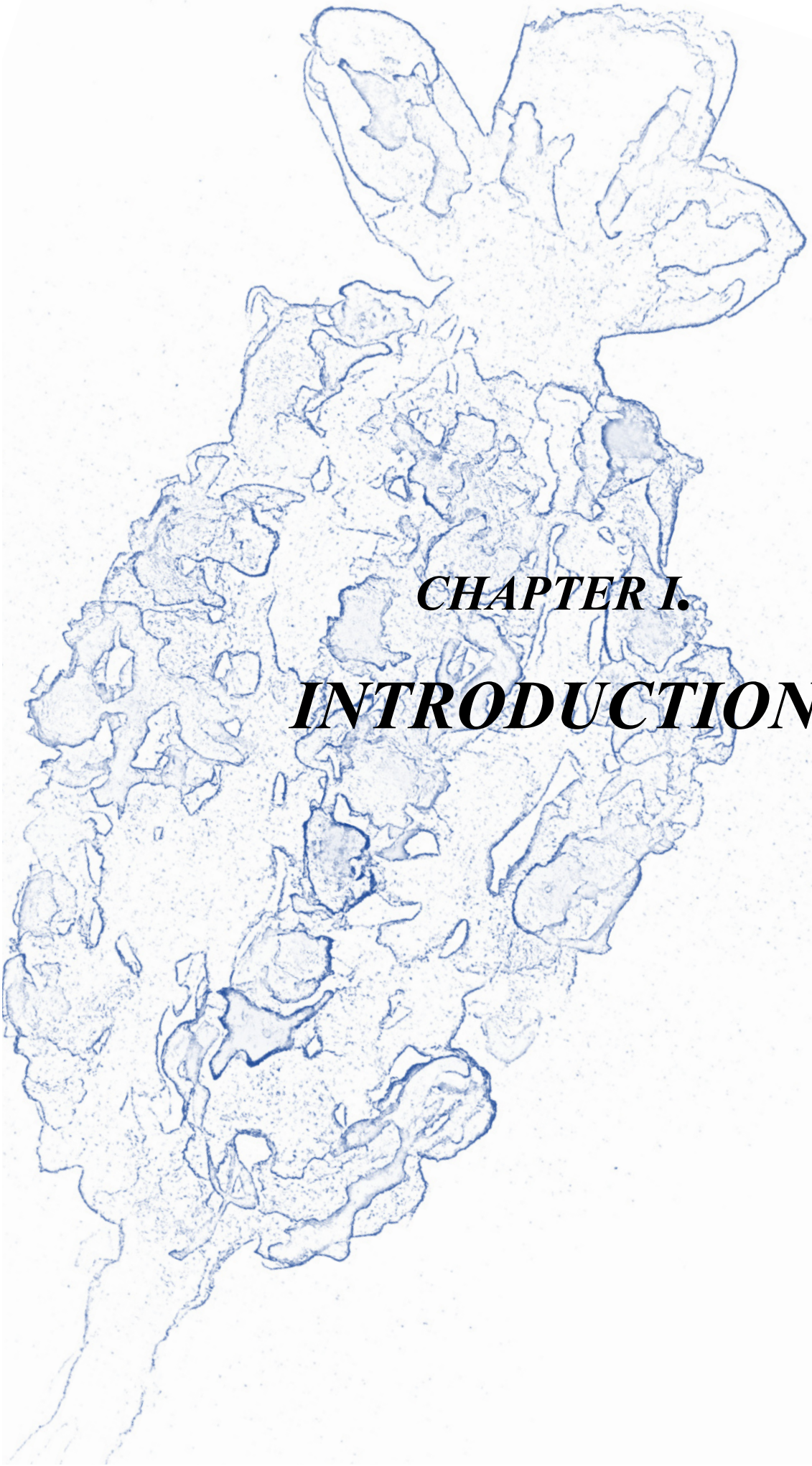
Abbreviations

AAPH	2,2′-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2′-azino-bis(3-ethylbenzothiazolline-6-sulfonic
ABPR	Automatic Back Pressure
ASAIS	Atomization of Supercritical Anti-solvent Induced Suspension
ASES	Aerosol Solvent Extraction System
ATP	Adenosine triphosphate
BPR	Back Pressure Regulator
CCD	Central Composite Device
CECT	Spanish Collection of Type Cultures
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CUPRAC	CUPric Reducing Antioxidant Capacity
DMSO	Dimethyl Sulfoxide
$DPPH^{\cdot}$	2,2-difemil-1-picrylhydracyl free radical
DSD	Droplets Size Distribution
DV	Downstream Vessel
EE%	Encapsulation Efficiency
EO	Essential Oil
$E_{RA\ PV/FS}$	Rosmarinic acid enrichment ratio in PV fraction
ET	electron transfer
EtAc	Ethyl Acetate
$E_{TC\ PV/FS}$	Total compound (rosmarinic, oleanolic and ursolic acids) enrichment ratio in PV fraction
$E_{OA\ DV/FS}$	Oleanolic acid enrichment ratio in DV fraction
$E_{OA\ PV/FS}$	Oleanolic acid enrichment ration in PV fraction
$E_{UA\ DV/FS}$	Ursolic acid enrichment ration in DV fraction
$E_{UA\ PV/FS}$	Ursolic acid enrichment ratio in PV fraction
DELOS	Depressurization of an Expanded Liquid Organic Solution
DPPH	α-diphenyl-β-picrylhydrazyl
FDA	Food and Drug Administration
FRAP	Ferric Reducing Antioxidant Power

FS	Feed solution
GAS	Gas Anti-solvent
GC-FID	Gas Chromatography-Flame Ionization Detector
GC-MS	Gas Chromatography-Mass Spectrometry
HAT	Hydrogen Atom Transfer
Hex	Hexane
HPLC	High Performance Liquid Chromatography
HPLC-MS	High Performance Liquid Chromatography-Mass Spectrometry
IC_{50}	Concentration required to inhibit the 50% of a free radical DPPH
LPIC	Lipid Peroxidation Inhibition Capacity
MetOH	Methanol
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MCP	Mixture Critical Point
P_c	Critical Pressure
ρ_c	Critical Density
PCA	Particles by Compressed Anti-solvent
PDA	Photo Diode Array
PLA	Polylactic acid
PLGA	Polylactic co-glycolic
P-LIQ	Liquid pump
PGSS	Particles from Gas Saturated Solutions
PSD	Particles Size Distribution
P-SCF	Supercritical Fluid Pump
PV	Precipitation Vessel
PVA	Polyvinyl Alcohol
RA	Rosmarinic acid
RESAS	Rapid Expansion from Supercritical to Aqueous Solutions
RESOLV	Rapid Expansion of Supercritical Solution into an Aqueous Solution

RESS	Rapid Expansion of Supercritical Solutions
RESS-SC	Rapid Expansion of Supercritical Solutions with solid co-solvent
RSM	Response Surface Methodology
SAF	Supercritical Antisolvent Fractionation
SAS	Supercritical Anti-Solvent
$scCO_2$	Supercritical CO_2
SCF	Supercritical Fluids
SEDS	Solution Enhanced Dispersion by Supercritical fluids
SEE	Supercritical fluid Emulsion Extraction
SEM	Scanning Electron Microscope
SFC	Supercritical Fluid Chromatography
SFE	Supercritical Fluid Extraction
SPAN	Sorbitan Monoestearate
T_c	Critical Temperature
TRAP	Total Radical-trapping Antioxidant Parameter
TSB	Tryptone Soy Broth
TSA	Tripticase Soy Agar
OA	Oleanolic acid
-OH	Hydroxyl group
ORAC	Oxygen Radical Absorbance Capacity
NMR	Nuclear Magnetic Resonance
UA	Ursolic acid
UV-vis	Ultraviolet-visible radiation
WHO	World Health Organization
X_P	Pressure variable
$X_{Q_{CO_2}}$	CO_2 flow rate variable
Y_{DV} (%)	Yield of mass recovered in the DV fraction after the SAF process
Y_{EtOH}(%)	Mass yield of the maceration in ethanol
Y_{hex} (%)	Mass yield of the maceration in hexane
Y_{PV} (%)	Yield of mass recovered in the PV fraction after the SAF process

Y_{SAF} (%)	Yield of the total mass recovered after the SAF process
α-TOC	α-Tocopherol
β-CA	β-carotene



CHAPTER I.

INTRODUCTION

1. Plants as a source of bioactive compounds

Traditionally, plant extracts have been used as health protectors in the treatment of numerous diseases and in food preservation. The use, extraction and preparation of plant raw material, such as roots, rhizomes, bulbs, leaves, stems, barks, wood, flowers, fruits and seeds, and its effects was passed from generation to generation by oral tradition and also printed in some texts. The Greek physician Dioscorides gathered information about these traditional remedies in a five-volume pharmacopeia named *De materia medica* (*On medical matter*) around 65 b. C. This document describes medicinal plant extracts along with animal and mineral active substances.

However, these ancient traditions were dismissed when western medicine grew in the modern era. Traditional medicine popularity fell into a category of useless, and unscientific, and its positive effects were considered "placebo".[1,2] Nevertheless, traditional medicine still plays an important role in the current health systems in developing countries and, in developed ones.[3] The traditional knowledge has offered important leads in the search of new drugs.[4]

Nowadays, the western herbal medicine has evolved towards a more scientific, evidence-based medicine model opposed to the traditional anecdotal knowledge gained through history, creating the term "*rational phytotherapy*", antecedent of today's conventional and complementary medicine.

Because of these practises through time in different civilizations and cultures, the World Health Organization (WHO) defined the term *Traditional Medicine* as: "*knowledge, skills and practises based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness*".[5] Because of scientifically demonstrated plant benefits and importance in health care, the WHO published: "*WHO Traditional Medicine Strategy 2014-2023*". This document about the traditional and complementary medicine encourages its global integration within health care systems, increases the knowledge to ensure its safety, efficacy and quality, ensures available and affordable products and promotes its rational use by practitioners and consumers.[6]

The compounds responsible for these therapeutic actions, called bioactive compounds or simply bioactives or actives, are usually the secondary metabolites of

plants. They are described as organic compounds produced by plants that are not directly involved in the normal growth, development, or reproduction of an organism.[2]

Unlike primary metabolites, any absence of secondary metabolites in plants does not result in their immediate death. Nevertheless, they are especially important for the long-term plant survival, since they play a protective role against a wide variety of microorganisms (virus, bacteria and fungi) and herbivores (arthropods and vertebrates).[7] These substances are often differentially distributed among limited taxonomic groups within the plant kingdom and usually classified according to their biosynthetic pathways[8] into three large groups: phenols, terpenes and alkaloids (Figure I.1).

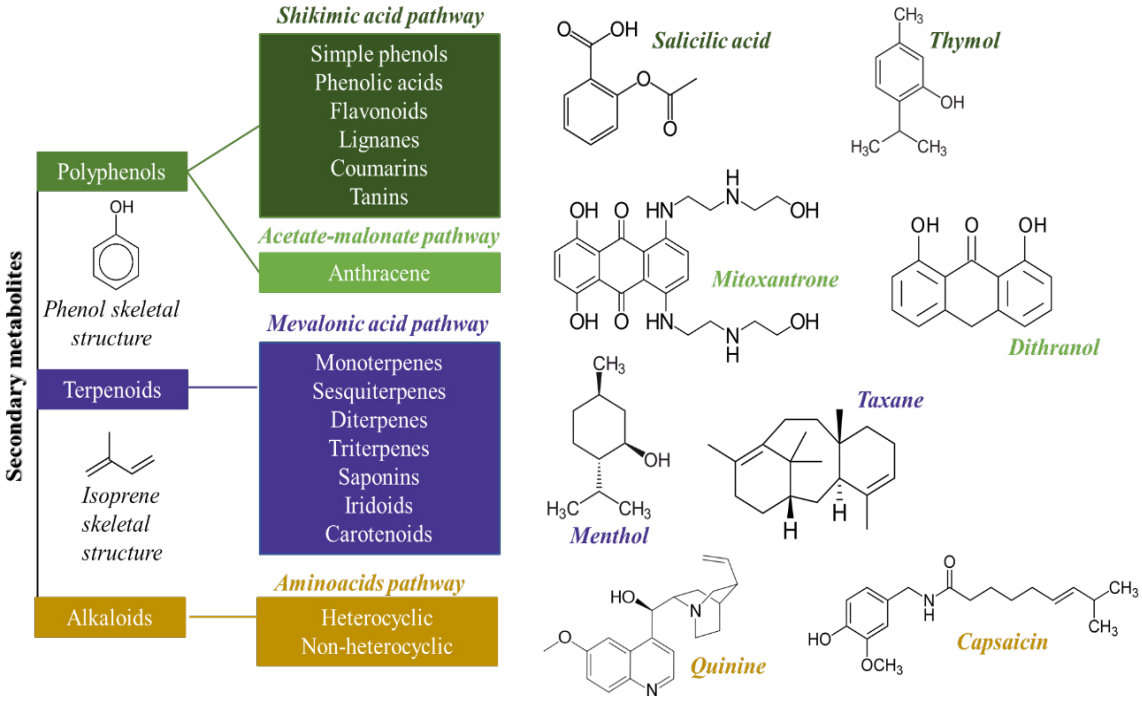


Figure I.1. Secondary metabolites classification and examples of each group.

Natural compounds have always been a source of new drug discovery and natural extracts have been and are studied in different research fields, specifically to find new molecules for clinical applications. It is estimated that over a hundred of new natural product-based leads are in clinical development.[9] Some examples of drugs used nowadays are gathered in the next Table I.1.

Table I.1 Example of bioactives from plants, their origin and medicinal use.

Compound	Vegetal drug	Medicinal uses
Salicylic acid (Phenolic acid)	*Salix alba* bark	**Skin**: Psoriasis, acne, keratosis, calluses etc **Blood system**: Antiplatelet, prophylactic heart attack or stroke **Pain**: Anti-inflammatory **Fever**: Antipyretic
Digoxin (Flavonoid)	*Digitalis* spp foxglove	**Blood system:** Cardiac insufficiency, heart failure and arrhythmia
Quinine and Quinidine (Alkaloids)	*Cinchona* spp bark	**Microorganisms:** Antimalaric **Blood system:** Anti-arrihythmic
Vincristine Vinblastine (Alkaloids)	*Catharanthus roseus*	**Cancer**: Lymphomas Hodgkin and no-Hodgkin, skin, testicular, bladder and brain cancer
Topotecan Irinotecan (Alkaloids)	*Campototheca accuminata* bark, root and fruits	**Cancer**: Uterus, ovaries, lungs, colon and rectum.
Atropine (Alkaloids)	*Atropa belladonna* leaves	**Poisoning**: Antidote for mushroom or pesticide intoxication
Morphine Codeine (Alkaloids)	*Papaver somniferum*	**Pain**: Acute or chronic pain **Respiratory system**: Antitussive **Digestive system**: Chronic diarrea
Taxano (Terpene)	*Taxus bacata* bark	**Cancer**: Breast, ovaries, lung, bladder, prostate skin and oesophagus.
Gingsenosids (Flavonoids)	*Ginko biloba*	**Nervous system**: Brain insuficiency and Alzheimer
Proantocianidine (flavonoid)	*Vaccinium macrocarpon fruit*	**Microorganisms**: Profilactic urine infections **Blood system**: venous and limphatic insuficiency

With the interest in healthier habits to prevent chronic diseases such as cardiovascular problems, diabetes or Alzheimer, nutraceuticals demand has increased to promote health, illness prevention and replacement of acute treatments intended for chronic exposure.[10] This same trend is followed by the cosmetic, food or agricultural industries, where the substitution of synthetic chemical additives or pesticides for natural ones has been demanded.[11] Some natural compounds allowed in EU are (Regulation (EC) No 1333/2008) are listed bellow:[12]

- Natural emulsifiers, stabilisers, thickeners and gelling agents: alginic acid (E400), agar (E406), processed Eucheuma seaweed (E407a), guar gum (E412), tragacanth (E4013), acacia gum, gum arabic (E414) or soybean hemicellulose (E426).
- Food colourantes: curcumin (E100), riboflavin (E101), carmines (E120), carotenes (E160a), paprika extract (E160c), lutein (E161b), anthocyanins (E163)

- Natural antioxidants: ascorbic acid (E300), tocopherols (E306) and extracts of rosemary (E392).
- Natural sweeteners: sorbitol (E420), mannitol (E421), neohesperidine DC (E959), steviol glycoside (E960), erythritol (E968).

In the food industry, these additives are investigated to be incorporated directly in the food products or even in the package that contains them, and, in some cases, they are expected to have also potential benefits in the consumers' health beyond its preservative properties.[13]

1.1. Lavandula luisieri

Historically, species of the family Lamiaceae have enjoyed a rich tradition of use for flavoring, food preservation, and medicinal purposes, due to both their curative and their preventive properties. Their value lays in the production of a wide range of secondary metabolites with potent antibacterial, antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer activities. [14]

Lavandula one of the largest genres of this family and its species, have been studies because of their perfumed essential oil. Its popularity derives from their multiple therapeutic properties as sedative effects on the nervous system, antibacterial, antifungal, antiviral, antiproliferative, antioxidant, antiallergic, and antiinflamatory.[15] Nevertheless, some species such as *Lavandula luisieri*, have not received that attention as it will be further describe in this work.

Lavandula (Linneo 1754) is one of the widest genres belonging to this family. It is formed by very aromatic specimens with a terminal inflorescence with different morphologies and chemotypes constituting a wide spectrum of species, hybrids, varieties and subspecies.[16] They are distributed mainly through the Mediterranean area along with North Africa, Arabian Peninsula, United States, Australia and Russia. The genus morphology is a mixed and divergent group so the number of recognised species keeps growing in the consecutive classifications along time.[17]

The Latin name of *Lavandula* comes from the early use of this plant to perfume water for bathing, being derived from the Latin word *lavare*, meaning "*to be washed*". In ancient civilizations, such as Greek or Roman, it was added as perfume and antiseptic to

baths and to laundry, and ancient Egyptians used it to the wrapped bodies during the mummification process.[18] *Lavandula* species essential oils are still appreciated in the perfume and cosmetic formulation because of their content in volatile compounds such as linalyl acetate or linalool.[19] In the fragrance industry *Lavandula* plants are of economic importance since their essential oil has been used in soaps, colognes, perfumes, skin lotions and other cosmetics, pillows, bath care, home and pet products.[20] Nevertheless, they are also being studied in aromatherapy and phytotherapy because of their anxiolytic and sleep aid effects,[21] as well as in the food industry, where the volatile compounds have been employed as flavouring since they provide a unique taste to many beverages, sweets, jellies, jams, marmalades, honey and condiments.[22]

The species *L. stoechas* derives from the Stoechades Islands situated off southern France (Îles d'Hyères), from where the plant was first described by the Greek physician Dioscorides and distributes along the Mediterranean area. Stoechas is a distinct group of small shrubs with linear-lanceolate leaves and dense and compact flowers toped on the apex by a distinctive tuft of enlarged and coloured sterile bracts (a coma).[15]

One of the vast number of medicinal and aromatic plants is the species *Lavandula stoechas luisieri,* which was first classified by Rozeira into the Lamiaceae family[16] (Table I.2) will be used in this work as a source of bioactives. It is an autochthonous lavender species endemic in the southwest of the Iberian Peninsula and has been traditionally used as antiseptic of the aerial ways or wounds or as antispasmodic and digestive. The essential oil of its inflorescences is sold by Nakai Cosmética natural, TerpenicLabs ®, as antiaging treatment for mature skins, scar healer, antalgic and antiseptic.

Lavandula lusieri was classified into the Lamiaceae family, an enlarged family of flowering plants. This family original name, Labiatae, was given by Lindley (1836) because the flowers typically have petals fused into an upper lip and lower lip.[23] Although it is still considered an acceptable name, most botanists now use the new term Lamiaceae. The species belonging to this family spread in the warm and template regions all over the world. They are mainly herbs and shrubs, very fragrant and rich in medicinal properties of great value in natural medicine and highly considered into the pharmacopeia by the medical society. Lamiaeae constitutents also perform as culinary herbs such as, basil, mint, rosemary, sage, oregano, thyme and lavender.[24]

Table I.2. *Lavandula luisieri* taxonomy.[25]

Lavandula luisieri	**Scientific classification**	
	Kingdom	Viridiplantae
	Phylum	Streptophyta
	Class	Magnoliopsida
	Clade	Asterids
	Clade	Lamids
	Order	Lamiales
	Frfr4amily	Lamiaceae
	Subfamily	Nepetoideae
	Tribe	Lavanduleae
	Genus	*Lavandula*
	Specie	*stoechas*
	Subspecie	*luisieri*

Lavandula stoechas subsp. *luisieri*, first classified by Rozeira Riv.-Mart (Rivas Martinez, 1979), is endemic in the Iberian Peninsula, common in the South of Portugal and in the Southwest of Spain. *L. luisieri* exhibits ornamental, melliferous and medicinal properties and serves as a natural insect repellent.[26]

Nevertheless, what makes *L. luisieri* dissimilar from other Stoechas species is the chemical composition of its essential oil. Differently to other *Lavandula* species, this subspecies from Stoechas produces atypical compounds with a five-carbon cycle terpene structure named necrodane and derivates. Necrodyl acetate and α-necrodol were first identified by García-Vallejo et al. [27] on the basis of their NMR spectra. This kind of compounds has been only identified so far in the Animalia kingdom, specifically in the defensive secretions of the carrion beetle *Necrodes surinamensis,*[26] and in the sex pheromone of the grape mealybug *Pseudococcus maritimus.*[28]

1.1.1. L. luisieri chemical composition

After García-Vallejo et al.,[27] several authors have characterized the chemical composition of different *L. luiseri* populations´ essential oil and other extracts, and their biological activities because of the production of these special aromatic monoterpenes.

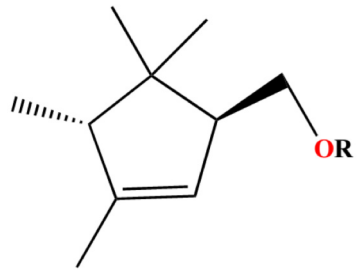
In the following Table I.3, all research works about different extracts composition of this lavender are chronologically gathered. As it can be observed, most authors

analysed the composition of the essential oil in which several chemotypes were observed. They also observed that the content of the main compounds, differ from one *L. luisieri* essential oil to another. The proportion of each compound quantified in the essential oil by the different authors quoted before is gathered in Annex A, where the actives have been classified according to their structure in monoterpenois, oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes and their acetates. The most abundant and common monoterpenes among specimes are α-pinene, 1,8-cineol, camphor, fenchone, lavandulool, linalool, terpineol, lavandulyl acetate, and necrodane derivates such as 1,1,2,3-tetramethyl-4-hydroxymethyl-2-cyclopentene, 2,3,5,5-tetramethyl-4-methylene-2-cyclopenten-1-one, 2,3,4,4-tetramethyl-5-methylene-cyclopent-2-enone or 5-hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one. In Figure I.2 the structure of these compounds is shown. Among the sesquiterpenes, the most abundant compounds are cadinol and viridiflorol.

Table I.3. Reported chemical composition of *L. luisieri* extracts.

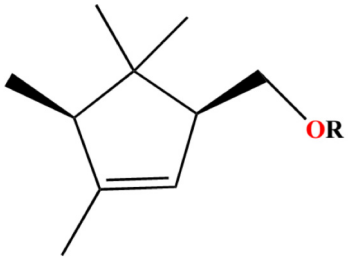
Plant extract	Analytical method	Bibliography
Essential oil	NMR	García Vallejo et al.,[27], Baldovini et al.,[29] Lavoine-Hanneguelle.,[30] Julio et al.[31]
	GC-MS or GC-FID	Matos et al.,[20] Zuzarte et al.,[22] Lavoine-Hanneguelle et al.,[30] Sanz et al., [32], Gonzalez-Coloma et al., [33] Delgado et al.,[34] Roller et al.,[35] Gonzalez-Coloma et al., [36] Videira et al.,[37] Rufino et al.,[38] Dias et al.,[39] Arantes et al.,[40] Pombal et al.,[41] Andrés et al.,[42] Costa et al.,[43] Queiroga et al.,[44]
Ethanol (s*oxhlet*)	HPLC-MS	Julio et al.,[45]
Hexane, dichlorometane, ethyl acetate, methanol (*aceration*)	Total phenol and flavonoid content	Pereira et al..[46]
Water and ethanol extracts (*maceration*)		Nunes et al.,[47]

Nevertheless, as in other aromatic plant species and as a consequence of variable environmental factors such as temperature, humidity, light intensity, water supply, minerals, and geographical region, the growth of a plant and its secondary metabolite production can change from one population to another.[48] This qualitative and quantitative differences can be observed in Annex A.



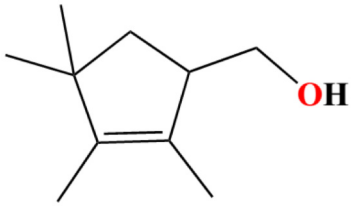
R =H Trans-α-necrodol

R=CH_3CO Trans-α-necrodyl acetate

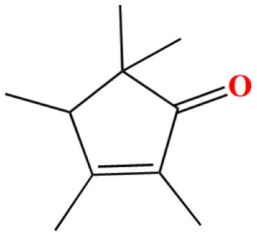


R =H cis-α-necrodol

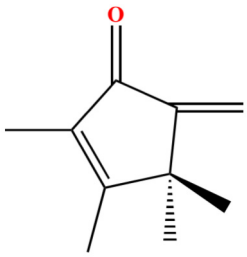
R=CH_3CO cis-α-necrodyl acetate



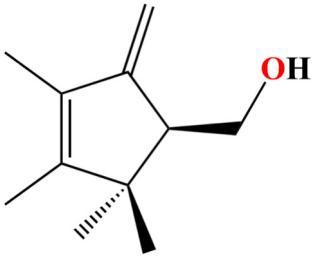
1,1,2,3-tetramethyl-4-hydroxymethyl-2-cyclopentene



2,3,5,5-tetramethyl-4-methylene-2-cyclopenten-1-one



2,3,4,4-tetramethyl-5-methylene-cyclopent-2-enone



5-hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one

Figure I.2. Necrodane derivates, the most abundant compounds of *L. Luisieri.*[28]

Although plants essential oil composition analysis is widely studied, other type of extracts is not. This difference is also very notable for *Lavandula luisieri*, which essential oil additionally presents a particularly interesting composition in necrodane-like actives. The study of its non volatile fraction, however, is very reduced, and it has been reported in two previous works. Nunes et al.[47] performed an electrothermal extraction from a Portuguese *L. luisieri* using ethanol-water mixtures as solvent and reported an enriched composition in phenolic acids, such as rosmarinic acid, ferulic acid and chlorogenic acid. Julio et al.[45] performed an ethanolic soxhlet extraction from cultivated and wild populations, and found also triterpene compounds. In table I.4, the identified compounds and their quantification from these two works are listed.

Table I.4. Identified compounds from extracts different from essential oil.[45,49]

		Julio et al.[45]	**Nunes et al.**[49]
		% relative abundance	*mg/100g dry extract*
Phenolic acids			
	Caffeic acid	-	3.94
	Chlorogenic acid	-	12.64
	Ferulic acid	-	17.30
	Protocatechuic acid	-	1.29
	Rosmarinic acid	3.41-7.32	301.71
	Vanillic acid	-	1.07
	3-hydroxybenzoic acid	-	1.7
Triterpene			
	Tormentic acid	0-13.85	
	Oleanolic acid	2.95-3.47	
	Ursolic acid	0-12.4	
Others			
	3-oxo-cadinol	0-10.03	
	5-hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one	4.33-7.93	

The differences in the extraction, separation and detection methods, plant cultivation and origin could be the reason of the different composition observed among them. Although the non-volatile fraction of this plant species has not received much attention, it seems to contain polyphenols and terpenes, besides other volatile compounds also founded in the essential oil.

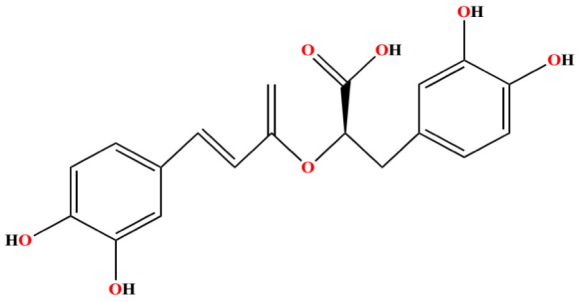
Rosmarinic acid (Figure I.3), dimer of caffeic acid is found in both cases, being the main compound (301.7 mg/g of dry extract) according to Nunes et al.[47] It is a phenolic acid widely distributed in the plant kingdom especially in the *Lamiaceae* family,

nepetoideae subfamily in species like basilicum, rosmarinus, sage or lavandula. It possesses several biological activities. As a potent antioxidant reduces oxidative stress and therefore reduces antiinflamatory response.[50] Besides, it also reacts rapidly with the viral coat proteins inactivating viruses.[51] Current research on rosmarinic acid centres on its physiological and pharmacological activities,[52] and in this work its concentration along with other *L. luisieri* non volatile compounds will be explored in order to be applied in cosmetical, nutraceutical and food products. Other phenolic acids such as, chlorogenic acid, caffeic acid, ferrulic acid, protocatechuic acid, vanillic acid and 3-hydroxybenzoic acid were detected in this Portuguese lavender.

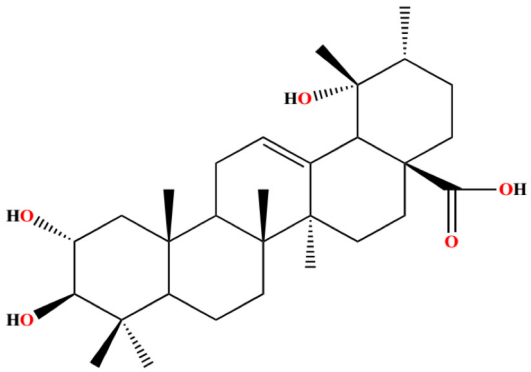
Julio et al.[45] identified also three triterpenes; tormentic, oleanolic and ursolic acids (Figure I.3). Oleanolic and its isomer ursolic acid are two molecules widely distributed in plant and frequently are founded together due to their similar structure. They have been isolated in other plant species such as olive leaves and fruits, pokeweed or myrtle plants and have showed hepatoprotective, anti-inflammatory, anticancer,[14] and antimicrobial effects.[54] Because of oleanolic and ursolic acid wide distribution among plant species and marked applications as potential drug prototypes, [53] in this work were selected to be tracked.

The ethanolic extraction performed by Julio et al.[45] obtained different kind of compounds from two different plant metabolic pathways; probably because the penetrating power of ethanol and the application of temperature. Nevertheless, temperature causes the degradation of rosmarinic, oleanolic, tormentic and ursolic acids other phenolic reducing the final yield of.[55]

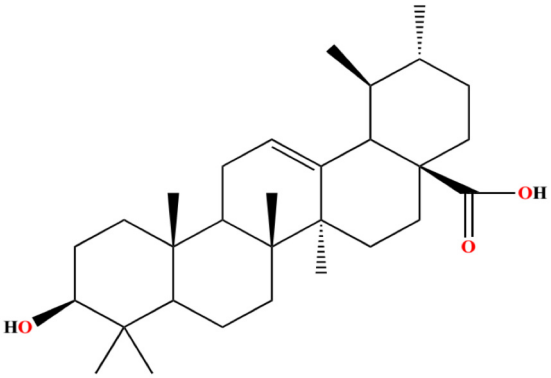
In tis thesis will be tracked three compounds from a *L. luisieri* population previously identified by Julio et al.:[45] rosmarinic, oleanolic and ursolic acids. The optimisation of an extraction of *L. luisieri* non-volatile fraction applying milder experimental conditions compared with the previous reported by Julio et al.[45] could have interest to obtain a concentrated product with both kind of secondary metabolites; phenolic acids, and triterpenes, because of its potential application with different purposes simultaneously.



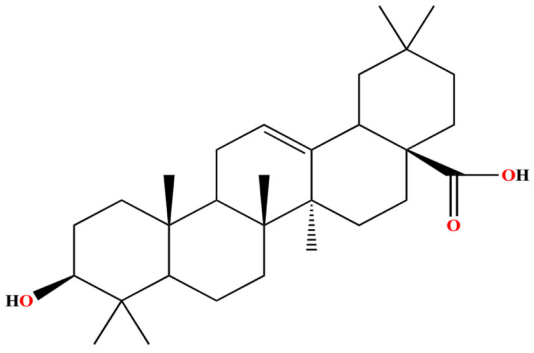
Rosmarinic acid



Tormentic acid



Ursolic acid



Oleanolic acid

Figure I.3. Rosmarinic, tormentic, oleanolic and ursolic acid molecules.

1.1.2. L. luisieri acivities

So far, several authors have tested diverse biological activities of *L. luisieri* extracts. In Table I.5, gathers *L. lusieri* biological activities reported in previous research. The essential oil has been the main focus of the activity assays.[20,22,29,33,35–38,40,45,46] Only one work has correlated the final bioactivity measured with the responsible compound, and most of them assign it to a group of not identified compounds. Only two previous studies have tested the activities of extracts different from essential oil.[46,56] These authors performed antimicrobial and antioxidant assays with the product obtaines by extracting with different organic solvents[46,56] and water,[56] and others with purified compounds from the plant.[31] As previously said, the secondary metabolites produced by a plant are usually involved in its survival against damaging agents of its environment such as light, temperature or microorganisms. This is the reason why the most common in vitro assays for plant activities are antimicrobial and antioxidant activities, which will also be studied in this work. For this plant species, the antifeedant effects against organisms affecting crops has also been studied, since necrodane-like compounds have been founded in the defensive secretion of the beetle *Necrodes surinamensis.* Besides other applications in human health, related with the traditional use of *Lavandula* species in aromatherapy, as anti-alzheimer, anti-inflammatory and analgesic have been reported.

Antimicrobial activity

The antimicrobial activity of *L. lusieri* extracts has been the most studied one so far. Nowadays, the number of microorganism strains that have developed resistance to the current antibiotic therapies is increasing and is one of the greatest threats for human health, food safety and development.[57] Antimicrobial resistance occurs when microbes, such as fungi, viruses and bacteria, develop defence mechanisms that make them safe from one or more antimicrobials. Some of the developed resistance mechanisms are enzyme inactivation, reduction in cell permeability, alteration in target site/enzyme, protection of target site by the formation of biofilms as mechanisms to resist antimicrobial agents.[58] Although all these processes occur naturally, they have been potentiated by the excessive antibiotic prescription in human and animal health, wrong use and adherence to the therapies by patients, lack of hygiene and inadequate control of infections in hospitals. The new cases of infectious diseases with difficult management with the current tools are increasing, causing longer hospital stays and perturbing morbidity and mortality statistics.[57]

Table I.5. Reported activities of *L. luisieri* extracts and their correlation with their composition.

Activity	*L. luisieri* extract	Responsible bioactives	Reference
Scavenging activity of DPPH free radical	EO	1,8-cineol trans-α-necrodyl acetate	Matos et al.[20]
Antioxidant by DPPH free radical scavenge, lipid peroxidation and DNA oxidation protection	EO and MetOH	polyphenols and flavonoids	Baptista et al.[46]
Antioxidant *vs* lipid peroxidation, antimicrobial, analgesic effect, anti-inflammatory and low toxicity	EO	oxygenated monoterpenes and necrodane derivates	Arantes et al.[40]
Antifungal *vs* the dermatophytes *Trichophyton rubrumand* and *Trichophyton mentagrophytes*	EO	oxigenated necrodane derivates and 1,8 -Cineol	Dias et al.[39]
Antimicrobial *vs Staphylococcus aureus Staphylococcus epidermidis, Enterococcus Faecalis*, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella* Thyphimurium and *Candida albicans*	Hex, CH_2Cl_2, EtAc, MetOH and H_2O	terpenes, phenolics and flavonoids	Lai et al.[56]
Antimicrobial *vs Candida albicans Staphylococcus aureus, Streptococcus. epidermidis* and *Streptococcus pyogenes*	EO	(n.s)	Baldovini et al.[29]
Antimicrobial *vs* methicillin sensitive and methicillin resistant *Staphylococcus aureus*	EO	1,8-cineol, α-necrodyl acetate	Roller et al.[35]
Antifungal *vs Candida strains, Aspergillus strains, Epidermophyton floccosum, Microsporum* strains and Trichophyton strains	EO	(n.s)	Zuzarte et al.[22]
Antifeedant *vs Spodoptera littoralis, Myzus persicae* and *Rhopalosiphum padi*	EO	trans-α-necrodyl acetate	Gonzalez-Coloma et al.[36]

EO, essential oil; Non-specified (n.s)

Table I.5. (continued) Reported activities of *L. luisieri* extracts and their correlation with their composition.

Activity	*L. luisieri* extract	Responible bioactives	Reference
Antifeedant *vs Spodoptera littoralis, Leptinotarsa decemlineata* and *Myzus persicae*	EO	(n.s.)	Gonzalez Coloma et al. [33]
Antifeedant *vs Spodoptera littoralis* and *Myzus persicae*	EO	necrodol derivates and camphor	Julio et al. [45]
Nematicidal *vs Meloydogine javanica*	Purified actives	necrodane derivates	Julio et al. [31]
Phytotoxic *vs Lactuca sativa* and *Lolium perenne*	Purified actives	necrodane derivates and cadinane-type sesquiterpenes	Julio et al. [31]
β-secretase in Alzheimer disease	EO	trans- α - necrodol and trans- α - necrodyl acetate	Videira et al. [37]
Inhibition of Interleukine-1 and nitric oxide production, anti-inflammatory, anti-catabolic and pro-anabolic	EO	α-pinene and necrodol	Rufino et al. [38]
Inhibition of nitric oxide synthase and nuclear factor-B from stimulated human chondrocytes and intestinal cell line C2BBe1	EO	necrodane derivates and α-pinene	Rufino et al. [38]

EO, essential oil; Non-specified (n.s)

Organisms, like the WHO, have expressed their concern about this matter, including the call for new and innovative agents and therapies in programs like Horizon 2020 and the following Horizon 2030, the biggest European Union Research and Innovation programme ever, with nearly 80 billion euro of funding available from 2014 to 2020 and 100 billion euro from 2021 to 2027. Horizon 2020 plan has five main strategic aims in this line of research; make awareness about antimicrobial resistant, reduce the incidence of infections, optimized antibiotic use and reinforce the vigilance and research.[59] Horizon 2030 claims an urgent need of new prevention, diagnostics, vaccines, therapies and alternatives to antibiotics.

To meet these increasing demands, biomedical researchers and pharmaceutical companies are combining advanced methods of drug discovery, such as combinatorial chemistry, high-throughput screening and genomics, with conventional approaches using natural products and traditional knowledge. In the food industry, the use of these natural products for the control of microorganisms and food preservation has already attracted attention to develop new food additives that replace synthetic preservatives, some of which are suspect of being toxic.[60]

Secondary metabolites of plants allow plant survival when they face damaging environmental factors, like certain pathogen microorganisms. Phenolic compounds, terpenoids and alkaloids can affect these surrounding microorganisms, causing changes in their microbial community, activity and/or chemotaxis (attraction and repulsion).[7,61]

Phenolic compounds, like rosmarinic acid, are a wide group of chemicals with many structural variations, being one of the most diverse groups of secondary metabolites. The hydroxyl groups on phenolic compounds are thought to be the cause of their inhibitory action. They can interact with the cell membrane and disrupt it, acting as proton exchanger and causing the depletion of ATP pump or the leakage of cellular components. Hydroxyl groups can also bind some enzymes and alter microorganisms' metabolism. Nevertheless, the position of this -OH can change the antimicrobial potential.

The antimicrobial activity of a compound also depends on the number of double bonds or their position. Terpenoid compounds, such as oleanolic and ursolic acids, have been reported to display antimicrobial activity because of their ability to increase membrane permeability and K^+ loss. Nevertheless, terpenes low water solubility limits their diffusion through agar medium and antimicrobial activity testing.[11,62] These terpenes

are often found in essential oils from plants, which antibacterial and antifungal properties were determined against some microorganisms of interest in the food industry, human health and agricultural fields, named previously in table I.5.

Gram positive bacteria such as *Staphylococcus* strains and *Enterococcus faecalis*;[56] gram negative bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella* species, *Escherichia coli*, *Morganella morganii* and *Proteus mirabilis,* [40,56] and fungal strains such as *Candida* species, *Rhodotorula rubra, Trichosporon* species*, Saccharomyces cerevisiae, Cryptococcus neoformans, Aspergillus strains* and *Trichophyton* species were tested against the *L. luisieri* essential oil. [22,39,46] According to the results obtained for the essential oils, those with higher content in necrodane derivates seem to influence the resulting antifungal activity against *Aspergillus* strains[22] and *Trichophyton* species[39] and antibacterial activity against methicillin sensitive and resistant *S. aureus*.[38] The different *L. luisieri* populations showed promising results when tested against several of the selected microorganism species.

The activity of different solid-liquid extracts was also tested on different microorganisms. The most used solvents were hexane, ethyl acetate, methanol, methylene chloride or just water. The biocidal results seem to depend on the extract chemical profile, but, in general, the bioactivity increases with the polarity of the extracts.[46,56]

Antioxidant activity

Another frequent analysis of plant extract is its antioxidant activity test. Many oxidative agents, such as temperature, ultraviolet radiation, air pollutants, nutrient depravation, pathogen attack or mechanical stress endanger plant survival. To counteract the effect of these factors, plants have developed a series of defence mechanisms, among them, the production of non-enzymatic antioxidants.[63]

Oxidative stress also occurs in humans as a consequence of the normal development of metabolic processes, producing free oxygen and nitrogen radicals like anion superoxide $O_2^{\cdot-}$, nitric oxide $NO^{\cdot}$ or hydroxyl $OH^{\cdot}$. When endogenous defensive mechanisms are not enough, those free radicals are accumulated, damaging lipids, proteins, DNA, lastly contributing to the progression of some chronic ailments. Besides, plant extract potential usefulness as natural preservative additives in processed foods, medicines or cosmetics has been explored when exposed to oxidizing processes that can modify their initial nutritional, pharmacological or organoleptic properties.[64] In this

regard, plant extracts are considered a potential and unlimited source of antioxidant actives for therapeutic and preservative applications.

As previous authors defined in their work,[65] an antioxidant is any substance that, when present at low concentration compared to that of an oxidizable substrate, significantly delays or prevents oxidation of that substrate, eliminates the oxidized substrate or increases the inner self-defence mechanisms.

There are several *in vitro* and *in vivo* methods to determine the antioxidant capacity of a substance or mixture.[66] However, antioxidants are classified in two mayor groups: enzymatic and non-enzymatic. Most of the *in vitro* assays of antioxidant activity of plant extracts are non-enzymatic. Secondary metabolites from plants may act as antioxidants because of their intervention at three possible levels: i) prevention of the production of new oxidants, ii) interruption of the chain initiation and/or propagation of the oxidizing reactions and iii) repair of the damage. These action mechanisms are of interest for their application in the food, cosmetic and pharmaceutical products.

Non-enzymatic antioxidant action mechanisms are radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, metal-chelation or a synergy among them.[67] In many occasions, a correlation between *in vitro* and *in vivo* is assumed; nevertheless, in *in vivo* tests, actives may suffer variations as a consequence of physiological processes that may reduce their antioxidant capacity, so these correlation should be confirmed.[68]

There are two main mechanisms of action to test non-enzymatic antioxidants are hydrogen atom transfer and single electron transfer. In the following table I.5, the different *in vitro* antioxidant activity assays mainly used in the characterization of natural compounds are gathered. There are other chemiluminescence or photoluminescence assays but their implementation is not as common because either special equipment is required or the reaction mechanism is not known, which makes the interpretation of results difficult.[66] As it can be observed in table I.6, the single electron transfer (ET), or hydrogen atom transfer assays (HAT) are the main mechanisms. The two main mechanisms of action to test non-enzymatic antioxidants are hydrogen atom transfer and single electron transfer, represented in Figure I.4.

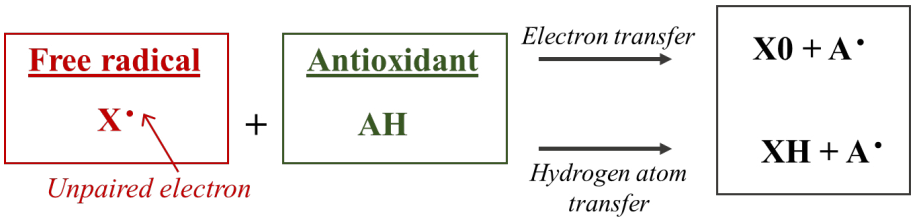


Figure I.4. Mode of action between a radical and an antioxidant.

The transference of both electron and hydrogen atom produces a measurable physical change. The α-diphenyl-β-picrylhydrazyl or DPPH, 2,2′-azino-bis(3-ethylbenzothiazolline-6-sulfonic acid or ABTS, ferric reducing/antioxidant power or FRAP, cupric reducing/antioxidant power or CUPRAC, total phenolic content assay and lipid peroxidation inhibition capacity or Lipid Peroxidation Inhibition Capacity (LPIC) assays, they all detect a change of absorbance at different wavelengths, depending on the substrate of the reaction, when the radicals are neutralized by the antioxidants. Besides, oxygen radical absorbance capacity or ORAC and total radical trapping antioxidant or TRAP measure the fluorescence reduction when the antioxidants transfer the hydrogen atom to free radicals such as AAPH (2,2′-azobis(2amidinopropane) dihydrochloride).

From all these assays, the DPPH test has a very wide spread use for the determination of the antioxidant activity by electron transference mechanism. It is a simple and rapid technique in alcoholic solution of the extracts, which can be applied over a large number of samples simultaneously using microplates and a spectrophotometer. In colorimetric methods, after the action of the natural compounds with the free radicals, a change of absorbance is expected at different wavelengths, as mentioned before. For DPPH, the working wavelength is 530 nm but it is also visible for human eyes since it changes from purple to yellow when the radical is scavenged. Although the method is widely used, it does have some limitations. The portion of the DPPH molecule responsible for its radical behaviour is a nitrogen atom located at the centre of the structure. While this centralized location is freely accessible to small molecules, larger molecules may have limited access to this moiety due to steric hindrances.[69]

Table I.6. Non-enzymatic antioxidant capacity in *in vitro* assays.

Name of the method	Mechanism of action	Nature of the substrate	Detection method
DPPH	ET	DPPH Organic radical	absorbance 520nm
ABTS	HAT	ABTS Organic cation radical	absorbance 600-750nm
FRAP	ET	Fe(III) complex	absorbance 592 nm
CUPRAC (Copper reduction assay)	ET	Cu (II) complex	absorbance 490 nm
ORAC/HORAC (oxygen/hydroxyl radical absorbance capacity)	HAT	AAPH radicals/ Hydroxyl radicals	Fluorescence/ Voltammetry
TRAP (total radical trapping antioxidant)	HAT	AAPH radicals	fluorescence
Total phenolic content assay reagent	ET	Folin-Ciocalteu reagent	absorbance 750nm
LPIC (lipid peroxidation inhibition capacity assay)	HAT	Linoneic acid or LDL oxidated	absorbance 234 nm

So far, Matos et al.,[20] Baptista et al.[46] and Arantes et al.,[40] have studied the antioxidant effect against DPPH and LPIC of *L. luisieri* essential oil and methanolic extracts. Besides, their properties to protect DNA from oxidation has been also tested.[20,40,46]

Matos et al.[20] reported that *L. luisieri* essential oil was the most effective antioxidant *vs* DPPH free radical when comparing different *Lavandula* species essential oils In this *L. luiseiri* species, its main actives were 1,8-cineol and trans-α-necrodyl acetate. Finally, Arantes et al.[40] reported that the inhibitory effect in lipid peroxidation of the *L. luisieri* essential oil was higher than its capacity to scavenge the DPPH free radical. In this research the main actives of the studied oil were 1,8-cineol, lavandulol and tras-α-necrodol. Any of these studies was capable of relating the observed activity to its

composition, but it can be observed that changes in the proportion of the essential oil main actives provoked differences on the studied activities.

Finally Baptista et al.[46] tested the antioxidant activity of the essential oil and the extracts obtained by maceration into different organic solvents, *L. luisieri* non-volatile fraction. The assays performed against the DPPH confirmed the activity of the essential oil, nevertheless the methanolic extract performed better. In this work, the inhibitory effect on lipid peroxidation was also tested, revealing that among all type of tested extracts, the methanolic one was the more active. The non volatile fraction of *L. luisieri* seems to perform better as antioxidant than the essential oil. Nevertheless, Baptista et al.[46] did not relate the any determined chemical composition of the *L. luisieri* population used to obtain the extracts of their study. The compounds rosmarinic, oleanolic and ursolic acids tracked in this work have been reported to present this antioxidant properties as previously explained.

Antifeedant, ixodicidal and nematocidal activities

As these necrodane-like compounds of *L. luisieri* are also present in insect defensive secretions, some authors studied the antifeedant, ixodicidal and nematocidal effects of this plant volatile fraction against organisms of interest such as the insects *Spodoptera littoralis*, *Myzus persicae*, *Rhopalosiphum padi, Leptinotarsa decemlineata* and the nematode *Meloydogine javanica*, in the agricultural field along with the plant chemical profile.[31,33,36,70] Any of the mentioned works could assigned the evaluated activity to the major essential oil constituents studied individually; they explained the obtained results to the synergistic effects among them.

Other biological activities

Rufino et al.[38] tested this plant species essential oil as inhibitor of inflammation markers. However, they could not attribute the effect to a single component, concluding that the result may be a consequence of the combine actions of individual compounds. Nevertheless, a posterior study by Arantes et al.[40] partially associated the anti-inflammatory activity of the essential oil to the presence of 1,8-cineol with a possible potentiation by other oxygenated monoterpenes necrodane derivate present in the extract. These authors also analysed other biological activities such analgesic and anti-inflammatory activity, and related them to the presence of the necrodane-like typical of this plant species. Finally, Videira et al.[37] studied the essential oil as possible treatment

for Alzheimer disease by quantifying its capability for the inhibition of β-secretase. Their experiments revealed that 2,3,4,4-tetramethyl-5-methylene-cyclopent-2-enone is the compound responsible for the activity and suggested further *in vivo* studies.

In this work the *Lamiacea* specie, *L. luisieri* will be used in this work as a source of bioactives compounds. Since, from this plant specie the non volatile fraction, extracts different from the essential oil, have received less attention, in this work will be obtained under mild experimental conditions to avoid the degradation of the extracted actives.

The phenolic acid, rosmarinic acid and the two isomer triterpenoids, oleanolic and ursolic acids, previously reported by Julio et al.[45] in this plant population, will be followed. Besides, as most common in vitro assays for plant activities, antimicrobial and antioxidant activities of the obtained extract will be determined and related to its composition in the three actives of interest.

2. Techniques for the obtaining of natural compounds: traditional vs supercritical

In this section they will be described the different traditional methods applied to plants and its comparison with supercritical techniques.

2.1. Traditional techniques

There are several techniques traditionally performed for the separation of natural bioactives from the plant matrix that produces them. Depending on the applied extraction method, the obtained product will have different chemical composition and, therefore, different bioactivity. These methods produce extracts that could be classified into 2 main groups: the essential oil, which is a complex mixture constituted by volatile compounds responsible for the plant aroma that are extracted with water but are not soluble in it, and the so-called aqueous or organic extracts, which composition will depend on the chemical characteristics of the extracting solvent.[71]

Essential oil

The concept "*essential oil*" is used to define the water-immiscible essence of a plant only when it is obtained through distillation. Essential oil compounds are located in impermeable granules placed in glandular hair, cells, or in secretory granules located in flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, or roots.[72] The main components of essential oils are terpenoid hydrocarbons, oxygenated terpenes, and sesquiterpenes, and their proportion may vary, even within the same plant species, depending on environmental and growth factors of the plant.[73] The principal historical method for essential oil isolation is hydrodistillation, in which the plant material is soaked for several hours in boiling water. The boiling water evaporates and its vapour drags the volatile components of the plant to be later condensed and separated thanks to their different densities.[74] According to the Pharmacopoiea Europea,[75] hydrodistillation is performed in a Clevenger-like apparatus, Figure I.5.

Nevertheless, hydrodistillation is a time and energetic-consuming process with a sustained application of high temperature during a long period of time to the vegetable matrix, which may cause, on some organic compounds, their decomposition. Apart from this process, hydrolysis reactions can occur too, due to the presence of water. Because of this, nowadays the most accepted method for the separation of these compounds from the plant matrix is the steam distillation, a low temperature distillation method in which the extracting agent is not boiling water but only steam.[76]

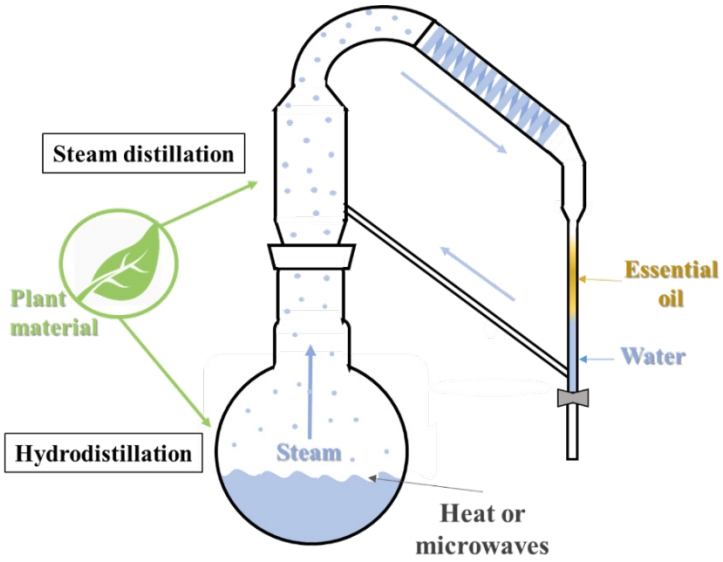


Figure I.5. Scheme of a Clevenger-type hydrodistiller device

Aqueous and organic extracts

They are a concentrated product obtained by a liquid-solid extraction process in which, firstly, the vegetable matrix is soaked into the suitable solvent and, finally, the liquid is separated from the compounds and eliminated (mainly with a rotary evaporator). This extract composition varies depending on the chemical nature of the selected extracting solvent, which can be water, an aqueous-soluble organic solvent such as ethanol, methanol or acetone or a non-aqueous-soluble organic solvent like hexane, toluene or dichloromethane.

During the extraction time, the soluble compounds from the plant diffuse to the selected liquid. To promote this diffusion and get higher yields, the time of exposure must be long (from 4h to 48h) as the particle size of the matrix has to be small (micrometer order), the mixture of the plant and the solvent must be stirred. Applying high temperature and/or pressure, microwaves or ultrasounds will help improving that yield too.[71]

Maceration, infusion, percolation (Figure I.6), decoction, hot continuous extraction, microwave assisted extraction or ultrasound-assisted extraction are some of the most common traditional methods. Nevertheless, it should be considered that the application of some of the above mentioned factors might have the ability to cause the degradation of some compounds.[77] Besides, although the selected solvent is normally the

most influential parameter, in some cases they are not environmentally respectful and leave traces in the final product, which could be a limiting factor depending on the final receptor.[78] Because of the inconvenients of compound degradation, presence of organic compounds in the final natural product, not to mention that some solvents are environmental pollutant, it has become necessary to apply other extraction methods that satisfy the cosmetic, pharmaceutical and food consumers necessities in terms of quality and safety.

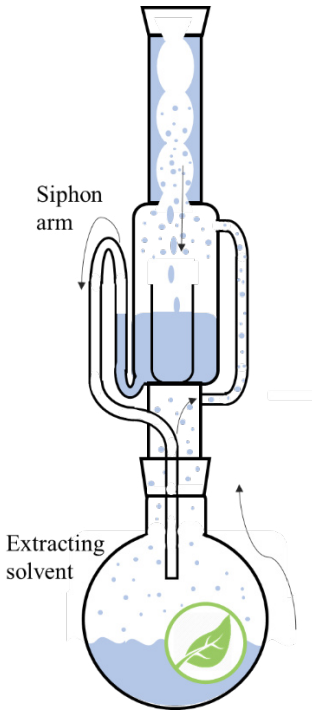


Figure I.6. Scheme of a Soxhlet device.

2.2. Supercritical fluids and Green Chemistry

At the end of 1990s, a movement towards pollution prevention through the minimization of environmental impact derived from chemical processes emerged in the chemical industry and research. This new trend was known as "Green Chemistry" or "Sustainable Chemistry".[79] It was born as a requirement to actively prevent pollution and resource depletion, besides the cost reduction and the complementation of the required environment normative established by the United Nations Organization in the Stockholm Declaration in 1972.[80] The definition of this concept was detailed into twelve principles by Anastas, P. & Warner J (Table I.7) [81]which are based on the design of new processes that maximized the final product, that were energetically efficient and reduced the

residues, as well as, the use of solvents or other substances with harmful environmental and health impact.

Table I.7. Twelve Green Chemistry principles elaborated by Anastas, P. & Warner, J. [81]

1. *It is better to prevent waste than to treat or clean up waste after it is formed.*
2. *Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.*
3. *Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment.*
4. *Chemical products should be designed to preserve efficacy of function while reducing toxicity.*
5. *The use of auxiliary substances (e.g., solvents, separation agents, and so forth) should be made unnecessary wherever possible and innocuous when used.*
6. *Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure.*
7. *A raw material or feedstock should be renewable rather than depleting wherever technically and economically practicable.*
8. *Unnecessary derivatization (blocking group, protection/deprotection, temporary modification of physical/chemical processes) should be avoided whenever possible.*
9. *Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.*
10. *Chemical products should be designed so that at the end of their function they do not persist in the environment and break down into innocuous degradation products.*
11. *Analytical methodologies need to be developed further to allow for real-time in-process monitoring and control before the formation of hazardous substances.*
12. *Substances and the form of a substance used in a chemical process should be chosen so as to minimize the potential for chemical accidents, including releases, explosions, and fires.*

(Green Chemistry: Theory and Practice, Oxford University Press, 2000).[81]

In the movement for the development of sustainable solvents, the use and research of supercritical fluids has been promoted and considered "green solvents for the future", which can be applied in lab or industrial scale.[82,83] Supercritical fluids were defined by Cagniard de la Tour (1822)[84] in the pressure-temperature phase diagram (Figure I.7). In it, there are three differentiated sections labelled as solid, liquid and vapour, which converge on the triple point, conditions at which the three phases coexist. The curves limiting these sections are the coexistence lines between solid-liquid, solid-vapour and liquid-vapour. A pure substance is supercritical when it is at pressure and temperature conditions above the critical point, which is at the end of the liquid-vapour coexistence line. Above the critical point, the pure substance is a supercritical fluid, where the liquid becomes less dense because of temperature increase and the gas becomes denser because of the increase in pressure.

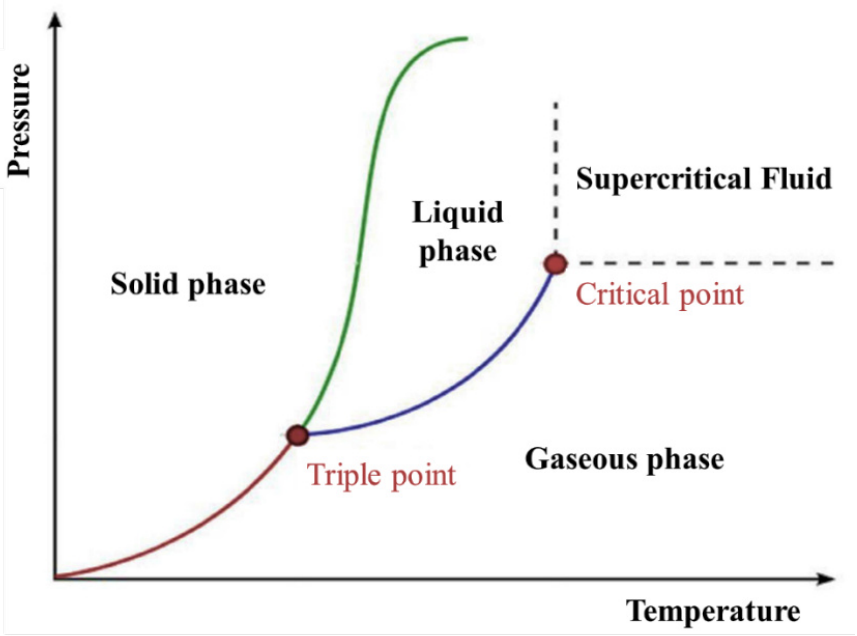


Figure I.7. Phase diagram of supercritical fluid region.

As table I.8 illustrates, a supercritical fluid has a density, viscosity and surface tension properties values intermediate between those for gases and liquids. It has no superficial tension, higher diffusivity coefficients and lower viscosity than a liquid, but possess density and solvating properties similar to a liquid. These intermediate properties make them useful in diverse applications.

Table I.8. Density, ρ, dynamic viscosity, η, diffusion coefficient, D, and superficial tension, γ, values for gases, supercritical fluids and liquid.

	ρ (g/ cm^3)	η (Pa·s)	D (cm^2/s)	γ *(dina/cm)*
Gas	10^{-3}	10^{-5}	10^{-1}	0
Supercritical	0.2-0.9	10^{-4}	10^{-4}	0
Liquid	0.6-1.6	10^{-3}	10^{-5}	20-50

Supercritical fluid properties are highly defined by its density, which is strongly dependent on pressure and temperature. At low temperatures but above the critical point, small changes of pressure provoke drastic density variations, which means that a substance solubility in supercritical fluid can be easily modified.[85]

The special combination of gas-like viscosity and diffusivity, liquid-like density and solvation properties of supercritical fluids have allowed the development of alternative technological processes to the traditional ones, to minimize environmental impact, such as reducing energy consumption, generating less toxic residues and increasing the quality and safety of the final products.[83]

In table I.9, the molar mass and the critical temperature, pressure and density of some compounds commonly used as supercritical fluids are listed. As it can be observed, lighter molecules have lower critical temperatures and pressures and higher critical densities than those of larger molecular weight (except for water). The presence of hydrogen bonds or the polarity of the molecules tend to increase the critical temperature or pressure of the compound.

Table I.9. Critical properties of selected compounds.[86]

Component	Molecular weight (g/mol)	T_c (K)	P_c (MPa)	ρ_c (g/mL)
Carbon dioxide	44.01	304.1	7.37	0.469
Water	18.02	647.3	22.12	0.348
Methane	16.04	190.4	4.60	0.162
Ethane	30.07	305.3	4.87	0.203
Propane	44.09	369.8	4.25	0.217
Ethylene	28.05	282.4	5.04	0.215
Methanol	32.04	512.6	8.09	0.272
Ethanol	46.07	513.9	6.14	0.276
Acetone	58.08	508.1	4.70	0.278

2.3. Supercritical CO_2 properties and applications

From all possible and useful substances to be applied as supercritical fluids, supercritical carbon dioxide ($scCO_2$) has received special attention for several advantages that distinguish it above others and allow its implementation at industrial level:[87,88]

- It is an inert, non-toxic (only large amounts of this gas could be a hazard because oxygen deprivation), non-inflammable and environmentally safe compound, so it is considered a harmless and sustainable solvent.
- Its critical temperature, T_c = 304 K, and pressure, P_c = 7.28 MPa, are moderate and easily reachable without an excessive energy consumption.
- Because of its low T_c, its suitable for the solvation of thermolabile compounds
- It creates an inert atmosphere that prevents the oxidation and the generation of highly reactive intermediate compounds.
- Its low viscosity, compared to other organic solvents, facilitates the contact among phases and increases the mass transference.
- It is easily eliminated by lowering the pressure, leaving no traces in the final product.
- It is produced in processes like combustion and fermentation, abundant in the atmosphere with a moderate cost that can be revalued.

Supercritical CO_2 has been applied at industrial level but also explored at research level for the obtaining of high value compounds from natural sources or the formulation

of certain products as a green alternative to the traditional techniques performed with organic solvents.[89] Its solvation properties have been widely studied in extraction processes and as chromatography mobile carrier phase. Other applications, considering $scCO_2$ as solvent and anti-solvent, are being investigated for particle production and encapsulation with materials and bioactives with interest in the pharmaceutical, cosmetic, agricultural and food fields. The success is a consequence of its possibility of producing homogeneous solids with controlled morphologies and particle size distributions to be directly used, impregnated or encapsulated into carriers for an active protection against degradation, the avoidance of undesired organoleptic properties or even controlled release, features of great interest to be applied in cosmetical, pharmaceutical and cosmetical products. Also because of their adjustable solvation power, it is applied for the separation and purification of certain compounds from complex mixtures. Supercritical CO_2 has been also considered for the sterilization of porous or thermosensitive materials because of its capacity of diffusion and elimination of microorganisms at low temperatures.[90] Finally, again with the aim of reducing the environmental damage, applications for these fluids as reaction media for chemical or biochemical synthesis and in processes for pollutants degradation have been found.[82]

2.3.1. Applications of supercritical CO_2 as solvent

Supercritical fluid extraction (SFE)

In the last decades, the use of supercritical fluids has not been only promoted in research, but also at industrial level for different applications all over the world (Table I.10).[83] Supercritical CO_2 has been applied in the extraction of interesting compounds from different matrices, such as caffeine from coffee and tea, fatty acids, vitamin E, nicotine, natural insecticides and pesticides, and spices, flavours, aromas or other natural products.[91]

Table I.10. Companies that apply SCF at industrial level for different purposes.[91]

Caffeine	KW-Trostberg AG Maximus Coffee Group LP
Fatty acids	Marbery, GmbH
Vitamin E	Wuhan Kaidi Fine Chemical Industrial Co
Nicotine	Philip Morrisp
Natural pesticides and insecticides	Hops Extraction Corp. of America
Flavours aromas and other natural products	Norac Technologies, Ogawa Flavours and Fragrances, SensientTechnologies, Kirin Food-Tech Co, Carlton & United Beverages Ltd

These products are obtained through a process called "*supercritical fluid extraction*" or SFE, in which, $scCO_2$ is used to dissolve compounds from a solid or liquid matrix. The extraction speed and selectivity of certain components can be modulated by changing the experimental conditions of pressure, temperature, time, $scCO_2$ flow rate, particle size of the matrix or the addition of co-solvents.[78] The extraction and subsequent separation of the desired compounds is possible thanks to the supercritical fluid properties between those of gas and liquid as previously described.

In a typical SFE procedure, represented in Figure I.8, the $scCO_2$ is pumped (usually from the bottom) into the extraction vessel, where the supercritical conditions are kept and where the solid or liquid matrix is placed. A co-solvent can be also added to induce the solvation of compounds. The solutes migrate from the matrix to the supercritical solvent and this solution is carried to the collectors, where pressure and temperature are lower than in the extraction vessel, provoking the fractionated precipitation of the solved compounds. It is the decrease in the CO_2 density, and so its lower solvation power, what induces the separation of the solutes and the CO_2. The working conditions in collector 1 are usually different from those in collector 2, causing a fractionated precipitation of the extracted compounds. When the pressure is reduced to the atmospheric one, the CO_2 is released, leaving a completely solvent-free extract in the last collector.

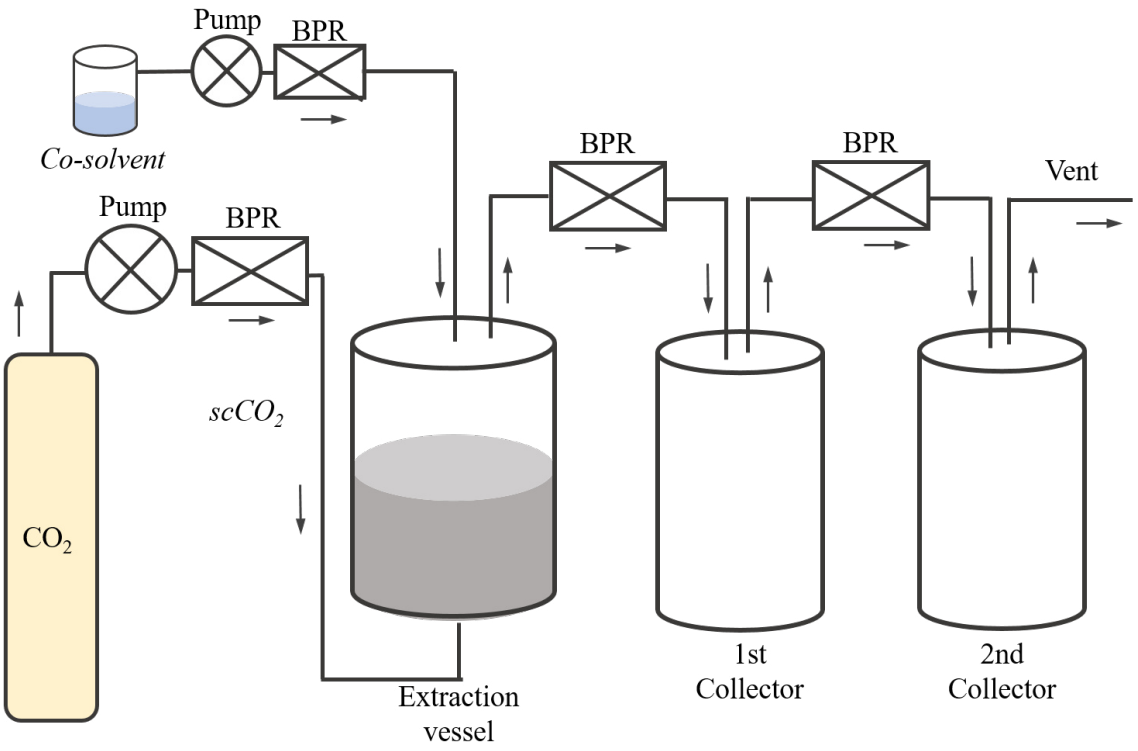


Figure I.8. Scheme of a supercritical fluid extraction apparatus. Co-solvent and CO_2 reservoirs, pumps and back pressure regulators (BPR) extraction vessel and collectors.

The $scCO_2$ allows the extraction of volatile compounds, mainly monoterpenoids and sesquiterpenoids, all typical from essential oils, traditionally extracted by hydrodistillation, overcoming the drawbacks of thermal degradation and/or hydrolysis and solubilisation in water of some compounds, adding the possibility of fractionation of the obtained product.[92] In the SFE process, a co-solvent can be also included for the extraction of more polar compounds with higher molecular weight, as shown in Figure I.8.

Besides its commercial applications, SFE is used in research to optimise the obtaining of other natural extracts free from organic solvents, because of their interest in the food, cosmetic or pharmaceutical industries. Basil leaves (*Ocimum basilicum*), grape seeds (*Vitis vinifera*), lemon balm (*Melissa officinalis*) or chamomile flowers (*Chamomilla recutita*) and many others extracts have been obtained with this technique.[93–95] Other authors have applied the supercritical CO_2 as solvent for the impregnation of different fibres with natural actives such as thymol or quercetin,[96,97] by introducing the material to be impregnated into the collector vessels.

