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A NEW FUNCTIONAL FOOD STARTING FROM BY-PRODUCTS OF THE OLIVE INDUSTRY

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Preface

The doctoral student **Carolina Liana Ronca**, the coordinator **Angelo Antonio Izzo**, and thesis tutor **Luana Izzo**, by signing this doctoral thesis, confirm that the work was carried out in co-tutelle between the Department of Pharmacy at the Food Chemistry Laboratory, University Federico II of Naples, and the Department of Nutrition and Bromatology of the Faculty of Pharmacy, University of Granada, to obtain the title of Doctor of Philosophy. We confirm that we have respected the copyright of other authors when using their results or publications.

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Granada, 27 Junio 2024

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Rafael Jesus Giménez Martínez
Tutor y director de la Tesis

_____

Carolina Liana Ronca
Doctoranda

In fin dei conti,

non temete i momenti difficili.

Il meglio scaturisce da lì.

Above all,

don't fear difficult moments.

The best comes from them.

Rita Levi Montalcini

A chi c'è sempre stato,

A chi ha creduto in me fin dall'inizio e fino alla fine,

A chi mi ama incondizionatamente fin da lassù,

A chi mi ha accompagnata durante questo meraviglioso percorso,

A mia figlia, Isabel Sofía

la mia ragione di vita,

che l'ha cambiata e ne ha fatto un capolavoro.

To all of you who have been there for me since the beginning,

who believed in me when I needed it most,

who love me unconditionally from up there,

who have been by my side on this incredible journey,

and to my daughter, Isabel Sofía,

who's my reason for living,

she's changed my life for the better and made it a masterpiece.

Abstract

In the food industry, functional foods are becoming increasingly popular. This trend is due to the fact that, in addition to providing the essential nutrients required for the development, preservation and optimal functioning of the body, these foods also include ingredients with health-promoting properties, along the lines of traditional foods. These ingredients include compounds with anti-hypertensive, anti-hypercholesterolemic, hypoglycaemic, anti-inflammatory and antioxidant effects.

Generally, these ingredients are obtained from plant matrices and are known as phytocomponents. Recent research has focused on polyphenolic compounds, which in addition to the aforementioned effects, have demonstrated antitumour, antimicrobial and protective action against cardiovascular and neurological degenerative processes. These polyphenolic compounds, mostly extracted from plants, fruits, vegetables, algae and also from their waste or by-products, can be incorporated into the formulation of functional foods, supplements or cosmetic products.

Conversely, olive leaves (*Olea Europaea L.*) present a significant environmental and sectoral challenge. They represent a considerable proportion of the solid waste generated in olive cultivation (25 kg per tree per year) and by the olive industry (approximately 10% of the weight of the fruit) and are typically discarded or used in the production of animal feed.

The European Union is the world's largest producer of olive by-products, with Spain, Italy, Greece and Portugal accounting for the majority of this production. In recent years, there has been a notable increase in interest in agri-food by- products, with the objective of exploiting this resource, which is rich in polyphenols and has thus far been underutilised. Olive leaves are a notable example of an abundant source of phenols, as widely reported in scientific literature. Nevertheless, this resource has not yet been fully exploited.

In line with the circular economy action plan for the recovery and reuse of food industry waste, olive leaves have been recovered for the generation of food ingredients with high added value. The extensive geographical extension and diversity of the land dedicated to olive production may be reflected in both the composition and the antioxidant activity of the polyphenols present in the leaves.

This research project involved the preparation and analysis of olive leaves extracts, both liquid and lyophilised. The methodology employed adhered to the most up-to-date protocols outlined in the relevant literature. The entire preparation process has been conducted with solvents, such as water and ethanol, that have been shown to be biodegradable, ecological, non-toxic, low cost, and respectful of the environment and human health.

A sample of olive leaves was gathered from olive trees in Italy, Portugal and Spain, and their polyphenol profiles and antioxidant activities were analysed. It was found that in the varieties under investigation, there is a correlation between a high polyphenol content and high antioxidant capacity.

Liquid olive leaf extracts have been freeze-dried and microencapsulated to improve the stability of the phytocomponents and to use them as an ingredient to be incorporated in food matrices. The freeze-drying process has not affected the major polyphenols (oleuropein and hydroxytyrosol), and the polyphenol content of the freeze-dried extract has remained stable during *in vitro* digestion tests.

However, research in the literature has indicated that environmental factors (such as heat, light, humidity) and factors related to absorption and metabolism by the human body (such as pH, enzymes, microorganisms) can affect the bioavailability and bioaccessibility of these components. Additionally, other challenges associated with the use of these plant extracts include their organoleptic properties, which in most cases make their direct incorporation into food unfeasible. In order to enhance the bioavailability, stability and

viability of antioxidant compounds in food matrices, the technique of microencapsulation has been employed.

Among the materials currently available on the market for the development of microparticles, sodium alginate has emerged as one of the most extensively researched natural anionic polymers, which can be incorporated into a variety of food products, supplements or pharmaceutical preparations in combination with a range of other ingredients due to its low manufacturing cost and low toxicity, combined with its biocompatibility, biodegradability and ability to form gel-like matrices in the presence of calcium ions.

This project demonstrates a promising microencapsulation rate of olive leaf polyphenolic compounds entrapped in sodium alginate, with approximately 98% of the polyphenols successfully micronencapsulated.

The food chosen to be enriched with the polyphenols extracted from olive leaves was yoghurt, for which goat's milk was used, given its characteristics in terms of composition and nutritional properties.

Further research should focus on the digestive processes and bioavailability of these fortified yoghurts, as well as the antioxidant activity and any potential beneficial effects on human health.

Resumen

En la actualidad, los alimentos funcionales están experimentando un aumento en su popularidad dentro de la industria alimentaria. Esta tendencia se debe a que, además de proporcionar los nutrientes esenciales requeridos para el desarrollo, mantenimiento y funcionamiento óptimo del organismo, estos alimentos también incluyen ingredientes con propiedades beneficiosas para la salud, siguiendo la línea de los alimentos tradicionales. Dentro de estos ingredientes se incluyen compuestos con efectos anti-hipertensivos, anti-hipercolesterolémicos, hipoglucemiantes, anti-inflamatorios y antioxidantes.

Por regla general, estos ingredientes se obtienen de matrices vegetales y se denominan fitocomponentes. Investigaciones recientes se han enfocado en los compuestos polifenólicos, los cuales, además de los efectos mencionados previamente, han demostrado actividad antitumoral, antimicrobiana y protectora contra procesos degenerativos de naturaleza cardiovascular y neurológica. Estos polifenoles, mayormente extraídos de plantas, frutas, verduras, algas y sus desechos o subproductos, pueden ser incorporados a la formulación de alimentos funcionales, suplementos o productos cosméticos.

Por otra parte, las hojas de olivo (*Olea Europaea L.*) presentan un desafío medioambiental y en el sector olivarero, dado que representan una constituyen una parte significativa de los residuos sólidos generados tanto en el cultivo del olivo (aproximadamente 25 kg por árbol anualmente) como en la industria olivarera (alrededor del 10% del peso de los frutos), y suelen ser descartadas o utilizadas en la producción de alimento para animales.

La Unión Europea ocupa la posición de mayor productor a nivel global de subproductos derivados del olivo, con España, Italia, Grecia y Portugal como principales actores en esta producción. En los últimos años, se ha observado un creciente interés en la utilización de los subproductos agroalimentarios, con el propósito de explotar este recurso tan rico en polifenoles y hasta el momento

ha sido subutilizado. Las hojas de olivo destacan como una fuente considerable de fenoles, como se ha documentado extensamente en la literatura científica. Sin embargo, a pesar de su potencial, este recurso no ha sido plenamente aprovechado.

En concordancia con las iniciativas del plan de acción de economía circular, orientadas hacia la valorización y reutilización de residuos de la industria alimentaria, las hojas de olivo han sido objeto de recuperación con miras a la generación de ingredientes alimentarios de alto valor añadido. La amplia extensión geográfica y la diversidad de terrenos destinados al cultivo del olivo puede influir en la composición y actividad antioxidante de los polifenoles presentes en las hojas.

Para llevar a cabo esta investigación se han preparados y analizados extractos líquidos y liofilizados de hojas de olivo, siguiendo los procedimientos más relevantes documentados en literatura científica. Durante todo el proceso de preparación, se emplearon solventes como agua y etanol, los cuales se caracterizan por ser altamente biodegradables, ecológicos, no tóxicos, de bajo coste, y respetuosos tanto con el medio medioambiente como con la salud humana. El muestreo se ha realizado recolectando hojas de olivos procedentes de Italia, Portugal y España, y se llevó a cabo un análisis exhaustivo de su perfil de polifenoles y actividad antioxidante. Entre las diferentes variedades evaluadas, se ha observado una correlación entre niveles elevados de compuestos polifenólicos y una actividad antioxidante significativa.

Los extractos líquidos de hojas de olivo han sido sometidos a procesos de liofilizados y microencapsulados con el fin de mejorar la estabilidad de los fitocomponentes y utilizarlos como ingrediente a incorporar en matrices alimentarias. Se ha constatado que proceso de liofilización no ha afectado los polifenoles mayoritarios, como la oleuropeína y el hidroxitirosol, y que el contenido en polifenoles del extracto liofilizado ha permanecido estable durante los ensayos de digestión *en vitro*.

Sin embargo, estudios recogidos en la literatura han señalado que, factores ambientales (como calor, luz, humedad), así como aquellos relacionados con la absorción y metabolización en el organismo humano (como pH, enzimas, microorganismos), pueden incidir en la biodisponibilidad y bioaccesibilidad de estos componentes. Además, otros problemas asociados al empleo de estos extractos vegetales son sus propriedades organolépticas, que, en su mayoría, dificultan su incorporación directa al alimento. Con el propósito de aumentar la biodisponibilidad, la estabilidad y la viabilidad en matrices alimentarias se ha explorado la técnica de microencapsulación de compuestos antioxidantes.

Actualmente, entre los materiales disponibles en el mercado para la elaboración de micropartículas, el alginato sódico ha surgido como uno de los polímeros aniónicos naturales más ampliamente investigados, que puede ser utilizado en productos alimentarios, suplementos o formulaciones farmacéuticas junto con otros ingredientes debido a su bajo coste, baja toxicidad, biocompatibilidad, biodegradabilidad y capacidad de formar matrices gelatinosas en presencia de iones calcio.

En el presente trabajo, se ha obtenido un porcentaje prometedor de microencapsulación de los compuestos polifenólicos de las hojas de olivo, atrapados en alginato sódico: casi el 98% de los polifenoles han sido exitosamente microencapsulados.

El alimento seleccionado como vehículo para enriquecerlo con los polifenoles extraídos de las hojas de olivo ha sido el yogurt, para el cual se ha empleado leche de cabra, debido a sus características en términos de composición y propiedades nutricionales.

Para futuras investigaciones, se sugiere dirigir los esfuerzos hacia el estudio de la digestión y bioaccesibilidad de estos yogures enriquecidos, además de evaluar la actividad antioxidante y posibles efectos beneficiosos sobre la salud humana.

List of Publication

Paper I
Graziani, G., Gaspari, A., Di Vaio, C., Cirillo, A., **Ronca, C. L**., Grosso, M., & Ritieni, A. **Assessment of *in vitro* bioaccessibility of polyphenols from annurca, limoncella, red delicious, and golden delicious apples using a sequential enzymatic digestion model**. *Antioxidants*, (2021). 10(4): p. 541.

Paper II
Ronca, C. L., Marques, S. S., Ritieni, A., Giménez-Martínez, R., Barreiros, L., & Segundo, M. A. **Olive Oil Waste as a Source of Functional Food Ingredients: Assessing Polyphenolic Content and Antioxidant Activity in Olive Leaves**. *Foods*, 13(2): p. 189.

Paper III
Ronca, C. L., Carmen Duque-Soto, Cristina Samaniego-Sánchez, María Encarnación Morales-Hernández, Manuel Olalla-Herrera, Jesús Lozano-Sánchez, and Rafael Giménez Martínez. **Exploring the Nutritional and Bioactive Potential of Olive Leaf Residues: A Focus on Minerals and Polyphenols in the Context of Spain's Olive Oil Production.** *Foods*, (2024). 13(7): p. 1036.

List of Abbreviations

AAPH 2,2′-azobis (2-amidinopropane) dihydrochloride

ACN acetonitrile

AIF all ion fragmentation

ANOVA analysis of Variance

AOX antioxidant capacity

CA caffeic acid

ChlA chlorogenic acid

CAT catechin

CUA p-cumaric acid

CUPRAC cupric reducing antioxidant capacity

DMSO dimethyl sulfoxide

DPPH 2,2-diphenyl-2-picrylhydrazyl

DS diosmin

DW dry weight

EtOH ethanol

EU European Union

FA formic acid

FC Folin-Ciocalteu

FCR phosphomolybdic/phosphotungstic acid reagent

FeA ferulic acid

FOSHU Foods for Specified Health Use

FRAP ferric ion reducing antioxidant power

FWHM Full Width at Half Maximum

FUSOSE European Commission Concerted Action Group on Functional Food Science in Europe

GA gallic acid

HCD High Collision Dissociation

HPLC-DAD High-performance liquid chromatography with diode array detector

HPLC-MS Ultra-high-performance liquid chromatography mass- coupled spectrometer

HPLC-UV-Vis High performance liquid chromatography ultraviolet visible

HTYR hydroxytyrosol

ISTAT Italian National Institute of Statistics

LIG ligstroside

LLOQ lower limit of quantification

LUT luteolin

LUT-R luteolin rutinoside

MC microparticles

MeOH methanol

NaOH sodium hydroxide

OL olive leaves

OLE olive leaves extract

OLE-A oleuropein aglycone

OLEU oleuropein

OO olive oil

ORAC oxygen radical absorption capacity

PINO pinoresinol

QUER quercetin

ROS reactive oxygen species

RUT rutin

S.D. standard deviation

SEG secologanoside

TE Trolox Equivalent

TEAC Trolox equivalent antioxidant capacity

TYR-G tyrosol-glucoside

UHPLC-MS Ultra-High Performance Liquid Chromatography and Mass Spectrometry Analysis

UHPLC-Q-Orbitrap-HRMS Ultra-High Performance Liquid Chromatography and Orbitrap High Resolution Mass Spectrometry Analysis

UHPLC-TQ-XS-MS-ESI Ultra-High Performance Liquid Chromatography Triple-Quadrupole Extra-Small Mass-Spectrometer, Electrospray Ionization.

VA vanillic acid

VB verbascoside

WHO World Health Organization

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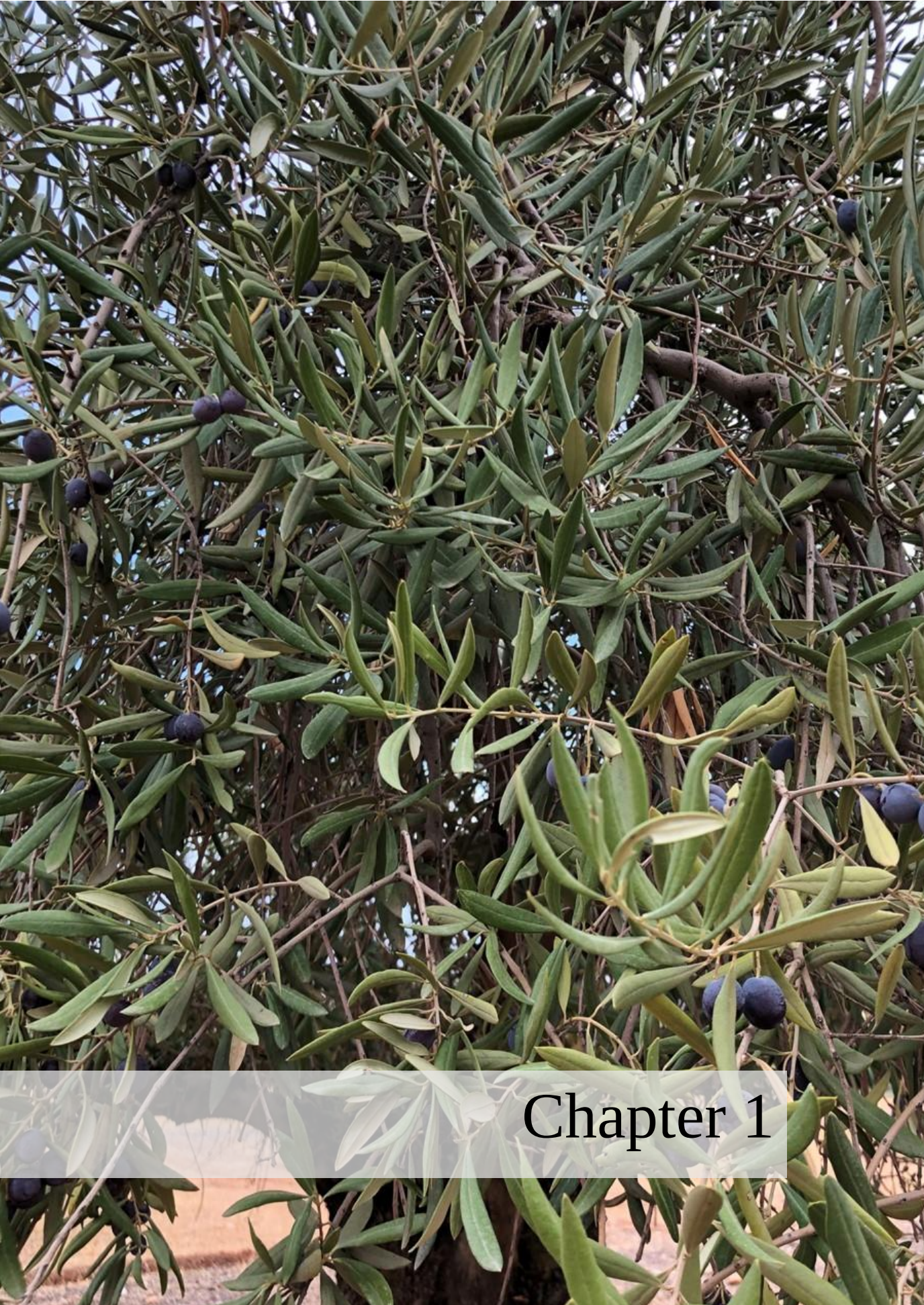


Chapter 1

Chapter 1

Introduction

1.1 A brief history of Olive Oil through the centuries.

Since ancient times, Olive oil (OO) has been considered a rich and symbolic ingredient, not only in terms of nutrition but also in anthropology. Alongside wine and bread, it forms the "Mediterranean triad" par excellence. Olive oil is widely regarded as a cornerstone of the Mediterranean diet due to its potential benefits in preventing cardiovascular and neurodegenerative diseases, certain types of cancer, and its positive effects on inflammatory processes and blood pressure reduction.

The olive tree, scientifically known as *Olea Europaea Linnæus,* is a small tree belonging to the Oleaceae family. It is native to the Mediterranean basin but is extensively cultivated worldwide, including in South America, South Africa, Australia, New Zealand, and the United States. The cultivation, harvesting, and extraction of liquids from olives for the production of olive oil are closely related to the history and culture of Mediterranean populations.

The importance of olive oil in culture and everyday life is evident from its early use in ancient Greece. Philosophers of that time researched and investigated its nutritional and therapeutic properties. Hippocrates called it "the great healer", Homer "liquid gold", and Galen decanted it for its pro-health effects. Both Aristotle and Hippocrates recognized the healthy and curative properties of olive oil recommending its ingestion and use as an unguent for the treatment of diseases, such as stomach and dermatological ulcers. This demonstrates the

historical use of olive oil for medicinal purposes [1]. Currently, olive oil is widely consumed as a healthy fat source due to its known associated health benefits. These include antioxidant [2, 3], hypoglycaemic [4], anti-inflammatory [3], and neuroprotective effects [5-7].

Worldwide, approximately 3 million tonnes of olive oil are produced annually [8, 9]. European countries produce about 2 million tonnes of olive oil, with Spain accounting for 66%, followed by Italy (15%), Greece (13%) and Portugal (5%) by the 2023/2024 season [10]. Nevertheless, over the last years, olive oil production has increased by 37% in Italy, 19% in Portugal and 27% in Spain (as shows in Figure 1). In general, the decline observed over the past decade can be attributed to two key factors: the global pandemic caused by the novel coronavirus (Covid-19) [11], and climate change . In the first instance, the restrictions on the movement of people and the difficulties in organising the workforce have made it challenging for farmers to cultivate and harvest olives. This has resulted in damage to distribution chains due to the difficulty in transporting olives from the fields to production facilities and from factories to markets. Furthermore, the economic difficulties faced by families and restaurateurs, who have been forced to reduce their expenses, have compounded the problem. In the second case, unfavourable climatic events, such as drought or frost, could damage olive crops, thereby reducing the amount of olive oil produced.

The European Union (EU) is the producer of about 67% of the world's olive oil and European countries are among the main consumers, with an annual consumption of around 1.5 million tonnes of olive oil (50% of world production). Italy and Spain are the largest consumers of olive oil, each consuming around five hundred thousand tonnes annually. Greece has the highest consumption per capita, with approximately 12 kg per person per year. The EU accounts for almost 53% of global consumption. Additionally, approximately 4 million hectares are dedicated solely to olive tree cultivation,

including traditional, intensive, and super-intensive groves [8, 9]. Of these areas, about 98% are in Mediterranean countries [9, 12].

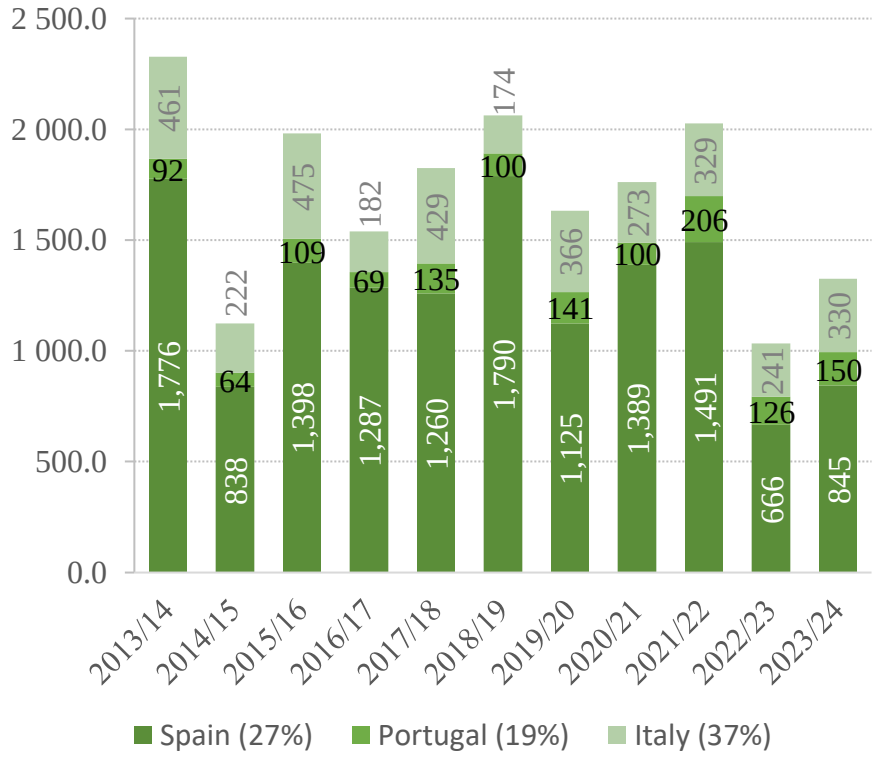


Figure 1. Olive Oil Production in Spain, Portugal and Italy in the last 10 years. Based on production by (1000t). Italy has experienced a 37% increase in olive oil production, Portugal a 19% increase, and Spain a 27% increase.

Particularly, in 2021, Portugal produced 784,340 tons of olives [13], with 97% of the yield dedicated to olive oil production. Between 120,000 and 150,000 tonnes of olive oil are forecast to be produced in 2023, and compared to 2021, there is not much change [14]. Currently, Portugal ranks among the top 5 European producers and the top 10 global producers [15, 16]. Olive plantations are widely spread across Portugal, with the majority located in Alentejo (50%), Trás-os-Montes (23%), Beira Interior (14%), and Ribatejo and Oeste (7.5%). Some activity in the field is also present in the Algarve and Beira Litoral regions [13]. Portugal is an emerging country in the olive oil production area,

thanks to extensive development in the sector, suitable ground, and remarkable climatic conditions [17]. By 2030, it is estimated that Portugal will be the world's third-largest olive oil producer [15]. The increase in olive oil production and subsequent olive tree harvesting presents environmental challenges due to the generation of significant amounts of waste, such as olive leaves and wood, and by-products, including olive pomace, mill wastewater, and olive stones [18].

Spain has the largest area dedicated to olive tree cultivation, with 2,507,684 hectares (equivalent to over 180 million trees). Tunisia follows with 1,746,360 hectares, and Italy with 1,143,363 hectares. In terms of virgin olive oil (VOO) production, Spain is the most significant producer, with over a million tons produced in the last five harvests (2017/18 to 2021/22). Italy and Greece follow, each producing an average of 300 thousand tons. [19]. The province of Jaén, located in Andalusia (southern Spain), is one of the most representative regions of olive monoculture worldwide. It boasts the highest olive oil production and the largest area of olive groves in Spain. Olive monoculture is the economic engine of this region, and heavily influences its economy. Jaén accounts for 20% of the global olive oil production, with over 66 million olive trees covering 550,000 hectares, which is more than 25% of Spain's total olive grove area and 42% of the cultivated land in Andalusia [19]. For this, Jaén is also the world's largest producer of olive oil.

The production of olive oil is distributed across several regions of Italy. Generally, Puglia is the largest producer, accounting for 45% of the country's olive oil production, followed by Calabria, Sicily and Tuscany with 15%. Meanwhile, Umbria, Liguria and Campania produce 10% of the total olive oil. Moreover, Campania produces olive oil from *Frantoio* and *Leccino* varieties, collected in the hills around Salerno, Avellino and Benevento. According to Italian National Institute of Statistics (ISTAT, 2024) reports, Italy cultivates over one million hectares and produces approximately 3,623.652 quintals of olive oil [20].

1.2 A brief history of Olive Leaves through the centuries.

The history of the olive leaf dates back to the Neolithic period. In the Mediterranean region, neolithic populations living in Greece, Anatolia (present-day Turkey), Spain, France, Italy, Tunisia, and parts of the Middle East (such as Israel and Morocco) switched from the art of hunting to the art of animal husbandry and plant cultivation, contributing to the emergence of the first agricultural communities. These were responsible for the beginning and spreading of olive growing. The cultivation of the olive tree is closely linked to the use of olive leaves in traditional medicine as a natural remedy for high blood pressure, inflammation and infections. Indeed, olive leaves, which contain many potentially bioactive compounds with antioxidant, antihypertensive, antiatherogenic, anti-inflammatory, hypoglycemic and cholesterol-lowering properties, have been used in the human diet in the form of extracts, teas and powders [21]. Historically, the victors of friendly games and bloody wars have been crowned with its leafy branches. In ancient Greek culture, the olive tree was associated with the goddess Athena, a symbol of peace, abundance, glory and wisdom. The Egyptians used olive leaves as a preservative in mummification, and also used to treat fevers and diseases, such as malaria [22]. In recent times, olive leaves have been used in the medical, cosmetic, pharmaceutical and food industries. It has a high potential for industrial exploitation in the food and beverage industry [23].

1.3 Therapeutic benefit of polyphenols

Several scientific studies have demonstrated the potentially beneficial effects of olive leaves polyphenols on human health (Figure 2). Indeed, olive leaves contain polyphenolic compounds, including phenolic acid (verbascoside), phenolic alcohols (hydroxytyrosol), secoiridoids (oleuropein), flavonoids (luteolin, rutin, quercetin), and lignans (pinoresinol) which have demonstrated therapeutic benefits [24, 25] both *in vitro* and *in vivo* [25-28]. Gallic acid has been identified as a potent antioxidant and an effective apoptosis-inducing

agent [29]. Rutin and luteolin have shown hypoglycaemic and antioxidant activities. Quercetin has shown anti-inflammatory and anti-tumor effects [30]. Similarly, oleuropein, which constitutes 6-9% of the dry matter in leaves, is a secoiridoid compound and the primary polyphenol in olive leaves. It exhibits protective effects against oxidative stress [31], anti-inflammatory activity [32],and positive effects on body weight control [33], glycemia [34], and intestinal microbiota [35, 36]. In recent years, hydroxytyrosol, the main metabolite of oleuropein, has also received increased attention. This compound has demonstrated beneficial effects in protecting cellular lipids, proteins, and DNA from oxidative damage, as well as preventing degenerative, cardiovascular, and carcinogenic diseases [37]. Furthermore, verbascoside and pinoresinol has exhibited significant antioxidant properties [38, 39]. Due to the valuable profile of olive leaves as a source of compounds with therapeutic properties, there is a demand for sustainable practices to promote the reuse of olive leaf waste from olive oil production for the extraction of these compounds.

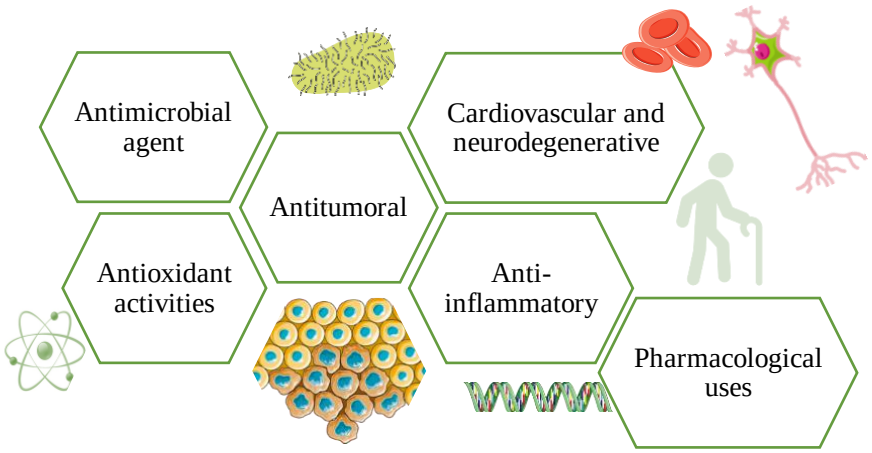


Figure 2. Potentially beneficial effects of olive leaves polyphenols on human health.

