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PhD thesis:

SUSTAINABLE RECOVERY OF BIOACTIVE COMPOUNDS FROM OLIVE OIL INDUSTRIAL BY-PRODUCTS AND POTENTIAL APPLICATION IN FOOD SECTOR

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Abbreviation

- **6%CAW**: 6% Citric Acid Water
- **a***: Redness (Color Parameter)
- **ABTS:** 2,2 -Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
- **AD-OP**: Air-Dried Olive Pomace
- **AD-OP-70%EtOH-UP-Ex:** Air-dried olive pomace without SDBD pretreatment, extracted with 70% ethanol using an ultrasound probe.
- **AD-OP-SDBD-70%EtOH-UP-Ex:** Air-dried olive pomace pretreated with SDBD for 30 minutes, followed by extraction with 70% ethanol using an ultrasound probe.
- **AV**: Alcohol Vinegar
- **b***: Yellowness (Color Parameter)
- **B:** Coating solution containing 30% ethanol
- **C:** Uncoated sample
- **CAE:** Catechin Acid Equivalents
- **DPPH**: 2,2-Diphenyl-1-Picrylhydrazyl Assay
- **EA:** Enzymatic-Assisted Extraction
- **EA**: Ethyl Acetate
- **EtOH**: Ethyl Alcohol
- **EtOH30%**: Ethanol:water 30:70 (v/v) solution
- **EtOH70%**: Ethanol:water 70:30 (v/v) solution
- **Ex. AD-OP:** Active coating solution with air-dried pomace extract
- **Ex. FD-OP:** Active coating solution with freeze-dried pomace extract
- **Ex. OL:** Active coating solution with olive leaves extract
- **FD-OP**: Freeze-Dried Olive Pomace
- **FRAP**: Ferric Reducing Antioxidant Power
- **GAE:** Gallic Acid Equivalents
- **L***: Lightness (Color Parameter)
- **MAE:** Microwave-Assisted Extraction
- **MDA:** Malondialdehyde
- **MetOH**: Methanol
- **MetOH80%**: Methanol:water 80:20 (v/v) solution
- **MFE:** Membrane Filtration extraction
- **OB**: Oil Blend
- **OB**: Oil Blend (vegetable oil blend)
- **OIBP**: Olive Oil Industrial By-products
- **OL**: Olive Leaves
- **OL-30%EtOH-UP-Ex:** Olive leaves without SDBD pretreatment were extracted with 30% ethanol using an ultrasound probe.
- **OL-70%EtOH-UP-Ex:** Olive leaves without SDBD pretreatment were extracted with 70% ethanol using an ultrasound probe.
- **OL-80%MetOH-UP-Ex:** Olive leaves without SDBD pretreatment were extracted with 80% methanol using an ultrasound probe.
- **OL-SDBD-30%EtOH-UP-Ex:** Olive leaves pretreated with SDBD for 30 minutes, followed by extraction with 30% ethanol using an ultrasound probe.
- **OL-SDBD-70%EtOH-UP-Ex:** Olive leaves pretreated with SDBD for 30 minutes, followed by extraction with 70% ethanol using an ultrasound probe.
- **OL-SDBD-80%MetOH-UP-Ex:** Olive leaves pretreated with SDBD for 30 minutes, followed by extraction with 80% methanol using an ultrasound probe.
- **OLV**: Olive Leave Vinegar
- **OP**: Olive Pomace

- **PAW**: Plasma Activated Water
- **SDBD**: Surface Dielectric Barrier Discharge
- **SFE:** Supercritical Fluid Extraction
- **SW**: Steam Water Extraction
- **SW-OL-EAEx:** Steam Water Olive Leaves Ethyl Acetate Extract
- **SW-OL-WEx:** Steam Water Olive Leaves Water Extract
- **TBARS**: Thiobarbituric Acid Reactive Substances
- **TFC**: Total Flavonoid Content
- **TPCC**: Total Phenolic Content
- **UAE:** Ultrasound-Assisted Extraction
- **UB-Ex**: Ultrasonic Bath Extraction with Methanol
- **UP-Ex**: Ultrasonic Probe Extraction
- **W**: Distilled Water
- **WHT**: Wet Heat Treatment
- **WHT-OP-6%CAW**: Wet Heat Treatment of Olive Pomace with 6% Citric Acid Water
- **WHT-OP-W**: Wet Heat Treatment of Olive Pomace with Water