Besides the essential oil volatile compounds, other high added value actives have been obtained with this technology such as the β-carotene, α-tocopherol and capsaicinoids antioxidants from paprika (*Capsicum annum*)[98] or catechins from green tea (*Cratoxylum prunifolium*).[99]

Nevertheless, as previously said supercritical extraction mainly extracts volatile compounds or non-polar compounds. The obtaining of non-volatile polar compounds from *Lavandula luisieri* such as rosmarinic, oleanolic or ursolic acid with this technology provides low recovery and the addition of a co-solvent has been proposed to increase the final extraction yield.[100] SFE without polar modifier was found not to be a suitable technology for the extraction of OA and UA from *Plantago mayor* leafs.[101] In fact, some authors have proposed SFE at low pressures to concentrate the initial plant material in these polar actives by eliminating other non-polar and volatiles.[51]

Supercritical fluid chromatography (SFC)

Supercritical CO_2 has also been implemented in the design of analytical supercritical chromatography as mobile phase, which is an intermediate modality between gas and liquid chromatography, for the separation of thermolabile and non-volatile compounds that cannot be separated with gases and not detected with liquid chromatography detectors.[102]

Supercritical fluid chromatography, SFC, is a separation technique that evolves from liquid chromatography but uses a supercritical fluid as mobile phase; being CO_2 the most used one. When supercritical conditions are applied to chromatography mobile phase, the speed of analysis is increased when compared to HPLC, mainly because of supercritical CO_2 weak intermolecular forces, and therefore lower viscosity than liquids, and high solute diffusion coefficients into the mobile phase.[102] It allows gas-like separation at low temperatures, which is ideal for the separation of volatile thermolabile molecules. A co-solvent can also be added to generate gradients and to obtain separation of certain compounds. These modifiers are normally more polar compounds and liquid at room temperature. The supercritical chromatography equipment is represented in Figure I.9, and it is mainly constituted by a CO_2 and modifier reservoirs, their high-pressure pumps, back pressure regulators to ensure the pressure required, an injector, a column inside an oven, and, finally, one or more detectors. The possibility UV-VIS absorbance

reader, flame ionized, or mass spectrometry detectors, among others, expands the applicability of this technique.[103]

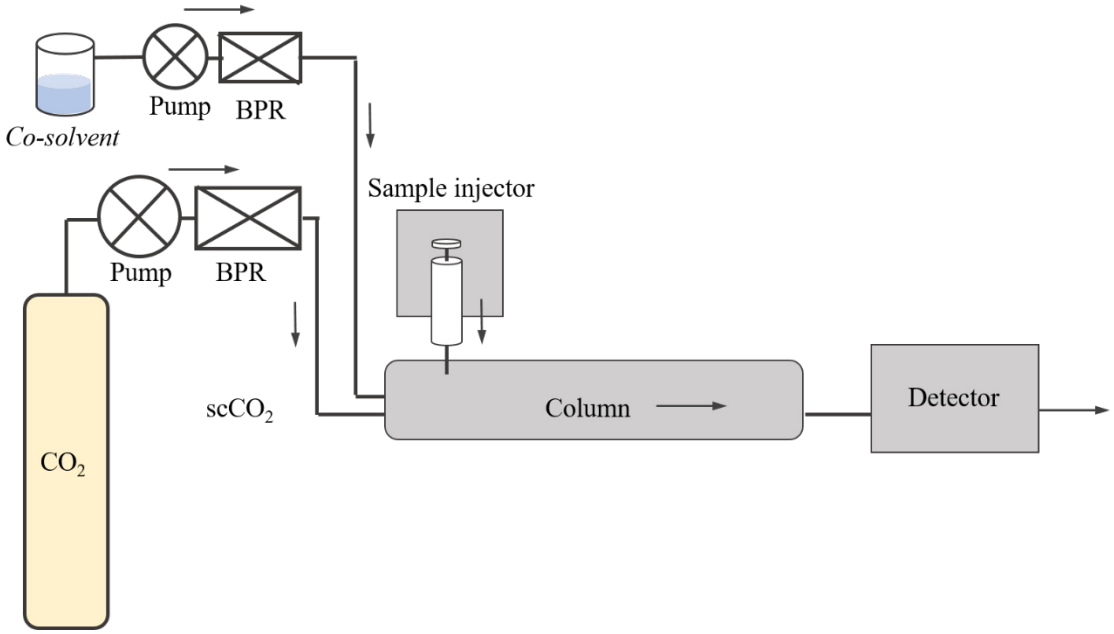


Figure I.9. Scheme of a supercritical chromatography apparatus. CO_2 and modifier reservoirs, pumps, back pressure regulators (BPR), sample injector, chromatographic column and detector.

This technology was applied to the challenging analysis of natural extracts, which are complex mixtures of compounds. Although in its origin it was only applied to non-polar compounds, later it evolved and expanded to a wider spectrum of chemical structures, being able to separate substances from different groups of secondary metabolites. Gibitz Eisath et al. [104] in their review work classified in different categories the results about natural compound analysis with this technology. Changing the analytical variables at supercritical conditions allows the separation, identification and quantification of natural compounds of a wide range of polarities; carotenoids from *red peper*; terpenes from *panax ginseng*; alkaloids from *Aconitum pedumculum;* or phenolic acids from *wine*.

Rapid Expansion of Supercritical Solutions (RESS)

RESS has been used for the micronization of solids for medical applications if the solid to micronize has compounds with high solubility in scCO2 or they are small molecules containing a few polar bonds.[105] In this process, the desired compounds are first dissolved into $scCO_2$ in a high-pressure vessel after which it is sprayed through a nozzle into a low-pressure precipitator where particles are formed spontaneously. Despite

the fact that the application of this technique is very limited, in terms of the spectrum of compounds to work on, and the high amount of CO_2 that is required, the experimental conditions can be adapted to solubilize other more polar and heavier compounds and to improve the produced particles while the depressurization. Nevertheless, the production of microparticles of several pharmaceuticals, such as ibuprofen (anti-inflammatory), benzoic acid (funostatic), cholesterol and β-sitosterol (sterols from animals and plants), have been already reported.[105]

Several modifications of the RESS process have been proposed to increase the range of compounds to solubilize into the first high pressured vessel. In Figure I.10 the following RESS variations are gathered in a schematic representation.

- RESS with co-solvent or RESS-SC: This method ads a liquid co-solvent and has been applied for the precipitation of phenytoin and salicylic acid and menthol and benzoic acid.[106,107]
- Particle formation from Gas Saturated Solutions or PGSS: Polar and high molecular weight substances are suspended in a solvent at a given temperature and then mixed with $scCO_2$. This technique has been applied for the encapsulation of theophylline into a palm oil matrix.[108]
- Depressurization of an Expanded Liquid Organic Solution or DELOS: The active substance is first dissolved in a proper organic solvent. $ScCO_2$ acts as a co-solvent in this process. In this case, milder temperatures than with PGSS can be applied to carry the active of interest since CO_2 acts as co-solvent.[109]

To improve the micronization step of the process, 2 variants have been proposed:

- Rapid Expansion of a Supercritical Solution into a Liquid Solvent (RESOLV) or Rapid Expansion of a Supercritical Solution into an Aqueous Solution (RESAS), both used for the production of poly(L-lactide) nanoparticles of retinyl palmitate.[110]
- Rapid Expansion from Supercritical to Aqueous Solutions (RESAS). In this process, the supercritical solution (SCF with polymer and drug) is expanded through a nozzle into an aqueous solution containing stabilizers. It has been applied for the production of nanoparticles of phytosterols.[111]

These precipitation techniques have been investigated for the impregnation of certain natural extracts onto polymeric carriers and formulated composites by the co-

precipitation of the desired active along with the polymer. Varona et al. [112] studied pressure, temperature and composition conditions on the impregnation process of modified starch with lavandin essential oil.

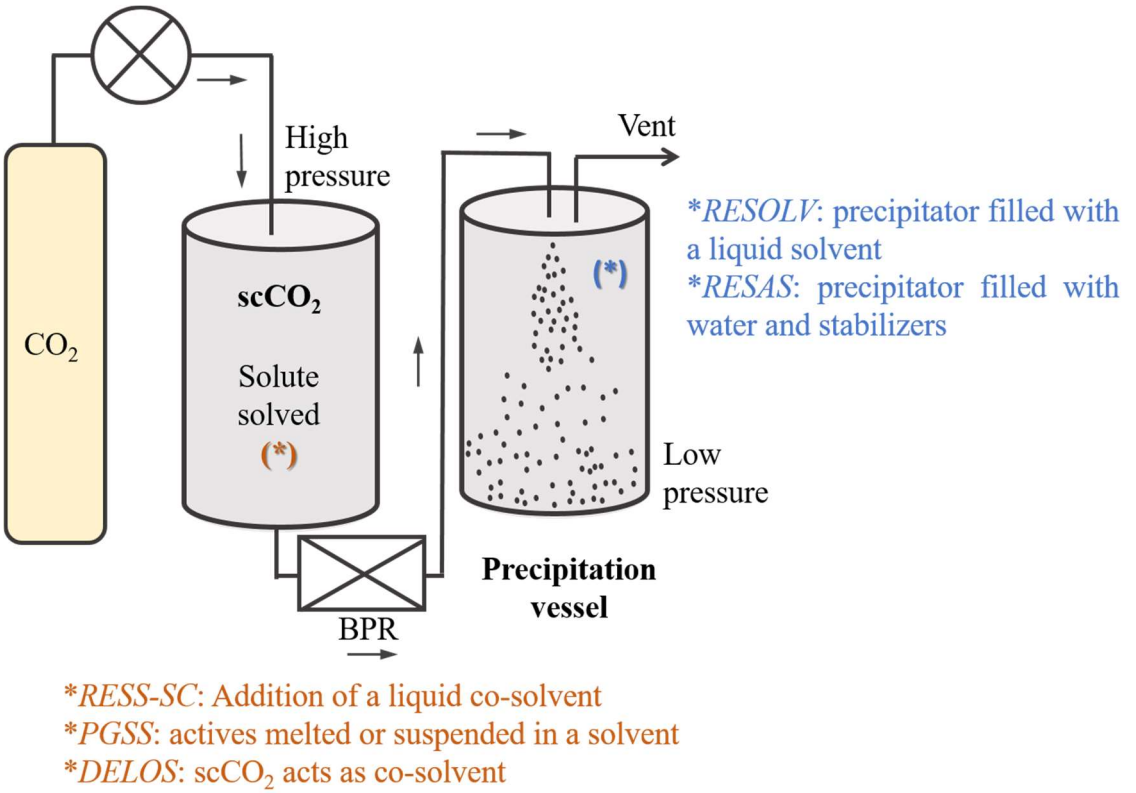


Figure I.10. Schematic representation of the RESS process and its variations. CO_2 reservoir, pump, solute dissolution vessel, back pressure regulator (BPR) and precipitation vessel.

These technologies allow the precipitation of $scCO_2$ soluble compounds and their co-precipitation along with polymers, not an encapsulation inside the polymer.

2.3.2. Applications of supercritical CO_2 as anti-solvent

The application of supercritical carbon dioxide to solve actives shows only one disadvantage: its low polarity. This means, as previously explained, that $scCO_2$ only extracts non-polar components or polar ones with low molecular weight.[88] Many compounds of medical interest show very limited or negligible solubility in scCO2. However, this lipophilic behavior has been useful for the development of other supercritical applications such as different actives separation, particle formation or encapsulation with homogeneous morphologies. In the anti-solvent techniques, the compounds of interest are dissolved in another liquid solvent different from $scCO_2$ but completely miscible in it.[89]

Gas Anti-Solvent (GAS)

Gas Anti-Solvent (GAS) is a batch process where the precipitation vessel is partially filled with the solution of solute of interest and the $scCO_2$ is pumped from the bottom into this vessel (Figure I.11). The compound precipitates when the $scCO_2$ proportion raises. Supercritical CO_2 solves and drags the organic solvent and at the end of the process, the CO_2 and the liquid solvent are eliminated.

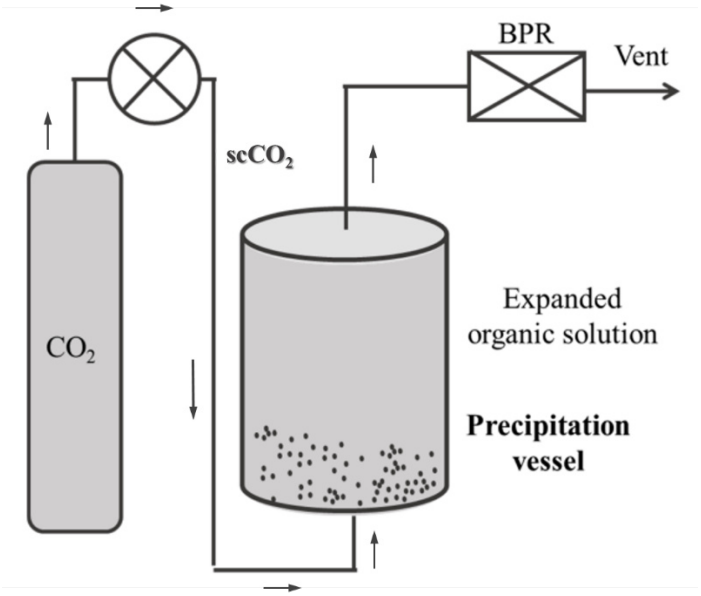


Figure I.11. GAS equipment schematic representation. CO_2 reservoir, pump, precipitation vessel and back pressure regulator (BPR).

Aerosol Solvent Extraction System (ASES), Particles by Compressed Anti-solvent (PCA) and Supercritical Anti-Solvent (SAS)

Aerosol Solvent Extraction System (ASES), Particles by Compressed Anti-solvent (PCA) and Supercritical Anti-Solvent (SAS) are continuous processes with three main steps (Figure I.12). Firstly, an organic solution of the actives of interest is sprayed into a pressurized vessel filled with $scCO_2$. Then, when the solution reaches this high-pressure vessel, small droplets are formed, the liquid is dissolved into $scCO_2$, so its dissolving power, regarding the solute, decreases. The actives are not soluble in this new environment of solvent-$scCO_2$ and precipitate. Finally, the liquid solvent is separated from the actives during the process, and dragged through a filter, located in the bottom of the pressurized container, to a second low pressure vessel from where it is released.[83] The equipment scheme is showed in Figure I.12. To remove the remaining solvent from the device, pure $scCO_2$ can be pumped to drag it. A solid powder is obtained in the high-

pressure vessel. PCA is a modification from ASES by the addition of a compressed antisolvent, which could perform more efficiently in the production of several biopolymer particles. There are some variations of these techniques that changes the method with which the solution and the supercritical fluid encounter or their application.

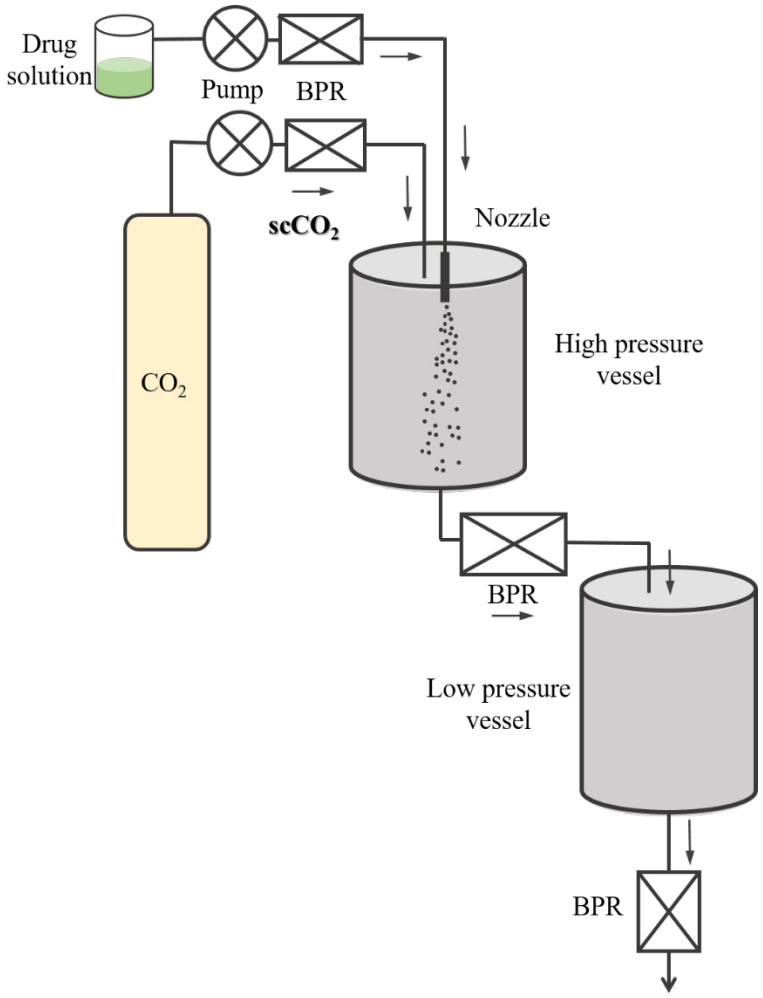


Figure I.12. General scheme of Anti-solvent techniques: Aerosol Solvent Extraction System (ASES), Particles by Compressed Anti-solvent (PCA) and Supercritical Anti-Solvent (SAS). CO_2 and drug solution reservoirs, pumps, back pressure regulators (BPR), nozzle, high- and low-pressure vessels.

The Atomization of Supercritical Anti-solvent Induced Suspension (ASAIS) is a variant of the Supercritical Anti-Solvent process. In this case, the precipitation takes place in a tube, before the spray, into the vessel.[113] In the Solution Enhanced Dispersion by Supercritical fluids (SEDS), both organic solution and supercritical fluid are introduced simultaneously through the same nozzle into the precipitation vessel. This method has

been applied in the production of nanoparticles of curcumin from *Curcuma longa*, a yellow-orange poorly water-soluble polyphenol, in order to enhance its bioavailability.[114]

The general applicability of these techniques has contributed to their increasing use for the micronization of different pharmaceutical polymeric compounds, colorants or inorganic components.

An example of this is the Supercritical Anti-Solvent process, SAS, sometimes named as Supercritical Anti-solvent Fractionation (SAF) or Supercritical Anti-solvent Extraction (SAE), where the solute is a mixture of different compounds (a plant extract, for example). This name changes according to the final purpose of the experimental procedure. In this situation, compounds are obtained in both vessels but in different proportions. Supercritical CO_2 as anti-solvent has been applied in research for the concentration of substances with antioxidant activity from natural extracts. For example Meneses et al.[115] fractionated a mango by-product extract with $scCO_2$ and concentrated mangiferin, isomangiferin, quiercetin and kaempferol into a dry powder spherical nanoparticles. Marqués et al.[116] applied SAE to extract grape seeds gallic acid, catechin, epicatechin and resveratrol antioxidant compounds, enriching its original ethanolic extract in a solid. Due to the potential applicability in compound concentration at mild experimental condition, this technology was used in this doctoral thesis for the enrichment of *L. luisieri* extract of non volatile compounds.

As with RESS and PGSS, it is possible to produce active-polymer composites. With SAS, composites can be produced by simultaneously co-precipitation of the active compound and the carrier, or precipitated a previously suspension of the active into a solution of the carrier.[117]

Supercritical Fluid Emulsion Extraction (SEE)

Supercritical Emulsion extraction technique emerged to replace the traditional techniques that involve emulsions to formulate capsules. For the encapsulation of bioactives, such as vitamins, the traditional solvent extraction technique can be applied. This process is based on the elimination of the internal phase of an emulsion by evaporation, applying temperature or by extraction with another solvent miscible into the dispersed phase. When the solvent is eliminated, the polymer hardness around the active, generating the capsule. Nevertheless, with traditional techniques, the evaporation usually proceeds at a slow rate and may also require high temperature or reduced pressure to

eliminate the organic solvent, or the extraction usually requires a large amount of an anti-solvent phase to extract the solvent.[118]

The use of supercritical CO_2 allows the fast production of micro and nanocapsules with a homogeneous particle diameter free from organic solvents at mild temperatures. The elimination of the phase, were the polymer is solved, occurs rapidly reducing the time of exposure to damaging experimental conditions,[119] reducing active degradation, and causing the precipitation of the coating material around the active of interest as a thin shell. Besides, the use of CO_2 flow to eliminate the solvent reduces the exposure to oxygen and therefore, the possible oxidation reactions capable to degrade the active of interest. The speed of SEE process, reduces the time for emulsion droplets to floccule and allows to produce active-carrier micro or nanocapsules with a controlled and homogeneous particle size distribution. Other supercritical techniques produce active-carrier composites by dispersing the active into a matrix of coating material, or by impregnating actives on particles of pure coating material.[120] Because of all the advantages that this technique offers in terms of encapsulation under mild experimental conditions, the encapsulation of non-volatile compounds from *L. luisieri* could be interesting because in this method the rapid evaporation of the solvent that vehicles the biopolymer allows it fast precipitation around the active, and not only its co-precipitation along with a polymer, ensuring its protection. In this work, it was proposed the encapsulation the antioxidant rosmarinic acid present in the plant understudy, *L. luisieri*.

Supercritical extraction processes have been proposed for an effective elimination of the organic solvents from single (*o/w*) or double emulsions (*w/o/w*), for the production of microparticle suspensions, using the Supercritical Fluid Emulsion Extraction process or SEE.[119] In this process, represented in Figure I.13, the solvent soluble in $scCO_2$ is extracted in a counter current column at a fixed temperature, pressure, emulsion and $scCO_2$ flow rate. The emulsion is pumped from the top of the column and the sc-CO_2 from the bottom. This counter current flow leads to the expansion of the organic solvent that carries the polymer in the emulsion, causing the precipitation of the polymer around the active and the formation of the capsules. This elimination occurs fast at mild temperature and allows the complete removal of the solvent. The speed of the process avoids the degradation of the bioactive compounds to encapsulate and the conglomerate of the droplets into larger ones, ensuring a reduced particle size distribution of the capsules.

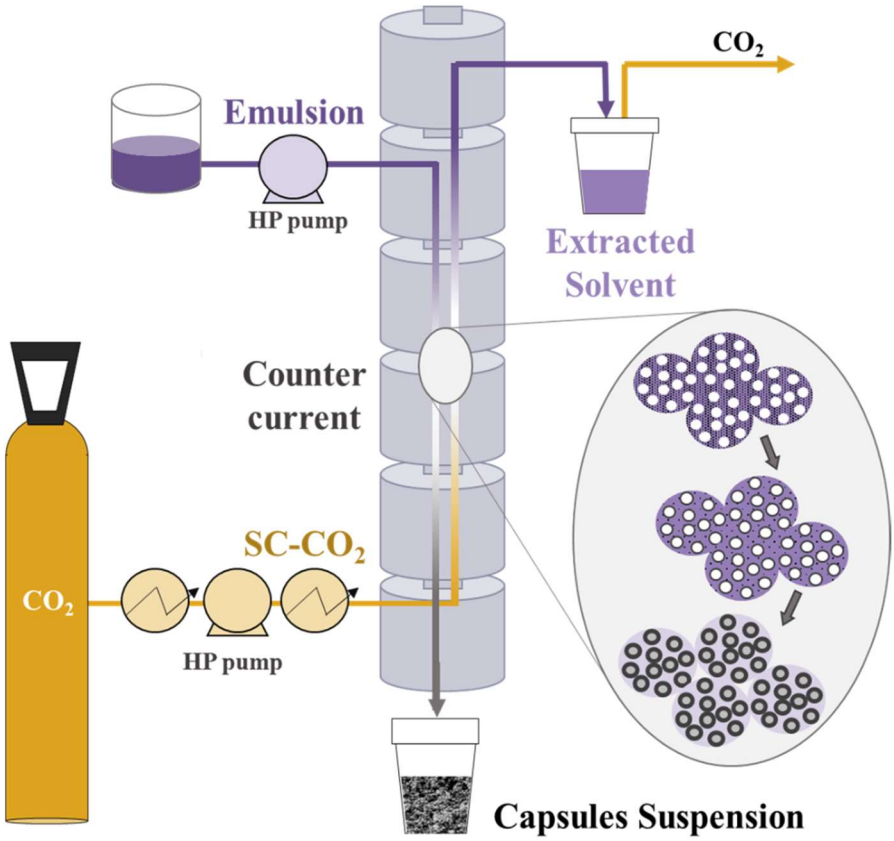
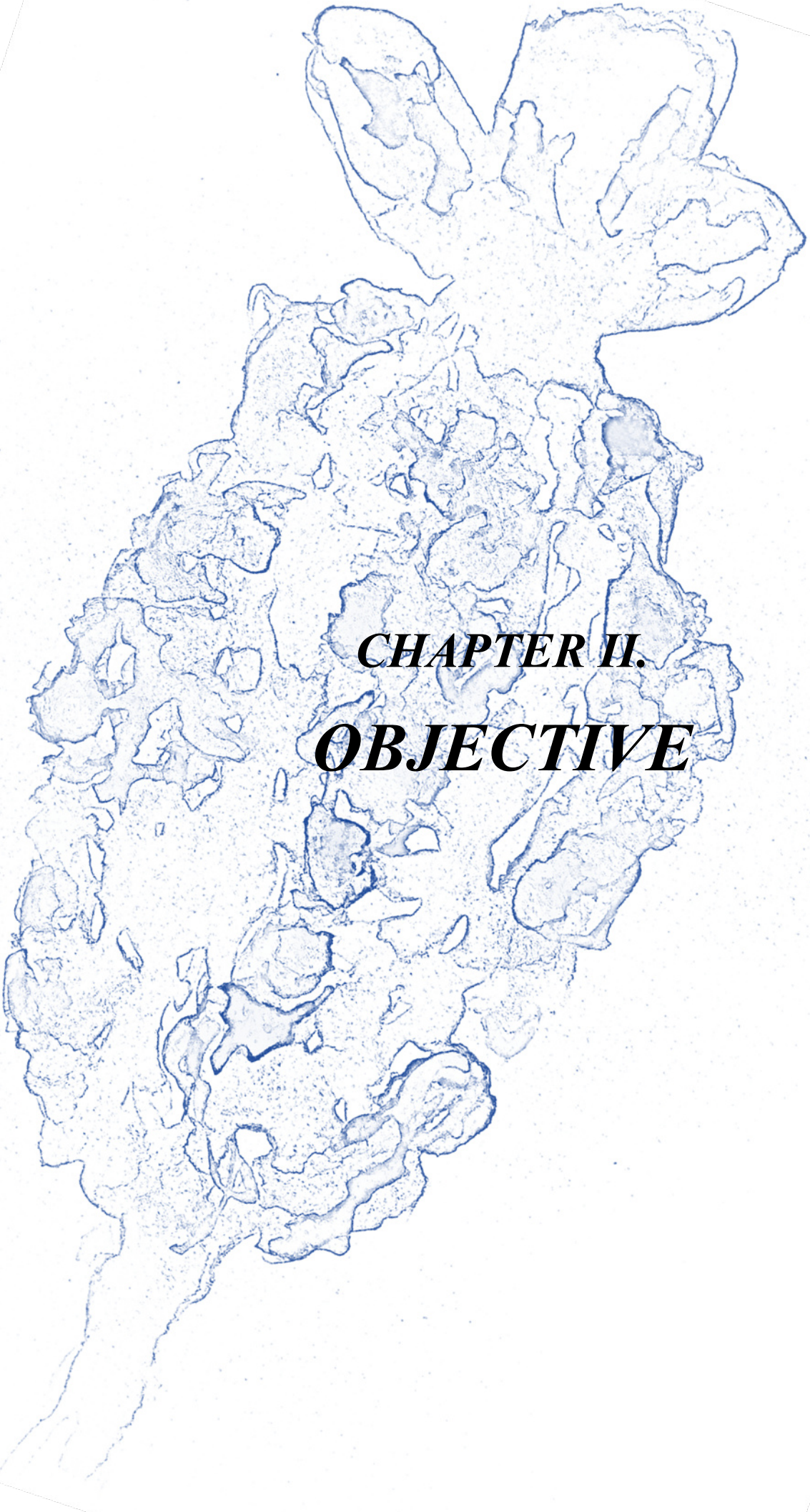


Figure I.13. Supercritical Fluid Emulsion Extraction (SEE) counter current tower. CO_2 and emulsion reservoirs, high pressure pumps (HP pumps), counter current, extracted solvent and capsules suspension vessels.

The coating materials are natural, such as cyclodextrins, starch or chitosan, semisynthetic, such as hydroxymethyl cellulose, or synthetic polymers, like polycaprolactone, poly(lactic acid) or poly(lactic-co-glycolic acid) and they must be selected according to different characteristics. They should be biocompatible and biodegradable, non-toxic and non-immunogenic, and their physicochemical characteristics should ensure the protection of the active as well as its biodisponibility. The synthetic polymer polycaprolactone, poly(lactic acid), poly(lactic-co-glycolic acid), or poly(methyl methacrylate) are usually selected over the natural ones because of their controlled and reproducible release of the bioactive.[121]



CHAPTER II.

OBJECTIVE

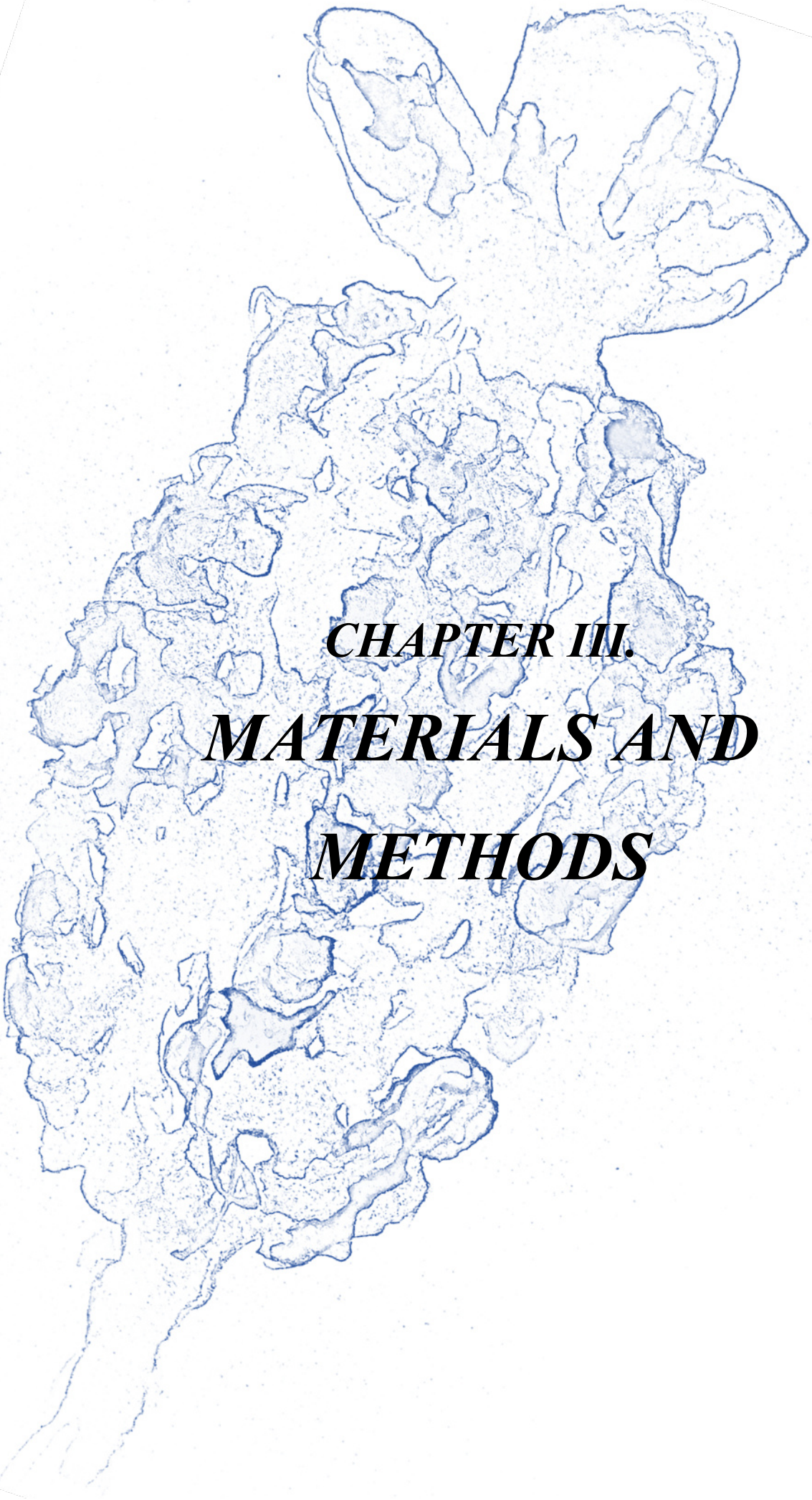
The general objective of this PhD work was to obtain concentrated products from the Spanish autochthonous *Lavandula luisieri* and study of the encapsulation of its bioactives using supercritical technologies.

This research has been developed as a part of the national project "*Supercritical Technologies and Biopesticides Development: Formulation, Diversification and Environmental Impact*" (Ministerio de Economía y Competitividad; CTQ2015-64049-C3-2-R), which is aligned with the Horizon 2020 goals highlighted by the European Union. An integral exploitation of aromatic medicinal plants from Spanish territory is pursued to obtain enriched and bioactive products from different plants to be used as biopesticides in the agroindustry field, or as nutraceutical or preservative additives in pharmaceutical, food or cosmetical industries. Besides, this project aims to give an effective contribution to the environmental protection against pollution and toxic effects on ecosystem organisms and human health by using sustainable supercritical technologies to formulate different bioactive products.

To reach this goal, the following specific objectives were stablished:

- Extraction and concentration of the natural bioactives of *L. luiseri* with the Supercritical Antisolvent Fractionation technique by the optimization with the statistical Response Surface Methodology based on Central Composite Design. The experimental conditions of pressure and CO_2 flow rate were optimized with this design for a higher mass recovery and concentration of the three actives; rosmarinic, oleanolic and ursolic acids, into a solid powder.
- Determination of the antimicrobial activity against bacterial strains of interest in human health and food safety, and antioxidant activity of different extracts of *L. luisieri*: essential oil, maceration extract and SAF fractions, and their correlation with their composition.
- Encapsulation of rosmarinic acid into biopolymer microcarriers as model of *L. luisieri* extract along with β-carotene and α-tocopherol using the Supercritical Emulsion Extraction technique to improve actives shelf life and preserve its antioxidant activity.

In the following chapters the specific methodology applied to achieve these objectives will be detailed as well as the results obtained and the conclusions derived from them.



CHAPTER III.

MATERIALS AND METHODS

1. *Extraction and concentration of Lavandula luisieri actives*

In this section the methods applied for the obtaining *of L. luisieri* extract will be described.

1.1. Lavandula luisieri plant material characterization and pre-treatment

The plant material was collected in 2009 from an experimental field in Aguarón, Zaragoza (Spain) from an adapted population of *L. luisieri*, original from Pueblo Nuevo del Bullaque, Ciudad Real (Spain). This adaptation was performed by the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA, Spain), as previously described.[31] The plant material was provided dried at room temperature in appropriate opaque bags.

1.1.1. Moisture content

The moisture analysis was performed with a *SARTORIUS* model *MA 40 Moisture Analyzer Software/hardware* 01.05.02 (Figure III.1). The equipment heats up to 110 ºC with infrared radiation to evaporate the water of the sample. The mass of the material is measured automatically until it reaches a non-changing value. Five replicates of the measurement with 1 g of plant material were performed and the moisture content (%Moisture) determined according to eq. III.1.

Figure III.1. Moisture analyser SARTORIUS MA40

$$\%\text{Moisture} = \left(m_{initial} - m_{final}\right) x\ 100 / m_{initial} \qquad \text{(eq. III.1)}$$

1.1.2. Grinding and sieving

The plant material, dried at room temperature, was then grinded. Its particle size distribution was carried out with a vibratory sieve shaker *CISA* model BA 300N (Figure III.2) and the average diameter (d_m) was calculated according to ASAEA S319.3 from the American National Standards Institute[122] as shown in the next equation eq.III.2.

Figure III.2. Siever CISA model BA 300

$$d_m = \log^{-1}\left[\frac{\sum_i^n (w_i \log \bar{d}_i)}{\sum_i^n w_i}\right];\ \bar{d}_i = (d_i \cdot d_{i+1})^{0.5} \qquad \text{(eq. III.2)}$$

where d_m is the average diameter obtained, n is the number of sieves, d_i is the nominal mesh of the i^{th} sieve (mm), $d_{(i+1)}$ is the nominal mesh of the next larger sieve after the i^{th} sieve (mm) and w_i is the mass (g) of the plant material retained by the i^{th} sieve.

1.2. Lavandula luisieri extracts

In this thesis two different *L. luisieri* extracts were obtained from the pre-treated plant material.

1.2.1. Hydrodistillation

L. lusieri essential oil (EO) was provided by the CITA and preserved in amber vials under refrigeration. This EO was obtained by Julio et al.,[70] with a hydrodistillation Clevenger-type apparatus. These authors also determined the EO chemical composition, which is shown under these lines. (Table III.1)

Table III.1. *L. luisieri* essential oil composition.[70]

Identified compounds	**EO GC-MS**
Camphene	1.6
1,8-cineol	2.0
Fenchone	2.9
Camphor	60.3
2,3,4,4-tetramethyl-5-methylidenecyclopent-2-en-1-one	8.5
D-Verbenone	1.2
Exobornyl acetate	4.6
Cis-α-Necrodyl acetate	1.9
3,4,5,5-tetramethylcyclopenta-1,3-diene-1-carboxylic acid	*Tr*
5-hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one	*Tr*
3,3,4,5-tetramethyl-2H-pyran-2,6(3H)-dione	*Tr*
2-(hydroxymethyl)-3,4,4-trimethyl-5-methylenecyclopent-2-en-1-one	*Tr*
5-hydroxy-5-(hydroxymethyl)-2,3,4,4-tetramethylcyclopent-2-en-1-one	*Tr*
(2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate	0.7
(1R,6R,7S,10R)-10-Hydroxy-4(5)-cadinen-3-one	*Tr*

Tr, trace amounts

1.2.2. Maceration

The plant material was summited to two serial macerations into two different extraction solvents: hexane (Panreac, 99%) and ethanol (Analar Norma, 99.96%). The first solvent was selected to degrease or eliminate the non-polar compounds such as cuticle waxes from the plant material to, ultimately, obtain the more polar compounds with ethanol.[123] To do so, 100 g of pre-treated dried *L. luisieri* plant material were stirred for 48 h at room temperature (25 °C) in 1 L of hexane. After the filtration of the hexane extract mixture with a Büchner funnel, the solvent was separated from this first extract at 178 mbar and 42 ºC using a rotary evaporator Büchi R-200, equipped with a heat bath B-490 and a controllable vacuum pump V-800. The remaining plant material exhausted from non-polar compounds was dried and afterwards macerated under the same experimental conditions but using ethanol this time. When the second ethanolic maceration mixture was filtered, the solvent was again separated from the extract, at 65 mbar and 42 ºC. [124] In order to avoid actives degradation because of heat and light, in this work the extraction was performed at room temperature and in amber bottles and the final extracts were kept in amber vials under refrigeration. Pictures of this process are shown in Figure III.3. The extraction *yield* for both macerations, Y_i (%), was calculated using equation eq. III.3.

$$Y_i(\%) = \left(\frac{mass_{\text{plant extract}}}{mass_{\text{plant material}}}\right) \cdot 100 \qquad \text{(eq. III.3)}$$

where *i* is the solvent of the extraction; hexane or EtOH, $mass_{\text{plant extract}}$ is the mass (g) of the dried extract after maceration (once the solvent had been removed) and $mass_{\text{plant material}}$ was the initial mass (g) of dried and pulverised plant.

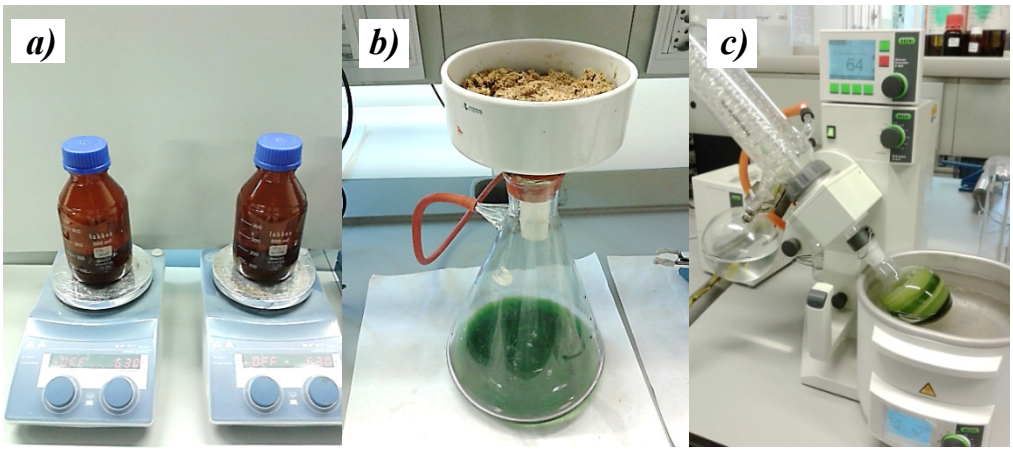


Figure III.3. Process of maceration extracts a) Soaking at room temperature under stirring; b) Filtration; c) Solvent evaporation.

1.3. Concentration of actives

1.3.1. Supercritical Antisolvent Fractionation (SAF)

Lavandula luisieri non-volatile compounds, were fractionated by Supercritical Antisolvent Fractionation. SAF experiments were performed in the "Green Chemistry Laboratory" (I3A Research Institute at University of Zaragoza) using a lab-scale apparatus (Figure III.4).

Figure III.4. Picture of the lab-scale Supercritical Anti-solvent Fractionation equipment located in the Green Chemistry Lab, I3A Institute, Zaragoza, Spain.

The main components of the device are represented in Figure III.5.[125] First of all the solvent-free extract obtained in the second maceration was dissolved into ethanol. This ethanolic feed solution (FS) was pumped by P-LIQ (Waters co-solvent pump series III maximum pressure 400 bar) and the sc-CO_2 (CO_2 Alpha Gaz 99.98%) was pumped with P-SCF (Thar SFC mod. P200 max pressure 600 bar).

The supercritical fractions were collected in two vessels: the precipitation vessel, PV, and the downstream vessel, DV, which pressure was controlled by an automated backpressure regulator (ABPR, TharSFC) and a manual backpressure regulator (BPR, CIRCOR Instrumentation Technologies) respectively. The working limits of the device

were 400 bar and 120 °C and the experimental conditions of the equipment were set with the controlling computer using the software Thar Instruments Process suite.

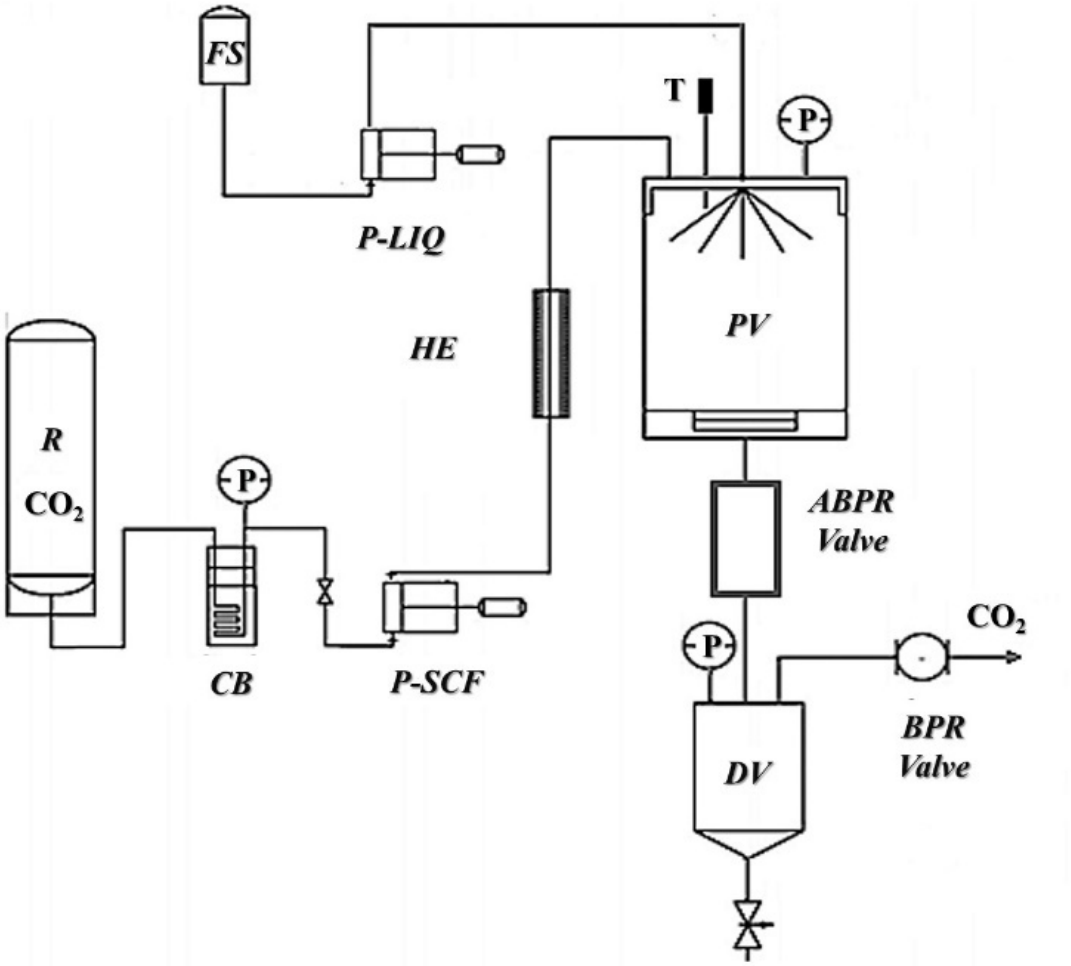


Figure III.5. Scheme of the SAF plant. Feed solution reservoir (FS); liquid pump (P-LIQ); CO_2 reservoir (R), cooling bath (CB); CO_2 pump (P-SCF); heat exchanger (HE); precipitation vessel (PV); Thermopar (T); automated back pressure regulator (ABPR); back pressure regulator (BPR); downstream vessel (DV).

In the first place, the experimental conditions were selected. sc-CO_2/ethanol ratio, pressure and temperature conditions were chosen according to the binary system ethanol-CO_2 reported by Marques et al.,[116] to ensure supercritical conditions in the PV chamber and a CO_2 molar fraction of 0.98 (Figure III.6). To do so, pressure and CO_2 flow rate parameters were varied always above 80 bar and 10 g/min scCO_2, into final ranges of 80-150 bar and 10-30 g/min respectively. The FS flow rate and PV temperature were fixed at 0.45 mL/min and 40 ºC respectively. The FS concentration was also fixed at 3% (wt.%) and filtered through *NYLON* 0.45 μm pore size.[124]

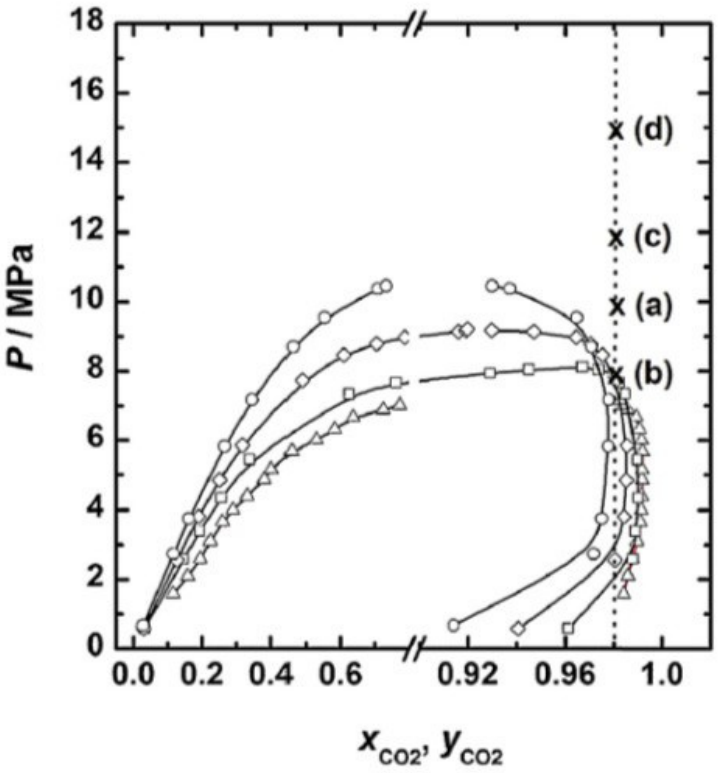


Figure III.6. Equilibrium curves of ethanol–CO_2 (x_{CO_2} = 0.98) at different temperatures and pressure (a) 100 bar, 50 ºC; (b) 80 bar, 35 ºC; (c) 100 bar 40 ºC; (d) 150 bar, 40 ºC, at which the mixture is a supercritical fluid.[2]

Every SAF process was performed following the procedure of Martin et al.[124] As the influence of pressure and $scCO_2$ flow rate influence on the final recovery yield and concentration of non-volatile actives was under study, the experimental conditions were selected for each experiment inside the range of 80-150 bar and 10-30 g/min. Once the experimental conditions were stabilized, the CO_2 was pumped into the system at the selected temperature, pressure and flow rate and, after filling the system with CO_2, pure ethanol solvent was pumped at 0.45 mL/min. Then the stabilization of the system was accomplished, and the feed solution of the extract was injected followed by 30 mL of ethanol. Finally, pure $scCO_2$ flowed for 30 min to wash out the residual pure solvent. Samples from the DV separator were collected every 20 min during the experiment. Finally, after the equipment de-pressurization, the PV fraction was collected. Although PV fraction was normally solid, when it did not precipitate in an easy recoverable solid product, it was taken by cleaning the PV content with ethanol and then dried with a rotary evaporator at 65 mbar and 42 ºC. The content of DV was collected too. It was a liquid mixture, which solvent, ethanol, was removed in this mentioned way. All samples of each

experiment and both vessels were kept under refrigeration at -20 ºC in amber vials for their posterior analysis.

The mass of each fraction was quantified and the yields in DV, $Y_{DV}(\%)$, and in PV, $Y_{PV}(\%)$, were calculated using equation eq. III.4.

$$Y_i(\%) = \left(\frac{\text{mass fraction collected in } i}{\text{mass of extract in the FS}}\right) \cdot 100 \qquad \text{(eq. III.4)}$$

where *i* is the place of collecting, that is, PV or DV.

Besides, the overall yield of the process, $Y_{SAF}(\%)$, was defined in eq. III.5, as follows,

$$Y_{SAF}(\%) = Y_{PV}(\%) + Y_{DV}(\%) \qquad \text{(eq. III.5)}$$

1.3.2. Experimental design and statistical analysis

The Response Surface Methodology (RSM) based on Central Composite Design (CCD) was employed to statistically design the experiments required to evaluate and optimise the conditions of pressure 80-150 bar and CO_2 flow rate 10-30 g/min, in order to obtain the maximum yield recovery, both in the precipitation (PV) and downstream (DV) vessels. The RSM was also applied to optimise the enrichment of the compounds of interest, rosmarinic acid (RA), oleanolic acid (OA) and ursolic acid (UA). The methodology applied by Langa et al.[125] was followed. A mathematical model for a two variable CCD is represented in Figure III.7 and by equation eq.III.6.

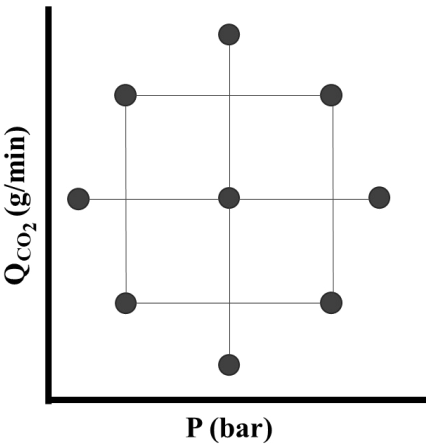


Figure III.7. Response surface methodology (RSM) based on central composite design (CCD) equation graphical representation. The points represent the experimental conditions of pressure and CO_2 flow rate.

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{2} \beta_{ij} X_i X_j \qquad \text{(eq. III.6)}$$

where Y is a dependent variable (e.g. extraction yield), β_0 is the constant coefficient, β_i is a linear coefficients, β_{ii} is a quadratic coefficients, β_{ij} is an interaction coefficient, and X_i and X_j are the independent variables under study, that is, pressure and CO_2 flow rate, coded in this work as XP and XQ_{CO_2}, respectively.

1.3.3. Microscopy observations

A Scanning Electron Microscope (SEM) was used to characterise the morphology of the substance obtained in the PV by SAF. It was performed by the Electron Microscopy Service from the Zaragoza University (Spain). For that purpose, a LEO 420 version V2.04, ASSING, was used. Precipitated and/or recovered solids in PV and DV were placed on a carbon tab previously stuck to an aluminium stub (Agar Scientific, Stansted, UK). Samples were overcoated with carbon using a sputter coater (mod. 108A, Agar Scientific).

1.4. Identification and quantification of actives

Rosmarinic, oleanolic and ursolic acids, RA, OA and UA respectively, were tracked along the SAF process. They were identified and quantified in the feed solution, FS, and in the supercritical fractions PV and DV.

Their chromatographic separation and quantification were performed by reverse phase high performance liquid chromatography in a HPLC *Waters® Alliance 2695* equipped with a *CORTECS®* C18 2.7 µm (4.6 × 150 mm) column, a CORTECS® Pre-column VanGuard C18 2.7 µm (2.1 × 5 mm) and a photodiode array detector *PDA Waters® 2998*. Figure III.8.

Figure III.8 HPLC Waters® Alliance 2695 equipment, PDA Waters®2998 detector and computer for the control and data processing.

In this work, the compounds were eluted following an adaptation of Chen et al.[126] with an isocratic mobile phase methanol (Scharlab 99.9% HPLC grade) and aqueous phosphoric acid (H_3PO_4 Fluka 85.9%) solution at 0.5% in a final mixture proportion of MetOH 88%: H_3PO_4 in Milli-Q water 12% for 10 min at 0.8 mL/min flow rate. The correlation of the obtained peaks was performed by internal standards. For the detection, PDA wavelength was fixed at 330 nm for the first 6 min and at 210 nm for the last 4 min, in order to detect and quantify RA, OA and UA. Extract solutions (100 ppm approximately) were filtered through a *GH Polypropylene membrane ACRODISC* 13 mm pore size 0.2 μm filter. RA (rosmarinic acid, Sigma Aldrich, 99%), OA (oleanolic acid, Sigma Aldrich, 99.8%) and UA (ursolic acid, Sigma Aldrich, 99.7%) standards were run under the same chromatographic conditions. All analyses were performed in triplicate.

2. Supercritical encapsulation of actives

2.1. Emulsion formulation

2.1.1. Reagents

In this work, the encapsulation of *L. luisieri* antioxidant, rosmarinic acid, was proposed alone and with other typical excipients used in the food, cosmetical and pharmaceutical industries, such as β-carotene and α-tocopherol.

For the encapsulation of the actives, several double ($w_1/o/w_2$, $o_1/w/o_2$) and single (o/w) emulsions were formulated. The emulsion oily phases were constituted with anhydrous chloroform, acetone and ethyl acetate, and the aqueous phases with distilled water, ethanol, and acetic acid (all of them of purity 99.9% and supplied by Carlo Erba Reagents).

The following surfactants were used to stabilize the emulsion formulation. PVA, Polyvinyl alcohol, is a water-soluble synthetic polymer with excellent film forming, emulsifying and adhesive properties, fully degradable and widely used in the food and pharmaceutical industries.[127] PVA (Sigma-Aldrich) was added as surfactant and emulsion stabilizer into the internal phase of the formulated emulsions. Tween 80 is a non-ionic surfactant and emulsifier, belonging to the polyethoxylated sorbitan family, a viscous yellow liquid at room temperature. It was purchased from Sigma Aldrich and used as surfactant in the external aqueous phase of the emulsions. Finally, sorbitan esters are non-ionic surfactants and, in this work, the sorbitan monolaurato, SPAN 20 (a viscous liquid of amber colour, from Sigma Aldrich) was used.

The pure actives to encapsulate in the carriers were rosmarinic acid (RA 96%, Sigma-Aldrich), β-carotene (β-CA, Sigma-Aldrich) and α-Tocopherol (α-TOC, Sigma-Aldrich). The biopolymers were poly-lactic acid (PLA) and poly-lactic-co-glycolic acid (PLGA).

Polylactic acid or PLA (Figure III.9) used to produce the microcapsules was purchased by Boehringer Ingelhein and it is of the RESOMER® RG 203S type. It exists in two enantiomeric forms (L-D), but only from the

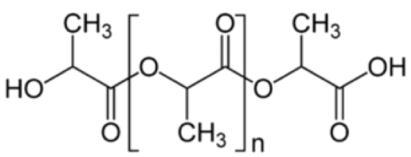


Figure III.9. PLA chemical structure.

optically active isomer (L) it is possible to obtain the polymer crystal clear. The polymer has the following characteristics:

- Molecular formula: $(C_3H_4O_2)_n$
- Molecular Weight: 18000 -28000 g/mol

PLGA or poly(lactic-co-glycolic acid) (Figure III.10) is a copolymer used in a host of the Food and Drug Administration (FDA) approved therapeutic devices and is synthesized by means of ring-opening co-polymerization the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid. During polymerization, successive monomeric units (of glycolic or lactic acid) are linked together in PLGA by ester linkages. A common form is composed by 75% of lactic acid and 25% of glycolic acid.[128] For the microcapsules production 75:25 Resomer PLGA (RESOMER® RG 752 H) from Boehringer Ingelheim was used. The polymer presents the following characteristics:

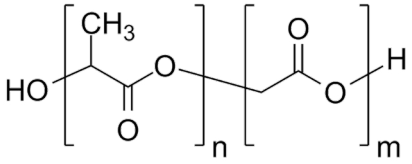


Figure III.10. PLGA formula.

- Molecular formula: $[C_3H_4O_2]_x\ [C_2H_2O_2]_y$
- Molecular weight: 20,000 g/mol

2.1.2. Emulsification procedure

The emulsification process requires energy to be provided to the emulsion, that is, to create instability at the O/W interface that leads to an increase of pressure on the surface, or to reduce the interfacial tension, which is the aim of the emulsification methods. To aim that, and for single emulsions, only rotation was applied, while for double emulsions, ultrasounds and rotation were used successively. In this work several emulsions were proposed in order to encapsulate three different antioxidants. An image of each apparatus is represented in Figure III.11.

Figure III.11. Ultrasonic probe mod S-450D, Branson on the left and high speed stirrer model L4RT, Silverson, on the right.

Single emulsions (Figure III.12) were prepared by adding the internal oily phase (actives, polymer and surfactant solved in an organic solvent) to the external aqueous phase (a water saturated solution with the organic solvent and a surfactant) under agitation. Single emulsions phases (*o/w*) composition ratio 20:80 wt/wt.

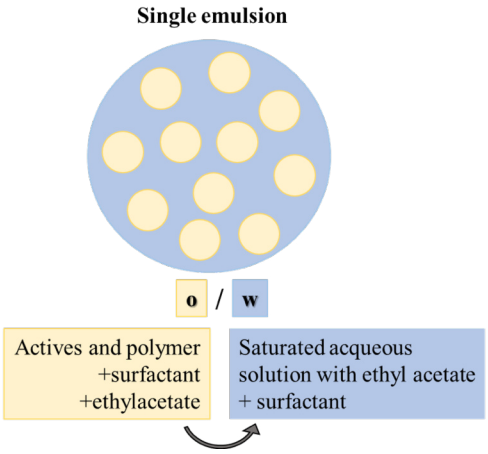


Figure III.12. Scheme of a single (*o/w)* emulsion formulation and the main components of each phase.

For double emulsions ($w_1/o/w_2$ and $o_1/o_2/w$) (Figure III.13), two steps were required. In the first place, a primary emulsion was prepared by adding the internal, *w* or *o* phase, which contains the actives and surfactant solved in an aqueous or organic solvent, to the oily phase with the polymer, under sonication conditions. And secondly, the primary emulsion was added to the saturated external water phase under agitation. Double emulsions were formulated with a composition ratio of 4:16:80 w/w/w.

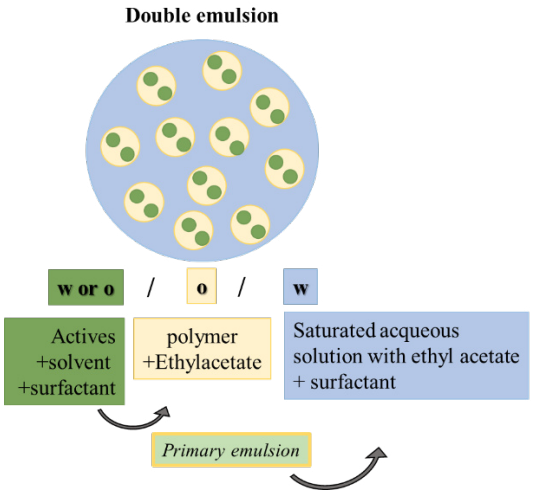


Figure III.13. Scheme of a double (*w/o/w* or *o/o/w*) emulsions formulation and the main components of each phase.

The emulsification via sonication used an ultra-sound probe Digital Sonifer Branson mod. 450 (Salerno, Italy). This method is used to disperse the internal phase in the oil phase, and it is based on propagation of ultra-sound waves in the liquid medium. Such propagation causes a cycle of low- and high-pressure states, depending on the wave amplitude of the programmed probe. The possibility of varying this amplitude allows the regulation of the final droplet mean dimension. During the depression phase of the cycle, the high-density ultra-sound waves create hollows and bubbles in the liquid medium. Another interesting feature of this method is the possibility of varying the amplitude of the probe, thus regulating the average dimension of the final drop.[129] The to-be-dispersed internal phase was added to the oily phase and the mixture was summited to 3 sets of sonication of 30 seconds at a fixed amplitude of 30 %.

Emulsification via high speed rotation was performed to disperse the internal oil phase, in single emulsions, or primary emulsion suspension into the external aqueous phase for double emulsions. A Silverson mod. L4RT emulsifier (Salerno, Italy) was used. The velocity of the sieve is the main parameter to take into account for control of the dimension of the droplets (and, consequently, of the microcapsules). Increasing this speed intensifies the shear stress and the turbulence, and therefore, diminishes the mean diameter of the droplets. The external phase was stirred at the set velocity to produce droplets of the desired shape and then, the to-be-dispersed internal phase or primary emulsion was added drop-by-drop.

2.3. Encapsulation

All formulated emulsions were processed through SEE in a continuous experimental laboratory apparatus located at the University of Salerno (Italy) (Figure III.14).

Figure III.14. SEE apparatus picture.

Some other emulsions were processed also by solvent evaporation in order to compare the particles produced with both techniques. The SEE process allows that the $scCO_2$ eliminates the solvent (ethyl acetate in this case) that carries the polymer in a fast and selective way. When the organic solvent, that must be soluble in $scCO_2$, is eliminated, the polymer hardens causing the encapsulation of the actives. In the traditional solvent

evaporation procedure, the elimination of the organic solvent from an emulsion is produced by heat evaporation while stirring. The experimental conditions to evaporate this solvent were 42 ºC and 110 rpm for 4 hours (Figure III.15).

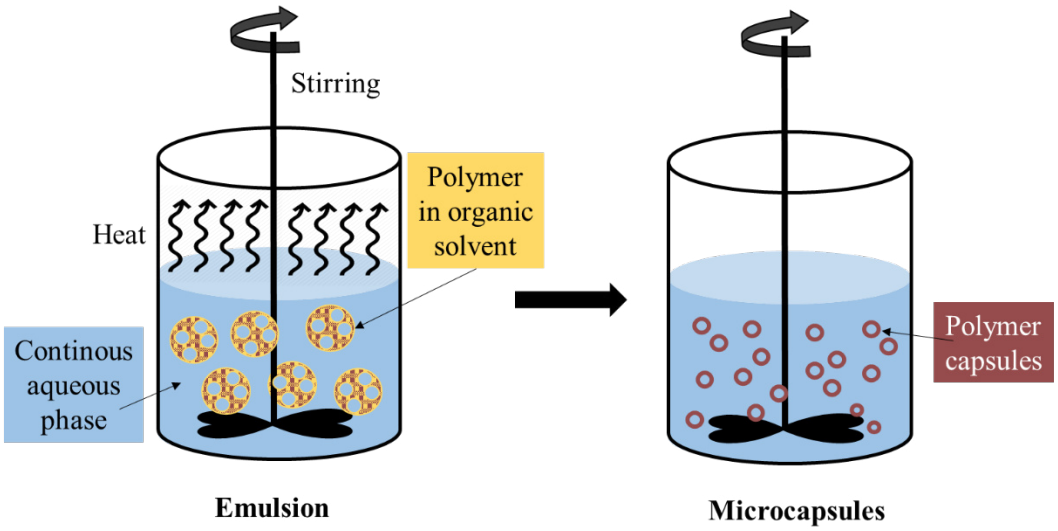


Figure III.15. Scheme of solvent evaporation procedure for encapsulation.

The SEE apparatus[119] consists of a column 1680 mm high, with a 13 mm internal diameter. The column is packed with stainless steel packings 4 mm nominal size with 1889 m^{-1} specific surface and 0.94 voidage (0.16-inch Pro-Pak, Scientific Development Company, State College, PA, USA), composed by five AISI 316 cylindrical sections, connected by four cross-shaped junctions. The column is thermally isolated by a fibreglass coating, with the temperature profile being controlled by six controllers.

The operating conditions and the procedure were performed following the methodology described by Della Porta et al[130] at 38 °C, 80 bar and, to ensure a good extraction efficiency and fluid dynamics of the counter current process, the Liquid/Gas ratio was set to 0.1, the gas feed to 1.4 kg/h, and emulsion feed rate to 2.4 mL/min.

$ScCO_2$ is sent upwards from the bottom of the column via a high-pressure membrane pump (Milroyal B, Milton Roy, Pont Saint-Pierre, France) at a constant rate. The emulsion is instead sent from the top, after being taken from a reservoir, through a high-pressure piston pump (mod. 305, Gilson, France). At the top exit of the column, a low-pressure operating separator is placed, for “oily” solvent recovery. The pressure of this separator is regulated via a backpressure valve (26-1700 Series, Tescom, Selmsdorf, Germany). At the exit of the separator, a rotameter (mod. N5-2500, ASA, Sesto San

Giovanni, Italy) and a dry test meter (mod. LPN/S80AL class G2.5, Sacofgas, Milan, Italy) are used to measure, respectively, the rate and the total amount of delivered CO_2. Microcapsules suspension is continuously gathered and periodically withdrawn at the bottom of the column by decompression, using a needle valve (mod. SS-31RS4, Swagelok, Brescia, Italy). A detailed representation of the SFEE process layout is reported in Figure III.16.

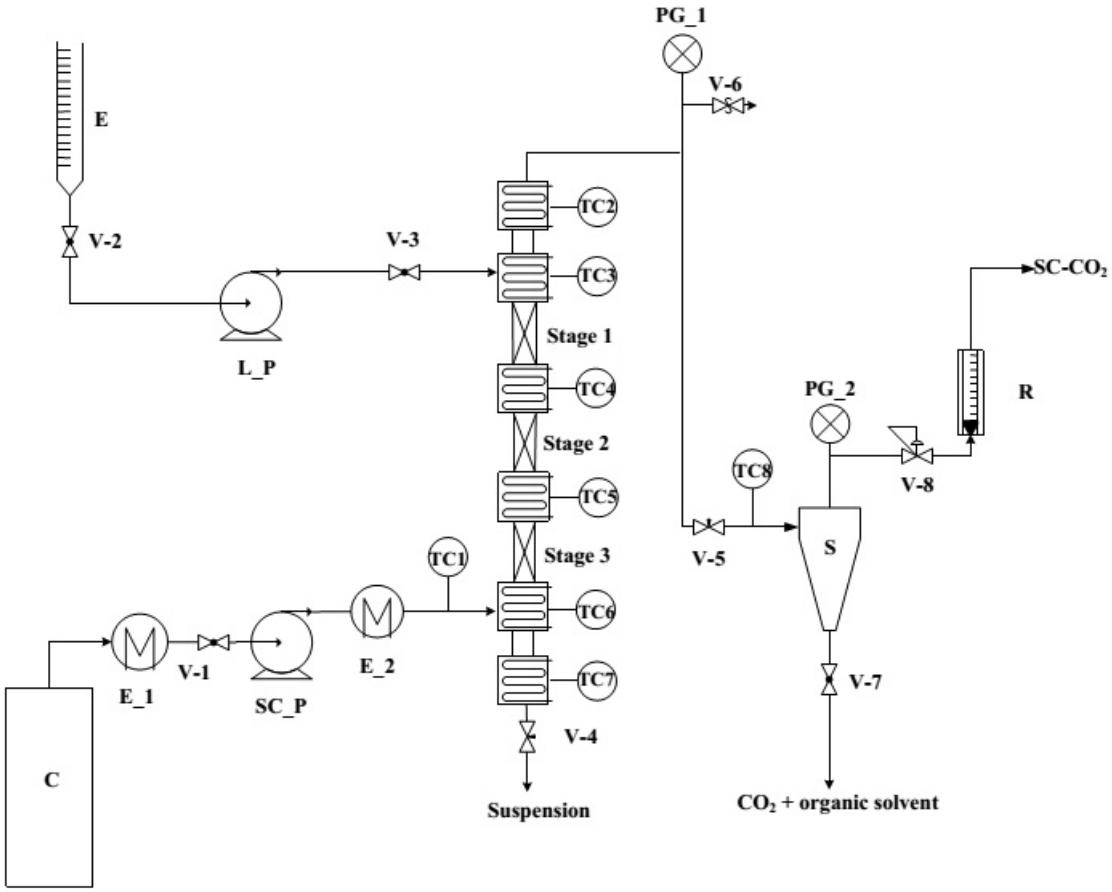


Figure III.16. SEE apparatus. C, CO_2 supply; E, emulsion; PG_1 and PG_2; pressure gauges; SC_P, diaphragm pump used for high-pressure sc-CO_2; L_P, liquid piston pump; E_1 and E_2, heat exchangers; V: valves; TC: thermocouples; S: separator; R: rotameter.

Operating conditions were set at a temperature of 38 °C and pressure of 80 bar. In these conditions, the solvent-CO_2 mixture is set above the MCP (Mixture Critical Point) for various solvents (ethyl acetate and acetone, for example). The Liquid/Gas ratio was 0.1 to ensure a good extraction efficiency and fluid dynamics of the counter current process. The gas feed was set to 1.4 kg/h, while the emulsion feed rate was 2.4 mL/min, calculated by the imposition of the feed ratio.