(Parts of the figure was created using images from the Server Medical Art database, which is licensed under a Creative Commons Attribution 4.0 Unported License).

1.4 Principal phenolic and polyphenolic compounds found in olive leaves.

Plants synthesize an extensive array of organic compounds traditionally classified as primary and secondary metabolites [40]. Primary metabolites are essential compounds involved in processes such as photosynthesis, respiration, and growth and development. These include phytosterols, acyl-lipids, nucleotides, aminoacids, and organic acids.

In contrast, secondary metabolites, are a heterogeneous group of compounds that exhibit considerable variation in concentration within different plant species. These compounds are often accumulated in surprisingly high concentrations, making them an important subject of study in plant biology.

The interest in these plants is considerable, given their potential as sources of new natural drugs, antioxidants, antibiotics, hypoglycaemic, anti-inflammatory, anti-tumour effects, and as agents that prevent degenerative, cardiovascular, and carcinogenic diseases.

In light of this, recent years have seen an increasing focus within human nutrition research on the role of certain secondary metabolites as protective dietary constituents. These can be divided into three main categories, based on their biosynthetic origins, as follows: (a) flavonoids, and related no-flavonoids phenolic and polyphenolic compounds, (b) terpenoids, and (c) nitrogen-containing alkaloids and sulphur-containing compounds.

The following section offers a brief description of the main Olea Europaea L. leaves polyphenols belonging to the first group, namely flavonoids and no-flavonoids compounds (phenolic acids, phenolic alcohols, secoiridoids, and lignans).

Flavonoid group represents a significant class of constituents found in olive leaves. Its structural composition comprises fifteen carbons, with two aromatic rings connected by a three-carbon bridge. They are found in high concentrations

in the epidermis of leaves and the skin of fruits, where they play an essential role in the plant's physiology and development [41]. They possess considerable radical scavenging activity, which is beneficial for human health and may contribute to the beneficial effects of olive leaf extract. The principal flavonoids present in olive leaf, as documented in the literature, include luteolin- 7- O- glucoside, luteolin-7-O-rutinoside, apigenin-7-O-glucoside, rutin, luteolin and apigenin [42].

The most abundant no-flavonoids compounds belong to the secoiridoids, phenolic acids, phenolic alcohols, and lignans group.

Secoiridoids are characteristic of the Oleaceae family and are designated as “oleosides”. These compounds are typically composed of a sugar, a glycoside derived from the hydrolysis of oleuropein, and a simple phenol (tyrosol or hydroxytyrosol). The most prevalent secoiridoid present in olive leaf extract is oleuropein. Isolated from the leaves of *Olea Europaea*, oleuropein is non-toxic and may be administered without experiencing any particular side effects. Due to the size and planar structure of the oleuropein molecule, it has low oral bioavailability; however, glycosylation may favour its absorption by epithelial cells in the small intestine [21]. In addition, olive leaves are also abundant in another secoiridoid such as verbascoside, a glycoside consisting of caffeic acid and hydroxytyrosol.

Phenolic acids are aromatic compounds with multiple functional groups, including a carboxylic acid group. They have been identified as potential markers for olive cultivar and harvest time. [43, 44]. The main phenolics acid in olive leaves are gallic acid, vanillic, p-coumaric acid and verbascoside.

Phenolic alcohols are aromatic compounds with multiple functional groups, including an oxydril group. The most representative examples are tyrosol and hydroxytyrosol. Olive leaves contain a high quantity of hydroxytyrosol, which is produced via the degradation of oleuropein. This process involves the cleavage of the glycosidic bond between the glucose molecule and the phenol,

resulting in the liberation of hydroxytyrosol. Once released, hydroxytyrosol is then available for absorption in the small intestine and subsequent distribution throughout the body, where it exerts its beneficial effects on health, such as including cardioprotective, anticancer, neuroprotective antimicrobial,

In this work, the principal flavonoids present in olive leaf are diosmin, luteolin, rutin, quercetin, and catechin; while the others main non-flavonoids subclasses are: phenolic acids (most notably is gallic acid, but also caffeic acid, ferulic acid, p-coumaric acid, chlorogenic acid, vanillic acid, syringic acid and verbascoside); phenolic alcohols (hydroxytyrosol); secoiridoids (oleuropein, oleuropein aglycol, secologaniside, and ligstroside) and lignans (pinoresinol), (Figure 3).

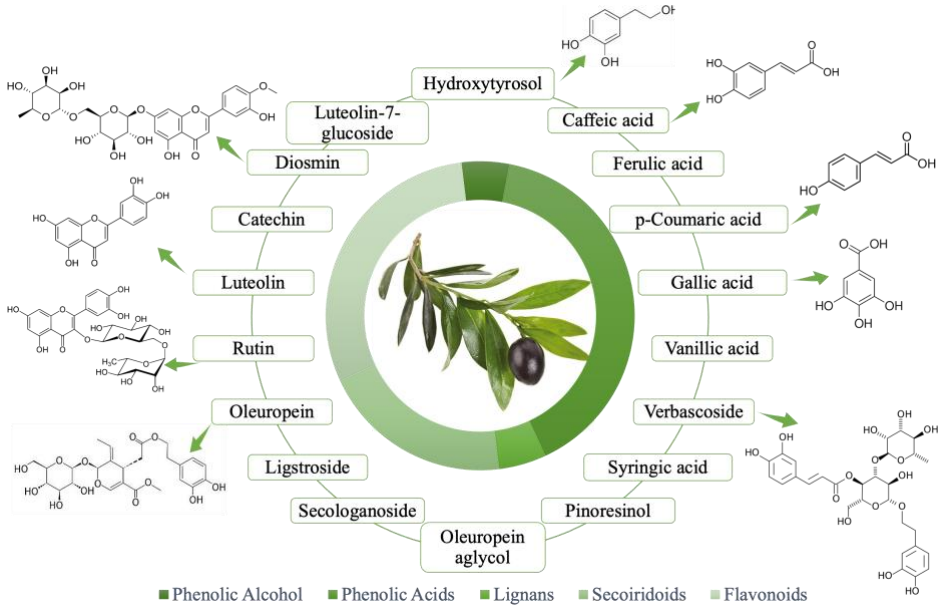


Figure 3. Olive leaf extract polyphenolic contents, classification by categories and the respective chemical structures of the principal compounds.

1.5 Worldwide Olive Leaves

The expression "olive leaves" means an amalgam of leaves and branches from both the pruning of the olive trees and the harvesting and cleaning of the olives. Today, it is possible to consider olive leaf (OL) a co-product of olive growing and processing. Indeed, the pruning and harvesting of the tree to produce olive oil and table olives generate a considerable amount of OL, which is used by some industries to obtain natural products rich in bioactive compounds for dietary supplements, food additives, nutraceuticals, and cosmetics. Each tree yields around 25 kg of by-products (branches and foliage) annually, and many of these (around 10% of the weight of the fruits) are discarded in the process of extracting the oil [45, 46]. It was only at the beginning of the 21st century that a chemist, William Fredrickson, began to explore the potential of using olive leaves for medicinal purposes. He patented his extraction process in 2000 [47].

1.6 Circular economy

Olive oil industry has increased the production of olive oil due to the worldwide recognition of its organoleptic characteristics, and health benefits associated with its consumption as part of the Mediterranean diet. Consequently, the production of residues has risen, including stones, pomace, wastewater, and olive leaves. In the spectrum of oil wastes, olive leaf (OL) is the first to be recovered during the pre-treatment of olives. This involves the disposal of olive leaf waste, an area where ecological issues are important. Given that olive leaves represent a valuable source of phenolic compounds [9, 46, 48-50] and in line with circular economy principles to minimize environmental impact and maximize raw material use, the potential of olive leaf waste is being harnessed [51, 52].

1.7 Methods to extract polyphenols from olive leaves

Polyphenolic compounds can be extracted from plants using different extracting solvent [53] (e.g., water, organic solvents, or hydroalcoholic mixtures)and a variety of methods, such as liquid-liquid extraction [54], microwave-assisted extraction [28, 53, 55], pressurized liquid extraction[56], supercritical fluid extraction[57], solid-phase extraction[53], and ultrasound-assisted extraction [58]. These methods have been proven effective and are widely used in the industry. To extract compounds of interest from agro-food matrices, such as olive leaves, greener extraction solvents can be used. These include water, 70:30 (v/v) water/ethanol solutions, or deep eutectic solvents [59]. Additionally, recent studies have proposed pressurized propane [60] or isopropanol water [61] for the extraction of bioactive compounds from olive leaves. By using these methods, we can confidently achieve environmentally friendly procedures for compound extraction. Polyphenols cannot be analysed using the initial method due to their high boiling point. Similarly, the second method and other commonly used solvents like methanol, ethyl acetate, hexane, diethyl ether, chloroform, and butanol [62] cannot be used directly for human or animal consumption.

Solid-liquid extraction was used to recover polyphenols from olive leaves. The chosen solvent was selected for its contribution to the greenness and sustainability of the process, as well as its compatibility with food applications. The optimal extraction solvent was determined by monitoring the polyphenol profile using high-performance liquid chromatography (HPLC) coupled with UV-Vis detection. The antioxidant capacity of various olive leaf samples was evaluated using several in vitro methods, including Folin-Ciocalteu, cupric-reducing antioxidant capacity (CUPRAC), 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and oxygen radical absorption capacity (ORAC).

The study aimed to investigate the relationship between quantitative analysis via HPLC and the antioxidant capacity assessed by various methods to determine any additive or synergistic effects. Additionally, bioaccessibility studies were conducted using the richest extract to assess the feasibility of incorporating the extracted compounds into food products through lyophilisation processes in the future.

1.8 Fundamentals of chromatography

1.8.1 HPLC: High Performance Liquid Chromatography

In order to quantify the different polyphenolic compounds, present in olive leaves, liquid chromatography is used as a separation technique. Specifically, high-performance liquid chromatography with diode array detector (HPLC-DAD) and ultra-high-performance liquid chromatography mass-coupled spectrometer (UHPLC-MS) have been employed.

HPLC-DAD involves the use of a liquid mobile phase, driven by a pump, and a solid stationary phase on which each compound is capable of adsorption. Compounds can be separated and distinguished based on their affinities to the mobile and stationary phases, resulting in different retention times. Various column materials with different hydrophobicities can be used as stationary phases. HPLC provides faster separations than column chromatography due to the mobile phase being driven by a pump, allowing for the use of smaller particles in the column packing material. The separation of components in a mixture is improved because the increased surface area facilitates interactions between the stationary phase and the molecules of interest. The mobile phase, typically a mixture of water and organic solvents such as acetonitrile and/or methanol, is used in both normal and reverse phase separations. The mobile phase, typically a mixture of water and organic solvents such as acetonitrile and/or methanol, is used in both normal and reverse phase separations. Normal-phase HPLC involves using a polar column made up of silica, silica-amino,

silica-diol or silica-cyano particles, along with a non-polar mobile phase. This type of separation retains polar compounds in the column to a greater extent than non-polar compounds, causing them to elute later. Reversed-phase HPLC, on the other hand, uses a non-polar stationary phase and a polar mobile phase. The columns consist of silica particles that have been modified by attaching hydrocarbon chains (8-18 C atoms) to their surface. This modification allows polar compounds present in a mixture to pass through the column faster, eluting before non-polar molecules. Normal- and reversed-phase HPLC columns usually have an internal diameter of 3.0 and 4.6 mm and a length of between 100 and 250 mm. The separation of compounds in HPLC is determined by their interactions with the stationary phase, specifically *Van der Waals* and hydrophobic interactions. Reversed-phase mode is the most commonly used form of HPLC [63-65].

1.9 From Olive Leaves Extract to the Formulation of Functional Foods

The discipline of food and nutrition science has undergone significant developments over the past decades. Initially, the focus was on fortified foods, but over time, the objective evolved to the construction of foods that promote health and are known as functional foods.

The term "functional food" has been defined in a multitude of ways across the globe. Japan was the first nation to coin the term in 1980 and to introduce regulations governing the production and sale of these products. Furthermore, the Japanese have designated the term Foods for Specified Health Use, or FOSHU, for foods that have been fortified with a functional ingredient to achieve a specific health benefit [66].

Whereas for the European Commission Concerted Action Group on Functional Food Science in Europe (FUSOSE), a functional food could be considered as one that has been satisfactorily demonstrated to have a beneficial effect on one or more functions in the body, beyond adequate nutritional effects, in a way

that is relevant to either an improved state of health and well-being and/or a reduction of risk [66].

It is commonly accepted by many researchers that a significant proportion of fruits, vegetables, grains, fish, dairy products and meats contain a number of naturally occurring substances, collectively know as "bioactives," that confer benefits beyond the basic nutritional value.

A considerable number of researchers believe that a significant proportion of fruits, vegetables, grains, fish, dairy products and meats contain a number of natural compounds that provide benefits beyond basic nutrition. These bioactives include, but are not limited to, the lycopene found in tomatoes, the omega-3 fatty acids present in salmon, the saponins found in soybeans, the tannins present in wine, the polyphenols present in olive oil, tea, coffee, and chocolate.

In contrast, other researchers propose that foods fortified, enriched or enhanced with components that confer a beneficial effect on human health beyond the basic nutritional value of their ingredients should also be regarded as functional [67].

The principal distinction between fortified food and enriched food lies in the reasons for the incorporation of nutrients and the original presence of these nutrients in the food.

The process of fortification involves the addition of nutrients that are not naturally present in the food or are only present in insignificant traces. This is typically carried out with the intention of improving public health and preventing nutritional deficiencies in the population. One example of this is the addition of iodine to salt, which helps to prevent iodine deficiency.

Enrichment, on the other hand, involves the addition of nutrients that have been lost during processing or are naturally present but are increased to improve

nutritional value. This is exemplified by the addition of B vitamins to white flour, which helps to improve the nutritional value of the flour.

In particular, fortified foods include the milk fortifies with minerals (such as calcium) and vitamin D, which helps to prevent rheumatism and osteoporosis and improves bone health. In the similar manner cereals fortify with B vitamins (such as B1, B2, B6 and B12), and minerals such as iron and folic acid, improve energy, nervous system function, and prevent anaemia. The iodised salt is also a fortified food, containing iodine, which helps prevent goitre and other thyroid diseases.

In the other hands, enriched foods include white flour enriched with B vitamins (such as niacin, riboflavin, thiamine), and folic acid, that play a role in preventing anaemia, supporting healthy pregnancy, and improving metabolism. Similarly, yoghurt enriched with probiotics and vitamin D improves digestive health, supports the immune system, and contributes to bone health. Likewise, soy milk enriched with calcium, vitamin D, and vitamin B12, is an excellent alternative to cow's milk for vegans or individuals with lactose intolerance, which supports bone health.

The increasing interest in the relationship between diet, food components and health has led to the development of functional foods. These foods, which can be defined as those that contribute to health and well-being, may offer a solution to the challenges of a busy lifestyle. They provide a convenient way to access ingredients that improve health, despite the limitations of time.

Indeed, consumer demand for foods with benefits beyond basic nutrition has increased the marketing opportunities for food manufacturers. Indeed, functional foods containing bioactive ingredients and nutraceuticals are becoming increasingly important in the marketplace.

One essential point of view is that functional foods have a significant physiological effect on human health, as they can be a source of essential nutrients.

In light of the aforementioned considerations, the present investigation involved the characterisation of olive leaves and the subsequent extraction of their phytochemical components, which was followed by the application of encapsulation techniques to trap the extracted components. The efficacy of this encapsulation process was subsequently evaluated using an effectiveness food matrix.

In this instance, microcapsules comprising alginate were employed for the microencapsulation process, while goat's yogurt was utilised as the foodstuff to create a novel functional food with enhanced nutritional value.

1.10 Polymeric Microparticles

1.10.1 Microparticles: definition and common characteristics

The term “microsphere” is often used interchangeably with the term “microparticle” in technical literature. However, it is important to note that “microparticle” specifically refers to spherical particles with diameters ranging from 1 μm to 1000 μm. Typically, polymeric microparticles consist of a polymer matrix in which a smaller amount of an active compound is immobilized. Two categories of microparticles can be differentiated in terms of the distribution of the active compound/beneficial-ingredient: “microspheres” and “microcapsules” [68].

“Microspheres” " consist of a homogeneous mixture of active ingredient and excipient, whereas “microcapsules” have a core (containing the active ingredient) surrounded by another substance (usually the excipient). The core can exist in a solid, liquid, or gaseous state. Additionally, the microcapsule core may also contain one or more distinct domains of active ingredients. Microparticles can be made from a wide range of natural and synthetic materials using various preparation techniques. This allows for a vast array of microparticles to be produced, differing in size, distribution, composition, surface chemistry, topography, and morphology. Moreover, microparticles are

manufactured from an extensive range of natural and synthetic materials using different preparation techniques, resulting in an extensive range of microparticles in terms of size, composition, distribution, surface chemistry, topographical and morphological spectrum [69, 70].

1.10.2 Microencapsulation

Microencapsulation (ME) is an effective method for protecting, transporting, and releasing bioactive molecules. It has gained attention for its potential applications in food and drug delivery systems, cosmetics, biomedical, and textile industries [71-75]. One of its many beneficial properties is the ability to trap fragile functional ingredients (bio-molecules) in microparticles, which are coated with additional protective layers/materials, in order to improve stability and protect the ingredients from hostile environments. This is particularly important as exposure to such environments can lead to alteration, modification, degradation or even deactivation of the ingredient's properties. By taking advantage of colloidal and interfacial interactions, ME can create a variety of carriers capable of delivering active ingredients to their intended sites of action [76].

1.10.3 Microparticles with sodium alginate and soybean oil

Incorporating polyphenols extracted from plant matrices into food products is a complex process due to the need to protect them from atmospheric factors such as light, heat, and humidity, as well as ensuring their bioavailability through factors such as solubility, pH, and the human microbiota of the gastrointestinal tract, human enzymes, and disorders that affect absorption [77]. Polyphenols are highly sensitive to oxidation and are easily degraded in the presence of heat sources or under very strong extraction conditions at high temperatures; they are photosensitive and must be protected from light sources by using dark glass containers or in the dark; they are unstable in aqueous solutions, so it is better to freeze-dry the plant matrix or the extract from it.

Additionally, several *in vitro* and *in vivo* studies [51, 78-82] have shown that polyphenols are affected by enzymatic degradation processes in the mouth, by the gastric acid pH and by several biochemical mechanisms, also depending upon the human gastrointestinal microbiota.

Other problems associated with the use of these plant extracts are related to their organoleptic properties, which do not allow them to be incorporated directly into foods, requiring the use of strategies to mask undesirable tastes and smells, and also to increase stability, viability and consumer acceptance. Currently, research in the field of food technology is focused on developing encapsulation techniques for antioxidant compounds. These compounds can be dissolved in liquid, solid or gaseous materials and then incorporated into food matrices using polymeric membranes [83]. To overcome the aforementioned drawbacks, freeze-dried olive leaf extracts were used as ingredients to prepare a microencapsulation with high antioxidant power.

The technique of internal ionic gelation in emulsion, previously adapted and developed by the research group at the Department of Pharmacy and Pharmaceutical Technology of the University of Granada, was chosen for the microencapsulation of lyophilised olive leaf extract.

The microparticle's structure is defined by the creation of an outer membrane, known as a wall, which is formed using a microencapsulating agent. This wall serves to protect the desired substance inside the core [84]. Sodium alginate, a polymer with a high affinity for water, is commonly used as a microencapsulating agent due to its ability to form a uniform, non-toxic, and biodegradable gel. Additionally, when ingested, it provides a prebiotic effect and acts as dietary fibre, reducing blood glycemia and cholesterol levels [85].

Moreover, sodium alginate has become one of the most extensively studied natural anionic polymers for use in the development of microparticles. It can be included in controlled-release pharmaceutical systems alongside other polymers due to its low cost, low toxicity, biocompatibility, biodegradability,

and gelatinous die-forming capacity in the presence of Ca^{2+} ions [70, 86]. Its popularity is due to these properties, which make it a versatile and effective material for this purpose.

The MC technique in use is based on the formulation of a water/oil (W/O) emulsion at room temperature. The aqueous phase was prepared by dissolving the lyophilised extract in water and then incorporating it with sodium alginate (mixture A). Simultaneously, the oil phase was prepared by mixing soybean oil with the surfactant Span80® (mixture B). Mixture B was then poured over mixture A while stirring constantly. An emulsion was formed, and a strong acid was added to initiate internal gelling. The resulting solid microparticles were separated from the oily phase and collected by vacuum filtration.

The microparticles were produced using two key components: sodium alginate, which is biodegradable and environmentally safe, and soybean oil, another discarded by-product of the food industry.

Several studies have demonstrated that this material is a natural, non-toxic polymer [70]. These systems allow for repeated administration of medicinal substances and protect them from enzymatic degradation, thereby increasing the half-life of the drug or prolonging the release of the active ingredient polymer [70]. Additionally, alginate can be used to microencapsulate polyphenolic compounds, which can be used to prepare supplements or functional foods with added benefits; and, recently alginate was used to perform a skin's patch that show a significant antioxidant and anti-inflammatory properties [87].

Sodium alginate is a natural linear polysaccharide that is biodegradable, biocompatible and safe for the body, and provides strength and flexibility to tissues [86]. Sodium alginate has industrial applications due to its gelling, viscosifying and stabilising properties as well as its ability to retain water. The cell wall of various species of brown algae can be used to synthesis alginate. These are *Laminaria hyperborea*, *Ecklonia maxima*, *Ascophyllum nodosum*,

Eisenia bicyclis, and *Macrocystis pyrifera*, as well as from bacteria such as Azotobacter and Pseudomonas. Alginate extracted from brown algae has commercial importance in the food, pharmaceutical, and cosmetic industries [70, 86].

Nowadays, the microencapsulated nutraceuticals with alginate[70-72] are a growing attractive field in food technology. This is due to the proven safety and effectiveness of pharmaceutical microencapsulates over time.

The development of novel biomaterials, which are processible, biocompatible, biodegradable, and nontoxic, enables the creation of formulations that are more efficient and advantageous than traditional formulations in the nutritional and medical fields. The category of new high-performance materials includes anionic natural polymers, such as sodium alginate. These are considered advantageous for microencapsulation matend. Studies have shown that they can influence the kinetics of drug release from the matrix, based on their degradation in the body.

1.11 Nutritional values of goat yoghurt

Over the past few decades, yoghurt has emerged as a popular fermented food product. It has been reported that fermented dairy products are more nutritious than the milk from which they are made. The increased availability of specific nutrients and the hydrolysis of milk components by lactic starter cultures contribute to the enhanced nutritional value of these products. Furthermore, yoghurt has been employed in the treatment of gastrointestinal conditions including diarrhea, infantile gastroenteritis, and constipation, demonstrating its therapeutic value [88].

In numerous countries, goat farming represents a significant economic activity, particularly in the Mediterranean and Middle Eastern regions. The goat farming industry is well-established in France, Italy, Spain, and Greece [89]. The industrialisation of goat milk has not been as successful as might have been

hoped, largely due to the poor and insufficient volume of the product. Nevertheless, from 1990, interest in the goat milk production is increased [90].

Indeed, goat's milk is a widely consumed dairy product, particularly in many regions of the globe. Its unique composition includes essential substances such as minerals, vitamins, polyphenols, enzymes, proteins, electrolytes and fatty acids, which are readily metabolised by the body.

Furthermore, goat milk differs from cow and human milk in terms of its digestibility, buffering capacity, alkalinity and therapeutic value [90].

The process of fermentation of milk is necessary for the production of yoghurt. According to International Dairy Goat Research Center, 1993 [88], goat yoghurt contains higher levels of the basic nutrients (including protein, carbohydrates , fat and minerals) and secondary metabolites (polyphenols) than cow yoghurt.

The composition of goat milk has been found to contain higher levels of potassium, calcium, chloride, phosphorus, selenium, zinc and copper than cow milk [90]. From an anthropological perspective, goat milk is a superior choice for human nutrition due to its higher content of most minerals. While goat milk and cow milk are similar in composition, goat milk cannot replace human milk in young children but can serve as an excellent complement to it.

In this study, goat milk was selected as a model due to its compositional and nutritional similarities to human milk [89, 90], as evidenced by its high concentration of polyphenols [91-93] and minerals [94, 95].

1.12 Recovery minerals from olive leaves and health benefits

Throughout recorded history, olive oil, fruit, and leaves have been used for medicinal purposes. More recently, olive leaves have also attracted considerable nutritional interest from the food industry due to their mineral and biophenolic content. In contrast to vitamins, proteins, carbohydrates and fats,

minerals and polyphenols do not provide energy, but they are essential nutrients that offer a wide range of health benefits. Polyphenols act as antioxidants, anti-inflammatory, anti-tumour and cardioprotective agents. Minerals are involved in various biochemical and physiological processes of cellular metabolism, including bone and tooth formation, muscle contraction, nerve transmission, blood clotting and hormone production [96]. Consequently, the minerals can enhance the antioxidant activity of the polyphenols.

Moreover, the World Health Organization (WHO) recommends that individuals consume a daily intake of calcium (1000-1300 mg/day), magnesium (300- 400 mg/day), and iron (8-18 mg/day), with values varying by age and gender.

In this work, identify and quantify trace-minerals (copper, zinc, selenium) and major minerals (calcium, iron, potassium) in dried olive leaves, lyophilised olive leaf extracts, microparticles and enriched yoghurt it was important for encouraging the recovery of waste that was not previously utilised by the olive oil industry. Indeed, numerous studies have demonstrated the significance of the minerals in the context of recovery [97, 98].

1.13 Objectives

The primary aim of this project is to optimize the extraction technique and identify bioactive phenolic and polyphenolic compounds derived from olive leaves of the most commonly cultivars grown in Italy, Spain, and Portugal. The compounds will then be evaluated for their potential use in the formulation of foods with functional properties.

The following secondary objectives are to be achieved as part of the development of this work:

1. Utilisation of a by-product of the olive oil industry that is abundant in quantity and causes significant disposal problems for the industry, with the objective of reducing the environmental impact.

2. Improvement of the recovery of bioactive compounds present in olive leaves, with the intention of increasing the value of the waste in question.

3. Determination and quantification of phytochemical compounds in this type of by-product.

4. Analysis and quantification of minority minerals (copper, zinc, selenium) and majority minerals (calcium, iron, potassium) in olive leaf extracts be undertaken, thereby enhancing the value of this type of material from the olive oil industry.

5. Formulation of functional and innovative foods by incorporating bioactive compounds from plant matrices.

6. Reduction of the environmental impact by using green technologies for nutraceutical or functional food products formulation.

7. Finally, reduction of the waste disposal costs for the industry.

1.14 Protocoles

The experiments were conducted in three different countries (Italy, Portugal and Spain) using varying protocols (as shown in Figure 4), reagents, and analytical instruments. The protocols used in this study were carried out in Italy at the Laboratory of Innovation and Research of the Linfa s.c.a.r.l. Consortium in Naples, and at the Laboratory of Food Chemistry, in the Department of Pharmacy at Federico II University of Naples (referred to as ***Protocol I***); in Portugal at the Laboratory of Applied Chemistry, in the Department of Chemical Sciences at the Faculty of Pharmacy at the University of Porto (referred to as ***Protocol P)***; and in Spain at the Laboratory of Nutrition and Bromatology and the Laboratory of Pharmaceutical Technology, in the Department of Nutrition and Bromatology and the Department of Pharmacy and Pharmaceutical Technology at the Faculty of Pharmacy at the University of Granada (referred to as ***Protocol S***).

The following protocol, as shown in Figure 4, is used once the leaves have been collected:

Protocol I) The Italian protocol provides for two types of solid-liquid extraction. The first is with HCl 0.1%, the second is with MeOH/H2O 80:20 with formic acid 0.1%. Following this, the liquid extract is freeze-dried, after which polyphenolic analysis and antioxidant assays are conducted.

Protocol P) The Portuguese protocol prescribes a solid-liquid extraction with EtOH/H2O 50:50 ratio, with the addition of formic acid at a concentration of 0.1%. This is followed by a freeze-drying of the liquid extract, which is then subjected to polyphenolic analysis, antioxidant assays and *in vitro* bioaccessibility.

Protocol S) The Spanish protocol prescribes a solid-liquid extraction EtOH/H_2O 50:50 with formic acid 0.1% and then a freeze-drying of the liquid extract, followed by microencapsulation of the freeze-dried extract to be

incorporated into a food matrix (in this case, yoghurt). A polyphenolic analysis is conducted on the lyophilised extract, microparticles and yoghurt. Antioxidant assays are performed on the lyophilised extract. Finally, mineralisation is conducted on the dried olive leaves, lyophilised extract, microparticles and yoghurt.

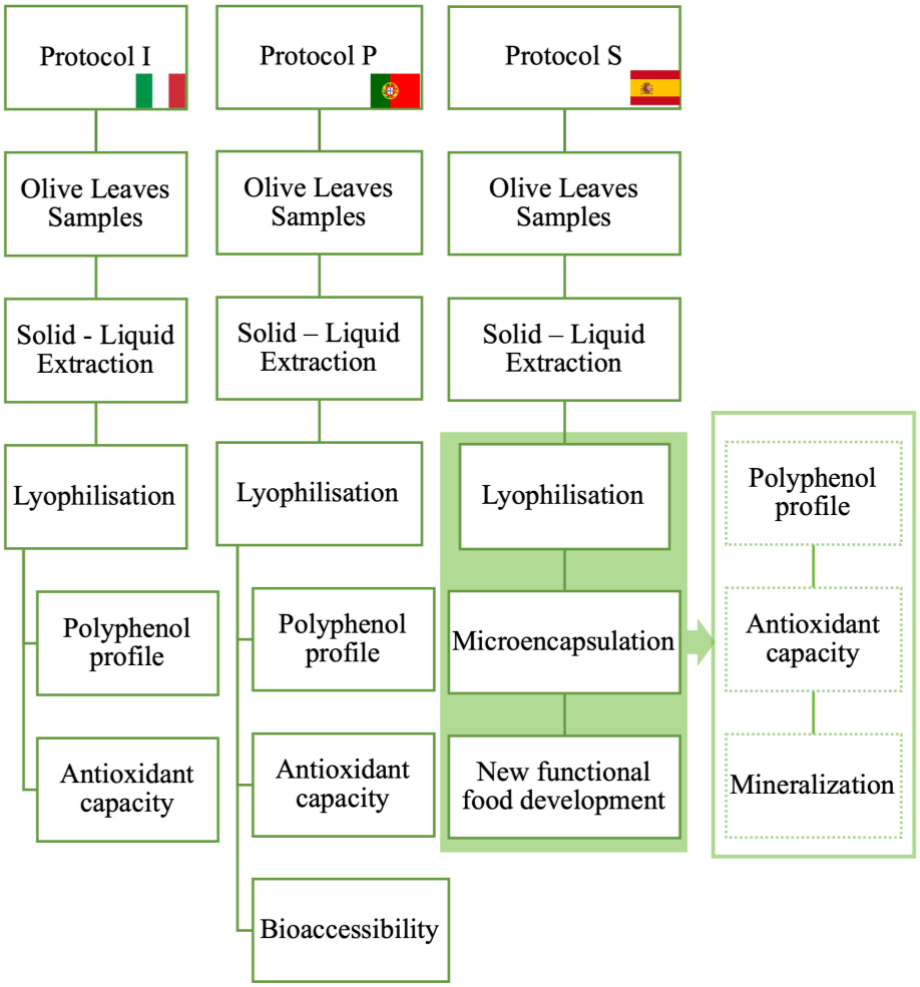


Figure 4. Diagram overview of three different protocols for extraction.