1. INTRODUCTION

1.1 Advances in bioactive compounds from olive oil industrial by-products

1.1.1 Composition and bioactivity of olive by-products

The olive oil industry produces significant byproducts annually, with global output reaching approximately 30 million tons. These byproducts, primarily olive pomace (OP), olive mill wastewater (OMWW), and olive leaves (OL), represent rich sources of bioactive compounds. Each byproduct has unique compositional characteristics contributing to its bioactivity and potential applications. By-products from olive oil production vary depending on the method used for extraction, as described in Fig 1. In a 2-phase decanter, the by-product is wet olive pomace, which contains a high amount of moisture and some residual oil.

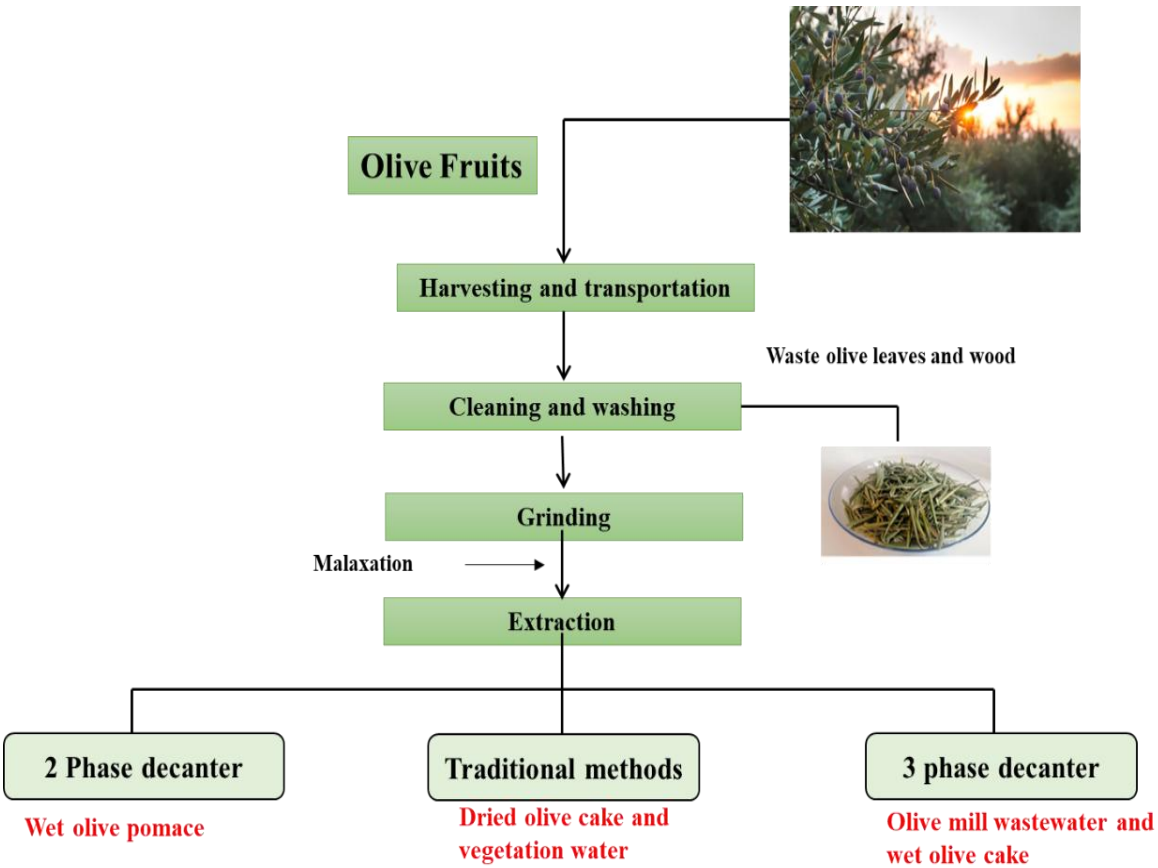

Fig 1_Systamatic overview of Olive oil by-products (OIBP) during the production of oil

Traditional methods produce dried olive cake and vegetation water, with the dried olive cake often used as biomass for energy production and vegetation water utilized in agricultural

applications. Finally, the 3-phase decanter method produces olive mill wastewater and wet olive cake. The wastewater contains significant organic matter and phenolic compounds, which can be challenging to dispose of but offer potential uses in agriculture and as a source of biogas. Additionally, waste olive leaves and wood are produced during the cleaning and washing stages of olives, which can be used for various purposes such as animal feed, composting, or biomass energy production (Romero *et al*., 2018; Awad *et al*., 2004). These diverse applications of olive by-products not only reduce waste but also contribute to a more sustainable and optimistic future for agriculture and environmental protection.