The microcapsules suspension was collected at the bottom of the column. The produced particles collected from each assay were washed with distilled water by 2 serial centrifugations for 20 min at 6500 rpm and at 4 °C; then, microparticles were recovered on a membrane filter (porosity 0.2 μm) and dried with vacuum filtration for its posterior evaluation.

2.4. Droplets and microcapsules morphology and size distributions

For the evaluation of the morphology and size of both droplets and formulated microcapsules, also called microspheres or particles in this work, visual images of them and the particle/droplet size mean and distribution were obtained.

The emulsion morphology and droplet size distribution were observed and measured immediately after its formulation. Emulsions were observed with an optical microscope (mod. BX 50 Olympus, Tokyo, Japan) equipped with a phase contrast condenser to ensure a homogeneous shape and stability. The microcapsules with the actives were studied after their cleaning and drying, previously described in section 2.3. A sample of these microspheres was coated with gold (layer thickness 250 Å) using a sputter coater (mod.108 A, Agar Scientific, Stansted, UK). Then the morphology was observed using a Field Emission-Scanning Electron Microscope (FE-SEM mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany).[130]

Droplets Size Distributions (DSD) and Particles Size Distributions (PSD) were measured by dynamic light scattering DLS, mod. Mastersizer S, Malvern Instruments Ltd., (Worcherstershire, UK) (Figure III.17) immediately after the preparation of the emulsion and the particles, using 1 mL of each sample. The distributions shown in this work are the average of 10 replicates. This equipment allows the measurement of the droplet dispersion in the emulsion or the dispersion of the suspended particles obtained by SFEE, via evaluation of the interaction between the droplets, or particles, with light. Light is diffracted by droplets or particles at an angle that is inversely proportional to their dimensions and detected by al multi-element reader made of 44 detectors. The detectors produce a very low electrical signal, proportional to the time-based average of the intensity of the light that hits them, and proportional to their surface (photodiode area). This signal is digitalized and inverted once it is sent to the computer. For the analysis of the formulated emulsion and particles , the method previously reported by Della porta et

al.[130] was applied. Every sample was solved in distilled water and introduced in glass cuvettes for its analysis. The scattering angle was fixed at 173 º at 25 ºC.

Figure III.17. Dynamic light scattering (DLS) apparatus for the droplet and particle size distribution determination.

2.5. Drug loading

For the quantification of encapsulated actives, a known mass (approximately 15 mg) of produced particles were dissolved in 0.5 mL of acetone in order to break the biopolymer and liberate the encapsulated molecules of active/s. Then, 3.5 mL of ethanol were added causing the biopolymer precipitation. This suspension was centrifuged for 30 min at 6500 rpm and 4 ºC. The supernatant was collected and the drug concentration was measured by HPLC-PDA (Agilent LC system 1100 and 1200 series) equipped with a Waters Spherisorb ODS-2 (Ø 5 µm 150x4.6 mm) column (Figure III.18).

Figure III.18. HPLC for the quantification of rosmarinic acid, β-carotene and α-tocopherol.

The mobile phase was 80:20 MetOH: H_2O acidified with 0.1% CH_3COOH under a flow rate of 1 mL/min. The injection volume was 20 µL in every test and the detection wavelength was of 450 nm for β-CA, 292 nm for α-TOC and 330 nm for RA. A calibration curve was built for each compound. The *encapsulation efficiency*, *EE* (%), was determined with equation eq. III.7.

$$EE\ (\%) = (measured\ actives\ amount / loaded\ actives\ amount)\ x\ 100 \quad \text{(eq. III.7)}$$

where *measured actives amount* is the mass of actives quantified with HPLC and *loaded actives amount* is the initial mass of actives introduced in the initial formulation before the supercritical process.

As β-CA is widely used in the food, cosmetic and pharmaceutical products as natural colorant and antioxidant, nevertheless is very susceptible to degradation by light, high temperature and oxygen.

In a previous study performed by the Supercritical Fluids group of Salerno University, PLA and PLGA capsules of β-CA were produced with SEE technique.[131] The degradation rate under UV radiation (λ=254 nm) for 1 hour a day for 10 days was evaluated. In this work, the remaining encapsulated β-CA after 2 years of storage at 4 ºC in the dark was also determined. To do so, samples were solved in acetone and the remaining or non-degraded quantity of β-CA was measured at 450 nm using a spectrophotometer UV-vis.

3. Activity assays

The antimicrobial and antioxidant activities from the different *L. luisieri* extracts and supercritical products was determined.

3.1. Antimicrobial activity of L. luisieri extracts and SAF fractions

The antimicrobial activity of *L. luisieri* maceration extract and its supercritical fractions obtained at the experiment performed at the optimised Supercritical Antisolvent Fractionation conditions. Besides, its essential oil was also tested.

3.1.1. Bacteria revivification and growth media

The bacterial strains assayed in this study included three gram-positive and two gram negative, gathered in Table III.2. All strains were obtained from the Spanish Collection of Type Cultures (CECT). They were all maintained frozen at −80 °C in cryovials. The media employed to grow bacteria and to adjust strain concentration were Trypticase Soy Agar (TSA) and peptone water (Buffered peptone water, Oxoid).

Table III.2. Bacterial strains selected for the antimicrobial assays.

Gram positive	*Denomination*
Listeria monocytogenes	CECT 911
Enterococcus faecium	CECT 410
Staphylococcus aureus	CECT 435
Gram negative	
Salmonella Typhimurium	CECT 443
Escherichia coli	CECT 516

Before the assays, the bacteria strains kept in cryovials were revivificated. From each cryovial, one porous ring coated with the microorganisms was transferred to a tube with 10 mL of tryptone soya broth (TSB, Oxoid) and incubated 24 hours at 37 ºC in a Memert and P-SELECTA oven. From this suspension each microorganism was incubated in Tripticase soy agar (TSA, Oxoid) at the same conditions, in order to obtain a fresh and pure cultures (Figure III.19).

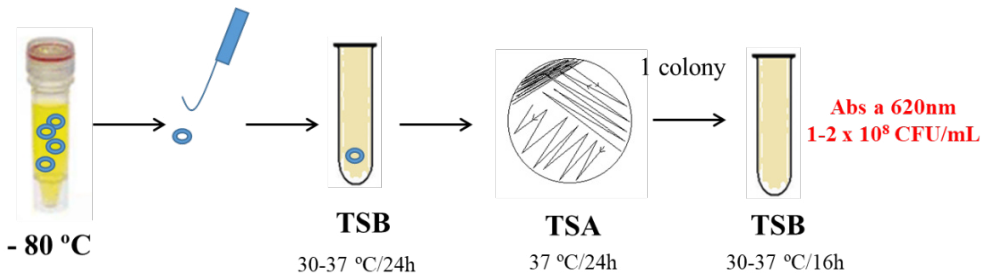


Figure III.19. Revivification process scheme.

The final working broth subcultures were prepared by inoculating, with one single colony from a TSA plate, a test tube containing 10 mL of sterile TSB. The inoculated tubes were incubated overnight (16 h) at 37 ºC. Then, the bacterial concentration was adjusted to an absorbance between 0.08 to 0.10 using a spectrophotometer (Jenway 3600, Tirana, Albania) at a wavelength of 620 nm, which corresponds to 1 x 10^8 CFU/mL according to McFarland Turbidity Scale (Standart N1 0.5, Becton Dickinson and Company, Madrid, Spain). Additionally, inoculum concentration was confirmed by the colonies counting in agar plates after planting 1:10 dilutions in peptone water.

3.1.2. Disk diffusion method

Antimicrobial activity of the EO was screened against the five cited microorganisms using the Kirby-Bauer sensitivity agar diffusion technique[132], methodology schematically represented in Figure III.20. Filter paper disks (Whatman No. 1, 6 mm diameter) containing 15 µL of EO were placed on the surface of agar plates of Mueller-Hinton (Merck), that were previously seeded by spreading one sterile hyssop impregnated in strain culture of 1 x 10^8 CFU/mL. Ampicillin disks (10 µg) (Oxoid) were used as positive control. The plates were incubated at 37 ºC for 24 hours and the diameter resulting from each inhibition zone (diameter of inhibition zone plus diameter of the disk) was measured in triplicate. The sensitivity was classified according to Rota et al.,[133] as follows:

- ≥ 20 mm is strongly inhibitory
- <20-12 mm moderately inhibitory
- < 12 mm is non-inhibitory

An average and standard deviation of the inhibition zone of the three replicates was calculated.

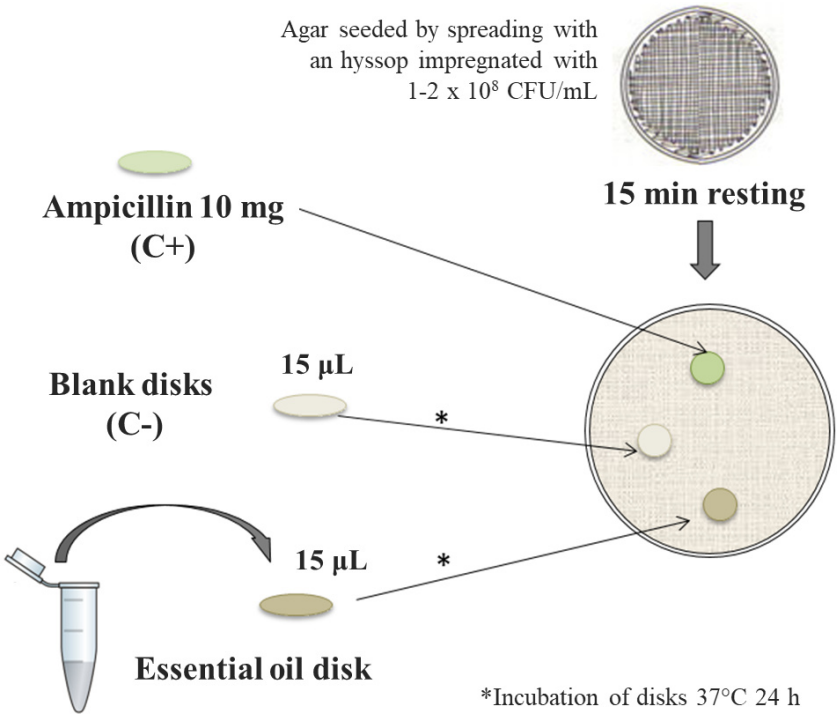


Figure III.20. Methodological scheme of the disk diffusion antimicrobial test.

3.1.3. Determination of the minimum inhibitory concentration and minimum bactericidal concentration

Antimicrobial activity of *Lavandula luisieri* extracts (essential oil, maceration extract, PV and DV fractions) was quantified against the five cited strains determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The MIC was defined as the lowest concentration of extract at which bacteria failed to grow, so no visible changes were detected in the broth medium. The MBC was defined as the concentration of substance at which it is lethal for the 99.9% of the bacterial concentration.

Macrodilution broth method

The essential oil activity was tested using the macrodilution method adapted from the Clinical and Laboratory Standards Institute (CLSI, M07-A10, 2018).[134] The assays were performed in 10 mL of TSB (ethanol 3%) and the tested concentrations were obtained by adding suitable amounts of essential oil to a final working range of 0.5-30 µL/mL. The final bacterial working suspension was adjusted to $5 \cdot 10^5$ - $1 \cdot 10^6$ CFU/mL by dilution from the measured 10^8 CFU/mL overnight culture. Positive controls contained TSB with microorganisms plus 3% ethanol. Negative control contained TSB plus 3%

ethanol and 5 µL/mL of *Satureja montana* essential oil, which biocide activity has been widely studied and proved.[135,136] After 24 h of incubation at 37 ºC in a shaking thermostatic bath (Bunsen, mod. BTG), the MIC was determined as the concentration at which no visible change of turbidity was detected. In order to evaluate MBC, 100 µL of each sample, in which microbial growth was not observed, was spread in TSA. After the incubation of plates at 37 ºC for 24h, MBC was the lowest concentration capable of inhibiting 99.9% of the bacterial growth. The evaluation of MIC and MBC values was carried out in triplicate.

Microdilution broth method

Lavandula luisieri ME, PV and DV fractions were tested against the same bacterial strains with the microdilution broth method. [137] The test was performed in 96-well sterile microplates. All wells received Mueller Hinton Broth (MHB, Merk) supplemented with 10% glucose and 1% phenol red broth (Merck, Madrid, Spain). Extracts working solutions were dissolved in water with DMSO (Sigma Aldrich Química) with a final well concentration of 2.5% (v/v), [138] from which two-fold serial dilutions were prepared. The solutions were sterilized by filtration with a sterile 0,2 µm pore membrane filter (GH Polypropylene membrane ACRODISC 13mm) and added to the first column of wells in the microplate.

Finally, inoculum suspension was added to all wells, being the final bacterial working suspension adjusted to $5 \cdot 10^5$ - $1 \cdot 10^6$ CFU/mL by dilution from the measured 10^8 CFU/mL overnight culture. The growth controls were constituted of medium with extract (negative control) and medium with bacterial inoculum (positive control).

Each microplate was incubated for 24 h at 37 ºC. A change of colour from red to yellow was interpreted as positive growth. For MBC determination, 10 µL from each microwell presenting no visible growth was inoculated on Mueller Hinton Agar (MHA, Merck) plates and incubated at 37 °C for 24 h. Each analysis was performed in triplicate.

Rosmarinic and ursolic acid standards (as triterpene representative) were also tested following the same microdilution procedure. For the results of extracts antimicrobial activity, the following considerations were taken into account: [139]

- significantly active when MIC < 100 µg/mL
- moderately active when 100 < MIC > 625 µg/mL
- weakly active when MIC > 625 µg/mL

3.2. Antioxidant activity

The capacity of *L. luisieri* maceration extract, its supercritical fractions and the formulated carriers to scavenge DPPH (2,2-diphenyl-1-picryl-hydrazil) free radicals was measured. DPPH is a stable free radical which possesses a deep purple colour and a strong absorption around 517 nm. The antioxidant compounds present in the medium convert DPPH radical to a more stable DPPH molecular product by donating an electron or a hydrogen atom. The colour changes from purple to pale yellow when the radical has been reduced. This fact allows the spectrophotometric determination of the antioxidant activity.[140] To determine the scavenging capacity or inhibition of the radicals, *DPPH Inhibition* (%), the following equation was applied (eq. III.8):

$$DPPH\ Inhibition\ (\%) = [(Abs_{control} - Abs_{sample})/(Abs_{control})] \times 100 \qquad \text{(eq. III.8)}$$

where $Abs_{control}$ is the measured absorbance of DPPH solution and Abs_{sample} the measured absorbance after the reaction between the extracts or control *vs* DPPH.

The antioxidant activity was given as IC_{50}, which is defined as the concentration of product (in μg/mL) required to inhibit 50% of DPPH radicals.

3.2.1. L. luisieri extracts and supercritical fractions antioxidant activity

L. luisieri maceration extract and its supercritical fractions, PV and DV, antioxidant activity was determined by an adaptation of Brand-Williams, Cuvelier and Berset (1995)[140] spectrophotometric method. This measurement was performed with a UV-V Multiskan EX mod. 355, Thermo Labsystems microplate spectrophotometer (Zaragoza, Spain). This device emits a beam of light from a suitable UV and/or visible light source, which first passes through a prism or diffraction grating monochromator, then, through the sample to be analysed and, finally, reaches the detector and quantifies the intensity as a function of wavelength.

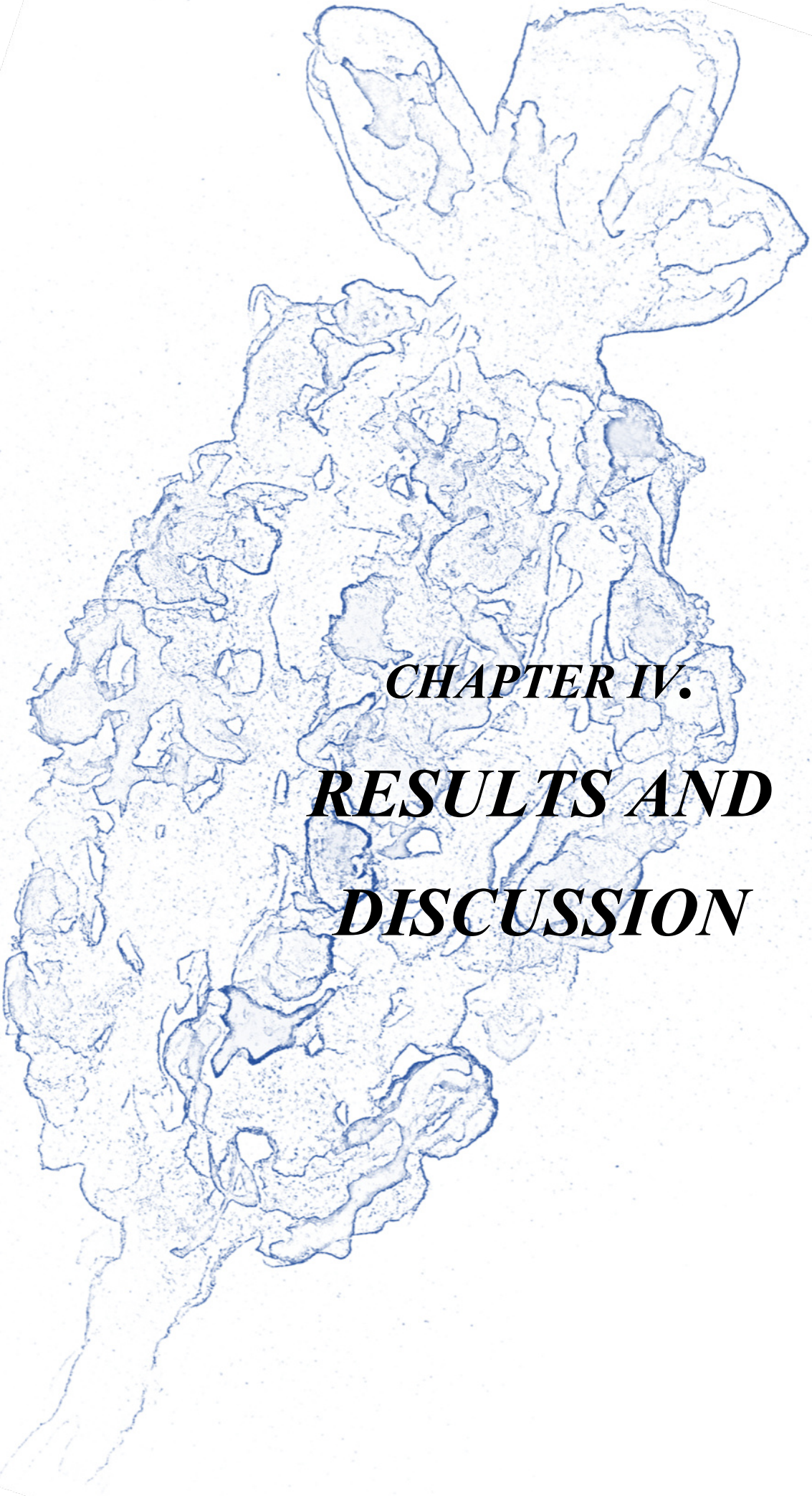
The ethanolic extract solutions were mixed 1:1 (v/v) with a DPPH ethanolic solution of 40 μg/mL. This was also confronted with another two solutions of pure rosmarinic acid and trolox (97% ACROS Organics), as positive control, and with ethanol as negative control. The final well concentration of ME, PV, DV and the positive controls rosmarinic acid and trolox ranged from 0.1-300 μg/mL. The absorbance was measured at 520 nm after 30 min of reaction at room temperature with the microplate photometer. To determine the scavenging capacity or inhibition of the radicals, eq. III.8 was applied.

IC_{50} values were estimated by a nonlinear regression (GraphPad Prism version 4.0). A low IC_{50} value indicates high antioxidant activity. The results are given as the mean ± standard deviation (SD) of experiments done in triplicate.

3.2.2. PLA and PLGA carriers' antioxidant activity

The DPPH radical scavenging activity of the microcapsules was determined with an adaptation of Gülcin et al.[141] method. In this case, firstly the calibration curve was performed for the study of the antioxidant activity of pure β-CA. A volume of 1.5 mL of β-CA solution was added to 1.5 mL of 0.1 mM DPPH, both dissolved in acetone:EtOH (1:7) and then kept in the dark for 18 hours at room temperature. The produced microcapsules were dissolved in 0.5 mL acetone and 3.5 mL of ethanol and after centrifugation at 6500 rpm for 30 min, 3 different concentrations of the supernatant were prepared. Finally, 1.5 mL of these solutions were added to 1.5 mL of 0.1 mM DPPH. The antioxidant activity was measured with a spectrophotometer (mod. Cary 50, Varian, PA, CA, USA) after 18 hours at 517nm.

Taking into account the measured quantity of β-CA encapsulated, the theoretical inhibition percentage was calculated considering no degradation of the active, and a theoretical inhibition curve was built for each SFEE product according to eq. III.8. The theoretical IC_{50} was compared with the IC_{50} obtained experimentally



CHAPTER IV.

RESULTS AND DISCUSSION

1. L. Luisieri concentration of actives and antimicrobial and antioxidant properties

In this section the results obtained from the plant material pre-treatment, extraction and supercritical fractionation are detailed, as well as the evaluation of the extracts antimicrobial and antioxidant properties.

1.1. L. luisieri pre-treatment results

The moisture content of the dried plant material, determined by mass difference in 5 replicates, was 10.2% (± 0.3%) and the values measured are shown in Table IV.1.

Table IV.1. Results of the moisture determination. Plant material initial and final mass and moisture content determined with eq. III.2.

Sample	$m_{initial}$ (mg)	m_{final} (mg)	*moisture* (%)
1	1096	972	**11.3**
2	1104	991	**10.2**
3	968	870	**10.1**
4	1986	909	**10.7**
5	1223	1103	**9.80**

The pulverised plant material was adjusted to a normal distribution and an approximately mean particle diameter of 300 µm to improve the extraction yield. Results of the considered final mass, regarding its diameter, are gathered in Table IV.2 and the histogram representation in Figure IV.1. This pre-treated plant material was kept in hermetically sealed food bags at −20 °C until the extraction.

Table IV.2 Mass retained on each sieve according to particle diameter after the sieving process.

Sieve	Ø (µm)	Mass* (g)
1	74 - 150	20.2247
2	150 - 246	40.1853
3	246 - 400	60.2375
4	400 - 495	39.6542
5	495 - 589	27.2078
6	589 - 710	20.1337
7	710 - 900	10.1502

**Mass ±0.0001*

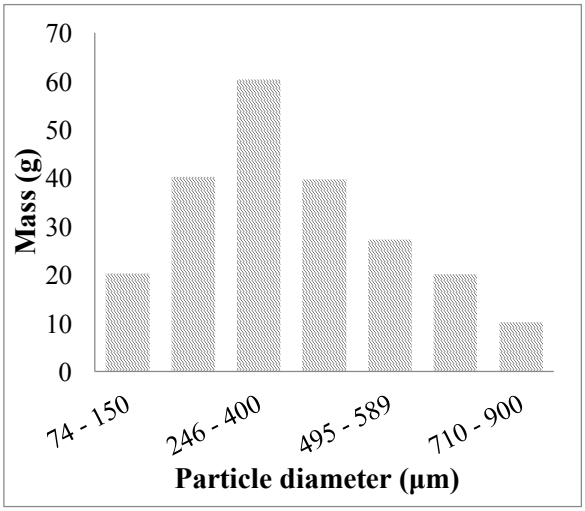


Figure IV.1. Pre-treated *L. luisieri* particle size distribution.

1.2. Maceration yield

Pre-treated plant material was submitted to two serial macerations, as mentioned in Chapter II, firstly with hexane in order to degrease or eliminate the non-polar compounds, such as cuticular wax.[123] The extraction yield of this first maceration, Y_{hex} (%), was 3.3%. The second maceration, in which polar actives were obtained, was performed with ethanol. It is nontoxic, easily biodegradable and it has a higher extractive capacity because it breaks the cell membrane of plant material.[142] The extraction yield obtained, Y_{EtOH} (%), was 12.2%. Results are gathered in the next Table IV.3.

Table IV.3. Yield extraction results after the serial macerations of *Lavandula luisieri.*

Soaking solvent	***Plant mass (g)***	***Extract mass (g)***	***Yield*** [a]
Hexane	74.24	2.45	3.30%
Ethanol	68.14	8.31	12.19%

[a] Mass $_{extract}$ x 100/ Mass $_{plant}$

Regarding this same *Lavandula luisieri* population, other authors[70] obtained similar extraction yield (12.5%), although it was obtained with ethanol in a Soxhlet apparatus without previous degrease treatment. This is a faster method that applies heat to enhance the extraction of actives, but it could also cause the degradation of thermosensitive compounds, besides, in this case, it does not provide better extraction yield.

1.3. SAF experiment design and yields

This ethanolic extract (ME) was dissolved in ethanol at 3% (w/w%), becoming the feed solution (FS) of the SAF process. In the fractionation process, the influence of pressure (XP) and CO_2 flow rate (XQ_{CO_2}) for a maximum yield recovery, both in precipitation and downstream vessel, as well as for a maximum concentration of active compounds (RA, OA and UA) was determined. The range and level of pressure and CO_2 flow rate variables were 80-150 bar and 10-30 g/min, respectively, and are gathered in Table IV.4.

Table IV.4. Codification and levels of the two independent variables for the SAF factorial design of experiments.

Variable	**Symbol**	**Factor levels**				
		{-1.44	−1	0	1	1.44}
Pressure (bar)	XP	80	90	115	140	150
CO_2 flow rate (g/min)	XQ_{CO_2}	10	13	20	27	30

The other experimental parameters were fixed for all experiments: FS flow rate 0.45 mL/min, precipitation vessel temperature 40 ºC, downstream vessel temperature and pressure 25 ºC and 35 bar.

Minitab® 17, propounded 11 random experiments with three replicates of the central point according to the range levels of both variables previously set (Tables IV.4 and IV.5).

Table IV.5. Central Composite Design proposed experiments.

Exp	X_P (bar)	$X_{Q_{CO_2}}$ (g/min)
5	**80**	**20**
1	**90**	**13**
10		**27**
11	**115**	**10**
6		**20**
7		**20**
9		**20**
8		**30**
3	**140**	**13**
2		**27**
4	**150**	**20**

After every experiment, a fine yellow-green powder and a green solution were obtained in the PV and DV fractions respectively. Besides, the PV fraction remaining content was also cleaned with ethanol and quantified after solvent removal with a rotary evaporator. Some pictures of the obtained products are gathered in Figures IV.2 and VI.3.

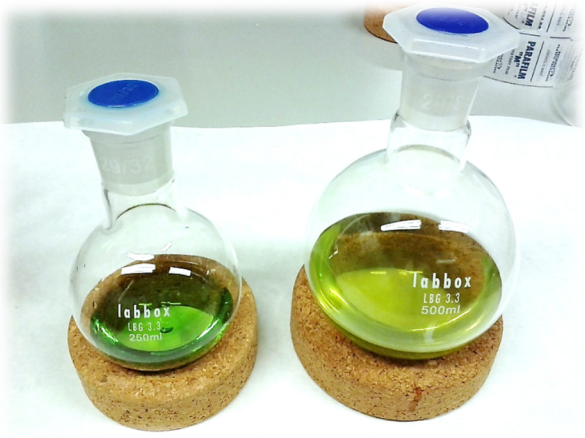


Figure IV.2 DV (left) and PV (right) fractions obtained in experiment 3 (p= 140 bar and Q_{CO_2} = 13 g/min).

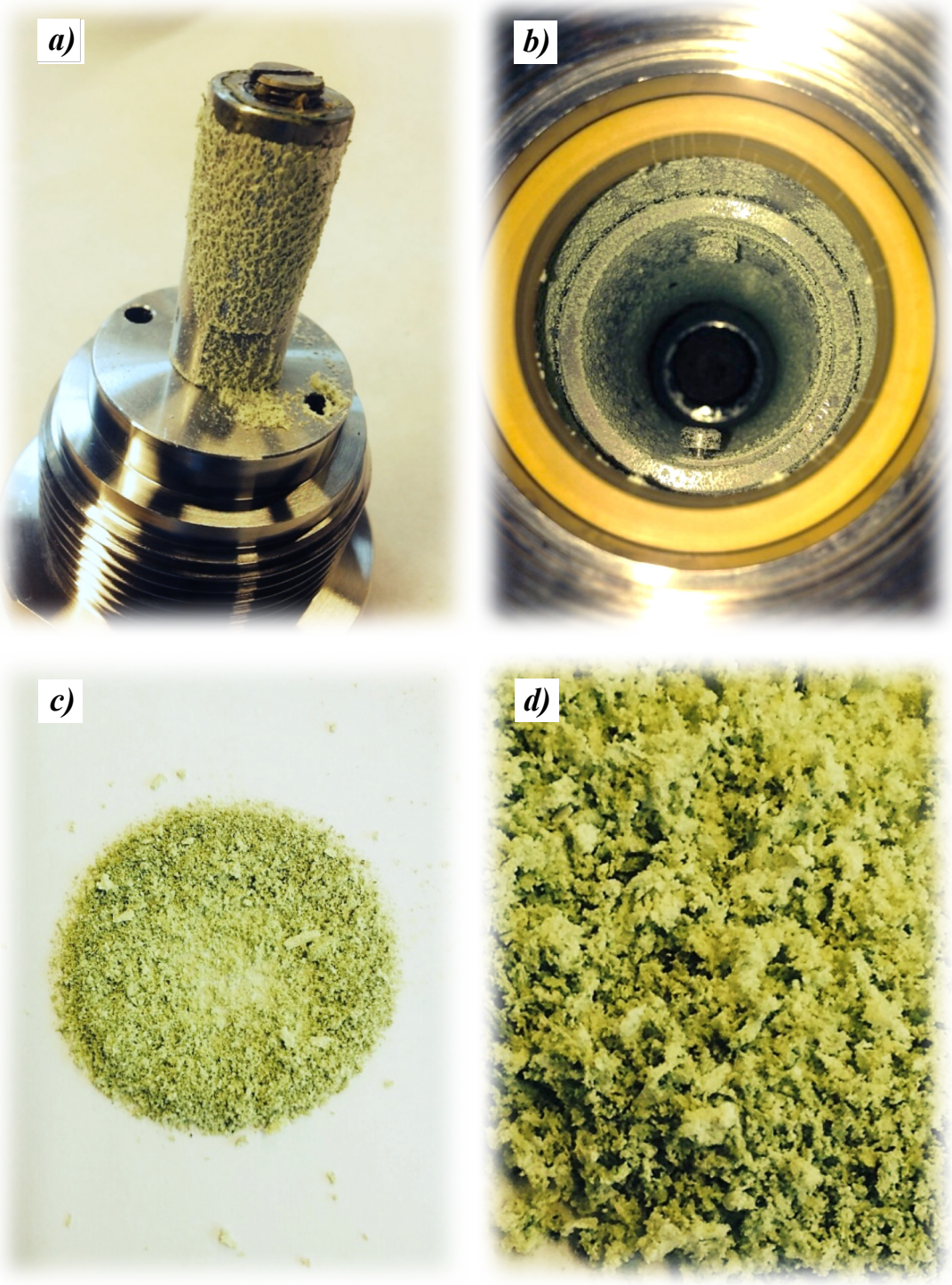


Figure IV.3. Pictures of the solid precipitated in PV fraction after experiment 9 (p = 115 bar and Q_{CO_2} = 20 g/min); a) Precipitation vessel injector; b) Precipitation vessel inside view once opened; c) and d) Powder recovered from PV.

The recovered mass in each fraction was quantified, and the yields Y_{PV} (%), Y_{DV} (%) and the sum of them, Y_{SAF} (%), were determined. These yield results are shown in Table IV.6, where they have been organised in ascending order of XP and XQ_{CO_2} for an easier understanding of data.

Regarding the amount of material recovered in PV and DV, it is noticeable that Y_{PV} (%) is always higher than Y_{DV} (%) independent of the XP and XQ_{CO_2} experimental conditions. The range of Y_{PV} (%) values was 27.7-55.6% (at 115 bar, 10 g/min and at 90 bar, 27 g/min, respectively), while the range of Y_{DV} (%) values was 8.8-30.9% (at 80 bar, 20 g/min and at 115 bar, 30 g/min, respectively). The range of Y_{SAF} (%) values was 46.9-82.6% (at 115 bar, 10 g/min and at 115 bar, 30 g/min, respectively).

Table IV.6. *L. luisieri* experimental SAF results. Experimental yields obtained in each experiment; Y_{SAF} (%), Y_{PV} (%) and Y_{DV} (%).

Exp	XP (bar)	XQ_{CO_2} (g/min)	Y_{PV} (%)	Y_{DV} (%)	Y_{SAF} (%)
5	**80**	**20**	48.0	8.8	56.8
1	**90**	**13**	39.0	15.4	54.4
10		**27**	55.6	20.1	75.7
11		**10**	27.7	19.2	46.9
6	**115**	**20**	38.7 (±3.2)	19.2 (±0.9)	57.9(±3.7)
7		**20**	36.3 (±3.2)	20.3(±0.9)	56.6(±3.7)
2		**20**	42.6 (±3.2)	20.9(±0.9)	63.6(±3.7)
8		**30**	51.7	30.9	82.6
3	**140**	**13**	34.2	22.7	56.9
2		**27**	50.6	29.6	80.2
4	**150**	**20**	33.9	20.3	54.2

As can be observed, for all measured yields, under the same XP the yield increases with XQ_{CO_2}. In the experiments performed at higher $scCO_2$ flow rate, experiments 90 bar-27 g/min, 115 bar- 30 g/min and 140 bar-27 g/min, the highest yields in PV and DV were obtained. According to these results, XQ_{CO_2} seems to have a marked effect in the mass recovery from *L. luisieri* ethanolic extract. There is a reduction in compound solubility in the final mixture EtOH-$scCO_2$, favouring compound precipitation in PV, and dragging compounds to DV, resulting in an increase of total mass recovery. Because of this, the overall mass losses in the SAF equipment are lower when CO_2 flow rate increases, besides, some extract is lost into the pipes, valves and filter of the supercritical plant. Nevertheless, the final mass recovery from an extract depends on the plant material under study. This correlation between $scCO_2$ flow rate and mass recovery was observed by Martín et al. (2011)[124], who fractionated ethanolic extract from *Persea indica*, while Langa et al. (2019)[125] fractionated *Artemisia absinthium* ethanolic extract.

On the other hand, in Table IV. 6, we can also observe that for the same value of ${}^{X}Q_{CO_2}$, 20 g/min, for example, Y_{PV} (%) slightly decreases with increasing pressure, changing from 48.0% at 80 bar to 33.9% at 150 bar. However, the behaviour for Y_{DV} (%) is just the opposite, changing from 8.8% at 80 bar and 20 g/min to 20.3% at 150 bar and the same CO_2 flow rate. This same trend was previously observed by Langa et al.[125] in the supercritical fractionation of ethanolic extract of *Artemisia absinthium L*, who obtained higher yields in the down stream vessel at higher pressures and, therefore, higher density,.

1.4. SEM observations

The microscopic images of the precipitated solid in the PV were obtained for some experiments to observe the produced solid. They mainly showed spherical morphologies but also, in some experiments, polyhedral structures (Figures IV.4 to IV.7). Although a particle mean size and size distribution were not determined, some particles were manually measured with the program smartSEM 05.07 Win 7. As an example, Figure IV.4.D is offered, where particle diameters of 67.91 and 69.78 nm can be observed. The morphology and the size are highly influenced by the droplet formed by the injector and the liquid surface tension.[143] At the precipitation process, the $scCO_2$ diffuses and eliminates very quickly the ethanol that surrounds the extract, forcing the solid to conserve its original shape and volume, which explains the small particle size diameter obtained.

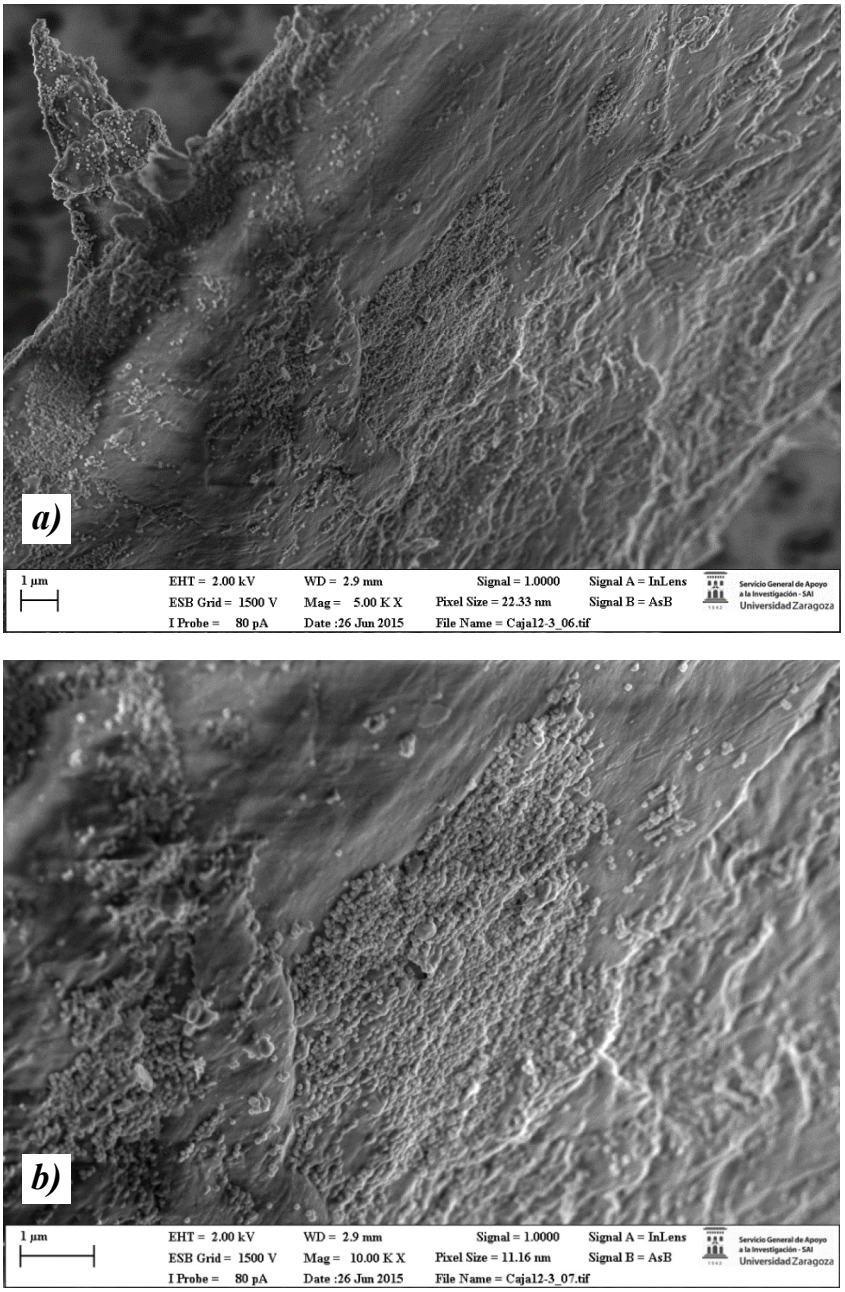


Figure IV.4. SEM Picture of the powder obtained in the precipitation vessel of experiment 10 (p = 90 bar and Q_{CO_2} = 27 g/min). a) 5.00 K X, b) 10.00 K X, c) 20.00 K X, and d) 40.00 K X particle diameters measured 82.66 nm and 71.16 nm.

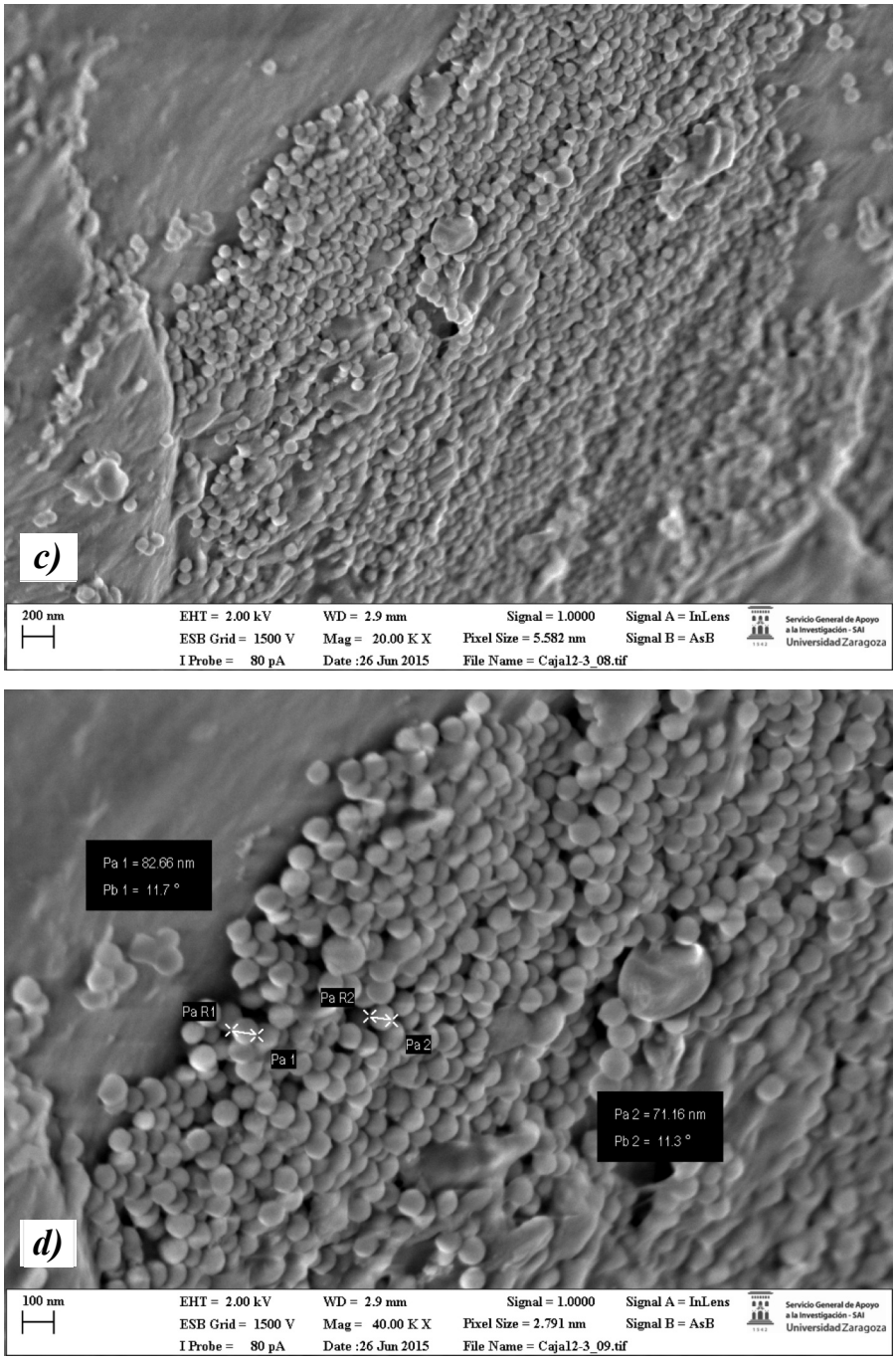


Figure IV.4 (continued). Image from the powder obtained in the precipitation vessel of experiment 10 (p = 90 bar and Q_{CO_2} = 27 g/min). a) 5.00 K X, b) 10.00 K X, c) 20.00 K X, and d) 40.00 K X particle diameters measured 82.66 nm and 71.16 nm

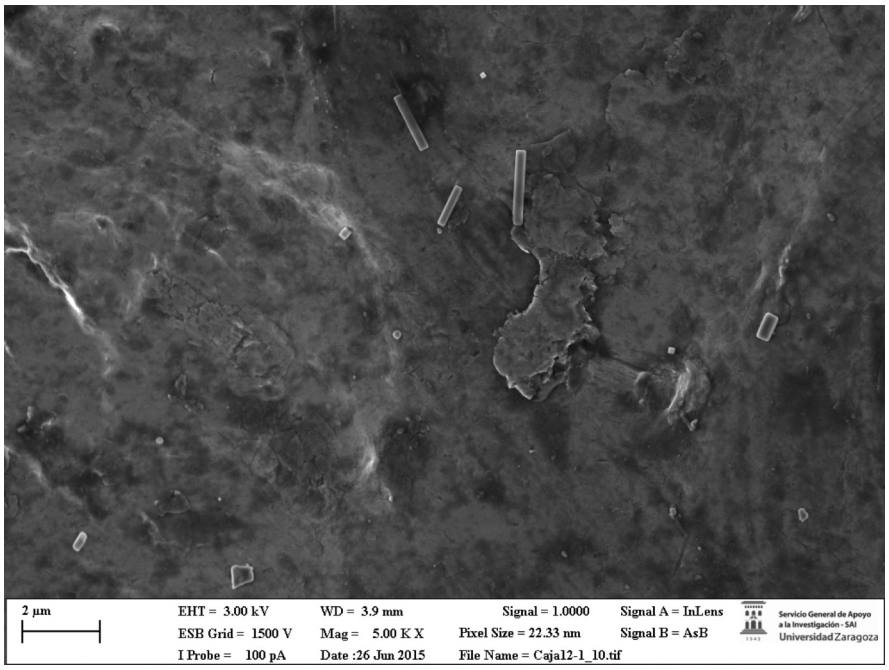


Figure IV.5. SEM Picture of the powder obtained in the precipitation vessel of experiment 10 (p = 90 bar and Q_{CO_2} = 27 g/min) (5.00 K X). Non-spherical, cuboid shape.

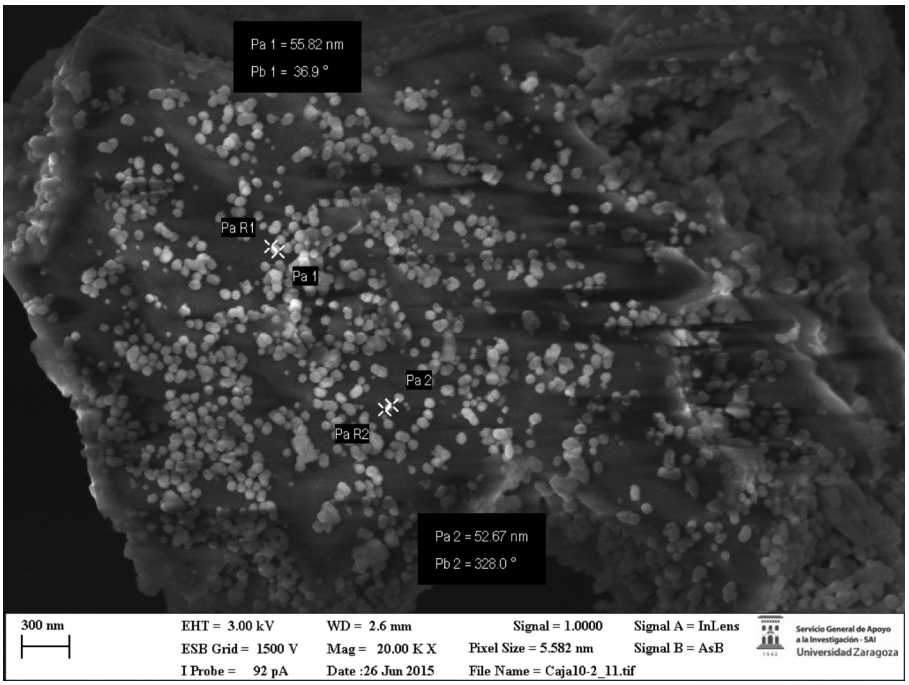


Figure IV.6. SEM Picture of the powder obtained in the precipitation vessel of experiment 11 (p = 115 bar and Q_{CO_2} = 10 g/min) (20.00 K X). Particle diameter measured 52.67 nm.

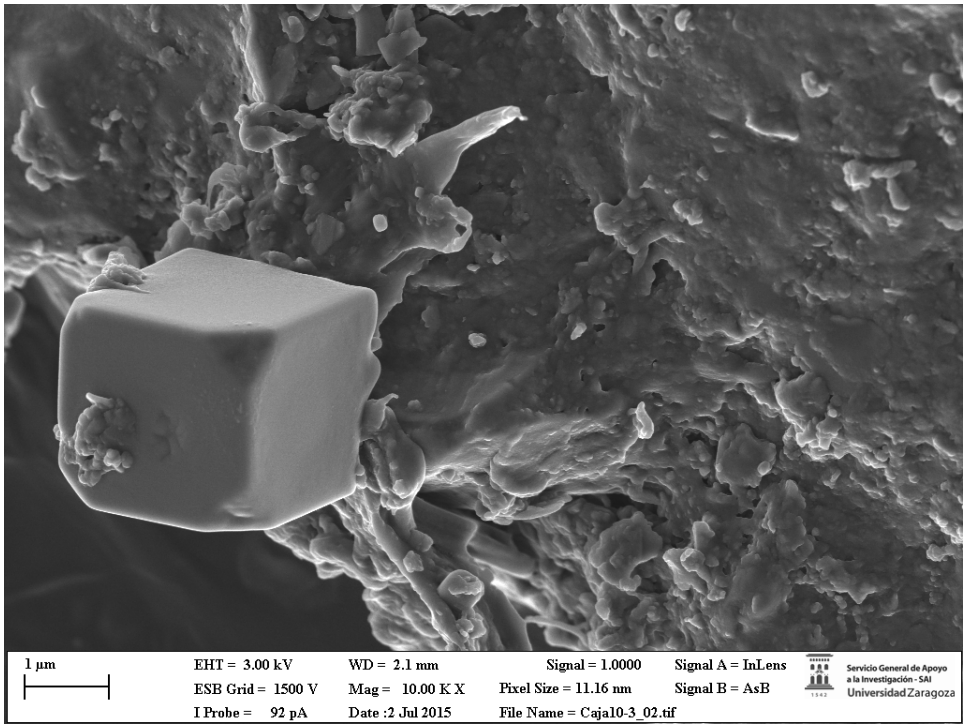


Figure IV.7. SEM Picture of the powder obtained in the precipitation vessel of experiment 11 (p = 115 bar and Q_{CO2} = 10 g/min) (10.00 K X). Polyhedral structure.

1.5. Rosmarinic, oleanolic and ursolic acids fractionation

HPLC-PDA was used to track and quantify the compounds RA, OA and UA from *L. luisieri* ethanolic extract FS and its supercritical fractions PV and DV of each experiment in triplicate (total chromatographic assays, n = 99). They were identified by retention time (min) and accordance of PDA spectrum (λ) to the standard of the cited pure compounds. The retention times for RA, OA and UA were 1.6 min, 7.5 min and 7.8 min, respectively, as can be observed in Figure IV.8. RA was measured at 330 nm, and OA and UA were measured at 210 nm for a better peak definition. For their quantification in the *L. luisieri* samples, a calibration curve for each compound was built and their equations are:

$$[\mathrm{RA}](\mu g/mL) = 1.714 \cdot 10^{-5} \cdot Area\ (\mu V * s) - 0.203 \quad \text{(eq. IV.1)}$$
$R^2 = 0.999$, s = 0.070, LOD = 0.18, LOQ= 0.59

$$[\mathrm{OA}](\mu g/mL) = 1.178 \cdot 10^{-4} \cdot \mathrm{Area}\ (\mu V*s) - 0.065 \quad \text{(eq. IV.2)}$$
$R^2 = 0.999$, s = 0.262, LOD= 0.65, LOQ= 2.17

$$[\mathrm{UA}](\mu g/mL) = 9.337 \cdot 10^{-5} \cdot \mathrm{Area}\ (\mu V*s) - 0.029 \quad \text{(eq. IV.3)}$$
$R^2 = 0.999$, s = 0.157, LOD=0.41, LOQ=1.35

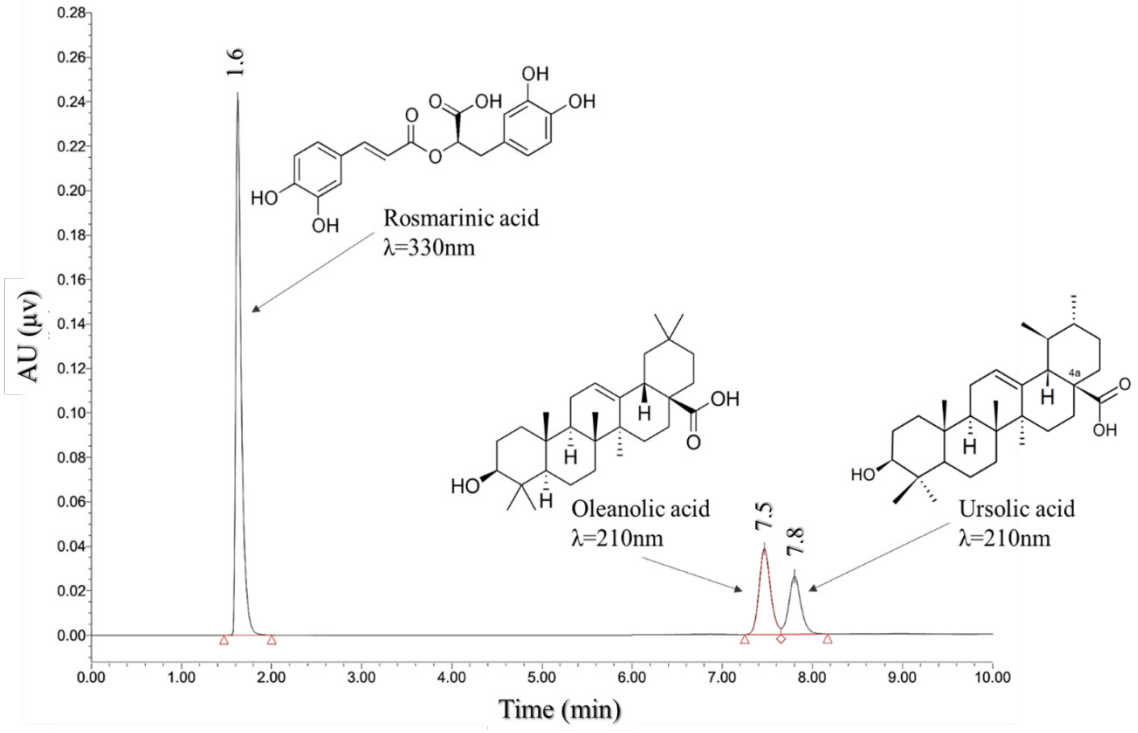


Figure IV.8. Chromatogram obtained after an injection of the 3 standards used. 1st peak RA (1.6 min), 2nd peak OA (7.5 min), and 3rd peak UA (7.8 min).

The chemical characterization of the *L. luisieri* ethanolic maceration dry extract provided values of 5.25% ± 1.2, 2.41% ± 0.7 and 5.12% ± 1.2 of RA, OA and UA, respectively.

In order to observe the behaviour of each compound during the fractionation process, its enrichment was determined in each fraction regarding the initial feed solution. Three overlaid chromatograms of *L. luisieri* and its supercritical fractions are represented in Figure IV.9.

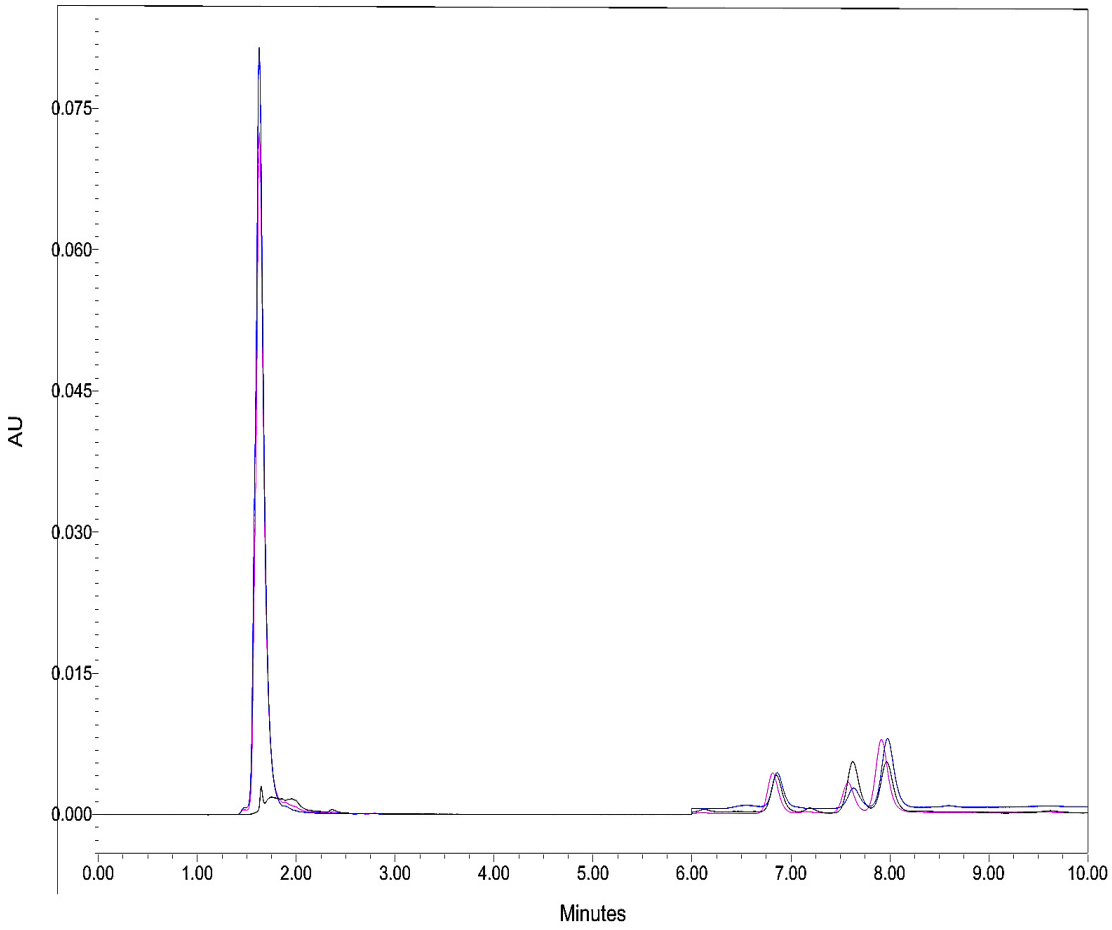


Figure IV.9. Overlaid chromatograms of PV (blue), ME (red) and DV (black) of experiment 2 (p = 140 bar and Q_{CO_2} = 13 g/min)

The three actives proportion in each fraction, X_j (%), was calculated according to eq. IV. 4.

$$Xj\ (\%) = \frac{mg\ of\ active\ X\ in\ fraction\ j}{mg\ of\ fraction\ j} x\ 100 \qquad \text{(eq. IV.4)}$$

where *X* is the compound of interest, RA, OA or UA, and *j* is the place where *X* was obtained and quantified from, that it, FS, PV or DV. Proportions for each compound are gather in Tables IV.7, IV.8 and IV.9.

For the enrichment of each component in PV and DV regarding FS, the Enrichment ratio ($E_{i\ j/FS}$) was defined according to eq. IV.5).

$$E_{i\frac{j}{FS}} = \frac{X_i\ in\ j}{X_i in\ FS} \qquad \text{(eq. IV.5)}$$

where *i* is the tracked compound, RA, OA or UA, *j* is the place where i was collected from, PV or DV, and *FS* is the initial feed solution. According to this, the

enrichment ratio for RA, OA and UA, regarding the initial feed solution, will be named $E_{RA\ PV/FS}$, $E_{OA\ PV/FS}$, $E_{UA\ PV/FS}$ in PV fraction and $E_{RA\ DV/FS}$, $E_{OA\ DV/FS}$, $E_{UA\ DV/FS}$ in DV fraction, respectively.

Nevertheless, since the concentration of the three actives was of interest, the behaviour of the three compounds together, RA, OA and UA was also studied and the parameter Total Compound Enrichment in PV fraction, $E_{TC\ PV/FS}$, was defined and calculated as follows:

$$E_{TC\ PV/FS} = \frac{\mathrm{RA(mg)}_{PV}+\mathrm{OA(mg)}_{PV}+\mathrm{UA(mg)}_{PV}\ /\mathrm{total\ fraction(mg)}_{PV}}{\mathrm{RA(mg)}_{FS}+\mathrm{OA(mg)}_{FS}+\mathrm{UA(mg)}_{FS}\ /\mathrm{total\ fraction(mg)}_{FS}} \qquad \text{(eq. IV.6)}$$

When any of these ratios is>1, an enrichment in a specific compound is assumed. Enrichment ratios for each compound are shown in Tables IV.7, IV.8 and IV.9 for rosmarinic, oleanolic and ursolic acids respectively.

Results of the chromatographic analysis of RA are gathered in Table IV.7. The quantification of this compound revealed that it is completely retained in PV, regardless the CO_2 pressure and flow rate conditions. Its enrichment in PV fraction, $E_{RA\ PV/FS}$, was always ≥1 (1.05-2.26) The fraction in PV was a solid extract with higher proportion of this antioxidant than in the original ethanolic extract. The highest enrichment of rosmarinic acid, $E_{RA\ PV/FS}$, was 2.26, which corresponds to 9.92% of RA_{PV}, at 140 bar and 27 g/min. According to these results, it can be said that RA, the most polar compound studied in this work, is insoluble in the scCO_2-EtOH mixture. Previous studies applying this technology to rosemary extract obtained also an RA enrichment in the PV. These authors concentrated RA 2.7 times from an initial 25.1 mg/g of rosemary extract up to 67.7 mg/g of SAF solid at 300 bar, having 20% of water in the initial solution.[144]

Table IV.7. Rosmarinic acid fractionation results for each experiment; its proportion in the initial extract, RA_{FS} *(%),* its proportion in the fraction PV, RA_{PV} *(%)*, and its enrichment ratio in PV $E_{RA\ PV/FS}$.

Exp	XP (bar)	XQ_{CO_2} (g/min)	RA_{FS}%	RA_{PV}%	$E_{RA\ PV/FS}$
5	80	20	5.07% (±0.06)	8.92% (±0.06)	1.76 (±0.07)
1	90	13	3.99% (±0.06)	7.88% (±0.06)	1.97 (±0.09)
10		27	6.41%(±0.06)	6.70% (±0.06)	1.05 (±0.04)
11	115	10	5.66%(±0.06)	8.04% (±0.06)	1.42 (±0.05)
6		20	4.04%(±0.06)	8.90% (±0.06)	2.20 (±0.10)
7			3.80%(±0.06)	7.33% (±0.06)	1.93 (±0.09)
2			6.30%(±0.05)	8.10% (±0.06)	1.29 (±0.04)
8		30	6.34%(±0.04)	8.07% (±0.06)	1.27 (±0.03)
3	140	13	7.04%(±0.07)	7.50% (±0.06)	1.06 (±0.04)
2		27	4.38%(±0.05)	9.92% (±0.06)	2.26 (±0.08)
4	150	20	4.57% (±0.06)	6.85% (±0.06)	1.50 (±0.07)

On the other hand, OA and UA were partially soluble in the mixture of solvents because they distributed between PV and DV, Tables IV.8 and IV.9. Furthermore, this separation between both fractions seems to be influenced by XP and XQ_{CO_2}, since their proportions (Xj (%)) and enrichment ratios ($E_{i\ j/FS}$) varied in every SAF experiment. $E_{OA\ PV/FS}$ values ranged from 0.5 to 2.3, while $E_{OA\ DV/FS}$ did it from 0 to 3.6. Although proportions in both vessels varied depending on the experimental conditions, OA was not soluble at 150 bar 20g/min in the mixture EtOH-scCO_2 leading to its complete absence in DV in this experiment, while UA was always present in both vessels (Table IV.9). $E_{UA\ PV/FS}$ varied from 0.9 to 2.7, which corresponds to 5.9-10.4% of the PV fraction, while $E_{UA\ DV/FS}$ was mainly >1 (0.9-2.7). Despite their similar chemical structure and same molecular weight, UA separates from OA under some of the experimental conditions. For example, at 115 bar 10 g/min UA seems to be retained in PV while OA is dragged to DV (Tables IV.8 and IV.9).

Table IV.8. Oleanolic acid fractionation results for each experiment; its proportion in the initial FS extract and in both supercritical fractions PV and DV, *OA_{FS} (%), OA_{PV} (%) and OA_{DV} (%)*, and its enrichment ratio in both fractions, *$EO_{OA\ PV/FS}$* and *$EO_{OA\ DV/FS}$*.

Exp	X_P (bar)	$X_{Q_{CO_2}}$ (g/min)	OA_{FS} %	OA_{PV} %	OA_{DV} %	$E_{OA\ PV/FS}$	$E_{OA\ DV/FS}$
5	80	20	1.9% (±0.2)	1.8% (±0.2)	5.5% (±0.2)	0.9 (±0.1)	2.9 (±0.1)
1	90	13	1.5% (±0.1)	3.3% (±0.2)	2.4% (±0.3)	2.3 (±0.5)	1.6 (±0.2)
10	90	27	2.4% (±0.2)	2.4% (±0.2)	1.2% (±0.2)	1.0 (±0.2)	0.5 (±0.1)
11	115	10	2.3% (±0.2)	1.9% (±0.2)	3.5% (±0.2)	0.8 (±0.2)	1.5 (±0.1)
6	115	20	1.5% (±0.2)	2.1% (±0.2)	5.0% (±0.2)	1.3 (±0.3)	3.2 (±0.1)
7	115	20	2.1% (±0.2)	2.4% (±0.2)	3.5% (±0.2)	1.1 (±0.2)	1.6 (±0.1)
2	115	20	3.7% (±0.2)	2.4% (±0.2)	3.2% (±0.2)	0.6 (±0.1)	0.9 (±0.1)
8	115	30	2.6% (±0.1)	3.0% (±0.2)	0.6% (±0.2)	1.2 (±0.2)	0.2 (±0.1)
3	140	13	2.7% (±0.3)	1.5% (±0.2)	4.6% (±0.2)	0.5 (±0.1)	1.7 (±0.1)
2	140	27	1.7% (±0.2)	2.0% (±0.2)	5.9% (±0.2)	1.2 (±0.3)	3.6 (±0.1)
4	150	20	1.7% (±0.2)	3.4% (±0.2)	0.0% (±0.2)	2.0 (±0.4)	0.0 (±0.1)

Table IV.9. Ursolic acid fractionation results for each experiment; its proportion in the initial FS extract and in both supercritical fractions PV and DV, UA_{FS} *(%),* UA_{PV} *(%),* UA_{DV}*(%),* and its enrichment ratio in both fractions, $EO_{UA\ PV/FS}$ and $EO_{UA\ DV/FS}$

Exp	XP (bar)	XQ_{CO_2} (g/min)	UA_{FS} %	UA_{PV} %	UA_{DV} %	$E_{UA\ PV/FS}$	$E_{UA\ DV/FS}$
5	80	20	4.6% (±0.1)	7.0% (±0.1)	4.6% (±0.3)	1.5 (±0.1)	1.0 (±0.1)
1	90	13	3.8% (±0.1)	10.4% (±0.1)	1.8% (±0.3)	2.7 (±0.1)	0.5 (±0.2)
10	90	27	5.6% (±0.1)	5.6% (±0.1)	1.0% (±0.2)	1.0 (±0.1)	0.2 (±0.1)
11	115	10	5.2% (±0.1)	7.1% (±0.1)	4.7% (±0.2)	1.4 (±0.1)	0.9 (±0.1)
6	115	20	3.9% (±0.1)	6.5% (±0.1)	3.9% (±0.2)	1.7 (±0.1)	1.0 (±0.1)
7	115	20	5.0% (±0.1)	8.2% (±0.1)	2.7% (±0.1)	1.6 (±0.1)	0.5 (±0.1)
2	115	20	7.7% (±0.1)	7.0% (±0.1)	2.3% (±0.2)	0.91 (±0.03)	0.3 (±0.1)
8	115	30	5.4% (±0.1)	8.8% (±0.1)	0.6% (±0.1)	1.61 (±0.05)	0.1 (±0.1)
3	140	13	6.4% (±0.2)	5.9% (±0.1)	4.2% (±0.2)	0.92 (±0.05)	0.7 (±0.1)
2	140	27	4.1% (±0.1)	10.3% (±0.1)	5.2% (±0.2)	2.5 (±0.1)	1.3 (±0.1)
4	150	20	4.3% (±0.1)	7.8% (±0.1)	0.2% (±0.2)	1.8 (±0.1)	0.1 (±0.1)

Although the analysis of the behaviour for each compound, the behaviour of the three compounds precipitated at the same time in the PV fraction was studied. The $E_{TC\ PV/FS}$ ratio of each experiment and the final mass yield are gathered in table IV.10. The three compounds concentrated in PV regarding the initial ethanolic solution at all experimental conditions. The highest enrichment of the three compounds together was obtained at 140 bar 27 g/min which corresponds with a high total mass recovery (Y_{SAF} (%) = 80.2%).

Table IV.10. PV, DV and SAF yields and RA OA UA total enrichment ($E_{TC\ PV/FS}$) in PV regarding FS.

Exp	XP (bar)	XQ_{CO_2} (g/min)	Y_{PV}%[1]	Y_{DV}%	Y_{SAF}%	$E_{TC\ PV/FS}$
5	80	20	48.0	8.8	56.8	1.4(±0.1)
1	90	13	39.0	15.4	54.4	1.7(±0.1)
10	90	27	55.6	20.1	75.7	1.2(±0.1)
11	115	10	27.7	19.2	46.9	1.4(±0.1)
6	115	20	38.7 (±3.2)	19.2 (±0.9)	57.9(±3.7)	1.4(±0.1)
7	115	20	36.3 (±3.2)	20.3(±0.9)	56.6(±3.7)	1.4(±0.1)
2	115	20	42.6 (±3.2)	20.9(±0.9)	63.6(±3.7)	1.4(±0.1)
8	115	30	51.7	30.9	82.6	1.6(±0.1)
3	140	13	34.2	22.7	56.9	1.2(±0.1)
2	140	27	50.6	29.6	80.2	1.8(±0.1)
4	150	20	33.9	20.3	54.2	1.4(±0.1)

1.6. Statistical analysis of results and optimization

In order to determine the statistical influence of XP and XQ_{CO_2} variables, a surface response analysis of all these results was performed with Minitab 17. Y_{PV} (%), Y_{DV} (%), Y_{SAF} (%) and $E_{TC\ PV/FS}$ were taken as response variables and used to determine the coefficients of the equation terms (eq. III.6). Yields study was considered for a maximum mass recovery and the total compound enrichment to determine the optimum conditions to obtain a product with the three actives concentrated and therefore, increased simultaneous potential bioactivities, for several future applications. The level of significance of each equation factor (linear, quadratic and interaction), the final coefficient of determination (R^2) and the standard deviation (s) were obtained.