Chapter 2

Chapter 2

Extraction of hydroxytyrosol according to European Patent (n. Ep 1 582 512 A1) developed by Linfa s.c.a.r.l. Consortium, ITALY.

Olive leaves (*Olea Europaea*) var. *Leccino* and *Frantoio* were collected from a farm in Eboli in the province of Salerno (Campania, Italy). A liquid extract highly rich in phenolic and polyphenolic compounds, including oleuropein aglycone (OLE-A), ligstroside (LIG), secologanoside (SEG), tyrosol-glucoside (TYR-G), hydroxytyrosol (HTYR), caffeic acid (CA), luteolin (LUT), luteolin rutinoside (LUT-R), p-cumaric acid (CUA), vanillic acid (VA), ferulic acid (FeA), pinoresinol (PINO), and verbascoside (VB) was performed. A satisfactory extraction technique is important because these bioactive substances have attested antioxidant, antimicrobial, antiproliferative and hypocholesterolemic properties. Therefore, it is very important to perform an exhaustive extraction process. This technique was performed at the *Linfa s.c.a.r.l. Consortium*. An extractive method in slightly acidic water has been used to obtain a high yield of phenolic and polyphenolic compounds contained in the leaves (and then a highly functionalized intermediate to be proposed as a nutraceutical, functional food, or cosmetic). The protocol was developed by making slight adjustments to the method reported by the European Patent Application "*n. EP 1 582 512 A1: Process for obtaining hydroxytyrosol from olive leaves extracts*" and entirely made at the Linfa

s.c.a.r.l. Consortium. Liquid extract was lyophilised and a powder with high polyphenol content was obtained.

Reversed-phase High-Performance Liquid Chromatography with diode array detector (HPLC-DAD) was used to analyze both extracts (liquid and lyophilised), and to quantify the main component hydroxytyrosol. Other bioactive compounds (such as oleuropein, tyrosol, caffeic acid, oleocanthal and verbascoside) present in the liquid and lyophilised extract were analyzed by Ultra-High Performance Liquid Chromatography and Orbitrap High Resolution Mass Spectrometry (UHPLC-Q-Orbitrap-HRMS). In addition, freeze-dried extracts were used for antioxidant capacity assays (AOX) to characterize highly functionalized intermediates for the formulation of nutraceuticals, supplements, or cosmetic products.

2.1 Materials and methods

2.1.1 Chemicals and Solutions

All reagents and chemicals were analytical grades. The standards of polyphenols (purity ³ 98%) for UHPLC-Q-Orbitrap HRMS analysis were purchased from Sigma Aldrich (Milan, Italy) and included: glucoside hydroxytyrosol (phenolic alcohols); oleuropein, oleuropein aglycon, ligstroside, secologanoside (secoiridoids); p-coumaric acid, ferulic acid, vanillic acid (hydroxycinnamic acids); (+)-pinoresinol (lignans); verbascoside (derivate of hydroxycinnamic acids); luteolin rutinoside and luteolin (flavonoids).Water (LC-MS grade), Methanol (MeOH), were acquired from Merk (Darmstadt, Germany), and formic acid (mass spectrometry grade) was purchased from Fluka (Milan, Italy). For liquid extraction, according to Linfa's European Patent "*n. EP 1 582 512 A1: Process for obtaining hydroxytyrosol from olive leaf extracts*," water (HPLC plus), formic acid (85% v/v), and hydrochloric acid (37% v/v) were obtained from Carlo Erba (Milan, Italy). The polyphenols profile was performed with a High-Performance Liquid Chromatography Diode-Array detector (HPLC-DAD), and water (LC-MA Grade) was purchased from Merk (Darmstadt, Germany), methanol and acetonitrile (HPLC plus gradient) was obtained from Carlo Erba (Val de Reuil Cedex, France).

2.1.2 Olive Leaves Samples

Olive branches from more than 30 leaves-rich trees (*Olea Europaea L.*) of *Leccino* and *Frantoio* cultivars, coming from a farm in Eboli (Salerno, Italy; geographical coordinates 40° 38' 32.2" N; 14° 55' 46.7" E), were collected in January 2020, after the olive harvest and during the most productive period for the plants. As a result of this timeliness, the sampling of olive leaves in which a greater amount of bioactive compounds were detected, was more accurate.

2.1.3 Preparation of Olive Leaves Extracts

HPLC-DAD. Approximately 6 kg of olive fresh leaves were provided from Santa Rita farm (Eboli, Campania, Italy), transferred to the laboratory and immediately detached by hand. All olive leaves were dried at 55°- 60°C in order to obtain dry leaves for one week (oven Binder GmbH, Tuttlingen, Germany). Dry residue of olive leaves was 4,961 Kg (weight loss of 18.13 %). After drying, dried leaves were milled into powder using a blender at room temperature. This material was transferred an opaque plastic bottle and stored in a cool and dry place until further use (freezer -20 °C). For liquid extraction, according to Linfa Patent, 50 grammas of leaves powder were extracted using 500 mL of water Milli-Q acidified with 0.1% of chloride acid (HCl 37% v/v). Autoclave cycle was used for 15 minutes at 121°C. The first gravity filtration, using Erlenmeyer flask and porous paper filter (Brentford, UK) to remove the solid residues. The solid residue was centrifuged with Thermo Fisher Scientific (Am Kalkberg, Germany), for 15-20 min at 5000 rpm at T 20°, in order to separate the liquid trapped in the wet solid. The resulting liquid extract was filtered using a vacuum system equipped with a borosilicate vacuum flask and porcelain Büchner filter. To separate the solid residue from the liquid extract and remove suspended particles, cellulose filters were placed in porcelain Büchner filters. Liquid extract was collected in two 250 mL beakers. Frozen extracts were lyophilised with freeze-dried Kambic Lio 5p (Semic, Slovenia) p = 0.0621, and at 36.2°C for one week.

UHPLC-Q-Orbitrap MS. Another extraction was performed to identify other polyphenols. Fifty grams of fresh olive leaves were frozen and then lyophilised with freeze-dried Kambic (Semic, Slovenia) p = 0.0621, and at 36.2°C for three days. Five hundred milligrams of lyophilised leaves were extracted using 30 mL MeOH/H_2O (80:20) with 0.1% of formic acid. The mixture was shaken in a vortex for 2 min and introduced in an ultrasound bath for 15 min at room temperature. Subsequently, the mixtures were mechanically shaken for 10 min and then centrifuged at 4000 rpm at 4°C for 10 min.

The supernatant was recollected and filtered with nylon filter 0.22 μm (Phenomenex, Castel Maggiore, Italy), prior to injection into the UHPLC-Q-Orbitrap-MS. The same extracts were used for antioxidant capacity (DPPH, ABTS, FRAPP) and total polyphenolic content determinations (Folin-Ciocalteu).

2.1.4 Chromatographic Analysis

The hydrophobic properties of polyphenolic compounds were considered for the separation and quantification of phenolic compounds.

Liquid and lyophilised extracts were analyzed by Reverse-phase High-Performance Chromatography with a diode array detector (HPLC-DAD), in order to separate and quantify hydroxytyrosol, whereas A UHPLC-MS system (Thermo Fisher Scientific, Waltham, MA, USA) was used for the quantification and separation of other polyphenolic compounds.

2.1.4.1 Chromatographic Conditions: High Performance Liquid Chromatography diode-array detector (HPLC-DAD)

HPLC-DAD Agilent 1200 (Santa Clara, California, USA) in order to segregate and quantify hydroxytyrosol was performed. Hydroxytyrosol 1ppm (1mg/1mL MeOH:H_2O) was used to performing calibration curve (y= 4964 x - 16.12; r^2= 0.999). The dilution of calibration curve (500, 250, 125, 64, 31.2 ppm) using MeOH:H_2O (1:1) as solvent. One milligram of lyophilised olive leaves extract was solubilized in 1mL MeOH/H_2O (1:1), while liquid olive leaves extract was analyzed as it was. A filter paper (Brantford, UK) was used to purify the OL extracts. Dilutions calibration curve of hydroxytyrosol and samples were injected (5 μL) with microsyringe Agilent (Australia, USA) into a reversed-phase Kinetex® core–shell C18 column (150 x 4.6 mm, 5 μm particle size; 110 Å). Chromatography separation was achieved in a gradient mode by mixing in-line mobile phase (A) consisted of 0.2% (v/v) formic acid in water and (B) 60:40 (v/v) MeOH:ACN (60:40, v/v).

The elution started with 90% of A and 10% B (0–5 min), followed by an decrease from 90 to 60% of A (5– 20 min). Then, a higher organic content [60:40 (v/v) A:B] was maintained for 5 min (20-30min), followed by a return to initial conditions (35–40 min) and 2 min of column equilibration (40–42 min) before the next run. Determinations were performed at room temperature. A flow rate of 0.800 mL min-1 was used. UV detection was performed in a UV-Vis diode array detector (DAD). The spectra acquisition was measured from 200 to 900 nm, with the quantification of phenolic compounds at 254 for oleuropein (the main component of OL), and 280 nm for hydroxytyrosol. The quantification of hydroxytyrosol in olive leaf extract was carried out by interpolating the peak area values of the calibration curve.

2.1.4.2 Chromatographic Conditions: Ultra-High Performance Liquid Chromatography and Orbitrap High Resolution Mass Spectrometry Analysis (UHPLC-Q-Orbitrap HRMS)

A UHPLC-MS system (Thermo Fisher Scientific, Waltham, MA, USA) was used for the quantification and separation of other polyphenolic compounds according to method of Graziani et al. [99]. The UHPLC system, equipped with a Dionex Ultimate 3000 Quaternary pump and a thermostated (25°C) Kinetex 1.7 µm biphenyl (10 x 2.1 mm) column (Phenomenex, Torrance, CA, USA), degassing system and autosampler device. The analytical eluents phase were: *phase A*, water/formic acid (99.9:0.1); *phase B*, methanol/formic acid (99.9:0.1). The flow rate was 0.2 mL/min., and the injection volume was 2 µL. The autosampler and column temperatures were set at 10°C and 25°C, respectively. A gradient elution program was applied, as follows: 0 min, 5% of phase B; 1.3 min, 30% of phase B; 9.3 min, 100% of phase B; 11.3 min, 100% of phase B; 13.3 min, 5% of phase B; 20 min, 5% of phase B. Due to the fact that some standards were not available, the quantification of some polyphenols was calculated applying calibration curves of structurally related substances having the same chemical group and a similar response.

The quali-quantitative analysis was performed by using the. Q-Exactive Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) operating in negative ion mode (Thermo Scientific, Bremen, Germany). A full MS/AIF mode that uses a full MS scan without higher energy collisional dissociation (HCD) fragmentation, followed by an all-ion-fragmentation (AIF) scan with a fragmentation energy applied was used for acquisition. Full MS experiments were carried out with under the following conditions: microscans, 1; AGC target, 1e6; maximum injection time, 200 ms; mass resolution, 35,000 FWHM at m/z 200; and AIF scan conditions: microscans, 1; AGC target, 10^5; maximum injection time, 200 ms; mass resolution, 17 500 FWHM at m/z 200; HCD energy, 10, 20 and 45. In both cases, the spray voltage was set to 3.5 kV; the capillary temperature was 275 C; sheath gas, was 45 (arbitrary units); auxiliary gas, was 10 (arbitrary units); m/z range, was 80–1200; and data acquisition was profile mode. The accuracy of the MS analysis was guaranteed by calibration of the detector with commercial calibration solutions supplied by the manufacturer. The mass tolerance was observed at 5 ppm in both full scan MS and AIF modes. Xcalibur software v. 3.1.66.10 (Xcalibur, Thermo Fisher Scientific, v. 3.0.63) was applied for data analysis and processing.

2.1.5 Antioxidant Assays

The antioxidant activity of liquid and lyophilised extracts was assessed and compared spectrophotometrically by three different procedures (FRAP, DPPH, and ABTS).

2.1.5.1 FRAPP: Ferric Reducing Ability of Plasma Antioxidant Capacity Assay

The Ferric Reducing Antioxidant Capacity (FRAP) assay measures the antioxidant reducing ability of iron and is based on the reduction, at low pH, of the ferric complex 2,4,6-tripyridyl-s-triazine (Fe3+-TPTZ) to the ferrous form (Fe2+-TPTZ), which develops a thick blue color. The absorbance of the blue

complex at 593 nm was measured using a PerkinElmer Lamba 25 UV/VIS spectrometer.

This method is performed as described by (I. F. Benzie & J. J. Strain, 1996) was used with slight modifications. The FRAP radicals were prepared by mixing 10:1:1 (v/v/v): acetate buffer 0.3 M, weighing 25.206 g sodium acetate anhydro and dissolved in 1000mL of MilliQ water, additionally the pH was adjusted to 3.6 with glacial acetic acid; solution of TPTZ 10 mM in HCl 40 mM, and for this 0.03123 g of TPTZ was weighed and dissolved in 10 mL of MilliQ water; and finally, 0.0312g of FeCl3, was weighed for dissolving in 10 mL of MilliQ water. To prepare HCl 40 mM 333 µL of HCl 37% v/v was deluded in 100 mL of MilliQ water.

The stock solution for calibration curve (Figure 5) was 1000 ppm of Trolox in MeOH absolute, and from this were prepared the dilutions (7.8, 15.6, 31.2, 62.5, 125, 250 ppm, Table 1). For the analysis of the olive leaf extract, 1 mg of the lyophilised extract was dissolved in 1 ml of MeOH/H2O (1:1), and 1 ml of the liquid extract was dissolved in 1:200. Appropriate sample dilutions of 100 µL were added to 2 mL of the radical FRAP. The absorbance at 593 nm after 4 min was monitored.

2.1.5.2 DPPH: 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay

DPPH is a stable radical in solution and appears purple colour absorbing at 515 nm in methanol. This assay is based on the principle that DPPH radical on accepting a hydrogen (H) atom from the scavenger molecule i.e. antioxidant, resulting into reduction of DPPH radical to $DPPH_2$, the purple colour changes to yellow with concomitant decrease in absorbance at 515 nm. The colour change is monitored by spectrophotometrically and utilized for the determination of parameters for antioxidant properties.

In this work, the DPPH free radical-scavenging activity was carried out according to the protocol proposed by [100], with minor modifications. Briefly, methanolic DPPH (4 mg in 10 mL) was diluted using methanol absolute until absorbance value 0.90 ± 0.02 at 517 nm, in this way DPPH radical working solution was obtained (1:15 dilution). Lyophilised extract was dissolved in MeOH/H2O 1:1 (1mg/mL), while the liquid extract was diluted 1:100 from the initial olive leaves extract.

The analysis was performed by adding 200 µL of each sample to 1 mL of DPPH radical working solution. The stock solution for calibration curve (Figure 5) was 1000 ppm of Trolox in MeOH absolute, and from this were prepared the dilutions (7.8, 15.6, 31.2, 62.5, 125, 250 ppm, Table 1). The decrease in absorbance was monitored after 10 min at 517 nm. Results were expressed as mmol Trolox equivalents (TE) per kg of sample extract.

2.1.5.3 ABTS: 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) Radical Cation Scavenging Capacity Assay

The ABTS assay was conducted based on the method reported by [101] with same modifications. Briefly, 9.6 g of ABTS radical was dissolved in 2.5 mL water (7 mM) and added to 44 µL of aqueous potassium persulfate (2.45 mM), following 16h of incubation at room temperature in the dark. The ABTS solution was diluted with ethanol (dilution 1:88) until an absorbance value of 0.700 (± 0.02) at 734 nm to obtain an ABTS radical working solution.

The stock solution for calibration curve (Figure 5) was performed with Trolox 2 mM (5 mg in 1 mL MeOH absolute), and from this 2mM was prepared; the latter stock solution was used for the calibration curve of Trolox (500, 250, 125, 62.5, 31.2, 1.6, 7.8 µM, Table 1). The analysis was performed by adding 100 µL of the sample to 1 mL of ABTS radical working solution. The absorbance was monitored after 3 min at 734 nm. Results were expressed as mmol Trolox equivalents (TE) per kg of sample extract.

2.1.6 Phenolic Content Colorimetric Method

2.1.6.1 Folin–Ciocalteu-Reducing Assay

Total phenolic content (TPC) was performed using the Folin-Ciocalteu colorimetric method described previously by Mai-Rui Gao et al. [102] with slight modifications. In a nutshell, the stock solution of gallic acid was prepared from 1000 ppm stock solution: 10mg of gallic acid were dissolved in 100mL of MilliQ in volumetric flask. The sodium carbonate solution 7.5% was prepared weighing 15g of Na_2CO_3 and dissolving in 200mL of warm MilliQ water in volumetric flask, to facilitate dissolution. The liquid extracts olive leaf (with 0.1 g/mL concentration, because 50g of olives leaves were extracted using 500 mL of H_2O acidified with 0.1 % HCl 37%); while the lyophilised extract was prepared dissolving 1mg in 1mL MeOH/H2O (1:1). The calibration curve of gallic acid (Figure 5) was performed from stock solution preparing solution a different concentration (0.25, 0.125, 0.0625, 0.0312, 0.0156, 0.0078, 0.0039 mg/mL, Table 1). For the colorimetric methods, 125 µL of hydro-ethanolic olive leaves extract was added to 500 µL of MilliQ water and 125 µL of Folin - Ciocalteu reagent.

Table 1. Specification calibration curve of Antioxidant assay for olive leaves var. *Leccino* and *Frantoio*. The values were expressed in µM Trolox for DPPH (b), ABTS (b) and FRAPP (b), and in µM GA for Folin (a).

AOX	Slope	Intercept	Coefficient of determination
FOLIN[a]	820.8	0.01	0.997
DPPH[b]	0.55	0.68	0.995
FRAPP[b]	0.002	0.071	0.993
ABTS[b]	0.38	0.74	0.989

The mixture was allowed to stand for 5 min, and then 2 mL of aqueous solution Na_2CO_3 10% was added. The olive leaf samples, allowed to stand for 6 min at room temperature, were added to 1.5 mL of sodium carbonate 7.5% (Na_2CO_3)

and 1 mL of water. Then the absorbance was measured at 760 nm using a Jasco V-730 ultraviolet-visible (UV–VIS) spectrophotometer (Tokyo, Japan). TPC is expressed as the form of mg standard equivalent per gram of sample (mg GAE/g DW).

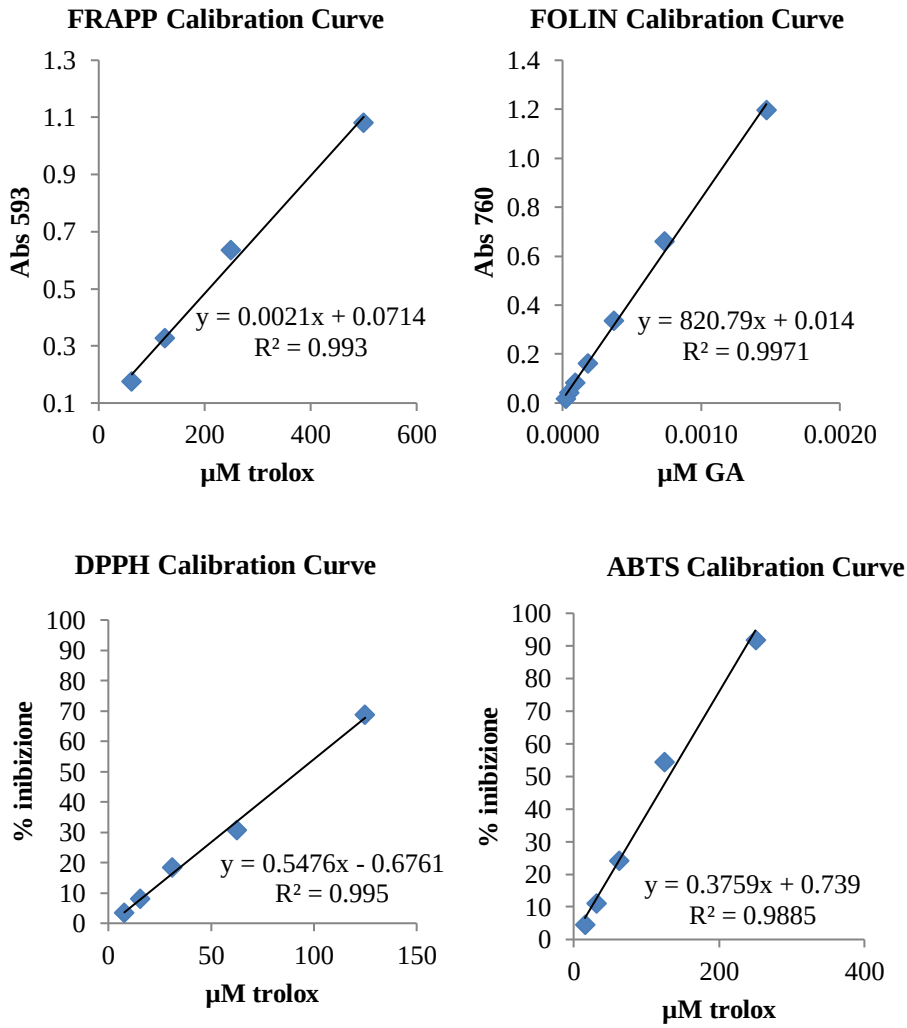


Figure 5. Calibration Curve of AOX for olive leaves var. *Leccino* and *Frantoio*.

2.2 Results

In order to re-evaluate extracts of the olive plant for the formulation of functional foods or supplements with pro-health properties, this part of the study evaluated the amount of hydroxytyrosol in olive leaves extracts.

2.2.1 Quantification of Polyphenols with HPLC-DAD

Olive plants produce large amounts of secondary metabolites, for better adaptation to environmental conditions, protection against microbial attack and resistance to biotic and abiotic stresses. Among these compounds, phenolics have received considerable attention in recent years due to their antioxidant, anti-inflammatory, anti-mutagenic and anticoagulant properties [50, 103-107], which have been correlated with a reduced risk of cardiovascular [108-110], of neurodegenerative diseases [111-113], cancer development [104] and obesity [109, 114].

Oleuropein is the major phenolic compound found in olive oil and its major by-products, olive leaves. Moreover, the hydrolysis of oleuropein produces another mayor hydroxytyrosol, noted as a major secondary metabolite with great potential healthy bioactive compounds [105, 106] .

Linfa European Patent "*n. EP 1 582 512 A1: Process for obtaining hydroxytyrosol from olive leaf extracts*," was performed on dried and milled olive leaves, in order to obtain the maximum quantity of hydroxytyrosol. The identified of this phenolic compound, such as the most abundant in olive plant and olive leaves according to literature [115-117], are presented in Figure 6**.** High quantity of the hydroxytyrosol was obtained through Linfa's extraction (36 % di hydroxytyrosol in 1000g of dried olive leaves).

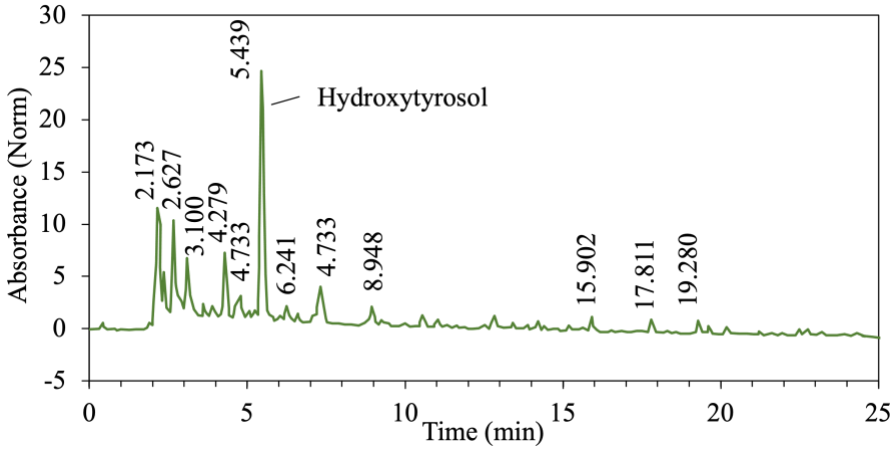


Figure 6. Identification of hydroxytyrosol in lyophilised extracts though HPLC-DAD. The lyophilised extract was diluted with methanol/water in a 1:1 ratio.

Moreover, comparing the liquid extract with the lyophilised one, by HPLC-DAD of Linfa s.c.a.r.l. laboratory, showed that both extracts have a very similar chromatographic peak for hydroxytyrosol Figure 7. This indicates two important points: on the one hand, that freeze-drying does not cause a severe breakdown of the phenolic molecule and, on the other hand, that this dehydration technique could be a possible alternative for obtaining intermediate rich in hydroxytyrosol, but also in other bioactive compounds with a high antioxidant activity (like show the Figure 6).

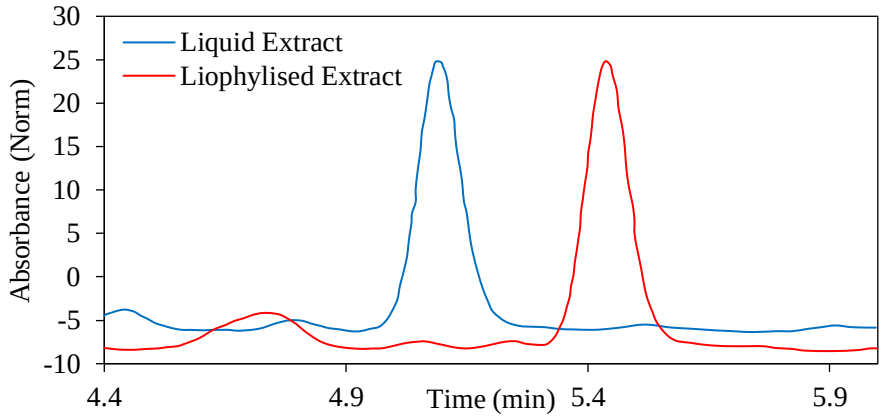


Figure 7. A comparison of the hydroxytyrosol peak in a liquid and lyophilised extract reveals that the two peaks are completely overlapping.

2.2.3 Quantification of Polyphenols with HPLC-Q-Orbitrap

Two different protocols were used: the first used dried leaves that were subsequently extracted according to Linfa Patent, and water Milli-Q acidified with 0.1% of chloride acid for the protocol; and the second method used fresh leaves that were freeze-dried and then extracted according to the protocol described for Graziani et al. [99], using MeOH/H_2O (80:20) with 0.1% of formic acid.

Through UHPLC-Q-Orbitrap-HRMS, it was possible to identify the presence of the following compounds: hydroxytyrosol glucoside, vanillic acid, coumaric acid, ferulic acid, luteolin rutinoside, verbascoside, oleuropein, oleuropein aglycone, ligstroside, luteolin, secologanoside.

A slightly higher total polyphenol is contained in the olive leaves liquid extract compared to the lyophilised, obtaining with Linfa Patent. The concentration of main compounds (such as hydroxytyrosol glucoside, oleuropein, oleuropein aglycone, verbascoside) shown in satisfactory amount to using for the next stages of the study (Table 2).

Environmental conditions during processing and storage, such as pH and temperature, affect the stability of phenolic compounds. A degradation of the compounds due to environmental conditions is to be expected and has already been reported for the storage of phenolic extracts from olive leaves [118]. However, in these cases, the leaves were dried and quickly milled for extraction. As a result, the degradation of the main compounds was minimal in liquid extract from fresh lyophilised olive leaves (as shown in Table 2).

Table 2. Comparison between fresh-lyophilised olive leaves (fresh-lyophilised OL[a]) extracted according to the Graziani et al. method; dried olive leaves liquid extract (OL liquid extract [b1]) and lyophilised extract (OL Lyophilised extract [b2]) extracted according to the Linfa Patent method.

Phenolic Compounds	Mean fresh lyophilised OL[a] (mg/g)	OL Liquid extract [b1] (mg/g)	OL Lyophilised extract [b2] (mg/g)
Hydroxytyrosol glucoside	3166	135741	21094
p-Coumaric acid	225	3068	1.4
Verbascoside	208	1139	0.7
Luteolin	17	17	0.09
Vanillic acid	0.1	4	nd
Ferulic acid	0.8	11	0.07
Oleuropein	457	384	4.7
Secologanoside	15	379	nd
Luteolin rutinoside	1	7	nd
Ligstroside	63	12	0.3
Oleuropein aglycon (DHPEA-EA)	104	930	1.4
Pinoresinol	nd	nd	nd

a: mg of polyphenols per grama of fresh-lyophilised olive leaves.
b1: mg of polyphenols per grama of dried olive leaves in liquid extract before the lyophilisation.
b2: mg of polyphenols per grama of dried olive leaves in lyophilised extract.

Hydroxytyrosol is a phenolic alcohol found in olive leaves, formed by the ring cleavage of oleuropein during endogenous or exogenous acidic or enzymatic hydrolysis. This compound is highly valued for its powerful antioxidant properties, which are present in many plant species [37, 105, 106, 115, 119-128].

Table 3. Phenolics' identification parameters.