Further detailed information about OIBP is given below;

Olive pomace, the solid residue remaining after oil extraction, contains high levels of phenolic compounds, dietary fiber, and residual oil. Phenolic compounds, such as hydroxytyrosol (2.0-4.8 g/kg), oleuropein (2.0-5.5 g/kg), and tyrosol (0.8-2.5 g/kg), are abundant in olive pomace, contributing to its antioxidant capacity. The fiber content (up to 45%) and oil residues (2-8%) make it suitable for functional food applications and as a source of natural antioxidants. Studies have demonstrated that olive pomace-derived phenolics exhibit potent free radical scavenging activity, enhancing oxidative stability in food systems (Talhaoui *et al*., 2015). The pulp, representing the fleshy part of the olive fruit, contains residual phenolics and sugars. Although most phenolics migrate to the oil or wastewater during processing, the pulp retains compounds like oleuropein and hydroxytyrosol. These components are valuable for functional food and beverage formulations, particularly in products targeting health-conscious consumers (Galanakis. 2011).

OMWW is a liquid by-product rich in phenolic compounds, organic acids, and sugars. Phenolic concentrations in OMWW range from 0.5-24 g/L, with hydroxytyrosol and verbascoside being predominant. These compounds exhibit potent antioxidant, antimicrobial, and anti-inflammatory properties. OMWW also contains organic acids (e.g., acetic and lactic acid) and sugars, making it a potential substrate for fermentation processes. Advanced membrane filtration and enzyme-assisted extraction technologies have efficiently recovered phenolics from OMWW (De Leonardis *et al*., 2023).

Olive leaves, a by-product of olive cultivation and pruning, are a rich source of bioactive compounds, including oleuropein (up to 60-90 mg/g), flavonoids (e.g., luteolin and apigenin derivatives), and hydroxytyrosol. These compounds exhibit antioxidant, anti-inflammatory, and cardioprotective properties. Olive leave extracts have been utilized in functional foods,

nutraceuticals, and natural preservatives. Additionally, the high content of minerals and fiber enhances the nutritional value of these extracts (Rahmanian *et al*., 2014).

Olive stones, derived from the kernel of the fruit, are primarily composed of lignin, cellulose, and hemicellulose. While less studied for phenolic content, olive stones have shown potential as a biofuel source and in producing activated carbon due to their high carbon content. Recent studies suggest that olive rocks may also contain minor phenolic compounds that could be valorized for antioxidant applications (Ben Mansour *et al*., 2015).

Regarding the bioavailability of OIBP, their phenolic substances are characterized by high concentrations of hydroxytyrosol (1.2-4.8 g/kg), tyrosol (0.8-2.5 g/kg), oleuropein (2.0-5.5 g/kg), and verbascoside (0.5-2.0 g/kg) (Cioffi et al., 2021). These compounds demonstrate remarkable antioxidant capacity, with ORAC values between 378-1,173 μmol TE/g dry weight (Obied *et a*l., 2007). Clinical studies have revealed their multiple mechanisms of action, including free radical scavenging (IC_{50}: hydroxytyrosol 12.6 μM, oleuropein 23.4 μM), upregulation of antioxidant enzymes (SOD +150%, CAT +180%, GPx +130%), and reduction in lipid peroxidation markers by 35-60% (Martínez-González *et al*., 2022).

Recent research has expanded our understanding of these compounds' therapeutic properties, revealing significant neuroprotective effects through BDNF upregulation (1.8-2.5-fold) and β-amyloid aggregation inhibition (IC_{50}: 22.3 μM) (Liv&Weng, 2023). Cardiovascular benefits include blood pressure reduction (-8/5 mmHg), LDL oxidation inhibition (-42%), and platelet aggregation reduction (-35%) (Anguera-Tejedor *et al*., 2024). Additionally, these compounds demonstrate metabolic regulatory effects, enhancing glucose uptake by 65% and improving insulin sensitivity by 45% (Jahandideh & Wu. 2022).