The equations IV.7, eq. IV.8 and eq. IV.9 define the response surface of the experimental yields, Y_{PV} (%), Y_{DV} (%), Y_{SAF} (%) and $E_{TC\ PV/FS}$ respectively, as a function of pressure and CO_2 flow rate.

$$Y_{PV}(\%) = 67.6 - 0.737XP + 1.196XQ_{CO_2} + 0.00256XP^2 \quad \text{(eq. IV.7)}$$
$(R^2 = 88.93\%, s = 3.5)$

$$Y_{DV}(\%) = -32.9 + 1.003XP - 1.920XQ_{CO_2} - 0.00364XP^2 + 0.0606XQ_{CO_2}{}^2 \quad \text{(eq. IV.8)}$$
$(R^2 = 88.59\%, s = 2.7)$

$$Y_{SAF}(\%) = 59.4 + 0.0164XP - 1.92XQ_{CO_2} + 0.0905XQ_{CO_2}{}^2$$
$(R^2 = 90.17\%, s = 4.4)$ (eq.IV.9)

Equations eq. IV.7, eq. IV.8 and eq. IV.9 are graphically represented in Fig IV.10. Total recovery of plant material (Y_{SAF} (%)) and its distribution between precipitation and downstream vessels (Y_{PV} (%) and Y_{DV} (%)) are represented as a function of XP and XQ_{CO_2}. The experimental conditions influenced the SAF processing of *L. luisieri* extract, since changes in yield recovery varied among the different experiments.

According to the statistical analysis, the mass recovered in the PV is influenced by both linear factors and only quadratic XP, eq.IV.7. The graphical representation of this equation in Figure IV.10A predicts a higher Y_{PV} (%) at the lowest values of XP and the highest XQ_{CO_2} of the studied range. In Figure IV.10.B, a maximum recovery in DV at the highest values of XQ_{CO_2} can be observed as well; however, in this case higher values of XP are also required. Y_{DV} (%) depends on both linear and quadratic XP and XQ_{CO_2} equation terms (eq. IV.8). The solubility of compounds extracted with ethanol from *L. luisieri* seems to increase with pressure, causing their passage through the PV filter towards the DV fraction. Finally, as Y_{SAF} (%) is the addition of both PV and DV yields, the influence of XP and XQ_{CO_2} is a combination of their effect in both fractions separately. In this case, Y_{SAF} (%) depends on both linear factors and the quadratic XQ_{CO_2} term (eq. IV.9). In the graphical representation of this equation, Figure IV.10.C, the total yield increases with the XQ_{CO_2} for a fixed pressure.

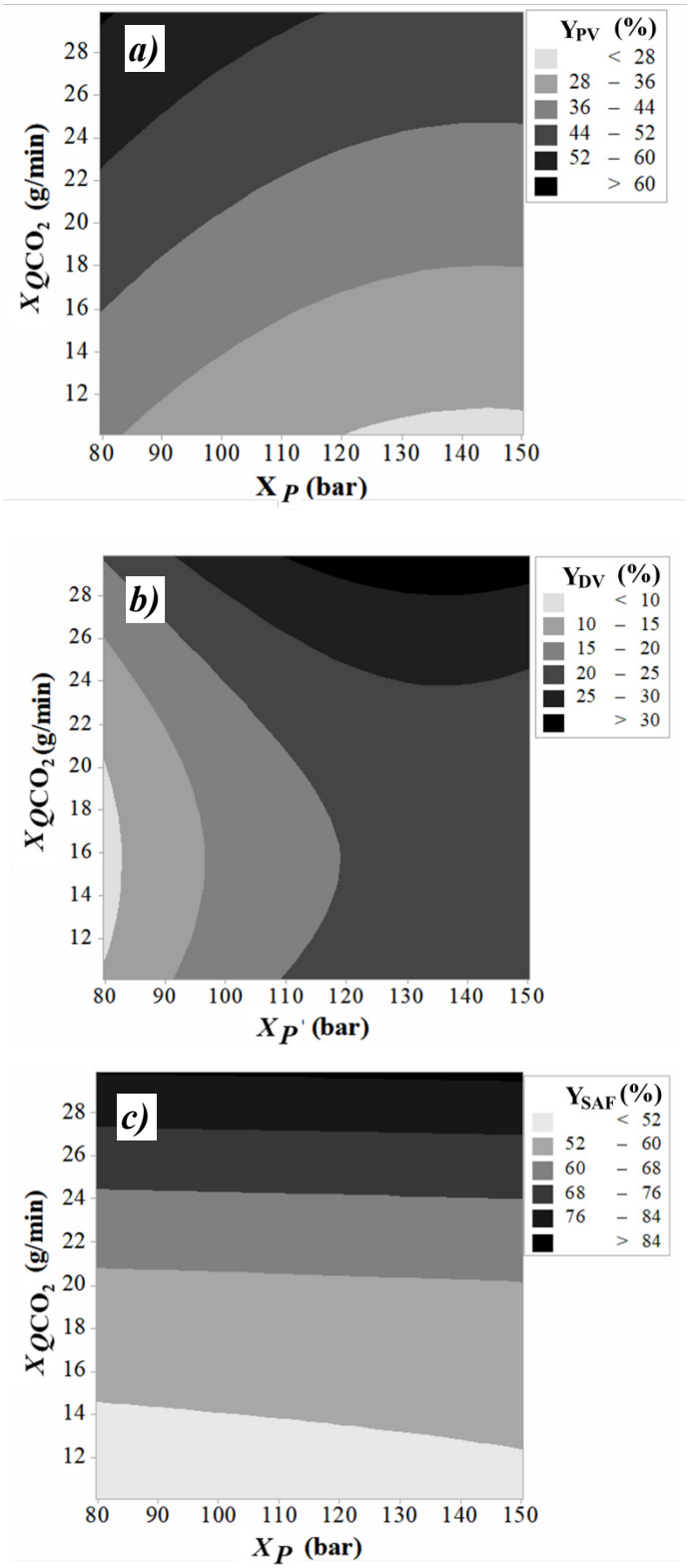


Figure IV.10. Response surface plots of the SAF fraction yields: a) Y_{PV} (%); b) Y_{DV} (%); c) Y_{SAF} (%) as a function of XP and XQ_{CO_2}

The influence of the experimental variables XP and XQ_{CO_2} on the simultaneous fractionation or enrichment of the identified compounds rosmarinic, oleanolic and ursolic acids was analysed too. According to the results, the behaviour of RA, OA and UA and their concentration in PV or DV vessels regarding the initial FS studied individually did not adjust to the CCD model applied in this work. Nevertheless, the parameter $E_{TC\ PV/FS}$ could be fitted to the proposed model, and its variation with experimental variables XP and XQ_{CO_2}could be studied. The dependence of RA, OA and UA simultaneous co-precipitation to XP and XQ_{CO_2} is defined in eq.IV.10, and graphically represented in Figure IV.11. No data of their concentration in DV is given since RA was not dragged to this fraction; besides, OA and UA concentration in DV did not adjust to the proposed model.

$$E_{TC\ PV/FS} = 5.257 - 0.03213XP - 0.2064XQ_{CO_2} + 0.000649XQ_{CO_2}^{\ 2} + 0.001625XP{\cdot}XQ_{CO_2} \quad \text{(eq.IV.10)}$$

$(R^2 = 96.24\%,\ s = 0.05)$

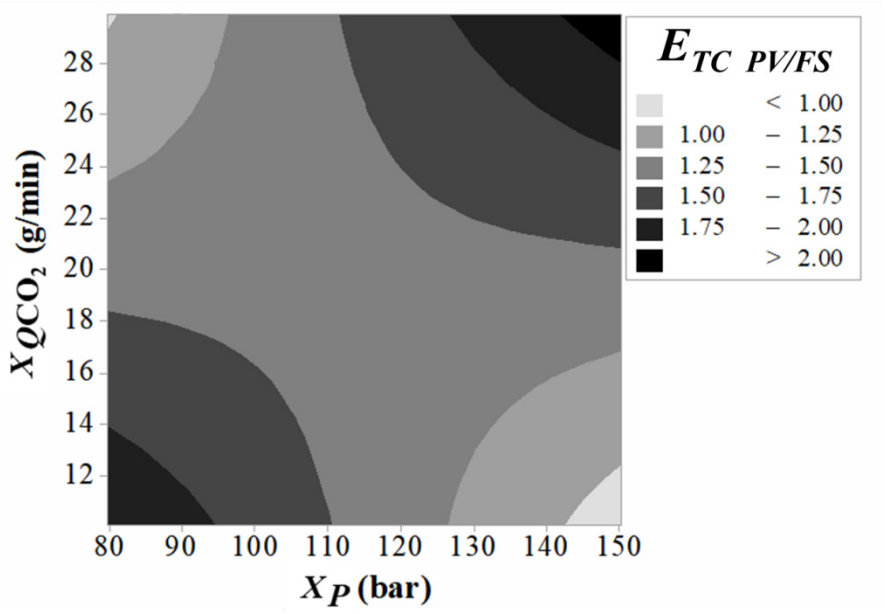


Figure IV.11. Response surface plots of $E_{TC\ PV/FS}$ as a function of XP and XQ_{CO_2}

According to this statistical prediction equation, the highest concentration of RA, OA and UA simultaneously in PV can be obtained at low XP and XQ_{CO_2} (XP < 90 bar and XQ_{CO_2}< 12 g/min) or at high XP and XQ_{CO_2} (XP > 140 bar and XQ_{CO_2} > 27 g/min) which corresponds with the results observed in table IV.10. Under these experimental conditions, the actives solubility in the $scCO_2$-EtOH mixture decreases. Their

concomitant precipitation is interesting since RA, OA and UA have been reported to present different biological activities, such as anti-inflammatory, antioxidant, antiviral, antidiabetic, antitumor, hepatoprotective and cardioprotective.[145,146] Due to the simultaneous presence of OA and UA in many medicinal plant species, some studies have reported their positive effects when applied together. They have shown chemo-protective effects against DNA damage through oxidation,[147,148] and in vitro and in vivo antiproliferatives.[149]

Compared to previous works, the SAF technique can be applied to obtain a dried solid product highly enriched in RA, OA and UA.[145] It could also be applied after the traditional extraction techniques. According to the final optimisation analysis performed in this work, the theoretical conditions for a maximum extract recovery in the SAF process (Y_{SAF} (%), Y_{PV} (%) and Y_{DV} (%) maximum) and a higher concentration of the three studied compounds in PV ($E_{TC\ PV/FS}$) would be 130 bar and 30 g/min (composite desirability 0.95), as represented in Figure IV.12.

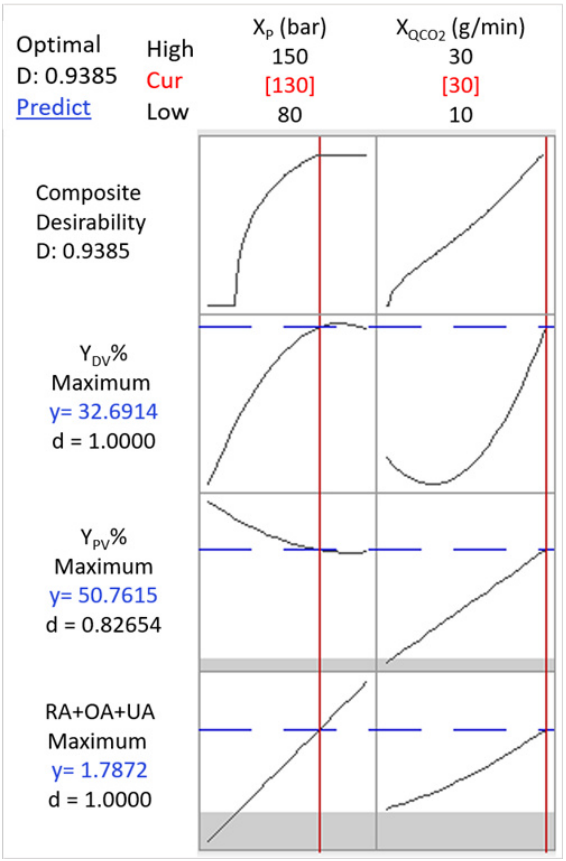


Figure IV.12. Optimal combined conditions for a maximum mass recovery in PV, DV and SAF and maximum $E_{TC\ PV/FS}$

The SAF was then performed at 130 bar and 30 g/min, being Y_{PV} (%) and Y_{DV} (%), 50.9% (w/w) and a 32.9% (w/w), respectively. The proportion of each active in each fraction was determined by HPLC-PDA as previously described and the results are gathered in table IV.11. In this optimization experiment, $E_{TC\ PV/FS}$ equalled 1.84, which was higher than any other value of total compound concentration obtained so far in the 11 designed experiments (Table IV.10).

Table IV.11. Rosmarinic, oleanolic and ursolic acids yield (%) quantified in each fraction at the optimum SAF experimental conditions.

	RA %	**OA %**	**UA %**	**RA+OA+UA %**
ME	5.1 ± 0.06	2.9 ± 0.2	5.2 ± 0.1	13.2 ± 0.4
PV	9.4 ± 0.05	2.6 ± 0.2	11.9 ± 0.1	23.9 ± 0.3
DV	-	4.9 ± 0.2	5.6 ± 0.1	10.5± 0.4
$E_{i\ PV/FS}$	**1.84**	**0.89**	**2.29**	**1.81**

1.7 L. luisieri extracts activities

The antimicrobial and antioxidant activities of the ethanolic maceration extract were tested and compared to the supercritical fractions PV and DV obtained at the optimised SAF conditions (130 bar and 30 g/min) in order to determine which fraction shows better antimicrobial and/or antioxidant properties. The essential oil was also tested for these two activities.

1.7.1. L. luisieri extracts antimicrobial activity

The antimicrobial tests of the different extracts were performed to determine the capacity to inhibit and kill the selected bacterial strains: *Listeria monocytogenes, Enterococcus faecium, Staphylococcus aureus, Salmonella* Typhimurium and *Escherichia coli.* For the essential oil, a previous test with the disk diffusion method was performed, then the minimum inhibitory concentration, MIC, and the minimum bactericidal concentration, MBC, were determined for all extracts as described in chapter III.

1.7.1.1 Essential oil

Essential oil antimicrobial activity was first tested using the paper disk Kirby-Bauer sensitivity agar diffusion technique.[150] The impregnated disks with the essential oil

were placed on agar plates seeded with the working strains and then incubated at 37 ºC for 24 hours.

Results from Table IV.12 show the bacteria sensitivity, according to the previous classification described in chapter III, 3.1.2. The most resistant bacteria were *E. faecium* (10.5 ± 0.3 mm) and *E. coli* (11.5 ± 0.2 mm), for which no inhibition by the *L. luisieri* essential oil was observed. On the other and, the most sensitive bacteria was *L. monocytogenes* (43 ± 3 mm), then *S. aureus* (36 ± 3 mm) and finally *S.* Typhimurium (21.4 ± 0.4 mm). In these cases, the essential oil had a strong inhibition activity.

Table IV.12. *Lavandula luisieri* essential oil antimicrobial results against bacterial strains. Inhibition zone (mm) and minimum inhibitory and bactericidal concentrations (µL/mL).

Strain	Inhibition zone (mm)*	MIC (µL/mL)	MBC (µL/mL)
L. monocytogenes	43.3 (++)*	0.5	3.0
E. faecium	10.5 (-)*	0.5	1.0
S. aureus	36.2 (++)*	0.5	0.5
S. Typhimurium	21.4 (++)*	5.0	5.0
E. coli	11.5 (-)*	30.0	-

*(-) <12 mm non inhibitory, (+) 12-20 mm moderately inhibitory (++) >20 mm strongly inhibitory Rota et al.[128]

With the agar diffusion technique, qualitative information of *L. luisieri* essential oil antimicrobial activity was obtained. Nevertheless, for quantitative results, the macrodilution broth method was applied and the MIC and MBC, given in *µL essential oil /mL medium*, were determined, following the methodology explained in section II. The macrodilution broth results are gathered in table IV.12.

In Figures IV.13 and IV.14 we can observe some pictures of the antimicrobial activity assay of the *L. luisieri* essential oil against *E. faecium* and *S.* Typhimurium after the incubation at 37 ºC for 24 hours. In these figures it can be seen how the turbidity of the solution changes when there is bacterial growth. *E. faecium* (Figure IV.13) showed turbidity only in the positive control. *S.* Typhimurium bacterial growth was only avoided at the highest concentrations tested (5 - 30 µL/mL) and at the lowest concentrations of essential oil turbidity was observed (Figure IV.14).

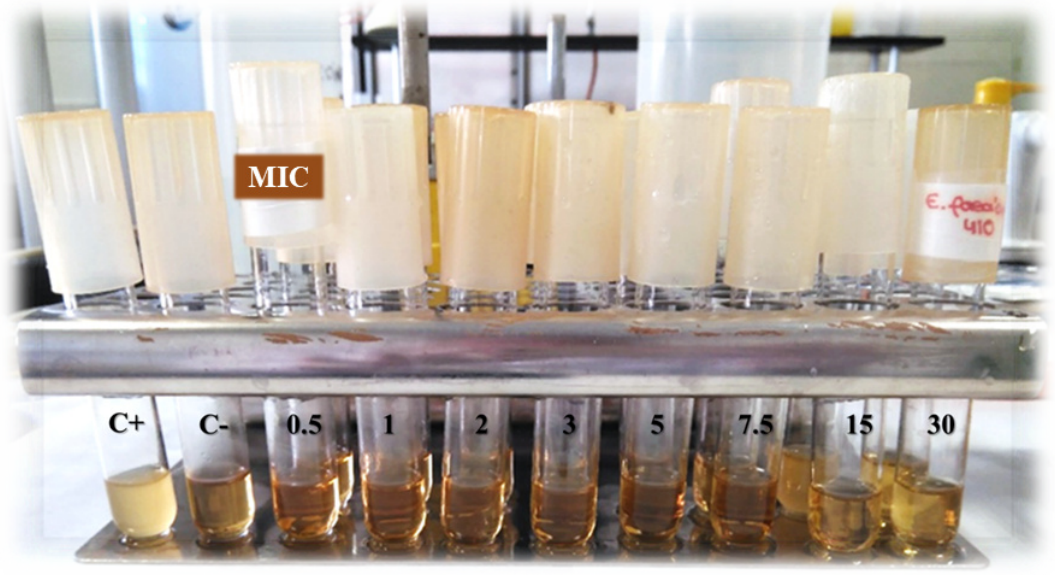


Figure IV.13. Macrodilution antimicrobial test of *L. luisieri* essential oil solutions against *Enterococcus faecium*. C+, Positive control, C-, Negative control *Satureja montana* essential oil, and the tested concentrations of *L. luisieri* essential oil 0.5, 1, 2, 3, 5, 7.5, 15 and 30 µL/mL.

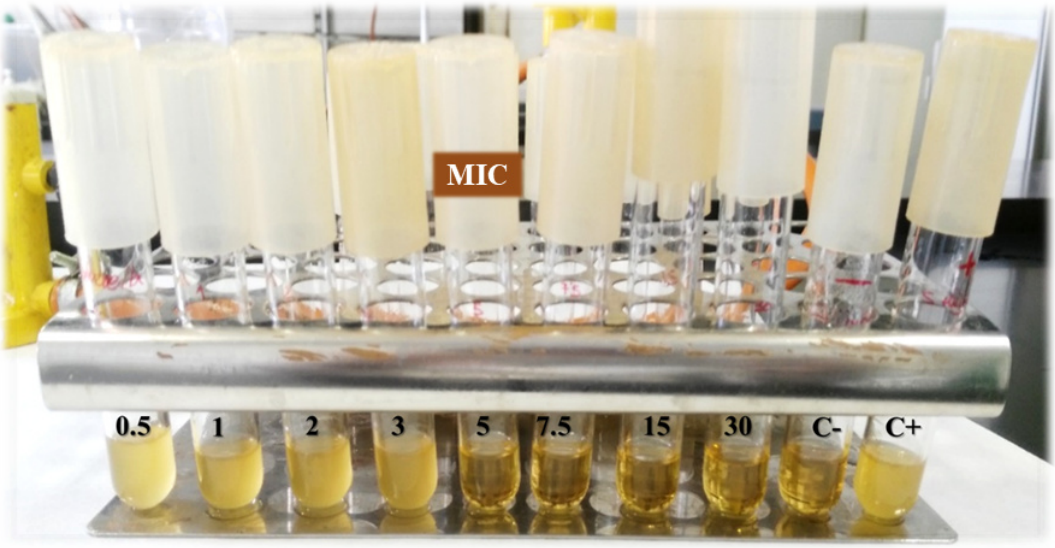


Figure IV.14. Macrodilution antimicrobial test of *L. luisieri* essential oil solutions against *Salmonella* Typhimurium. C+, Positive control, C- Negative control of *Satureja montana* essential oil, and the tested concentrations of *L. luisieri* essential oil 0.5, 1, 2, 3, 5, 7.5, 15 and 30 µL/mL.

According to the results gathered in Table IV.12, *L. luisieri* essential oil had bacteriostatic activity against all bacteria tested, showing a wide antibacterial spectrum, inhibiting both gram-positive and gram-negative bacteria.

Bacteriostatic activity was observed against gram positive strains, with MIC values of 0.5 µL essential oil/mL medium, (*L. monocytogenes, E faecium* and *S. aureus*), whereas higher concentrations were required to inhibit gram negative ones:

5.0 µL essential oil /mL medium against *S.* Typhimurium and 30 µL essential oil /mL medium *vs E. coli*.

The evaluation of MBC revealed that *L. luisieri* essential oil was bactericidal against 4 of the 5 bacteria at the assayed concentrations. The MBC values ranged from 0.5 µL essential oil/mL medium against *S. aureus*, to 5.0 µL essential oil/mL medium against *S.* Typhimurium. *E. coli* seems to be a more resistant strain to our oil composition, since it showed bacteriostatic but not bactericidal activity at the highest tested ratio, MIC = 30 µL essential oil/mL medium (MBC >30 µL/mL).

This activity was also observed by other authors,[29] when applying the diffusion agar method with a chemically defined necrodyl-rich essential oil from *L. luisieri* against different bacterial and yeast strains. Although the test was performed with a different method, it was also observed that the gram positive strains were more sensitive to the essential oil than the negative ones, and the *E. coli* strains had sensitivity to *L. luisieri* essential oil, but also at the highest tested concentration. *L. luisieri* essential oil has a higher antimicrobial activity compared to other *Lavandula* species, such as *L. lavandulifolia, L. angustifolia,* Lavandin super, Lavandin Abrialis*,* which only had activity against *S. aureus*.[136] Nevertheless, Rota et al.,[136] reported that other species, such as *L. latifolia* and Lavandin grosso*,* had similar MIC and MBC results to *L. luisieri* against several strains, such as *S.* Typhimurium, *E coli* and *L. monocytogenes*. *Lavandula* species essential oils have sensitivity differences among gram positive and gram negative strains, being *E. coli* the most resistant one. Sensitivity differences among gram negative strains could be a consequence of the restricted penetration because of their different and more complex cell wall, since they have a second external phospholipid bilayer designed to reduce the permeability to all compounds.[151] However, the chemical composition of this other antimicrobial species differ from *L. luisieri*, and their activity was related to their content in 1,8-cineol, linalool and camphor.[152]

The composition of *L. luisieri* (2009 Aguaron adapted by CITA) oil was reported in a previous study.[70] Its main components were camphor (60.3%) and 2,3,4,4-tetramethyl-5-methylidenecyclopent-2-en-1-one (8.5%), a necrodane-type compound, along with other substances found in this *Lavandula* species: fenchone (2.9%) and 1,8-cineol (2.0%). Other authors studying *L. luisieri* essential oil composition observed that the differences among populations appear to be quantitative, the same volatiles were identified although their proportion changed between *L. luisieri* populations.[3,14, 18,19,20,

So far, only Baldovini et al. (2005),[29] Roller, Ernest, & Buckle. (2009)[35] and Zuzarte et al. (2012)[20] have tested *L. luisieri* essential oil antimicrobial activity. They obtained good results against all tested strains, especially against gram positive bacterial and fungal ones, such as *Candida albicans* and *Aspergillus* species, and related these results to its necrodane terpenoids content. Roller, Ernest, & Buckle, (2009)[35] compared *Lavandula* species essential oil at different impregnation volumes antimicrobial activity against methicillin resistant and sensitive S*taphylococcus aureus* with disk diffusion methods. *L. luisieri* essential oil, highly concentrated in necrodane monoterpenoids, 34.5% relative content in α-necrodyl acetate, was the most active at lower proportions. In addition, its combination 50:50 with *L. stoechas* and *L. angustifolia* essential oils produced inhibitory zones twice the diameter that the zones obtained with both single oils. A possible synergy among necrodane terpenoids and 1,8-cineole, fenchone, and camphor from *L. stoechas*, or linalool and linalyl acetate from *L. angustifolia*, was suggested, and may be also the cause of the results obtained in this work. Similarly, Zuzarte et al., (2012)[22] also related *L. luisieri* essential oil antimicrobial activity to its particular content in necrodane-type compounds when studied two different populations from central and south regions of Portugal against different *Aspergillus* strains. This activity results were considered relevant since this strain is usually less sensitive to essential oils, and the essential oil of this work, containing necrodane compounds, could also be applied to fungal microorganisms such as *Aspergillus*.

The antimicrobial activity results obtained in this work could be a consequence of its high content in camphor, which is a well-known antimicrobial compound,[153] besides, its content in atypical necrodane compounds and the combination with other volatiles, could also be contributing to this activity.

1.7.1.2. Maceration extract and SAF fractions

The antimicrobial activity of the maceration extract, ME, and its supercritical fractions PV and DV (at the optimized conditions 130 bar and 30 g/min), were also tested. The activity of the extracts was compared with the one of pure rosmarinic and ursolic acid. To our knowledge, this is the first work to study the antimicrobial activity of polar and non-volatile fractions of this plant. For this type of extracts, the disk diffusion method could not be applied because of the poor diffusion from the cellulose disks to the agar of this kind of compounds. The antimicrobial activity of ME, PV and DV, as well as the

pure compounds rosmarinic and ursolic acids, was tested and quantified directly with the microdilution broth procedure. The final extracts concentrations assayed ranged from 2 to 2000 μg/mL, from 0.3 to 300 μg/mL for ursolic acid and from 0.1 to 200 μg/mL for rosmarinic acid, see Table IV.13. The concentrations were obtained by twofold serial dilution from the first column.

Table IV.13. Assayed *Lavandula luisieri* concentrations of the ethanolic maceration extract (ME), its supercritical fractions PV and DV, and pure ursolic and rosmarinic acid in each well of the plate.

	Concentrations of tested extracts (μg/mL)										
ME	1146.7	573.4	286.7	143.3	71.7	35.8	17.9	9.0	4.5	2.2	1.1
PV	1932.9	966.5	483.2	241.6	120.8	60.4	30.2	15.1	7.6	3.8	1.9
DV	1862.5	931.3	465.6	232.8	116.4	58.2	29.1	14.6	7.3	3.6	1.8
UA	263.0	131.5	65.8	32.9	16.4	8.2	4.1	2.1	1.0	0.5	0.3
RA	125.0	62.5	31.3	15.6	7.8	3.9	2.0	1.0	0.5	0.2	0.1

In the following figure (Figure IV.15), the antimicrobial activity of the PV fraction is represented against three bacterial strains. *E. faecium, S. aureus and L. monocytogenes*. In the first row of the microwell plate was included a negative control containing MH broth with red phenol, glucose and the PV fraction solution, in order to control crossed contaminations or presence of microorganisms in the obtained extracts (no change along the experiment). In the last column two wells of each microorganism into MH broth supplemented with red phenol and glucose were included as positive control, in order to evaluate the microorganism growth (change of colour from red to yellow). And finally, PV fraction antimicrobial activity was tested against *E. faecium* (rows 2 and 3), *S. aureus* (rows 4 and 5) and *L. monocytogenes* (rows 6 and 7). It can be observed how the highest concentrations of PV fraction bacterial growth is inhibited. The bactericidal activity (MBC) was measured afterwards by inoculating in MH agar plates 100 μL of each well.

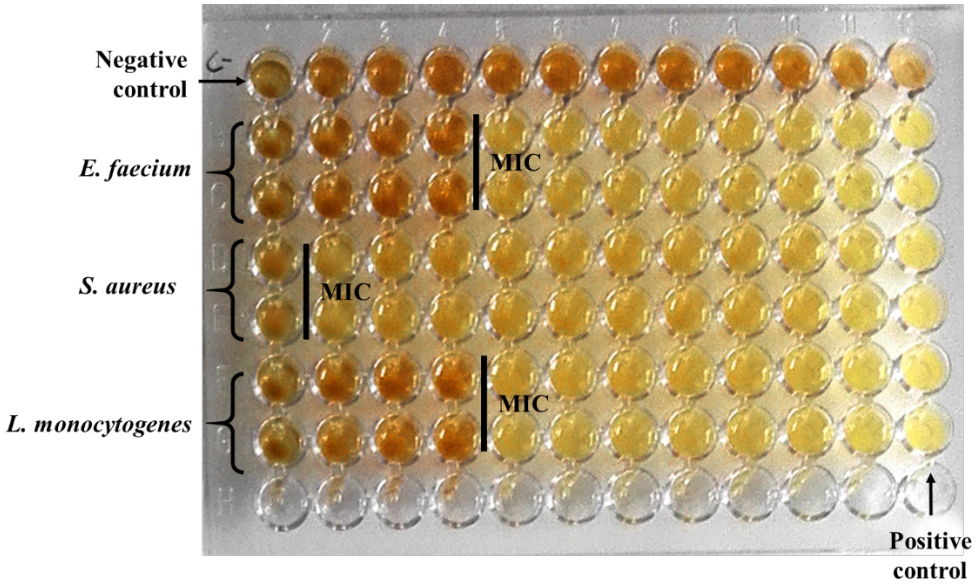


Figure IV.15 Microdilution antimicrobial test of *L. luisieri* PV fraction solutions against *Enterococcus faecium. Staphilococcus aureus* and *L. monocytogenes*.

From all studied strains, only *L. monocytogenes, E. faecium* and *S. aureus* showed sensitivity to the *L. luisieri* maceration extract and its supercritical fractions. The obtained values of ME, PV DV and pure ursolic acid MIC and MBC against these three bacteria have been ordered left to right from more to less sensitive strain in Table IV.14. *S.* Typhimurium and *E. coli* did not showed sensitivity to the extracts at the assayed concentrations.

Table IV.14. Minimum inhibitory (MIC) and bactericidal (MBC) concentrations (µg/mL) of ursolic acid (UA), *L. luisieri* ethanolic feed solution (ME), and its supercritical fractions PV and DV, against *L. monocytogenes, E. faecium and S. aureus.*

	L. monocytogenes		***E. faecium***		***S. aureus***	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
UA	32.9	65.8	65.8	65.8	263.0	263.0
ME	286.7	557.4	286.7	1146.7	-	-
UA concentration in ME	*14.9**	*28.9**	*14.9**	*59.6**	-	-
PV	241.6	483.2	241.6	241.6	1932.9	-
UA concentration in PV	*28.7**	*57.3**	*28.7**	*28.7**	*229.2**	-
DV	232.8	931.3	-	-	-	-
UA concentration in DV	*11.3**	*45.4**	-	-	-	-

** UA concentration into the correspondent extract concentration according to its previous quantification expressed in* µg/mL.

Regarding the two pure compounds assayed, RA and UA, rosmarinic acid did not show any antimicrobial activity in the studied range. Although other authors have confirmed this lack of activity against food bacteria,[154] it has been also reported rosmarinic acid antimicrobial activity but only against phytopathogenic bacteria.[155] This compound is produced and released by some plants as a natural microbe defence in challenging environments.

Pure ursolic acid had inhibitory and bactericidal activity, against the three gram positive bacteria, *L. monocytogenes*, *E. faecium* and *S. aureus*. However, while *L. monocytogenes* and *E. faecium* MIC and MBC values ranged from 32.9 to 65.8 μg/mL, *S. aureus* was more resistant with MIC and MBC values up to 263.0 μg/mL. (Table IV.14).

L. luisieri maceration extract, PV and DV were also active in inhibiting these bacteria (Table IV.14). The lack of activity against gram negative strains could be a consequence of the cell wall structural differences previously named.[151]

The maceration extract was only active against *L. monocytogenes* and *E. faecium* which MIC and MBC values were 286.7-557.4 μg/mL and 286.7-1146.7 μg/mL, respectively (Table IV.14). According to the measured ursolic acid proportion, these MIC values corresponded to 11.3 μg/mL of UA and MBC concentrations corresponded to a range of 45.4-59.6 μg/mL of UA.

After the SAF process, the PV fraction had better antimicrobial results. The precipitated solid showed activity against the three gram positive bacteria. The MIC and MBC values against *L. monocytogenes* (241.6-483.2 μg/mL) and *E. faecium* (241.6-241.6 μg/mL) were lower than the obtained with the maceration extract, and had inhibitory properties against *S. aureus* (1932.9 μg/mL). (Table IV.14)

According to the measured ursolic acid proportion (Section 1.6 Table IV.11), the maceration extract MIC and MBC values against *L. monocytogenes* and *E. faecium* corresponded to 14.9 μg/mL and 28.9-59.6 μg/mL of UA respectively, Table IV.14. PV inhibitory and bactericidal concentrations corresponded to a final UA concentration of UA 28.7-57.3 μg/mL. In all cases, the final proportion of this triterpene in *L. luisieri* samples was lower than the inhibitory and biocidal concentrations of the pure active (32.9-65.8 μg/mL, Table IV.14).

So far, only Lai et al (2012)[56] have tested the antimicrobial activity of extracts different from the essential oil. They extracted *L. luisieri* actives by soaking the dried plant material into hexane, methylene chloride, ethyl acetate, methanol and water. The methanolic extract MIC values were 62 μg/mL *vs S. aureus*, 62 μg/mL *vs S. epidermidis*, 250 μg/mL *vs S.* Typhimurium. There were not differences in MIC values among the extracts from the different solvents. This could be because the antimicrobial test was performed by solving the extract in pure DMSO which avoids bacterial growth inhibition at high concentrations and falsify the obtained results.[156] This inhibitory results were not correlated to a certain composition.

So, although ursolic acid was concentrated in PV, the activity cannot entirely be attributed to it. Oleanolic acid, the other triterpene tracked in this work, with a very similar molecular structure, may be contributing to the final activity by addition or synergic effect. Indeed, several studies have reported the antimicrobial activity of oleanolic and ursolic acids.[54,157] Do Nascimeinto et al.[157] observed an inhibitory capacity of ursolic acid against both gram negative and positive and a synergistic effect between it and aminoglucoside antibiotics when tested against 12 bacterial strains. Besides, Wolska et al.[54] related oleanolic and ursolic acids antimicrobial activity to the peptidoglycan structure, bacterial gene expression and biofilm formation.[54] Even though rosmarinic acid did not showed activity by its own, its combination with the other compounds could enhance it.

In any case, the concentrations required to inhibit and kill bacterial strains by *L. luisieri* maceration and supercritical extracts were higher than those obtained with the essential oil (Table IV.12 and IV.14). This difference could be a consequence of the different compounds that constitute these extracts. Their penetration into bacterial cells and their mode of action to inhibit and kill could be different.

1.7.2. L. luisieri extracts antioxidant activity

The antioxidant activity of ME, PV, DV and pure compounds RA, UA and OA was evaluated against the free radical DPPH. Only results for ME, PV, DV and RA are shown because, as will be commented later, UA and OA did not show antioxidant activity. As can be observed in Figure IV.16, the DPPH antioxidant activity was concentration-dependent.

L. luisieri essential oil, ethanolic maceration extract and its SAF fractions showed antioxidant activity, but always lower than the positive controls trolox (IC_{50} = 3.5 ± 0.3 µg/mL) and rosmarinic acid (IC_{50} = 1.7 ± 0.1 µg/mL). The antioxidant evaluation of *L. luisieri* maceration extract showed an IC_{50} equals 30.7 ± 1.9 µg/mL and the EO IC_{50} was 7 ± 3 µL/mL. Although the results are not comparable with other authors, since they applied different DPPH methods, the good antioxidant activity of the *L. luisieri* essential oil has been reported and correlated to its high phenol content. Nevertheless, these results are similar to others previously published about different *Lavandula* species and their extracts. The DPPH scavenging activity of methanolic extracts of *L. stoechas* was also studied before,[158] being its IC_{50} 34.2 ± 3.1 µg/mL. This result was related to the extracted phenolic acids, 25.2 ± 0.4 mg GAE/g.

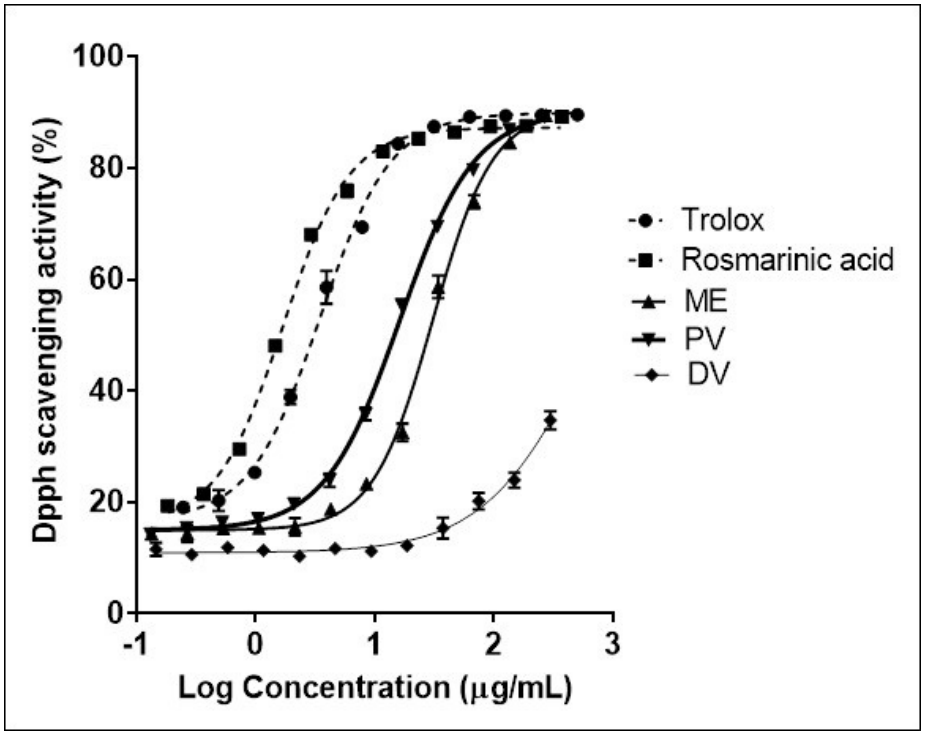


Figure IV.16. Logarithmic curves representation of the antioxidant activity of *Lavandula luisieri* ethanolic maceration extract (ME) and its SAF fractions: precipitation vessel fraction (PV) and downstream vessel fraction (DV). Positive controls 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and rosmarinic acid (RA).

Other authors[158–160] reported also free radical inhibition of other *Lavandula* spp extracts. *L. x intermedia* ethyl acetate and ethanolic extracts, with IC_{50} =50.4 µg/mL and IC_{50}=15.1-45.3 µg/mL, respectively. *L. angustifolia* ethanolic extract IC_{50} was 10.6-33.9 µg/mL, ethanolic extracts from *L. coronopifolia* and *L. multifida* IC_{50}=15.8 µg/mL and IC_{50}=19.3 µg/mL, respectively.[158–160] In contrast, other authors [161] reported for the *L.*

stoechas methanolic extract an IC_{50} of 300±10 µg/mL. This reported decreased antioxidant activity may be a consequence of the heat application in the extraction process and the different proportion of extract:DPPH applied.

After the supercritical processing of the maceration extract, the antioxidant activity was higher in PV, IC_{50} of 16.2 ± 0.7 µg/mL. DV fraction, however, was the least active, not inhibiting the 50% of the DPPH free radicals at the studied range of concentrations. These results can be graphically observed in Figure IV.16, where the PV and DV antioxidant curves are in the left and right respectively from the maceration extract curve.

According to these results and to the chemical analysis (table IV.11, section 1.6), after the supercritical fluid process, the antioxidant compounds extracted with the maceration are mainly concentrated in the solid of the PV fraction. The increased scavenging activity of PV and the lack of activity in DV may be a consequence of one of the main compounds identified, rosmarinic acid, which completely precipitates during the supercritical process when converges with CO_2, increasing its proportion in this fraction at the optimal conditions from 5.14% of maceration extract to 9.35 % of PV (table IV.15). The IC_{50} of pure rosmarinic acid was 1.7 µg/mL, which correlates with the antioxidant results IC_{50} results obtained in the maceration extract and PV. Considering an IC_{50} value of ME, 30.7 µg/mL and PV 16.2 µg/mL, and the RA proportion in these fractions 5.1% and 9.4% respectively, the corresponding rosmarinic acid concentration into ME and PV IC_{50}, is 1.51 and 1.58 µg/mL. (Table IV.15).

Table IV.15. Antioxidant activity of the *L. luisieri* maceration extract and its fractions IC_{50} value and corresponding concentration of RA.

	IC_{50} (µg/mL)	RA%	RA concentration (µg/mL)
ME	30.7	5.14	1.58
PV	16.2	9.35	1.51
DV	-	-	-

On the other hand, oleanolic and ursolic acids did not seem to scavenge DPPH free radicals since the 50% of the inhibition was not reached even at the highest tested extract concentration, 300 µg/mL, where the content of OA and UA was 4.9 % and 5.6% respectively (table IV.11, section 1.6). Other authors reported that ursolic acid had antioxidant activity but at higher concentrations, IC_{50} = 59.70 µg/mL.[157]

L. luisieri scavenging activity seems to be related to its content in rosmarinic and not in oleanolic and ursolic acids. Other *Lavandula* species extracts have also been reported to display several antioxidant mechanisms such as antioxidant potential, organic, cation and superoxide free radicals scavenging, electron or metal cation chelation [161–164] and has always been related to their content in phenolic compounds such as protocatechuic acid, caffeic acid and rosmarinic acid.

2. Encapsulation and co-encapsulation of rosmarinic acid through SEE

Because of potential antioxidant applications in food, cosmetic or pharmaceutical products of *L. lusieri* precipitated SAF fraction, its encapsulation was proposed in this work. Nevertheless, rosmarinic acid was selected to perform the assays among the three tracked compounds of this project because it was the most antioxidant component of *L. luisieri* extracts. The encapsulation of the pure active was proposed alone and with other two antioxidants widely used in the pharmaceutical, cosmetical and food industries, β-CA and α-TOC. The emulsion formulas applied in each case were based in the previous study performed by Odierna (2014) [165] who encapsulated, β-CA and α-TOC into PLA and PLGA from double (o_1/ o_2/w) emulsions. Rosmarinic acid encapsulation was attempted in double emulsions $w_1/o/w_2$, in this case, to increase its initial loading into the formulation.

After the production of the particles, their particles size, antioxidant activity against DPPH and shelf life were evaluated. The encapsulation assays were performed with the Supercritical Fluid group of research from the University of Salerno (Italy).

2.1. Rosmarinic acid encapsulation

Several double and single emulsions were prepared to produce microspheres loaded with RA into biopolymer carriers using SEE technique as described in section 2.2 from chapter III. Table IV.16 shows data about all emulsions formulated, all of them containing RA, their composition phase by phase (w_1, o, w_2), the emulsification conditions (w_1/o and w_1/o/w_2), the active theoretical loading per gram of polymer (TL), the measured loading per gram of polymer (ML), the encapsulation efficiency (EE%) and the produced droplets and microspheres mean size. All double emulsions were formulated with a composition ratio of 4:16:80 w/w/w and the single emulsion 20:80 wt/wt. As it can be seen, 10 different experiments were carried out. For the first 4 experiments ($RA1_{SEE}$ to $RA4_{SEE}$) only SEE was used to create the microspheres, however for the other 3 experiments (RA5 to RA7) both SEE and the conventional solvent evaporation (SE) were proposed in order to compare and justify the obtained results of the previous experiments ($RA1_{SEE}$ to $RA4_{SEE}$) in terms of encapsulation efficiency.

The first set of SEE experiments, $RA1_{SEE}$ to $RA5_{SEE}$, was performed changing the proportions of EtOH:H_2O (0.06% PVA) as solvent of the internal phase w_1, and RA

concentration (Table IV.16). The oily phase o_2 contained 1 g of PLA into EA (Ethyl acetate); whereas, the external w_2 phase was EA-saturated aqueous Tween 80 solution (0.6%, w/w of Tween 80). These variations on the internal w_1 phase of the emulsion were applied in order to solve the active into the internal phase and favour the encapsulation within the polymer PLA. Nevertheless, in these 5 experiments the encapsulation efficiency, EE %, obtained was very low in every one, ranging from 1.3% to 3.9%. The measured particle size diameter of the microspheres was generally reduced from the emulsion droplets as it happens, for example, in $RA1_{SEE}$ from 1.3 ±0.2 to 0.3 ±0.1 μm. The general appearance of the produced particles of experiments was spherical, nevertheless the product obtained from emulsions $RA1_{SEE}$ and $RA3_{SEE}$ had a more homogeneous particle size than $RA2_{SEE}$ and $RA4_{SEE}$ (Figure IV.17, B and D). For a better understanding of the reason why this very low encapsulation efficiency was obtained (table IV.16), the same emulsion used in $RA5_{SEE}$ experiment was processed to get microparticles but implementing the conventional solvent evaporation this time, run RA5 (SE), table IV.16. The supercritical processing reduced particles mean size regarding the initial droplets, while the traditional evaporation increased it (Figure IV.17 E *vs* F). Nevertheless, the encapsulation efficiency was also very low in both cases, 3.1% and 2.4%.

Table IV.16. RA emulsions composition, formulation conditions, Theoretical Loading (TL), Effective Loading (EL), Encapsulation Efficiency (EE%), droplet mean size diameter and final particles mean size diameter produced by SEE and solvent evaporation (SE).

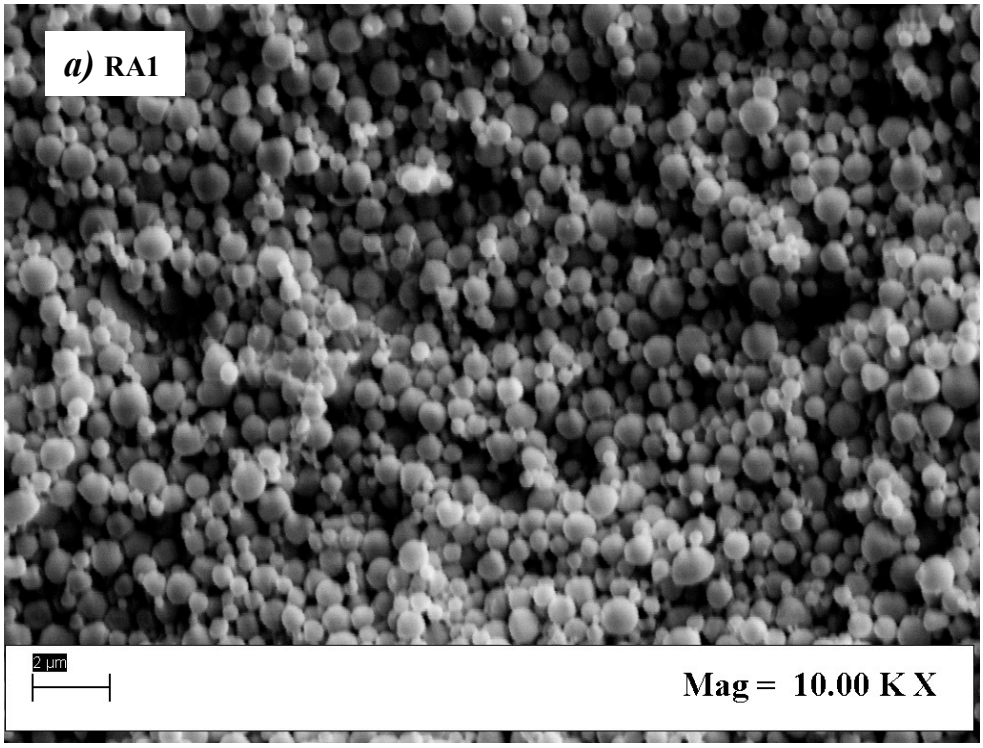
Run	w_1 *active+solvent +surfactant*	o *solvent +biopolymer*	w_2 *solvent +surfactant*	w_1/o	$w_1/o/w_2$	TL mg/g	ML mg/g	EE %	Droplets mean size (sd) µm	Particles mean size (sd) µm
$RA1_{SEE}$	RA 20 mg, 20:80 EtOH:H_2O and 0.06% PVA	1g PLA 20g EA	0.6% of TWEEN 80 in water saturated EA	30 s at 30% amplitude 3 times	4min at 2900 rpm	20	0.8	3.9	1.3 ±0.2	0.3 ±0.1
$RA2_{SEE}$	RA 37 mg, 20:80 EtOH:H_2O and 0.06% PVA	1g PLA 20g A EA	0.6% of TWEEN 80 in water saturated EA	30 s at 30% amplitude 3 times	3min at 2800 rpm	37	0.5	1.3	0.8±0.1	0.4 ±0.1
$RA3_{SEE}$	RA 20 mg, 30:70 EtOH:H_2O and 0.06% PVA	1g PLA 20g EA	0.6% of TWEEN 80 in water saturated EA	30 s at 30% amplitude 3 times	4min at 2900 rpm	20	0.7	3.5	1.0±0.1	0.3 ±0.2
$RA4_{SEE}$	RA 100 mg, 100% EtOH and 0.06% PVA	1g PLA 20g EA	0.6% of TWEEN 80 in water saturated EA	30 s at 30% amplitude 3 times	4min at 2900 rpm	100	2.2	2.2	1.3 ± 0.2	1.3±0.2

SEE. Supercritical Emulsion Evaporation

Table IV.16 (continued). RA emulsions composition, formulation conditions, Theoretical Loading (TL), Effective Loading (EL), Encapsulation Efficiency (EE%), droplet mean size diameter and final particles mean size diameter produced by SEE and solvent evaporation (SE).

Run	***w_1*** *active+solvent +surfactant*	***o*** *solvent +biopolymer*	***w_2*** *solvent +surfactant*	***w_1/o***	***$w_1/o/w_2$***	***TL*** ***mg/g***	***ML*** ***mg/g***	***EE %***	***Droplets mean size (sd) µm***	***Particles mean size (sd) µm***
RA5 SEE	RA 10 mg, 100 H_2O and 0.06% PVA	1g PLA 20g EA	0.6% of TWEEN 80 in water saturated EA	30 s at 30% amplitude 3 times	4min at 2900 rpm	10	0.3	3.1	0.8±0.1	0.6±0.1
RA5 SE							0.2	2.4		1.4 ±0.1
RA6 SEE	-	150 mg RA 1g PLA 20g EA	0.6% of TWEEN 80 in water saturated EA	30 s at 30% amplitude 3 times -	3 min at 3400 rpm	150	0	0	1.2 ± 0.2	-
RA6 SE							14.2	9.4		0.40 ±0.03
RA7 SEE	RA 125 mg, HCl solution pH 2.8, 0.4%Chitosan and 0.06% PVA	2g PLA 18g EA	0.6% of TWEEN 80 in water saturated EA	30 s at 30% amplitude 3 times	4min at 2900 rpm	75	3.3	4.4	0.8±0.2	0.20 ±0.04
RA7 SE							1.9	2.6		1.4 ±0.1

SE Solvent evaporation
EA Ethyl Acetate
PVA Polyvinyl alcohol
sd standard deviation



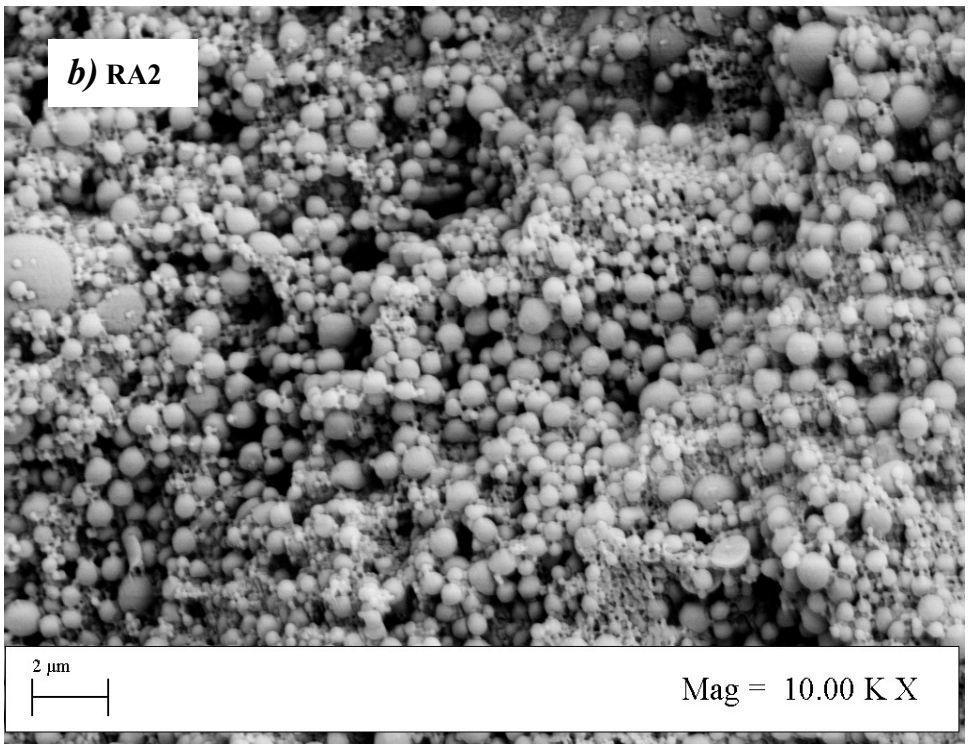
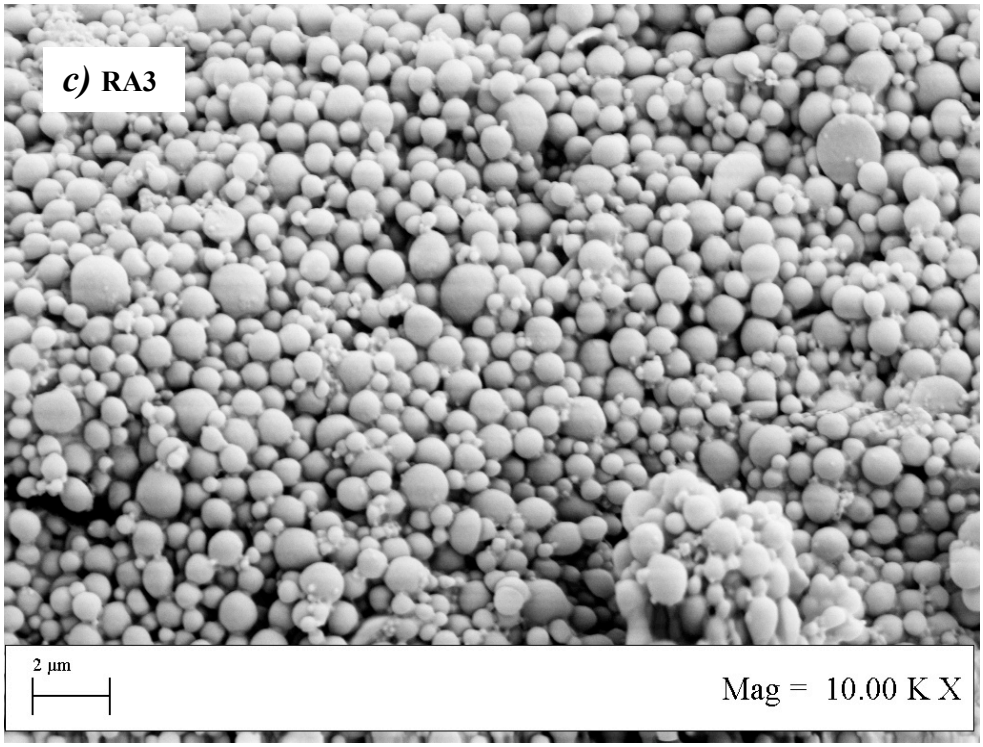


Figure IV.17. a) SEM images obtained after SEE processing of the emulsions RA1 and b) RA2



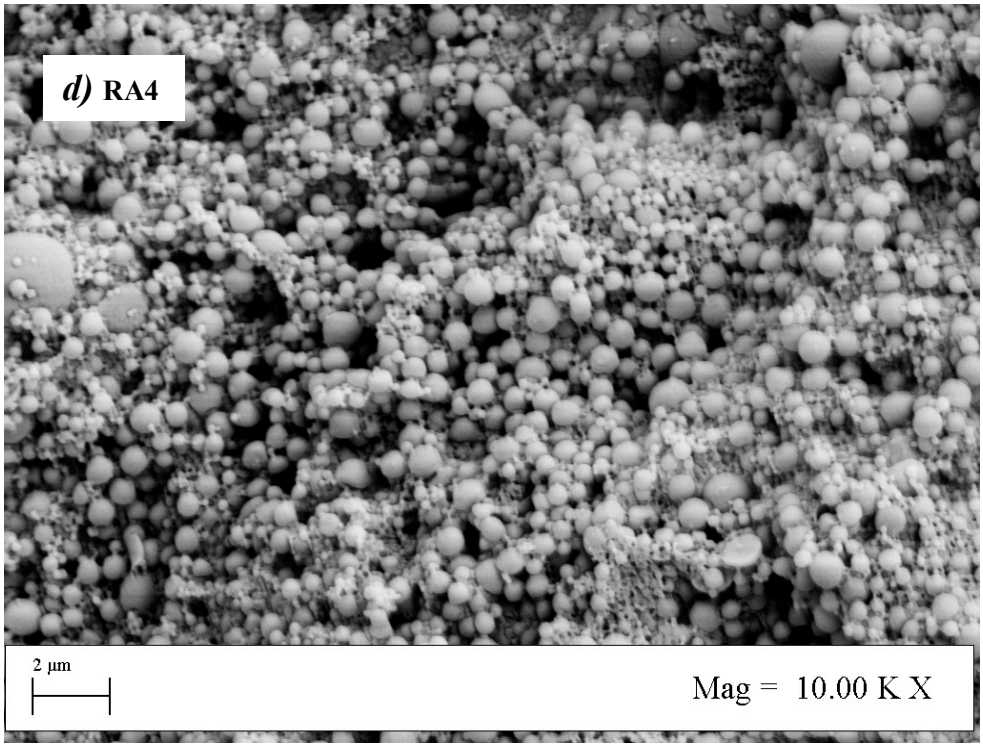


Figure IV.17 (continued). c) SEM images obtained after SEE processing of the emulsions RA3 and d) RA4

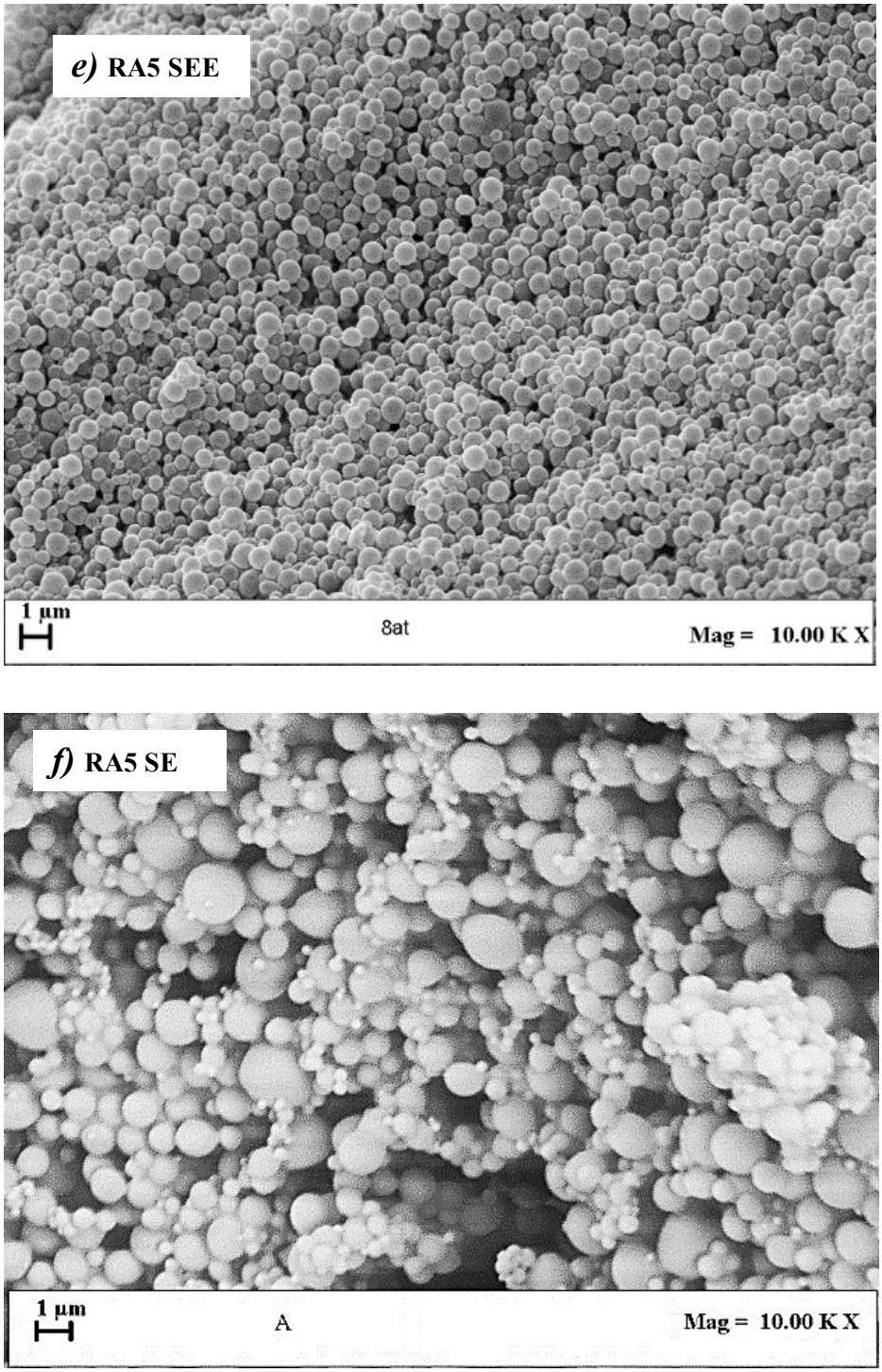


Figure IV.17 (continued). e) SEM images of the obtained microspheres obtained after SEE from RA 5, and f) obtained microspheres after solvent evaporation from RA 5

Rosmarinic acid was relatively soluble in ethyl acetate at room temperature (20 ºC), this solubility allowed the formulation of a single emulsion of *o*/*w* ($RA6_{ESS}$), where the internal oily phase contained the rosmarinic acid and polymer solved into ethyl acetate and the external aqueous phase saturated water with EA 0.6% of TWEEN 80. Since the proportion oil in water was 20:80, the final rosmarinic loading into 20 g of oily phase and

1g of PLA was 150 mg (Table IV.16). This single emulsion was processed through both, supercritical emulsion extraction, $RA6_{SEE}$, and the traditional solvent evaporation, RA6. The suspension obtained after the SEE process contained visible clots of polymer and the external water phase was yellowish. Besides, after its centrifugation, only 14.2% of the initial mass was recovered. On the contrary, in the traditional solvent evaporation method the visual aspect of the particles was more homogeneous (figure IV.18) and the encapsulation efficiency was the highest obtained so far, 9.4% in RA6 SE (Table IV.16).

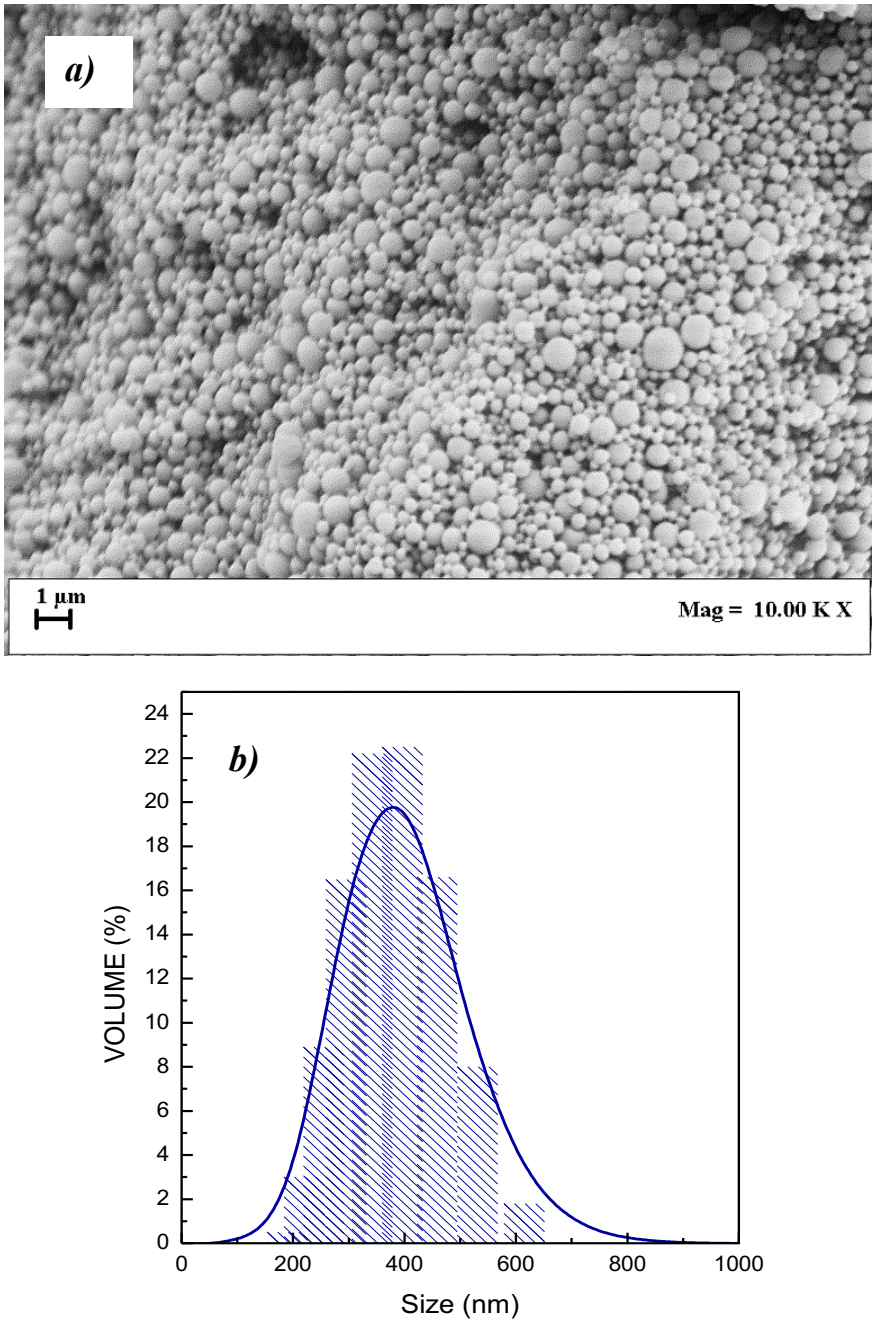


Figure IV.18. RA6 SE a) SEM image obtained after single emulsion solvent evaporation of the emulsion RA 6 and b) the obtained partible size distribution.

Considering the results obtained from the processing of emulsions $RA1_{SEE}$ - $RA5_{SEE}$, it was clear that the RA could not be encapsulated in the polymer with SEE with this procedure. The best encapsulation efficiency, 9.4% was obtained with the traditional solvent evaporation (RA6 SE). The system ethyl acetate-$scCO_2$ may generate an expanded solution that can co-extract RA during the process. Indeed, since the encapsulation of this active was not possible at the tested conditions, 38 ºC and 80 bar (see chapter III section 2.3), it could be because the ternary system formed by ethyl acetate+$scCO_2$+RA may have a miscibility hole, not enough to avoid the co-extraction of RA. Besides, the partial solubility of RA into the different solvents of the emulsion formula may produce its solvation in the different phases that conform the emulsion.

Further investigations of different emulsion formulations were proposed in order to modify the miscibility hole of the overall system introducing chitosan inside the aqueous internal phase and increasing the biopolymer amount up to 2 g, in order to improve RA encapsulation. Indeed, the goal of including a higher concentration of polymer was to increase the viscosity of the oily phase. This emulsion was also processed through both methods, SEE ($RA7_{SEE}$) and the traditional solvent evaporation (RA7), Table IV.16. The obtained particles through traditional solvent evaporation were bigger than 10µm, probably because of the presence of a higher quantity of polymer and chitosan. The higher viscosity of the overall system and the longer evaporation time intrinsic to this method favoured the formation of bigger spheres.

The different size of the microspheres observed between the supercritical and the traditional method confirmed the possibility that the emulsions can be further modified by passing through the SEE process, reducing the droplet sizes, besides, the solvent elimination occurs faster. Nevertheless, the addition of chitosan to the formula ($RA7_{SEE}$) did not improve the encapsulation efficiency regarding the previous experiments (RA1-RA6), 4.4% through SEE and 2.6% through SE (Table IV. 16).

In the end, RA encapsulation could not be successfully achieved. RA may be extracted by the expanded liquid ethylacetate-$scCO_2$ generated at supercritical conditions of the SEE process. [166] In the traditional solvent evaporation method, the long time it takes to evaporate the oily solvent and the partial solubility of RA in all solvents constituting the emulsion favour the diffusion of the active from the internal phase to the external water.

The obtained results were almost in contrast with the ones reported by several authors that described RA encapsulation efficiencies of 60-78% in poly-caprolactone and sodium carboxymethyl cellulose, respectively when processed with emulsion evaporation.[167,168]

The inclusion of rosmarinic acid into carriers has been previously attempted with methods such as supercritical anti-solvent technique into polycaprolactone[169] and conventional emulsion solvent evaporation into polycaprolactone [39,40] or spray drying within cellulose. [170] Yesil-Celtikas et al. performed the encapsulation into polycaprolactone of the active by both, supercritical anti-solvent technique and solvent evaporation. While supercritical anti-solvent co-precipitation allowed the encapsulation of the 83% of the active. the traditional solvent evaporation achieved 62%. [169] Although the supercritical process is a co-precipitation and not an encapsulation *per se*, a high encapsulation efficiency was possible with these published formulations if compared with the results obtained in this work. Nevertheless, the emulsion produced to perform the encapsulation used high volumes of dichloromethane, which is a toxic and contaminant organic solvent. Further investigations should be performed in order to determine if the encapsulation of RA by evaporation of an emulsion phase is possible into this polylactic acid, or other biopolymers.

2.2. Co-encapsulation of rosmarinic acid with β-carotene and α-tocopherol

Rosmarinic acid encapsulation along with other two antioxidants was attempted since it has been reported that the presence of several antioxidants in a formulation increases β-CA stability,[171] besides, their concomitant presence in the formula could increase RA EE%. RA was co-encapsulated with the natural antioxidants β-CA and α-TOC using the SEE technique and following a previous work by Angelo Odierna (2014).[131,165]

Based on these previous studies with β-CA in, several double $o_1/o_2/w$ emulsions were formulated. Their composition ratio was 4:16:80 w/w/w, where the internal o_1 phase contained the three active principles dissolved into chloroform with 0.06% Span 20; the second oily phase, o_2, was formed with EA with a given amount of biopolymer dissolved in it (0.4 to 0.8 g of PLA or PLGA) (Table IV.17).