Phenolic Compounds	RT (min)	Formula	a)	b)	c)	d)	e)
Phenolic Acids							
p-Coumaric a.	1.51	C9H8O3	163	163	0.9	119	20
Vanillic a.	4.17	C8H8O4	167	167	-4.3	152	20
Ferulic a.	11.67	C10H10O4	193	193	-4.8	178	20
Phenolic Alcohols							
OH-tyrosol glucoside		C13H18O8	301	301	-1.8	123	12
Flavonoids							
Luteolin	19.07	C15H10O6	285	285	0.7	133	35
Luteolin rut.	15.08	C27H30O15	593	593	1.7	285	30
Lignans							
(+) Pinoresinol	16.98	C20H22O6	357	357	-1.8	151	40
Derivative of hydroxycinnamic acid							
Verbascoside	14.7	C29H36O15	623	623	2.2	459	15
Secoiridoids							
Oleuropein	16.56	C25H32O13	539	539	1.7	377	15
DHPEA-EA	20.17	C19H22O8	377	377	0.6	308	15
Ligstroside	18.15	C25H32O12	523	523	-2.5	361	15
Secologanoside	10.76	C16H22O11	389	389	-1.4	178	15

a) Theoretical m/z of deprotonated molecular ions [M−H]-
b) Experimental m/z of deprotonated Errors molecular ions [M−H]-
c) Calculated Errors Δppm
d) Fragment
e) Collision Energy (eV)

The UHPLC-MS analysis suggested that the dried leaves, extracted according to Linfa Patent, present 16% hydroxytyrosol versus 1.2 % oleuropein (Table 4). This showed that this method is specific for extracting hydroxytyrosol. Furthermore, the amount of other polyphenols present in the liquid extract, obtained from dried leaves using the Linfa Patent method, was found to be significantly higher than that of the freshly freeze-dried leaves extracted

according to the methodology proposed by Graziani et al. This included the presence of additional polyphenols such as coumaric acid, verbascoside, secologanoside, oleuropein, and oleuropein aglycon [99].

Table 4. Percentage of polyphenolic compounds preserved during the freeze-drying process, in olive leaves extracted according to Linfa Patent.

Bioactive Compounds	**% recovery [Lyophilised extract]/ [Liquid extract*100]**
Hydroxytyrosol glucoside	16%
p-Coumaric acid	0.04%
Verbascoside	0.1%
Luteolin	0.5%
Vanillic acid	nd
Ferulic acid	0.6%
Oleuropein	1.2%
Secologanoside	nd
Luteolin rutinoside	nd
Ligstroside	2.5%
DHPEA-EA	0.2%
Pinoresinol	ND

2.2.4 Antioxidant Activity

The antioxidant activity (DPPH, FRAPP, ABTS) and total phenol content (Folin) of the liquid and lyophilised extract were also evaluated. The results for the extract are expressed in mmol Trolox/g for DPPH, FRAPP and ABTS, and mmol GA/g for Folin. These results were in relation to the extraction of dried olive leaves in accordance with the Linfa Patent. For the lyophilised extract, the result is reported in milligrams of lyophilised material, while for the liquid extract, it is reported in relation to the weight of dried olive leaves (Table 5).

Table 5. Antioxidant assays for olive leaves var. *Leccino* and Frantoio. The values expressed in µmol Trolox/g for DPPH, ABTS and FRAPP, and in µmol GA/g for Folin.

AOX/TFC		**FOLIN**	
sample	**mg GA /g**	**mmol GA /g**	**µmol GA /g**
Liophylized Extract (a)	49 ± 7	0.29 ± 0.04	286 ± 40
Liquid Extract (b)	36 ± 11	0.23 ± 0.07	211 ± 66
		DPPH	
sample	**mg trolox /g**	**Mmol /g**	**µmol /g**
Liophylized Extract (a)	113 ± 37	0.51 ± 0.01	505 ± 10
Liquid Extract (b)	31 ± 1	0.11 ± 0.03	125 ± 5
		FRAPP	
sample	**mg trolox /g**	**mmol /g**	**µmol /g**
Liophylized Extract (a)	30 ± 3	0.12 ± 0.01	119 ± 11
Liquid Extract (b)	63 ± 1	0.251 ± 0.004	251 ± 4
		ABTS	
sample	**mg trolox /g**	**mmol /g**	**µmol /g**
Liophylized Extract (a)	38 ± 2	0.15 ± 0.01	153 ± 10
Liquid Extract (b)	52 ± 4	0.21 ± 0.02	206 ± 16

(a) result expressed in mg or mmol or µmol of trolox or GA per grama of olive leaves Liophylized Extract

(b) result expressed in mg or mmol or µmol of trolox or GA per grama of dried olive leaves for Liquid Extract

The results of the antioxidant activity of liquid and lyophilised extracts, are in line with the previously described yield of phenolic extraction. The evaluated assays showed high antioxidant activity, with the best results observed for DPPH, followed by Folin, ABTS and FRAP.

Data obtained from the liquid extract are comparable or lower than those reported in previous literature for liquid ethanolic extracts and methanolic, and the results were expressed per grama of dry weight (DW).

The results for liquid extract are lower than those observed in previous literature for liquid methanolic extracts (MeOH 70%) [129]. DPPH results have ranged from 69.7 ± 0.2 mmol TE/g to 111.37 ± 0.2 mmol Trolox/g dry weight, ABTS results have ranged from 76.2 ± 0.9 to 109.8 ± 0.8 mmol Trolox/g dry weight, FRAP results have ranged from 51.3 ± 0.6 to 91 ± 0.5 mmol Trolox/g dry weight, and Folin results have ranged from 9.2 ± 0.5 to 16 ± 0.5 mgGAE/g dry weight [129].

When compared with the existing evidence, the TPC of the Italian samples was comparable or lower than those found in the literature for liquid ethanolic extracts with EtOH 80% [103], and EtOH 70%[130]. Folin results have ranged from 31.7 ± 2.0 mg GA/g to 41.0 ± 5.0 mg GA/g olive leaves dry weight, ABTS results have ranged from 372 ± 61 µmol Trolox/g to 591 ± 60 µmol Trolox/g olive leaves dry weight, FRAP results have ranged from 266 ± 60 µmol Trolox/g to 505 ± 124 µmol Trolox/g olive leaves dry weight, DPPH results have ranged from 168 ± 32 µmol Trolox/g to 240 ± 82 µmol Trolox/g olive leaves dry weight [103].

Similar TPC (Folin) ranges have been reported for cultivar *Leccino* and *Frantoio* olive leaves (TPC: 21.6–106.9 mg GAE/g DW [131]); and for another Italian olive leaves (7.76–8.79 mg GAE/g DW [130]). While respect Portuguese and Spanish olive leaves, the result are lower (2.31–3.78 g GAE/100g DW, and 251-353 mmolGA/Kg of dry olive leaf respectively [132, 133]). Data obtained from the lyophilised extract are comparable or lower than those reported in previous literature for liquid water extract.

Lower TPC (Folin) has been reported for cultivar *Frantoio* (TPC: 99.95 µg GAE/mg DW, [134]), while respect Spain, Turkey and Syria olive leaves results have ranged from 78.52 ± 2.18 to 102.69 ± 1.63 mgGAE/g dry weight.

Extracts produced under different extraction techniques, should show completely different qualitative phenolic composition [24, 55, 59, 103, 117, 134-140], however, the chromatographic profiles show a high levels of hydroxytyrosol (Table 2), the polyphenol of interest.

2.3 Conclusion

The patented process developed by Linfa employed a highly specific methodology for the extraction of the polyphenol hydroxytyrosol. This secondary metabolite is primarily found in olive leaf extracts. It is derived by means of acid hydrolysis, which involves the dissolution of the plant's major component, oleuropein, as previously described in some literature [116]

Due to its bioavailability, chemical properties and easy formulation along with its lack of toxicity, hydroxytyrosol is considered an excellent food supplement by the nutraceutical and food industries[141]. In a recent study, Papageorgiou et al. [142] investigated the optimal conditions for the production of an extract rich in hydroxytyrosol from OL.

A freeze-drying process was employed to preserve the polyphenolic compounds present in the liquid extract obtained from dried leaves using the Linfa Patent method. The HPLC-DAD analysis demonstrated that the process was highly effective in maintaining the integrity of these compounds, including hydroxytyrosol.

Conversely, HPLC-MS analysis revealed that the polyphenol concentration in the lyophilised extract decreased significantly following prolonged storage in a freezer. This phenomenon can be attributed to the facile oxidation of polyphenolic compounds, which is accelerated by prolonged freezing, humidity, temperature fluctuations, and solar exposure. These are normal oxidation reactions due to the lability of polyphenolic compounds.

Consequently, the extraction process developed by Linfa Patent represents an optimised approach for the extraction of hydroxytyrosol in large quantities. The freeze-drying process maintains the presence of a certain number of polyphenols, although it may require optimisation of the method of storage or utilisation of the freeze-dried extract.

Hydroxytyrosol exhibits anti-inflammatory [103, 115, 119], anti-tumour [65, 124, 126, 143, 144], antiviral, antibacterial and antifungal properties [65, 144-149]. Furthermore, it improves endothelial dysfunction, decreases oxidative stress and is neuro- and cardio-protective. Given the aforementioned biological properties, hydroxytyrosol is currently the most actively investigated natural phenol [122, 141].

The utilisation of an extract derived from leaves, which is rich in hydroxytyrosol, could represent an optimal starting point in the formulation of food products with functional characteristics. Such products include those that are enriched or fortified.

Chapter 3

Chapter 3

Characterization of the polyphenol profile and studies of bioaccessibility, PORTUGAL

The study analyzed olive leaves collected from various regions in Portugal, including Arcos de Valdevez, Mirandela, Vila Pouca de Aguiar, Braga, Vila Nova de Gaia, Buçaco, Aveiro, and Algarve. Different extraction methods will be used to determine the most effective procedure for obtaining extracts with a higher yield of compounds. The fresh leaves will undergo varying extraction steps to achieve this goal.

The study aims to compare the content of phenolic compounds extracted using ethanol:water and methanol:water mixtures. The quantification of the compounds were evaluated using HPLC. Additionally, the impact of the leaves storage procedure on the extracted compounds were evaluated. It is common practice to lyophilize leaves before the extraction process. The fresh leaves were undergo different storage and milling procedures before the extraction process, including: a) storage at -20°C followed by lyophilisation and milling; b) storage at -20°C and milling; c) storage at room temperature (dried in the dark) and milling; d) drying at 40°C and milling.

Based on the number of compounds obtained from each strategy, the most effective extraction procedure were selected. Following the methodology described in the previous section, the extracts were analysed by HPLC-DAD for oleuropein, hydroxytyrosol, rutin, caffeic acid, catechin, luteolin and pinoresinol. Additionally, the extracts will be analysed for antioxidant capacity using methods such as Folin, DPPH, ABTS, CUPRAC, and ORAC.

In order to establish a correlation between specific compounds and the antioxidant capacity profile, the number of compounds present in the extracts as determined by HPLC-DAD will be compared with the antioxidant activity data. Additionally, we will study the presence of synergistic effects. In the second stage, the phenolic compounds and antioxidant activity in the leaves are tested to determine their bioavailability and stability after exposure to gastrointestinal mimics (mimetic fluids).

3.1 Materials and methods.

3.1.1 Chemicals and Solutions

Sigma-Aldrich provided the reactive 3-hydroxytyrosol (purity ≥ 98%), (+)- catechin hydrate, oleuropein, caffeic acid (purity ≥ 98%), (+)-pinoresinol, gallic acid (purity ≥ 98%), rutin hydrate (purity ≥ 94%), quercetin (purity ≥ 95%), and verbascoside (purity ≥ 99%). Alfa Aesar GmbH & Co provided luteolin (purity ≥ 97%). Ultrapure water with a resistivity greater than 18 MΩ cm was obtained from the AriumPro system by Sartorius in Göttingen, Germany. Methanol (MeOH) and acetonitrile (ACN), both of HPLC grade by LiChrosolv, were purchased from VWR Chemicals in Radnor, PA, USA and used for HPLC and extraction solutions. Sigma-Aldrich provided formic acid (purity ≥ 95%) and dimethyl sulfoxide (DMSO, purity ≥ 99.5%). Ethanol absolute (EtOH) was obtained from Chem-Lab NV (Zedelgem, Belgium). Ultrapure water was used to prepare stock solutions of 3-hydroxytyrosol (1 mg mL^{-1}), catechin (1 mg mL^{-1}), oleuropein (1 mg mL^{-1}), and gallic acid (1 mg mL^{-1}). Stock solutions of rutin hydrate, caffeic acid, pinoresinol, and luteolin were prepared in a mixture of methanol and water (70:30, v/v) at concentrations of 1 mg/mL, 1 mg/mL, 0.3 mg/mL, and 1 mg/mL, respectively. A quercetin stock solution (1 mg/mL) was prepared in a mixture of DMSO and water (50:50, v/v). All stock solutions were stored at 4°C and protected from light. Daily working standard solutions (0.25-25 mg/L) were prepared in a mixture of methanol, acetonitrile, and 0.02% (v/v) formic acid in a ratio of 85:7.5:7.5 (v/v/v). Calibration curves were performed at 80% (v/v) methanol with 0.02% (v/v) formic acid, and 80%, 50%, and 10% (v/v) ethanol with 0.02% (v/v) formic acid for comparison purposes.

Antioxidant studies were conducted using Folin & Ciocalteu's phenol reagent, 2,2-Diphenyl-1- picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethyl-benzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, and neocuproine (Nc) hydrochloride monohydrate, copper (II) chloride

dihydrate, fluorescein sodium salt, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), ammonium acetate and potassium phosphate were acquired from Sigma. Fluka (Buchs, Switzerland) supplied sodium acetate trihydrate and sodium carbonate decahydrate.

In order to perform the bioaccessibility studies, we prepared simulated gastric and intestinal fluids in accordance with the guidelines set forth in the U.S. Pharmacopeia 38 - National Formulary 33 [150]. To prepare the gastric-simulated fluid, dissolve 0.1 g of NaCl (VWR Chemicals, Radnor, PA, USA), 0.16 g of porcine pepsin (Sigma-Aldrich, St. Louis, MO, USA), and 0.68 mg of lecithin (Sigma-Aldrich) in water up to 40 mL. Adjust the pH to 1.2 with HCl 2 M (VWR Chemicals) and complete the volume to 50 mL. The simulated intestinal fluid was created by combining the gastric digest with a phosphate-buffered solution (0.2 M, pH 6.8) made by dissolving 0.85 g of potassium phosphate dibasic (Sigma-Aldrich) and 0.69 g of potassium phosphate monobasic (Sigma-Aldrich) in 100 mL of ultrapure water, along with pancreatin (Sigma-Aldrich).

3.1.2 Olive Leaves Samples

Freshness of olive leaves from several trees in different locations in Portugal (see Table 6) were collected, dried at room temperature, and protected from light until a constant weight. In order to achieve this, 7g of each sample was separated from the pool of leaves and weighed on arriving at the laboratory. Throughout the drying process, each aliquot was weighed frequently (e.g. once a day) until no further variation in mass was recorded for three consecutive days.

The moisture content of the olive leaves was determined by measuring the loss of mass during drying. A moisture content of between 12 % and 34 % of the initial moisture content was used.

Table 6. Geographical locations of olive leaves samples.

Sample ID	City	GPS coordinates
1	Arcos de Valdevez	41° 51' 24.4" N, 8° 25' 46.7"W
2	Arcos de Valdevez	41° 51' 25.8"N, 8° 25' 46.9"W
3	Arcos de Valdevez	41° 51' 17.6"N, 8° 26' 09.9"W
4	Vila Nova de Gaia	41° 03' 52.1"N, 8° 32' 52."W
5	Braga	41° 32’ 23,66"N, 8° 23’ 34"W
6	Luso	40° 23' 15.2"N, 8° 23' 17.8"W
7	Vila Real	41° 15' 47"N, 7° 44' 5"W
8	Mirandela	41° 26' 23.8"N, 7° 17' 34.5"W
9	Cervães, Vila Verde	41°35'58.8"N 8°31'08.8"W
10	Porto	41° 08' 58" N, 8° 36' 39"W
11	Paços de Ferreira	41°14'40.1"N 8°24'12.8"W
12	Celorico de Basto	41°23'57.6"N 8°00'05.4"W

3.1.3 Preparation of Olive Leaves Extracts or (Preparation of extracts from the olive leaf)

From each sample, 20 g of dried olive leaves were collected and ground in a conventional coffee mill (Model Nevir, 230V, 130W, Alfonso Gomez, Madrid, Spain). Of the powder obtained, 0,5 g was weighed and mixed with 12.5 mL of a 50:50 (v/v) solution of EtOH:water with 0.1% (v/v) formic acid (extraction solvent C) for one minute using a vortex. The mixture was then placed in an ultrasonic bath for 15 min, followed by orbital shaking at 210 rpm, 20 °C for 20 min (incubator shaker RO 10 S000, IKA®, Staufen im Breisgau, Germany). The resulting solution was then centrifuged at 4750× g at 20°C for 20 minutes using an ALLEGRA X-15R Centrifuge from Beckman Coulter, Brea, CA, USA. The supernatant was collected in a volumetric flask. The pellet obtained from this step underwent a second extraction, and the resulting supernatant was combined with the first fraction. The final volume was adjusted to 25 mL using

a volumetric flask. Solvent A was 80:20 (v/v) MeOH:water with 0.1% (v/v) formic acid, solvent B was 80:20 (v/v) EtOH:water with 0.1% (v/v) formic acid, solvent D was 10:90 (v/v) EtOH:water with 0.1% (v/v) formic acid, and solvent E was only water with 0.1% (v/v) formic acid. Extraction solvents A, B, D, and E were used for the procedure. The procedure using 80:20 (v/v) MeOH:water with 0.1% (v/v) formic acid was only included for comparative purposes, as MeOH is not sufficient in terms of extraction greenness. After centrifugation at 18,000× g for 10 minutes at 4°C using a Microfuge 22R Centrifuge (Beckman Coulter, Landshut, Germany), every olive leaf extracts was analysed by HPLC-UV-Vis.

3.1.4 Chromatographic Conditions

The Jasco HPLC system (Easton, PA, USA) was used for chromatographic analysis. The system was equipped with a PU-2089 pump, AS-2057 autosampler, LC-Net II/ADC controller, and Jasco MD-2015 photodiode array detector. A reversed-phase Kinetex® core–shell C18 column (250 × 4.6 mm; 5 μM particle size; 100 Å) was used for injecting standards and samples (20 μL). The sample was separated using a gradient method. In this method, two solutions were mixed in-line: solution A, which was an aqueous solution containing 0.02% (v/v) formic acid (pH ≈ 3.0), and solution B, which was a mixture of 50:50 (v/v) MeOH:ACN with 0.02% (v/v) formic acid. The flow rate was set at 0.8 mL/min. Elution began with 15% of solution B and continued for 4 minutes. From 4 to 26 minutes, the percentage of solution B was increased from 15% to 60%. The organic content was increased to 40:60 (v/v) A:B and maintained for 5 minutes, followed by a return to initial conditions (31- 36 minutes) and 10 minutes of column equilibration (36-46 minutes) before the next run.

The determinations were performed at room temperature and the phenolic compounds were quantified using UV detection with spectra acquisition from 200 to 400 nm, specifically at 280, 320, and 350 nm (Figure 9).

The olive leaf-extracted polyphenols were quantified by interpolating peak area values for each compound in the calibration curve obtained with standards (refer to Table 7). The analysed extracts were fortified with a standard mixture at 5 mg L^{-1} to assess compound identification, complemented by inspection of UV spectra. The HPLC analysis of olive leaf extracts was conducted directly after centrifugation at 18,000× g for 10 minutes at 4°C (Microfuge 22R Centrifuge, Beckman Coulter, Germany). The analysis was also performed after lyophilisation using a Benchtop Freeze Dryer from Telstar, Spain, under the following conditions: These results were then compared with those before lyophilisation.

Table 7. HPLC calibration curves for standard compounds.

Standards	Slope	Intercept	R	R^2
Gallic acid	48595	-13528	0.998	0.996
Hydroxytyrosol	9724	-704	1.000	0.999
Catechin	11545	-1786	1.000	0.999
Oleuropein	4956	-1771	0.994	0.988
Pinoresinol	34957	-12450	0.997	0.994
Caffeic acid	98558	17293	1.000	1.000
Rutin	28909	6008	0.987	0.974
Quercetin	23356	-9533	1.000	0.999
Luteolin	45584	-3772	0.989	0.978

3.1.5 Antioxidant Assays: CUPRAC, FCR, DPPH, and ABTS.

Assays of antioxidant capacity were performed in a 96-well microplate reader (Synergy HT or Cytation3® microplate reader, both from Bio-Tek Instruments, Winoosky, VT, USA) controlled by Gen5 software (Bio-Tek Instruments).

Analyses were performed in triplicate or quadruplicate. Reducing and scavenging antioxidant capacity assays were performed on different extract dilutions (at least 3) as described below. Furthermore, for each method, the antioxidant capacity of standards of the phenols of interest was evaluated.

3.1.5.1 CUPRAC: Cupric-Reducing Antioxidant Capacity Assay

With minor adjustments, the CUPRAC assay was performed according to the procedure described in [151]. In brief, 50 μL of a 10 mM aqueous copper (II) solution, 50 μL of a 7.5 mM aqueous neocuproine (Nc) solution, and 50 μL of 1.0 M ammonium acetate (pH 7.0) were combined in the microplate wells to create the CUPRAC (Cu(II)-Nc) chromogenic reagent. Then, 100 μL of olive leaf extracts at different concentration levels (corresponding to 10–1000× dilution) or Trolox standard solutions (15–200 μM), both prepared in a 10% (v/v) ethanol aqueous solution, were added to the wells (4 replicates for each dilution). The antioxidant capacity was determined by measuring the reduction in Cu (II)-Nc to Cu(I)-Nc with the formation of a yellow-orange product. The measurement of absorbance values at 450 nm was carried out for 60 minutes. Instead of the sample or Trolox, 100 μL of ethanolic solution (10%, v/v) was added for blank control experiments. By interpolating the absorbance values obtained for the samples on the Trolox standard curve, the antioxidant capacity values were expressed in Trolox equivalents, as mmol Trolox g^{-1} of sample.

3.1.5.2 Folin–Ciocalteu-Reducing Assay

The Folin method, which is used to measure total phenolic content [151], was performed according to [151, 152] with some modifications. Briefly, 150 μL of sample aqueous solutions at different concentration levels (10–1000× dilution) or the gallic acid standard aqueous solution (6–90 μM) were placed in each well. Then, 50 μL of the Folin reagent was added, followed by 100 μL of the carbonate solution (9% w/v, pH ≈ 10). Reagent blank was performed by replacing sample with 150 μL water. The ability of phenolic compounds to

reduce phosphomolybdic/phosphotungstic acid reagent was monitored at 760 nm every 10 min for 2 h at room temperature. Results were expressed as mmol gallic acid per gram (mmol/g) in the sample by interpolation of absorbances measured for samples on the gallic acid calibration curve.

3.1.5.3 DPPH: 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay

The procedure described in [151]. for the microplate DPPH antioxidant assay was followed. Briefly, 150 μL of olive leaf extracts at different dilutions (10–1000× dilution) or Trolox standard solutions (9–70 μM) prepared in 50% (v/v) ethanol/water were added to the microplate wells. Then, 150 μL of the DPPH• solution (prepared at 50% v/v ethanol) at a concentration of 30–210 μM was added to achieve an initial absorbance value of ≈0.90, as previously reported [151]. The study monitored the antioxidant capacity by measuring the decrease in the purple chromogenic substrate 2,2-diphenyl-1-picrylhydrazyl (DPPH•) every 10 minutes for 120 minutes. The absorption at 515 nm was used to determine the corresponding pale-yellow hydrazine. Blank experiments were conducted using 150 μL of the ethanolic solution (50%, v/v) instead of a sample, or Trolox was used to monitor the stability of the DPPH• radical during the reaction time [151, 153, 154]. By subtracting the absorbance values obtained in the presence of Trolox from those obtained in blank experiments, net absorbance values were calculated for the samples and for Trolox. By interpolating the values obtained for the samples on the Trolox standard curve, the antioxidant capacity of the olive leaf sample extracts was expressed as mmol Trolox g^{-1} of sample.

3.1.5.4 ABTS: 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) Radical Cation Scavenging Capacity Assay

The ABTS assay was used to measure the antioxidant capacity to scavenge ABTS•+ radical, following the method described in [151, 152] with slight

modifications. The ABTS•+ radical solution was prepared by mixing equal volumes of the ABTS aqueous solution (7 mM) and potassium persulfate aqueous solution (2.45 mM) and left to stand for 12-16 hours prior to use [151]. Prior to the assay, the solution was diluted in an acetate buffer (50 mM, pH 4.6) in order to obtain 88-350 mM solutions and to establish a calibration curve. The concentration of an ABTS•+ radical solution prepared in acetate buffer to use in the assay was defined using this calibration curve. Microplate wells were filled with 150 μL of olive extract dilutions (10-1000-fold) or Trolox standards (7-50 μM) prepared in 10% (v/v) ethanol solution. Then, 150 μL of ABTS+ radical solution prepared in acetate buffer (50 mM, pH 4.6) was added to give an initial absorbance of 0.90 ± 0.02. The assessment of antioxidant capacity involved monitoring the reduction of the green-blue-coloured ABTS•+ radical to a colorless product through absorbance at 734 nm every 10 minutes for 5 hours. To monitor the absorbance of the ABTS•+ radical (blank experiment) over time, a 10% (v/v) ethanolic solution was added instead of a sample or Trolox. The net absorbance values for Trolox and olive leaf extracts were determined by subtracting the absorbance values after 5 h from those obtained in the blank experiments. Antioxidant capacities of olive leaf extracts were expressed as mmol Trolox per gram of the sample (mmol Trolox/g).

3.1.5.5 ORAC: Oxygen Radical Absorbance Capacity Assay

The ORAC assay was conducted following the methodology described in [151, 155], with some modifications. Specifically, 20 μL of olive leaf extracts at varying dilutions (10–1000×) or Trolox standard solutions (10–200 μM) were incubated with a fluorescein solution (117 μM) prepared in a phosphate buffer (75 mM, pH 7.4) for 15 minutes at 37 °C. Subsequently, 60 μL of the AAPH solution (40 mM, prepared in phosphate buffer, pH 7.4) was added to the microplate wells. Fluorescein oxidation was assessed in blank experiments without a sample/Trolox. Control experiments were also conducted to monitor fluorescein's stability and reaction time by replacing the sample/Trolox and AAPH with phosphate buffer. Fluorescence values were measured every

minute for 4 hours (240 minutes) at 37°C with λ_{exc} 485 nm and λ_{em} 528 nm. The area under the curves (fluorescence vs. time) was calculated for both Trolox standard solutions and olive leaf extracts. The antioxidant capacity values were expressed as mmol Trolox g^{-1} of the sample, using Trolox equivalents obtained by interpolating the area under the curve for the sample extracts in the Trolox standard curve.

3.1.5.6 Samples Theoretical Trolox Equivalent Antioxidant Capacity (TEAC) Values

In the study, the relative antioxidant capacity of each of the phenolic compounds was evaluated using the CUPRAC, ABTS, DPPH, ORAC and Folin assays, according to [156]. The results were compared to Trolox for the CUPRAC, ABTS, DPPH, and ORAC assays, and to gallic acid for the Folin assay. The slope of the linear responses obtained for the standard compounds was divided by the slope obtained for the Trolox Calibration Curve [156]. Experimental TEACs for each compound were multiplied by the concentration of the compound in olive leaf extracts using HPLC. Theoretical TEACs for each sample were obtained by summing all individual contributions [156].

3.1.6 Bioaccessibility Assays

The olive leaf extract powder, obtained via lyophilisation, was incubated with 5.3 mL of gastric fluid containing pepsin for 2 hours at 37°C under orbital mixing (incubator shaker RO 10 S000, IKA®, Staufen im Breisgau, Germany). A total of 35 milligrams of the powder was used. After incubation with the surrogate gastric fluid, the powder was then subjected to the intestinal fluid surrogate. This was achieved by adding 2.7 mL of the phosphate buffer (pH 6.8, 0.2 M) to the gastric digest, followed by pH adjustment to 6.8 using a 2 M NaOH (Pronalab, Lisboa, Portugal) solution. Subsequently, 80 mg of pancreatin was added to the solution, and intestinal digestion was performed for 4 hours at 37°C under orbital mixing. Before conducting HPLC analysis,

the olive leaf extract incubation result underwent a sample treatment procedure [157]. Briefly, samples were treated with ACN until an organic solvent content of 80% (v/v) was achieved. After 30 seconds of vortexing, 1.44 g of zinc sulphate was added. The mixture was then vortexed again and centrifuged (18,000× g, 10 min, 4 °C). The resulting supernatant was collected and diluted to match the mobile phase composition before HPLC analysis. Experiments were conducted to test the effects of olive leaf extract powder samples on gastric surrogate fluid. Control experiments were also performed, where gastric and intestinal simulated fluids were fortified with the polyphenols under study, including gallic acid, hydroxytyrosol, catechin, caffeic acid, verbascoside, rutin, oleuropein, pinoresinol, quercetin, and luteolin, at the same concentration levels as the extracts under analysis. A comparable experiment was also conducted, substituting the fluids with water to assess the stability of the compounds at the 37 °C temperature used during the assays.

3.1.7 Statistical Analysis

The mean ± standard deviation (S.D.) for each assay's values is presented. HPLC was used to analyse each extract in triplicate (n = 3). Each extract dilution or standard compound was analysed in quadruplicate (n = 4) for the antioxidant assays in ABTS, DPPH, CUPRAC, and Folin assays and in triplicate (n = 3) for the ORAC assay. At least two dilution factors were used to obtain values for the different antioxidant assays. A one-way ANOVA test (95% confidence level, GraphPad Prism software) was used to evaluate significant differences among the mean values when extraction was performed using different solvents. Additionally, the same methodology was used to measure antioxidant capacity for different samples.