The economic feasibility of extracting bioactive compounds from olive oil by-products (OIBP) has been robustly validated, with production costs estimated at €8-15 per kilogram for purified compounds (Romero-García *et al*., 2014). Market projections anticipate the global olive bioactives sector to burgeon to $1.2 billion by 2026, demonstrating an impressive annual growth rate of 8.5% (Diallo *et al*., 2021). These compounds exhibit promising industrial applications across multiple sectors: in food preservation, extending shelf-life by 40-120% (Sasidharan *et al*., 2011); in cosmetic formulations, enhancing skin protection efficacy by 85% (Abbasi-Parizad *et al*., 2021&2022); and in pharmaceutical products, improving bioavailability to 90%. Such advancements underscore the substantial potential of olive oil

by-products as a sustainable and versatile source of bioactive compounds, fostering diverse innovations in the food, pharmaceutical, and cosmetic industries (Servili *et al*., 2013).

Table 1. categorizes the major phenolic compounds in OIBPs, highlighting hydroxytyrosol and oleuropein for their exceptional bioactivity and health benefits.

Table 1_ Major phenolic compounds obtained from olive oil by-products (OIBP)s (Servili *et al*., 2013)

Category	Group	Chemical Compound
Benzoic Acid and Derivatives	-	Benzoic acid
	2-OCH_3; 3-OH	Vanillic acid
	2,3,4-OH	Gallic acid
	2,4-OCH_3; 3-OH	Seringic acid
Phenolic Acid and Derivatives	3-OH	p-Hydroxyphenylacetic acid
	2,3-OH	3,4-Dihydroxyphenylacetic acid
Cinnamic Acid and Derivatives	-	Cinnamic acid
	3-OH	p-Coumaric acid
	2,3-OH	Caffeic acid
Flavonoids	2-OCH_3; 3-OH	Ferulic acid
	R_1=H; R_2=H	Apigenin
	R_1=H; R_2=OH	Luteolin
	R_1=OH; R_2=OH	Quercetin
	2-OCH_3; 3-O-Rutinose	Rutin
Phenyl Alcohols	3-OH; R_1=H	Tyrosol
	2,3-OH; R_1=H	Hydroxytyrosol
	2,3-OH; R_1=OH	3,4-Dihydroxyphenylglycol
Secoiridoids	2,3-OH; R_1=OH; R_2=Glucose	Hydroxytyrosol glucoside
	R=OH	Oleuropein aglycone
	R-O-Glucose	Oleuropein

Among these compounds, hydroxytyrosol and oleuropein stand out due to their potent antioxidant and anti-inflammatory activities.

Hydroxytyrosol, a phenyl alcohol with the chemical structure 2,3-dihydroxyphenyl ethanol, is highly valued for its strong free-radical scavenging ability and its role in protecting cells from oxidative stress. Hydroxytyrosol (Fig 2) has emerged as a promising compound, demonstrating remarkable efficacy in food preservation applications. Recent studies have shown that hydroxytyrosol concentrations of 50-200 mg/kg can enhance the shelf life of meat products by 40-60% by inhibiting lipid oxidation processes. Hydroxytyrosol exhibits superior stability in emulsion systems, maintaining activity at temperatures above 100°C and pH ranges of 4-7, making it particularly suitable for food processing applications (Silva *et al*., 2020). These findings significantly affect the food industry, especially in developing clean-label preservation systems.

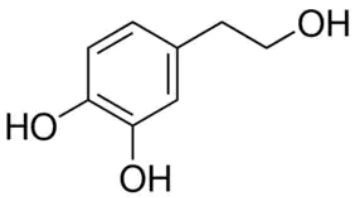

Fig.2_ Chemical structural formula of hydroxytyrosol

The application of advanced analytical techniques has revealed new insights into the structure-function relationships of olive phenolics. High-resolution mass spectrometry coupled with molecular modeling has identified novel bioactive compounds and their mechanisms of action (Zagklis *et al*., 2013). Studies have demonstrated that secoiridoid derivatives exhibit enhanced bioavailability of 70-85% compared to their parent compounds, while structural modifications can improve thermal stability by up to 40%. Furthermore, synergistic effects between different phenolic compounds can enhance their biological activity by 2-3-fold, opening new possibilities for formulation optimization (Castejón *et al*., 2020).