Table IV. 17. Emulsion phase's composition and formulation sonication and stirring conditions of RA β-CA and α-TOC PLA and PLGA formulations.

o_1 *active+solvent +surfactant*	o_2 *solvent +biopolymer*	w *solvent+surfactant*	w_1/o	$w_1/o/w_2$
4mL chloroform SPAN 20 0.06%	16g EA	0.6% of TWEEN 80 in water saturated EA	30 s, 30% amplitude 3 times	6min 3600 rpm

For emulsions formulation, the inner phase o_1 was mixed with the second oily phase o_2, to form the o_1/o_2 emulsion by sonication. The o_1/o_2 emulsions were, then, immediately added into a known amount of EA-saturated aqueous Tween 80 solution (0.6%, w/w of Tween) with the high-speed stirrer for 6 min and at 3600 rpm. The actives and polymer in each formulation are gathered in Table IV.18.

Table IV.18. Different PLA and PLGA emulsions charged with β-CA, α-TOC and RA formulations: Polymer mass, Theoretical loading (TL), effective loading (EL) and Encapsulation Efficiency (EE%) using SEE.

	Polymer	*actives*	*TL mg/g*	*EL mg/g*	*EE %*	*Droplets mean size (sd) µm*	*Particles Mean size (sd) µm*
PLA1	PLA 0.4	β-CA and α-TOC	β-CA : 10 α-TOC: 10	β-CA: 6.1 α-TOC:	β-CA : 61.8 α-TOC:53.3	1.3 ± 0.1	0.3 ±0.1
PLA2	PLA 0.4	β-CA, α-TOC and RA	β-CA : 10 α-TOC: 10 RA:10	β-CA: 3.5 α-TOC:3.1 RA: 0.5	β-CA :35 α-TOC:30.7 RA:5.0	1.4±0.2	0.6 ±0.1
PLGA1	PLGA 0.8	β-CA and α-TOC	β-CA : 10 α-TOC: 10	β-CA :3.1 α-TOC:	β-CA :61 α-TOC:76.6	1.2 ± 0.2	0.3 ± 0.1
PLGA2	PLGA 0.8	β-CA, α-TOC and RA	β-CA : 10 α-TOC: 10 RA:	β-CA:2.7 α-TOC:2.8 RA:0.6	β-CA :53.0 α-TOC:52.6 RA:12.1	1.3 ± 0.2	0.5 ±0.1

sd standard deviation

In the case of PLA, (PLA1 and PLA2) 400 mg of polymer were always solubilized in the oily phase. In PLA1 microcarriers of 0.3± 0.1 µm were obtained, Table

IV.18, with 61.8% of β-CA and 53.3% of α-TOC encapsulation efficiency. In figure IV.19, a picture of the PLA1 emulsion droplet (Figure IV.19 A) is shown together with a SEM image of the microspheres produced (Figure IV.19 B). The final product has a homogeneous particle size and spherical shape distribution. In this work, the stirring conditions were intensified compared with previous works.[131, 165] The new formulation conditions allowed a considerable reduction of the particle size mean diameter (0.3 ±0.1 *vs* 1.4 ±0.5 μm) maintaining a good EE% (62% *vs* 72%).[131]

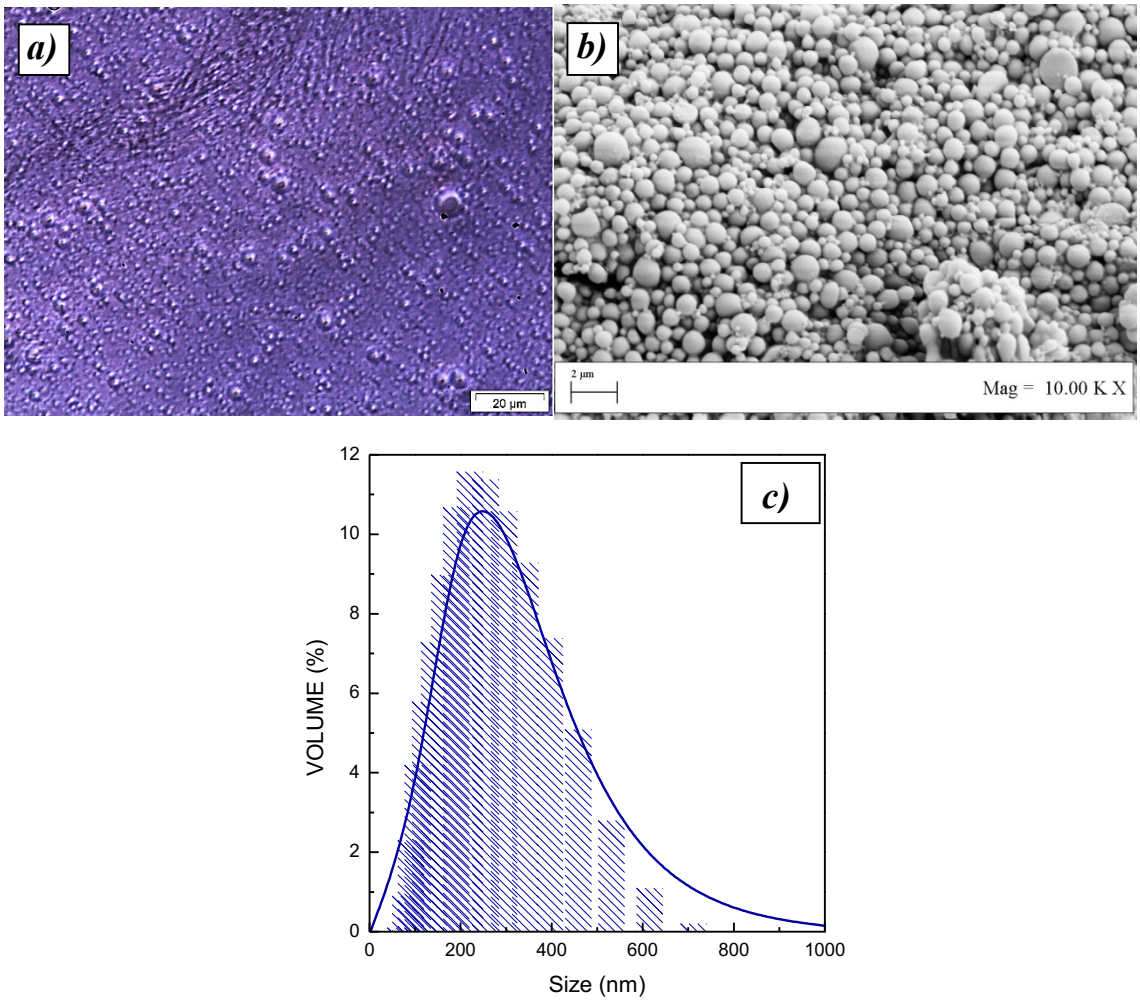


Figure IV.19. β-carotene, α-tocopherol PLA1 experiment. a) Emulsion optical microscope image; b) SEM image of the produced microspheres; c) droplet and particle size distribution.

The encapsulation of β-CA, α-TOC was also tested with the polymer PLGA (Table IV.18). The PLGA1 emulsion, compared with the previous results,[131] reduced the microspheres particle size diameter from 2.0 ±0.6 μm to 0.3 ±0.1 μm (PLGA1), maintaining the β-CA EE% (58% *vs* 62%). The amount of PLGA polymer was increased up to 800 mg in the oily phase (PLGA1 and PLGA2) regarding the PLA emulsion formula (PLA1 and PLA2) because the molecular weight of PLGA was three times lower. The

increased amount was required to obtain comparable EE% results among polymers since a higher molecular weight provides higher active entrapment.

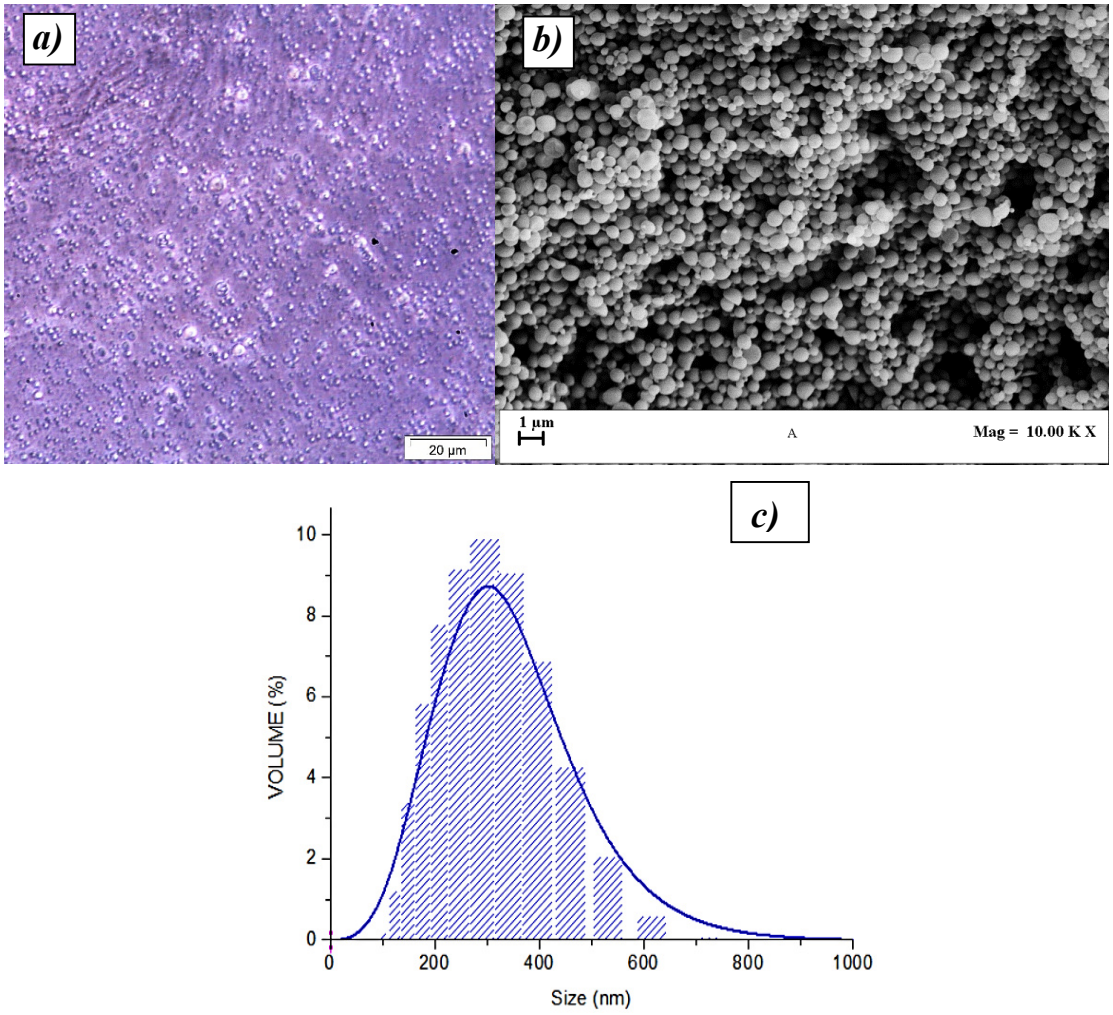


Figure IV.20. β-carotene, α-tocopherol PLGA a) emulsion optical microscope image, b) SEM image of the produced microspheres and their droplet and c) particle size distribution.

Because of the RA low encapsulation potential (section 2.1), further investigations were carried out in order to modify the overall system composition by suspending RA along with β-CA and α-TOC into $o_1/o_2/w$ emulsion formulations, of PLA and PLGA, since the presence of the two other actives may improve their entrapment into the microspheres produced by SEE[171]. Besides, RA may have a potential synergetic effect as antioxidant into the formula stabilizing the final antioxidant activity.[171]

The inclusion of RA into the PLA and PLGA emulsions increased the produced microspheres. Additionally, the inclusion of RA into PLA microspheres did not improve its EE% and caused the reduction of β-CA EE% from 62% to 35%, and of α-TOC from

53.3 to 30.7. Nevertheless, when the co-encapsulation was performed into PLGA, RA EE% was 12% and β-CA and α-TOC EE% were maintained at 53.0% and 52.6%.

As β-CA is an interesting antioxidant compound because of its bioactivity but is highly labile under light and heat, other authors have proposed its encapsulation into different polymeric carriers. In order to prevent molecule degradation, different polymers were studied as carriers for β-CA encapsulation such as, tapioca starch, maltodextrin or oleoresin all using spray drying technology,[162–164] or casein using solid lipid nanoparticles (SLNs) protocol and galactan by evaporation technology.[165,166] Faria et al. [162] proposed its encapsulation with spry-drying technique into gum Arabic and maltodextrines to be applied by the food and cosmetic industries. The average diameter of the produced particles obtained with this technique was between 7 and 10 μm and they achieved a 64% and 95% of encapsulation efficiency into maltodextrine and gum arabic respectively. Loksuwan did also apply the spry drying technique to encapsulate this natural antioxidant into modified and native tapioca. Modified tapioca starch had a wider particle size distribution than its native form but also a higher encapsulation efficiency, 82.18% *vs* 68.35%.[164] Nevertheless, these high encapsulation efficiencies of β-CA obtained with spray drying, correspond with a co-precipitation not an encapsulation.

The content of β-CA into the microcapsules of β-CA+ α-TOC produced by Odierna (2014) [165] after 2 years of preservation at 4 °C in the dark was measured too. Pure β-CA needs freezing conditions for its conservation. Odierna (2014)[165] formulated PLA and PLGA particles with the same procedure as PLA1 and PLGA1 of the present work. They varied only de speed of agitation of the emulsion and obtained microcapsules larger than the ones obtained in the present work (PLA: 1.5±0.5 μm *vs* 0.3±0.1 μm, PLGA: 2.0±0.6 μm *vs* 0.3±0.1 μm), with an encapsulation efficiencies of β-CA into PLA and PLGA of 72% and 52% respectively. After their evaluation 2 years later, these values that were reduced to 22% and 30% (reduction of 32% with PLGA and 52% with PLA), showing a better protection with PLGA.

Odierna (2014)[165] also evaluated the remaining β-CA into the capsules after their exposure to UV radiation for 3 days, and observed that, while pure β-CA degraded down to 17%, the capsules of PLA and PLGA kept the 78.3% and 83.2% of the active inside. After ten days of forced light oxidation, pure β-CA degraded completely while the PLA and PLGA capsules still contained 4% and 3%. The presence of the antioxidant α-TOC

in the capsules along with β-CA prevented its degradation and after ten days of exposure of the PLA and PLGA capsules containing both actives, the remaining β-CA was 22% and 30% respectively.

Both studies confirmed that encapsulation into these two biopolymers increases β-CA protection against oxidation being the particles of β-CA+ α-TOC into PLGA the most stable carrier formulation.

2.3. Microspheres antioxidant activity

The co-encapsulation of β-CA with α-TOC has been reported to block the oxygen radicals chain reaction, prolonging its shelf life in colloidal lipid particles of fat-in-water dispersions.[177] In addition, solid lipid microparticles with both actives presented more antioxidant stability too in terms of β-CA loading.[168]

In order to determine the functionality of the capsules after their exposition to the experimental encapsulation conditions, their antioxidant activity was assayed against DPPH radical, applying the method described in chapter III. 2.6. According to β-CA, α-TOC and RA calibration curve *vs* DPPH and its encapsulation efficiency in each SEE product (Table IV.18), the theoretical antioxidant activity of microspheres was determined by interpolation of the encapsulation efficiency result in the calibration curve and compared with the one obtained experimentally, which was measured immediately after their formulation. Antioxidant results are represented in Figure IV.21.

PLA and PLGA microspheres of β-CA+ α-TOC theoretical IC_{50}, according to the EE% 62% in both cases, was 2.7 mg/mL and 5.6 mg/mL respectively, but the measured antioxidant activity was always higher, 1.8 mg/mL and 3.0 mg/mL respectively, as it can be seen in Figure IV.21. When RA was included in the formulation, because of the reduction of β-CA loading, a higher theoretical IC_{50} was expected, 4.2 mg/mL and 6.3 mg/mL for PLA and PLGA microcapsules, respectively. Nevertheless, as it can be observed in in Figure IV.21, the measured activity or the three antioxidant in both, PLA and PLGA, was higher than expected, again probably because of the combination of the three actives, β-CA+α-TOC+RA even at lower concentrations. The particles that showed the highest antioxidant activity were PLA microcapsules of β-CA+ α-TOC.

The antioxidant stability of β-CA was also observed after its encapsulation into stearyl ferulate-based solid lipid nanoparticles. [166] The co-encapsulation with other

antioxidant excipients, such as α-tocopherol and ascorbic acid was reported too to provide a better protective effect against oxidation.[169168]

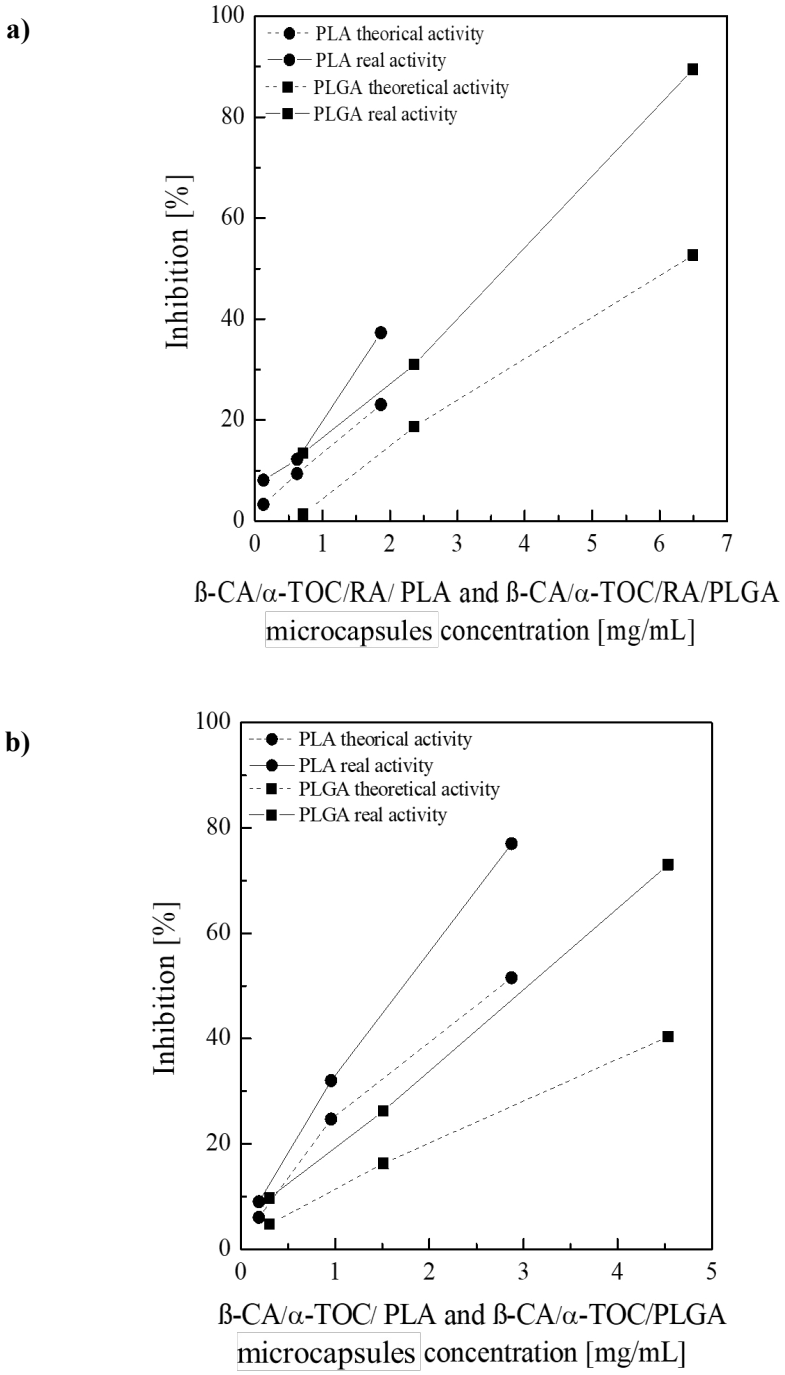
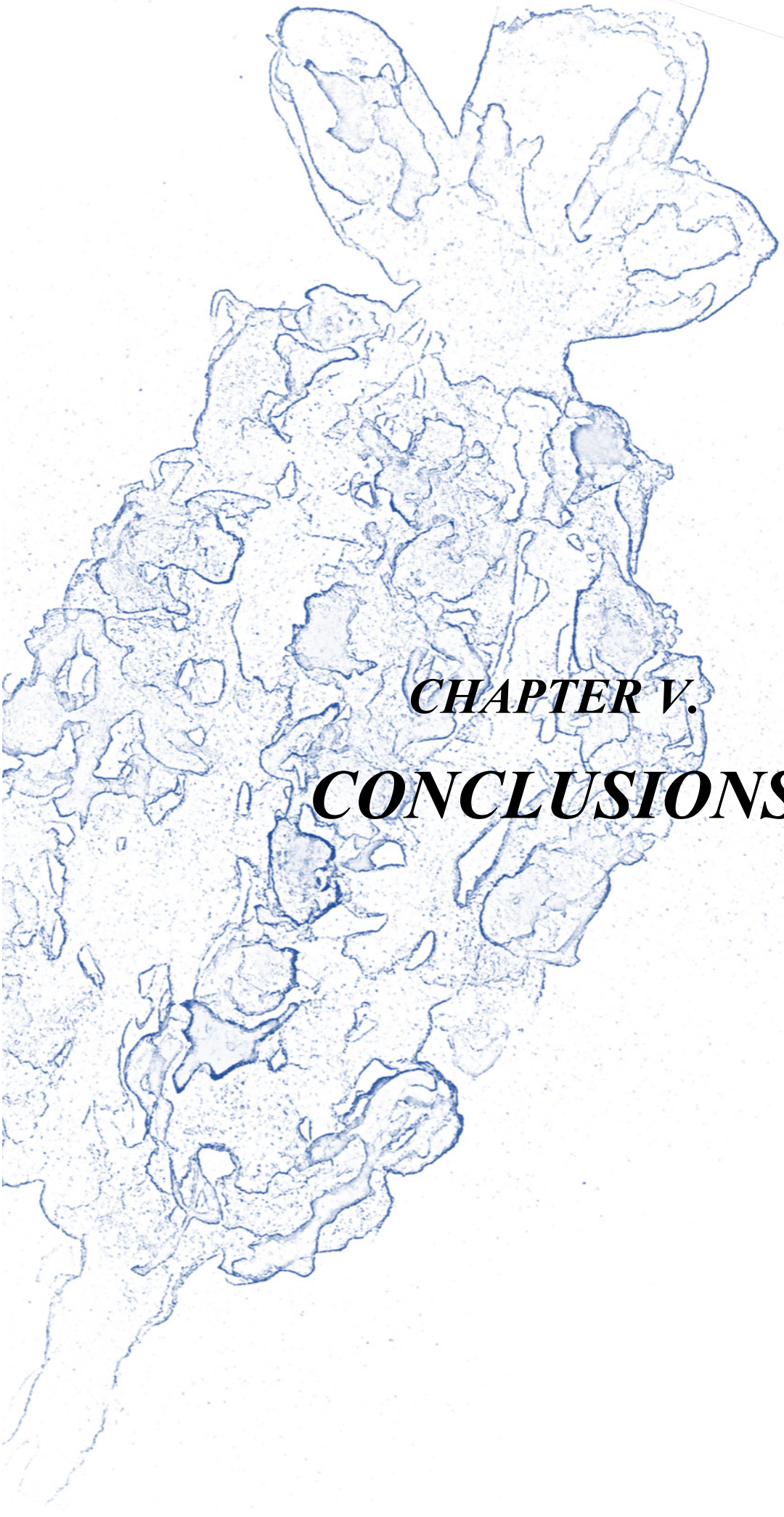


Figure IV.21. % DPPH Inhibition *vs* microcapsules concentration (mg/mL) for and α-TOC, a) PLA and PLGA nanocapsules loaded with β-CA, α-TOC and RA and b) PLA and PLGA nanocapsules loaded with β-CA



CHAPTER V.

CONCLUSIONS

As previously exposed, the obtaining of concentrated products from the Spanish autochthonous *Lavandula luisieri* and the study of the encapsulation of its bioactives using supercritical technologies was pursued in this work. Once the results have been presented and considering the proposed aims of this work, the following conclusions can be drawn.

First. The variation of the experimental conditions of pressure and $scCO_2$ flow rate in the range 80-150 bar and 10-30 g/min respectively, at fixed ethanolic solution flow rate (0.45ml/min) and temperature (40℃) in the Supercritical Antisolvent Fractionation of *L. luisieri* ethanolic extract, influenced on the yields recovered in the precipitation vessel and downstream vessel. For a given pressure, the PV and DV yields increased with the $scCO_2$ flow rate. In addition, for a fixed value of $scCO_2$ flow rate, PV yield increases at lower pressure. The highest total yield obtained in this work was 82.6% at 115 bar 30 g/min.

Second. The Supercritical Antisolvent Fractionation process caused the complete retention of rosmarinic acid into the precipitation vessel under all experimental conditions tested, concentrating it, regarding the initial ethanolic extract, up to 2.26 times at 140 bar and 27 g/min. This behaviour was not observed for the triterpenes OA and UA since the triterpenes oleanolic and ursolic acids distributed between both fractions, PV and DV, depending on the experimental conditions.

Third. The Central Composite Design based on a Response Surface Methodology allowed the statistical design of 11 aleatory experiments with three central replicates for the assessment of the conditions of pressure and CO_2 flow rate of the Supercritical Antisolvent Fractionation of *L. luisieri* ethanolic extract in the range of 80-150 bar and 10-30 g/min, respectively. Furthermore, the statistical analysis of the results predicted a higher precipitate yield in PV at the lowest values of pressure and at the highest of $scCO_2$ flow rate; a maximum recovery in DV at higher values of $scCO_2$ flow and pressure, and an increase in global recovery yield with $scCO_2$ flow rate with independence from the pressure.

Fourth. According to the statistical analysis of the composition for the three actives of interest, it is predicted that the highest simultaneous concentration of RA, OA and UA in the PV can be obtained at the lowest or highest combination of values of *pressure* and $scCO_2$ flow rate into the range assayed.

Fifth. For a maximum concentration of the three actives in PV and maximum recovery in PV, DV and total yield, the predicted optimum conditions of pressure and $scCO_2$ flow rate, were 130 bar and 30 g/min. At these optimum conditions, the total compound PV and DV yields were 32.7% and 50.8% respectively, and the concentration of actives was 1.8 times higher than the initial ethanolic extract.

Sixth. The antimicrobial properties of the ethanolic extract and its supercritical fractions at the optimized conditions were determined against *Listeria monocytogenes, Enterococcus faecium, Staphylococcus aureus, Salmonella* Typhimurium and *Escherichia coli* with the microdilution broth method. The assay showed that three gram-positive bacterial strains were more sensitive to *L. luisieri* ethanolic extract and its supercritical fractions. While, *L. monocytogenes* was sensitive to all extracts assayed, having inhibitory and bactericidal properties, PV the most active with a MIC of 241 µg/mL and an MBC of 483 µg/mL. *E. faecium* and *S. aureus* sensitivity was limited, being the concentrated PV fraction again the more active.

Seventh. To determine the compound responsible for the antimicrobial activity, the pure actives of interest in this work were tested. Pure ursolic acid showed antimicrobial activity against the three gram-positive strains showing to be more effective against *L. monocytogenes* and *E. faecium* (MIC: 32.9-65.8 µg/mL and MBC: 65.8 µg/mL). Nevertheless, rosmarinic acid did not showed any inhibitory or bactericidal activities. These results revealed that the triterpenes from the *L. luisieri* extracts were responsible for the antimicrobial activity.

Eight. The antimicrobial evaluation of *L. luisieri* essential oil performed with the macrodilution broth method on the cited bacteria showed an important antimicrobial activity against the five strains; nevertheless, the sensitivity of gram-positive was again higher than that of gram-negative, with a MIC of 0.5 µg/mL and MBC under 3 µg/mL.

Ninth. The antioxidant activity evaluation of the ethanolic extract and its supercritical fractions obtained at the optimized conditions against the free radical DPPH showed that, the concentrated PV fraction was again the most active, with an IC_{50} of 16.2 µg/mL. In in this case the antioxidant assays performed with the pure active, showed that the increase of antioxidant activity was a consequence of rosmarinic acid enrichment in this fraction.

Tenth. All the bioactivity tests showed that the SAF process experimental conditions are mild enough to allow the concentration of the followed actives while maintaining their antioxidant and antimicrobial activities.

Eleventh. The encapsulation of rosmarinic acid as model of the *L. luisieri* extract was proposed into PLA and PLGA polymers. The Supercritical Emulsion Extraction did not allow the encapsulation of this active in any of the seven experiments performed, probably because its co-extraction along with the organic solvent that constitutes the oily phase of the emulsion. Nevertheless, this technology was successful for the encapsulation of β-CA and α-TOC from double emulsions in both polymers and in both experiments performed. The inclusion of RA into β-CA and α-TOC formulations increased the encapsulation efficiency of RA up to 12% into PLGA, but decreased the encapsulation of the other antioxidants, β-CA from 62% to 35%, and α-TOC from 53% to 31%. Besides, with the use of the traditional Solvent Evaporation allowed its encapsulation up to 9%.

Twelfth. The microcapsules mean diameter were reduced regarding to the emulsion droplets mean size with both, the traditional Solvent Evaporation and the Supercritical Emulsion Extraction. Nevertheless, the supercritical process reduction was more pronounced and with a homogeneous particle distribution. The difference among obtained particle size to both methods is observable in the encapsulation experiments performed with RA and is a consequence of the inherent speed at which the solvent is extracted.

Thirteenth. The antioxidant activity of PLA and PLGA microcapsules containing β-CA and α-TOC and RA, β-CA and α-TOC against the free radical DPPH showed that the Supercritical Emulsion Extraction did not degraded the actives properties. According to the encapsulation efficiency of β-CA, the obtained antioxidant activity was higher than the expected theoretical value, because of the influence of the other actives included into the formulation.

Fourteenth. The evaluation of PLA and PLGA particles containing β-CA after 2 years of conservation under refrigeration conditions showed that the encapsulation protected the actives against degradation. Although with both formulations the encapsulation efficiency of β-CA was reduced, 32% with PLGA and 52% with PLA, they provided protection against oxidation, being the particles of β-CA+ α-TOC into PLGA the most stable carrier formulation.

Fifteenth. Two isocratic reverse phase chromatographic methods were optimised. The first one for the separation, identification and quantification of the three non-volatile actives of interest, rosmarinic, oleanolic and ursolic acids of *L. luisieri* ethanolic extract. Under this optimal experimental condition, the separation of ursolic and oleanolic acid, two triterpenes with the same molecular weight and differentiated by the position of one -H group, was possible. And the second one allowed the extraction and the chromatographic separation of rosmarinic acid, β-CA and α-TOC from the PLA and PLGA capsules, and the quantification and determination of the encapsulation efficiency of the three antioxidants.

The application of supercritical CO_2 as antisolvent within the Supercritical Antisolvent Fractionation and Supercritical Emulsion Extraction allowed the obtaining of two products: i) a concentrated powder enriched in rosmarinic, oleanolic and ursolic acids, with potentiated antimicrobial and antioxidant activities, and ii) stable microcapsules of three co-encapsulated natural actives with antioxidant properties, with potential use in the pharmaceutical, cosmetical or food fields.

References

1. De Vos, P. European Materia Medica in Historical Texts: Longevity of a Tradition and Implications for Future Use. *J Ethnopharmacol* **132**, 28–47 (2010).
2. Shapiro, A. K. The placebo effect in the history of medical treatment: implications for psychiatry. *Am J Psychiatry* **116**, 298–304 (1959).
3. Gupta, R., Gabrielsen, B. & Ferguson, S. M. Nature's medicines: traditional knowledge and intellectual property management. Case studies from the National Institutes of Health (NIH), USA. *Curr Drug Discov Technol* **2**, 203–219 (2005).
4. Patwardhan, B. & Vaidya, A. D. B. Natural products drug discovery: Accelerating the clinical candidate development using reverse pharmacology approaches. *IJEB Vol.48(03) [March 2010]* (2010).
5. Traditional Medicine. *WHO | Regional Office for Africa* https://www.afro.who.int/health-topics/traditional-medicine.
6. WHO | Publications and databases. *WHO* http://www.who.int/traditional-complementary-integrative-medicine/publications/en/.
7. Wink, M. Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. *Theoret. Appl. Genetics* **75**, 225–233 (1988).
8. Harborne, J. B. Classes and functions of secondary products from plants. in *Chemicals from Plants* 1–25 (WORLD SCIENTIFIC / IMPERIAL COLLEGE PRESS, 1999). doi:10.1142/9789812817273_0001.
9. Harvey, A. L. Medicines from nature: are natural products still relevant to drug discovery? *Trends Pharmacol. Sci.* **20**, 196–198 (1999).
10. Kalim, M. D., Bhattacharyya, D., Banerjee, A. & Chattopadhyay, S. Oxidative DNA damage preventive activity and antioxidant potential of plants used in Unani system of medicine. *BMC Complement Altern Med* **10**, 77 (2010).
11. Gyawali, R. & Ibrahim, S. A. Natural products as antimicrobial agents. *Food Control* **46**, 412–429 (2014).

12. BOE.es - Documento DOUE-L-2008-82640. https://www.boe.es/buscar/doc.php?id=DOUE-L-2008-82640.
13. Friedman, M. Chemistry, Antimicrobial Mechanisms, and Antibiotic Activities of Cinnamaldehyde against Pathogenic Bacteria in Animal Feeds and Human Foods. *J. Agric. Food Chem.* **65**, 10406–10423 (2017).
14. Tzima, K., Brunton, N. P. & Rai, D. K. Qualitative and Quantitative Analysis of Polyphenols in Lamiaceae Plants—A Review. *Plants* **7**, 25 (2018).
15. Lis-Balchin, M. *Lavender: The Genus Lavandula*. (CRC Press, 2003).
16. María Isabel García Vallejo. Aceites esenciales de las Lavandulas ibéricas ensayo de la quimiotaxonomía. (Universidad Complutense de Madrid, 1992).
17. Aprotosoaie, A. C., Gille, E., Trifan, A., Luca, V. S. & Miron, A. Essential oils of Lavandula genus: a systematic review of their chemistry. *Phytochem Rev* **16**, 761–799 (2017).
18. Basch, E. *et al.* Lavender (Lavandula angustifolia Miller). *Journal of Herbal Pharmacotherapy* **4**, 63–78 (2004).
19. Herraiz-Peñalver, D. *et al.* Chemical characterization of Lavandula latifolia Medik. essential oil from Spanish wild populations. *Biochemical Systematics and Ecology* **46**, 59–68 (2013).
20. Matos, F. *et al.* Antioxidant Capacity of the Essential Oils From Lavandula luisieri, L. stoechas subsp. lusitanica, L. stoechas subsp. lusitanica x L. luisieri and L. viridis Grown in Algarve (Portugal). *Journal of Essential Oil Research* **21**, 327–336 (2009).
21. Fismer, K. L. & Pilkington, K. Lavender and sleep: A systematic review of the evidence. *European Journal of Integrative Medicine* **4**, e436–e447 (2012).
22. Zuzarte, M. *et al.* Lavandula luisieri essential oil as a source of antifungal drugs. *Food Chem* **135**, 1505–1510 (2012).
23. Philip D. Cantino. Evidence for a polyphyletic origin of the labiatae. *Annals of the Missouri Botanical Garden* **79**, 361–379 (1992).

24. Medicinally Potential Plants of Labiatae (Lamiaceae) Family: An Overview. *Science Alert* https://scialert.net/fulltext/?doi=rjmp.2012.203.213 doi:10.3923/rjmp.2012.203.213.

25. Taxonomy browser (Lavandula stoechas subsp. luisieri). https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=1461649.

26. Eisner, T., Deyrup, M., Jacobs, R. & Meinwald, J. Necrodols: Anti-insectan terpenes from defensive secretion of carrion beetle (Necrodes surinamensis). *J Chem Ecol* **12**, 1407–1415 (1986).

27. M.I. García-Vallejo, M. C., García-Vallejo, Sanz, J., Bernabe, M. & Velasco-Negueruela, A. Necrodane (1,2,2,3,4-pentamethylcyclopentane) derivatives in Lavandula luisieri, new compounds to the plant kingdom. *Phytochemistry* **36**, 43–45 (1994).

28. Figadère, B. A. *et al.* trans-α-Necrodyl isobutyrate, the sex pheromone of the grape mealybug, Pseudococcus maritimus. *Tetrahedron Letters* **48**, 8434–8437.

29. Baldovini, N., Lavoine-Hanneguelle, S., Ferrando, G., Dusart, G. & Lizzani-Cuvelier, L. Necrodane monoterpenoids from Lavandula luisieri. *Phytochemistry* **66**, 1651–1655 (2005).

30. Lavoine-Hanneguelle, S. & Casabianca, H. New Compounds from the Essential Oil and Absolute of Lavandula luisieri L. *Journal of Essential Oil Research* **16**, 445–448 (2004).

31. Julio, L. F. *et al.* Phytotoxic and Nematicidal Components of Lavandula luisieri. *J. Nat. Prod.* **79**, 261–266 (2016).

32. Sanz, J., Soria, A. C. & García-Vallejo, M. C. Analysis of volatile components of Lavandula luisieri L. by direct thermal desorption–gas chromatography–mass spectrometry. *Journal of Chromatography A* **1024**, 139–146 (2004).

33. González-Coloma, A., Martín-Benito, D., Mohamed, N., García-Vallejo, M. C. & Soria, A. C. Antifeedant effects and chemical composition of essential oils from different populations of Lavandula luisieri L. *Biochemical Systematics and Ecology* **34**, 609–616 (2006).

34. Delgado, F. M. G. *et al.* Seed germination and essential oil of Lavandula luisieri from Central Eastern Portugal. *1st International Symposium on Labiatae, SanRemo, 22 a 25 February* 283–287 (2005).

35. Roller, S., Ernest, N. & Buckle, J. The antimicrobial activity of high-necrodane and other lavender oils on methicillin-sensitive and -resistant Staphylococcus aureus (MSSA and MRSA). *J Altern Complement Med* **15**, 275–279 (2009).

36. González-Coloma, A. *et al.* Chemical and biological profiles of Lavandula luisieri essential oils from western Iberia Peninsula populations. *Biochemical Systematics and Ecology* **39**, 1–8 (2011).

37. Videira, R. *et al.* A necrodane monoterpenoid from Lavandula luisieri essential oil as a cell-permeable inhibitor of BACE-1, the β-secretase in Alzheimer's disease. *Flavour and Fragrance Journal* **28**, 380–388 (2013).

38. Rufino, A. T. *et al.* Differential effects of the essential oils of Lavandula luisieri and Eryngium duriaei subsp. juresianum in cell models of two chronic inflammatory diseases. *Pharm Biol* 1–11 (2015) doi:10.3109/13880209.2014.970701.

39. Dias, N. *et al.* Oxygenated monoterpenes-rich volatile oils as potential antifungal agents for dermatophytes. *Natural Product Research* **31**, 460–464 (2017).

40. Arantes, S. *et al.* Pharmacological and Toxicological Studies of Essential Oil of Lavandula stoechas subsp. luisieri. *Planta Med* **82**, 1266–1273 (2016).

41. Pombal, S. *et al.* Antibacterial and antioxidant activity of Portuguese Lavandula luisieri (Rozeira) Rivas-Martinez and its relation with their chemical composition. *Springerplus* **5**, (2016).

42. Andrés, M. F. *et al.* Nematicidal potential of hydrolates from the semi industrial vapor-pressure extraction of Spanish aromatic plants. *Environ Sci Pollut Res Int* **25**, 29834–29840 (2018).

43. Costa, S., Cavadas, C., Cavaleiro, C., Salgueiro, L. & do Céu Sousa, M. In vitro susceptibility of Trypanosoma brucei brucei to selected essential oils and their major components. *Experimental Parasitology* **190**, 34–40 (2018).

44. Queiroga, M. C., Coelho, M. P., Arantes, S. M., Potes, M. E. & Martins, M. R. Antimicrobial Activity of Essential Oils of Lamiaceae Aromatic Spices Towards Sheep mastitis-Causing Staphylococcus aureus and Staphylococcus epidermidis. *Journal of Essential Oil Bearing Plants* **21**, 1155–1165 (2018).

45. Julio, L. F. *et al.* Comparative chemistry and insect antifeedant effects of conventional (Clevenger and Soxhlet) and supercritical extracts (CO2) of two Lavandula luisieri populations. *Industrial Crops and Products* **58**, 25–30 (2014).

46. Baptista, R. *et al.* Antioxidant and Antimycotic Activities of Two Native Lavandula Species from Portugal. *Evidence-Based Complementary and Alternative Medicine* https://www.hindawi.com/journals/ecam/2015/570521/abs/ (2015) doi:10.1155/2015/570521.

47. Nunes, S. *et al.* Therapeutic and nutraceutical potential of rosmarinic acid—Cytoprotective properties and pharmacokinetic profile. *Critical Reviews in Food Science and Nutrition* **57**, 1799–1806 (2017).

48. Akula, R. & Ravishankar, G. A. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior* **6**, 1720–1731 (2011).

49. Nunes, R. *et al.* Antibacterial, antioxidant and anti-proliferative properties and zinc content of five south Portugal herbs. *Pharmaceutical Biology* **55**, 114–123 (2017).

50. Godzien, J. *et al.* Metabolomic approach with LC-QTOF to study the effect of a nutraceutical treatment on urine of diabetic rats. *J. Proteome Res.* **10**, 837–844 (2011).

51. Ribeiro, M. A., Bernardo-Gil, M. G. & Esquível, M. M. Melissa officinalis, L.: study of antioxidant activity in supercritical residues. *The Journal of Supercritical Fluids* **21**, 51–60 (2001).

52. Petersen, M. & Simmonds, M. S. J. Rosmarinic acid. *Phytochemistry* **62**, 121–125 (2003).

53. Jesus, J. A., Lago, J. H. G., Laurenti, M. D., Yamamoto, E. S. & Passero, L. F. D. Antimicrobial Activity of Oleanolic and Ursolic Acids: An Update. *Evidence-Based Complementary and Alternative Medicine* https://www.hindawi.com/journals/ecam/2015/620472/ (2015) doi:10.1155/2015/620472.

54. Wolska, K. I., Grudniak, A. M., Fiecek, B., Kraczkiewicz-Dowjat, A. & Kurek, A. Antibacterial activity of oleanolic and ursolic acids and their derivatives. *cent.eur.j.biol.* **5**, 543–553 (2010).

55. Zhang, Y. *et al.* Degradation Study of Carnosic Acid, Carnosol, Rosmarinic Acid, and Rosemary Extract (Rosmarinus officinalis L.) Assessed Using HPLC. *J. Agric. Food Chem.* **60**, 9305–9314 (2012).

56. Lai, B. *et al.* Evaluation of the antimicrobial activity in species of a Portuguese Montado ecosystem against multidrug resistant pathogens. *JMPR* **6**, 2381–2387 (2012).

57. Martínez, J. L. Natural Antibiotic Resistance and Contamination by Antibiotic Resistance Determinants: The Two Ages in the Evolution of Resistance to Antimicrobials. *Front Microbiol* **3**, (2012).

58. Baptista, P. V. *et al.* Nano-Strategies to Fight Multidrug Resistant Bacteria—"A Battle of the Titans". *Front Microbiol* **9**, (2018).

59. kugleta. What is Horizon 2020? *Horizon 2020 - European Commission* https://ec.europa.eu/programmes/horizon2020/en/what-horizon-2020 (2013).

60. Nychas, G. J. E. Natural antimicrobials from plants. in *New Methods of Food Preservation* (ed. Gould, G. W.) 58–89 (Springer US, 1995). doi:10.1007/978-1-4615-2105-1_4.

61. Musilova, L., Ridl, J., Polivkova, M., Macek, T. & Uhlik, O. Effects of Secondary Plant Metabolites on Microbial Populations: Changes in Community Structure and Metabolic Activity in Contaminated Environments. *Int J Mol Sci* **17**, (2016).

62. Lai, P. K. & Roy, J. Antimicrobial and chemopreventive properties of herbs and spices. *Curr. Med. Chem.* **11**, 1451–1460 (2004).

63. Mittler, R. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7**, 405–410 (2002).

64. Santos-Sánchez, N. F., Salas-Coronado, R., Valadez-Blanco, R., Hernández-Carlos, B. & Guadarrama-Mendoza, P. C. Natural antioxidant extracts as food preservatives. *Acta Sci Pol Technol Aliment* **16**, 361–370 (2017).

65. Halliwell, B. Biochemistry of oxidative stress. *Biochemical Society Transactions* **35**, 1147–1150 (2007).

66. Alam, M. N., Bristi, N. J. & Rafiquzzaman, M. Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharm J* **21**, 143–152 (2013).

67. Lobo, V., Patil, A., Phatak, A. & Chandra, N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev* **4**, 118–126 (2010).

68. Kasote, D. M., Katyare, S. S., Hegde, M. V. & Bae, H. Significance of Antioxidant Potential of Plants and its Relevance to Therapeutic Applications. *Int J Biol Sci* **11**, 982–991 (2015).

69. Prior, R. L., Wu, X. & Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **53**, 4290–4302 (2005).

70. Julio, L. F. *et al.* Ixodicidal compounds from pre-domesticated Lavandula luisieri. *Industrial Crops and Products* **110**, 83–87 (2017).

71. Azwanida, N. A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. in (2015). doi:10.4172/2167-0412.1000196.

72. Chamorro, E. R., Zambón, S. N., Morales, W. G., Sequeira, A. F. & Velasco, G. A. Study of the Chemical Composition of Essential Oils by Gas Chromatography. *Gas Chromatography in Plant Science, Wine Technology, Toxicology and Some Specific Applications* (2012) doi:10.5772/33201.

73. Tisserand, R. & Young, R. 2 - Essential oil composition. in *Essential Oil Safety (Second Edition)* (eds. Tisserand, R. & Young, R.) 5–22 (Churchill Livingstone, 2014). doi:10.1016/B978-0-443-06241-4.00002-3.

74. Beoletto, V. G., de las Mercedes Oliva, M., Marioli, J. M., Carezzano, M. E. & Demo, M. S. Chapter 14 - Antimicrobial Natural Products Against Bacterial Biofilms. in *Antibiotic Resistance* (eds. Kon, K. & Rai, M.) 291–307 (Academic Press, 2016). doi:10.1016/B978-0-12-803642-6.00014-9.

75. EDQM - European Directorate for the Quality of Medicines |. https://www.edqm.eu/en.

76. Dilworth, L. L., Riley, C. K. & Stennett, D. K. Chapter 5 - Plant Constituents: Carbohydrates, Oils, Resins, Balsams, and Plant Hormones. in *Pharmacognosy* (eds. Badal, S. & Delgoda, R.) 61–80 (Academic Press, 2017). doi:10.1016/B978-0-12-802104-0.00005-6.

77. Zhang, Q.-W., Lin, L.-G. & Ye, W.-C. Techniques for extraction and isolation of natural products: a comprehensive review. *Chin Med* **13**, (2018).

78. da Silva, R. P. F. F., Rocha-Santos, T. A. P. & Duarte, A. C. Supercritical fluid extraction of bioactive compounds. *TrAC Trends in Analytical Chemistry* **76**, 40–51 (2016).

79. Poliakoff, M., Fitzpatrick, J. M., Farren, T. R. & Anastas, P. T. Green Chemistry: Science and Politics of Change. *Science* **297**, 807–810 (2002).

80. Declaration of the United Nations Conference on the Human Environment - A/CONF.48/14/Rev.1 Chapter I - UN Documents: Gathering a body of global agreements. http://www.un-documents.net/unchedec.htm.

81. Anastas, P. & Warner, J. *Green Chemistry: Theory and Practice*. (Oxford University Press, 2000).

82. Knez, Ž. *et al.* Industrial applications of supercritical fluids: A review. *Energy* **77**, 235–243 (2014).

83. Parhi, R. & Suresh, P. Supercritical Fluid Technology: A Review. *JAPST* **1**, 13 (2013).

84. Clifford, A. A. & Williams, J. R. Introduction to Supercritical Fluids and Their Applications. in *Supercritical Fluid Methods and Protocols* (eds. Williams, J. R. & Clifford, A. A.) 1–16 (Humana Press, 2000). doi:10.1385/1-59259-030-6:1.

85. Martin, A. N. & Sinko, P. J. *Martin's Physical Pharmacy and Pharmaceutical Sciences: Physical Chemical and Biopharmaceutical Principles in the Pharmaceutical Sciences*. (Lippincott Williams & Wilkins, 2011).

86. Poling, B. E., Prausnitz, J. M. & O'Connell, J. P. *Properties of Gases and Liquids, Fifth Edition*. (McGraw-Hill Education: New York, Chicago, San Francisco, Athens, London, Madrid, Mexico City, Milan, New Delhi, Singapore, Sydney, Toronto, 2001).

87. Budisa, N. & Schulze-Makuch, D. Supercritical Carbon Dioxide and Its Potential as a Life-Sustaining Solvent in a Planetary Environment. *Life* **4**, 331–340 (2014).

88. Beckman, E. J. Supercritical and near-critical CO2 in green chemical synthesis and processing. *The Journal of Supercritical Fluids* **28**, 121–191 (2004).

89. Reverchon, E. & De Marco, I. Supercritical fluid extraction and fractionation of natural matter. *The Journal of Supercritical Fluids* **38**, 146–166 (2006).

90. Zhang, J. *et al.* Sterilization using high-pressure carbon dioxide. *The Journal of Supercritical Fluids* **38**, 354–372 (2006).

91. Supercritical CO2: A Green Solvent - Chemical Engineering. https://www.chemengonline.com/supercritical-co2-a-green-solvent/?printmode=1.

92. Sharif, K. M. *et al.* Experimental design of supercritical fluid extraction – A review. *Journal of Food Engineering* **124**, 105–116 (2014).

93. Özer, E. Ö., İin, S. P., Akman, U. & Hortaçsu, Ö. Supercritical carbon dioxide extraction of spearmint oil from mint-plant leaves. *The Canadian Journal of Chemical Engineering* **74**, 920–928 (1996).

94. Marongiu, B. *et al.* Antioxidant activity of supercritical extract of Melissa officinalis subsp. officinalis and Melissa officinalis subsp. inodora. *Phytother Res* **18**, 789–792 (2004).

95. Cao, X. & Ito, Y. Supercritical fluid extraction of grape seed oil and subsequent separation of free fatty acids by high-speed counter-current chromatography. *J Chromatogr A* **1021**, 117–124 (2003).

96. Dias, A. M. A. *et al.* Development of natural-based wound dressings impregnated with bioactive compounds and using supercritical carbon dioxide. *International Journal of Pharmaceutics* **408**, 9–19 (2011).

97. Milovanovic, S., Stamenic, M., Markovic, D., Radetic, M. & Zizovic, I. Solubility of thymol in supercritical carbon dioxide and its impregnation on cotton gauze. *The Journal of Supercritical Fluids* **84**, 173–181 (2013).

98. Daood, H. *et al.* Extraction of pungent spice paprika by supercritical carbon dioxide and subcritical propane. *The Journal of Supercritical Fluids* **23**, 143–152 (2002).

99. Cao, X. L., Tian, Y., Zhang, T. Y. & Ito, Y. Supercritical fluid extraction of catechins from Cratoxylum prunifolium dyer and subsequent purification by high-speed counter-current chromatography. *J Chromatogr A* **898**, 75–81 (2000).

100. Zhannan, Y., Shiqiong, L., Quancai, P., Chao, Z. & Zhengwen, Y. GC-MS Analysis of the Essential Oil of Coral Ginger (Zingiber corallinum Hance) Rrhizome Obtained by Supercritical Fluid Extraction and Steam Distillation Extraction. *Chroma* **69**, 785 (2009).

101. Tarvainen, M., Suomela, J.-P., Kallio, H. & Yang, B. Triterpene Acids in Plantago major: Identification, Quantification and Comparison of Different Extraction Methods. *Chroma* **71**, 279–284 (2010).

102. Berger, T. A. CHROMATOGRAPHY: SUPERCRITICAL FLUID | Instrumentation. in *Encyclopedia of Separation Science* (ed. Wilson, I. D.) 1–8 (Academic Press, 2007). doi:10.1016/B0-12-226770-2/00471-3.

103. Inamdar, P. K. & Chatterjee, S. TERPENOIDS: LIQUID CHROMATOGRAPHY. in *Encyclopedia of Separation Science* (ed. Wilson, I. D.) 4354–4363 (Academic Press, 2000). doi:10.1016/B0-12-226770-2/01511-8.

104. Gibitz Eisath, N., Sturm, S. & Stuppner, H. Supercritical Fluid Chromatography in Natural Product Analysis - An Update. *Planta Med.* **84**, 361–371 (2018).

105. Thakur, R. & Gupta, R. B. Rapid Expansion of Supercritical Solution with Solid Cosolvent (RESS−SC) Process: Formation of Griseofulvin Nanoparticles. *Ind. Eng. Chem. Res.* **44**, 7380–7387 (2005).

106. Thakur, R. & Gupta, R. B. Formation of phenytoin nanoparticles using rapid expansion of supercritical solution with solid cosolvent (RESS-SC) process. *Int J Pharm* **308**, 190–199 (2006).

107. Domingo, C., Wubbolts, F. E., Rodríguez-Clemente, R. & van Rosmalen, G. M. Solid crystallization by rapid expansion of supercritical ternary mixtures. *Journal of Crystal Growth* **198**, 760–766 (1999).

108. Rodrigues, M. *et al.* Microcomposites theophylline/hydrogenated palm oil from a PGSS process for controlled drug delivery systems. *The Journal of Supercritical Fluids* **1–2**, 175–184 (2004).

109. Ventosa, N., Sala, S., Veciana, J., Torres, J. & Llibre, J. Depressurization of an Expanded Liquid Organic Solution (DELOS): A New Procedure for Obtaining Submicron- or Micron-Sized Crystalline Particles. *Crystal Growth & Design* **1**, 299–303 (2001).

110. Sane, A. & Limtrakul, J. Formation of retinyl palmitate-loaded poly(l-lactide) nanoparticles using rapid expansion of supercritical solutions into liquid solvents (RESOLV). *The Journal of Supercritical Fluids* **51**, 230–237 (2009).

111. Türk, M. & Lietzow, R. Stabilized nanoparticles of phytosterol by rapid expansion from supercritical solution into aqueous solution. *AAPS PharmSciTech* **5**, 36–45 (2004).

112. Varona, S., Rodríguez-Rojo, S., Martín, Á., Cocero, M. J. & Duarte, C. M. M. Supercritical impregnation of lavandin (Lavandula hybrida) essential oil in modified starch. *The Journal of Supercritical Fluids* **58**, 313–319 (2011).

113. M.A Rodrigues *et al.* Theophylline polymorphs by atomization of supercritical antisolvent induced Suspensions. *J. Supercrit. Fluids* **58**, 303–312 (2011).

114. Zhao, Z. *et al.* Formation of curcumin nanoparticles via solution-enhanced dispersion by supercritical CO2. *Int J Nanomedicine* **10**, 3171–3181 (2015).

115. Meneses, M. A., Caputo, G., Scognamiglio, M., Reverchon, E. & Adami, R. Antioxidant phenolic compounds recovery from Mangifera indica L. by-products by supercritical antisolvent extraction. *Journal of Food Engineering* **163**, 45–53 (2015).

116. Marqués, J. L., Porta, G. D., Reverchon, E., Renuncio, J. A. R. & Mainar, A. M. Supercritical antisolvent extraction of antioxidants from grape seeds after vinification. *The Journal of Supercritical Fluids* **82**, 238–243 (2013).

117. Kalani, M. & Yunus, R. Application of supercritical antisolvent method in drug encapsulation: a review. *Int J Nanomedicine* **6**, 1429–1442 (2011).

118. Campardelli, R., Porta, G. D. & Reverchon, E. Solvent elimination from polymer nanoparticle suspensions by continuous supercritical extraction. *The Journal of Supercritical Fluids* **Complete**, 100–105 (2012).

119. Della Porta, G., Falco, N. & Reverchon, E. Continuous Supercritical Emulsions Extraction: A New Technology for Biopolymer Microparticles Production. *Biotechnology and bioengineering* **108**, 676–86 (2011).

120. Cocero, M. J., Martín, Á., Mattea, F. & Varona, S. Encapsulation and co-precipitation processes with supercritical fluids: Fundamentals and applications. *The Journal of Supercritical Fluids* **47**, 546–555 (2009).

121. Mora-Huertas, C. E., Fessi, H. & Elaissari, A. Polymer-based nanocapsules for drug delivery. *Int J Pharm* **385**, 113–142 (2010).

122. ANSI/ASAE S319.3 JUL97 - Method of Determining and Expressing Fineness of Feed Materials by Sieving. https://webstore.ansi.org/standards/asabe/ansiasaes319jul97.

123. de Melo, M. M. R., Silvestre, A. J. D. & Silva, C. M. Supercritical fluid extraction of vegetable matrices: Applications, trends and future perspectives of a convincing green technology. *The Journal of Supercritical Fluids* **92**, 115–176 (2014).

124. Martín, L. *et al.* Supercritical antisolvent fractionation of ryanodol from Persea indica. *The Journal of Supercritical Fluids* **60**, 16–20 (2011).

125. Elisa Langa *et al.* Supercritical anti-solvent fractionation of Artemisia absinthium L. conventional extracts: tracking artemetin and casticin | Elisa Langa | Request PDF. *Journal of Supercritical Fluids* **151**, (2019).

126. Chen, J. H., Xia, Z. H. & Tan, R. X. High-performance liquid chromatographic analysis of bioactive triterpenes in Perilla frutescens. *Journal of Pharmaceutical and Biomedical Analysis* **32**, 1175–1179 (2003).

127. PubChem. Polyvinyl alcohol. https://pubchem.ncbi.nlm.nih.gov/compound/11199.

128. Astete, C. E. & Sabliov, C. M. Synthesis and characterization of PLGA nanoparticles. *Journal of Biomaterials Science, Polymer Edition* **17**, 247–289 (2006).

129. Bilati, U., Allémann, E. & Doelker, E. Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles. *Eur J Pharm Sci* **24**, 67–75 (2005).

130. Della Porta, G. *et al.* Injectable PLGA/Hydroxyapatite/Chitosan Microcapsules Produced by Supercritical Emulsion Extraction Technology: An In Vitro Study on Teriparatide/Gentamicin Controlled Release. *Journal of Pharmaceutical Sciences* **105**, 2164–2172 (2016).

131. Gimenez-Rota, C. *et al.* β-Carotene, α-tocoferol and rosmarinic acid encapsulated within PLA/PLGA microcarriers by supercritical emulsion extraction: Encapsulation efficiency, drugs shelf-life and antioxidant activity. *The Journal of Supercritical Fluids* **146**, 199–207 (2019).

132. Bauer, A. W., Kirby, W. M., Sherris, J. C. & Turck, M. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* **45**, 493–496 (1966).

133. Rota, M. C., Herrera, A., Martínez, R. M., Sotomayor, J. A. & Jordán, M. J. Antimicrobial activity and chemical composition of Thymus vulgaris, Thymus zygis and Thymus hyemalis essential oils. *Food Control* **19**, 681–687 (2008).

134. Melvin P. Weinstein. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*. (Clinical and Laboratory Standards Institute, 2018).

135. Mihajilov-Krstev, T. *et al.* Chemical composition, antimicrobial, antioxidative and anticholinesterase activity of Satureja Montana L. ssp montana essential oil. *Open Life Sciences* **9**, 668–677 (2014).

136. Rota, C., Carramiñana, J. J., Burillo, J. & Herrera, A. In vitro antimicrobial activity of essential oils from aromatic plants against selected foodborne pathogens. *J. Food Prot.* **67**, 1252–1256 (2004).

137. Tekwu, E. M., Pieme, A. C. & Beng, V. P. Investigations of antimicrobial activity of some Cameroonian medicinal plant extracts against bacteria and yeast with gastrointestinal relevance. *Journal of Ethnopharmacology* **142**, 265–273 (2012).

138. Basch, H. & Gadebusch, H. H. In vitro antimicrobial activity of dimethylsulfoxide. *Appl Microbiol* **16**, 1953–1954 (1968).

139. Kuete, V. & Efferth, T. Cameroonian Medicinal Plants: Pharmacology and Derived Natural Products. *Front Pharmacol* **1**, (2010).

140. Brand-Williams, W., Cuvelier, M. E. & Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology* **28**, 25–30 (1995).

141. Gülçin, İ., Huyut, Z., Elmastaş, M. & Aboul-Enein, H. Y. Radical scavenging and antioxidant activity of tannic acid. *Arabian Journal of Chemistry* **3**, 43–53 (2010).

142. Wüst Zibetti, A., Aydi, A., Arauco Livia, M., Bolzan, A. & Barth, D. Solvent extraction and purification of rosmarinic acid from supercritical fluid extraction fractionation waste: Economic evaluation and scale-up. *The Journal of Supercritical Fluids* **83**, 133–145 (2013).

143. Reverchon, E. & De Marco, I. Mechanisms controlling supercritical antisolvent precipitate morphology. *Chemical Engineering Journal* **169**, 358–370 (2011).

144. Sánchez-Camargo, A. P. *et al.* Supercritical antisolvent fractionation of rosemary extracts obtained by pressurized liquid extraction to enhance their antiproliferative activity. *The Journal of Supercritical Fluids* **107**, 581–589 (2016).

145. Bernatoniene, J. *et al.* Novel approaches to optimize extraction processes of ursolic, oleanolic and rosmarinic acids from Rosmarinus officinalis leaves. *Industrial Crops and Products* **84**, 72–79 (2016).

146. J. C. Furtado, N. A. *et al.* Pentacyclic Triterpene Bioavailability: An Overview of In Vitro and In Vivo Studies. *Molecules* **22**, 400 (2017).

147. Aparecida Resende, F. *et al.* Antimutagenicity of ursolic acid and oleanolic acid against doxorubicin-induced clastogenesis in Balb/c mice. *Life Sciences* **79**, 1268–1273 (2006).

148. Musabayane, C. T., Tufts, M. A. & Mapanga, R. F. Synergistic antihyperglycemic effects between plant-derived oleanolic acid and insulin in streptozotocin-induced diabetic rats. *Ren Fail* **32**, 832–839 (2010).

149. Soica, C. *et al.* The Synergistic Biologic Activity of Oleanolic and Ursolic Acids in Complex with Hydroxypropyl-γ-Cyclodextrin. *Molecules* **19**, 4924–4940 (2014).

150. Bauer, A. W., Kirby, W. M., Sherris, J. C. & Turck, M. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* **45**, 493–496 (1966).

151. Lewis, K. & Ausubel, F. M. Prospects for plant-derived antibacterials. *Nature Biotechnology* **24**, 1504–1507 (2006).

152. Guillén, M. D., Cabo, N. & Burillo, and J. Characterisation of the Essential Oils of some Cultivated Aromatic Plants of Industrial Interest. *Journal of the Science of Food and Agriculture* **70**, 359–363 (1996).

153. Soković, M., Glamočlija, J., Marin, P. D., Brkić, D. & van Griensven, L. J. L. D. Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an in vitro model. *Molecules* **15**, 7532–7546 (2010).

154. Moreno, S., Scheyer, T., Romano, C. S. & Vojnov, A. A. Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. *Free Radic. Res.* **40**, 223–231 (2006).

155. Bais, H. P., Walker, T. S., Schweizer, H. P. & Vivanco, J. M. Root specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of Ocimum basilicum. *Plant Physiology and Biochemistry* **40**, 983–995 (2002).

156. Kirkwood ZI, Millar BC, Downey DG & Moore JE. Antimicrobial effect of dimethyl sulfoxide and N, N-Dimethylformamide on Mycobacterium abscessus: Implications for antimicrobial susceptibility tes... - PubMed - NCBI. *Int J Mycobacteriol.* **7**, 134–136 (2018).

157. do Nascimento, P. G. G. *et al.* Antibacterial and Antioxidant Activities of Ursolic Acid and Derivatives. *Molecules* **19**, 1317–1327 (2014).

158. Messaoud, C., Chograni, H. & Boussaid, M. Chemical composition and antioxidant activities of essential oils and methanol extracts of three wild Lavandula L. species. *Natural Product Research* **26**, 1976–1984 (2012).

159. Blažeković, B., Vladimir-Knežević, S., Brantner, A. & Štefan, M. B. Evaluation of Antioxidant Potential of Lavandula x intermedia Emeric ex Loisel. 'Budrovka': A Comparative Study with L. angustifolia Mill. *Molecules* **15**, 5971–5987 (2010).

160. Torras-Claveria, L., Jauregui, O., Bastida, J., Codina, C. & Viladomat, F. Antioxidant Activity and Phenolic Composition of Lavandin (Lavandula x intermedia Emeric ex Loiseleur) Waste. https://pubs.acs.org/doi/abs/10.1021/jf070236n (2007) doi:10.1021/jf070236n.

161. Ceylan, Y., Usta, K., Usta, A., Maltas, E. & Yildiz, S. Evaluation of Antioxidant Activity, Phytochemicals and ESR Analysis of Lavandula Stoechas. *Acta Physica Polonica A* **128**, (2015).

162. Apak, R., Güçlü, K., Özyürek, M., Karademir, S. E. & Erçağ, E. The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. *International Journal of Food Sciences and Nutrition* **57**, 292–304 (2006).

163. Carrasco, A., Martinez-Gutierrez, R., Tomas, V. & Tudela, J. Lavandula angustifolia and Lavandula latifolia Essential Oils from Spain: Aromatic Profile and Bioactivities. *Planta Med.* **82**, 163–170 (2016).

164. Vasileva, I. *et al.* Effect of lavender (Lavandula angustifolia) and melissa (Melissa Officinalis) waste on quality and shelf life of bread. *Food Chemistry* **253**, 13–21 (2018).

165. Angelo Odierna. Incapsulamento di beta-carotene in microsfere biopolimeriche mediante estrazione supercritica di emulsioni. *Universita deglu studi di Salerno* (2014).

166. Smith, R. L., Yamaguchi, T., Sato, T., Suzuki, H. & Arai, K. Volumetric behavior of ethyl acetate, ethyl octanoate, ethyl laurate, ethyl linoleate, and fish oil ethyl esters in the presence of supercritical CO2. *The Journal of Supercritical Fluids* **13**, 29–36 (1998).

167. da Silva, S. B., Ferreira, D., Pintado, M. & Sarmento, B. Chitosan-based nanoparticles for rosmarinic acid ocular delivery—In vitro tests. *International Journal of Biological Macromolecules* **84**, 112–120 (2016).

168. Kim, H.-J., Kim, T.-H., Kang, K.-C., Pyo, H.-B. & Jeong, H.-H. Microencapsulation of rosmarinic acid using polycaprolactone and various surfactants. *International Journal of Cosmetic Science* **32**, 185–191 (2010).

169. Yesil-Celiktas, O. & Cetin-Uyanikgil, E. O. In vitro release kinetics of polycaprolactone encapsulated plant extract fabricated by supercritical antisolvent process and solvent evaporation method. *The Journal of Supercritical Fluids* **62**, 219–225 (2012).

170. Aguiar, J., Costa, R., Rocha, F., Estevinho, B. N. & Santos, L. Design of microparticles containing natural antioxidants: Preparation, characterization and controlled release studies. *Powder Technology* **313**, 287–292 (2017).

171. Zhang, P. & Omaye, S. T. Beta-carotene and protein oxidation: effects of ascorbic acid and alpha-tocopherol. *Toxicology* **146**, 37–47 (2000).

172. Faria, A. F., Mignone, R. A., Montenegro, M. A., Mercadante, A. Z. & Borsarelli, C. D. Characterization and Singlet Oxygen Quenching Capacity of Spray-Dried Microcapsules of Edible Biopolymers Containing Antioxidant Molecules. *J. Agric. Food Chem.* **58**, 8004–8011 (2010).

173. Rodríduez- Huezo, M. E., Pedroza, R., Prado, L. A., Beristain, C. I. & Vernon-Carter, E. J. Microencapsulation by Spray Drying of Multiple Emulsions Containing Carotenoids. *Journal of Food Science* **69**, 351–359 (2006).

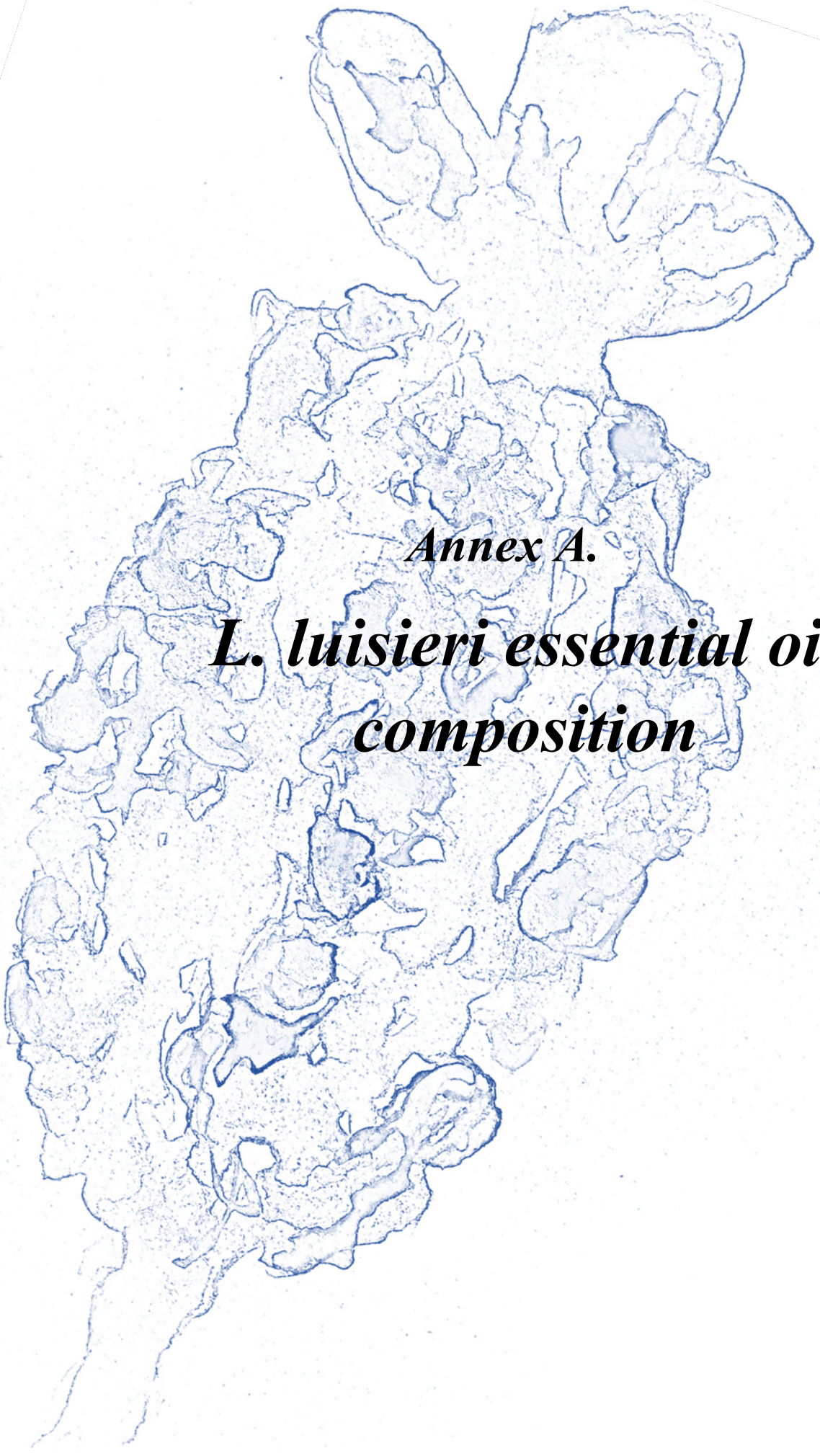
174. Loksuwan, J. Characteristics of microencapsulated β-carotene formed by spray drying with modified tapioca starch, native tapioca starch and maltodextrin. *Food Hydrocolloids* **21**, 928–935 (2007).