3.2 Results

3.2.1 Selection of the Solid–liquid Extraction Solvent

The influence of the organic solvent on the extraction of gallic acid, hydroxytyrosol, catechin, oleuropein, pinoresinol, caffeic acid, verbascoside, rutin and luteolin was assessed using olive leaves from sample 1 (Table 8). The study did not assess quercetin. The compounds selected for monitoring were chosen due to their potential for incorporation into food products, including their antioxidant properties and other claimed health benefits such as anti-tumoral, hypoglycaemic, and anti-cholesterolemic effects [24].

The major compounds found in the leaves were oleuropein, hydroxytyrosol, verbascoside, luteolin, and rutin (Figure 8). The most efficient extraction of oleuropein was achieved using 80% (v/v) MeOH, resulting in 2.3 ± 0.2 mg extracted per g of leaves. This was followed by 80% (v/v) EtOH and 50% (v/v) EtOH (ANOVA, $p < 0.0001$), with 1.72 ± 0.01 and 1.61 ± 0.02 mg/g of leaves, respectively. For hydroxytyrosol (log P = 0.89), the best extraction yields were obtained using 80% (v/v) MeOH or 50% (v/v) EtOH as the extraction solvents, resulting in 1.3 ± 0.1 and 1.1 ± 0.1 mg/g of leaves (Figure 8). The highest extraction yields for verbascoside were achieved using solvents containing 80% (v/v) MeOH or 50% (v/v) EtOH, with no significant differences observed between the two solvents (ANOVA, $p < 0.0001$). Likewise, there were no significant differences in the extraction yields for luteolin and rutin when using 80% (v/v) MeOH or 50% (v/v) EtOH.

The results clearly demonstrate that extraction solvents containing ≥ 50% organic solvent are essential for achieving higher extraction yields. It is important to note that lower contents of organic solvent can result insignificant losses for hydroxytyrosol, oleuropein, verbascoside and luteolin, with losses of up to 33%, 92%, 56% and 86%, respectively.

A 50% (v/v) EtOH solution was chosen as the extraction solvent, considering the intended incorporation of the extracted compounds in food products and the achieved extraction yields. This choice represents a more environmentally friendly and safer profile compared to the use of MeOH, while still maintaining high compound extraction yields. The decision to use EtOH with confidence was based on careful consideration of all factors.

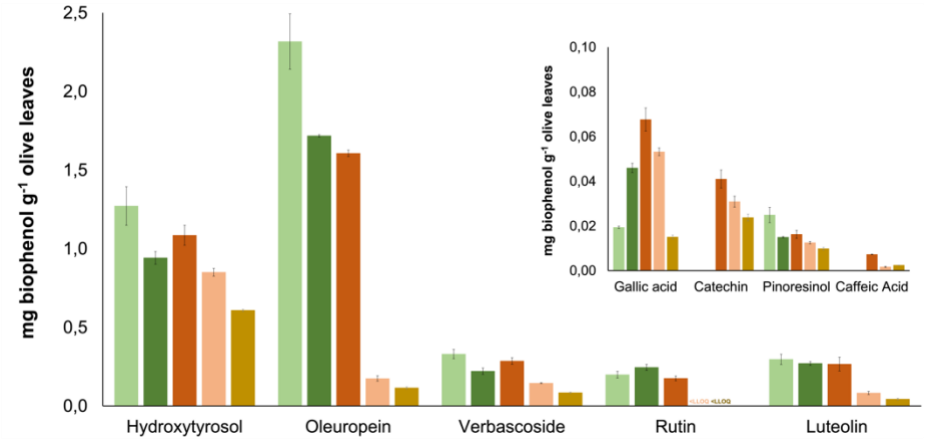


Figure 8. The study determined the number of polyphenolic compounds (mg) in 1 g of olive leaves from sample 1 using different extraction solvents. The solvents used were 80% (v/v) MeOH (light green), 80% (v/v) EtOH (dark green), 50% (v/v) EtOH (dark orange), 10% (v/v) EtOH (light orange), and 100% water (brown), each containing 0.1% (v/v) formic acid. An insert at a reduced scale shows the values for gallic acid, catechin, pinoresinol, and caffeic acid. Quercetin was not evaluated in this study.

3.2.2 Quantification of Polyphenols

The phenolic compounds in olive leaves showed variability in type and amount due to several factors, including solar exposure, soil nutrient composition, olive tree age and cultivar, climate conditions, geographic location, and agricultural practices [24]. To assess the impact of polyphenol content on antioxidant capacity values, we analysed twelve samples from various locations in Portugal (see Table 8) using the previously selected extraction solvent. The quantities and types of polyphenols found in the samples are presented in Table 8, while Figure 9 displays the chromatograms from a standard solution and sample 5.

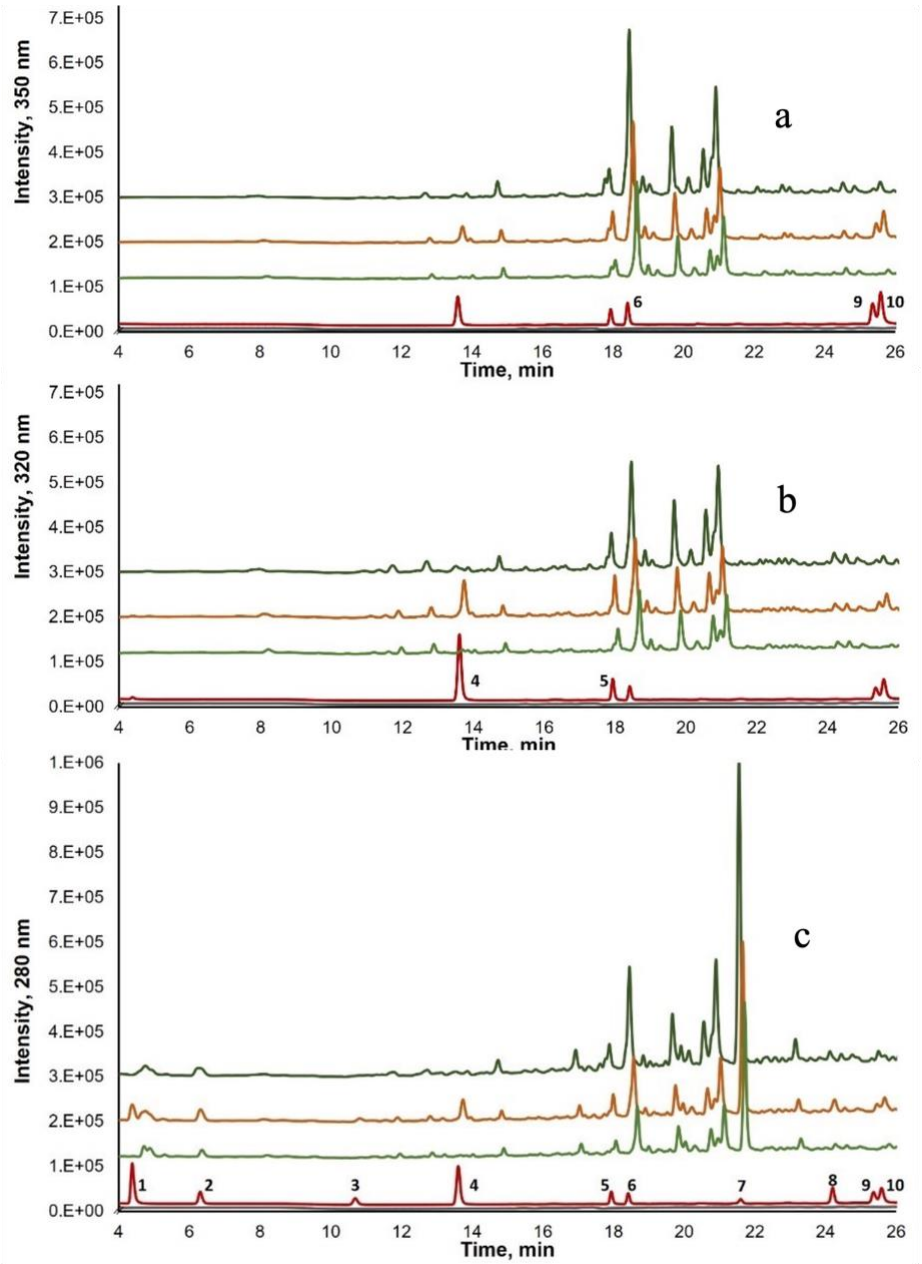


Figure 9. Chromatograms at (a) 350 nm, (b) 320 nm, and (c) 280 nm depicting: a standard solution containing 10 mg L^{-1} of the following compounds (red line): 1) gallic acid, 2)hydroxytyrosol, 3) catechin, 4) caffeic acid, 5) verbascoside, 6) rutin, 7) oleuropein, 8) pinoresinol, 9) quercetin, and 10) luteolin; 50% (v/v) EtOH extract from sample 5, undiluted (dark green line) and diluted 2× (light green line) and, for the same extract, diluted 2× supplemented with 5 mg L^{-1} of each compound (orange line). Compounds 1-3, 7, 8 were monitored at 280 nm; compounds 4 and 5 were monitored at 320 nm; compounds 6, 9, 10 were monitored at 350 nm. The grey line represents the injection of mobile phase.

The results demonstrate a clear and comprehensive understanding of the polyphenol content in the samples. The quantities and types of polyphenols found in the samples are presented in Table 8, while Figure 8 shows the chromatograms from a standard solution and sample 5.

All samples were found to contain hydroxytyrosol, catechin, rutin, oleuropein, luteolin, verbascoside, and quercetin, as indicated in Table 1. Gallic acid was present in samples 1-6 and 8, while only sample 12 did not contain pinoresinol. Caffeic acid was only present in eight out of the tested samples (samples 2, 3, 5, 6, 7, 8, 9, 11). The leaves were found to contain oleuropein as the major compound, followed by verbascoside, hydroxytyrosol, luteolin, and rutin. Gallic acid and caffeic acid were identified as the least representative polyphenols. It also had the highest levels of oleuropein and caffeic acid (approximately 29 and 0.1 mg g^{-1} olive leaves, respectively) and was the second sample with higher levels of hydroxytyrosol, verbascoside, and pinoresinol (approximately 0.9, 1.5, and 0.2 mg g^{-1} leaves, respectively).

Sample 5 contained all the quantified polyphenols and had the highest percentage of polyphenols per g^{-1} of leaves (3.26%, w/w). Likewise, sample 7 exhibited the second-highest quantity of total polyphenols (2.30%, w/w). Additionally, it demonstrated the highest levels of verbascoside (1.8 ± 0.2 mg g^{-1} leaves) and ranked second in amounts of oleuropein (19 ± 1 mg g^{-1} leaves), rutin (0.90 ± 0.07 mg g^{-1} leaves), and caffeic acid (0.08 ± 0.01 mg g^{-1} leaves).

Table 8. Mass of polyphenols (mg per 100 g of leaves) found in the liquid extracts of the different samples of olive leaves under analysis; n/d, not determined.

BC	S1	S2	S3	S4	S5	S6
Gallic Acid	3.2 ± 0.1	1.6 ± 0.2	2.03 ± 0.05	2.2 ± 0.3	3.3 ± 0.1	3.57 ± 0.05
Hydroxytyrosol	22 ± 3	25.1 ± 0.3	27 ± 2	21 ± 2	86 ± 8	56 ± 6
Catechin	1.9 ± 0.1	4.8 ± 0.2	7 ± 1	9 ± 1	4.1 ± 0.4	6 ± 1
Oleuropein	164 ± 22	583 ± 15	131 ± 8	71 ± 6	2894 ± 214	103 ± 9
Pinoresinol	8.9 ± 0.4	5.3 ± 0.7	3.4 ± 0.1	29 ± 4	22 ± 2	10.1 ± 0.1
Caffeic Acid	n/d	3.04 ± 0.02	2.72 ± 0.01	n/d	9 ± 1	0.39 ± 0.01
Rutin	12 ± 2	35 ± 3	52.6 ± 0.2	63 ± 3	52 ± 5	18 ± 1
Quercetin	3.2 ± 0.2	12.6 ± 0.3	10 ± 1	35 ± 3	12 ± 2	10 ± 1
Luteolin	32 ± 2	70 ± 2	78 ± 3	96 ± 6	25 ± 1	40 ± 2
Verbascoside	14 ± 2	13 ± 1	7.8 ± 0.4	52 ± 3	150 ± 13	106 ± 11

BC	S7	S8	S9	S10	S11	S12
Gallic Acid	n/d	0.5 ± 0.1	n/d	n/d	n/d	n/d
Hydroxytyrosol	70 ± 5	146 ± 12	62 ± 8	49 ± 4	84 ± 3	13 ± 1
Catechin	22 ± 2	25.48 ± 0.01	3.7 ± 0.3	4.4 ± 0.1	15.5 ± 0.1	8.1 ± 0.5
Oleuropein	1852 ± 141	474 ± 56	1040 ± 131	135 ± 17	518 ± 40	165 ± 12
Pinoresinol	8 ± 1	6 ± 1	7.4 ± 0.2	1.8 ± 0.1	3.3 ± 0.2	n/d
Caffeic Acid	8 ± 1	0.6 ± 0.1	2.1 ± 0.2	n/d	0.58 ± 0.05	n/d
Rutin	90 ± 7	101 ± 12	31 ± 4	17.3 ± 2.1	67 ± 6	20 ± 2
Quercetin	19 ± 2	26 ± 2	10 ± 1	10.2 ± 1.1	17 ± 2	3.8 ± 0.4
Luteolin	58 ± 8	63 ± 4	28 ± 2	30 ± 3	62 ± 5	6 ± 1
Verbascoside	183 ± 21	67 ± 7	66 ± 7	67 ± 7	77 ± 5	3.3 ± 0.1

Sample 8 had the highest levels of hydroxytyrosol (1.5 ± 0.1 mg g^{-1} leaves) and rutin (1.0 ± 0.1 mg g^{-1} leaves) and was the second highest in quercetin (0.3 ± 0.01 mg g^{-1} leaves). This sample presented 0.91% (w/w) of polyphenols. Notably, samples 5, 7, 8 and 9 had higher contents of polyphenols, including

hydroxytyrosol, oleuropein, verbascoside, rutin and quercetin. These were also within the top 4 samples with the highest quantities of total polyphenols per g^{-1} of the leaves (sample 5 > sample 7 > sample 9 > sample 8), Figure 10.

The increased polyphenol content in these samples is not attributed to geographic location or climate, but rather to other factors such as olive tree variety (cultivar), age, soil composition, and agricultural practices.

Samples 7 and 8 were collected from locations closer to samples 11 and 12 (refer to Table 6). Despite the proximity, the polyphenolic content of samples 7 and 8 varied significantly from that of samples 11 and 12, indicating that geographical location is not a significant factor for these samples.

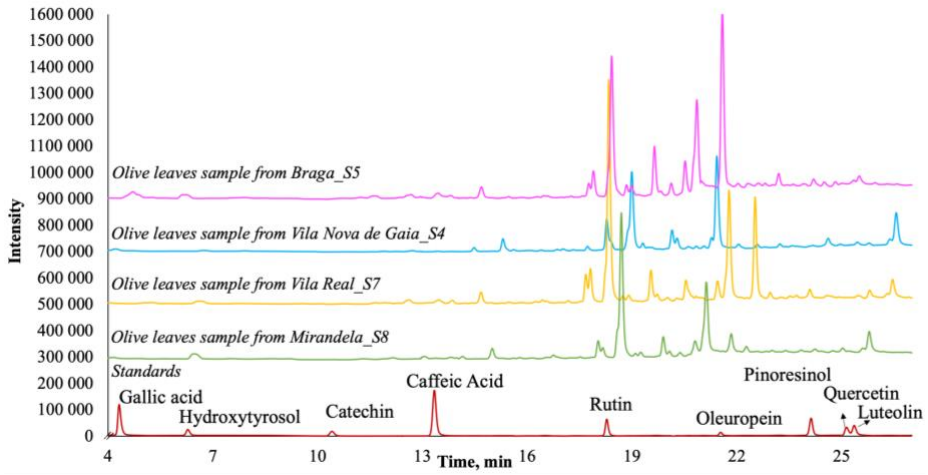


Figure 10. A chromatogram is presented for liquid extracts that are richest in polyphenols (S4, S5, S7, and S8). The red line represents the standard polyphenol. The highest peaks are observed for rutin and oleuropein, which are the most abundant polyphenols in the extracts.

3.2.3 Antioxidant Activity

Various antioxidant capacity assays were used to evaluate the antioxidant capacity of each sample (refer to Table 9). The Folin, CUPRAC, ABTS, and DPPH antioxidant capacity assays transfer an electron from the antioxidant species to the spectrophotometric probe/chromogenic reagent (in the case of Folin and CUPRAC) or to the coloured radical reagent (in the case of ABTS and DPPH) [152].

Table 9. Antioxidant capacity values determined for the extracts of the different samples under analysis using each methodology**.**

	FOLIN [a]	**DPPH** [b]	**ABTS** [b]	**CUPRAC** [b]	**ORAC** [b]
S1	0.18 ± 0.03	0.11 ± 0.01	0.28 ± 0.03	0.30 ± 0.01	0.9 ± 0.1
S2	0.31 ± 0.05	0.17 ± 0.02	0.37 ± 0.05	0.43 ± 0.06	0.22 ± 0.04
S3	0.24 ± 0.03	0.14 ± 0.02	0.36 ± 0.04	0.31 ± 0.02	0.53 ± 0.09
S4	0.22 ± 0.01	0.09 ± 0.01	0.25 ± 0.04	0.27 ± 0.01	1.4 ± 0.2
S5	0.38 ± 0.05	0.19 ± 0.01	0.51 ± 0.05	0.52 ± 0.04	0.9 ± 0.1
S6	0.15 ± 0.01	0.06 ± 0.01	0.19 ± 0.02	0.22 ± 0.01	1.0 ± 0.1
S7	0.33 ± 0.04	0.16 ± 0.02	0.46 ± 0.02	0.43 ± 0.02	0.61 ± 0.07
S8	0.22 ± 0.01	0.12 ± 0.01	0.31 ± 0.04	0.36 ± 0.02	1.8 ± 0.1
S9	0.28 ± 0.01	0.15 ± 0.01	0.35 ± 0.02	0.35 ± 0.01	0.78 ± 0.08
S10	0.18 ± 0.02	0.16 ± 0.02	0.23 ± 0.02	0.22 ± 0.01	0.25 ± 0.05
S11	0.31 ± 0.04	0.16 ± 0.01	0.41 ± 0.04	0.42 ± 0.02	0.9 ± 0.1
S12	0.18 ± 0.01	0.142 ± 0.004	0.21 ± 0.01	0.25 ± 0.01	0.27 ± 0.01

a Values expressed as mmol of gallic acid g^{-1} sample; b Values expressed as mmol Trolox g^{-1} sample.

Samples 2, 5, 7, and 11 were the top four samples with the highest antioxidant capacity values for these assays. Notably, sample 5 had the highest quantity of total polyphenols (Table 8) and demonstrated significantly superior antioxidant capacity values ($p < 0.05$, one-way ANOVA) compared to the other three samples (Figure 11). Among the top 4 samples, sample 7 showed significantly

higher antioxidant capacity values for the ABTS assay ($p = 0.0392$, one-way ANOVA) compared to sample 2 and sample 11, despite having the second highest quantity of total polyphenols.

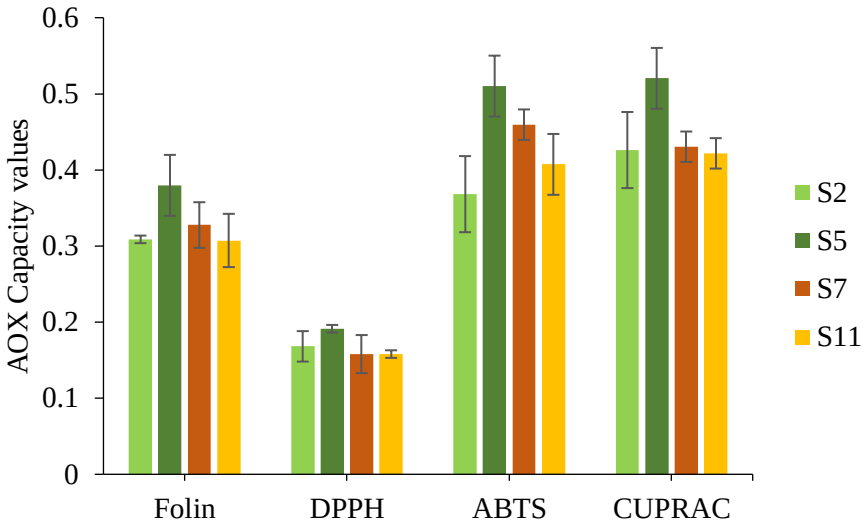


Figure 11. Antioxidant capacity values (mean ± SD) for the samples 2, 5 7 and 11 as determined by Folin, DPPH, ABTS and CUPRAC methods.

Sample 5 has the highest quantity of polyphenols among all the samples (32.6 mg per g^{-1} olive leaves). However, the determined antioxidant capacity values do not correlate linearly with the total quantity of polyphenols. Despite having a total amount of polyphenols approximately 4 times higher than sample 2, the latter only presented slightly lower antioxidant capacity for the Folin, CUPRAC, ABTS, and DPPH assays.

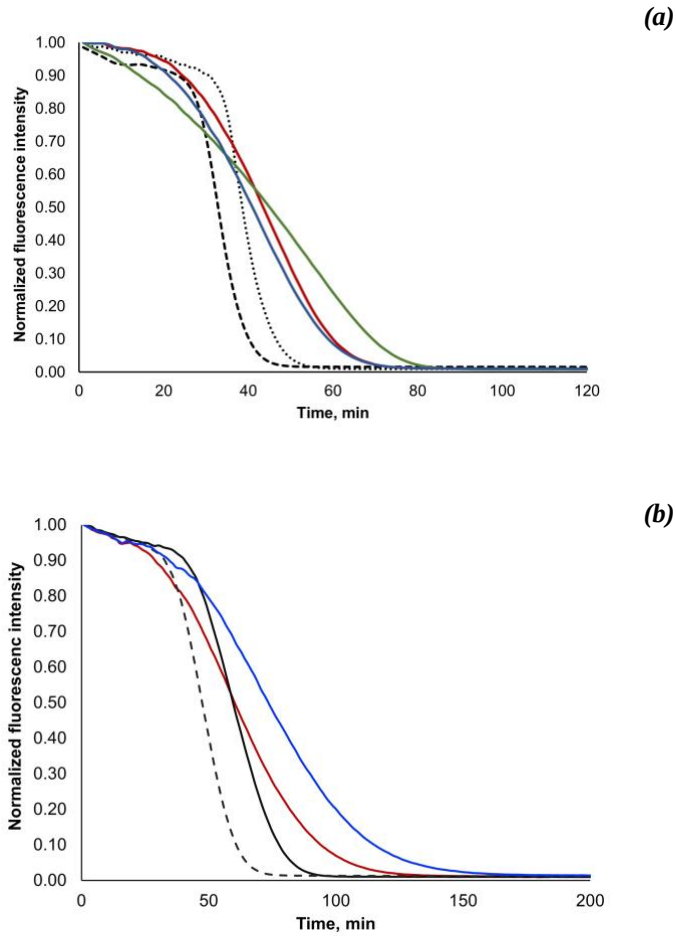


Figure 12. ORAC curves obtained for **(a)** the standard antioxidant compounds oleuropein (10 μM, dashed black line), hydroxytyrosol (8 μM, dotted black line), rutin (10 μM, solid red line), luteolin (10 μM, solid blue line) and quercetin (9 μM, solid green line) and for **(b)** samples 4 (solid red line), 5 (solid black line), 7 (dashed black line) and 8 (solid blue line) analyzed at the same dilution.

ORAC is a method of measuring antioxidative protection against oxidation by bio-relevant free radicals through sequential proton-loss electron transfer [151, 155, 158].

Antioxidant species constantly tackle the generated radicals until depletion [155] ,after which the radicals oxidize the fluorescent probe, resulting in photobleaching. The ORAC methodology evaluates antioxidant capacity values based on the stoichiometry and reactivity of antioxidant compounds, including their kinetic profile. This approach differs from other antioxidant methodologies used in this study. Furthermore, the formation of by-products resulting from the reaction of antioxidants with radicals also contributes to the measured values [155].

In ORAC assays, the total quantity of polyphenols is not a reliable predictor of antioxidant capacity. Samples 5 and 7, despite having markedly higher quantities of total polyphenols, did not exhibit higher antioxidant capacity values, while samples 4 and 8 did.

Sample 4 and sample 8 had higher levels of several compounds compared to sample 5, including pinoresinol, rutin, quercetin, luteolin, verbascoside, hydroxytyrosol, and catechin (Table 1). These differences likely contributed to the observed results. The antioxidant capacity values of different compounds vary depending on the methodology used for the antioxidant capacity assay. This fact was demonstrated in Table 10, where the TEAC values for each standard compound were assessed using different antioxidant capacity assays. It is expected that there will be differences in relation to the values assessed by Folin, CU-PRAC, ABTS, and DPPH assays when compared to ORAC [151]. This is because ORAC operates through different antioxidant capacity mechanisms in comparison to these assays. Additionally, the importance of antioxidant kinetic aspects and formation of antioxidant by-products for this methodology [155] further contributes to the expected differences.

Table 10. TEAC values determined by each methodology for the biophenolic compounds under analysis. Folin expressed in mmol*g AG and DPPH, ABTS, CUPRAC, ORAC expressed in mmol*g Trolox.

BC	Folin[a]	CUPRAC[b]	ABTS[b]	DPPH[b]	ORAC[b]
GA	1.00[a]	2.0 ± 0.1	4.9 ± 0.1	5.3 ± 0.5	1.9 ± 0.1
HTYR	0.92 ± 0.01	1.60 ± 0.04	1.50 ± 0.01	0.99 ± 0.04	6.1 ± 1.4
CAT	1.58 ± 0.01	2.39 ± 0.04	4.3 ± 0.1	3.3 ± 0.1	8.9 ± 0.6
CA	0.98 ± 0.01	1.11 ± 0.03	2.11 ± 0.02	1.04 ± 0.03	6.5 ± 0.8
RUT	1.57 ± 0.01	1.9 ± 0.1	2.55 ± 0.03	2.1 ± 0.2	10.2 ± 0.8
OLEU	0.99 ± 0.01	2.07 ± 0.03	1.32 ± 0.02	0.99 ± 0.04	5.6 ± 0.2
PINO	1.19 ± 0.01	1.19 ± 0.03	2.6 ± 0.1	1.1 ± 0.1	8.3 ± 1.2
QUER	2.0 ± 0.1	1.64 ± 0.02	3.3 ± 0.1	2.4 ± 0.1	10.8 ± 1.6
LUT	2.71 ± 0.02	2.08 ± 0.04	3.43 ± 0.06	2.0 ± 0.1	9.5 ± 1.25
VB	1.9 ± 0.1	4.2 ± 0.1	2.48 ± 0.04	2.09 ± 0.08	5.7 ± 0.18

Results are presented as the mean ± error (the last calculated by propagation error equation). [a] Expressed in relation to gallic acid (mmol*g AG). [b] Expressed in relation to Trolox (mmol*g Trolox).

Quercetin is a slow-reacting antioxidant compound, as previously identified in the ORAC assay [155]. The ORAC assay curve does not exhibit a lag phase, which is typically observed when fast-reacting antioxidant species interact with AAPH-generated radicals to protect fluorescein fluorescence decay. Instead, the fluorescence probe signal decays in a tailed manner, indicating a slow- acting antioxidant mechanism. Samples 4 and 8 exhibit an ORAC profile similar to that of quercetin (Figure 12.a), as do rutin and luteolin, indicating that these compounds also possess a slow-reacting antioxidant mechanism (Figure 12.a).

Opposite ORAC profiles were obtained for oleuropein and hydroxytyrosol (Figure 12.a). They presented a clearly defined lag phase followed by faster fluorescein photobleaching.

The increased content of quercetin, luteolin, and rutin in samples 4 and 8 may justify the higher antioxidant capacity values for these samples under the

ORAC methodology compared to samples 5 and 7. Samples 4 and 8 exhibited ORAC curve profiles with shorter lag phases and a more gradual decay of fluorescent probe signals [155] compared to those shown in Figure 12.b.

To determine the predictability of the antioxidant capacity for each methodology based on the polyphenolic content, the TEAC value for each compound was multiplied by its concentration in the olive leaf samples. This calculation was performed using the details provided in the materials and methods section. The TEAC value determined experimentally for each sample was then compared to the theoretical value across different antioxidant methodologies. This was done to explore the correlations between polyphenolic quantity and TEAC values with antioxidant capacity, as well as to evaluate possible synergetic antioxidant effects.

The theoretical TEAC values, which are calculated by summing the individual contributions of the assessed polyphenols, were found to be lower than the experimentally determined values (data not shown). Since not all compounds present in olive leaves are quantified, this was expected. In addition, the presence of different antioxidative compounds could promote synergism, with slow-reacting compounds regenerating the antioxidative capacity of other sample constituents [155].

Samples 4 and 8 exhibited lower total theoretical TEAC values compared to sample 5 and other antioxidant capacity assays (data not shown). The ORAC assay showed the most significant differences between theoretical and experimental TEAC values for these samples, with experimental values 9 to 16 times higher than theoretical values. The increased antioxidant protection of samples 4 and 8 under the ORAC methodology could not be predicted based solely on the TEAC values of individual compounds and their concentration.

It is important to comprehend the chemistry underlying each polyphenolic compound's antioxidant mechanism and its impact on the overall antioxidant protection provided. The findings demonstrate the significance of conducting

antioxidant research in conjunction with quantitative analysis of polyphenols when selecting samples for food enrichment purposes. For instance, Sample 5, is the most relevant for obtaining a greater amount of total polyphenolic compounds for future incorporation into foods (Table 8).

Table 9 demonstrates that this sample exhibits higher antioxidant capacity values in assays involving reducing and radical scavenging electron-transfer mechanisms. Notably, samples 4 and 8, which had higher contents of slow-reacting antioxidants, showed interesting antioxidant capacities in more complex environments where the antioxidant capacity was measured against biologically relevant radicals and where the formation of by-products with antioxidant activity could occur. The varying O-H dissociation proton affinity and electron-transfer enthalpies of different olive leaf polyphenols have a significant impact on antioxidant protection mechanisms and, consequently, on the antioxidant capacity measured by different methods [159-161].