175. Pan, X., Yao, P. & Jiang, M. Simultaneous nanoparticle formation and encapsulation driven by hydrophobic interaction of casein-graft-dextran and β-carotene. *Journal of Colloid and Interface Science* **315**, 456–463 (2007).

176. Trombino, S. *et al.* Stearyl ferulate-based solid lipid nanoparticles for the encapsulation and stabilization of β-carotene and α-tocopherol. *Colloids and Surfaces B: Biointerfaces* **72**, 181–187 (2009).

177. Boon, C. S., McClements, D. J., Weiss, J. & Decker, E. A. Factors Influencing the Chemical Stability of Carotenoids in Foods. *Critical Reviews in Food Science and Nutrition* **50**, 515–532 (2010).

178. Santos, D. T., Martín, Á., Meireles, M. A. A. & Cocero, M. J. Production of stabilized sub-micrometric particles of carotenoids using supercritical fluid extraction of emulsions. *The Journal of Supercritical Fluids* **61**, 167–174 (2012).



Annex A.

L. luisieri essential oil composition

Table A.1. Relative abundance of monoterpenes, oxygen containing monoterpenes and their acetates of L. luisieri

	Arantes et al. [40]	**Andrés et al.** [42]	**M.I Garcia Vallejo** [27]	**Dias et al.** [39]	**Matos et al.** [20]			**Videira et al.** [37]	**Lavoine-Hanneguelle Casabianca** [30]	**Zuzarte et al** [22]		**Queiroga et al.**[44]	**Roller et al.** [35]	**Sanya et al. 2018**	**Julio et al.**[45]	
					LIM	LIS	Liv			A	B				A	B
Monoterpenes																
3,5-dimethylene-1,4,4-trimethylcyclopentene								2.5							-	2.8
Camphene			0.2		0.2	0.1	0.1	0.2	0.1				0.1			
Carene			0.1					0.1	0.1							
Limonene			0.2		0.8	0.4	0.3	0.1	0.3							
Myrcene						0.1	0.1		0.2							
p-cymene			0.1		0.3	0.4	0.3	0.1								
Sabinene			1.6		0.2	0.1	0.1	0.1	0.2	0.6	0.2					
Terpinolene			0.2					0.2								
Z-β-ocymene	0.39		0.2		0.1	0.4	0.2	0.4	0.8	0.5	0.9					
α-pinene	1.29	2.3	1.9	2.3	3.4	2.8	3.4	2.3	2.4			1.2	2.6	2.3	1.2	-
β-pinene	4.46		0.1		0.6	0.2	0.4	0.1	0.4			4.5				
β-trans-ocymene										-	0.2					
γ-terpinene								0.1	0.3	-	0.3					

	Arantes et al. [40]	Andrés et al. [42]	M.I Garcia Vallejo [27]	Dias et al. [39]	Matos et al. [20]			Videira et al. [37]	Lavoine-Hanneguelle Casabianca [30]	Zuzarte et al [22]		Queiroga et al.[44]	Roller et al. [35]	Sanya et al. 2018	Julio et al.[45]	
					LIM	LIS	LIV			A	B				A	B
Oxygenated monoterpenes																
1,1,2,3-tetramethyl-4-hydroxymethyl-2-cyclopentene	2.38								2.4	2.0	2.0	1.1			2.4	
1,8-cineol	18.80		13.6	18.9	26.3	25.7	34.3		2.1	16.1			18.8	17.6	18.9	4.8
2,3,5,5-tetramethyl -4-methylene-2-cyclopenten-1-One					2.4	5.1	3.7			4.1						
2,4,5,5-tetramethyl -1,3-cyclopentadien-1-carboxylic acid										0.6						
2,3,4,4-tetramethyl-5-methylene-ciclopent-2-enone	2.12	19.7						5.2	0.7		2.8	0.3			5.2	2
3,4,5,5-tetramethylcyclopentana-1,3-dienecarboxylic acid								0.6								
5-hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one		10.10														7.93 4
Borneol			0.8										1.2			
Camphor	1.12	49.4	2.2		2.9	1.2	1.0		1.4	0.9	2.5	2.2	11.1	3.5		7
Carvone											.	0.2				

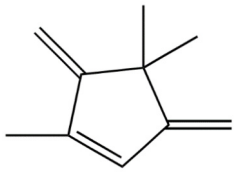
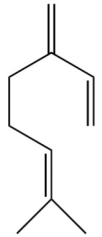
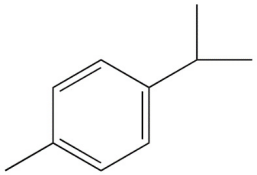
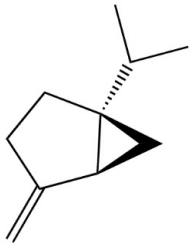
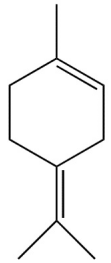
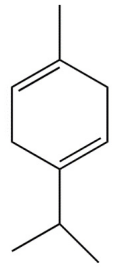
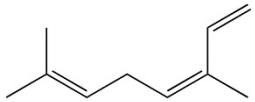
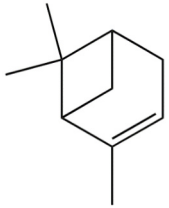
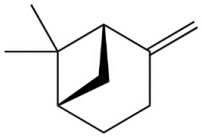
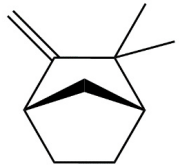
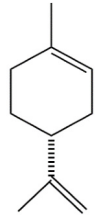
	Arantes et al. [40]	Andrés et al. [42]	M.I Garcia Vallejo [27]	Dias et al. [39]	Matos et al. [20]			Videira et al. [37]	Lavoine-Hanneguelle Casabianca [30]	Zuzarte et al [22]		Queiroga et al.[44]	Roller et al. [35]	Sanya et al. 2018	Julio et al.[45]	
					LIM	LIS	LIV			A	B				A	B
Cis-linalool oxyde						0.9	0.7	1.0	0.6		0.9	0.5				3.2
Cis α-Necrodol																3.3
Cis-verbenol						0.6	1.2		-		0.2	-				
Fenchone	2.01		0.6			6.6	1.3	0.2		0.3	-	18.2	1.9	2.4		-
Isopulegol																
Lavandulol	11.68		2.4			1.7	0.9	1.3	0.6		0.3	0.5	11			
linalool	4.11		2.4	3.1		0.2	1.6	1.4	3.1	2.3	6.2	3.0	4.1	2.3	3.1	1.4
Myertenal											0.3	-				
Myrtenol											-	0.8				
P-cymen-8-ol			0.3						0.1							
Pinocarvone									0.2		-	0.4				
Teresantolol											1.7	1.3				
Terpinene-4-ol	3.56								0.5		-	1.2	3.6			
Terpineol	2.60					1.0	1.5	1.1		0.4	0.2	1.2	2.6			
Trans-linalool oxide									0.3	0.1	0.7	0.4				
Trans-pinocarveol			3.6						0.2		0.2	0.3				
Trans-sabinene hydrate			0.4													
Trans-verbenol			0.5													
Γrans-α-necrodol	10.63		6.4			8.2	5.4	2.8	8.4	5.7	7.1	4.5	10.1			7.7
Verbenone		2.5				0.1	0.1	0.4	0.7		0.4	-				
α-campholenal						0.2	0.2	0.2	0.1		0.1	-				
α-terpineol						0.4	0.1	0.4								

	Arantes et al. [40]	Andrés et al. [42]	M.I Garcia Vallejo [27]	Dias et al. [39]	Matos et al. [20]			Videira et al. [37]	Lavoine-Hanneguelle Casabianca [30]	Zuzarte et al [22]		Queiroga et al.[44]	Roller et al. [35]	Sanya et al. 2018	Julio et al.[45]	
					LIM	LIS	LIV			A	B				A	B
Monoterpene acetates																
2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-methyl acetate		7.7	0.2													
3,4,4-trimethyl-2-cyclohexanone	0.72							0.9	0.9	0.8	0.7					
cis- α -necrodyl acetate	1.23							1.2	0.4			1.2			5.9	-
Fenchyl acetate										-	0.6					
Geranyl acetate					0.4	0.4	0.2								14.2	-
Lavandulyl acetate	3.32			7.2	4.3	3.6	3.3	6.1	5.3	7.6	2.2	3.3		7.2		
Linalyl acetate			0.4													
Lyratyl acetate								3.5		2.4	0.3			3.5		
Myrtenyl-acetate	2.67		1.1						0.6	-	0.2		0.6			
neryl acetate								1		0.6	-					
Trans verbenyl acetate					1.6	2.6	2.5									
Trans-α-necrodyl acetate	16.16		19		17.5	11.3	11.3	16.0	22.7	17.4	3.2	15.6	34.5	16	33	8.2
α-terpinyl acetate												0.6				

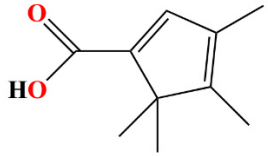
Matos et al. [20]: LIM, *L. luisieri* (Rozeira) Rivas-Martinez from Montenegro, Faro, Portugal; LIS, *L. luisieri* (Rozeira) Rivas-Martinez from Salir, Loulé, Portugal; LIV, *L. luisieri* (Rozeira) Rivas-Martinez from Villa Real de Santo António, Portuga.. Zuzarte et al [22] field-growing plants A, from Piódão región, Portugal; B, Cabo São Vicente region, Portugal. Julio et al.[45] *L. luisieri* A, population from Sevilla, Spain; B, population from Ciudad Real, Spain.

Table A.2 Chemical structure of monoterpenes, oxygenated monoterpenes and monoterpene acetates of L. luisieri

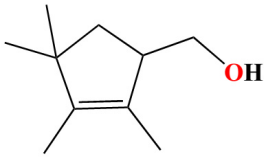
Monoterpenes

					
3,5-dimethylene-1,4,4-trimethylcyclopentene	Myrcene	p-cymene	Sabinene	Terpinolene	Γ-terpinene
					
Z-β-Ocymene	3-carene	α-pinene	β-pinene	Camphene	Limonene

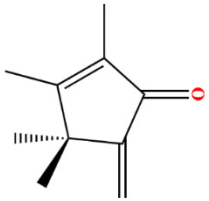
Oxygenated monoterpenes



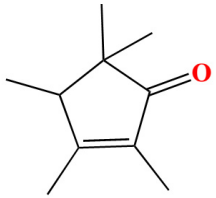
3,4,5,5-tetramethylcyclopentana-1,3-dienecarboxylic acid



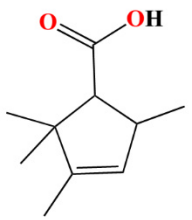
1,1,2,3-tetramethyl-1,4-hydroxymethyl-2-cyclopentene



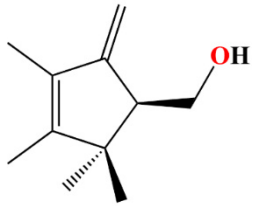
2,3,4,4-tetramethyl-5-methylene-cyclopent-2-enone



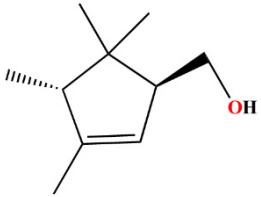
2,3,5,5- tetramethyl -4-methylene-2-cyclopente-1-one



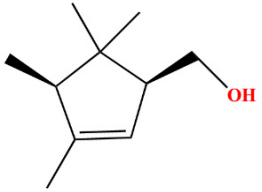
2,4,5,5- tetramethyl -1,3-cyclopentadien-1-carboxylic acid



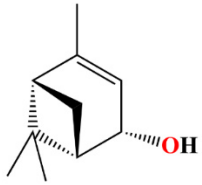
5-hydroxymethyl-2,3,4,4-tetramethylcyclpent-2-en-1-one



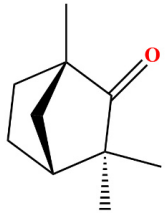
Trans-α-necrodol



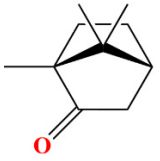
Cis-α-necrodol



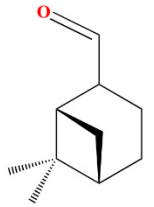
Cis-verbenol



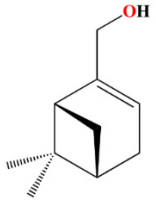
Fenchone



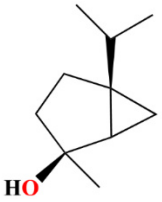
Camphor



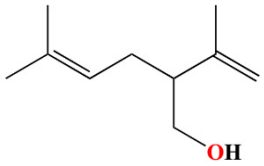
Myrtenal



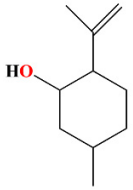
Myrtenol



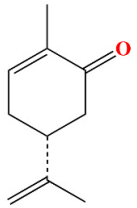
Trans sabinene



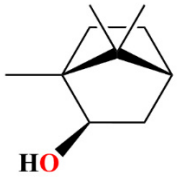
Lavandulol



Isopulegol

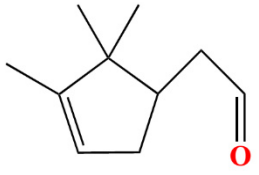


Carvone

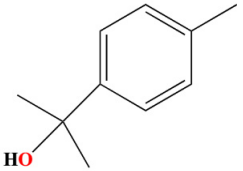


Borneol

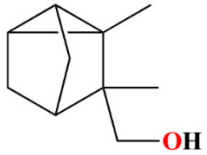
Oxygenated monoterpenes



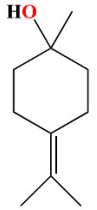
Campholenal



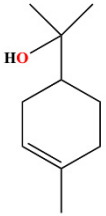
p-cymenol



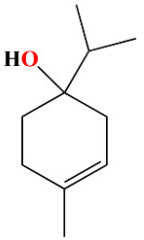
Teresantolol



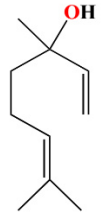
4-terpineol



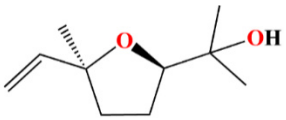
Terpineol



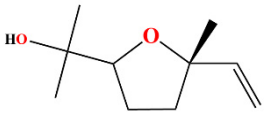
Terpinen-4-ol



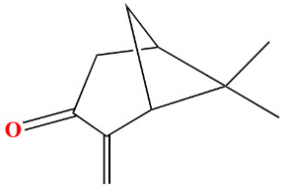
Linalool



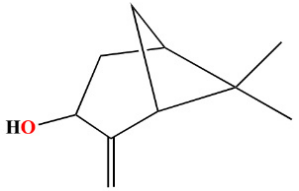
Cis-linalool oxyde



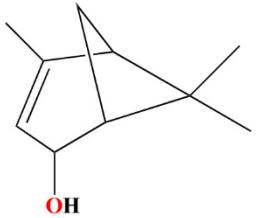
Trans linalool



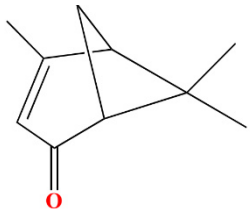
Pinocarvone



Trans pinocarvone

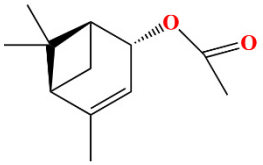


Trans verbenol



Verbenone

Monoterpene acetates



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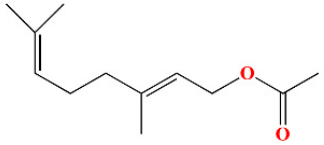
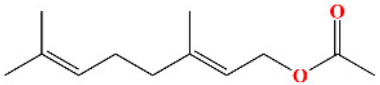
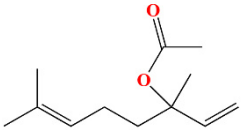


Neryl acetate



Geranyl acetate



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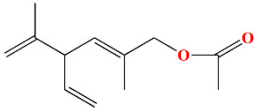
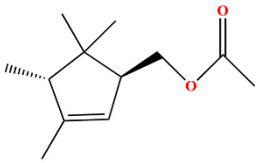
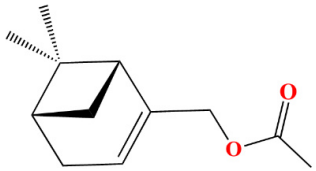


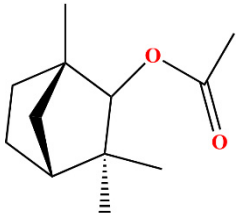
Lyratyl acetate



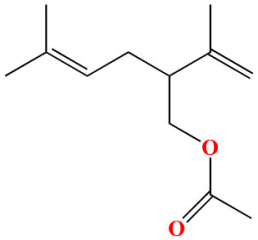
Trans α-necrodyl acetate



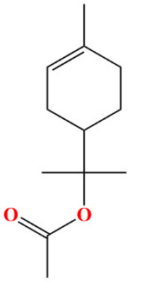
Myrtenyl acetate



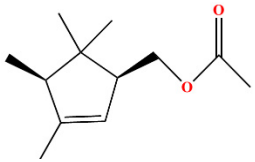
Fenchyl acetate



Lavandulyl acetate



α-terpinyl acetate



Cis- α-necrodyl acetate

Table A.3. Relative abundance of sesquiterpenes, oxygenated sesquiterpenes of L. luisieri

	Arantes et al. [40]	Andrés et al. [42]	M.I Garcia Vallejo [27]	Dias et al. [39]	Matos et al. [20]			Videira et al. [37]	Lavoine-Hanneguelle Casabianca [30]	Zuzarte et al [22]		Queiroga et al.[44]	Roller et al. [35]	Sanya et al. 2018	Julio et al.[45]	
					LIM	LIS	LIV			A	B				A	B
Sesquiterpenes																
Allo-aromadendrene	0.49							0.4	0.7	0.7	-					
cis-calamenene								0.6		0.5	-					
Copaene			1.2					0.4	0.4	0.3	-					
E-caryophyllene	6.00							0.6		0.9	0.2	6				
Germacrene D	0.70															
Nerolidol			0.6													
Sativene								0.4		1.4	-					
selina-3,7(11)-diene					1.7	1.0	0.4	1	1.9	1.4	0.3					
α-calacorene			0.6					0.4								
α-cubebene										0.2	-					
α-Gurjunene			0.4							1.4	-					
α-muurolene								0.2		0.2	-					
α-selinene			0.1					0.8								
β-bourbunene										0.1	-					
β-cubebene								0.3		0.4	-					
β-selinene					0.5	0.4	0.3	0.4							4.3	
γ -cadinene								0.8	1.1	0.7	-					
δ - cadinene								0.6								

	Arantes et al. [40]	Andrés et al. [42]	M.I Garcia Vallejo [27]	Dias et al. [39]	Matos et al. [20]			Videira et al. [37]	Lavoine-Hanneguelle Casabianca [30]	Zuzarte et al [22]		Queiroga et al.[44]	Roller et al. [35]	Sanya et al. 2018	Julio et al.[45]	
					LIM	LIS	LIV			A	B				A	B
Oxygenenated sesquiterpenes																
Cadinol			1.8		2.6	0.5	5.4	2		0.7	-					
Caryophyllene-oxyde	0.62							0.7		0.8	0.3	0.6				
Caryophyllenol			1.0													
Epi-cubenol					0.2	0.6	0.3	0.5	0.3							
Humulene epoxide								0.3		-	0.1					
Ledol								0.9		1.3	0.3					
Muurol								0.6		-	0.2					
Palustrol								0.2								
Spathulenol								0.2								
t-muurol								0.5	0.4	-	0.2					
Viridiflorol	1.65		2.3		0.2	1.5	0.2	3.3	1.1	2.1	1.4	1.5				
α-cis-copaene-8-ol								1.2		1.0	0.7					

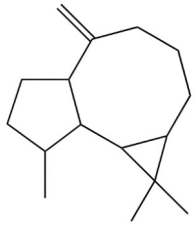
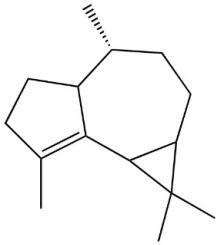
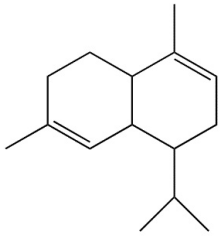
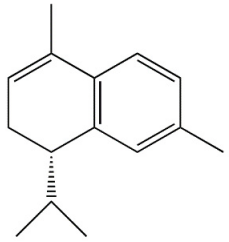
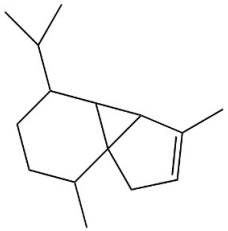
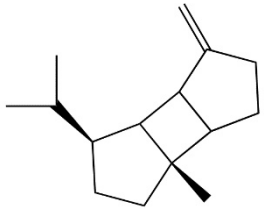
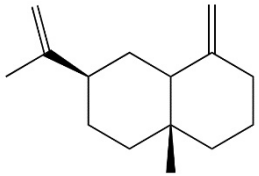
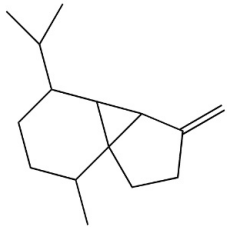
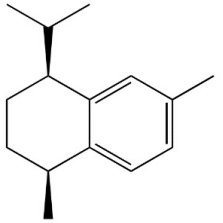
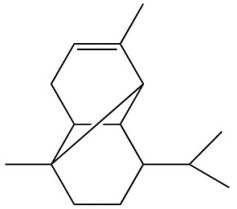
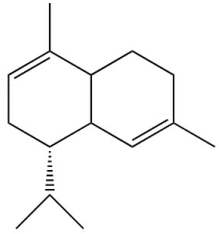
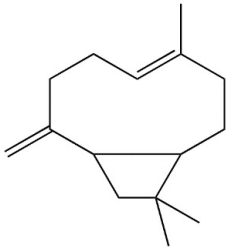
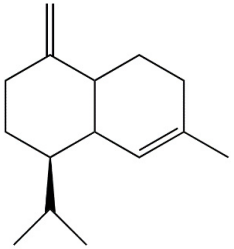
Matos et al. [20]: LIM, *L. luisieri* (Rozeira) Rivas-Martinez from Montenegro, Faro, Portugal; LIS, *L. luisieri* (Rozeira) Rivas-Martinez from Salir, Loulé, Portugal; LIV, *L. luisieri* (Rozeira) Rivas-Martinez from Villa Real de Santo António, Portugal

Zuzarte et al [22] field-growing plants A, from Piódão región, Portugal; B, Cabo São Vicente region, Portugal.

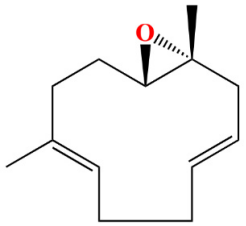
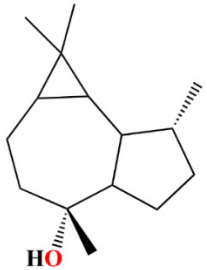
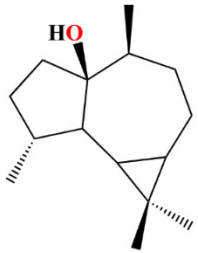
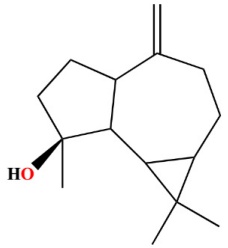
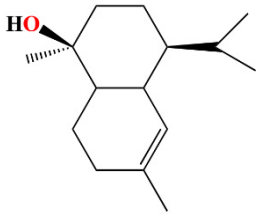
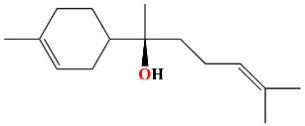
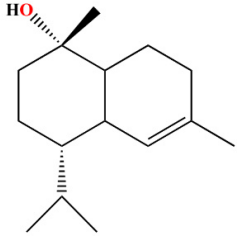
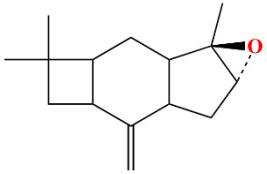
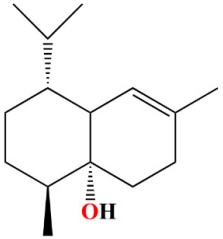
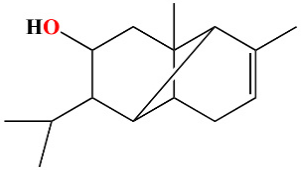
Julio et al.[45] *L. luisieri* A, population from Sevilla, Spain; B, population from Ciudad Real, Spain.

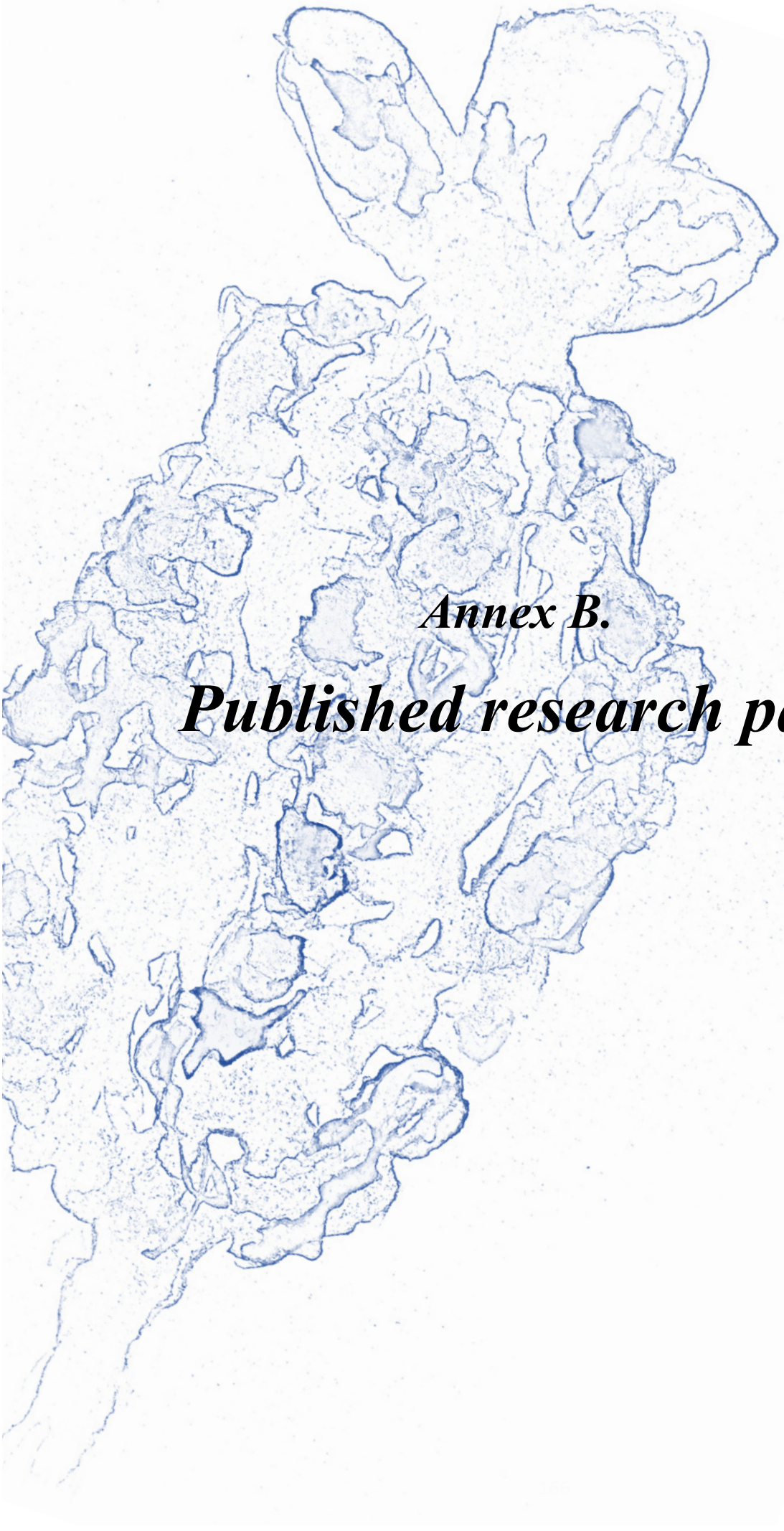
Table A.4 Chemical structure of sesquiterpenes and oxygenated sesquiterpenes of L. luisieri

Sesquiterpenes

				
Allomadrene	α-Gurjunene	α-muurolene	α-calacorene	α-cubenene
				
β-bourbonene	β-selinene	β-cubenene	Cis-calamenene	Copaene
				
δ-cadinene	E caryophyllene	γ-cadinene		

Oxygenated sesquiterpenes

				
Humulene epoxide	ledol	palustrol	Spatulenol	t-muurol
				
α-bisabolol	cadinol	Caryophyllene epoxide	Epi-cubenol	Cis- α-copaene



Annex B.

Published research papers

J. of Supercritical Fluids 161 (2020) 104821

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

journal homepage: www.elsevier.com/locate/supflu

Supercritical antisolvent fractionation of antioxidant compounds from *Lavandula luisieri* (Rozeira) Riv.-Mart.

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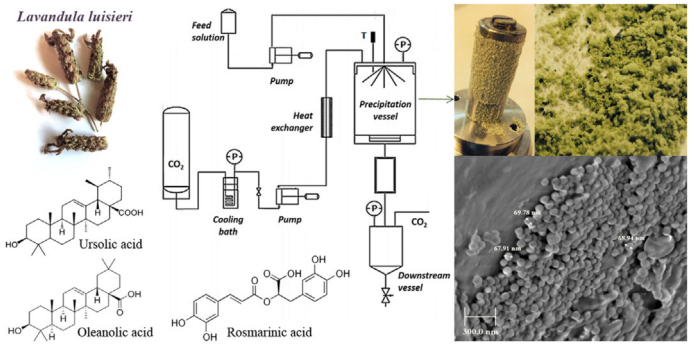
HIGHLIGHTS

- Supercritical antisolvent fractionation was applied to *Lavandula luisieri* extract.
- Pressure and CO_2 flow rate influence in the fractionation process was evaluated.
- The fractionation of rosmarinic, oleanolic and ursolic acids was tracked.
- The best conditions for the supercritical fractionation were 130 bar and 30 g/min.
- A fine concentrated powder of *Lavandula luisieri* actives was produced.

GRAPHICAL ABSTRACT



ARTICLE INFO

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Chemical compounds studied in this article:
Rosmarinic acid (PubChem: 5281792)
Oleanolic acid (PubChem: 10494)
Ursolic acid (PubChem: 64945)

Keywords:
Supercritical antisolvent fractionation
HPLC
Rosmarinic acid
Oleanolic acid
Ursolic acid

ABSTRACT

There is a renewed research interest in food industry in natural additives to improve food shelf life and provide preventive or therapeutic effects on chronic ailments. The aim of this study was to optimise the concentration of three bioactives, rosmarinic, oleanolic and ursolic acids from *Lavandula luisieri* ethanolic extracts using the supercritical antisolvent fractionation technique. In order to evaluate the influence of pressure and CO_2 flow rate in the process, response surface methodology was employed. Actives quantification was accomplished using HPLC-Photodiode array. Rosmarinic acid, was completely retained and concentrated in the precipitation vessel, while oleanolic and ursolic acids distributed between both fractions. The supercritical antisolvent fractionation process is a useful and green technology to concentrate bioactive in a fine solid to be applied as natural preservative in food products. The optimum conditions for higher mass recovery and concentration of actives were 130 bar and CO_2 feeding flow rate 30 g/min, respectively.

Abbreviations: $scCO_2$, supercritical CO_2; SAF, Supercritical Antisolvent Fractionation; RA, Rosmarinic acid; OA, Oleanolic acid; UA, Ursolic acid; PDA, photodiode array; RSM, Response Surface Methodology; CCD, Central Composite Design; *XP*, Pressure; XQ_{CO_2}, CO_2 flow rate; FS, Feed Solution; PV, Precipitation Vessel; DV, Downstream Vessel; Y_{PV}%, Y_{DV}% and Y_{SAF}%, yields recovered in PV, DV and SAF fractions.

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0896-8446/

1. Introduction

Technological advances in food processing have increased the number and variety of additives used to produce the desired preservation or improvement of flavour, texture, appearance and nutritional value maintaining its safeness. Nevertheless, daily consumption of products with these substances have raised the consumers concern about their long-term consequences, demanding the substitution of artificial additives for natural ones [1]. In addition, society purpose to optimise wellbeing through healthy habits and diets with health-promoting properties, are a growing trend [2,3]. The focus of many studies across the world, is targeted in searching natural alternatives to preserve food, and demonstrate their beneficial functionalities on food, not only improve or maintain their nutritional quality, but also add positive effect beyond its nutritional value [1,4].

Plants are an inexhaustible natural source of compounds, such as polyphenols and terpenes with a wide range of beneficial bioactivities in human health: antioxidant, anticancer, anti-inflamatory, antimicrobial, antiviral, cardioprotective, neuro- and hepatoprotective [3,5]. *Lamiaceae* is a highly distributed plant family, and one of the biggest in the plant kingdom, known for its content in polyphenolic compounds. In this work a member of this family, *Lavandula luisieri* (Rozeira) Riv.-Mart., [6] an aromatic shrub endemic from to the south Iberian Peninsula, is studied. Although the essential oils of other *Lavandula* species present importance in the fragrance industry, *L. luisieri*, has not because of the presence of camphor. Nevertheless, it has a curious composition in a series of volatile substances with a 1,2,2,3,4-pentamethylcyclopentane (necrodane) structure [6], which gives to this plant the category of species since these compounds have only been found in the defensive secretions of the beetle *Necrodes surinamensis* [7,8], and in the sex pheromone of the grape mealybug Pseudococcus maritimus [9]. Different extracts obtained from it have shown antifeedant, insecticide and antimicrobial effects [10,11]. In addition rosmarinic acid (RA), tormentic acid, ursolic acid (UA) and oleanolic acid (OA) were isolated from the nonvolatile fraction [11,12]. These polyphenol and triterpenoids have been reported to have several beneficial bioactivities such as antioxidant, anti-inflamatory, neuroprotective and hepatoprotective [13–15]. Rosmarinic acid is contained into Rosemary extract, another plant from the *Lamiaceae* family, which has been accepted by the EU food additive legislation as an effective and natural alternative to synthetic antioxidants [16]. As a consequence, there are several studies that analyse its extraction and concentration techniques [17], stability and pharmacokinetic profile that ensure its antioxidant properties [18]. Regarding the identified triterpenes Ursolic and Oleanolic acids, it has been reported their potential application as antimicrobials because of their capacity to disrupt the peptidoglycan structure, and inhibit bacterial gene expression and biofilm formation [19]. Therefore, *L. luisieri* extracts containing a combination of these compounds could have several applications in the pharmaceutical, cosmetical or food fields.

Nevertheless, plant extracts have been obtained traditionally using techniques with two main limitations: high temperatures, as in hydrodistillation, which can cause actives principles degradation, and the use of organic solvents that are environmental pollutants. The use of supercritical CO_2 ($scCO_2$) is an alternative to obtain natural antioxidants from herbs and plants [19,20]. The application of supercritical carbon dioxide as extraction solvent shows only one disadvantage: its low polarity. This means that the compounds that $scCO_2$ can extract are limited to nonpolar components with less antioxidant bioactivity or small volatile compounds [21,22]. However, this lipophilic behaviour is useful when polar compounds have to be concentrated from an organic solution extract, as in Supercritical Antisolvent Fractionation (SAF) [23]. The obtained product is a dried powder avoiding solvent residues, whose shape and diameter can be modulated to improve its solubility or vehiculization along with polymers [24,25].

In SAF technique an organic solution is continuously pumped and sprayed into a vessel with $scCO_2$. Molecules that are insoluble in this new solvent mixture of ethanol-$scCO_2$ precipitate as a solid, and the rest of them are dragged downstream. This technique has been applied by many researchers to fractionate and concentrate natural compounds, such as lignans from flaxseeds [26], flavonoids and polyphenols from *Vitis vinifera* seeds [27], or flavonoids from *Arrabidaea chica* leaves [28]. Sánchez-Camargo et al. and Visentin et al., and Quintana et al. [17,29,30], applied SAF process to rosemary extracts in order to concentrate their polyphenols, RA among them, and produced *raffinate* fraction with a higher antiproliferative and antioxidant activity than the original extract. Because of the distribution of RA, OA and UA in the plant kingdom and their many probed activities, also traditional techniques, such as ultrasound-assisted extraction and maceration, have been applied by other authors. Bernatoniene et al. [13] studied different techniques for the extraction of these three actives from Rosmarinus officinalis and achieved a highest yield of UA (15.8 ± 0.2 mg/g), RA (15.4 ± 0.1 mg/g), and OA (12.2 ± 0.1 mg/g).

According to these previous works on extraction and concentration of natural bioactives with supercritical techniques, the aim of this study was to optimise the pressure and CO_2 flow rate conditions in the Supercritical antisolvent fractionation of *L. luisieri* extract for a higher mass recovery and concentration of the three actives; rosmarinic, oleanolic and ursolic acids, into a solid powder.

2. Material and methods

2.1. Plant material

Plant material was collected in 2009 in Zaragoza (Spain) from an adapted population of *L. luisieri*, original from Toledo (Spain). This adaptation was performed by Centro de Investigación y Tecnología de Aragón (CITA) (Spain).

Plant material was dried at room temperature and then pulverised. Its particle size distribution was carried out by a vibratory sieve shaker *CISA* model BA 300 N, and the average diameter was calculated according to ASAEA S319.3 from the American National Standards Institute as shown in Eq. (1).

$$d_{mg} = \log^{-1}\left[\frac{\sum_{l}^{n}\left(w_i \log \bar{d}_i\right)}{\sum_{l}^{n} w_i}\right]; \ \bar{d}_i = (d_i \cdot d_{i+1})^{0.5} \quad (1)$$

where d_i is the nominal mesh of the i^{th} sieve (mm), $d_{(i+1)}$ is the nominal mesh of the next larger sieve after the i^{th} sieve (mm) and w_i is the mass (g) of plant material retained by the i^{th} sieve.

The pulverised plant material was adjusted to a normal distribution and an approximately mean particle diameter of 0.33 mm to improve the extraction yield. Moisture content was tested five times using a *Sartorious* model MA 40 *Moisture Analyzer*, and the standard deviation was determined (10.6 %, s = 0.3 %). This pretreated plant material was kept in hermetically sealed food bags at −20 °C.

2.2. Chemicals and reagents

The solvents used in the extraction process were hexane (Panreac 99.0 %) and ethanol (AnalaR NORMAPURE 99.96 %). The SAF process was performed with CO_2 (ALPHA GAZ 99.8 %) and ethanol

(AnalaR NORMAPURE 99.96 %). The chromatography mobile phase solvents were methanol (Scharlab 99.9 %), water (MilliQ 18.2 MΩ·cm), phosphoric acid (Fluka 85.9 %) and acetonitrile (Scharlab 99.9 %). The HPLC-PDA standards used were rosmarinic acid (RA, 99 %), oleanolic acid (OA, 99.8 %) and ursolic acid (UA, 99.7 %), supplied by Sigma-Aldrich.

2.3. Maceration

100 g of plant material were stirred for 48 h at room temperature (25 °C) in 1 L absolute ethanol, after a previous clearance extraction using hexane under the same conditions. The extraction yields for the macerations, Y_i (wt.%), were calculated using Eq. (2). The extract obtained (ME) was used to prepare the feed solution (FS) for the SAF experiments.

$$Y_i\,(\text{wt.\%}) = \left(\frac{mass\ (g)_{\text{plant extract}}}{mass\ (g)_{\text{plant material}}}\right) \cdot 100 \qquad (2)$$

where i is the solvent of the extraction; hexane or EtOH, $mass_{\text{plant extract}}$ (g) is the mass of the dry extract after maceration, once the solvent had been removed, and $mass_{\text{plant material}}$ (g) was the initial mass of dried and pulverised plant.

2.4. Supercritical antisolvent fractionation process

L. luisieri ethanolic extract was fractionated using the SAF technique. The experiments of were performed in the "Green Chemistry Laboratory" (I3A Researching Institute at University of Zaragoza) using a scale apparatus previously described [31,32]. A schematic structure is represented in Fig. 1. The main components of the device are: a CO_2 pump (mod. P200 max pressure 600 bar), an extract solution pump (Waters co-solvent pump series III maximum pressure 400 bar), a 0.5 L precipitation high pressure vessel (PV) with an injection nozzle (Ø = 100 μm) in the top and a collection filter in the bottom, and a 0.5 L downstream low pressure separation vessel (DV). The pressure in PV was set with automated backpressure regulator (ABPR, TharSFC) and in DV with a manual backpressure regulator (BPR, CIRCOR Instrumentation Technologies). The experimental parameters of temperature, CO_2 flow rate, liquid solution flow rate, and PV pressure were controlled with the computer software Thar Instruments Process Suite. The equipment working limits are 400 bar and 120 °C.

Different SAF experiments were performed varying the PV pressure, from 80 to 150 bar, and the CO_2 flow rate from 10 to 30 g/min, the rest of variables were set at: extract solution concentration, 3% (wt.%); ethanolic solution flow rate, 0.45 mL/min and PV temperature, 40 °C. These settings were chosen according to previous experience with the SAF equipment [32]. The fixed settings of ethanolic solution flow rate and temperature were chosen in order to maintain always a CO_2 molar fraction over 0.98 and ensure supercritical conditions of the CO_2-ethanol mixture in the precipitation vessel in all experiments performed [27]. The operational conditions of DV were also fixed at 35 bar and 25 °C, to achieve the recovery of the solvent and its separation from gaseous CO_2.

The experiments procedure, which was previously described by Langa et al. [32], consisted in three steps. Firstly, the experimental conditions were stabilized, pressure (bar), CO_2 flow rate (g/min), temperature (40 °C) and liquid flow rate (0.45 mL/min) with pure ethanol (≈60 min). Secondly, the ethanolic extract was dissolved in 30 mL of ethanol at 3% (wt.%) and filtered through *NYLON* 0.45 μm pore size to constitute the feed solution (FS) to be pumped towards the precipitation vessel (PV) with a flow rate of 0.45 mL/min (≈60 min). The insoluble compounds in the supercritical mixture precipitated in this high-pressure vessel. Those compounds that were still soluble in the ethanol-scCO_2 mixture were collected in the downstream vessel (DV). The manual backpressure regulator allowed the exit of the gas through the top of the vessel and the ethanolic solution of the dragged actives was recovered from the bottom. Finally, after the FS is entirely pumped, 30 mL of pure ethanol were pumped to ensure the complete injection of the FS, and later on, pure scCO_2 was injected (≈90 min), to eliminate the residual solvent and ensure its complete dragging to the DV. The ethanolic solution recovered in this vessel was dried using a rotavapor (model R-200) equipped with a heat bath (model B-490) a controller vacuum (model V-800) and a vacuum pump (model V-700) (Büchi, Marshall Scientific) at 70 mbar and 42 °C and weight to determine the mass. The solid fraction from PV was directly weighted. The yields Y_{SAF}%, Y_{DV}%, Y_{PV}% were calculated using Eqs. (3) and (4).

Table 1
Codification and levels of the two independent variables for the SAF factorial design of experiments.

Variable	Symbol	Factor levels				
		{−1.44	−1	0	1	1.44}
Pressure (bar)	XP	80	90	115	140	150
CO_2 flow rate (g/min)	$X_{Q_{CO_2}}$	10	13	20	27	30

$$Y_i\,(\text{wt\%}) = \left(\text{mass fraction collected}_i/\text{mass of FS}\right) \cdot 100 \qquad (3)$$

where i is the place of collecting: PV or DV

$$Y_{SAF}\,(\text{wt\%}) = Y_{PV}\,(\text{wt\%}) + Y_{DV}\,(\text{wt\%}) \qquad (4)$$

The confidence interval of the obtained yields Y_{PV}%, Y_{DV}% and Y_{SAF}% from three experiment replicates was determined to measure the reproducibility of the SAF process applied.

Fractions from PV and DV and 1 mL sample from FS were collected and kept in ambar vials at −20 °C until their analysis with HPLC-PDA.

2.5. HPLC analysis

The FS and its supercritical fractions PV and DV, were analysed by HPLC-PDA on a HPLC *Waters® Alliance 2695* with a *PDA Waters® 2998* detector. A *CORTECS®* C18 2.7 μm (4.6 × 150 mm) with a pre-column CORTECS® Pre-column VanGuard C18 2.7 μm (2.1 × 5 mm) was used. The compounds were eluted with an isocratic mobile phase methanol (MeOH): 0.5 % H_3PO_4 in Milli-Q water (88:12) for 10 min at 0.8 mL/min flow rate. The detection wavelength was fixed at 330 nm for the first 6 min and at 210 nm for the last 4 min, in order to detect and quantify RA, OA and UA. Extract solutions (100 ppm approximately) were filtered through a *GH Polypropylene membrane ACRODISC* 13 mm pore size 0.2 μm filter. RA, OA and UA standards were run under the same chromatographic conditions in order to obtain their calibration regression which allows their quantification in the samples. The analyses were performed in triplicate.

2.6. Experimental design and statistical analysis

Response surface methodology (RSM) based on central composite design (CCD) [33] was employed to statistically evaluate and optimise the conditions of pressure (bar) in PV and CO_2 flow rate (g/min), for maximum yield recovery, in both vessels (Y_{SAF}%, Y_{DV}% and Y_{PV}%), as well as for a maximum concentration of *L. luisieri* bioactive compounds (RA, OA and UA). *Pressure* and *CO_2 flow rate* were coded as X_P and $X_{Q_{CO_2}}$, respectively. The range and levels of the variables used are gathered in Table 1.

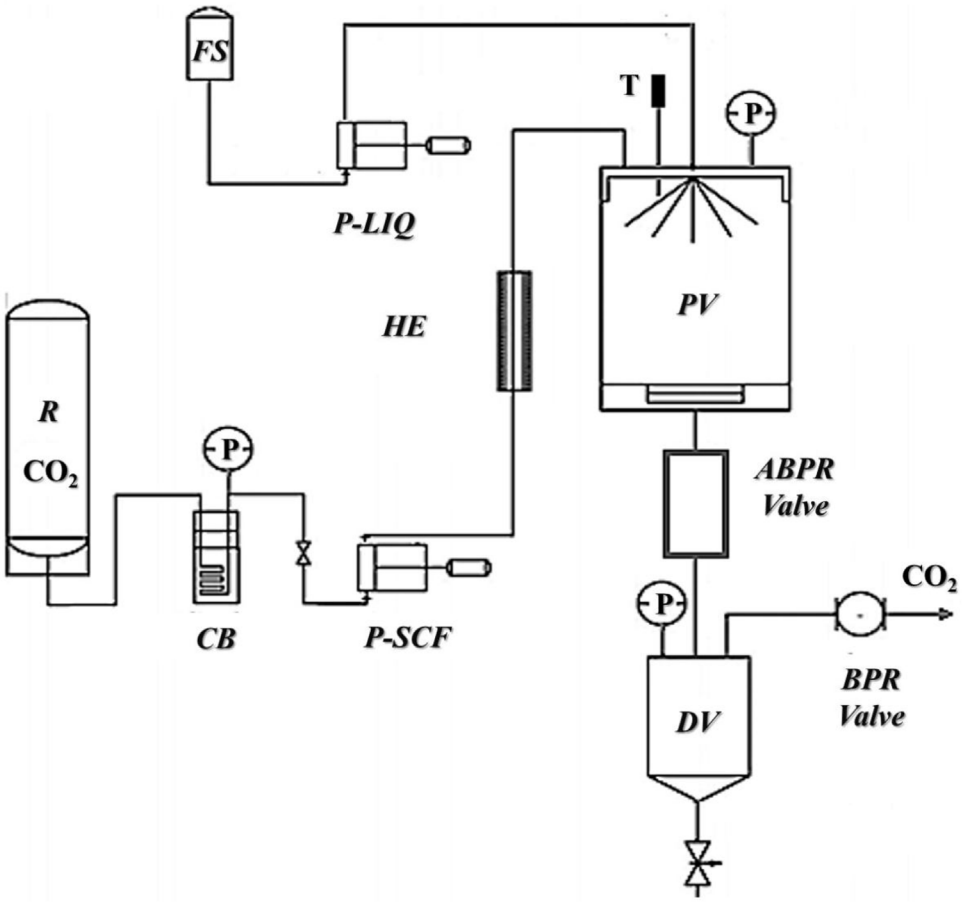

Fig. 1. Scheme of the SAF plant. Feed solution reservoir (FS); liquid pump (P-LIQ); CO_2 reservoir (R), cooling bath (CB); CO_2 pump (P-SCF); heat exchanger (HE); precipitation vessel (PV); Thermopar (T); automated back pressure regulator (ABPR); back pressure regulator (BPR); downstream vessel (DV).

A mathematical model for a two variable CCD is represented by Eq. (5)

$$Y = \beta_0 + \sum_{i=1}^{2}\beta_i X_i + \sum_{i=1}^{2}\beta_{ii}X_i^2 + \sum_{i\neq j=1}^{2}\beta_{ij}X_iX_j \quad (5)$$

where Y is an independent variable *(extraction yield)*, β_0 is the constant coefficient, β_1 and β_2 are linear coefficients, β_{11} and β_{22} are quadratic coefficients and β_{12} is an interaction coefficient, and X_i and Xj (X_P and $X_{Q_{CO_2}}$) are the independent variables whose influence is under study.

Response surface design following a central composite design was performed using the software *Minitab 17,* which propounded 11 random experiments with three central replicates (115 bar, 20 g/min) according to the range levels of both variables previously set (Table 1). The statistical software was also used to determine the significance ($p < 0.05$) of each coefficient in the model (Eq. (5)) and the optimal conditions for the maximum yield recovery or maximum bioactive compound concentration in *L. luisieri* extracts (RA, OA and UA).

2.7. Microscopy observations

A scanning electron microscope (SEM) was used to characterise the solid morphology obtained in the PV by SAF. It was performed by the Electron Microscopy service from Zaragoza University (Spain). To that extent, a LEO 420 version V2.04, ASSING, was used. Extracted solids were placed on a carbon tab previously stuck to an aluminium stub (Agar Scientific, Stansted, UK). Samples were overcoated with carbon using a sputter coater (mod. 108A, Agar Scientific), to have an approximated idea of the particles observed, some spheres of the obtained images were measured using the software *Smartiff image estimator*.

3. Results and discussion

3.1. L. luisieri *maceration extraction yield and chemical characterization*

Plant material was submitted to two serial macerations. First of all, plant material was soaked into hexane in order to eliminate volatiles and degrease non-polar compounds such as cuticular waxes [20]. The extraction yield of this maceration with hexane, Y_{hex}, was 3.3 %. This pretreatment reduce the dilution of the final bioactivity of the second maceration extract. Then, polar and bioactive compounds were obtained in a second maceration performed with ethanol. It is nontoxic solvent, easily biodegradable and it has a higher extractive capacity because it breaks the cell membrane of plant material [34]. This second extract was afterwards processed through Supercritical Antisolvent Fractionation, which only uses Carbon dioxide, also non toxic. The seriated treatment of natural resources allows its complete exploitation and it has been observed in a previous work performed [35,36].

The extraction yield obtained, Y_{EtOH}%, was 12 %, a total content in actives of 120 mg/g of dried *L. luisieri*. Julio et al. [11] obtained similar results when extracting from 2 different *L. luisieri* populations with a Soxhlet apparatus, 18 % and 12 % respectively, methodology that applies heat. In this work the extraction was performed at room

temperature and in ambar bottles to avoid degradation from heat and light, obtaining extraction yield results comparable with soxhlet procedure. The different *L. luisieri* populations presented mainly quantitative differences among the founded actives, for example, in the relative content in rosmarinic (3.4 % vs 7.3 %) and oleanolic acid (3.5 % vs 3.0 %), but it was also noticeable the qualitative difference between them regarding ursolic acid, absent in one of the populations [11].

In this work, the ethanolic extract composition analysis, performed applying the method described in 2.7, allowed the identification and quantification of rosmarinic acid (RA), oleanolic acid (OA), and ursolic acid, (UA). Their retention times were 1.6 min, 7.5 min and 7.8 min, respectively, as can be observed in Fig. 2. RA was measured at 310 nm, and OA and UA were measured at 210 nm for a better peak definition and quantification. All identified compounds were quantified: RA 5.3 % ±1.2, OA 2.4 % ±0.8 and UA 5.1 % ±1.2. Although in this work only three compounds were identified, Upson et al. [37] reported that an methanolic extract form Portuguese *L. luisieri* contained several types of flavonoids. Further studies should be perform to elucidate completely the composition of this extract. This maceration extract was submitted to SAF under different experimental conditions of pressure and CO_2 flow rate.

3.2. SAF mass recovery yields

Several experiments of supercritical antisolvent fractionation of *L. luisieri* ethanolic extract were performed varying the pressure and CO_2 flow rate conditions inside the ranges 80–150 bar and 10–30 g/min respectively. The other experimental parameters were fixed; temperature 40 °C to avoid degradation, liquid flow rate 0.5 mL/min to maintain CO_2 molar fraction over the critical point, and FS concentration 3% (wt.%). After every SAF experiment, the mass recovered in each fraction was quantified, and the yields Y_{PV}%, Y_{DV}% and the sum of them Y_{SAF}% were determined according to Eq. (4). These yield results are shown in Table 2, where they have been organised in ascending order of X_P and $X_{Q_{CO_2}}$ for an easier understanding of the data. The central experimental replicates yield measures with 95 % confidence intervals, were Y_{PV}% 39.2 % ± 3.6, Y_{DV}% 20.1 % ± 0.98 and Y_{SAF}% 59.4 % ± 4.2.

The conditions at which the highest mass recovery (Y_{SAF}%) was obtained were; 90 bar-27 g/min, 140 bar-27 g/min, and at 115 bar-30 g/min. Besides, as can be observed in Table 2, for all measured yields, under the same X_P the yield increases with the $X_{Q_{CO_2}}$. According to these results, $X_{Q_{CO_2}}$ seems to have a marked effect in the mass recovery from *L. luisieri* ethanolic extract. A higher proportion of $scCO_2$ favoured compound precipitation in PV as well as dragging compounds to DV, resulting in an increased total mass recovery. Consequently, the overall mass losses in the SAF equipment are lower when $X_{Q_{CO_2}}$ is increased. Nevertheless, it was not achieved a complete recovery of the matter introduced in the equipment, since, it was always some material retained into the valves, pipes or filter of the SAF equipment. These results correspond with a previous study reported by Martín et al. [38], although there have been reported other behaviours like with *Artemisia absinthium* [32]. These differences reported depend on the original plant material understudied, the composition of the extract to be fractionated and its solubility into the mixture ethanol-$scCO_2$.

Regarding the amount of material recovered in PV and DV, it is noticeable that Y_{PV}% is always higher than Y_{DV}% independent of the X_P and $X_{Q_{CO_2}}$ experimental conditions. The range of Y_{PV}% values was 27.7–55.6 % (at 115 bar, 10 g/min and at 90 bar, 27 g/min, respectively), and the range of Y_{DV}% values was 8.8–30.9 % (at 80 bar, 20 g/min and at 115 bar, 30 g/min, respectively). The range of Y_{SAF}% values was 46.9–82.6 % (at 115 bar, 10 g/min and at 115 bar, 30 g/min, respectively). This can be interpreted as the ethanolic maceration extract of *L. luisieri* having a low solubility in ethanol-$scCO_2$ mixture. The initial composition of the FS affects to the final mass yields obtained in SAF fractions. While Marqués et al. [27] who studied the *Vitis vinifera* seeds extract, yield results correlate with the ones obtained in this work, in a previous study with *Artemisia absithium* ethanolic extract, the mass recover results were very different since the DV fraction yield was always higher than the PV fraction yield [32].

3.3. Actives supercritical fractionation

The ethanolic extract, containing RA 5.3 % ±1.2, OA 2.4 % ±0.8 and UA 5.1 % ±1.2, was dissolved in ethanol at 3% (wt.%) to constitute the feed solution (FS) for each one of the 11 SAF experiments. FS provided two fraction after every experiment: PV, a solid that precipitates in the mixture ethanol-sc-CO_2, and DV, soluble compounds in the mixture collected as an ethanolic solution after depressurization and separation of CO_2. After each experiment, a fine yellow-green powder and a green solution were obtained in the PV and DV fractions respectively, and their content in RA, OA and UA quantified with HPLC-PDA and express in percentage. All chromatographic assays were performed in triplicate (total chromatographic assays n = 99). In order to analyse the behaviour of each compound during the supercritical process, it was measured the concentration in PV and DV of each component regarding to the ethanolic extract used as feed solution (FS). To do so, the ratios PV/FS and DV/FS were defined according to Eq. (6) and when any of these ratios was >1 an enrichment of a compound was assumed. The percentage of RA, OA and UA in each fraction and their ratio regarding the initial FS, are shown in Table 2, where results have been organised in ascending order of X_P and $X_{Q_{CO_2}}$ for an easier understanding of the data.

$$(PV\ or\ DV)/FS = \frac{mg\ \text{of active}/g\ \text{of}\ PV\ \text{or}\ DV}{mg\ \text{of active}/g\ \text{of}\ FS} \quad (6)$$

The chromatographic analysis revealed that RA is completely retained in the PV, regardless of the CO_2 pressure and flow rate conditions. RA% in the solid fraction was always higher than in the initial FS therefore, RA ratio PV/FS was always ≥1 (1.1–2.3) (Fig. 2). Due to RA complete precipitation in the first vessel, PV, it concentrates its quantity regarding to the FS, providing a solid extract with higher proportion in this antioxidant than in the ethanolic extract. At the working conditions of 140 bar 27 g/min of CO_2 flow rate, it was achieved the highest enrichment of RA regarding the initial FS (PV/FS), 2.3 times higher, which correspond with 99.2 mg/g of PV. According to these results, it can be said that RA, the most polar compound studied in this work, is insoluble in the ethanol-$scCO_2$ mixture. Previous studies applying this technology to Rosmarinus extract obtained also an RA enrichment regarding the initial feed solution, in the PV, *raffinate or precipitate* [17,29]. Quintana et al. [17] produced a precipitated with a 2–3 fold enrichment of rosmarinic acid at a work range of 80–200 bar and 40–60 °C.

On the other hand, OA and UA are partially soluble in the mixture of solvents because they distributed between PV and DV (Fig. 2). The solubility of this triterpenes in the mixture ethanol-$scCO_2$ has been previously observed in the supercritical extraction of apple pomace and *Hedoytis diffusa* or snake needle grass [39,40]. The highest UA extraction yield extracted from apple pomace was at 60 °C, 550 bar and ethanol 25 % (w/w), while for OA from snake needle grass was 282 bar, 56 °C and ethanol 12.5 % (v/v).

Besides, this separation between both fractions seems to be influenced by X_P and $X_{Q_{CO_2}}$ because their concentration distribution varied in every SAF experiment performed. This is shown in Table 2, where OA and UA concentrations (%) and therefore their ratios (PV/FS and DV/FS) varied. OA concentration ratios in PV were

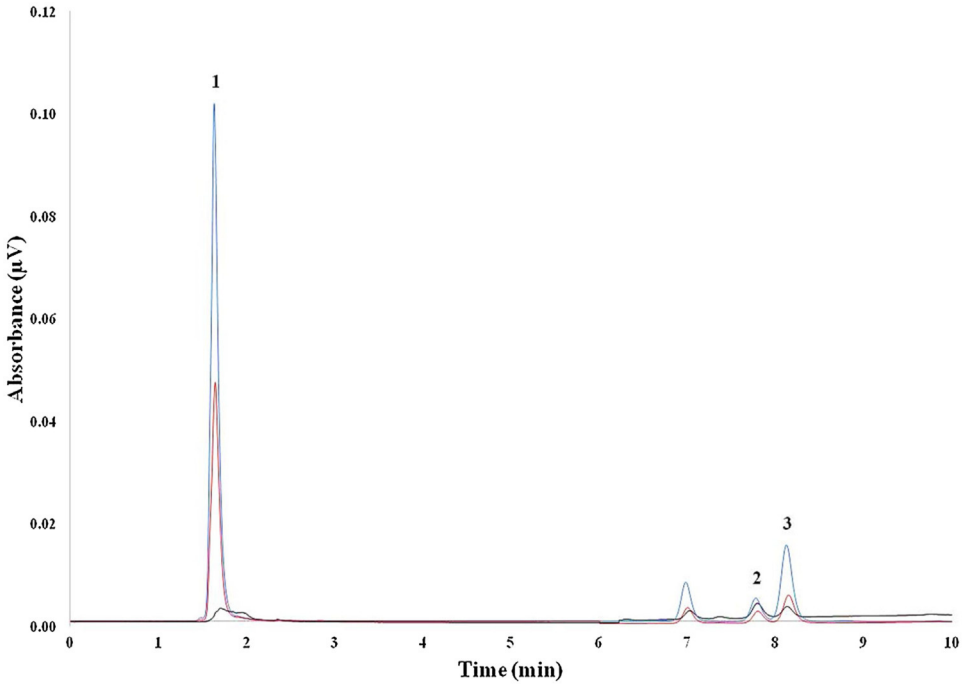

Fig. 2. Overlayed chromatograms of ME (red) PV (blue) and DV (black) at 115 bar and 20 g/min. Peak 1 RA (T_R =1.626 min, λ =310 nm), peak 2 OA (T_R =7.468 min, λ =210 nm) and peak 3 UA (T_R =7.802 min, λ =210 nm).

Table 2
L. luisieri experimental SAF results. Experimental yields obtained in each experiment; Y_{SAF} %, Y_{PV} % and Y_{DV}% RA, OA and UA in % quantified in FS, PV and DV obtained in each experiment; and RA, OA and UA concentration in PV and DV regarding FS.

X_P (bar)	$X_{Q_{CO_2}}$ (g/min)	Y_{PV}%	Y_{DV}%	Y_{SAF}%	RA			OA					UA					PV/FS
					FS (%)	PV(%)	PV/FS	FS(%)	PV(%)	DV(%)	PV/FS	DV/FS	FS(%)	PV(%)	DV(%)	PV/FS	DV/FS	
80	**20**	48.0	8.8	56.8	5.1	8.9	**1.8**	1.9	1.8	5.5	**1.0**	**2.9**	4.6	7.0	4.6	**1.5**	**1.0**	**1.4**
90	**13**	39.0	15.4	54.4	4.0	7.9	**2.0**	1.5	3.3	2.4	**2.3**	**1.6**	3.8	10.4	1.8	**2.7**	**0.5**	**1.7**
	27	55.6	20.1	75.7	6.4	6.7	**1.1**	2.5	2.5	1.2	**1.0**	**0.5**	5.6	5.6	1.0	**1.0**	**0.2**	**1.2**
115	**10**	27.7	19.2	46.9	5.7	8.0	**1.4**	2.3	1.9	3.5	**0.8**	**1.5**	5.2	7.1	4.7	**1.4**	**0.9**	**1.4**
	20	38.7	19.2	57.9	4.0	8.9	**2.2**	1.5	2.1	5.0	**1.3**	**3.2**	3.9	6.5	3.9	**1.7**	**1.0**	**1.4**
	20	36.3	20.3	56.6	3.8	7.3	**2.0**	2.2	2.4	3.5	**1.1**	**1.6**	5.1	8.2	2.7	**1.6**	**0.5**	**1.4**
	20	42.6	20.9	63.6	6.3	8.1	**1.3**	3.7	2.4	3.2	**0.7**	**0.9**	7.7	7.0	2.3	**0.9**	**0.3**	**1.4**
	30	51.7	30.9	82.6	6.3	8.1	**1.3**	2.6	3.0	0.6	**1.2**	**0.2**	5.5	8.8	0.6	**1.6**	**0.1**	**1.6**
140	**13**	34.2	22.7	56.9	7.2	7.5	**1.1**	2.8	1.5	4.6	**0.5**	**1.7**	6.5	5.9	4.2	**0.9**	**0.7**	**1.2**
	27	50.6	29.6	80.2	4.4	10.0	**2.3**	1.7	2.0	5.9	**1.2**	**3.6**	4.1	10.3	5.1	**2.5**	**1.3**	**1.8**
150	**20**	33.9	20.3	54.2	4.6	6.9	**1.5**	1.7	3.4	0.0	**2.0**	**0.0**	4.3	7.8	0.2	**1.8**	**0.1**	**1.5**

0.53–2.00 which correspond to 1.49–3.40 % of PV, while DV/FS ranged from 0.00–3.56, which corresponds to 0.00–5.86 % DV fraction. UA and OA have a very similar chemical structure and same molecular weight, only differs on the position of a methyl group. As we can see in Table 2, at some experimental conditions such as 115 bar 30 g/min or 150 bar 20 g/min both terpenes behaves similarly, they precipitate in the first vessel when they find sc-CO_2, and only a few proportion of them is dragged to the final DV fraction.

UA PV/FS varied from 0.90 to 2.72, which corresponds to 5.86–10.44 % of PV fraction, while DV/FS was mainly <1 (0.05–1.28) which corresponds to 0.20–5.22 %. Between all experiment performed, at 140 bar 27 g/min OA and UA concentrates in both fractions along with RA in PV and a high mass recovery is obtained. The different distribution into SAF fractions of natural extracts differ among plant species depending on the solubility into the mixture ethanol-scCO_2 of their main compounds. An analysis of the possible traces of the liquid solvent employed should be performed in further studies since the simultaneous concentration of this three natural bioactives could have interest for its industrial production and application in the alimentary or pharmaceutical fields as natural preservatives.

3.4. SEM image analysis/characterization

The microscopic observation of the precipitated solid obtained in the PV showed spherical morphologies and particles of nanometric order. Although an analysis of particle distribution was not performed, some micrographs of the powder recovered in some experiments for its observation. As an example, Fig. 3 is provided, where particle measured diameter observed was of 68−70 nm at 90 bar 27 g/min. The morphology and the size are highly influenced by the droplet formed by the injector and the liquid surface tension [41] but also by the experimental conditions. During the precipitation process, the scCO_2 diffuses and eliminates the ethanol that surrounds the extract very quickly forcing the solid to conserve its original shape and volume, and when the mixture ethanol-scCO_2 is over the critical point an increase of pressure leads to smaller particles would [42]. The mean size and particle size distribution of the precipitate should be further characterize to determine the influence of X_P and $X_{Q_{CO_2}}$ in the precipitation process for this *L. luisieri* extract.

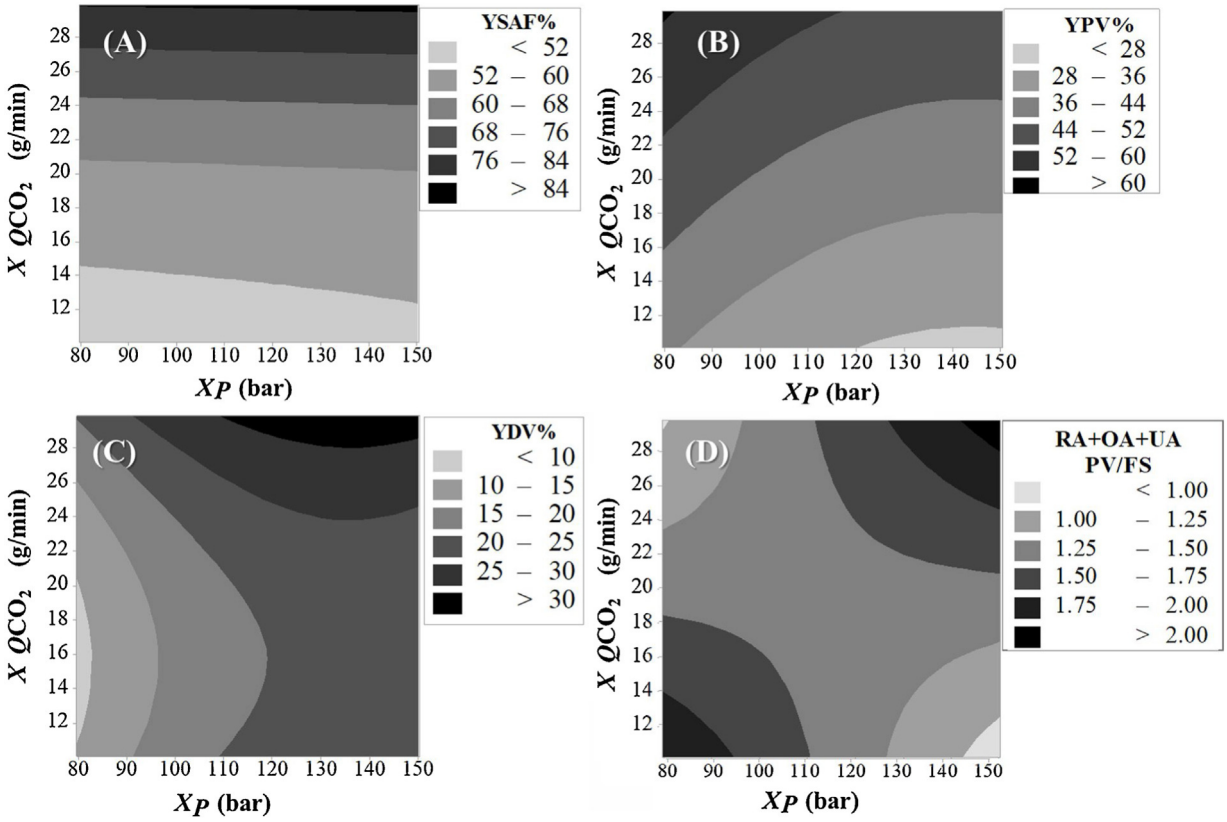

Fig. 4. Response surface plots of the SAF fraction yields: A) Y_{PV}%; B) Y_{DV}%; C) Y_{SAF}% as a function of X_P and $X_{Q_{CO_2}}$; and D) concentration of RA+OA+UA in PV regarding FS as a function of X_P and $X_{Q_{CO_2}}$.