Therefore, when selecting extracts for food fortification, it is important to consider the specific purposes for which they are to be used, as some compounds may be more relevant to food stability during storage, while others may have a higher antioxidant capacity in biological media.

3.2.4 Stability of Lyophilisation Process and Bioaccessibility

The liquid extract from sample 5 was chosen to produce powder via lyophilisation due to its high content of total polyphenols, oleuropein, and antioxidant activity as determined by Folin, CUPRAC, ABTS, and DPPH. It is important to note that this extract will be incorporated into food products in the future. The 25 mL ethanolic extract was obtained from 0.5 g of dried olive leaves, yielding approximately 110 mg of powder, which represents 20% of the mass of leaves used.

The results obtained were comparable to those of Sample 7, which exhibited the second-highest quantity of total polyphenols, with 149 mg of powder being

recovered. The dried extracts were assessed for the content of hydroxytyrosol, oleuropein, pinoresinol, verbascoside, rutin, quercetin, and luteolin (Table 11), revealing higher quantities of polyphenols per gram of dried extract than those described in the literature [51]**.** Oleuropein represented 84% and 14% of the mass in the dried extract obtained from samples 5 and 7, respectively.

Table 11. Quantity of polyphenolic compounds (mg) found per g of the dried extract.

Bioactive Compounds	**Sample 5**	**Sample 7**
Hydroxytyrosol	14.4 ± 0.1	5.5 ± 0.3
Oleuropein	837 ± 1	135 ± 3
Pinoresinol	7.2 ± 0.1	1.93 ± 0.2
Verbascoside	21.7 ± 0.1	6.4 ± 0.1
Rutin	15.1± 0.1	8.2± 0.3
Quercetin	3.30 ± 0.01	2.0 ± 0.1
Luteolin	4.1 ± 0.1	4.9 ± 0.1

However, the lyophilisation process used for the liquid extracts has affected the quantity of polyphenols. The content of polyphenols for all the assessed compounds (Table 12) in both samples has decreased. Therefore, it may be necessary to optimize the lyophilisation procedure for industrial applications.

The aim of incorporating olive leaf-extracted polyphenols in food products is a future goal. To assess the bioaccessibility of oleuropein, the major compound present in the lyophilised powder, assays were conducted. These assays unveil the portion of oleuropein capable of being released from the powder for intestinal absorption [51].

The lyophilised powder was incubated with surrogate gastric fluid (pH 1.2) containing pepsin for 2 hours at 37°C. Similarly, analysis was conducted after the powder was incubated with intestinal surrogate fluid (pH = 6.8, containing pancreatin, 4 h at 37 °C). In sample 5, approximately 50% of the oleuropein present in the powder was detected in the gastric surrogate fluid (Figure 13).

Table 12. Content of biophenols (mg) per g of olive leaves determined in liquid extracts before and upon lyophilisation.

	Sample 5		Sample 7	
BC	**Liquid extract**	**Lyophilised powder**	**Liquid extract**	**Lyophilised powder**
Hydroxytyrosol	7.4 ± 0.7	2.9 ± 0.1	6.4 ± 0.3	1.6 ± 0.1
Oleuropein	485 ± 56	167 ± 1	202 ± 6	40 ± 1
Pinoresinol	3.6 ± 0.3	1.45 ± 0.01	2.3 ± 0.2	0.58 ± 0.01
Verbascoside	11.6 ± 0,3	4.35 ± 0.02	7.5 ± 0.5	1.88 ± 0.01
Rutin	5.0 ± 0.5	3.0 ± 0.1	4.4 ± 0.4	2.5 ± 0.1
Quercetin	1.4 ± 0.1	0.70 ± 0.01	1.2 ± 0.1	0.59 ± 0.1
Luteolin	1.7 ± 0.1	0.83 ± 0.03	4.9 ± 0.2	1.45 ± 0.04

The content of determined oleuropein was not significantly affected by the subsequent incubation with intestinal surrogate fluid. For sample 7, approximately 87% of the oleuropein in the lyophilised powder was found in the gastric surrogate fluid (Figure 13). In sample 5, the content of oleuropein was not significantly affected by further incubation with intestinal fluid.

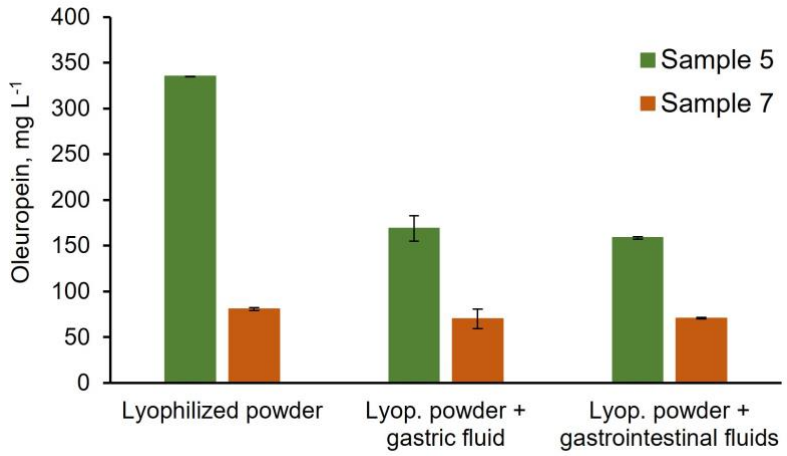
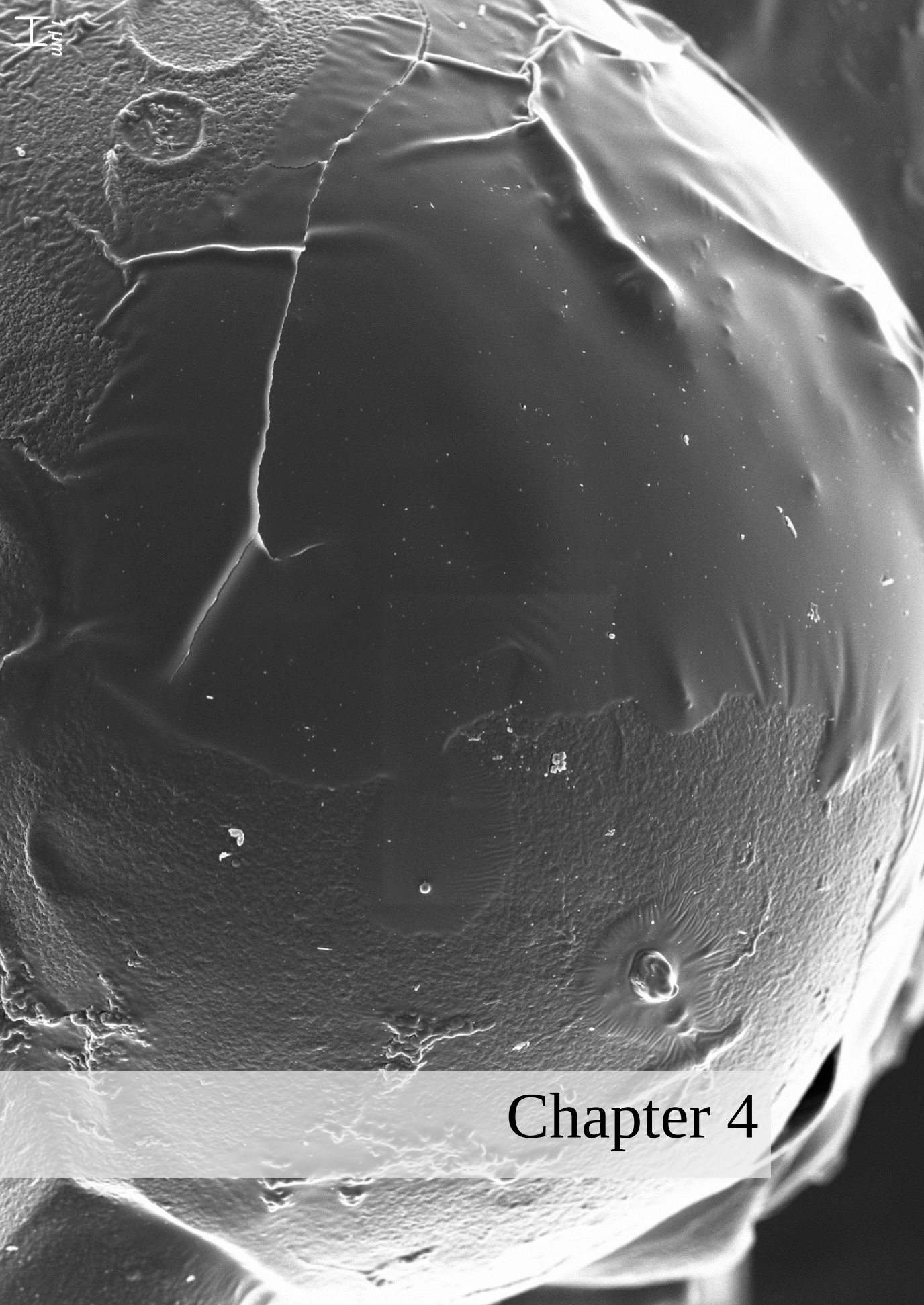


Figure 13. The concentration (mg L−1) of oleuropein in the lyophilised powder was measured before and after incubation with gastrointestinal surrogate fluids (left panel vs. the concentration found upon incubation with (i) gastric fluid (middle panel) and (ii) gastric fluid followed by intestinal fluid (right panel) for samples 5 and 7, respectively.

The study indicates that exposure to gastric fluid has a negative impact on the release of oleuropein from the powder. There were no significant differences in the levels of oleuropein observed after gastric-simulated digestion and after gastric+intestinal-simulated digestion for both samples. The results also suggest that oleuropein is stable in intestinal surrogate fluid. Furthermore, there was a difference in the amount of oleuropein released from the matrix during simulated gastric digestion between samples 5 and 7. This difference could be attributed to the distinct oleuropein release kinetics from the powder, which may be due to differences in the composition of samples 5 and 7 [162]. As different bioaccessibility values have been reported for different oleuropein isomers, another possible explanation is that samples 5 and 7 contain different amounts of oleuropein isomers [51].

3.3 Conclusion

The HPLC-UV-Vis method was used to evaluate the presence of 10 polyphenolic compounds, namely gallic acid, hydroxytyrosol, catechin, oleuropein, pinoresinol, caffeic acid, rutin, quercetin, luteolin and verbascoside, in olive leaves collected from 12 different locations in northern Portugal. The choice of solvent used for extracting these compounds was found to affect the levels of hydroxytyrosol, oleuropein, verbascoside, and luteolin. Extraction solvents containing 50% or more organic solvent resulted in higher yields of these compounds. A 50% (v/v) ethanolic solution was found to be a suitable compromise for extracting polyphenolic compounds and for future food fortification with the extracted compounds. In addition to quantitatively assessing polyphenolic compounds in the samples, we evaluated their antioxidant capacity using various methodologies. This approach was taken because different methods provide distinct information about antioxidant mechanisms and capacity. While these assays confirmed the significance of total polyphenols in determining antioxidant capacity, as measured by Folin, CUPRAC, ABTS, and DPPH assays, the results obtained through the ORAC methodology highlighted the importance of compounds with slow-reacting antioxidant mechanisms that produce by-products with antioxidant capacity in environments similar to biological ones. Furthermore, the study evaluated the feasibility of utilising extracted compounds from olive leaf waste for lyophilisation and intestinal absorption, emphasizing crucial characteristics and optimisation requirements for food fortification purposes. Further research is necessary to assess the effect of extracted compounds on food stability during storage and their potential benefits for the development of functional foods. Moreover, the use of olive leaf-extracted compounds in specific food processes, such as emulsification and encapsulation, warrants further investigation.



Chapter 4

Chapter 4

Development of functional food: Goat Yoghurt Enriched with Picual Olive Leaves, SPAIN

Olive trees are one of the main crops in the European countries of the Mediterranean basin. In this sense, olive oil production has held significant influence over the history, economy and culture of Western civilization. The association of its consumption with numerous positive effects on human health linked with the benefits of the Mediterranean diet, has led to its increased consumption. Among the Mediterranean countries, Spanish production involves a high percentage of olive-oil are produced worldwide. Olive cultivation covers an area of 2.75 million hectares, which accounts for 70% of EU production and 45% of global production [9, 12].

As a consequence of the increased demand and production, vast quantities of derived residues are produced, being their accumulation a pressing environmental issue. Olive leaves, an abundant waste product of tree-pruning, stand out as one of the primary contributors to this waste generation.

The conventional method for their removal has entailed the grinding and incineration of these by-products, with a derived severe environmental impact. Thus, the optimal management of these wastes has gained interest in the scientific community, with a great interest in the development of a circular economy [52, 163].

In this sense, olive leaves are presented as an unexploited and novel source of bioactive compounds, with both interesting technological and health properties, as they have been traditionally used for medicinal applications in folk medicine. The effects have been related to its high content in phenolic compounds, including phenolic alcohols, phenolic acids, flavonoids and secoiridoids, being oleuropein its main representative. In relation to its bioactive composition, olive leave extracts have been related to a variety of biological activities such as antioxidant, anti-inflammatory, anti-microbial and antiviral as well as anti-atherogenic, anti-cancer, hypolipidemic and hypoglycemic effects and has had an impact on several intestinal pathologies as related to a modulatory effect on intestinal microbiota due to their prebiotic effect. Additionally, extracts from these by-products have also been applied in the cosmetic and food industry, as additives to increase food shelf-life and safety [164]. Therefore, its revalorization as a potential source of bioactive molecules could lead to the obtention of high added-added value products, boosting the industry, and reducing the environmental impact derived of their accumulation [165, 166].

Given their bioactive properties, one of the main areas of potential applications is in their incorporation to functional foods, in which phenolic compounds could serve both health and technological purposes. In this sense, fermented products such as dairy products have surged as some of the most interesting incorporating matrices due to the potential interaction of these bioactive compounds with the inherently present probiotic strains. However, although highly interesting, incorporation of olive leaf extracts into raw materials to produce functional foods is underexplored. In this sense, incorporation of these bioactive compounds in early stages of industrial production may be detrimental, as phenolic compounds are highly unstable under environmental and processing conditions, being sensitive to both light, pH and high temperatures. Additionally, interaction between the addition of olive leaf phenolic compounds and microbial strains present in yoghurt is still scarce [93].

Encapsulation techniques have been developed as alternative delivery strategies allowing for the protection of phenolic compounds during processing before their introduction into a food matrix. This delivery system can allow for the incorporation of bioactive compounds without altering their attributes, its protection from external conditions, and a controlled release which may positively influence the food matrix in which it is introduced. In this sense, alginate-based ionic gelation encapsulation systems present an incredible potential as this polysaccharide has been widely used in the food industry and its simplicity. Sodium alginate is a polymer widely used as a microencapsulating agent, since it has a high affinity for water, forming a homogeneous, non-toxic and biodegradable gel [86]. In addition, it provides a prebiotic effect if ingested and also acts as dietary fiber, reducing blood glucose and cholesterol [70] .

Therefore, the aim of this study is the obtention of an olive-leaf phenolic rich extract and its encapsulation through alginate-in-oil emulsion coupled with internal ionic gelation for their later incorporation into the development of a functionalized yoghurt, with the intention of evaluating the potential of this encapsulation strategy on the development of a functionalized food product.

4.1 Materials and methods

Figure 14 show the experimental design, starting with the collection of the samples, following with the solid–liquid extraction, lyophilisation and encapsulation through alginate-in-oil emulsion coupled with ionic gelation were proposed for developing functional ingredients. Validation of the microencapsulated formulation was carried out via incorporation into the goat yoghurt production process with the intention of evaluating the potential of this encapsulation strategy on the development of a functionalized food product. Additionally, the effect of the inclusion of the obtained microparticles into the micronutrient content of the functional yoghurt was evaluated.

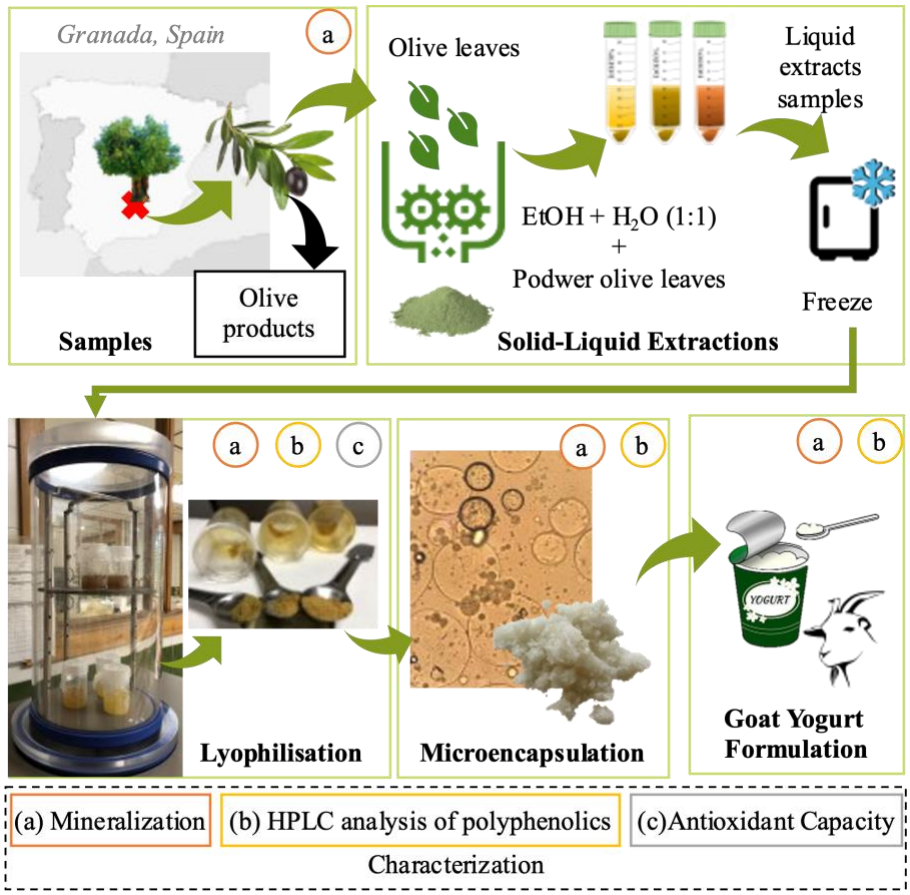


Figure 14. A schematic illustration of the experimental design employed for the olive leaves from Spain.

4.1.1 Chemicals and Solutions.

All reagents and solvents were of analytical or HPLC grade. Standards of gallic acid, 3-hydroxytyrosol, oleuropein, luteolin, rutin hydrate, p-coumarin, and diosmin was provided from Sigma-Aldrich SL (Madrid, Spain); verbascoside was acquired from PhytoLab GmbH (Vestenbergsgreuth, Germany); ferulic acid, and chlorogenic acid (purity ≥ 94%) were obtained from Fluka Chemicals (Buchs, Switzerland).

For phenolic extraction, absolute ethanol (EtOH) was provided from JT Baker (Gliwice, Poland), formic acid, hydrochloric acid (37% w/w) were purchased from Panreac (Barcelona, Spain), and ultrapure water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

Acetonitrile UHPLC-MS (purity ≥ 99.9%), methanol UHPLC-MS (purity ≥ 99.9 %) and formic acid (purity ≥ 98-100%) were provided from Scharlau (Sentmenat, Spain), and used in the preparation of HPLC-MS analysis.

For microparticles preparation, the following reagents were provided by the mentioned suppliers: glacial acetic acid (CH_3COOH) from VWR Chemicals BDH (Milan, Italy), sodium alginate from Fagron Ibérica S. A. (Barcelona, Spain), soya oil from GUINAMA (Valencia, Spain); SPAN 80, calcium chloride ($CaCl_2$) and calcium Carbonate ($CaCO_3$) from Sigma-Aldrich (MO, USA).

Concerning the preparation of the yoghurt commercial semi-skimmed goat (caprine) milk was used, yoghurt cultures YO-MIX 205 LYO 250 DCU were obtained from Danisco (Dange-Saint-Romain, France), microparticles with olive leaves extract was prepared in the laboratory of Pharmaceutical Technology Department (Faculty of Pharmacy, Granada University).

Additionally, ethyl acetate and hexene were provided from Panreac Quimica SL (Barcelona, Spain), and sodium sulphate from by Honeywell (Seelze, Germany) for phenolic extraction from this matrix (yoghurt).

Regarding antioxidant capacity assays, Trolox standard (6- hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate)], TPTZ (2,4,6-tripyridyl-S-triazine) used for the ferric iron (FRAP), were all supplied by Sigma-Aldrich SL (Madrid, Spain); potassium peroxodisulphate ($K_2S_2O_8$), sodium acetate trihydrate, sodium carbonate anhydrous (Na_2CO_3), glacial acetic acid, ferric chloride 6-hydrate were supplied by Panreac Quimica SL (Barcelona, Spain).

Folin- Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany), and the anhydrous sodium carbonate (Na_2CO_3) used to determine the total phenolic components (TPC) was obtained from Panreac Quimica SL (Barcelona, Spain).

For mineral analysis, certified single-element standards provided by Perkin-Elmer (Perkin Elmer, Inc., Waltham, USA) were used for calibration line and nitric acid was provided from Honeywell (Seelze, Germany). The samples dried olive leaves, lyophilised olive leaves extract, commercial semi-skimmed goat (caprine) milk supplement with microparticles, microparticles with olive leaves extract were analysed by Inductively Coupled Plasma Optical Emission Spectroscopy ICP-OES (Perkin Elmer, Optima 8300 model, Waltham, USA) and by Inductively Coupled Plasma Optical Emission Mass Spectrometry ICP-OES-MS (Perkin Elmer, Inc., Waltham, USA). Sample microwave digester equipment is Multiwave 5000HZ PACKAGE24HVT50 (Anton Paar, Graz, Austria).

4.1.2 Olive Leaves Samples.

In March 2022, fresh olive leaves rich in polyphenols were collected from more than 30 trees (*Olea Europaea L. Sativa Picual* variety) in the Granada region of Spain. The leaves were dried at room temperature for 2-3 days in a cool, non-humid place away from sources of light or heat to remove water and concentrate the compounds present inside. Prior to extraction, the dried olive leaves (DOL) were ground into a powder using a conventional coffee mill. The leaves were then stored for 2 months to analyze the evolution of phenolic compounds.

4.1.3 Preparation of Olive Leaves Extracts

The macerations were carried out in triplicate, using the optimum solvent conditions as described in Ronca et al. [50]. Olive leaf powder (2, 5, 12.5 g) was mixed with 100 mL of 1:1 EtOH/H2O containing 0.1% formic acid. The solutions were vortexed for 2 minutes, sonicated for 30 minutes, mechanically shaken for 40 minutes, and then centrifuged at 5000 rpm for 30 minutes at 20°C. The liquid portions were dried using a Zirbus freeze-dryer (Zirbus Vaco 2, Bad Grund, Germany) for 96 hours and kept for future analysis of antioxidant capacity and HPLC.

4.1.4 Microparticles.

The freeze-dried extracts of Picual variety olive leaves (*Olea Europea Sativa*) have been used to prepare microparticles with high antioxidant power. A microparticle's structure is characterized by the formation of an outer membrane, called a wall, which is obtained by microencapsulating agent. The wall's function is to protect the desired substance inside the core [84]. Sodium alginate is a polymer commonly used as a microencapsulating agent due to its high affinity for water, which allows it to form a homogeneous, non-toxic, and biodegradable gel (Parra Huertas 2010). Additionally, it provides a prebiotic effect when ingested and acts as a dietary fibre, reducing blood sugar and

cholesterol levels [85]. The technique used in the study involves formulating a water/oil (W/O) emulsion at room temperature.

4.1.4.1 Preparation of microparticles

For preparation of the aqueous phase for microemulsion in a water/oil (W/O) emulsion, freeze-dried extract (50mg), sodium alginate (1g) and calcium carbonate (100mg) are dissolved in 20 mL deionised water. For the oil phase, incorporate 1.8 g of the Span80® surfactant in 40 mL of soybean oil. Next, pour the oil phase over the water phase while constantly stirring (using the Eurostar Power CV IKA, Spain). To start the internal gelation process, 2.5 mL of 4.25% v/v acetic acid was added. The mixture was stirred continuously for 10 minutes before adding 2.5 mL of 0.45 M calcium chloride. The resulting microparticles were collected through vacuum filtration and washed with MilliQ water (Figure 15).

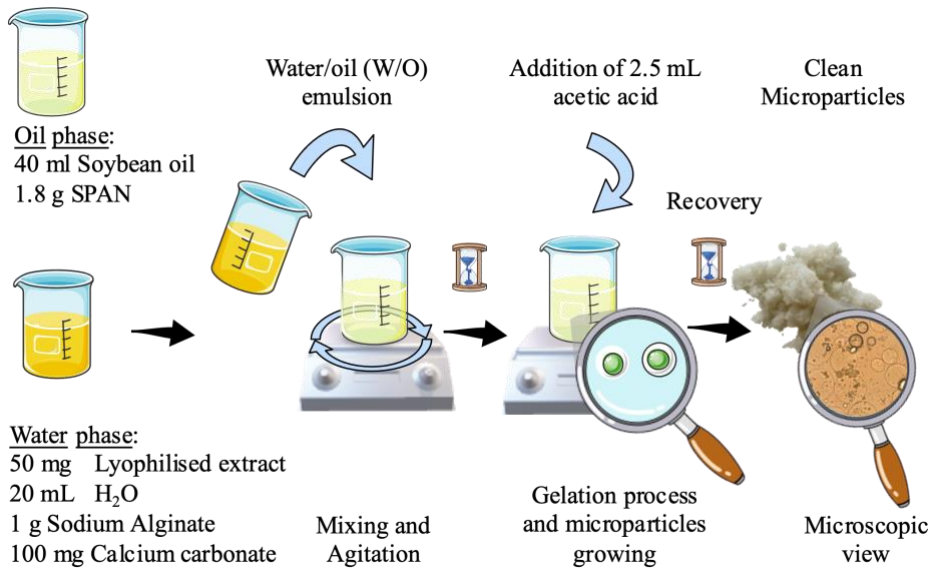


Figure 15. Scheme for the preparation of microparticles with freeze-dried extract.

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4.1.4.2 Granulometry of microparticles (mean diameter and size distribution) and optical microscopy.

The mean diameters and size distribution were analyzed using a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). The mean diameter was expressed as the median value (D50). The polydispersity index (PDI) was calculated using the equation (1) described in De Moura (2018) [167]:

$$PDI = \frac{d_{90} - d_{10}}{d_{50}} = 1.83. \quad (1)$$

Being d10, d50 and d90 the diameters at 10%, 50% and 90% of accumulated volume, respectively.

Morphology: The morphology of the microparticles was analyzed using a GEMINI (FESEM) CARL ZEISS High-Resolution Scanning Electron Microscope (SEM) at magnifications of 500×, 2000×, and 10,000×.

Encapsulation efficiency: The phenolic content of the respective extract administered for encapsulation and the washing water of the microparticles obtained were analysed as described in section 4.1.10. called "Characterization of Phenolic Compounds by HPLC-TQ-XS-MS-ESI", to determine the total and surface phenolic content, respectively. Encapsulation efficiency (EE) was determined using the equation (2).

$$EE\ (\%) = \frac{PC_{total} - PC_{free}}{PC_{total}}\ x\ 100 \quad (2)$$

Where PC_{total} indicates the total amount of phenolic compounds for encapsulation and PC_{free} indicates the non-encapsulated phenolic.

4.1.5 Preparation of goat yoghurt.

The functional yoghurt was prepared using goat (caprine) milk obtained from Murciano-Granadina breed goats that were raised in extensive farming conditions. The milk was divided into 500 mL batches, replicated three times, and compared with the milk control (100% goat milk).

To prepare the milk, it was preheated on the stove to 40-45°C and then maintained at 40°C for 20 minutes in a thermostatic bath (J.P. Selecta, Abrera, Barcelona, Spain). Probiotic yoghurt cultures YO-MIX 205 LYO 250 DCU (0.024 mg), composed of *Streptococcus thermophilus, Lactobacillus delbrueckii subsp. Bulgaricus, Lactobacillus acidophilus, Bifidobacterium lactis* and microparticles rich in polyphenols (3 mg), were inserted and dissolved in the warm milk. The mixture was then incubated in sterile, enclosed bottles at 42°C for 5 hours to promote fermentation. After incubation, the yoghurt was cooled to 4°C.

4.1.6 Phenolic extraction in goat yoghurt.

To extract phenolic compounds from the yoghurt preparation, 25 g of the sample were shaken for 10 minutes. The sample was then acidified to pH 2.5 using 6 M HCl and centrifuged at 5000 rpm for 20 minutes at 20°C. The resulting pellet was resuspended in 50 mL of distilled water and centrifuged under the same conditions. The supernatants were combined and filtered, and then mixed in duplicate with 10 mL of hexane. The samples were decanted and the lower layer was extracted three times with 10 mL of ethyl acetate. The resulting supernatant was filtered using Na2SO4, evaporated in a rotary evaporator, and resuspended in 1 mL of MeOH:H2O (50:50, v/v). Prior to analysis, the extracted samples were filtered using a 0.22 µm nylon filter. The extraction process was carried out in triplicate.

4.1.7 Antioxidant Assays: FRAPP, DPPH, and ABTS.

The study evaluated the antioxidant capacity using three assays: FRAP (Ferric Reducing Antioxidant Capacity), DPPH (2,2-diphenyl-1-picrylhydrazyl radical), and TEAC (Trolox Equivalent Antioxidant Capacity). The analyses were performed in triplicate using a microplate reader from Synergy MX, BioTek (Winooski, VT, USA). The FRAP assay was conducted according to Benzie and Strain et al., 1996, with slight modifications [168].

Briefly, FRAP reagent was prepared by mixing acetate buffer pH 3.6, TPTZ 10 mM in HCl 40 mM, and 20 mM $FeCl_3$-6-H2O (10:1:1, v/v/v). The extract dilutions (200 µL) were added to 1.5 mL of the radical FRAP reagent and incubated for 10 minutes at room temperature. The absorbance of the solutions was measured at 593 nm to determine the antioxidant capacity. The calibration curve was obtained using different Trolox concentrations (10, 20, 25, 30, 50, 70, 100 mM) ($y = 6.1115x – 0.0235$; $R2 = 0.999$). FRAP values were expressed as µmol $FeSO_4$ equivalents per gram of lyophilised extract.