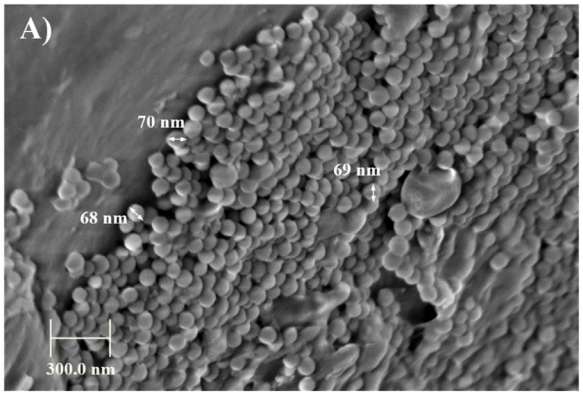

Fig. 3. Scanning electron micrograph from the solid obtained in the precipitation vessel. Minimum particle measured diameter of 68 nm at 90 bar 27 g/min (Mag=40.00 K X).

3.5. Statistical analysis and SAF conditions optimization

In order to determine the statistical influence of X_P and $X_{Q_{CO_2}}$ variables, a surface response analysis of all these results was performed with the software *Minitab 17*. Y_{SAF}% Y_{PV}% Y_{DV}% and RA+OA+UA PV/FS were adopted as response variables and used to determine the coefficients of the equations. The level of significance of each equation factor (linear, quadratic and interaction), the final coefficient of determination (R^2) and the standard deviation (s) were obtained. The Eqs. (6)–(8) define the response surface of the experimental yields, Y_{PV}% Y_{DV}% and Y_{SAF}% respectively, as a function of pressure and CO_2 flow rate. These equations are graphically represented in Fig. 4.

Total recovery of plant material (Y_{SAF}%) and its distribution between precipitation and downstream vessels (Y_{PV}% and Y_{DV}%) depended on X_P and $X_{Q_{CO_2}}$ experimental conditions.

According to the statistical analysis, the mass recovered in the PV is influenced by both linear factors and only quadratic *XP* (Eq. (7)). The graphical representation of this equation in Fig. 4B predicts a higher Y_{PV}% at the lowest values of X_P and the highest $X_{Q_{CO_2}}$ of the range studied. At this same figure, a maximum recovery in DV at the highest values of $X_{Q_{CO_2}}$ can be observed, however in this case higher values of *XP* are also required. Y_{DV}% depends on both linear and quadratic X_P and $X_{Q_{CO_2}}$ (Eq. (8)). The solubility of compounds extracted with ethanol from *L. luisieri* seems to increase their solubility along with pressure, causing their pass through to the PV filter towards the DV fraction. See Fig. 4C.

Finally, as Y_{SAF}% is the sum of both PV and DV yields, the influence of X_P and $X_{Q_{CO_2}}$ is a combination their effect in both fractions separately. In this case, Y_{SAF}% depends on the linear factors and the quadratic $X_{Q_{CO_2}}$ (Eq. (9)). In the graphical representation, Fig. 4A, of this equation, for a fixed pressure the yield increases with the $X_{Q_{CO_2}}$.

$$Y_{PV}\% = 67.6 - 0.737X_P + 1.196X_{Q\,CO2} + 0.00256X_P{}^2 (R^2 = 88.93\%, s = 3.5) \quad (7)$$

$$Y_{DV}\% = -32.9 + 1.003X_P - 1.920X_{Q\,CO2} - 0.00364X_P{}^2 + 0.0606X_{Q\,CO2}{}^2 (R^2 = 88.59\%, s = 2.7) \quad (8)$$

$$Y_{SAF}\% = 59.4 + 0.0164X_P - 1.92X_{Q\,CO2} + 0.0905X_{Q\,CO2}{}^2 (R^2 = 90.17\%, s = 4.4) \quad (9)$$

It was also analysed how experimental variables influenced the fractionation chemical composition. According to the results, the behaviour of RA, OA and UA did not respond individually to the CCD model applied in this work, nor its % in PV or DV, neither their concentration regarding FS, PV/FS or DV/FS. Nevertheless, when the three compounds are analysed together RA+OA+UA, their concentration in PV regarding the initial FS depend on the studied experimental variables *XP* and XQ_{CO2}. This dependence, defined in Eq. (10), is graphically represented in Fig. 4D.

$$RA + OA + UAPV/FS = 5.257 - 0.03213X_P - 0.2064X_{Q_{CO_2}} + 0.000649X_{Q\,CO2}{}^2 + 0.001625X_P \cdot X_{Q\,CO2} (R^2 = 96.24\%, s = 0.05) \quad (10)$$

According to this statistical prediction equation, the highest concentration of RA, OA and UA in the PV can be obtained at low X_P and $X_{Q_{CO_2}}$ (X_P < 90 bar and $X_{Q_{CO_2}}$ < 12 g/min) or at high X_P and $X_{Q_{CO_2}}$ (X_P > 140 bar and $X_{Q_{CO_2}}$ > 27 g/min) which correspond with the results observed in Table 2.

Under these experimental conditions, the actives solubility in the ethanol-scCO_2 mixture decreases. RA, OA and UA have been reported to present different biological activities; RA as anti-inflammatory, anti-allergy [43] and cytoprotective [18]; and OA and UA as hepatoprotective [44], antiinflamatory [15], antimutagenic [45], and antimicrobial [18,35,46]. Besides, because of the concomitant presence of OA and UA in many medicinal plant species, some studies have reported their positive effects when applied together. They have shown chemo-protective effects against DNA damage through oxidation [34,35], and in vitro and in vivo anti-proliferatives [47]. Because of this, their concomitant concentration may be interesting for several applications. Thus, the antioxidant and antimicrobial properties of *L. luisieri* supercritical fractions have been reported. Both bioactivities were concentrated into the PV fraction regarding the initial ethanolic extract. The antioxidant activity was related to the enrichment in rosmarinic acid, while the terpenes oleanolic and ursolic acids seemed to be responsible of the inhibitory and bactericidal properties [31]. Therefore, the final enriched multifunctional product can be used for several applications.

According to the final optimisation analysis performed in this work, the theoretical conditions for a maximum extract recovery after the SAF process (Y_{SAF}%, Y_{PV}% and Y_{DV}% maximum) and a higher concentration of the three studied compounds in PV (RA+OA+UA PV/FS) are 130 bar and 30 g/min (composite desirability 0.9385).

4. Conclusions

In the present work, an integrated process based on the use of supercritical antisolvent fractionation has been optimised to obtain *L. luisieri* extracts with concentrated composition in bioactives. By employing an RSM CCD it has been possible to optimise the yield recovery by modifying important factors involved in the SAF process (pressure X_P and scCO_2 flow rate $X_{Q_{CO_2}}$). Besides, the behaviour of three tracked compounds identified from the ethanolic extract of *L. luisieri* along the supercritical process was also followed. The optimised conditions for a higher mass recovery and RA, OA and UA enrichment in PV were 130 bar 30 g/min. The application of the green SAF technology lets us obtain in the PV a fine solid product highly enriched, with potential for its industrial production and application in the alimentary or pharmaceutical fields as natural preservatives.

Declaration of Competing Interest

The corresponding author, on behalf of all authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] R. Gyawali, S.A. Ibrahim, Natural products as antimicrobial agents, Food Control 46 (December) (2014) 412–429, http://dx.doi.org/10.1016/j.foodcont.2014.05.047.
[2] R. Campos-Vega, G. Loarca-Piña, B.D. Oomah, Minor components of pulses and their potential impact on human health, Food Res. Int. 43 (March (2)) (2010) 461–482, http://dx.doi.org/10.1016/j.foodres.2009.09.004.
[3] S. Krüger, M. Mirgos, G.E. Morlock, Effect-directed analysis of fresh and dried elderberry (Sambucus nigra L.) via hyphenated planar chromatography, J. Chromatogr. A 1426 (December) (2015) 209–219, http://dx.doi.org/10.1016/j.chroma.2015.11.021.
[4] A. Oniszczuk, M. Olech, T. Oniszczuk, K. Wojtunik-Kulesza, A. Wójtowicz, Extraction methods, LC-ESI-MS/MS analysis of phenolic compounds and antiradical properties of functional food enriched with elderberry flowers or fruits, Arab. J. Chem. (2016), http://dx.doi.org/10.1016/j.arabjc.2016.09.003.
[5] M.D. Kalim, D. Bhattacharyya, A. Banerjee, S. Chattopadhyay, Oxidative DNA damage preventive activity and antioxidant potential of plants used in Unani system of medicine, BMC Complement. Altern. Med. 10 (December (1)) (2010), http://dx.doi.org/10.1186/1472-6882-10-77, p. 77.
[6] M.C.M.I. García-Vallejo, J. Sanz, M. Bernabe, A. Velasco-Negueruela, Necrodane (1,2,2,3,4-pentamethylcyclopentane) derivatives in *Lavandula luisieri*, new compounds to the plant kingdom, Phytochemistry 36 (May (1)) (1994) 43–45, http://dx.doi.org/10.1016/S0031-9422(00)97009-2.
[7] T. Eisner, M. Deyrup, R. Jacobs, J. Meinwald, Necrodols: anti-insectan terpenes from defensive secretion of carrion beetle (Necrodes surinamensis), J. Chem. Ecol. 12 (June (6)) (1986) 1407–1415, http://dx.doi.org/10.1007/BF01012360.
[8] B. Roach, T. Eisner, J. Meinwald, Defense mechanisms of arthropods. 83. alpha.- and. beta.-Necrodol, novel terpenes from a carrion beetle (Necrodes surinamensis, Silphidae, Coleoptera), J. Org. Chem. 55 (June (13)) (1990) 4047–4051, http://dx.doi.org/10.1021/jo00300a020.
[9] B.A. Figadère, J.S. McElfresh, D. Borchardt, K.M. Daane, W. Bentley, J.G. Millar, trans-α-Necrodyl isobutyrate, the sex pheromone of the grape mealybug, Pseudococcus maritimus, Tetrahedron Lett. 48 (2020) 8434–8437.
[10] N. Baldovini, S. Lavoine-Hanneguelle, G. Ferrando, G. Dusart, L. Lizzani-Cuvelier, Necrodane monoterpenoids from *Lavandula luisieri*, Phytochemistry 66 (July (14)) (2005) 1651–1655, http://dx.doi.org/10.1016/j.phytochem.2005.04.040.
[11] L.F. Julio, et al., Comparative chemistry and insect antifeedant effects of conventional (Clevenger and Soxhlet) and supercritical extracts (CO2) of two *Lavandula luisieri* populations, Ind. Crops Prod. 58 (July) (2014) 25–30, http://dx.doi.org/10.1016/j.indcrop.2014.03.021.
[12] A. González-Coloma, F. Delgado, J.M. Rodilla, L. Silva, J. Sanz, J. Burillo, Chemical and biological profiles of *Lavandula luisieri* essential oils from western Iberia Peninsula populations, Biochem. Syst. Ecol. 39 (February (1)) (2011) 1–8, http://dx.doi.org/10.1016/j.bse.2010.08.010.
[13] J. Bernatoniene, U. Cizauskaite, L. Ivanauskas, V. Jakstas, Z. Kalveniene, D.M. Kopustinskiene, Novel approaches to optimize extraction processes of ursolic, oleanolic and rosmarinic acids from *Rosmarinus officinalis* leaves, Ind. Crops Prod. 84 (June) (2016) 72–79, http://dx.doi.org/10.1016/j.indcrop.2016.01.031.
[14] M. Ghasemzadeh Rahbardar, B. Amin, S. Mehri, S.J. Mirnajafi-Zadeh, H. Hosseinzadeh, Anti-inflammatory effects of ethanolic extract of *Rosmarinus officinalis* L. and rosmarinic acid in a rat model of neuropathic pain, Biomed. Pharmacother. 86 (February) (2017) 441–449, http://dx.doi.org/10.1016/j.biopha.2016.12.049.

[15] J. Liu, Pharmacology of oleanolic acid and ursolic acid, J. Ethnopharmacol. 49 (December (2)) (1995) 57–68, http://dx.doi.org/10.1016/0378-8741(95)90032-2.
[16] M. Petersen, M.S.J. Simmonds, Rosmarinic acid, Phytochemistry 62 (2) (2003) 121–125, http://dx.doi.org/10.1016/S0031-9422(02)00513-7, ene.
[17] S.E. Quintana, D. Villanueva-Bermejo, G. Reglero, M.R. García-Risco, T. Fornari, Supercritical antisolvent particle precipitation and fractionation of rosemary (Rosmarinus officinalis L.) extracts, J. CO2 Util. 34 (December) (2019) 479–489, http://dx.doi.org/10.1016/j.jcou.2019.07.032.
[18] S. Nunes, et al., Therapeutic and nutraceutical potential of rosmarinic acid—cytoprotective properties and pharmacokinetic profile, Crit. Rev. Food Sci. Nutr. 57 (June (9)) (2017) 1799–1806, http://dx.doi.org/10.1080/10408398.2015.1006768.
[19] K.I. Wolska, A.M. Grudniak, B. Fiecek, A. Kraczkiewicz-Dowjat, A. Kurek, Antibacterial activity of oleanolic and ursolic acids and their derivatives, Cent. Eur. J. Biol. 5 (October (5)) (2010) 543–553, http://dx.doi.org/10.2478/s11535-010-0045-x.
[20] M.M.R. de Melo, A.J.D. Silvestre, C.M. Silva, Supercritical fluid extraction of vegetable matrices: applications, trends and future perspectives of a convincing green technology, J. Supercrit. Fluids 92 (2014) 115–176, http://dx.doi.org/10.1016/j.supflu.2014.04.007, ago.
[21] E. Reverchon, I. De Marco, Supercritical fluid extraction and fractionation of natural matter, J. Supercrit. Fluids 38 (September (2)) (2006) 146–166, http://dx.doi.org/10.1016/j.supflu.2006.03.020.
[22] V. Pérez-Tortosa, A. López-Orenes, A. Martínez-Pérez, M.A. Ferrer, A.A. Calderón, Antioxidant activity and rosmarinic acid changes in salicylic acid-treated Thymus membranaceus shoots, Food Chem. 130 (2) (2012) 362–369, http://dx.doi.org/10.1016/j.foodchem.2011.07.051, ene.
[23] M.A. Meneses, G. Caputo, M. Scognamiglio, E. Reverchon, R. Adami, Antioxidant phenolic compounds recovery from Mangifera indica L. By-products by supercritical antisolvent extraction, J. Food Eng. 163 (October) (2015) 45–53, http://dx.doi.org/10.1016/j.jfoodeng.2015.04.025.
[24] P. Franco, E. Reverchon, I. De Marco, PVP/ketoprofen coprecipitation using supercritical antisolvent process, Powder Technol. 340 (December) (2018) 1–7, http://dx.doi.org/10.1016/j.powtec.2018.09.007.
[25] G. Ozkan, P. Franco, I. De Marco, J. Xiao, E. Capanoglu, A review of microencapsulation methods for food antioxidants: principles, advantages, drawbacks and applications, Food Chem. 272 (2019) 494–506, http://dx.doi.org/10.1016/j.foodchem.2018.07.205, ene.
[26] G. Perretti, C. Virgili, A. Troilo, O. Marconi, G.F. Regnicoli, P. Fantozzi, Supercritical antisolvent fractionation of lignans from the ethanol extract of flaxseed, J. Supercrit. Fluids 75 (March) (2013) 94–100, http://dx.doi.org/10.1016/j.supflu.2012.12.028.
[27] J.L. Marqués, G.D. Porta, E. Reverchon, J.A.R. Renuncio, A.M. Mainar, Supercritical antisolvent extraction of antioxidants from grape seeds after vinification, J. Supercrit. Fluids 82 (October) (2013) 238–243, http://dx.doi.org/10.1016/j.supflu.2013.07.005.
[28] J.T. Paula, I.M.O. Sousa, M.A. Foglio, F.A. Cabral, Selective fractionation of extracts of *Arrabidaea chica* Verlot using supercritical carbon dioxide as antisolvent, J. Supercrit. Fluids 133 (March) (2018) 9–16, http://dx.doi.org/10.1016/j.supflu.2017.09.021.
[29] A.P. Sánchez-Camargo, et al., Supercritical antisolvent fractionation of rosemary extracts obtained by pressurized liquid extraction to enhance their antiproliferative activity, J. Supercrit. Fluids 107 (2016) 581–589, http://dx.doi.org/10.1016/j.supflu.2015.07.019, ene.
[30] A. Visentin, S. Rodríguez-Rojo, A. Navarrete, D. Maestri, M.J. Cocero, Precipitation and encapsulation of rosemary antioxidants by supercritical antisolvent process, J. Food Eng. 109 (March (1)) (2012) 9–15, http://dx.doi.org/10.1016/j.jfoodeng.2011.10.015.
[31] C. Giménez-Rota, S. Lorán, A.M. Mainar, M.J. Hernáiz, C. Rota, Supercritical carbon dioxide antisolvent fractionation for the sustainable concentration of Lavandula luisieri (Rozeira) riv.- mart antimicrobial and antioxidant compounds and comparison with its conventional extracts, Plants 8 (November (11)) (2019), http://dx.doi.org/10.3390/plants8110455, p. 455.
[32] Elisa Langa, Juan Ignacio Pardo, Carlota Giménez-Rota, Azucena González-Coloma, María J. Hernáiz, Ana M. Mainar, Supercritical anti-solvent fractionation of *Artemisia absinthium* L. conventional extracts: tracking artemetin and casticin, J. Supercrit. Fluids 151 (May) (2019), http://dx.doi.org/10.1016/j.supflu.2019.05.003.
[33] C. Douglas, Montgomery, Design and Analysis of Experiments, 9th ed., Wiley, 2020.
[34] A. Wüst Zibetti, A. Aydi, M. Arauco Livia, A. Bolzan, D. Barth, Solvent extraction and purification of rosmarinic acid from supercritical fluid extraction fractionation waste: Economic evaluation and scale-up, J. Supercrit. Fluids 83 (November) (2013) 133–145, http://dx.doi.org/10.1016/j.supflu.2013.09.005.
[35] Hanna Pieper, The Utilization of the Residues of Viticulture Via Supercritical Extraction: Antioxidative Capacity of the Extracts, Bachelor Essay, 2020.
[36] Juan I. Pardo, et al., Té de roca: Extractos supercriticos y actividad antioxidante, in: vol. VII reunión de expertos en tecnologías de fluidos comprimidos, 2020, 10-13 Junio.
[37] T.M. Upson, R.J. Grayer, J.R. Greenham, C.A. Williams, F. Al-Ghamdi, F.-H. Chen, Leaf flavonoids as systematic characters in the genera Lavandula and Sabaudia, Biochem. Syst. Ecol. 28 (December (10)) (2000) 991–1007, http://dx.doi.org/10.1016/S0305-1978(00)00013-2.
[38] L. Martín, A. González-Coloma, C.E. Díaz, A.M. Mainar, J.S. Urieta, Supercritical CO2 extraction of Persea indica: effect of extraction parameters, modelling and bioactivity of its extracts, J. Supercrit. Fluids 57 (June (2)) (2011) 120–128, http://dx.doi.org/10.1016/j.supflu.2011.03.004.
[39] E. Ordonez-Quintana, et al., Supercritical and subcritical extraction of ursolic acid and polyphenols from apple pomace: effect of variables on composition and antioxidant capacity, J. Food Process. Preserv. (2020), http://dx.doi.org/10.1111/jfpp.14296, p. e14296.
[40] M.-C. Wei, Y.-C. Yang, S.-J. Hong, Determination of oleanolic and ursolic acids in Hedyotis diffusa using hyphenated ultrasound-assisted supercritical carbon dioxide extraction and chromatography, Evid. Based Complement. Altern. Med. ECAM 2015 (2015), http://dx.doi.org/10.1155/2015/450547.
[41] E. Reverchon, I. De Marco, Mechanisms controlling supercritical antisolvent precipitate morphology, Chem. Eng. J. 169 (May (1)) (2011) 358–370, http://dx.doi.org/10.1016/j.cej.2011.02.064.
[42] A. Montes, C. Pereyra, E.J.M. de la Ossa, Mean aspects controlling supercritical CO2 precipitation processes, Heat Mass Transf. Adv. Sci. Technol. Appl. (May) (2019), http://dx.doi.org/10.5772/intechopen.85735.
[43] J. Weiss, P. Takhistov, D.J. McClements, Functional materials in food nanotechnology, J. Food Sci. 71 (November (9)) (2006) R107–R116, http://dx.doi.org/10.1111/j.1750-3841.2006.00195.x.
[44] G.A. Gutiérrez-Rebolledo, A.G. Siordia-Reyes, M. Meckes-Fischer, A. Jiménez-Arellanes, Hepatoprotective properties of oleanolic and ursolic acids in antitubercular drug-induced liver damage, Asian Pac. J. Trop. Med. 9 (july (7)) (2016) 644–651, http://dx.doi.org/10.1016/j.apjtm.2016.05.015.
[45] F. Aparecida Resende, C.A.M. de Andrade Barcala, M.C. da Silva Faria, F.H. Kato, W.R. Cunha, D.C. Tavares, Antimutagenicity of ursolic acid and oleanolic acid against doxorubicin-induced clastogenesis in Balb/c mice, Life Sci. 79 (13) (2006) 1268–1273, http://dx.doi.org/10.1016/j.lfs.2006.03.038, ago.
[46] J.A. Jesus, J.H.G. Lago, M.D. Laurenti, E.S. Yamamoto, L.F.D. Passero, Antimicrobial activity of oleanolic and ursolic acids: an update, Evid. Based Complement. Altern. Med. (2015) [En línea]. Disponible en: https://www.hindawi.com/journals/ecam/2015/620472/. [Accedido: 10-jul-2018].
[47] C. Soica, et al., The synergistic biologic activity of oleanolic and ursolic acids in complex with Hydroxypropyl-γ-Cyclodextrin, Molecules 19 (4) (2014) 4924–4940, http://dx.doi.org/10.3390/molecules19044924, abr.

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Supercritical Carbon Dioxide Antisolvent Fractionation for the Sustainable Concentration of *Lavandula luisieri* (Rozeira) Riv.- Mart Antimicrobial and Antioxidant Compounds and Comparison with Its Conventional Extracts

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Abstract: *Lavandula stoechas* subsp. *luisieri* is a Spanish subspecies from the *Lamiaceae* family. Its essential oil has been traditionally used for several medical applications though little is known about other extracts. Similar to many other studies aiming to obtain traditional plant extracts to be used in different applications, this work evaluated the antioxidant and antimicrobial activities of *Lavandula luisieri* extracts and the correlation with their composition. Traditional hydrodistillation and ethanolic maceration were used to obtain the essential oil and the maceration extract, respectively. A green and sustainable methodology was applied to the maceration extract that was under a Supercritical Antisolvent Fractionation process to obtain a fine solid enriched in rosmarinic acid and the terpenes oleanolic and ursolic acids. Antimicrobial activities of all extracts and pure identified compounds (rosmarinic and ursolic acids) were evaluated against five bacterial strains; *Listeria monocytogenes*, *Enterococcus faecium*, *Staphylococcus aureus*, *Salmonella* Typhimurium and *Escherichia coli* and were compared with the pure compounds identified, rosmarinic and ursolic acids. All strains were sensitive against *L. luisieri* essential oil. The solid product obtained from the supercritical process was concentrated in the identified actives compared to the maceration extract, which resulted in higher antimicrobial and DPPH scavenging activities. The supercritical sustainable process provided *L. luisieri* compounds, with retention of their antimicrobial and antioxidant activities, in a powder exemptof organic solvents with potential application in the clinical, food or cosmetic fields.

Keywords: SAF; antioxidant activity; antimicrobial activity; ursolic acid; oleanolic acid; rosmarinic acid

1. Introduction

Plants have been used since ancient times for their perfume, flavor, and preservative properties in a variety of products and applications with medicinal and cosmetic uses [1] because of their secondary metabolites with diverse bioactivities [2]. Nowadays, population concern about healthier and more natural habits has promoted the study of plant extracts in different research fields, specifically

to find new molecules for various applications. Indeed, it is estimated that over a hundred of new natural product-based leads are under clinical development [3] to prevent and treat chronic illnesses, whose physiopathology is based on oxidative stress, such as cardiovascular problems, diabetes or Alzheimer disease [4]. This same trend is followed by the cosmetic, food or agricultural industries, where the substitution of synthetic chemical additives or pesticides for natural ones has been demanded [5].

The Spanish lavender or *Lavandula luisieri* (Rozeira) Riv. Mart. is an aromatic *Lamiaceae* widespread in the southwest of the Iberian Peninsula. It has been traditionally used as an antiseptic of for wounds, antiaging for mature skin and scar healer, or as an antispasmodic and digestive. Its study has been centered on its essential oil, because of the atypical compounds that produces. Besides 1,8-cineole, lavandulol, linalool and their acetates, also found in other *Lavandula* species, *L. luisieri* possesses a series of compounds with a 1,2,2,3,4-pentamethylcyclopentane (necrodane) structure [6–9]. These constituents have only been previously found in the defensive secretions of the beetle *Necrodes surinamensis* and in the sexual pheromone of the grape mealybug *Pseudococcus maritimus* [10]. The potential bioactivity of this particular plant species has been focused so far on its essential oil, for which several bioactivities have been reported; antioxidant [11–13]; antimicrobial [6,13–17] antifeedant [7,8]; ixodicidal [18]; antiparasitic [19]; and antiinflamatory [13,20]. However, information about other *L. luiseri* extracts is scarce. So far, only two other studies have obtained its extract but a high temperature was applied [8,21]. Nunes et al., [21], reported a high content in polyphenols such as rosmarinic, chlorogenic, and ferulic acids in *L. luiseri* ethanolic extracts. Julio et al., [8] also identified terpene compounds such as oleanolic ursolic and tormentic acids. Rosmarinic acid, the most abundant compound in both studies, is a dimer of caffeic acid, possesses potential biological activities such as antiviral, antibacterial, anti-inflammatory and antioxidant activities [22]. In addition, oleanolic and ursolic acids, two isomer triterpenoid compounds, have hepatoprotective, anti-inflammatory, antimicrobial and anticancer effects. [23,24].

Plant extracts are usually obtained by traditional techniques, operating at high temperatures, as in hydrodistillation to obtain the essential oil or soxhlet to obtain its polar compounds. These methods can cause either the degradation of actives from plant material because of the harsh conditions and environmental contamination or toxicity as well as the use and traces of organic solvent. The design of green and sustainable processes is currently a hot research topic in food, cosmetic, agronomic and pharmaceutical industries. In this regard, supercritical CO_2 ($scCO_2$) is a suitable extractor to obtain natural actives from vegetable sources [25,26] since its supercritical conditions, 72 bar and 32 °C, are mild enough to avoid their degradation. In addition, CO_2 is harmless and it can be easily separated from the extract by a simple change of pressure, which generates a final product without residual presence of this solvent [27]. $ScCO_2$ has been studied in research and applied at large scale by the industry for the extraction of natural compounds from plant matrices. Its adjustable solvation power can be applied in the separation and purification of certain compounds from complex mixtures [28]. Supercritical antisolvent fractionation (SAF) with CO_2 has been reported to be a promising technique to reach this purpose. It allows the concentration of actives from an organic solution at mild conditions and precipitates them into solid particles with controlled shape and diameter without damaging organic solvents [29]. In SAF, a solution of the organic extract is continuously pumped and sprayed into a vessel where it converges with $scCO_2$. The fraction of compounds insoluble in this new mixture of solvent-$scCO_2$ precipitate, while the remaining portion, along with the solvent, is collected in the second vessel as a solution. This technique has been applied to obtain enriched fractions that could enhance certain bioactivities [30–33].

The aim of this work was the determination of the antimicrobial and antioxidant activities of different extracts, essential oil, maceration extract and SAF fractions of *L. luisieri*, and their correlation with their composition.

2. Materials and Methods

2.1. Reagents

Solvents used were hexane (99.0% Panreac, Barcelona, Spain), ethanol (EtOH, 99.8%, Sigma Aldrich, Madrid, Spain), Carbon dioxide (CO2, 99.8% ALPHA GAZ, Madrid, Spain) and dimethyl sulfoxide (DMSO, SigmaAldrich Química, Madrid, Spain). For the chromatographic analysis mobile phase, the following solvents were used: water ultrapure 18.2 MΩ·cm filtered through 0.2 µm (Milli-Q-Plus apparatus from Millipore, mod Milford, MA), phosphoric acid (85.9% Fluka), methanol and acetonitrile (MetOH and ACN, 99.9% Scharlab, Barcelona, Spain). Chromatographic standards, rosmarinic oleanolic and ursolic acids (99% 99% and 99.8%) were purchased from Sigma-Aldrich, Madrid, Spain. For the antioxidant activity assay, we used the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich, Madrid, Spain) and as a positive control, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97% ACROS Organics, Madrid, Spain).

2.2. Plant Material

Lavandula luisieri plant material was collected from Cariñena, Zaragoza (Spain) and provided by the Agrifood Research and Technology Centre of Aragon (CITA, Spain). This plant was adapted to the Cariñena experimental field in 2008 from a Toledo (Spain) wild population. It was cultivated and collected as reported by Julio et al., [34]. Plant material was dried at room temperature, pulverised and sieved. Its mean diameter was adjusted to 0.330 mm (ASAEA S319.3 from the American National Standards Institute).

2.3. Lavandula luisieri Extracts

2.3.1. Essential Oil

The essential oil was obtained by hydrodistillation in a Clevenger-type apparatus from *Lavandula luisieri* previously described. The essential oil was provided by the Agrifood Research and Technology Centre of Aragón (CITA), and preserved in an amber vial under refrigeration. Its chemical composition has been reported in a previous study [18].

2.3.2. Maceration Extract

The polar fraction of *L. luisieri* was obtained by submitting the pulverized plant material to two serial macerations: first with hexane, to eliminate the apolar compounds such as cuticular wax, and second with ethanol, to obtain the polar and active compounds. Macerations were carried out at room temperature for 48 hours, with a plant material:solvent ratio of 1:10 (w/w). Both solvents were removed with a rotary evaporator (Büchi R-200) to obtain the dry extract. Only the ethanolic extract (ME) was considered to perform the tests.

The yield of the maceration extract was determined according to Equation (1).

$$Y_{ME}(wt.\%) = \left(\frac{mass_{\ plant\ extract}(g)}{mass_{\ plant\ material}(g)} \right) \times 100 \quad (1)$$

where $mass_{plant\ extract}$ is the mass of the extract obtained (dry extract, with the solvent removed), and $mass_{plant\ material}$ was the initial mass of dried and pulverised plant submitted to the extraction process.

2.3.3. Supercritical Antisolvent Fractionation

Part of the ethanolic maceration extract was dissolved in ethanol 3% (w/w), and filtered through a 0.45 µm pore size to constitute the feed solution (FS) of the SAF process. SAF was performed using a laboratory-scale apparatus (Green Chemistry Laboratory, I3A Researching Institute at University of

Zaragoza) equipped with a CO_2 pump, an extract solution pump, a 0.5 L precipitation vessel and a downstream collector as main components (Figure 1).

The FS was pumped towards the precipitation vessel with a flow rate of 0.5 mL/min. The SC-CO_2 flow rate was 30 g/min and the final pressure was 130 bar, i.e., conditions selected for a higher mass recovery according to previous SAF optimization (Figure 1). The insoluble compounds in the mixture ethanol-supercritical CO_2 precipitate in this vessel constitute the precipitation vessel fraction (PV). Those compounds that were still soluble in the mixture were collected, along with the solvent ethanol, as the downstream vessel fraction (DV). The mass recovery yields in PV and DV were determined according to Equation (2).

$$Y_i(wt\%) = \left(\frac{mass\ fraction\ collected_i}{mass\ of\ FS}\right) \times 100 \quad (2)$$

where i is the location of PV or DV collection.

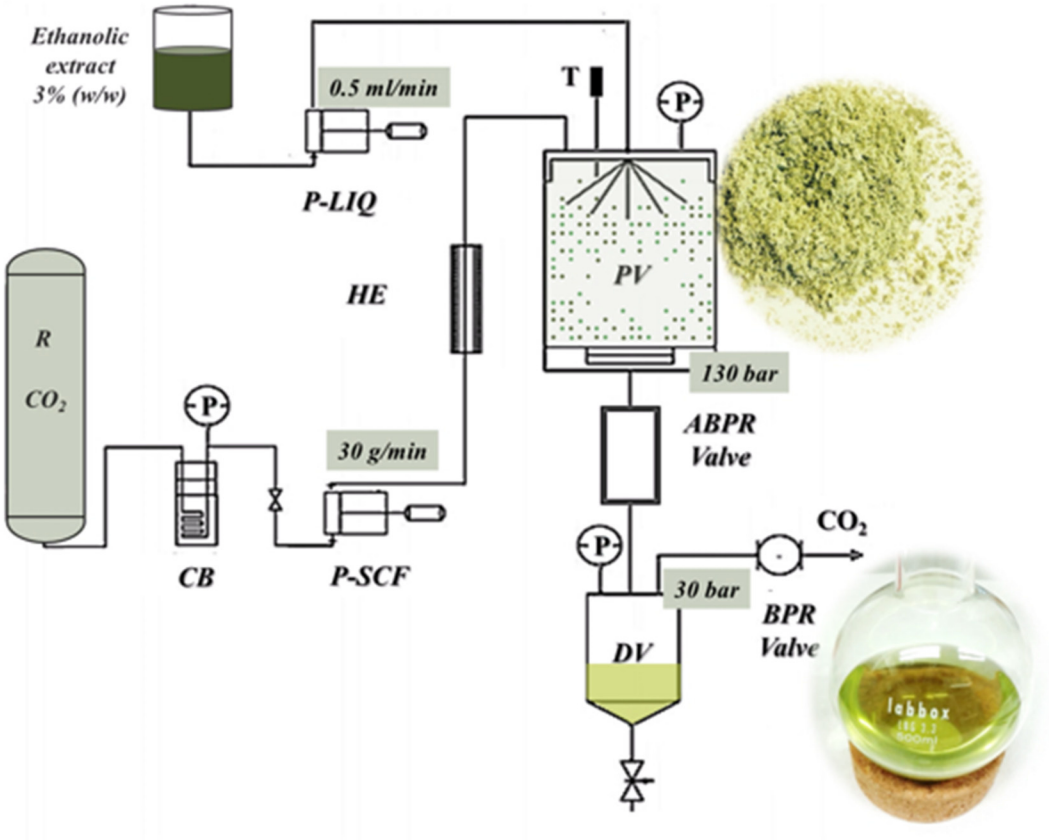

Figure 1. Scheme of the SAF plant. Feed solution reservoir (FS); liquid pump (P-LIQ); CO_2 reservoir (R), cooling bath (CB); CO_2 pump (P-SCF); heat exchanger (HE); precipitation vessel (PV); Thermopar (T); automated back pressure regulator (ABPR); back pressure regulator (BPR); downstream vessel (DV).

2.4. Chemical Composition Analysis

Lavandula luisieri maceration extract and its supercritical fractions were analysed with the following chromatographic procedure. The equipment used was an High Performance Liquid Chromatography (HPLC) Waters® Alliance 2695 with a PDA Waters® 2998 detector provided with the column CORTECS® C18 2.7 µm (4.6 × 150 mm) and a CORTECS® Pre-column VanGuard C18 2.7 µm (2.1 × 5 mm) (Barcelona, Spain). For the chromatographic analysis, an isocratic mobile phase of 88:12, methanol (MeOH): H_3PO_4 0.5% in Milli-Q water, was pumped for 10 min at 0.8 mL/min. Extracts were dissolved in ethanol to 100 ppm (approximately), filtered through a GH Polypropylene membrane (ACRODISC 13 mm, 0.2 µm, Waters, Barcelona, Spain) and finally 10 µL was injected. In order to quantify the maceration

extract, PV and DV the identified rosmarinic, oleanolic and ursolic acids, standards were used to build the calibration curves. Rosmarinic acid was measured at 330 nm, whereas ursolic and oleanolic acids were measured at 210 nm. All analyses were performed in triplicate. The actives content was expressed as a percentage of the dry extract or SAF fraction analyzed.

2.5. Antimicrobial Activity Assays

2.5.1. Microorganisms and Growth Conditions

The bacterial strains assayed in this study were obtained from the Spanish Collection of Type Cultures (CECT) included and maintained frozen at −80 °C in cryovials until the sensitivity tests. Three gram-positive bacteria, *Listeria monocytogenes* (CECT 911), *Enterococcus faecium* (CECT 410) and *Staphylococcus aureus* (CECT 435), and two gram-negative bacteria, *Salmonella* Typhimurium (CECT 443) and *Escherichia coli* (CECT 516), were selected for the assays.

Broth subcultures were prepared by inoculating, with one single colony from a Tryptic Soy Agar (TSA, Oxoid, Madrid, Spain) plate, a test tube containing 10 mL of sterile Tryptic Soy Broth (TSB, Oxoid, Madrid, Spain). The inoculated tubes were incubated overnight (16 hours) at 37 °C. Then, the bacterial concentration was adjusted to an absorbance between 0.08 to 0.1 using a spectrophotometer (Jenway 3600, Tirana, Albania) with a wavelength of 620 nm which corresponds to 1×10^8 UFC/mL according to McFarland Turbidity scale (Standart N1 0.5, Becton Dickinson and Company, Madrid, Spain). Additionally, inocula concentration was confirmed by colony counting in agar plates after performing 1:10 dilutions in peptone water (Buffered peptone water, Oxoid, Madrid, Spain).

2.5.2. Disk Diffusion Method

Antimicrobial activity of the essential oil was tested against the five bacterial strains using the agar disk diffusion technique. Filter paper disks (Whatman 6 mm diameter, Sigma- Aldrich, Madrid, Spain) containing 15 μL of essential oil were placed on the surface of agar plates of Mueller-Hinton (Merck, Spain) that were previously seeded by spreading one sterile swab impregnated in strain culture of 1×10^8 UFC/mL. Ampicillin disks (10 μg, Oxoid, Madrid, Spain) were used as a positive control. The plates were incubated at 37 °C for 24 hours, and the diameter resulting from each inhibition zone (diameter of inhibition zone plus diameter of the disk) was measured in triplicate in millimeters. The scale of measurement was the following: ≥ 20 mm is strongly inhibitory, < 20-12 mm moderately inhibitory, and < 12 mm is non-inhibitory [35]. An average and standard deviation of the inhibition zone from three replicates were calculated.

2.5.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC)

Antimicrobial activity of *Lavandula luisieri* extracts (essential oil, maceration extract, PV and DV) was quantified against the five working strains by the determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). These values were assessed for *Lavandula luisieri* essential oil by the macrodilution broth technique, whereas for ME, PV and DV, the microdilution broth technique was applied. The final bacterial working suspension for both assays was adjusted to 5×10^5–1×10^6 CFU/mL by dilution from the measured 10^8 UFC/mL overnight culture. For both procedures, macrodilution and microdilution, the MIC was the lowest concentration of extract at which bacteria failed to grow, so no visible changes were detected in the broth medium, and the MBC was defined as the concentration at which bacteria were reduced by 99.9%.

The essential oil activity was tested using the macrodilution method adapted from Clinical and Laboratory Standards Institute (CLSI, M07-A10, 2018). The assays were performed in 10 mL of TSB (ethanol 3%), and the tested concentrations were obtained by adding suitable amounts of essential oil to a final working range of 0.5–30 μL/mL. Positive controls contained TSB with microorganisms plus 3% ethanol. Negative controls contained TSB plus 3% ethanol and 5 μL/mL of *Satureja montana*

essential oil, whose activity has been widely studied and proved [35,36]. After a 24 h incubation at 37 °C in a shaking thermostatic bath (Bunsen, mod. BTG), the MIC was read as the concentration with no visible growth. In order to evaluate MBC, 100 µL of each case in which microbial growth was not observed was spread plated in TSA. Plates were incubated at 37 °C for 24 h. The evaluation of MIC and MBC values was carried out in triplicate.

Lavandula luisieri ME, PV and DV extracts were tested against the same bacterial strains with the microdilution broth method [37]. The test was performed in 96-well sterile microplates. All wells received Mueller Hinton Broth (MHB) supplemented with 10% glucose and 1% phenol red broth (Merck, Madrid, Spain). Extract working solutions were dissolved in water with DMSO 5% with a final well highest concentration of 2.5% [38]. The solutions were sterilized by filtration with a 0.2 µm pore membrane filter and added to the first column of wells in the microplate. The final extract concentration assayed ranged from 2000 to 2 µg/mL and was obtained by twofold serial dilution from the first column.

Finally, inoculum suspension was added to all wells. The growth controls constituted medium with extract (negative control) and medium with bacterial inoculum (positive control).

Each microplate was incubated for 24 h at 37 °C. A change of color from red to yellow was interpreted as positive growth. For MBC determination, 10 µL from each well presenting no visible growth was inoculated on Mueller Hinton agar plates and incubated at 37 °C for 24 h. Each analysis was performed in triplicate.

The standards rosmarinic and ursolic acids were also tested following the same microdilution procedure with final concentrations of 125 to 0.06 µg/mL and 1000 to 0.5 µg/mL, respectively, according to its proportion in the *L. luisieri* studied extracts. For the results of antimicrobial activity, the following were considered: significantly active when MIC < 100 µg/mL, moderately active 100 < MIC > 625 µg/mL and weakly active MIC > 625 µg/mL [37].

2.6. Antioxidant Activity

The capacity of the *L. luisieri* maceration extract and its supercritical fractions to scavenge DPPH free radicals was measured by an adaptation of the Brand-Williams, Cuvelier and Berset (1995) [39] spectrophotometric method. The extract solutions were mixed 1:1 (v/v) with a DPPH ethanol solution of 40 µg/mL. The DPPH solution was also confronted with pure RA and trolox (97% ACROS Organics) as a positive control and with ethanol as a negative control. The final well concentrations of ME, PV, DV and the positive controls rosmarinic acid and trolox ranged from 300 - 0.1 µg/mL. The absorbance was measured at 520 nm after 30 min of reaction at room temperature with a microplate photometer (Multiskan EX mod. 355, Thermo Labsystems, Zaragoza, Spain). To determine the scavenging capacity the following equation was applied, Equation (3):

$$Radical\ scavenging\ activity\ (wt\%) = \left[\left(Abs_{control} - Abs_{sample}\right)/\left(Abs_{control}\right)\right] \times 100 \quad (3)$$

where $Abs_{control}$ is the measured absorbance of the DPPH solution and Abs_{sample} is the measured absorbance after the reaction between the extracts or control vs. DPPH.

The antioxidant activity of plant extracts was expressed as IC_{50}, which is defined as the concentration of extracts (in µg/mL) required to inhibit 50% of DPPH radicals. IC_{50} values were estimated by a nonlinear regression (GraphPad Prism version 4.0). A lower IC_{50} value indicates higher antioxidant activity. The results are given as the mean ± standard deviation (SD) of experiments performed in triplicate.

3. Results and Discussion

3.1. Chemical Composition of Lavandula luisieri Extracts

The non-volatile and polar fraction of this plant was obtained by ethanolic maceration. The final extraction yield obtained with this maceration was 12.2%. Regarding this same *Lavandula luisieri*

population, other authors [18] obtained similar yield extraction (12.5%), although it was obtained with ethanol in a Soxhlet apparatus. This method applies heat for a long time and enhances the extraction of actives but it also can cause the degradation of the thermolabile compounds; it does not seem to provide better extraction yield. In this work, the extraction was performed at room temperature and in amber bottles to prevent degradation from heat and light.

The ethanolic maceration extract, ME, was fractionated through SAF into two fractions.It was recovered 50.9% (w/w) in the PV fraction and a 32.9% (w/w) in the DV fraction. According to these results, a high quantity of compounds was insoluble in the mixture $scCO_2$-EtOH under these experimental conditions of pressure and CO_2 flow rate, since a 50.9% of the initial mass precipitated when the ethanolic solution encountered the $scCO_2$ in the PV.

The composition of ME, PV and DV was studied by means of HPLC and only the polyphenol rosmarinic acid and the two triterpenes ursolic acid and oleanolic acid were identified (Figure 2). The content of these actives was previously reported [8]. The concentration of rosmarinic, oleanolic and ursolic acids was quantified in each fraction and compared to the initial ME (Table 1).

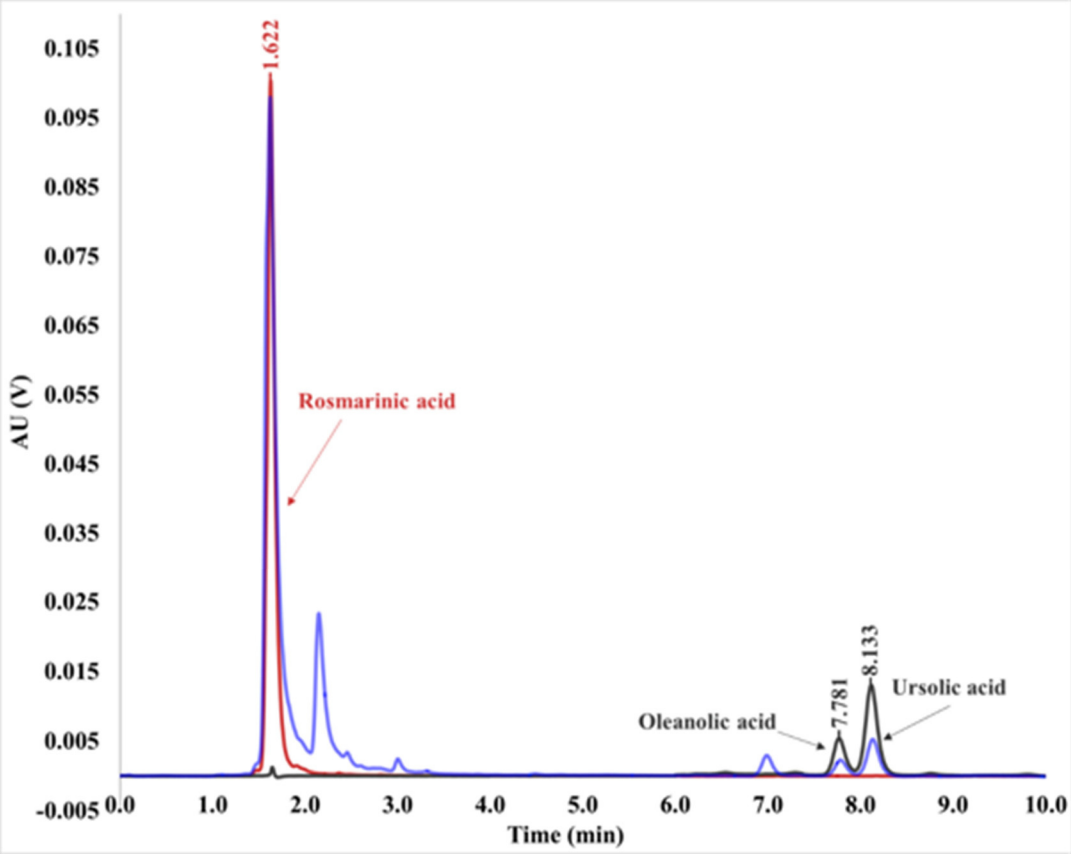

Figure 2. Overlayed chromatograms at 330 nm (0–6 min) and 210 nm (6–10 min) of pure rosmarinic acid (red, retention time 1.622 min), pure oleanolic and ursolic acids (black, retention times 7.781 min and 8.133 min) and the ethanolic extract of *L. luisieri* (blue).

Table 1. Rosmarinic, oleanolic and ursolic acids mg quantified in the maceration extract and each supercritical fraction.

	RA (mg/g)	OA (mg/g)	UA (mg/g)
ME	51.4 ± 0.6	28.8 ± 2.3	52.0 ±1.4
PV	93.5 ±0.5	26.4 ± 1.8	118.6 ± 1.1
DV	-	48.7 ± 1.8	56.1 ± 1.1

ME: Maceration Extract; PV: Precipitation Vessel; DV: Downstream vessel.

The rosmarinic acid concentration was 51.4 mg/g of the initial maceration extract. After the supercritical fractionation process, rosmarinic acid was entirely retained in the PV fraction, at a final concentration of 93.5 mg/g of PV. Although ursolic acid and oleanolic acid were distributed between both fractions, ursolic acid also precipitated mostly in the first fraction, reaching a concentration of 118.6 mg/g of PV; meanwhile, oleanolic acid seem to be dragged to the downstream vessel. The total quantity of the studied compounds in the PV was 238.5 mg/g, 1.9 times more concentrated compared to the initial ME.

Other authors have previously performed this process of supercritical concentration for natural compounds. The application of SAF to rosemary extracts in order to concentrate its polyphenols, rosmarinic acid among them, produced a fraction with a higher antiproliferative and antioxidant activity. Sánchez-Camargo et al., [32] and Visentin et al., [33]. Bernatoniene et al., [40] also studied different techniques for the extraction of these three active constituents from *Rosmarinus officinalis*. The highest mass of active per gram of extract was achieved with ultrasound-assisted ethanolic extraction for ursolic acid (15.8 ± 0.2 mg/g) and rosmarinic acid (15.4 ± 0.1 mg/g), and with ethanolic maceration for oleanolic acid (12.2 ± 0.1 mg/g).

In our work, the ethanolic extraction revealed that *L. luisieri* is a rich source of RA, OA and UA, and the supercritical antisolvent fractionation process is a useful technique for the co-precipitation of the followed actives into a solid enriched product, with 93.5 mg/g, 118.5 mg/g and 26.4 mg/g of RA, OA and OA, respectively.

3.2. Essential Oil Antimicrobial Activity

Bacterial susceptibility of *L. luisiseri* essential oil was first tested with the paper disk agar diffusion method and then quantified with the broth dilution method. The results indicated in Table 2 represent bacterial sensitivity according to the classification previously described [35].

E. faecium (10.5 ± 0.3 mm) and *E. coli* (11.5 ± 0.2) showed no susceptibility to *L. luisieri* essential oil. *L. monocytogenes* was the most susceptible bacteria (43.3 ± 2.8 mm), followed by *S. aureus* (36.2 ± 2.9 mm) and finally *S.* Typhimurium (21.4 ±0.4 mm). With the agar disk diffusion technique, qualitative information of *L. luisieri* essential oil antimicrobial activity was obtained.

Regarding MIC and MBC values, *L. luisieri* essential oil had bacteriostatic activity against all bacteria tested, showing a wide antibacterial spectrum inhibiting both, gram-positive and gram-negative bacteria (Table 2). The highest bacteriostatic activity obtained was against gram-positive tested (MIC = 0.5 µL/mL), whereas higher MIC values were acquired against gram-negative ones: 5 µL essential oil /mL against *S.* Typhimurium and 30 µL essential oil /mL vs *E. coli*. The evaluation of MBC revealed that *L. luisieri* essential oil was bactericidal against four of the five bacteria at the assayed concentrations. The bactericidal MBC values ranged from 0.5 µL/mL (*S. aureus*) to 5µL/mL (*S.* Typhimurium). *E. coli* seems to be the most resistant strain to this oil composition, since it showed bacteriostatic but not bactericidal activity, at the highest concentration tested, 30 µL. This behavior was also observed by Baldovini et al., [6], by applying a dilution agar method with a chemically defined necrodyl-rich essential oil from *L. luisieri* against different bacterial and yeast strains. Although the activity was tested with a different method, it was also observed that gram-positive strains were more sensitive to the essential oil than the gram-negative ones. Some *E. coli* strains had sensitivity to *L. luisieri* essential oil, but also at the highest concentration tested.

Table 2. *Lavandula luisieri* essential antimicrobial results against bacterial strains. Inhibition zone (mm) and minimum inhibitory and bactericidal concentrations (μL/mL).

Strain	Inhibition Zone (mm)	MIC (μL/mL)	MBC (μL/mL)
L. monocytogenes	+++	0.5	3
E. faecium	+	0.5	1
S. aureus	+++	0.5	0.5
S. Typhimurium	+++	5	5
E. coli	+	30	-

(-) not halo (+) non-inhibitory (++) moderately inhibitory (+++) strongly inhibitory.

L. luisieri essential oil has a higher antimicrobial activity compared to other *Lavandula* species, which only had activity against *S. aureus* such as *L. lavandulifolia*, *L. angustifolia*, Lavandin Super and Lavandin Abrialis [41]. Nevertheless, other species such as *L. latifolia* and Lavandin Grosso had similar MIC and MBC results to *L. luisieri* against several strains such as *S.* Typhimurium, *E coli* and *L. monocytogenes*. Generally, *Lavandula* species essential oil, as other plant families, has different sensitivity among gram-positive and gram-negative strains, with the gram-negative *E. coli* as the most resistant one. Nevertheless, the chemical composition of this other antimicrobial species differs with *L. luisieri*, and the activity was related to their content in 1,8-cineol, linalool and camphor [42].

So far, only Baldovini et al. [1] Roller et al. [16] and Zuzarte et al. [17] have tested *L. luisieri* essential oil antimicrobial activity. They obtained good results against all strains tested, especially against gram-positive bacterial and fungal strains, such as *Candida albicans* and *Aspergillus* species, and related these results to *L. luisieiri* essential oil necrodane terpenoid content. Roller, Ernest and Buckle [16] compared *Lavandula* species antimicrobial activity against methicillin resistant and sensitive *Staphylococcus aureus* with the disk diffusion method. *L. luisieri* essential oil, highly concentrated in necrodane monoterpenoids, with 34.5% relative content in α-necrodyl acetate, was the most active at lower volumes. In addition, its combination (50:50) with *L. stoechas* and *L. angustifolia* essential oils produced inhibitory zones twice the diameter than the zones obtained with both single oils. A possible synergy among necrodane terpenoids and 8-cineole, fenchone, and camphor from *L. stoechas*, or linalool and linalyl acetate from *L. angustifolia*, is suggested. Similarly, Zuzarte et al., [17] also related *L. luisieri* essential oil antimicrobial activity to its particular content in necrodane-type compounds when studied in two different populations from central and southern regions of Portugal against different *Aspergillus* strains. The activity results were considered relevant since this strain is usually less sensitive to essential oils.

In our work, the obtained essential oil also had a good antimicrobial activity. Its composition was reported in a previous study [18]. Its main compounds were camphor (60.3%) and 2,3,4,4-tetramethyl-5-methylidenecyclopent-2-en-1-one (8.5%), a necrodane-type compound, along with other substances such as fenchone (2.9%) and 1,8-cineol (2.0%). Other authors studying *L. luisieri* essential oil composition observed that the differences among populations appeared to be only quantitative [6,8,9,18,43]. The antimicrobial activity results obtained in this work could be a consequence of *L. luisieri* essential oil high content in camphor, which is a well-known antimicrobial compound, obtained mainly from *Cinnamomum camphora* but widely distributed in the plant kingdom [44]. According to other authors' results, besides camphor, the *L. luisieri* content in atypical necrodane compounds and the combination with other volatiles could also be contributing to this activity.

3.3. Antimicrobial Activity of SAF Fractions

The antimicrobial activity of the ethanolic extract and its supercritical fractions PV and DV was tested. To our knowledge, this is the first work to study the antimicrobial activity of polar non-volatile fractions of this plant. For this type of extract, the disk diffusion method could not be applied as a screening method because of the poor diffusion from the cellulose disks to the agar. The antimicrobial activity of ME, PV and DV, as well as the pure compounds rosmarinic and ursolic

acids, was tested and quantified with the microdilution broth procedure. From all studied strains assayed, only *L. monocytogenes*, *E. faecium* and *S. aureus* showed sensitivity to the *L. luisieri* maceration extract and its supercritical fractions. However, *Salmonella* Typhimurium and *Escherichia coli* did not show sensitivity to the extracts at the assayed concentrations (Table 3).

Table 3. Minimum inhibitory and bactericidal concentrations (μL/mL) of usolic acid, *L. luisieri* ethanolic maceration extract (ME), and the supercritical fractions PV and DV against *L. monocytogenes*, *E. faecium* and *S. aureus*.

Extract or Pure Active	*L. monocytogenes*		*E. faecium*		*S. aureus*	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Ursolic acid	33	66	66	66	263	263
ME	286	557	286	1146	-	-
PV	242	483	242	242	1933	-
DV	232	931	-	-	-	-

Rosmarinic acid did not show any antimicrobial activity in the studied range (0.06–125 μg/mL). Although other authors have confirmed this lack of activity against food bacteria [45], antimicrobial activity against fitopathogenic bacteria was reported [46]. This compound is produced and released by some plants only as a natural defense in challenging environments.

Pure ursolic acid had inhibitory and bactericidal activity against the three gram-positive bacteria, *L. monocytogenes*, *E. faecium* and *S. aureus*; however, while *L. monocytogenes* and *E. faecium* MIC and MBC values ranged from 33 to 66 μg/mL, *S. aureus* was more resistant, with MIC and MBC values up to 263 μg/mL.

L. luisieri maceration extracts, PV and DV, were also active in inhibiting these bacteria. The lack of activity against gram-negative strains could be a consequence of the restricted penetration because of their different and more complex cell walls, since they have a second external phospholipid bilayer designed to reduce permeability to all compounds [47].

The initial maceration extract was only active against *L. monocytogenes* and *E. faecium*, MIC 286–286 μg/mL and MBC 557–1146 μg/mL, respectively. The corresponding ursolic acid concentration in MIC and MBC was 11.3 μg/mL and 45.4–59.6 μg/mL.

After the fractionation process, the PV fraction had better antimicrobial results than ME results. The precipitated solid showed activity against the three gram-positive bacteria. The MIC and MBC values against *L. monocytogenes* (242–83 μg/mL) and *E. faecium* (242–242 μg/mL) were lower than those obtained with the maceration extract and also had inhibitory properties against *S. aureus* (1933 μg/mL). The corresponding ursolic acid concentration in the PV inhibitory and bactericidal values ranged from 28.6 to 56.9 μg/mL. In this case, the final terpene concentration in PV MIC (UA 28.7–57.3 μg/mL) was lower compared with the inhibitory and biocidal concentrations of the pure active (33–66 μg/mL). Although this compound is concentrated in PV regarding the initial maceration extract, the activity cannot entirely be attributed to it. Nevertheless, oleanolic acid, the other triterpene in this work, with a very similar molecular structure, may contribute to the final activity by addition or synergistic effect. Indeed, several studies have reported the antimicrobial activity of oleanolic and ursolic acids. There is an inhibitory capacity against both gram-negative and gram-positive and a synergetic effect between ursolic acid and aminoglucoside antibiotics [48] when tested against 12 bacterial strains. Indeed, oleanolic and ursolic acids have antimicrobial activity which has been related to the peptidoglycan structure, bacterial gene expression and biofilm formation [23].

These differences between gram-positive and gram-negative bacteria was observed by Lai et al., [15] who performed a methanolic extraction of the aerial parts of *L. luisieri.* In this work, the extract was dissolved in DMSO 100%, which may affect the strain sensitivity to the extracts [38].

Even though rosmarinic acid did not show activity on its own, its combination with other non-identified compounds could enhance it. In any case, the concentrations required to inhibit and kill

bacterial strains by *L. luisieri* maceration and supercritical extracts were higher than those obtained with the essential oil. This decreased antimicrobial activity could be a consequence of the diverse compounds that constitute these extracts. Their penetration into bacterial cells and action mechanism to inactivate them could be different. Some authors studying new treatments for food preservation have observed a synergistic effect between heat treatment in combination with the addition of natural plant extracts, allowing the reduction of the heat conditions [27].

3.4. Antioxidant Activity

The antioxidant activity was evaluated against the free radical DPPH. Results represented in Figure 3 showed that for every compound and extract, the activity was concentration dependent. *L. luisieri* ethanolic maceration extract and its supercritical fractions showed antioxidant activity, but always lower than the positive controls trolox (IC_{50} 3.5 ± 0.3 µg/mL) and rosmarinic acid (IC_{50} 1.7 ± 0.1 µg/mL). The antioxidant measure of *L. luisieri* maceration extract IC_{50} was 30.66 ± 1.9 µg/mL. This result is similar to other published results about different *Lavandula* species and extracts. The DPPH scavenging activity of the methanolic extract of *L. stoechas* was also studied previously [49]; its IC_{50} value was 34.2 ± 3.1 µg/mL, which was related to its phenolic acid content, 25.2 ± 0.4 mg GAE/g.

Other authors reported free radical inhibition of other *Lavandula* spp extracts. *L. x intermedia* ethyl acetate and ethanolic extracts reported results were IC_{50} = 50.4 µg/mL and IC_{50} = 15.1–45.3 µg/mL, respectively. *L. angustifolia* ethanolic extract IC_{50} was 10.6–33.9 µg/mL; ethanolic extracts from *L. coronopifolia* and *L. multifida* IC_{50} = 15.8 µg/mL and IC_{50} = 19.3 µg/mL, respectively [49–51]. In contrast, other authors [52] reported for the *L. stoechas* methanolic extract an IC_{50} of 300 ± 10 µg/mL. The decreased antioxidant activity reported could be a consequence of the heat application in the extraction process and the different proportion of extract: DPPH applied.

After the supercritical processing of maceration extract, the antioxidant activity showed an increase in PV, IC_{50} to 16.17 ± 0.7 µg/mL. The DV fraction, however, was the less active, not inhibiting the 50% of DPPH free radicals in the studied range of concentrations. These results can be graphically observed in Figure 3, where the PV and DV antioxidant curves are in the left and right, respectively, of the maceration extract curve.

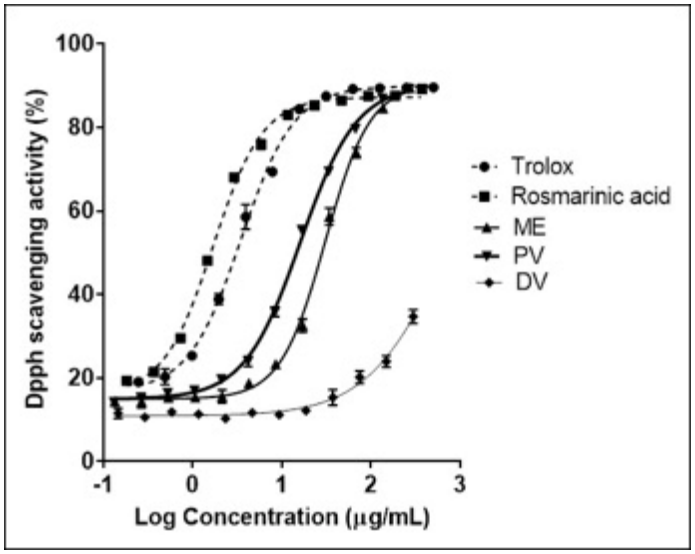

Figure 3. Logarithmic curve representation of the antioxidant activity of *Lavandula luisieri* ethanolic maceration extract (ME) and its SAF fractions: precipitation vessel fraction (PV) and downstream vessel fraction (DV). Positive controls 6-hdroxy-2,5,7,8-tetramethylchromane- 2-carboxylic acid (Trolox) and rosmarinic acid (RA).

According to these results and the chemical analysis, after the supercritical fluid process, the antioxidant compounds extracted in maceration extract were mainly concentrated into the solid of PV fraction. The increased scavenging activity of PV regarding the initial maceration extract and the lack of activity in DV may be a consequence of rosmarinic acid, which completely precipitates during the supercritical process when converging with CO_2, increasing its proportion in this fraction from 51.5 mg/g of maceration extract to 93.5 mg/g of PV. The IC_{50} of pure rosmarinic acid was 1.7 µg/mL, which correlates with the rosmarinic acid concentration in the maceration extract and PV IC_{50}, 1.5 µg/mL.

On the other hand, oleanolic and ursolic acids did not seem to scavenge DPPH free radicals since the DPPH inhibition (%) did not reach 50% at the highest concentration tested, even though their concentration in the second fraction was 48.7 mg/g and 56.1 mg/g, respectively. This result did not correspond to other authors, who reported that ursolic acid IC_{50} was 59.70 µg/mL [48].

L. luisieri scavenging activity seems to be related to its content in rosmarinic and not in oleanolic and ursolic acids. Other *Lavandula* species extracts have also been reported to display several antioxidant mechanisms such as reductive potential, organic, cation and superoxide free radical scavenging, electron or metal cation chelation [52–55] and has always been related to their content in phenolic compounds such protocatechuic acid, caffeic acid and rosmarinic acid.

4. Conclusions

L. luisieri extracts showed antioxidant activity against the free radical DPPH and antimicrobial activity against *Listeria monocytogenes*, *Enterococcus faecium* and *Staphylococcus aureus*, and two gram-negative bacteria, *Salmonella* Typhimurium and *Escherichia coli*. Although the essential oil showed a strong antimicrobial activity against the studied bacterial strains, its application could be limited because of its organoleptic properties, which should be further evaluated. The application of the Supercritical antisolvent green technology to the non-volatile fraction of *L. luisier*, not only allowed the concentration of bioactive compounds in a final solid product but also the enhancement of the studied bioactivities in the precipitated solid fraction. The increased antimicrobial activity seems to be a consequence of the ursolic acid enrichment and the antioxidant activity because of rosmarinic acid. Nevertheless, a thorough analysis by HPLC-MS to know the full chemical composition of the ethanolic extract and its supercritical fraction could be interesting for identifying other bioactive compounds. This improved final product could have potential applications in food, cosmetic and pharmaceutical industries as a preservative or nutraceutical. However, it is recommended to perform additional studies in order to assess the preservative effect after its application in the final product as well as its safety for consumers.