The DPPH assay was performed according to the procedure described by [168]. Briefly, 50 µL of extract (3 mg in 1 mL EtOH/H_2O 50:50), were added to 1.45 mL of DPPH solution (2.3 mg in 100 mL MeOH). Solutions were incubated for 15 min at room temperature and the absorbance was measured at 515 nm. A calibration curve was obtained using different Trolox concentrations (50, 100, 250, 500 mM) ($y = 146.71x + 2.5309$; $R^2 = 0.997$). The results were expressed as µmol Trolox equivalent per gram (µmol TE/g).

The TEAC (Trolox Equivalent Antioxidant Capacity) assay was developed as previously reported [169]. The ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS radical with 88 µL of 140 mM potassium persulphate $K_2S_2O_8$ (1:0.5, v/v). For maximum stability and absorbance of the radical, the mixture was kept in the dark for 12-16 hours. After formation of the radical, it was diluted until its absorbance at 734 nm was 0.700 ± 2. Two hundred microliters (200 µL) of appropriate sample dilutions were added to 2 mL of ABTS·+.

The absorbance was measured at 734 nm once per min for 30 min at 25 °C and compared to a Trolox calibration curve (0.01, 0.1, 0.2, 0.3, 0.4 mM) ($y = 237.19x + 1.0381$; $R^2 = 0.9975$). Results were expressed as µmol Trolox equivalents/g of lyophilised extract.

4.1.7.1 FRAP: Ferric Reducing Antioxidant Capacity

The FRAP assay measures the antioxidant reducing ability of iron by reducing the ferric complex 2,4,6-tripyridyl-s-triazine (Fe^{3+}-TPTZ), to the ferrous form (Fe^{2++}-TPTZ) at low pH, resulting in a blue colour. This reduction results in the development of a thick blue colour, which is measured at 593 nm using a PerkinElmer Lamba 25 UV/VIS spectrometer.

The method described by [170], was used with slight modifications by Samaniego-Sanchez et al. [168, 171, 172] to perform the FRAP reagent. The reagent was prepared by mixing 10:1:1 (v/v/v) of acetate buffer, which was made by dissolving 2.4609 g of sodium acetate anhydrous in 100 mL of MilliQ water and adjusting the pH to 3. To prepare the solution, mix 333 µl of 37% v/v HCl in 100 mL of MilliQ water with 0.03123 g of TPTZ (dissolved in 10 mL of 40 mM HCl) and 54.06 mg of FeCl3-6-H2O (dissolved in 10 mL of MilliQ water).

For the calibration curve, prepare a stock solution of 20 mM Trolox by dissolving 5 mg of Trolox in 1 mL of 100% MeOH. From this, prepare a 2 mM stock solution, which will be used for the calibration curve with concentrations of 10, 20, 25, 30, 50, 70, and 100 mM (Table 13). In relation to the ethanolic extract of olive leaves, 3 mg of lyophilised extract was dissolved in 1 mL of a 1:1 mixture of ethanol and water. The liquid extract was analysed as is. Diluted samples were used, and 200 µL of these were added to 1.5 mL of the FRAP reagent. The samples were left to stand for 10 minutes at room temperature, and the absorbance was measured at 593 nm using a spectrophotometer (Shelton, USA).

4.1.7.2 DPPH: 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay

The DPPH assay was carried out following the procedure outlined by [153],with some modifications by [168, 172]. In brief, 2.3 mg of the DPPH radical was dissolved in 100 mL of absolute MeOH. This solution is stable for 12 hours, and the absorbance ranges from 600-900 Abs at a length of 515 nm. The calibration curve's stock solution (Table 13) consisted of 2 mM of Trolox, which was prepared by weighing 5 mg in 1 mL MeOH 100%. This stock solution was then used for the Trolox calibration curve (50, 100, 250, 500 mM). For the olive leaf extracts, 3 mg of lyophilised extract was dissolved in 1 mL of EtOH/H2O (1:1), while the liquid extract was analysed as is. From these solutions, 50 µL was added to 1.450mL of radical DPPH after appropriate dilution. The samples were left to stand for 15 minutes at room temperature, and their absorbance was measured at 515 nm using a spectrophotometer from (Shelton, USA).

4.1.7.3 ABTS: 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) Radical Cation Scavenging Capacity Assay

The antioxidant equivalent capacity as radical scavenging activity, based on the reduction of the radical cation ABTS, following the procedure of [173], lightly modified by Samaniego-Sánchez et al. [174], was adapted to samples of olive leaf. The radical was generated using potassium persulphate: 0.096 g of ABTS radical was dissolved in 25 mL of MilliQ water, with final concentration 7 mM; while 0.945 g of $K_2S_2O_8$ (140 mM) in 25 mL of MilliQ water. The ABTS reagent was performed by mixing 5 mL of ABTS radical 7mM with 88 µL of potassium persulphate $K_2S_2O_8$ 140 mM (in the ratio 1:0.5). The oxidation of the radical is immediate, but the maximum absorbance and stability of ABTS·+ is not reached until 6 hours.

The mixture was kept in the dark for 12-16 hours. Once the radical was formed, was diluted until its absorbance was 0.700 ± 2 at 734 nm. The stock solution for calibration curve (Table 13) was 5mM of Trolox: 0.0125 mg was weighed and added to 10 mL EtOH 100%; the latter stock solution was used for the Trolox calibration curve (0.01, 0.1, 0.2, 0.3, 0.4 mM).

Regarding the olive leaves extract, 3 mg of lyophilised extract was dissolved in 1mL of EtOH/H_2O (1:1), while the liquid extract was analysed as it was. Appropriate samples dilutions (200µL) were added to 2 mL of ABTS·+. The absorbance was measured at 734 nm once per min for 30 min at 25ºC.

Table 13. FRAP, DPPH, and ABTS calibration curves.

Antioxidant Assay	Calibration curve
FRAP	$y = 6.1115x – 0.0235$; $R^2 = 0.9998$
DPPH	$y = 146.71x + 2.5309$; $R^2 = 0.9974$
ABTS	$y = 237.19x + 1.0381$; $R^2 = 0.9975$

4.1.8 Mineralization.

4.1.8.1 Mineralization Conditions

ICP-OES (Inductive Coupling Plasma Optical Emission Spectrometry) quantitation is based on measuring excited atoms and ions at specific wavelengths for the elements being measured. In contrast, ICP-MS (Inductively Coupled Plasma-Mass Spectrometry), measures an atom's mass using mass spectrometry. ICP-OES is a technique that can identify and quantify all elements of the periodic table, except nitrogen, oxygen, and noble gases, through its emission spectrum, using plasma as a source of excitation. The instrument measures the light produced by a liquid sample when introduced into an inductively coupled argon gas plasma. The metals in the sample can be quantified by measuring the intensity of the light emitted with a specific optical

bank for each metal. Liquid samples are required for ICP-OES determination, while solid matrices require a preliminary mineralization phase to disintegrate the solid matrix into a liquid solution. ICP-OES-MS is an inorganic analysis technique that can determine and quantify most elements of the periodic table. It uses an ICP plasma torch for ionization and a mass spectrometer for ion separation and detection. Isotopic analysis is also possible with ICP-MS.

4.1.8.2 Preparation of mineralization samples

The mineral analysis was conducted following the procedure outlined in [175]. For lyophilised extract samples, 0.250 g was used, while 1 g was used for liquid milk samples, microparticles, and crushed leaves. The samples were mixed with 6 mL of nitric acid and placed in the digestion equipment (Multiwave 5000, Anton Paar, Austria). To remove organic matter from the samples, the temperature was raised to 180°C within 20 minutes and maintained for another 20 minutes. After digestion, the mineralized liquid from the borosilicate tubes was collected and resuspended in 10 mL of MilliQ water. Mineral analysis was then conducted using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) and Inductively Coupled Plasma Optical Emission Mass Spectrometry (ICP-OES-MS) on a Perkin Elmer Optima 8300 model in Waltham, MA, USA.

4.1.9 Characterization of Phenolic Compounds by UHPLC-TQ-XS-MS-ESI

The phenolic composition of olive leaf extracts, encapsulated formulations, and yoghurt samples was assessed using HPLC-ESI-MS, as described in [176]. The analysis was performed with an Acquity UPLC System I-Class system (Waters Corporation, Milford, MA, USA), a Z-Spray electrospray ionization interface (ESI, Waters Corporation, Milford, MA, USA), and a Triple Quadrupole Mass Spectrometer Xevo TQ-XS (Waters Corporation, Milford, MA, USA).

The sample was separated using an Acquity UPLC BEH C18™ column (2.1 × 100 mm, 1.7 µm) with a constant column oven temperature of 50 °C. The mobile phases were 0.5% acetic acid in water (A) and methanol (B). A multistep linear gradient was applied over 11 minutes: 1% B for 5 minutes, 30% B for 2 minutes, 60% B for 0.1 minutes, 100% B for 2 minutes, and 1% B for 2 minutes. The flow rate was 0.4 mL/min, and the injection volume was 10 µL.

The separated compounds were analysed using a TQ-XS analyser equipped with an ESI interface operating in negative ion mode, with a mass range of 72-2020 m/z. Nitrogen was used as the nebulising, ionising, and drying gas at conditions of 7 bar and 0.15 L/min. The drying temperature was set to 150/600 °C, with a capillary voltage of +2.20 kV and an End Plate Offset of 30 V.

To achieve a mass precision of 5 ppm, a sodium formate solution was used as a calibration agent at the beginning of each analysis (m/z range of 50 – 1500 Da). The ion mass data were processed using MassLynx 4.1 software (Waters Corporation, Milford, CT, USA). Identification was performed by comparing the retention time and MS spectral data of the samples with those of the available commercial standards.

Phenolic compounds in the extract, microparticles, and yoghurt samples were quantified using HPLC-MS analysis. The samples were analyzed in triplicate to ensure accuracy and consistency. Oleuropein, rutin, hydroxytyrosol, gallic acid, diosmin, p-coumaric acid, ferulic acid, chlorogenic acid and verbascoside stock solutions were prepared, filtered using 0.22 µm nylon filters, and stored at −18 °C until analysis. Calibration curves were calculated using seven points at different concentrations (10, 25, 50, 100, 250, 500 and 1000 ppb). The concentration of phenolic compounds was determined by creating a calibration curve based on peak area. All calibration curves demonstrated excellent linearity ($R^2 > 0.99$)

4.1.10 Statistical Analysis

The study evaluated significant differences in phenolic content, antioxidant activity, and mineral content using SPSS statistical software (version 28; SPSS Inc., Chicago, IL, USA). Statistical differences were determined using analysis of variance (ANOVA) and Tukey's post hoc tests with a 95% confidence level.

4.2 Results

This study proposes the revalorization of an industrial by-product, olive leaves, by obtaining a phenolic-rich extract and encapsulating it for later use in the development of functional yoghurt.

4.2.1 Extraction and analytical characterization of olive leaf phenolic compounds.

Phenolic content of a rich olive by-product (olive leaves) can be extracted and microencapsulated into a fortified functional yoghurt with great potential to improve nutritional value and provide health benefits.

When developing new functional foods, it is important to consider the scalability of the process and its safety for human consumption. Solid-liquid extraction is an industrial extraction process that is still relevant today, as it requires less specific equipment.

Conventional solid-liquid extraction was performed on dried and milled olive leaves using varying times and sample:solvent ratios. This was done to assess the evolution of phenolic compounds in the matrix and the extractive power of a specific amount of solvent. The identified and quantified phenolic compounds, which are the most abundant in olive leaves according to literature, are presented in Table 14.

Oleuropein, hydroxytyrosol, and verbascoside have demonstrated significant antioxidant, anti-inflammatory, immunomodulatory, and cardioprotective

effects [115, 177-179]. The monitored compounds, including phenolic acids (coumaric, ferulic, and chlorogenic acid) and flavonoids (rutin and diosmetin), could also serve as indicators of the phenolic extract's stability.

No significant differences were observed in the global phenolic extraction when increasing the sample amount while maintaining the extraction volume for most of the phenolic compounds. This suggests that the extraction solvent may be limiting the extractability of the desired compounds.

However, high quantities of the compounds of interest were obtained through extraction when not stored for extended periods of time. Oleuropein is the most abundant compound, comprising 95% of the total identified content, followed by verbascoside (4%) and hydroxytyrosol (0.6%). The amount of oleuropein found in the dried leaf, ranging from 97.07 to 122.77 mg/g, is higher than previous literature on solid-liquid conventional extraction with solvents of varying methanol content. Previous studies have reported amounts ranging from 0.54 mg (80% methanol) to 14.4 mg/g dried leaf (50% methanol) [180]. Microwave Assisted Extraction (MAE) with 80% methanol resulted in 72.08 mg/g dried leaf [181]. Furthermore, it appears to align with results from macerations using 80% ethanol, which yielded up to 109.6 mg/g of dried leaves [118, 182].

As for verbascoside, obtained in 4.38-5.32 mg/g dried leaf, it seems to be similar to that reported for macerations with 80% ethanol, which obtained a range of 2.08-2.32 mg/g dried leaf [118]. Finally, the hydroxytyrosol content (0.66-0.77 mg/g dried leaf) seems to be slightly higher than similar extractions reported in the literature, which ranged from 0.14 (methanol) to 0.43 (80% methanol) mg/g dried leaf [181, 183]. In this sense, the extraction procedure has been shown to allow an adequate extraction of phenolic compounds.

In addition, the effect of storage of the olive leaves on the stability of the phenolic compounds, and therefore on their extractability, was also evaluated in extractions carried out over a period of 2 months. In this case, a significant,

although not intense, degradation of phenolic compounds was observed over time, regardless of the extraction volumes chosen.

In particular, this degradation seems to be more pronounced during the first month of storage of the leaves and is mainly concentrated in the degradation of oleuropein (reduced to 1% over a period of 2 months), while it becomes less intense in the third extraction.

Table 14. Polyphenolic content, expressed in mg/g dried olive leaf using different sample-solvent proportions.

BC	S1	S2	S3	S4	S5
HTYR	0.72 ± 0.05 ^{ab}	0.77 ± 0.02 ^{a}	0.67 ± 0.05 ^{ab}	0.61 ± 0.07 ^{b}	0.49 ± 0.04 ^{c}
CUA	0.028 ± 0.003 ^{a}	0.032 ± 0.002 ^{a}	0.029 ± 0.002 ^{ad}	0.03 ± 0.01 ^{ab}	0.027 ± 0.001 ^{bc}
FeA	0.011 ± 0.001 ^{a}	0.01 ± 0.002 ^{a}	0.0066 ± 0.0002 ^{b}	0.006 ± 0.001 ^{b}	0.0067 ± 0.0005 ^{b}
ChlA	0.09 ± 0.01 ^{ab}	0.102 ± 0.003 ^{a}	0.06 ± 0.01 ^{c}	0.08 ± 0.01 ^{b}	0.06 ± 0.01 ^{c}
OLEU	113 ± 5 ^{a}	123 ± 6 ^{a}	97 ± 3 ^{b}	81 ± 7 ^{c}	81 ± 7 ^{c}
DS	0.09 ± 0.01 ^{ab}	0.11 ± 0.01 ^{a}	0.102 ± 0.004 ^{ab}	0.07 ± 0.01 ^{c}	0.08 ± 0.01 ^{c}
RUT	0.56 ± 0.03 ^{ab}	0.69 ± 0.03 ^{c}	0.57 ± 0.03 ^{a}	0.44 ± 0.05 ^{bc}	0.45 ± 0.04 ^{abc}
VB	4.4 ± 0.3 ^{ab}	5.3 ± 0.2 ^{c}	4.5 ± 0.2 ^{ab}	3.8 ± 0.5 ^{bd}	3.7 ± 0.3 ^{bd}

BC	S6	S7	S8	S9
HTYR	0.58 ± 0.06 ^{bc}	0.5 ± 0.05 ^{c}	0.44 ± 0.01 ^{c}	0.48 ± 0.07 ^{c}
CUA	0.026 ± 0.001 ^{bd}	0.0218 ± 0.0005 ^{a}	0.03 ± 0.001 ^{c}	0.036 ± 0.002 ^{e}
FeA	0.0062 ± 0.0002 ^{b}	0.009 ± 0.001 ^{ac}	0.007 ± 0.002 ^{bc}	0.0075 ± 0.0005 ^{b}
ChlA	0.08 ± 0.01 ^{b}	0.031 ± 0.004 ^{d}	0.047 ± 0.003 ^{cd}	0.05 ± 0.01 ^{c}
OLEU	85 ± 10 ^{c}	62 ± 6 ^{d}	69 ± 1 ^{cd}	88 ± 13 ^{bc}
DS	0.09 ± 0.01 ^{b}	0.06 ± 0.01 ^{c}	0.063 ± 0.003 ^{c}	0.09 ± 0.02 ^{ab}
RUT	0.52 ± 0.07 ^{ab}	0.38 ± 0.05 ^{c}	0.4 ± 0.01 ^{c}	0.5 ± 0.08 ^{abc}
VB	4.4 ± 0.5 ^{ab}	2.9 ± 0.3 ^{d}	3.37 ± 0.09 ^{d}	5.2 ± 0.3 ^{a}

S1: 2 g/100 mL, no storage time; S2: 5 g/100 mL, no storage time; S3: 12.5 g/100 mL, no storage time; S4: 2 g/100 mL, 1 month storage; S5: 5 g/100 mL, 1 month storage; S6: 12.5 g/100 mL, 1 month storage; S7: 2 g/100 mL, 2 months storage; S8: 5 g/100 mL, 2 months storage; S9: 12.5 g/100 mL, 2 months storage. Different letters among the same row indicate presence of statistically significant differences.

The stability of phenolic compounds has been shown to depend on the environmental conditions during processing and storage, which include pH and

temperature. In fact, degradation of compounds as a result of their evolution under environmental conditions is to be expected and has been previously reported for the storage of phenolic olive leaf extracts [118].

This may be related to the exposure of phenolic compounds to environmental conditions and the effect of the drying process on their resulting stability. In addition, the presence of enzymes found in olive structures may have an effect on the observed results. In this sense, some phenolic degrading enzymes could be compartmentalised in the leaf, as was observed for the olive fruit cell [184]. However, drying and storage under the conditions studied may increase the rupture of cell structures, allowing interaction with the polyphenols studied. Thus, even with a reduced activity, enzymes present in these plant structures could also be considered as polyphenol oxidase. In this sense, although dehydrated, the phenolic content could evolve further due to the environmental storage conditions.

4.2.2 Antioxidant Activity

Evaluation of the antioxidant activity of the obtained extracts by different antioxidant methods, namely DPPH, ABTS and TEAC, was also carried out. Results for the extracts obtained at different storage times have been presented in Table 15.

The antioxidant activity results of the extracts are consistent with the phenolic extraction yield described above. The evaluated assays showed high antioxidant activity, with the best results observed for FRAP. The data obtained from the first extraction are similar to or higher than those observed in previous literature for ethanolic extracts. FRAP results have ranged from 75.7 ± 0.9 to 109.5 ± 5 mg TE/g dry weight, and ABTS results have ranged from 4.5 ± 0.4 to 8.3 ± 0.5 mg TE/g dry weight [118]. Furthermore, these findings align with Hayes et al. (2011) who reported an antioxidant capacity of 379.3 mg TE/g DW for ABTS and 300.1 mg TE/g DW for FRAP in a commercial olive leaf extract [185]. Similarly, various alcoholic extracts of olive leaves have been found to

have DPPH inhibition percentages of around 90%, consistent with the 86.4% presented in this study [186, 187] . This highlights the potential of a simple and scalable extraction process for obtaining bioactive compounds from olive by-products.

Table 15. *In vitro* antioxidant activity of extracts obtained at different times and sample:solvent ratios determined by DPPH, FRAP and TEAC, expressed as µmol of Trolox Equivalent (TE) per g of dried olive leaf.

AOX	S1	S2	S3	S4	S5
DPPH	632 ± 54 ^{a}	608 ± 12 ^{a}	474 ± 6 ^{bc}	554 ± 26 ^{ad}	451 ± 15 ^{bc}
FRAP	858 ± 19 ^{a}	834 ± 5 ^{ab}	822 ± 56 ^{ab}	623 ± 7 ^{c}	628 ± 1 ^{c}
TEAC	448 ± 4 ^{a}	401 ± 7 ^{b}	407 ± 1 ^{b}	414 ± 7 ^{b}	382 ± 5 ^{c}

AOX	S6	S7	S8	S9
DPPH	405 ± 32 ^{b}	557 ± 14 ^{ad}	505 ± 41 ^{cd}	432 ± 7 ^{bc}
FRAP	836 ± 92 ^{ab}	776 ± 46 ^{b}	723 ± 6 ^{b}	1040 ± 4 ^{d}
TEAC	486 ± 17 ^{d}	408 ± 22 ^{b}	369 ± 2 ^{c}	395 ± 6 ^{bc}

S1: 2 g/100 mL, no storage time; S2: 5 g/100 mL, no storage time; S3: 12.5 g/100 mL, no storage time; S4: 2 g/100 mL, 1 month storage; S5: 5 g/100 mL, 1 month storage; S6: 12.5 g/100 mL, 1 month storage; S7: 2 g/100 mL, 2 months storage; S8: 5 g/100 mL, 2 months storage; S9: 12.5 g/100 mL, 2 months storage. Different letters among the same row indicate presence of statistically significant differences.

The antioxidant capacity was highest when extracting lower amounts of olive leaves, emphasizing the importance of sample-solvent proportions in observed bioactivity.

The highest antioxidant activity for the DPPH assay was found in the first extractions at different proportions, with no significant differences observed. For 2 g/100 mL, storage did not affect this value, while a reduction was observed for 5 g/100 mL after 1 and 2 months of storage, and for 12.5 g/100 mL after 1 month of storage. However, when comparing the behaviour of these proportions at different storage times, it appears that 2 g/100 mL consistently maintains the highest antioxidant activity. This difference is even more significant at 12.5 g/100 mL.

Nevertheless, the FRAP assay has produced varying results. While the antioxidant activity appears to decrease at a concentration of 2 g/100 mL, the highest activity among the evaluated samples was observed for the other proportions, specifically for extractions at a storage time of 2 months (S8 and S9).

In contrast, the ABTS assay showed a decrease in antioxidant activity for concentrations of 5 g/100 mL and 12.5 g/100 mL after one month, followed by a slight increase in the second month. The best antioxidant results were reported with 2 g/100 mL as the initial extraction conditions.

Antioxidant activity generally decreases with increased storage time, except for FRAP results. Variations in activity among assays are expected due to the different nature of the evaluated assays and their dependence on the specific action of the presented compounds. The activity of each compound appears to be related to its specific structure. The structure-antioxidant activity of phenolic compounds is linked to the number and location of hydroxyl groups in the phenolic ring.

The potential of these compounds increases when hydroxyl groups are present in the ortho- or para- positions (catechol), as this enhances their electron donating ability [78, 188]**.** All the phenolic compounds identified in the extract contain hydroxyl groups in this configuration, which is a determinant factor for the observed results. Hydroxytyrosol, chlorogenic acid, oleuropein, rutin and verbascoside are phenolics that exhibit two catechol groups and can be found with a presence in the orto-position. Other functional structures such as C=O (oxo) or OAc have also demonstrated an association with high antioxidant potential and are found in some of the mentioned phenolics. It is important to note that the use of subject-specific vocabulary is crucial for precise communication.

Considering the extraction technique and sample, it appears that the data are related to the specific concentration and modification of phenolic content in

stored olive leaves. This favours the degradation of identified compounds into other molecules that differentially contribute to the observed antioxidant results. Each specific compound has a distinct antioxidant capacity for different assays, as previously reported. The degradation of these compounds may be responsible for the observed decrease in antioxidant activity after 1 month of storage.

Nevertheless, compounds obtained during longer storage periods may contribute to a higher electron-transference antioxidant activity, resulting in increased FRAP values. Since the DPPH and ABTS assays are based on different molecular processes, it is unlikely that these degradation products contributed in the same way. However, the specific phenolic composition may have had a synergistic effect on the observed results. Understanding the molecular mechanisms behind the antioxidant properties of phenolic compounds and their interactions is crucial for their observed bioactivity.

In addition, it is important to consider environmental and technological factors, such as the impact of the drying process on the stability of phenolics and the effect of storage temperature on enzymatic activity. Studies have shown that enzymes like polyphenol oxidase and peroxidase have significantly reduced activity at lower temperatures [189]. In addition, it is important to note that storage temperature alone cannot prevent degradation. Research has shown that even when stored at lower temperatures (4°C), lyophilised olive phenolics experience significant reductions in antioxidant activity after 21 days or more [118].

4.2.3 Microparticles mean diameter and size distribution.

The positive data in the various assays indicate that S1 is the optimal extract to produce microcapsules and the development of functionalised dairy products, based on the previously reported results for phenolic content and antioxidant capacity of the evaluated extracts under different conditions. Incorporating the phenolic-rich extract into an encapsulated formulation is expected to enhance

the stability and structural integrity of the bioactive compounds during industrial processes and in the gastrointestinal environment.

The physical characterization of the microparticles is presented in Figure 16, showing the size distribution of the particles generated by encapsulating the phenolic compounds. The mean diameter (D) was 191.48 µm with a median diameter (D50) of 180.91 µm. The polydispersity index (PDI) was 1.48.

Microparticles with a size range of less than 330 µm were obtained under the experimental conditions of emulsion production, including maintenance of surfactant concentration, and stirring. The produced emulsion particles exhibited a multimodal size distribution, consistent with previous literature [190, 191]. Variation in the gelation process may be due to differential release of calcium ions, although the parameters of the process were kept constant [192]. However, this effect does not seem to be significant as the polydispersity (PDI) is low, with most of the volume found between 100-300 µm.

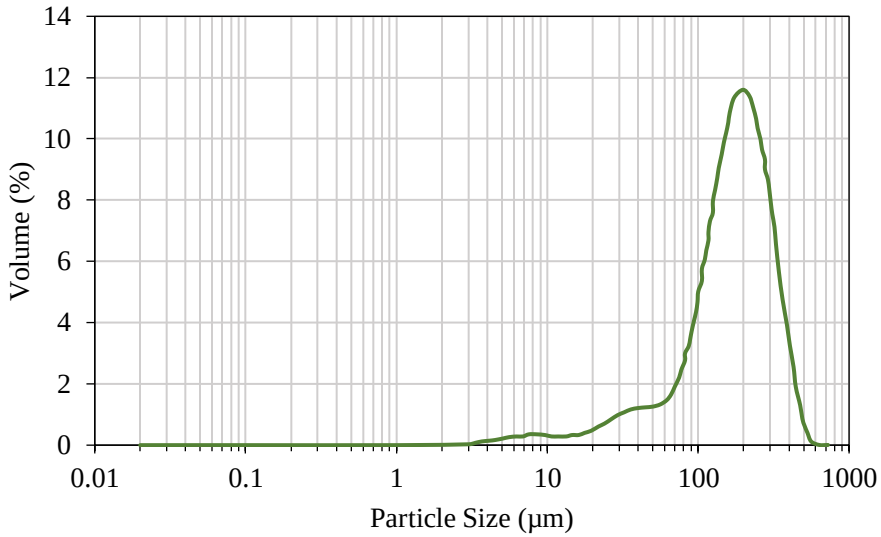

Figure 16. Particle size distribution of the olive leaf-extract microcapsules.

This encapsulation procedure provides a similar size range to that reported in previous literature for ionic gelation. The results presented here fall within the

D50 values (400 μm) reported by Yamdech et al. (2012) [193] for encapsulating mulberry fruit extract, although slightly lower [193]. Furthermore, chitosan encapsulation also produced microparticles within the size range observed in this study (160.58–206.52 μm) [194]. Overall, the size distribution obtained in this study appears to be smaller than that reported in other studies, where the size ranged up to millimetres [191, 195, 196].

4.2.4 Microparticles morphology.

Regarding the morphology, phenolic loaded microparticles with a spherical shape, few surface irregularities and porosity were successfully produced by the ionotropic gelation process, resulting in high smoothness (see Figure 17). Irregularities in the desired morphology have been observed in previous studies. For example, Flamminii et al. (2021) [197] found irregularities in alginate-based ionic gelation microparticles of olive leaf extract, and Belščak-Cvitanović et al. (2016) [192] observed irregularities in dandelion phenolic extract encapsulated with alginate and pectin.

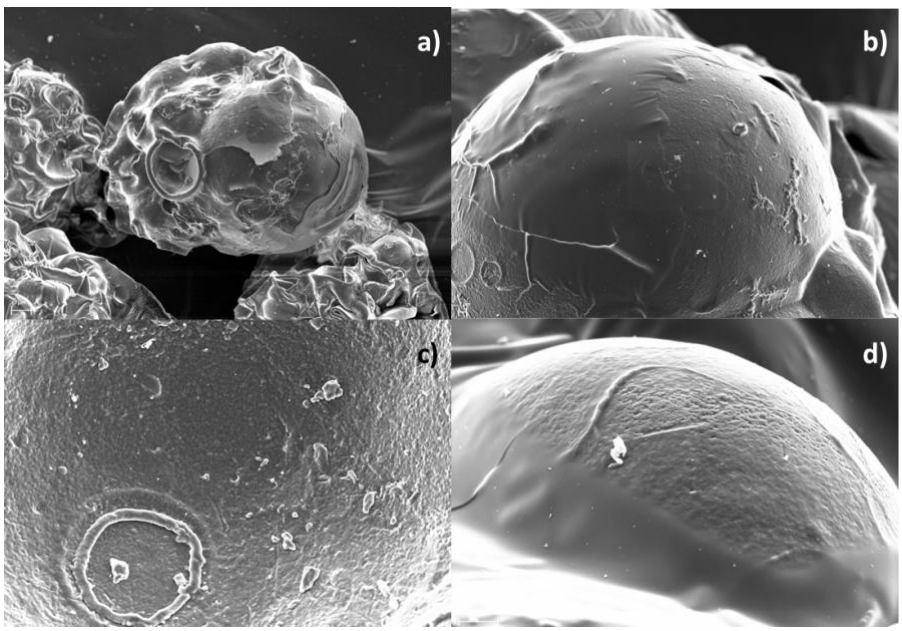

Figure 17. SEM microphotograph of olive leaf-extract alginate microcapsules at different magnifications: (a) 500×, (b) 2000×, (c) 5000×, (d) 10,000×.

These structural aspects appear to be influenced by varying $CaCl^2$ percentages and different encapsulation agents [192, 194]. Additionally, the drying process increased the likelihood of aggregation between structures. This process significantly altered the morphology of alginate-based microparticles, resulting in collapsed structures, irregular shapes, and heterogeneous surfaces, as previously observed [192, 197, 198]. However, the study found that the capsules maintained their structural integrity during the drying process, demonstrating their resilience to these demanding procedures.

4.2.5 Encapsulation efficiency of the phenolic-rich olive leaf extract

The optimal extraction conditions for sample S1 were found to be those with the least storage time and concentration of olive leaf. These conditions were used for the encapsulation process. Table 16 presents the total phenolic content, encapsulated amount, and efficiency percentage of the extract.

Table 16. Extract composition encapsulated phenolic content and encapsulation efficiency in the obtained microcapsules.

Bioactive Compounds	Extract Phenolic Content (mg/g of Extract)	Encapsulated Content (mg/g Microparticles)	Encapsulation Efficiency (%)
Hydroxytyrosol	2.2 ± 0.2	2.05	91.72
P-coumaric acid	0.09 ± 0.01	0.09	100.00
Ferulic acid	0.04 ± 0.002	0.04	100.00
Chlorogenic acid	0.28 ± 0.02	0.27	97.60
Oleuropein	353 ± 15	339.22	96.10
diosmin	0.29 ± 0.02	0.28	96.26
Rutin	1.7 ± 0.1	1.69	96.81
Verbascoside	13.7 ± 0.9	13.25	97.01

Preliminary morphological characterization results have demonstrated the potential of the chosen encapsulation process to produce microparticles with a

desired morphology and size. However, it is also important to consider its ability to incorporate the desired compound into a protective structure. Therefore, encapsulation efficiency has been used as an insightful parameter to evaluate its potential and adequacy for the extract used, as presented in Table 16**.** The analysis has focused on the most abundant compounds detected in the extract, which are related to its positive health benefits.

Encapsulation efficiency values are remarkable, consistently above 90% for all cases, indicating the suitability of the selected phenolic extract.

For hydroxycinnamic acids, such as p-coumaric acid and ferulic acid, all of their content has been incorporated into the encapsulated formulations, making the process highly effective for these compounds. There is only a slight reduction for chlorogenic acid (97.60%). This positive effect has been previously observed for ionotropic gelation with alginate in combination with whey protein as a co-structural agent, reaching up to 89% [192].

Globally, the encapsulation efficiency for the selected phenolic extract is remarkable, with values above 90% for all cases. It is worth noting that all of the content of hydroxycinnamic acids, such as p-coumaric acid and ferulic acid, has been incorporated into the encapsulated formulations, making the process highly effective for these compounds. There is only a slight reduction for chlorogenic acid (97.60%). Previous studies have shown a positive effect of ionotropic gelation with alginate and whey protein as a co-structural agent, achieving up to 89% encapsulation efficiency [192] .

The presented extract contains oleuropein, hydroxytyrosol, and verbascoside as the most abundant bioactives, which also exhibit exceptional encapsulation efficiency. In contrast to Flamminii et al. (2021) [197], where hydroxytyrosol was encapsulated with 80% efficiency, the encapsulation efficiency for both oleuropein and verbascoside in alginate micro-particles was lower. The presented results are more similar to those obtained for co-encapsulation with pectin [197].

These values are higher than the range observed in literature for other phenolic extracts, where total phenolic encapsulation has ranged from 41% to 85%. In some cases, the observed efficiency is similar to that obtained with co-encapsulation of alginate with other wall materials, which is promising.

The nature of the matrix material has been shown to be an essential factor in ensuring the entrapment and stability of bioactive compounds. Therefore, it may affect the variations in encapsulating efficiency between studies.

Alginate has been successful in encapsulating phenolic compounds from various sources using ionotropic gelation. However, the efficiency of alginate in encapsulating phenolic compounds may vary depending on their structure. This variation is due to the interactions established between the molecules, such as hydrogen bonds. The presence of hydroxyl groups has been found to positively correlate with a higher formation of hydrogen bonds with polysaccharides [199]. The abundance of this functional group in the identified molecules may explain the high efficiency of the presented process. Verbascoside has previously shown increased values of encapsulation efficiency with alginate compared to other olive leaf phenolic compounds, such as oleuropein, due to this aspect [194, 200].

Furthermore, the efficiency of the encapsulation process may be affected by the ability of the encapsulating material to produce smooth structures. Porous structures resulting from specific interactions of the protective material may cause a loss of bioactive molecules, leading to a reduction in observed encapsulation efficiency [201]. Therefore, the smooth surface of these microparticles may contribute to their overall efficacy.

The presented encapsulation efficiencies appear to be promising when considering the encapsulation efficiency of oleuropein (the major phenolic compound present in olive leaf extracts) using different methods. The highest encapsulation efficiency for oleuropein was achieved using the freeze-drying encapsulation method, with an efficiency of 99.23 ± 0.16%, which is

comparable to that proposed in this study [201]. Furthermore, the study found that the encapsulation efficiency for oleuropein was 60.8% in olive leaf microcapsules that were encapsulated using spray drying with alginate[202] .

These results demonstrate the potential of the encapsulation process for the selected olive leaf extract. The effectiveness of the capsules was established by the matrix material's ability to retain bioactive compounds, confirming the success of the process.

4.2.6 Functional-Goat Yoghurt Formulation by the Incorporation of Microencapsulated Olive-Leave Phenolic Extract

The study evaluated the applicability of microencapsulated olive-leaf phenolic extract in food products by incorporating it into functional goat yoghurt. The stability of the microcapsules during the production process was tested. The main phenolic compounds in the extract were quantified and compared to a control sample, as shown in

Table 17. The presence of several phenolics in the control yoghurt may be attributed to the specific type of farming that the goats were subjected to, and therefore directly derived from their diet rather than synthesized by the mammal. This could be due to the abundance of olive plantations in the area, which may have led to an increase in oleuropein, a secoiridoid mainly found in different structures derived from the olive tree, as it could have been ingested by the mammals [91]. Regarding the functional yoghurt, only hydroxytyrosol and oleuropein were found among the selected phenolics, and in low quantities. The presence of these compounds, which are the most abundant in the olive leaf extract, could be attributed to their observed encapsulation efficiency. This presence was not observed in the control yoghurt sample for hydroxytyrosol. As a result, these compounds may be superficially present and could diffuse into the yoghurt media. Furthermore, regarding oleuropein, its presence in the yoghurt is natural and the encapsulation efficiency is related to this natural occurrence.

Table 17. Phenolic content of the obtained enriched goat yoghurt as expressed as µg of polyphenols per 100 g of yoghurt.

Bioactive Compounds	Control Yoghurt	Functional Yoghurt
Hydroxytyrosol	nd	0.062 ± 0.004
P-coumaric acid	nd	nd
Ferulic acid	nd	nd
Chlorogenic acid	0.774 ± 0.007	nd
Oleuropein	0.69 ± 0.03	2.75 ± 0.192
Diosmin	nd	nd
Rutin	1.24 ± 0.04	nd
Verbascoside	Nd	nd

nd: non detected.

The results demonstrate the stability of the encapsulated extract formulation in the acidic environment of the fermented product and its ability to withstand the technological production process. This makes the microcapsules obtained optimal for the selected product [203].

Encapsulation of these compounds is desirable in a food matrix with a high presence of lactic acid bacteria. Phenolic compounds are molecules with a high degree of prebiotic properties and have attracted the interest of the public because of their bioactive properties and because they can be easily metabolized by bacteria. If these compounds were accessible and freely present in the food matrix, they would be fermented and therefore not available in the final product. The microcapsules are incorporated into the yoghurt obtention at the same time as the starter cultures. Encapsulation effectively protected these compounds from being degraded by bacteria, ensuring that the product remained functional. These results highlight the protective effect of the obtained formulations on the selected phenolic extract, allowing its preservation and controlled release, potentially increasing its bioaccessibility.

Sodium alginate has been identified as a safe encapsulating agent that also plays a role in the preservation of phenolic compounds during industrial processes.

However, there is a lack of literature on the use of ionic gelation encapsulation for olive leaf extracts in dairy products. However, previous studies have shown that Ceratonia siliqua L. hydroethanolic extract can be effectively protected and stabilized through encapsulation. This was demonstrated in a functional yoghurt formulation where the encapsulated formulation efficiently preserved the bioactive compounds [204]. Similar results were also found for the encapsulation of *Rubus ulmifolius* flower extracts [205]. However, in both studies, the microparticles were added to pre-existing yoghurt. In this study, we have expanded on this research by identifying the primary phenolic compounds present in olive leaf extracts, including secoiridoids, phenolic alcohols, and phenilethanoids/phenilpropanoids, throughout the production process of functional yoghurt with olive leaf microcapsules. These results demonstrate that stability is maintained when microparticles are introduced during processing, indicating the potential for scalability of these formulations.

In addition, as observed for encapsulated aromatic plant extracts [201], this stabilization may also extend to storage time. Furthermore, the formulations have also increased the stability of phenolic compounds upon their incorporation into several fortified cheeses [169, 201].

The study has demonstrated not only the potential of olive leaf extracts as a source of bioactive compounds with outstanding applications, but also the suitability of the encapsulation process to ensure the stability of these compounds when developing functionalised yoghurts. This statement lays the foundation for further research on the revalorization of industrial by-products into functionalised dairy fermented products.

4.2.7 Mineral Content in the Analyzed Samples

Besides the content of bioactive plant compounds, the effect of the inclusion of the microparticles obtained in the microelement content of the functional yoghurts was also assessed. Table 18 presents the concentrations of mineral elements in the olive leaves, extracts, microparticles, and enriched yoghurts,

classified into macroelements and microelements. The potential of the studied by-product as a mineral source is being analysed, as the presented minerals are essential for the correct functioning of biological responses and enzyme activity.

Table 18. Mineral content presented in the dried leaves, selected extract and microparticles (MC) as well as the functionalized and control yoghurts.

	Minerals	Dried Olive Leaves (µg/g)	Extract Lyophilised (µg/g)	MC (µg/g)	CTR Yoghurt (mg/100g)	Functional Yoghurt (mg/100 g)
Macrominerals	**B**	10.7 ± 0.2 [a]	119 ± 1 [b]	3.293 ± 0.002 [c]	0.175 ± 0.001 [d]	2.01 ± 0.08 [e]
	Ca	12792 ± 231 [a]	3838 ± 69 [b]	6963 ± 37 [c]	80.1 ± 1.2 [d]	82.9 ± 1.2 [e]
	Fe	291 ± 9 [a]	13.7 ± 0.9 [b]	9.6 ± 0.1 [b]	0.098 ± 0.001 [d]	0.073 ± 0.003 [e]
	K	4767 ± 139[a]	10459 ± 109[b]	525 ± 1 [c]	122.9 ± 0.6 [d]	132.6 ± 1.8 [e]
	Mg	1224 ± 47 [a]	2625 ± 40 [b]	21.2 ± 0.1 [c]	11.491 ± 0.197 [d]	12.1 ± 0.3 [e]
	Na	69 ± 6 [a]	311 ± 4 [b]	3090 ± 2 [c]	61.3 ± 0.2 [d]	66.3 ± 2.7 [e]
	P	611 ± 12 [a]	572 ± 17 [a]	25.1 ± 0.1 [b]	67.3 ± 0.6 [d]	64.4 ± 1.9 [e]
	S	1265 ± 39 [a]	1076 ± 34 [b]	64.3 ± 0.01 [c]	25.0 ± 0.1 [d]	25.5 ± 0.8 [d]
Microminerals	**Mn**	32.93 ± 9.4[a]	56.57 ± 4.5 [b]	0.35 ± 0.04 [c]	0.0031 ± 0.0003 [d]	0.0039 ± 0.0011 [d]
	Cu	38 ± 7 [a]	41 ± 2 [a]	0.308 ± 0.027 [b]	0.0038 ± 0.001 [d]	0.0044 ± 0.0005 [d]
	Zn	8 ± 4 [a]	33 ± 8 [b]	0.84 ± 0.07 [a]	0.33 ± 0.04 [d]	0.41 ± 0.02 [d]
	Se	0.12 ± 0.02[a]	0.13 ± 0.01 [a]	0.013 ± 0.002 [c]	0.0037 ± 0.0004 [d]	0.0036 ± 0.0008 [d]
	Rb	1.9 ± 0.6 [a]	4 ± 0.1 [b]	0.04 ± 0.002 [c]	0.15 ± 0.01 [d]	0.17 ± 0.01 [d]
	Pb	0.42 ± 0.1 [a]	0.34 ± 0.08 [a]	0.11 ± 0.01 [b]	0.0004 ± 0.0001 [d]	0.0008 ± 0.0005 [d]
	U	0.012 ± 0.002 [a]	0.0021 ± 0.0001 [b]	0.0138 ± 0.0003 [a]	0.00003 ± 0.00001 [d]	0.000045 ± 0.00001 [d]

Note: Different letters within the same row indicate the presence of statistically significant differences between the data.

Among the macroelements analysed, calcium, potassium, magnesium and sulphur were the most abundant in all the samples considered. These were followed by phosphorus and iron. Calcium appears to be the predominant mineral in olive leaves, with a concentration of 9.409 ± 0.045 mg/g, consistent with the findings of Lee et al. (2009) [206] (9.296 mg/g dried leaves) and

Bahloul et al. (2014) [207] (9.25 mg/g dried leaves). The presence of this compound in dietary supplements may be useful for preventing calcium deficiencies and improving bone health. However, it is important to consider the presence of Fe in dried olive leaves, despite it not being the most abundant element. Consuming 1 g of olive leaves provides 2% of the Population Reference Intake (PRI), while consuming 50 g provides 100%, as shown in Cavalheiro et al.'s (2015) research [208].

The microelements present in the leaves were Cu, with a similar content to that previously observed, and Mn [208].Additionally, trace amounts of Pb and uranium (U) are present. This may indicate soil contamination, as Pb accumulation is often linked to anthropogenic sources and its affinity with organic and colloidal materials enhances plant uptake [209]. However, the quantities present are relatively low compared to those reported in the literature for spice crops, such as rosemary, where Pb concentrations of up to 9.38 mg/kg have been observed [209]. In the present study, the concentration of uranium in food was found to be lower than that of several other foods [210].

Therefore, its consumption should not raise any health concerns. The no-observed-adverse-effect levels (NOAEL) for uranium depend on its specific form and the observed alterations. One of the lowest NOAEL concentrations observed is 40 mg U/kg/day for uranium peroxide, while for Pb, this value is 57 µg Pb/kg/day [210, 211]. The concentrations observed are below the recommended levels, indicating that it is safe for consumption.

However, the concentration of the quantified compounds varies in the extract. This difference in mineral extraction could be due to the nature and affinity of the extraction solvent. Previous studies have shown that the distribution of minerals in moringa extracts varies depending on the extraction solvent used [212].

Different sample-solvent proportions were evaluated. This parameter does not appear to significantly influence the presence of some macro- and micro-

minerals, such as Fe, P, S, Mn, Cu, Rb and U, which coincides with the above-stated extractability of phenolic compounds. Although significant differences have been observed between different ratios for some compounds, their content mainly decreases as sample content increases, as is the case for B, Zn and Se.

Regarding microminerals, differences in content were observed, resulting in a reduction of content with a higher presence of olive leaves in the extraction. Therefore, the lowest concentration of olive leaves appears to have a more desirable effect, consistent with what was mentioned in previous sections.

Furthermore, the content of all major minerals in the microparticles differs significantly from that observed in the different extracts. The encapsulation of microparticles appears to be molecule-specific, with Na and Ca being the most effectively encapsulated, while B is less prevalent in the extracts.

In the case of functionalized yoghurt, the inclusion of these microparticles led to an increase in the content of most macrominerals, while microminerals remained similar to the control, except for Fe and P, which were slightly lower in the yoghurt.

The observed content in both the extract and encapsulated formulations appears to be associated with the possible effect of its incorporation. The increased presence of the former minerals in the microparticles allows for a significant effect on the yoghurt composition, even when administered at low quantities, as is the case here. However, microminerals are present in trace amounts and are not significant enough to efficiently affect the nutritional profile of the functionalized yoghurt. Therefore, although introduced in small increments, the inclusion of microparticles in the proposed functional food not only allows for the inclusion of bioactive compounds that may have a positive impact on health but also has a positive influence on its mineral profile.

Overall, the preliminary study results have highlighted the potential of the selected extract and microencapsulated formulation for developing a functional product with potential health benefits. Further studies are necessary to evaluate

the impact of scaling up the production process on bioactive phenolics. Additionally, it is important to establish the behaviour of the goat yoghurt formulation under gastrointestinal conditions, both *in vitro* and *in vivo*. Finally, the bioactive potential of consuming the yoghurt should be evaluated.

4.3 Conclusions

This work evaluates the influence of sample:solvent ratios and storage times on the green extraction of phenolic compounds from olive leaves, which are industrial by-products with great potential for encapsulation and incorporation [148] into a functionalized yoghurt formulation. The optimal extraction conditions involved using low sample proportions and minimizing storage time. This is due to the risk of solvent saturation and degradation of bioactive compounds when stored in their original plant structures. The encapsulation process resulted in a high incorporation of the main phenolic compounds, which protected the fermentation process. Therefore, the microencapsulated extract presented demonstrates significant potential for integration into the initial industrial stages of obtaining fermented dairy products, resulting in the production of functional foods with high health benefits.

Chapter 5

Chapter 5

General Discussion and Conclusion

Olive leaf polyphenols are currently attracting a great deal of scientific attention because of their benefits for human health [213, 214]. Recently they are also considered a by-product of the table olive and the oil industries [215]. To explore the potential health benefits of this waste, the study investigated the phytochemical profiles and antioxidant activities of several leaf samples (*Olea Europaea L.*) from three different countries: Italy (n>30), Portugal (n>12), and Spain (n >30). The Italian cultivars, including *Leccino* and *Frantoio*, were from a farm in Eboli (Salerno, Campania, Italy), and more than 30 trees were sampled. The Portuguese cultivar came from regions including Arcos de Valdevez, Mirandela, Vila Pouca de Aguiar, Braga, Vila Nova de Gaia, Buçaco, Aveiro, and Algarve, and covers 12 different sites, although the sampling was done by collecting leaves from several trees. The Spanish cultivar included Sativa Picual variety from Granada (Andalucía, Spain), and the sampling was carried out on more than 30 trees.

This study involved preparing a liquid olive leaf extract, which was subsequently freeze-dried to obtain a potential functional ingredient for enriching foods. To support this hypothesis, various antioxidant studies were carried out, and the chromatographic profile and quantity of the main polyphenolic compounds were determined. The freeze-dried extract's chromatographic profile was also analysed after undergoing *in vitro* analysis to mimic gastrointestinal condition. Additionally, a mineral study was conducted to valorise the extract, which was then used to enrich a goat's yoghurt after encapsulating the phenolic compounds it contained.

The initial Italy results suggest that the lyophilised extract is a promising ingredient for the formulation of new functional foods or dietary supplements, due to its high concentration of hydroxytyrosol (Figure 6). Indeed, previous studies have shown that hydroxytyrosol concentrations can vary significantly depending on the experimental approach and conditions used. In the research of Ghomari et al. [216], using 80% ethanol and distilled water, the concentrations of hydroxytyrosol were 15.17 and 27.20 mg g−1 (through sonication), and 0.04 and 0.95 mg g−1 of dry weight (through maceration). María Kourti et al. [116], demonstrate a holistic approach to the evaluation of the *in vitro* antioxidant capacity of an extract derived from the direct hydrolysis of olive leaves from Greek cultivars, and also investigate its ability to protect against oxidative DNA damage. Hydroxytyrosol is a small molecule, which means that its bioactivity to weight ratio is greater than that of OLE. It exhibits potent antioxidant activity and is closely associated with beneficial effects in several human diseases [141] including anti-tumor [177, 217-219], cardioprotection [37, 120, 128, 220, 221] and neuroprotection [222, 223]. HTYR also displays strong protective activity against damage caused by heavy metals. HTYR prevented mercury-induced oxidative stress in red blood cells (RBCs) and mitochondrial dysfunction in doxorubicin-induced cardiotoxicity in rats with breast cancer [120, 224]. It is generally recognized as safe by the European Food Safety Authority (EFSA) and the Food and Drug Administration (FDA). According to EU Regulation 432/2012, consumption of a minimum dose of 5 mg per day is asserted to improve human health [225]. In addition, the European Union has recently approved the use of HTYR as a novel food ingredient on the market, in accordance with Regulation of the European Parliament (EC) No. 258/97 [226] This decision creates new opportunities for revalorizing olive by-products, which are rich in this compound [24].

Due to its multifaceted biological activity, HTYR is a well-studied component of olive trees. Therefore, the production of OL extracts rich in HTYR has garnered interest from the scientific community, as well as food,

pharmaceutical, and cosmetic companies, due to the high demand for products enriched with natural antioxidants. The literature proposes an often-cited methodology for producing HTYR-rich extracts, which is acidic hydrolysis. This methodology involves a two-step procedure for the hydrolysis of Oleuropein. Firstly, OLE is extracted from dried OLs. Secondly, it is hydrolyzed to obtain HTYR under acidic conditions. Papageorgiou et al. [142] recently investigated the optimal conditions for producing an extract that is rich in HTYR from OLs. The study investigated several parameters such as the particle size of the plant material, the liquid/material ratio (g/L), the extraction solvent, the effect of temperature during solid-liquid extraction, the temperature and duration of hydrolysis, the hydrolysis medium (acidic or alkaline hydrolysis) and the type of catalyst (H2SO4 or HCl), as well as the pH of the aqueous phase before liquid-liquid extraction with ethyl acetate. Specifically, the protocols developed by Linfa Patent utilize acidic hydrolysis with water extraction to produce an extract rich in hydroxytyrosol, which has a high antioxidant capacity.

The result from Portugal confirms the strong antioxidant activity of the sample, as well as a high concentration of phytochemical compounds. However, the issue of gastrointestinal bioaccessibility remains a challenge. In the liquid extract from Portuguese olive leaves, oleuropein followed in abundance by luteolin, hydroxytyrosol, verbascoside, rutin, and quercetin. (Table 8). Subsequent to the lyophilisation process, the content of polyphenols for all the assessed compounds, in both the Italy and Portugal samples, has significantly decreased (Table 2, and Table 11). The results demonstrate that the lyophilisation process used for the liquid extracts has resulted in a reduction in the quantity of polyphenols present.

Nevertheless, Portuguese olive leaves showed a higher polyphenol content per gram of lyophilised extract (Table 11) than reported in the literature. [51, 227] including oleuropein, pinoresinol, verbascoside, rutin, quercetin, and luteolin.

Likewise, Italian olive leaves (Table 2) showed a higher amount of hydroxytyrosol per gram of dried extract for lyophilised extract [65], and higher concentration of hydroxytyrosol, oleuropein, coumarin, verbascoside, and secologanoside for liquid extract than reported in the literature [131, 227].

The study of gastrointestinal bioavailability in Portuguese olive leaves lyophilised extract revealed that exposure to gastrointestinal fluids significantly reduces the bioavailability of most polyphenols. However, oleuropein was found to be the least affected, with a concentration decrease of only 50-87%.

Similarly, Daniel Martín-Vertedor et al. [227], demonstrate that the quantity of individual phenols (such as hydroxytyrosol, tyrosol, oleuropein, luteolin, apigenin, and verbascoside) present in lyophilised extract exhibits significant variations in polyphenols following gastric and intestinal digestion steps. Subsequent to the digestion process, the initial amount of phenolic compounds was reduced by an average of 60% following the action of gastric acids *in vitro*. Consequently, 40% of the phenolic compounds would be available for absorption by the organism and would enter the intestinal tract. The lowest values were observed for olive leaf extract subjected to intestinal digestion, with a reduction of 90% of phenolic compounds with respect to the olive leaf fresh extract. This suggests that 10% of phenolic compounds would become available for assimilation in the intestinal tract.

Another investigation conducted by J. Rocha-Pimienta et al. [144], revealed that the quantity of individual phenolic compounds (including gallic acid, caffeic acid, hydroxytyrosol, tyrosol, oleuropein, luteolin, apigenin, verbascoside and quercetin) present in liquid olive leaf extract exhibited a gradual decline in polyphenol content following the oral, gastric and intestinal digestion phase. Furthermore, the Italian and Portugal results for olive leaf liquid extract are in con concordance with antioxidant activity measured by ABTS method [144].

Moreover, olive leaf liquid extract shows high antibacterial activity against *Escherichia coli* (Gram-negative bacteria) and *Listeria innocua* (Gram-positive bacteria), and after gastric digestion it retained more than 90% of its initial activity. However, in the final intestinal digestion stage, the olive leaf extract lost approximately 40% of its antimicrobial activity[144]. Strong antibacterial activity against five pathogenic bacteria (*Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Listeria monocytogenes*) was also demonstrated in literature[65]. A comparison of the TPC, ABTS and CUPRAC, and TPC, DPPH and ABTS values of the Spanish and Italian samples, respectively, with those reported in the literature revealed that they were either similar or higher [144]. The content of phenolics and flavonoids in olive leaf (OL) extracts is susceptible to modification by a multitude of variables, including the harvesting season, drying conditions and the extraction solvent, which can influence comparative analyses across national borders [228].

It has been established that plants produce a range of polyphenols as secondary metabolites to protect themselves and interact with other plants. In addition, it has been demonstrated that polyphenols affect the formation of a bitter taste in plants. The most important secondary metabolites synthesised by plants include terpenes, saponins, glycosides and polyphenols. Of these polyphenols, those produced by plants are particularly noteworthy, as they are important functional foods in human nutrition. Due to their diverse chemical structures, they are classified according to their chemical properties. The main groups of polyphenols are flavonoids, lignans, stilbenes and phenolic acids. Currently, *in vivo* and *in vitro* studies have investigated the health effects of these compounds. Furthermore, polyphenols have been shown to have protective effects against specific diseases and to halt their progression through a number of mechanisms, including the prevention of tumour, degenerative diseases and inflammatory pathways.

These antioxidant compounds, which are recovered from by-products, represent a valuable resource for the food industry. However, they frequently exhibit poor solubility, high susceptibility to environmental stress factors (temperature, pH, light and oxygen levels, moisture), and are prone to degradation during gastrointestinal digestion, with detrimental consequences on their bioavailability and bioactivity[197]. Microencapsulation may be a promising tool, as it can provide bio-compounds with appropriate protection against instability phenomena, thus increasing their potential application. A variety of microencapsulation technologies are available, including spray drying, freeze drying, coacervation, liposomes, ionic gelation, emulsions, and many others, which have been well described and detailed in numerous reviews. [229-232]

In this study, the effectiveness of MC in mitigating these deficiencies was investigated. Results from the preliminary study suggest that the selected extract and microencapsulated formula may be utilized to develop a functional product with beneficial health effects.

The most common emulsion type used in recent times is the conventional oil-in-water (O/W) emulsion, as described in numerous studies [230] Many studies have demonstrated the positive effects of microencapsulation for polyphenols [192, 197].

Furthermore, emulsion encapsulation has emerged as a particularly promising approach for the protection and delivery of polyphenols. This is due to its high encapsulation efficiency, maintenance of chemical stability.

5.1 Conclusions

The use of non-toxic, biodegradable, and environmentally and human-friendly solvents to obtain liquid extracts of olive leaves rich in phenolic and polyphenols compounds by extractive methods has been demonstrated to be a cost-effective approach to obtain a high value-added resource.

The HPLC-DAD and UHPLC-MS techniques employed to analyse the liquid and freeze-dried extracts of olive leaf have permitted the quantification and analysis of not only the major compounds (oleuropein, hydroxytyrosol, verbascoside, luteolin and rutin), but also minor compounds such as diosmin, quercetin, gallic acid, caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid and ferulic acid.

Specifically, oleuropein and its principal secondary metabolite, hydroxytyrosol, were identified as the most abundant polyphenols in the extract. The maximum oleuropein concentration was 2,894 mg/100 g dry weight of dried leaves, while the maximum hydroxytyrosol concentration was 146 mg/100 g. This evidence suggests that olive leaves could be a valuable, cost-effective resource with a high concentration of polyphenols.

Moreover, the development of ICP-OES techniques (for the identification of macrominerals) and ICP-MS techniques (for microminerals) allowed the determination of high levels of minerals, which can have antioxidative capacity due to their role as antioxidants or enzyme co-factors. The calcium content was found to be 12,792 µg/g in the dried leaves, 3,838 µg/g in the freeze-dried extract and 82.9 mg/100 g in the enriched yoghurt. Similarly, the potassium, magnesium, phosphorus and selenium contents were also high, with respective values of 4767 µg/g, 1224 µg/g, 611 µg/g and 1265 µg/g in dried olive leaves; and 10459 µg/g, 2625 µg/g, 572 µg/g, 1076 µg/g in freeze-dried extract; in contrast, the highest value observed in the enriched yoghurt was for potassium at 132.6 mg per 100 grams, followed by sodium at 66.3 mg per 100 grams, phosphorus at 64.4 mg per 100 grams, selenium at 25.5 mg per 100 grams, and

magnesium at 12.1 mg per 100 grams. This indicates that olive leaves are not only rich in polyphenols but also in minerals, thereby further enhancing their nutritional value.

The high antioxidant power of the lyophilised extract has been shown to be compatible with the microencapsulation method, which involves the use of sodium alginate microspheres. This has resulted in a 91% microencapsulation rate for hydroxytyrosol and a 96% rate for oleuropein.

In view of these considerations, olive leaves, a by-product present in large quantities, inexpensive, and rich in phytochemical compounds and minerals, could become a high-value resource useful in reducing waste disposal costs for the olive industry.

In conclusion, the results of the preliminary study have indicated that the selected extract and microencapsulated formulation have the potential to develop a functional product that may have health benefits.

Further research is required to evaluate the effect of the extended production process on the bioactive phenols and minerals. It is also crucial to ascertain the behaviour of the goat yoghurt formulation within gastrointestinal environments, including *in vitro* and *in vivo* studies. Additionally, future research is required to validate consumer acceptance of the proposed product. This should entail a taste testing and a sensory analysis to ascertain whether the product meets the desired standards. Finally, the bioactive potential of yoghurt consumption should be assessed.

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