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References

1. Friedman, M. Antibiotic-resistant bacteria: Prevalence in food and inactivation by food-compatible compounds and plant extracts. *J. Agric. Food Chem.* **2015**, *63*, 3805–3822. [CrossRef] [PubMed]
2. Domingo, D.; López-Brea, M. Plants with antimicrobial action. *Rev. Esp. Quim. Publ. Soc. Esp. Quim.* **2003**, *16*, 385–393.
3. Harvey, A.L. Medicines from nature: Are natural products still relevant to drug discovery? *Trends Pharm. Sci.* **1999**, *20*, 196–198. [CrossRef]
4. Kalim, M.D.; Bhattacharyya, D.; Banerjee, A.; Chattopadhyay, S. Oxidative DNA damage preventive activity and antioxidant potential of plants used in Unani system of medicine. *BMC Complementary Altern. Med.* **2010**, *10*, 77. [CrossRef]
5. Gyawali, R.; Ibrahim, S.A. Natural products as antimicrobial agents. *Food Control* **2014**, *46*, 412–429. [CrossRef]
6. Baldovini, N.; Lavoine-Hanneguelle, S.; Ferrando, G.; Dusart, G.; Lizzani-Cuvelier, L. Necrodane monoterpenoids from Lavandula luisieri. *Phytochemistry* **2005**, *66*, 1651–1655. [CrossRef]
7. González-Coloma, A.; Martín-Benito, D.; Mohamed, N.; García-Vallejo, M.C.; Soria, A.C. Antifeedant effects and chemical composition of essential oils from different populations of *Lavandula luisieri* L. *Biochem. Syst. Ecol.* **2006**, *34*, 609–616. [CrossRef]
8. Julio, L.F.; Martín, L.; Muóz, R.; Mainar, A.M.; Urieta, J.S.; Sanz Perrucha, J.; Burillo, J.; Alqueza, J.; Gonzalez-Coloma, A. Comparative chemistry and insect antifeedant effects of conventional (Clevenger and Soxhlet) and supercritical extracts (CO_2) of two Lavandula luisieri populations. *Ind. Crop. Prod.* **2014**, *58*, 25–30. [CrossRef]
9. Sanz, J.; Soria, A.C.; García-Vallejo, M.C. Analysis of volatile components of Lavandula luisieri L. by direct thermal desorption–gas chromatography–mass spectrometry. *J. Chromatogr. A* **2004**, *1024*, 139–146. [CrossRef]
10. Roach, B.; Eisner, T.; Meinwald, J. Defense mechanisms of arthropods. 83 α- and β-Necrodol, novel terpenes from a carrion beetle (Necrodes surinamensis, Silphidae, Coleoptera). *J. Org. Chem.* **1990**, *55*, 4047–4051. [CrossRef]
11. Matos, F.; Miguel, M.G.; Duarte, J.; Venâncio, F.; Moiteiro, C.; Correia, A.I.D.; Figueiredo, A.C.; Barroso, J.G.; Pedro, L.G. Antioxidant Capacity of the Essential Oils from *Lavandula luisieri* L. stoechas subsp. *lusitanica*, L. stoechas subsp. *lusitanica* × L. *luisieri* and L. viridis Grown in Algarve (Portugal). *J. Essent. Oil Res.* **2009**, *21*, 327–336. [CrossRef]
12. Baptista, R.; Madureira, A.M.; Jorge, R.; Adão, R.; Duarte, A.; Duarte, N.; Lopes, M.M.; Teixeira, G. Antioxidant and Antimycotic Activities of Two Native Lavandula Species from Portugal. *Evid. Based Complement. Altern. Med.* **2015**, *2015*, 570521. [CrossRef] [PubMed]
13. Arantes, S.; Candeias, F.; Lopes, O.; Lima, M.; Pereira, M.; Tinoco, T.; Cruz-Morais, J.; Martins, M.R. Pharmacological and Toxicological Studies of Essential Oil of Lavandula stoechas subsp. luisieri. *Planta Med.* **2016**, *82*, 1266–1273. [CrossRef] [PubMed]
14. Dias, N.; Dias, M.C.; Cavaleiro, C.; Sousa, M.C.; Lima, N.; Machado, M. Oxygenated monoterpenes-rich volatile oils as potential antifungal agents for dermatophytes. *Nat. Prod. Res.* **2017**, *31*, 460–464. [CrossRef] [PubMed]
15. Lai, B.G.; Teixeira, G.; Moreira, I.; Correia, A.I.; Duarte, A.; Madureira, A.M. Evaluation of the antimicrobial activity in species of a Portuguese Montado ecosystem against multidrug resistant pathogens. *J. Med. Plants Res.* **2012**, *6*, 2381–2387. [CrossRef]
16. Roller, S.; Ernest, N.; Buckle, J. The antimicrobial activity of high-necrodane and other lavender oils on methicillin-sensitive and -resistant Staphylococcus aureus (MSSA and MRSA). *J. Altern. Complement. Med.* **2009**, *15*, 275–279. [CrossRef]
17. Zuzarte, M.; Gonçalves, M.J.; Cruz, M.T.; Cavaleiro, C.; Canhoto, J.; Vaz, S.; Pinto, E.; Salgueiro, L. Lavandula luisieri essential oil as a source of antifungal drugs. *Food Chem.* **2012**, *135*, 1505–1510. [CrossRef]
18. Julio, L.F.; Díaz, C.; Assani, N.; Valcarcel, F.; Burillo, J.; Olmeda, S.; González-Coloma, A. Ixodicidal compounds from pre-domesticated Lavandula luisieri. *Ind. Crop. Prod.* **2017**, *110*, 83–87. [CrossRef]
19. Costa, S.; Cavadas, C.; Cavaleiro, C.; Salgueiro, L.; do Céu Sousa, M. In vitro susceptibility of Trypanosoma brucei brucei to selected essential oils and their major components. *Exp. Parasitol.* **2018**, *190*, 34–40. [CrossRef]

20. Rufino, A.T.; Ferreira, I.; Judas, F.; Salgueiro, L.; Lopes, M.C.; Cavaleiro, C.; Mendes, A.F. Differential effects of the essential oils of Lavandula luisieri and Eryngium duriaei subsp. juresianum in cell models of two chronic inflammatory diseases. *Pharm. Boil.* **2015**, *53*, 1220–1230. [CrossRef]
21. Nunes, R.; Pasko, P.; Tyszka-Czochara, M.; Szewczyk, A.; Szlosarczyk, M.; Carvalho, I.S. Antibacterial, antioxidant and anti-proliferative properties and zinc content of five south Portugal herbs. *Pharm. Biol.* **2017**, *55*, 114–123. [CrossRef] [PubMed]
22. Petersen, M.; Simmonds, M.S.J. Rosmarinic acid. *Phytochemistry* **2003**, *62*, 121–125. [CrossRef]
23. Wolska, K.I.; Grudniak, A.M.; Fiecek, B.; Kraczkiewicz-Dowjat, A.; Kurek, A. Antibacterial activity of oleanolic and ursolic acids and their derivatives. *Cent. Eur. J. Biol.* **2010**, *5*, 543–553. [CrossRef]
24. Jesus, J.A.; Lago, J.H.G.; Laurenti, M.D.; Yamamoto, E.S.; Passero, L.F.D. Antimicrobial Activity of Oleanolic and Ursolic Acids: An Update. *Evid. Based Complement. Altern. Med.* **2015**, *2015*, 620472. [CrossRef]
25. de Melo, M.M.R.; Silvestre, A.J.D.; Silva, C.M. Supercritical fluid extraction of vegetable matrices: Applications, trends and future perspectives of a convincing green technology. *J. Supercrit. Fluids* **2014**, *92*, 115–176. [CrossRef]
26. Meneses, M.A.; Caputo, G.; Scognamiglio, M.; Reverchon, E.; Adami, R. Antioxidant phenolic compounds recovery from Mangifera indica L. by-products by supercritical antisolvent extraction. *J. Food Eng.* **2015**, *163*, 45–53. [CrossRef]
27. Burt, S. Essential oils: Their antibacterial properties and potential applications in foods—A review. *Int. J. Food Microbiol.* **2004**, *94*, 223–253. [CrossRef]
28. Parhi, R.; Suresh, P. Supercritical Fluid Technology: A Review. *J. Adv. Pharm. Sci. Technol.* **2013**, *1*, 13. [CrossRef]
29. Reverchon, E.; De Marco, I. Supercritical fluid extraction and fractionation of natural matter. *J. Supercrit. Fluids* **2006**, *38*, 146–166. [CrossRef]
30. Martin, L.; Gonzalez-Coloma, A.; Adami, R.; Scognamiglio, M.; Reverchon, E.; Della Porta, G.; Urieta, J.; Mainar, A. Supercritical antisolvent fractionation of ryanodol from Persea indica. *J. Supercrit. Fluids* **2011**, *60*, 16–20. [CrossRef]
31. Perretti, G.; Virgili, C.; Troilo, A.; Marconi, O.; Regnicoli, G.F.; Fantozzi, P. Supercritical antisolvent fractionation of lignans from the ethanol extract of flaxseed. *J. Supercrit. Fluids* **2013**, *75*, 94–100. [CrossRef]
32. Sánchez-Camargo, A.; Mendiola, J.A.; Valdés, A.; Castro-Puyana, M.; Garcia-Cañas, V.; Cifuentes, A.; Herrero, M.; Ibáñez, E.; Sánchez-Camargo, A.D.P. Supercritical antisolvent fractionation of rosemary extracts obtained by pressurized liquid extraction to enhance their antiproliferative activity. *J. Supercrit. Fluids* **2016**, *107*, 581–589. [CrossRef]
33. Visentin, A.; Rodríguez-Rojo, S.; Navarrete, A.; Maestri, D.; Cocero, M.J. Precipitation and encapsulation of rosemary antioxidants by supercritical antisolvent process. *J. Food Eng.* **2012**, *109*, 9–15. [CrossRef]
34. Julio, L.F.; Barrero, A.F.; Herrador, M.M.; Arteaga, J.F.; Burillo, J.; Andres, M.F.; Díaz, C.E.; Azucena, G.-C. Phytotoxic and Nematicidal Components of Lavandula luisieri. *J. Nat. Prod.* **2016**, *79*, 261–266. [CrossRef]
35. Rota, M.C.; Herrera, A.; Martínez, R.M.; Sotomayor, J.A.; Jordán, M.J. Antimicrobial activity and chemical composition of Thymus vulgaris, Thymus zygis and Thymus hyemalis essential oils. *Food Control* **2008**, *19*, 681–687. [CrossRef]
36. Mihajilov-Krstev, T.; Radnovic, D.; Kitić, D.; Jovanović, V.; Mitić, V.; Stojanović-Radić, Z.; Zlatković, B. Chemical composition, antimicrobial, antioxidative and anticholinesterase activity of *Satureja Montana* L. ssp montana essential oil. *Open Life Sci.* **2014**, *9*, 668–677. [CrossRef]
37. Tekwu, E.M.; Pieme, A.C.; Beng, V.P. Investigations of antimicrobial activity of some Cameroonian medicinal plant extracts against bacteria and yeast with gastrointestinal relevance. *J. Ethnopharmacol.* **2012**, *142*, 265–273. [CrossRef]
38. Basch, H.; Gadebusch, H.H. In vitro antimicrobial activity of dimethylsulfoxide. *Appl. Microbiol.* **1968**, *16*, 1953–1954. [CrossRef]
39. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT Food Sci. Technol.* **1995**, *28*, 25–30. [CrossRef]
40. Bernatoniene, J.; Cizauskaite, U.; Ivanauskas, L.; Jakstas, V.; Kalveniene, Z.; Kopustinskiene, D.M. Novel approaches to optimize extraction processes of ursolic, oleanolic and rosmarinic acids from Rosmarinus officinalis leaves. *Ind. Crop. Prod.* **2016**, *84*, 72–79. [CrossRef]

41. Rota, C.; Carramiñana, J.J.; Burillo, J.; Herrera, A. In vitro antimicrobial activity of essential oils from aromatic plants against selected foodborne pathogens. *J. Food Prot.* **2004**, *67*, 1252–1256. [CrossRef] [PubMed]
42. Guillén, M.D.; Cabo, N.; Burillo, J. Characterisation of the Essential Oils of some Cultivated Aromatic Plants of Industrial Interest. *J. Sci. Food Agric.* **1996**, *70*, 359–363. [CrossRef]
43. González-Coloma, A.; Delgado, F.; Rodilla, J.M.; Silva, J.; Burillo, J. Chemical and biological profiles of Lavandula luisieri essential oils from western Iberia Peninsula populations. *Biochem. Syst. Ecol.* **2011**, *39*, 1–8. [CrossRef]
44. Soković, M.; Glamočlija, J.; Marin, P.D.; Brkić, D.; van Griensven, L.J.L.D. Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an in vitro model. *Molecules* **2010**, *15*, 7532–7546. [CrossRef]
45. Moreno, S.; Scheyer, T.; Romano, C.S.; Vojnov, A.A. Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. *Free Radic. Res.* **2006**, *40*, 223–231. [CrossRef]
46. Bais, H.P.; Walker, T.S.; Schweizer, H.P.; Vivanco, J.M. Root specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of Ocimum basilicum. *Plant Physiol. Biochem.* **2002**, *40*, 983–995. [CrossRef]
47. Lewis, K.; Ausubel, F.M. Prospects for plant-derived antibacterials. *Nat. Biotechnol.* **2006**, *24*, 1504–1507. [CrossRef]
48. do Nascimento, P.; Lemos, T.; Bizerra, A.; Arriaga, Â.; Ferreira, D.; Santiago, G.; Raimundo, B.F.; José, G.M.C. Antibacterial and Antioxidant Activities of Ursolic Acid and Derivatives. *Molecules* **2014**, *19*, 1317–1327. [CrossRef]
49. Messaoud, C.; Chograni, H.; Boussaid, M. Chemical composition and antioxidant activities of essential oils and methanol extracts of three wild *Lavandula* L. species. *Nat. Prod. Res.* **2012**, *26*, 1976–1984. [CrossRef]
50. Blažeković, B.; Vladimir-Knežević, S.; Brantner, A.; Štefan, M.B. Evaluation of Antioxidant Potential of Lavandula x intermedia Emeric ex Loisel. 'Budrovka': A Comparative Study with L. angustifolia Mill. *Molecules* **2010**, *15*, 5971–5987. [CrossRef]
51. Torras-Claveria, L.; Jauregui, O.; Bastida, J.; Codina, C.; Viladomat, F. Antioxidant Activity and Phenolic Composition of Lavandin (Lavandula x intermedia Emeric ex Loiseleur) Waste. *J. Agric. Food Chem.* **2007**, *55*, 8436–8443. [CrossRef] [PubMed]
52. Ceylan, Y.; Usta, K.; Usta, A.; Maltas, E.; Yildiz, S. Evaluation of Antioxidant Activity, Phytochemicals and ESR Analysis of Lavandula Stoechas. *Acta Phys. Pol. A* **2015**, *128*, 483–487. [CrossRef]
53. Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S.E.; Erçağ, E. The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. *Int. J. Food Sci. Nutr.* **2006**, *57*, 292–304. [CrossRef] [PubMed]
54. Carrasco, A.; Martinez-Gutierrez, R.; Tomas, V.; Tudela, J. Lavandula angustifolia and Lavandula latifolia Essential Oils from Spain: Aromatic Profile and Bioactivities. *Planta Med.* **2016**, *82*, 163–170. [CrossRef] [PubMed]
55. Vasileva, I.; Denkova, R.; Chochkov, R.; Teneva, D.; Denkova, Z.; Dessev, T.; Denev, P.; Slavov, A. Effect of lavender (Lavandula angustifolia) and melissa (Melissa Officinalis) waste on quality and shelf life of bread. *Food Chem.* **2018**, *253*, 13–21. [CrossRef] [PubMed]

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β-Carotene, α-tocoferol and rosmarinic acid encapsulated within PLA/PLGA microcarriers by supercritical emulsion extraction: Encapsulation efficiency, drugs shelf-life and antioxidant activity

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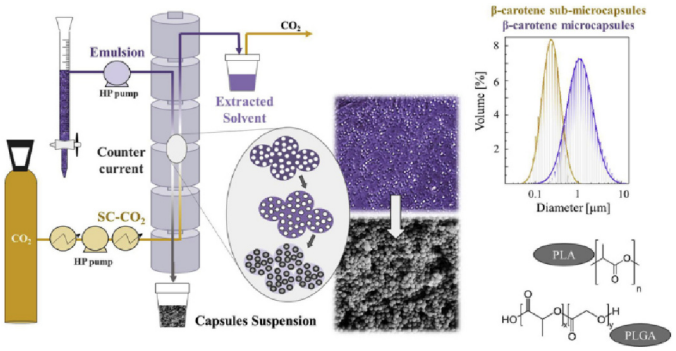
GRAPHICAL ABSTRACT



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ABSTRACT

β-Carotene (β-CA) is largely used antioxidant with a high sensibility to oxidation when in contact with light and temperature. To improve its shelf life and develop a nutraceutical formulation it was encapsulated into *poly-lactic-co-glycolic acid* (PLGA) and *poly-lactic acid* (PLA) carriers using Supercritical Emulsion Extraction (SEE). α-Tocoferol (α-TOC) and Rosmarinic Acid (RA) were proposed as excipients to improve product shelf-life. Different emulsion formulation conditions, such as compositions and mixing rate were used; whereas, SEE operating conditions in the counter-current tower were fixed at 80 bar and 38 °C with an L/G ratio of 0.1. In these conditions, PLA and PLGA carriers with sizes ranges between 1.5 ± 0.5 μm and 0.3 ± 0.1 μm were fabricated with Encapsulation Efficiencies (EEs) between 50–80%. The co-encapsulation of α-TOC with β-CA gained to prolonged drug shelf life; whereas, RA co-encapsulation was not successfully and, in some cases, also reduced β-CA-EE. The poor loading experienced, in the case of RA, was probably due to its high solubility into the high-pressure mixture of carbon dioxide and organic solvent formed during the emulsion extraction. This behaviour was defined "*co-extraction effect*" and may limit the application of SEE technology in the encapsulation of molecules soluble in the mixture obtained during oily phase extraction. Shelf-life studies were performed after UV irradiation for ten days and after carriers storage for 2 years at 4 °C. The better performance in terms of shelf life was observed for PLGA capsules loaded with β-CA/α-TOC; whereas, PLA formulation with β-CA/α-TOC showed

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a superior antioxidant activity with an half minimal Inhibitory Concentration (IC_{50}) against the free radical DPPH of 1.8 mg/mL of carriers.

1. Introduction

β-Carotene (β-CA) is widely used in the food, cosmetic and pharmaceutical industries, as natural photo protector colorant and antioxidant [1]. β-CA has a high free radical scavenging and antioxidant activity due to their multiple conjugated double bonds; moreover, it is the major precursor of vitamin A [2,3]. Others biological effects of β-CA are: decrease of cancer risks enhancing iμμune responses [1], stimulation of gap-junctional communication [4], protection from arterial disease [5] and induction of a light barrier avoiding cell damaging [6]. Concerning food applications, its inclusion in food matrix allows to obtain the *so-called* functional or nutraceutical food; moreover, carotenoids are also used as colorants to recover the colour lost during food processing and storage [7].

β-CA molecule is highly hydrophobic and difficult to disperse in water [2]. For this reason, to improve the bioavailability and enhance its water dispersion, it is often micronized [2,8] or co-precipitated with protein [3]. β-CA is also very susceptible to degradation under temperature, light and oxygen [9,10]; clinical studies revealed that its degradation products are highly reactive and can shift properties from antioxidant to pro-oxidant [11]. In order to prevent molecule degradation, different polymers were studied as carrier for β-CA encapsulation such as, tapioca starch, maltodextrin or oleoresin using spray drying technology [12–14], or casein using solid lipid nanoparticles (SLNs) protocol and galactan by evaporation technology [9,15]. The co-encapsulation with other antioxidant excipients, such as α-tocopherol (α-TOC) and ascorbic acid can also provide a better protective effects on oxidation [16].

Poly-lactic acid (PLA) and *poly-lactic-co-glycolic acid* (PLGA) are biocompatible and biodegradable polymers which have recently been the subject of extensive investigation [17–20]. α-Tocopherol (α-TOC), also known as vitamin E, is a fat-soluble highly potent antioxidant, abundant in vegetable oils or wheat germs; it is widely used by the pharmaceutical, cosmetic and food industries because of its clinical and preservative applications [21,22]. Rosmarinic Acid (RA) is a polyphenol with important biological activities, such as anti-inflammatory, antiagregant and antioxidant [23]. β-CA and carotenoids loading into PLA and PLGA biopolymers have been already proposed by different fabrication technologies for micro-capsules preparation [10]. The emulsification-evaporation method [24–27], spontaneous emulsification solvent diffusion method (SESD) [18], nanoprecipitation method [2,28] are all widely used in preparing PLA/PLGA micro-carriers of various mean sizes. Each of these methods employs a similar first step, where β-CA organic solution is emulsified in a water phase to form an *oil-in-water* dispersion (*o–w*). If appropriate, the molecule may also be dispersed as a solid powder in an organic polymer solution, or co-dissolved in a common solvent with the polymer. The solution or dispersion is then processed according to one of the aforementioned methods. During the solid carriers formation using emulsification-evaporation and precipitation approaches, organic solvents such as dichloromethane and chloroform are usually employed. To meet the requirement for the food and pharmaceutical use, residual solvents should be completely removed from fabricated carriers [29].

Supercritical Emulsion Extraction (SEE) technique has been proposed to encapsulate several drugs and molecules [30,31]. In the process, the dense gas is used to extract the oily phase from a pre-formulated emulsion and obtain solvent free micro and nano-carriers [32–34]. The use of dense gas, such as Supercritical Carbon Dioxide (SC-CO_2) is an alternative to almost all the conventional processes because it is possible to work at near-ambient temperatures, avoiding the degradation of thermolabile substances; SC-CO_2 also provides an inert medium suitable for processing oxidable substances.

In this paper, the β-CA encapsulation into *poly-lactide* (PLA) and *poly-L-lactide-co-glycolide* (PLGA) is proposed using SEE technique with the aim of improving its shelf-life and preserving its antioxidant activity. The co-encapsulation with other natural antioxidants, such as α-TOC and RA is also described to improve micro-capsules shelf-life and activity. The possibility of fabricate carriers with different sizes will be also explored and the related encapsulation efficiency and antioxidant performance will be tested. Products shelf-life will be monitored after their degradation induced by UV exposure for 10 days and after a storage of 2 years. The combination of β-CA with other antioxidants will be also tested in order to understand the formulations stability and their biological activity and to demonstrate the versatility of the SEE technology in producing complex formulation.

2. Materials and methods

2.1. Materials

CO_2 (99.9%, Morlando Group, Naples, Italy), chloroform anhydrous (CL), methanol (ME), acetone (AC), ethanol (ET), acetonitrile (ACN), acetic acid (ACE), ethyl acetate (EA) all of purity 99.9% were supplied from Carlo Erba Reagents (Milan, Italy). Water (HPLC grade), glycerol (GLY, purity 99%, Aldrich Chemical Co.), sorbitan monolaurate (Span 20 Sigma-Aldrich), Tween 80 (Sigma-Aldrich), β-carotene (β-CA, Aldrich Chemical Co), α-Tocoferol (α-TOC, Sigma-Aldrich), Rosmarinic acid (RA, 96% Sigma-Aldrich), *poly-lactic acid* (PLA, MW: 60,000 g/mol, Resomer RG 708H Boehringer Ingelhein), *poly-lactic-co-glycolic acid* (PLGA, 75:25 MW: 20,000 g/mol, Resomer RG 752S, Boehringer), 1,1-diphenyl-2-picrylhydrazine (DPPH, 97% Sigma-Aldrich), were used as received.

2.2. Emulsions preparation with water as external phase

To encapsulate β-CA, α-TOC and RA, several double $o_1/o_2/w$ emulsions were formulated with a composition ratio of 4:16:80 w/w/w. The internal o_1 phase contained the active principles solved into CL with 0.06% Span 20; the second oily phase, o_2, was formed by EA with a given amount of biopolymer dissolved (0.4 to 0.8 g of PLA or PLGA). For emulsions formulation, the inner phase o_1, was mixed with the second oily phase o_2 to form the o_1/o_2 emulsion by ultrasonication (mod. S-450D, Branson Ultrasonics Corporation, Danbury, CT, USA). The o_1/o_2 emulsion was, then, immediately added into a known amount of EA-saturated aqueous Tween 80 solution (0.6%, w/w of Tween) with the high-speed stirrer (model L4RT, Silverson Machines Ltd., Waterside, Chesham Bucks, United Kingdom) for a time ranges between 4–6 min and at with stirring ranges of 2800–3600 rpm. Other procedures such as direct solvent mixture preparation (CL: EA ratio 4:16) did not worked properly and β-CA precipitation was observed; indeed, the surfactant (Span 20) added in CL oily phase improved its mixing with the EA oily phase assuring: (i) an excellent solubilisation of all the antioxidants in the o_1 phase; (ii) the good solubilisation of the biopolymers in the second o_2 phase.

Different formulations of RA/PLA were also prepared. In this case $w_1/o_1/w_2$ emulsions were formulated with a ratio of 1:19:80 w/w/w. ET/RA solution plus 0.06% w/w of PVA was used as internal w_1 phase; o_2 phase contained 1 g of PLA into EA; w_2 external phase was formed of EA-saturated aqueous Tween 80 solution (0.6% w/w).

2.3. Emulsions preparation with water-glycerol as external phase

Some runs were performed fixing the external water phase composition at 80% (w/w) glycerol and 20% (w/w) distilled water plus 0.6% w/w of Tween 80; in this condition we used AC as solvent for the oily phase. The overall ratio *o/w* was always maintained at 20/80 for all the emulsion prepared and PLGA amount in the oily phase was fixed at 1 g, while the β-C concentration was fixed at 6 mg/g; the use of AC allowed the solubilization of both compounds (β-CA/PLGA) in the oily phase. AC was not used to form emulsion with water because of their mutual solubilisation in all compositions, which prevent the correct emulsion formulation.

2.4. Supercritical emulsion extraction (SEE)

All emulsions formulated were processed through SEE in a continuous experimental laboratory apparatus, as better described elsewhere [34–37]. Briefly, the apparatus consists of a stainless steel packed column with an internal diameter of 13 mm, in which carbon dioxide is fed from the bottom of the column using a high-pressure diaphragm pump, and emulsions are delivered from the top of the column using a high pressure piston pump. The column is formed by three AISI 316 stainless steel cylindrical sections of 30 cm height, connected by four cross-unions and is filled with stainless steel packing elements of 4 mm nominal size with 1889 m^{-1} specific surface and 0.94 of voidage (0.16 inch Pro-Pak, Scientific Development Company, State College, PA, USA). The apparatus is thermally insulated by ceramic cloths and its temperature is controlled by six controllers (TC1-TC6, Gordon J/Series 93, Watlow, Milan, Italy) at different heights of the column. Oily phase solvent is recovered in a separator located downstream the top of the column, in which the pressure is regulated by a backpressure valve (V5, 26-1700 Series, Tescom, Selmsdorf, Germany). A rotameter (mod. N5-2500, ASA, Sesto San Giovanni, Italy), located at the exit of the separator, measures the CO_2 flow rate used. A schematic representation of the SEE layout is also reported in Fig. 1a. The operating pressure and temperature were fixed at 80 bar and 37 °C. SC-CO_2 flow rate was 1.4 kg/h and emulsion flow rate 0.14 kg/h maintaining a ratio between liquid and SC-CO_2 (L/G) of 0.1. At this experimental conditions, all the organic solvents used to form the oily phase of the emulsions showed a large miscibility in SC-CO_2 [38–42]. A mean recovery efficiency of 80% was measured in each run. The lost material was due to the shutdown protocol, which avoid the recovering the last washing fractions because it may give less accurate data on carriers particle size, due to the not steady state conditions operation during the final washing. The biopolymer suspensions were collected at the bottom of the column. They were washed with distilled water by 2 serial centrifugations for 20 min at 6500 rpm at 4 °C and then, recovered on a membrane filter (porosity 0.2 μm). When water-glycerol external phase was used, a multiple dilution in pure water was necessary before the filtering step to recover the carriers.

2.5. Droplets and microspheres morphology & size distributions

The droplets formed in the emulsion were observed using an optical microscope (mod. BX 50 Olympus, Tokyo, Japan) equipped with a phase contrast condenser. A Field Emission-Scanning Electron Microscope (FE-SEM mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany) was used to study the morphology of the produced capsules. Samples were coated with gold (layer thickness 250 Å) using a sputter coater (mod.108 A, Agar Scientific, Stansted, UK). *Droplets Size*

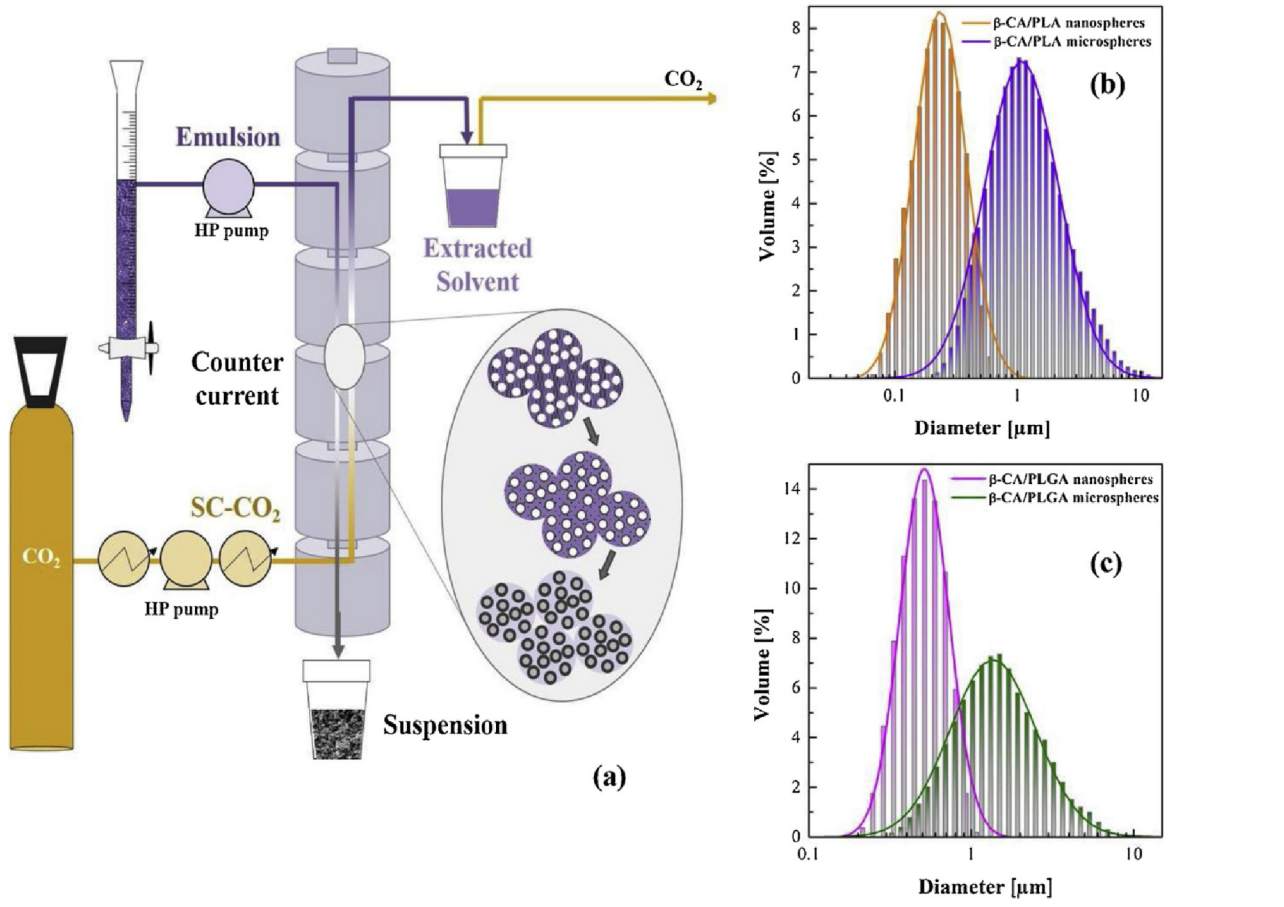

Fig. 1. Schematic representation of SEE apparatus layout with description of some emulsions used (a). Particle Size Distributions (PSDs) of PLA and PLGA carriers (b–c); the possibility of varying carriers size modifying the droplet sizes in emulsion was demonstrated for both biopolymers.

Distributions (DSD) and *Particles Size Distributions* (PSD) were measured by dynamic light scattering (DLS, mod. Mastersizer S, Malvern Instruments Ltd., Worcherstershire, UK) immediately after the preparation of the emulsion and the suspension, using 1 mL of each sample. The distributions proposed in this paper are the mean of 10 measurements. The shrinkage factor percentage (SF%), defined as the ratio between the particle mean size and droplets mean size percentage [34] was also measured for each run.

2.6. Solvent residue analysis

Solvents (EA, AC, CL, ET,) contents in the water suspensions at the exit of the column was analysed, to monitor the efficiency of solvent removal by SC-CO_2 extraction. The solvent residue was measured using a head space sampler (mod. 50 Scan, Hewlett & Packard, Palo Alto, CA, USA) coupled to a gas chromatograph interfaced with a flame ionization detector (GC-FID, mod. 6890 Agilent Series, Agilent Technologies Inc., Wilmington, DE). Organic solvents were separated using a fused-silica capillary column 30 m length, 0.25 mm internal diameter, 0.25 μm film thickness (mod. DB-1, J&W, Folsom, CA, USA). GC conditions were: oven temperature at 40 °C for 8 min. The injector was maintained at 180 °C (split mode, ratio 1:1) and Helium was used as the carrier gas (7 mL/min). Head space conditions were: equilibration time 60 min at 100 °C, pressurization time 2 min, loop fill time 1 min. Head space samples were prepared in 10 mL vials filled with 3 mL of suspension. Analyses were performed on each sample in three replicates.

2.7. Drug loading

For the loading quantification, a known mass (approximately 15 mg) of recovered capsules were dissolved in 0.5 mL of AC in order to break the biopolymer and solubilize the encapsulated molecules. Then 3.5 mL of ET were added to precipitated the biopolymer. The suspension was then centrifuged for 30 min are 6500 rpm at 4 °C and the supernatant was collected for concentration measurements. The HPLC-PDA (mod. 1100 and 1200 series; Agilent LC system) was equipped with a Waters Spherisorb ODS-2 (Ø 5 μm 150 x 4.6 mm) column. The mobile phase used was 80:20 ME:WATER acidified 0.1% ACE; the flow rate was of 1 mL/min. The injection volume was 20 μL in every test and the detection wavelength of: 450 nm for β-CA, 292 nm for α-TOC and 330 nm for RA. A calibration curve was built for each compound. The encapsulation efficiency percentage (EE%) was calculated as amount (mass) measured/amount (mass) loaded x100.

2.8. Shelf life study

The assays were performed on capsules containing β-CA and β-CA with α-TOC. In the first case, by quantifying the remaining β-CA in the capsules compared with the pure compound after exposed to UV radiation (λ = 254 nm) for 1 h a day for 10 days. In the second case, by measuring the remaining encapsulated β-CA after 2 years of storage at 4 °C in the dark. In both shelf life studies, samples were solved in AC and the amount of active β-CA was measured at 450 nm using a spectrophotometer UV–vis (mod. Cary 5000, Agilent LC system).

2.9. Antioxidant activity

The 2,2-diphenyl-1-picryl-hydrazil (DPPH) radical scavenging activity was determined [36]. Firstly, the calibration curve was performed monitoring the antioxidant activity of pure β-CA. 1.5 mL of β-CA solution were added to 1.5 mL of 0.1 mM DPPH both dissolved in mixture of AC:ET (1:7) and then kept in the dark for 18 h at room temperature. The biopolymer capsules were solved in 0.5 mL AC first and then 3.5 mL of ET was added; the mixture was centrifuged at 6500 rpm for 30 min. 3 different concentrations of the supernatant were prepared. Finally, 1.5 mL of these solutions were added to 1.5 mL of 0.1 mM DPPH. The antioxidant activity was measured 18 h at 517 nm. The DPPH inhibiton percentage (%) was defined as in eq. (1):

$$DPPH\ inhibition\ \%= 100\,x(Abs\ DPPH - Abs\ sample)/Abs\ DPPH \quad (1)$$

where *Abs sample* is the absorbance measured for the sample solution with DPPH after the reaction and *Abs DPPH* is the absorbance of DPPH solution. The theorical inhibition percentage was calculated for each concentration of capsules and a theorical inhibition curve was built for each product or for their mixtures (measured by simple mixing the two or three components). Theorical half minimal Inhibitory Concentration (IC_{50}) was compared with the IC_{50} obtained experimentally.

3. Results and discussion

Samples of water suspension recovered at the bottom of the column showed solvent residue values between 50–100 ppm for EA and AC; no other solvents were found in all the formulations processed. Repeatability of about 90% was always observed in all the experiment described. Indeed, the relatively high amount of emulsion pumped for each run (100 mL) and the steady state conditions set before the emulsion processing by feeding external water phase for a proper time, provided a good simulation of a continuous operating system.

3.1. SEE-C encapsulation of β-CA and α-TOC in PLA and PLGA carriers

Different emulsion formulations were explored in order to assure the fabrication of microcarriers with different sizes. All the emulsion formulations, carrier size, and antioxidant loading are summarized in Table 1. In the case of PLA, 400 mg of biopolymer were always solubilized in the oily phase, obtaining micro-carriers of 1.4 ± 0.5 μm with 68% of EE. When α-TOC was included in the formulation (ratio 9:1, β-CA: α-TOC) along with β-CA, the microcapsules mean diameter did not changed and the EE was of 72%. The same formulation with 400 mg of biopolymer in the oily phase was also tested for PLGA; in this case, microcarriers with mean size of 1.5 ± 0.6 μm were obtained but with a poor EE of only 22%; the use of α-TOC in this formulation did not strongly improved the EE. When, the amount of PLGA in the oily phase of emulsion was increased up to 800 mg, microcarriers of 2.0 ± 0.6 μm were obtained with an improved EE of 58%. A shrinkage was always observed between the droplets and the solid particles in all experiments performed by SEE [36]. Nevertheless, in the case of emulsion formulations with PLA the SF was almost always of 24%; whereas, for PLGA a reduced shrinkage factor was observed of 37% and 10%, respectively when 400–800 mg of biopolymer were solubilized in the oily

Table 1
Different emulsion formulations tested by SEE-C: antioxidants loaded in the o_1 phase and biopolymer loaded in the o_2 phase; mean sizes of the obtained droplets and particles with standard deviation. Encapsulation Efficiency (EE, %) and overall Antioxidant Loading (AL, mg/g). Ratio β-CA:α-TOC was of 9:1; Ratio β-CA:α-TOC:RA was of 8:1:1.

Polymer in o_2 (g)	Antioxidant in o_1	Droplets size (μm)	Carriers size (μm)	EE (%)	AL (mg/g)
PLA					
0.4	β-CA	2.1 ± 0.5	1.4 ± 0.5	68	6.8
0.4	β-CA/α-TOC	2.1 ± 0.5	1.4 ± 0.5	72	5.0
0.4*	β-CA/α-TOC	1.2 ± 0.1	0.3 ± 0.1	62	6.2
0.4	β-CA/α-TOC/RA	1.4 ± 0.2	0.5 ± 0.1	35	3.5
PLGA					
0.4	β-CA	2.4 ± 0.6	1.5 ± 0.6	22	2.1
0.8	β-CA/α-TOC	2.1 ± 0.6	2.0 ± 0.6	58	2.6
0.8*	β-CA/α-TOC	1.2 ± 0.1	0.3 ± 0.1	62	3.1
0.8	β-CA/α-TOC/RA	1.3 ± 0.2	0.5 ± 0.1	53	2.7
1**	β-CA/α-TOC	7.8 ± 0.9	4.3 ± 0.7	82	25

* *Higher value of rpm were used in emulsion formulation to reduce droplets size.*
** *AC:GLY/WATER emulsion.*

phase.

In order to reduce the droplet mean sizes and, therefore, to fabricate smaller carriers, the rotation per minute of the emulsifier was increased during emulsion preparation [37]. For both PLA (400 mg load in oily phase) and PLGA (800 mg load in oily phase), the droplets mean sizes were reduced to 1.2 ± 0.1 μm and 1.2 ± 0.1 μm, respectively, and the resulting carriers mean diameters were of 0.3 ± 0.1 μm in both cases, respectively, after SEE processing. Both EE data were measured at 62%. Fig. 1b–c illustrated the PSDs of the carriers fabricated using both PLA and PLGA, biopolymers. The emulsion optical microscope images and the SEM images of the produced microsystems are illustrated in Figs. 2a–d and 3 a–d. Spherical shaped devices were always fabricated.

The improved EE% observed in the PLGA carriers when the amount of polymer was doubled in the oily phase may be due to the extremely low molecular weight of PLGA used in this work. Indeed, it was of 20,000 g/mol for PLGA, so 3 times lower with respect the one of PLA that was 60,000 g/mol; as a consequence, we supposed that the dynamic viscosity of the two oily phase solutions could be extremely different, when the same amount of biopolymer was dissolved in it. The large difference in oily phase dynamic behaviour may strongly influence the capability of the oily phase in holding the antioxidant molecules during the emulsion preparation (sonication and stirring phases) and, therefore, it may explain the necessity of a double amount of PLGA, in the oily phase, to assure better encapsulation efficiency. However, further investigation involving the experimental measurement of dynamic viscosity for the oily phase solutions in relation of the amount of polymer solubilized within and its molecular weight should provide a better understanding of the described behaviour.

3.2. RA encapsulation into PLA and PLGA

Several runs were performed to test the RA encapsulation into PLA and PLGA systems; then, it was co-encapsulated with β-CA and α-TOC (ratio 8:1:1; β-CA: α-TOC:RA). The first set of experiments used a $w_1/o/w_2$ double emulsion where RA was solved in the aqueous internal phase formed by ET and water (w_1). The proportion of ET/WATER in the internal water phase was varied together with RA concentrations. All the conditions explored are described in Table 2. In all cases, very low EE were experienced ranging between 1.3% to 3.9%. Further investigations were performed in order to modify the overall system compositions and gain a good RA encapsulation; indeed, emulsion type $o_1/o_2/w$ using β-CA and α-TOC (with Span20 0.06% w/w), with both PLA and PLGA were tested and carriers with a mean diameter of 0.5 ± 0.1 μm for PLA and PLGA, respectively were obtained with an RA-EE% always no higher than 5%. Additionally, in the case of PLA processing, the inclusion of RA in the emulsion formulation, caused a further reduction of β-CA-EE% from 62% to 35%. When the co-encapsulation was performed into PLGA carriers, RA-EE was of 12% and β-CA-EE was maintained at 53%. The described data seemed in contrast with the ones reported by others authors that indicated RA-EE of 60–78% in *poly-capro-lactone* and *carboxy-methyl cellulose*, respectively; however, these results were obtained by using conventional evaporation/extraction processes [38,39]. Taking into account the SEE operative conditions used of 80 bar and 37 °C, the low RA-EE cannot be due to its solubilisation in SC-CO_2 because higher P and T values are required to properly solubilized it [39]. However, considering the presence of the high pressure mixtures formed in the column during the oily phase extraction, the RA solubilisation can still occur. Indeed, in the literature, it is largely reported that mixtures of organic solvent such as EA and CL with CO_2, lead to expanded liquid formation, at the P and T conditions used for SEE processing [40–42]; this mixtures can extract the RA before or meanwhile the biopolymer is hardened. A better understanding of the behaviour observed can be possible with an accurate and deeper knowledge of the behaviour of the complex mixture CO_2/EA/RA, (ET and CL may be neglected due to the very low amount in the emulsion formulation) at high pressure. Indeed, we hypothesized that the complex composition system, formed at the P and T conditions tested, has a miscibility hole very sharp or not large enough to prevent the molecule co-extraction in every conditions explored. This behaviour, described as "*co-extraction effect*" may limit the application of SEE technology in the encapsulation of compounds with high solubility in the high-pressure mixture formed during the oily phase extraction. On the other hand, different molecular affinity between RA and the two biopolymers tested, may explain the higher encapsulation rate observed in the case of PLGA, which showed more polar functional groups with respected to PLA.

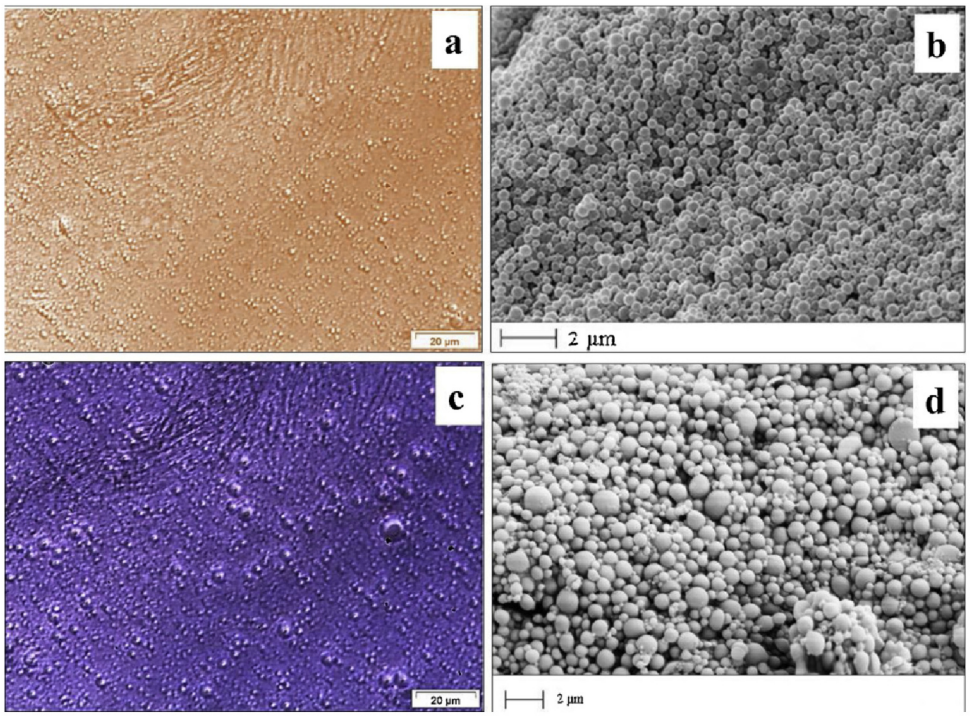

Fig. 2. Optical microscope images of droplets in emulsions (a, c) and SEM images of PLA/β-CA/α-TOC carriers fabricated after emulsion processing by SEE technology (b, d). Carriers with mean size of 0.3 ± 0.1 μm with antioxidant loading of 6.2 mg/g (b) and of 1.5 ± 0.5 μm with an antioxidant loading of 6.8 mg/g (d) were obtained. In order to reduce droplets and, therefore, particle size, higher value of rpm were used in emulsion formulation.

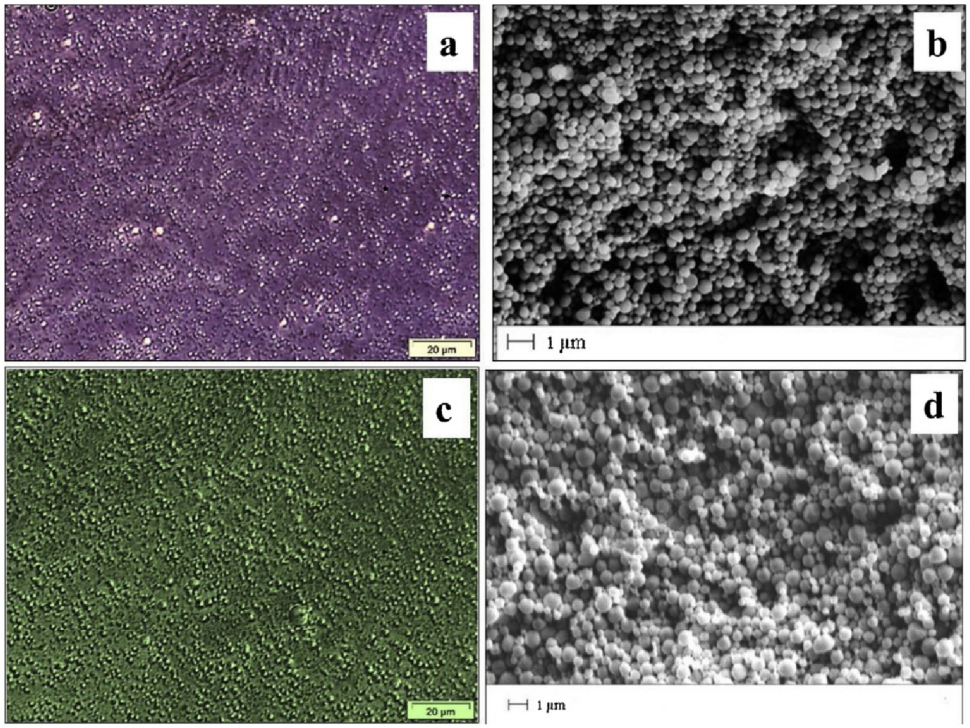

Fig. 3. Optical microscope images of droplets in emulsions (a, c) and SEM images of PLGA/β-CA/α-TOC carriers fabricated after emulsion processing by SEE technology (b, d). Carriers with a mean size of 0.5 ± 0.1 µm with antioxidant loading of 2.7 mg/g (b) and of 2.0 ± 0.6 µm with antioxidant loading 2.6 mg/g (d) were obtained. In order to reduce droplets and, therefore, particle size, higher value of rpm were used in emulsion formulation.

Table 2
Different emulsion formulations processed to RA encapsulation; oily phase was formed of EA with 1 g of PLA; the external water phase was always formed by saturated water with 0.6% w/w of Tween 80.

RA loaded (mg)	*Composition of* w_1 (1 mL)	*Composition of* o_2 (19 mL)	EE (%)
20	20:80 ET:Water	PLA	3.8
100	ET	PLA	2.1
37	20:80 ET:Water	PLA	1.3
20	30:70 ET:Water	PLA	3.5
10	Water	PLA	3.5
150	–	PLA	–
150	–	PLGA	–

3.3. SEE processing of emulsions with glycerol-water phase

Some runs were performed fixing the water phase composition at GLY:WATER of 80:20 with 0.6% (w/w) of Tween 80; the overall ratio *o/w* was always maintained at 20/80 for all the emulsion prepared. Using GLY in the water phase, AC can be used as solvent of the oily phase, due to the immiscibility hole described in the ternary diagrams reported in Fig. 4b. PLGA amount in the oily phase was fixed at 1 g, while the β-C loading was fixed at 30 mg/g. From the optical image of the emulsion droplets in Fig. 4a it is possible to observe the presence of crystals of β-CA in the oily saturated phase. For this reason, the emulsion can be more correctly described as *solid-oil-water s/o/w*. In Fig. 4c–d a SEM image of the fabricated carriers is also reported with the PSD that showed a mean size of 4.4 ± 0.7 µm; encapsulation rate of 82% has been also reported. SEE pressure and temperature conditions used, were of 80 bar and 40 °C with an L/G ratio of 0.3; however, difficulties in SEE process managing have to be reported because of the high viscosity of the GLY-WATER phase that caused severe column blockage, preventing a good plant operation. For this reason this emulsion formulation was considered not good, even if excellent EE was monitored.

3.4. Shelf life study

Shelf life studies were proposed. Firstly, it was quantified the remaining β-CA into all PLA and PLGA capsules containing β-CA and β-CA + α-TOC after its exposure to the light; the control test with pure β-CA was also performed. Results are illustrated in Fig. 5a. The 83% of pure β-CA degrades after 3 days of UV exposure, while only the 21.7% and 16.8%, respectively, of the encapsulated drug degraded when loaded in PLA and PLGA capsules. After 10 days of UV exposure, pure β-CA was completely degraded; whereas, 4% and 3% of drug was still monitored, when encapsulated into PLA and PLGA, respectively. If β-CA was co-encapsulated with α-TOC, the remaining active drug was of 22% and 30% in PLA and PLGA, respectively after 10 days. This result is in agreement with several authors which reported the beneficial combination of α-TOC presence to prevent degradation of the carotenoid [43–45]. Indeed, the co-encapsulation of β-CA with α-TOC has been reported to block the oxygen radicals chain reaction, prolonging its shelf life in colloidal lipid particles of fat-in-water dispersions [46,47]. In the second shelf life study, the remaining β-CA was quantified after 2 years of storage in the dark at 4 °C when co-encapsulated with α-TOC in both PLA and PLGA capsules. As we can see from Fig. 5b, the 563% of the active was still not degraded in PLA and the 63% one when encapsulated in PLGA. Both studies confirmed a better protection against oxidation for β-CA and good performances of the carriers fabricated by SEE; the best carrier formulation seemed to be the ones with β-CA/α-TOC in PLGA.

3.5. Antioxidant activity

In order to measure the functionality of the micro capsules, their antioxidant activity was assayed against DPPH radical. According to β-CA calibration curve and the loading in each carriers tested, the theorical antioxidant activity of micro capsules was measured and compared. The antioxidant activity of PLA and PLGA capsules was first monitored after 2 years of storage, as illustrated in Fig. 6a. For PLA formulation, the activity measured was lower than expected ($IC_{50expected}$ 3.2 mg/mL vs $IC_{50measured}$ 6.7 mg/mL) because of a higher β-CA degradation in these carriers; whereas for PLGA carriers the

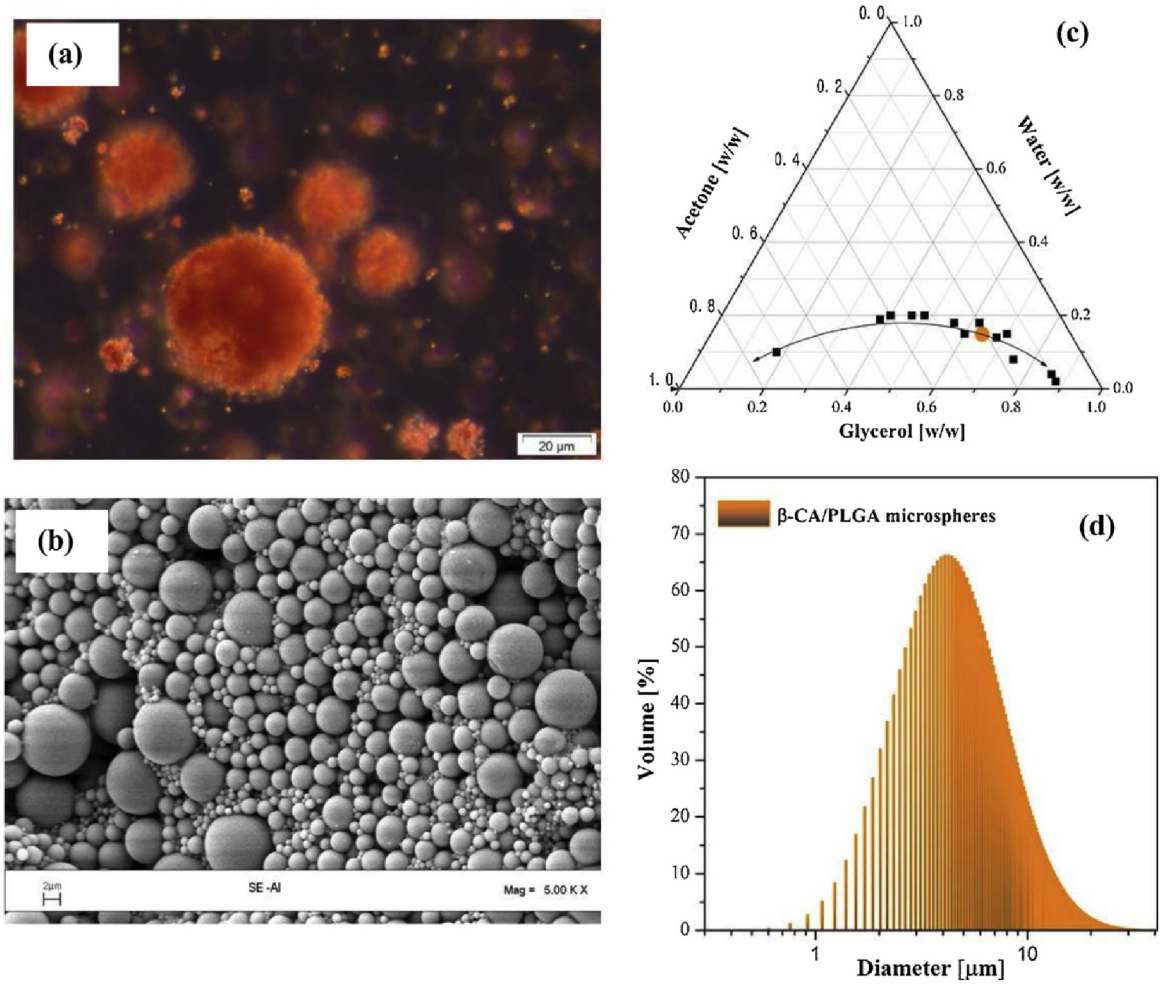

Fig. 4. Optical microscope images of the emulsions (a) and SEM images of the related micro-capsules (b) produced by SEE using an *o–w* emulsions (20/80) AC:GLY/WATER. The emulsion composition is represented in the ternary diagram (c). Particle Size Distribution (PSD) of PLGA micro-capsules fabricated is also reported (d); mean size of 4.3 µm with β-CA loading of 30 mg/g was obtained.

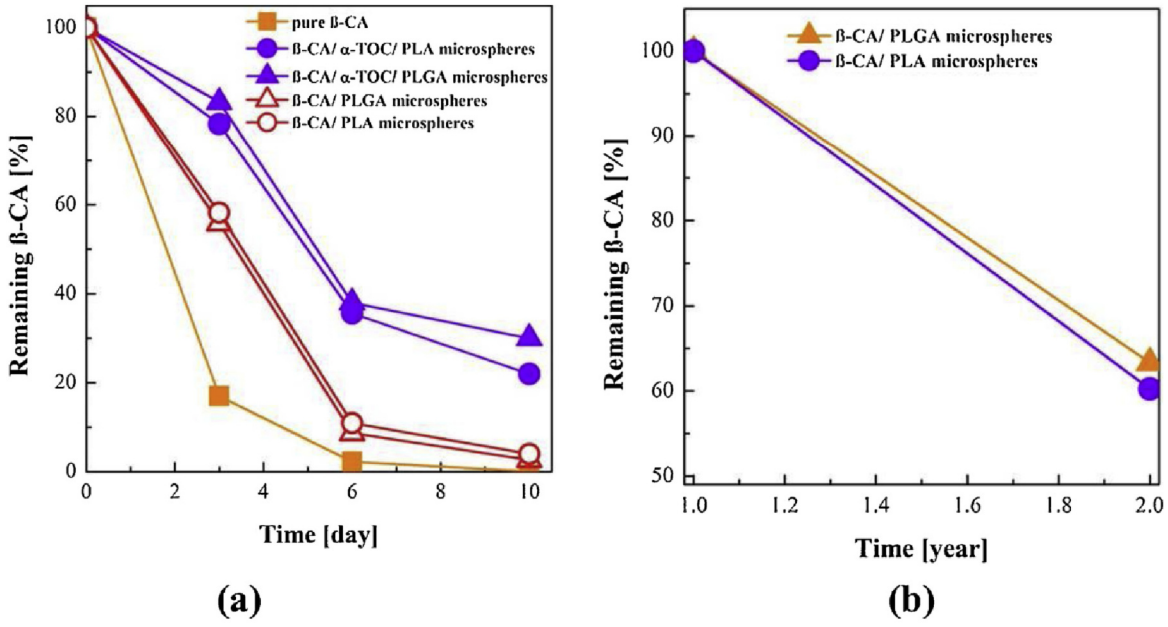

Fig. 5. Shelf-life studies results. Pure β-CA and encapsulated β-CA degradation tendencies along time after its exposure to UV radiation (UV length 259 nm) for one hour during ten days (a); encapsulated β-CA degradation tendencies along two years after its storage at 4 °C in the dark (b).

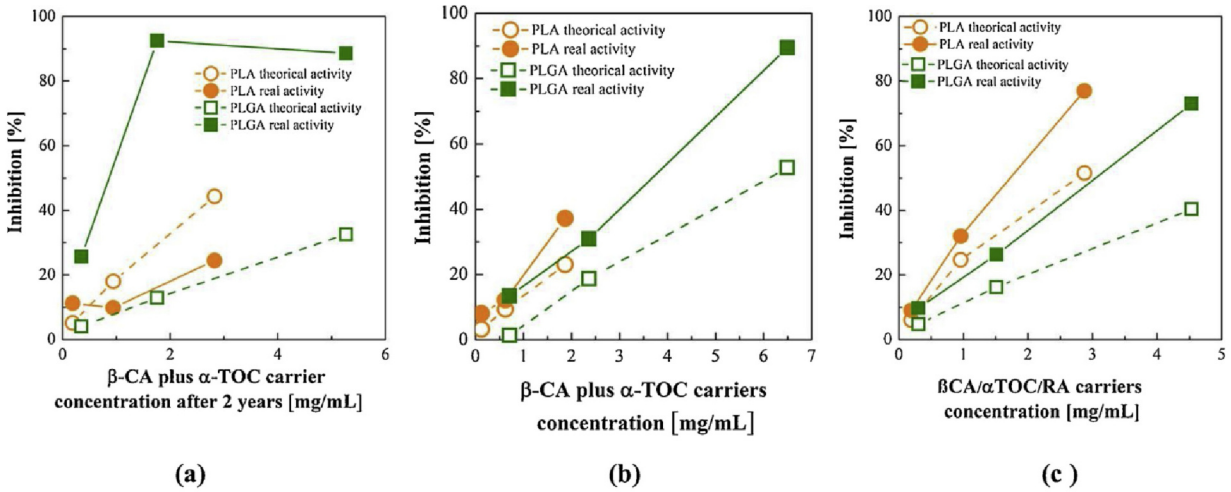

Fig. 6. Antioxidant activity theorical vs real. PLA and PLGA carriers loaded with β-CA and α-TOC, after 2 years (a); loaded with β-CA and α-TOC, just after their fabrication (b); loaded with β-CA, α-TOC and RA, just after their fabrication (c).

performance was better, confirming the lower degradation of β-CA in the system after two years with an activity maintained at IC_{50} 2.3 mg/mL. From Fig. 6a, although the theorical antioxidant activity of PLA capsules seemed higher than the ones of PLGA capsules, it is only due to the higher loading of β-CA in the PLA system. This difference could be also a consequence of a higher content of the co-encapsulated α-TOC (26.78% into PLA vs 52.97% into PLGA). Looking at the real activity, and in accordance with the shelf life results, PLGA seemed to be the more protecting biopolymer, preserving the β-CA activity along the time.

PLA and PLGA antioxidant activity was also measured immediately after their formulation for both PLA and PLGA systems. β-CA + α-TOC theorical IC_{50} according to their loading (6.2 mg/g and 3.1 mg/g, respectively) was of 2.7 mg/mL and 5.6 mg/mL; however, the measured antioxidant activity was always higher and of 1.8 mg/mL and 3 mg/mL, respectively, as reported (in percentage) in Fig. 6b. When RA was included in the formulation, a higher theorical IC_{50} was expected, and 6.3 mg/mL and 4.2 mg/mL for PLA and PLGA carriers was measured; however, their antioxidant activity was lower than the equivalent PLA and PLGA carriers without RA because of the overall reduction of β-CA loading. Nevertheless, as described in Fig. 6c, the measured activity is higher than the expected, when carriers with the co-encapsulation of the three bio-actives are tested. This is probably due to the combination of the three actives (β-CA + α-TOC + RA) even if they are entrapped at an overall lower concentration. β-CA/α-TOC/PLA capsules showed a superior antioxidant activity with an IC_{50} against the free radical DPPH of 1.8 mg/mL.

4. Conclusions and perspectives

The Supercritical Emulsion Extraction (SEE-C) allowed the formulation of β-CA/PLA and β-CA/PLGA carriers with a wide range of sizes and with good EEs. The fabricated microsystem demonstrated to provide drug protection from UV radiation and along two years of storage, improving drug shelf life. α-TOC co-encapsulations gives an extra protection to β-CA degradation in both oxidizing conditions; whereas, encapsulation within PLGA gave better β-CA preservation and an improved shelf-life. A SEE limitation was detected when RA was processed to be encapsulated; indeed a lack of EE in the case of molecules extremely soluble in the high-pressure mixture formed during the oily phase extraction may be described in that case. More data on oily phases dynamic viscosity and high pressure behaviours of complex mixture will improve SEE technology managing and may provide deeper understanding of process performances.

It is also worth of note that the emulsions formulated using AC as a solvent of the oily phase gave the best results in terms of EE, but required the presence of an external water phase formed by GLY/Water, which was difficult to manage within the high pressure packed column and, therefore, this solution was discarded. AC was able to dissolve great amount of β-CA and biopolymer and several authors reported nano-emulsions obtained using AC and water [48–50]; however, AC completely miscibility with water prevented the formation of stable emulsion that can be properly processed by our SEE layout. Indeed, when AC oily phase (loaded with polymer) was mixed with water (ratio 20:80), the resulted emulsion was always unstable and polymer precipitation/aggregation was observed in all cases, even if surfactant was added in both phases.

References

[1] E. Franceschi, et al., Co-precipitation of beta-carotene and bio-polymer using supercritical carbon dioxide as antisolvent, Open Chem. Eng. J. 5 (2014).
[2] H.S. Ribeiro, B.-S. Chu, S. Ichikawa, M. Nakajima, Preparation of nanodispersions containing β-carotene by solvent displacement method, Food Hydrocoll. 22 (2008) 12–17.
[3] M.J. Cocero, Á. Martín, F. Mattea, S. Varona, Encapsulation and co-precipitation processes with supercritical fluids: fundamentals and applications, J. Supercrit. Fluids 47 (2009) 546–555.
[4] K. Nesaretnam, E. Jin Lim, K. Reimann, L.C. Lai, Effect of a carotene concentrate on the growth of human breast cancer cells and pS2 gene expression, Toxicology 151 (2000) 117–126.
[5] A. D'Odorico, et al., High plasma levels of α- and β-carotene are associated with a lower risk of atherosclerosisResults from the Bruneck study, Atherosclerosis 153 (2000) 231–239.
[6] M. Gonnet, L. Lethuaut, F. Boury, New trends in encapsulation of liposoluble vitamins, J. Controll. Release 146 (2010) 276–290.
[7] H. Silva, et al., Nanoemulsions of β-carotene using a high-energy emulsification–evaporation technique, J. Food Eng. 102 (2011) 130–135.
[8] B.-S. Chu, S. Ichikawa, S. Kanafusa, M. Nakajima, Stability of protein stabilised β-carotene nanodispersions against heating, salts and pH, J. Sci. Food Agric. 88 (2008) 1764–1769.
[9] X. Pan, P. Yao, M. Jiang, Simultaneous nanoparticle formation and encapsulation driven by hydrophobic interaction of casein-graft-dextran and β-carotene, J. Colloid Interface Sci. 315 (2007) 456–463.
[10] M. Stevanović, D. Uskokovi, Poly(lactide-co-glycolide)-based micro and nanoparticles for the controlled drug delivery of vitamins, Curr. Nanosci. 5 (2009).
[11] L. Cao-Hoang, R. Fougère, Y. Waché, Increase in stability and change in supramolecular structure of β-carotene through encapsulation into polylactic acid nanoparticles, Food Chem. 124 (2011) 42–49.
[12] J. Loksuwan, Characteristics of microencapsulated β-carotene formed by spray drying with modified tapioca starch, native tapioca starch and maltodextrin, Food Hydrocoll. 21 (2007) 928–935.
[13] M.E. Rodríduez-Huezo, R. Pedroza, L.A. Prado, C.I. Beristain, E.J. Vernon-Carter, Microencapsulation by spray drying of multiple emulsions containing carotenoids, J. Food Sci. 69 (2006) 351–359.
[14] A.F. Faria, R.A. Mignone, M.A. Montenegro, A.Z. Mercadante, C.D. Borsarelli, Characterization and singlet oxygen quenching capacity of spray-dried microcapsules of edible biopolymers containing antioxidant molecules, J. Agric. Food Chem. 58 (2010) 8004–8011.

[15] S. Trombino, et al., Stearyl ferulate-based solid lipid nanoparticles for the encapsulation and stabilization of β-carotene and α-tocopherol, Colloids Surf. B Biointerfaces 72 (2009) 181–187.
[16] P. Zhang, S.T. Omaye, Beta-carotene and protein oxidation: effects of ascorbic acid and alpha-tocopherol, Toxicology 146 (2000) 37–47.
[17] L.S. Nair, C.T. Laurencin, Biodegradable polymers as biomaterials, Prog. Polym. Sci. 32 (2007) 762–798.
[18] H. Zhang, W. Cui, J. Bei, S. Wang, Preparation of poly(lactide-co-glycolide-co-caprolactone) nanoparticles and their degradation behaviour in aqueous solution, Polym. Degrad. Stab. 91 (2006) 1929–1936.
[19] J. Lademann, et al., Nanoparticles–an efficient carrier for drug delivery into the hair follicles, Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Pharm. Verfahrenstechnik EV 66 (2007) 159–164.
[20] M. Stevanović, J. Savić, B. Jordović, D. Uskoković, Fabrication, in vitro degradation and the release behaviours of poly(DL-lactide-co-glycolide) nanospheres containing ascorbic acid, Colloids Surf. B Biointerfaces 59 (2007) 215–223.
[21] J.M. Tucker, D.M. Townsend, Alpha-tocopherol: roles in prevention and therapy of human disease, Biomed. Pharmacother. 59 (2005) 380–387.
[22] G. Tomassi, V. Silano, An assessment of the safety of tocopherols as food additives, Food Chem. Toxicol. 24 (1986) 1051–1061.
[23] A. Panya, et al., Interactions between α-tocopherol and rosmarinic acid and its alkyl esters in emulsions: synergistic, additive, or antagonistic effect? J. Agric. Food Chem. 60 (2012) 10320–10330.
[24] S. Prabha, V. Labhasetwar, Nanoparticle-mediated wild-type p53 gene delivery results in sustained antiproliferative activity in breast cancer cells, Mol. Pharm. 1 (2004) 211–219.
[25] K.C. Song, et al., The effect of type of organic phase solvents on the particle size of poly(d,l-lactide-co-glycolide) nanoparticles, Colloids Surf. Physicochem. Eng. Asp. 276 (2006) 162–167.
[26] S.K. Sahoo, V. Labhasetwar, Enhanced antiproliferative activity of transferrin-conjugated paclitaxel-loaded nanoparticles is mediated via sustained intracellular drug retention, Mol. Pharm. 2 (2005) 373–383.
[27] C.E. Astete, C.S.S.R. Kumar, C.M. Sabliov, Size control of poly(d,l-lactide-co-glycolide) and poly(d,l-lactide-co-glycolide)-magnetite nanoparticles synthesized by emulsion evaporation technique, Colloids Surf. Physicochem. Eng. Asp. 299 (2007) 209–216.
[28] T. Govender, S. Stolnik, M.C. Garnett, L. Illum, S.S. Davis, PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug, J. Controll. Release 57 (1999) 171–185.
[29] F.-Y. Cheng, et al., Stabilizer-free poly(lactide-co-glycolide) nanoparticles for multimodal biomedical probes, Biomaterials 29 (2008) 2104–2112.
[30] G. Della Porta, N. Falco, E. Reverchon, NSAID drugs release from injectable microspheres produced by supercritical fluid emulsion extraction, J. Pharm. Sci. 99 (2010) 1484–1499.
[31] T. Uchida, et al., Preparation and characterization of polylactic acid microspheres containing bovine insulin by a w/o/w emulsion solvent evaporation method, Chem. Pharm. Bull. (Tokyo) 45 (1997) 1539–1543.
[32] N. Falco, E. Reverchon, G. Della Porta, Injectable PLGA/hydrocortisone formulation produced by continuous supercritical emulsion extraction, Int. J. Pharm. 441 (2013) 589–597.
[33] G. Della Porta, et al., Injectable PLGA/hydroxyapatite/chitosan microcapsules produced by supercritical emulsion extraction technology: an in vitro study on teriparatide/gentamicin controlled release, J. Pharm. Sci. 105 (2016) 2164–2172.
[34] G. Della Porta, R. Campardelli, N. Falco, E. Reverchon, PLGA microdevices for retinoids sustained release produced by supercritical emulsion extraction: continuous versus batch operation layouts, J. Pharm. Sci. 100 (2011) 4357–4367.
[35] G. Della Porta, R. Campardelli, E. Reverchon, Monodisperse biopolymer nanoparticles by continuous supercritical emulsion extraction, J. Supercrit. Fluids 76 (2013) 67–73.
[36] N. Falco, E. Reverchon, G. Della Porta, Injectable PLGA/hydrocortisone formulation produced by continuous supercritical emulsion extraction, Int. J. Pharm. 441 (2012) 589–597.
[37] G. Della Porta, N. Falco, E. Reverchon, Continuous supercritical emulsions extraction: A new technology for biopolymer microparticles production, Biotechnol. Bioeng. 108 (2011) 676–686.
[38] R.N. Carvalho, L.S. Moura, P.T.V. Rosa, M.A.A. Meireles, Supercritical fluid extraction from rosemary (Rosmarinus officinalis): kinetic data, extract's global yield, composition, and antioxidant activity, J. Supercrit. Fluids 35 (2005) 197–204.
[39] M.V. da Silva, D. Barbosa, P.O. Ferreira, J. Mendonça, High pressure phase equilibrium data for the systems carbon dioxide/ethyl acetate and carbon dioxide/isoamyl acetate at 295.2, 303.2, and 313.2 K, Fluid Phase Equil. 175 (1-2) (2000) 19–23.
[40] R.L. Smith, T. Yamaguchi, T. Sato, H. Suzuki, K. Arai, Volumetric behavior of ethyl acetate, ethyl octanoate, ethyl laurate, ethyl linoleate and fish oil ethyl esters in the presence of supercritical CO2, J. Supercrit. Fluids 13 (1998) 29–36.
[41] H. Pöhler, E. Kiran, Volumetric properties of carbon dioxide + acetone at high pressures, J. Chem. Eng. Data 42 (2) (1997) 379–383.
[42] N. Falco, E. Kiran, Volumetric properties of ethyl acetate + carbon dioxide binary fluid mixtures at high pressures, J. Supercrit. Fluids 61 (2012) 9–24.
[43] G. Scita, The stability of β-carotene under different laboratory conditions, J. Nutr. Biochem. 3 (1992) 124–128.
[44] C.S. Boon, D.J. McClements, J. Weiss, E.A. Decker, Factors influencing the chemical stability of carotenoids in foods, Crit. Rev. Food Sci. Nutr. 50 (2010) 515–532.
[45] D.T. Santos, Á. Martín, M.A.A. Meireles, M.J. Cocero, Production of stabilized submicrometric particles of carotenoids using supercritical fluid extraction of emulsions, J. Supercrit. Fluids 61 (2012) 167–174.
[46] A. Hentschel, S. Gramdorf, R.H. Müller, T. Kurz, Beta-carotene-loaded nanostructured lipid carriers, J. Food Sci. 73 (2008) N1–6.
[47] T.C. Brito-Oliveira, C.V. Molina, F.M. Netto, S.C. Pinho, Encapsulation of beta-carotene in lipid microparticles stabilized with hydrolyzed soy protein isolate: production parameters, alpha-tocopherol coencapsulation and stability under stress conditions, J. Food Sci. 82 (2017) 659–669.
[48] C. Prieto, L. Calvo, Supercritical fluid extraction of emulsions to nano-encapsulate vitamin E in poly-caprolactone, Supercrit. Fluids J. 119 (2017) 274–282.
[49] C. Prieto, C.M.M. Duarte, L. Calvo, Performance comparison of different supercritical fluid extraction equipments for the production of vitamin E in poly-caprolactone nanocapsules by supercritical fluid extraction, Supercrit. Fluids J. 122 (2017) 70–78.
[50] C. Prieto, L. Calvo, C.M.M. Duarte, Continuous supercritical fluid extraction of emulsions to produce nanocapsules of vitamin E in poly-caprolactone, Supercrit. Fluids J 124 (2017) 72–79.