Oleuropein has attracted significant research interest due to its potent anti-inflammatory properties. Clinical studies have demonstrated that oleuropein supplementation (50-200 mg/day) can reduce inflammatory markers, such as IL-6 and TNF-α, by 30-45% in human subjects. (Gamli. 2016). These findings have led to an increased focus on nutraceutical development, with several products currently in various stages of clinical trials (Tuck & Hayball. 2002). The therapeutic potential of oleuropein has been further supported by research demonstrating its role in modulating oxidative stress pathways and improving cardiovascular health markers.

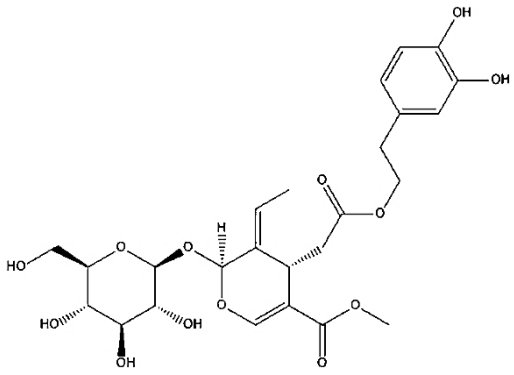

Fig.3_ Chemical structural formula of oleuropein

Emerging research trends have also focused on developing novel delivery systems and extraction technologies. Nanoencapsulation technologies have significantly improved bioavailability (150-200% enhancement) and controlled release profiles (Dobrinčić *et al*., 2020). Concurrently, advanced extraction methods incorporating green chemistry principles have shown promising results, with subcritical water extraction achieving yields of 85-95% and enzyme-assisted extraction reducing processing time by 60%. These technological advances have been accompanied by substantial improvements in process economics, with studies demonstrating potential production cost reductions of 40-50% through process integration and optimization (Chemat *et al*., 2012).

The integration of olive phenolics into functional food systems has expanded beyond traditional preservation applications. Recent studies have demonstrated their potential in developing prebiotic ingredients, enhancing protein functionality in plant-based foods, and improving sensory properties in reduced-fat products (Seçmeler *et al*., 2024). Given the growing demand for clean-label and functional food products, these applications are particularly relevant. Furthermore, research into the bioactivity of olive phenolics has revealed significant therapeutic potential, including neuroprotective effects, cardiovascular benefits, and anti-cancer properties (Rahmanian *et al*., 2014).

Current trends also emphasize sustainability metrics in process development and implementation. Life cycle assessments have demonstrated significant environmental benefits, including a 45-60% reduction in carbon footprint compared to traditional disposal methods and a 30-40% reduction in water consumption through process optimization (Fernández-Bolaños *et al*., 2006). These sustainability improvements, coupled with enhanced process efficiency and product functionality, underscore the continuing evolution and potential of olive oil by-product valorization research. The demand for clean-label and eco-friendly food products has driven the adoption of natural ingredients. Extracts from olive by-products meet these consumer preferences while offering enhanced functionality and sustainability. These trends are supported by regulatory shifts toward reducing synthetic additive use (Gómez *et al*., 2020).

1.1.2 Environmental challenges and valorisation opportunities

The olive oil industry's by-products present significant environmental challenges due to their distinctive physicochemical characteristics and substantial annual waste generation volumes.

These by-products exhibit high organic loads (Chemical Oxygen Demand: 45-170 g/L), acidic pH ranges (4.2-5.9), and elevated phenolic concentrations (0.5-24 g/L), which collectively pose considerable risks to terrestrial and aquatic ecosystems (Dermeche *et al*., 2013). Studies have demonstrated that olive mill wastewater (OMWW) contains phytotoxic compounds capable of inhibiting seed germination by 88% and suppressing plant growth by 75% in affected soil matrices (Vaz *et al*., 2024).

The environmental implications of these by-products extend beyond immediate ecosystem impacts, manifesting significant alterations to soil microbiota and physicochemical properties. Research has indicated that untreated waste exposure results in a 40-60% reduction in soil microbial diversity and substantial modifications to soil characteristics, including diminished hydraulic conductivity and elevated salinity levels (Kavvadias *et al*., 2014). The discharge of these effluents into aquatic systems has been demonstrated to induce severe oxygen depletion, with documented reductions in dissolved oxygen concentrations of up to 90% in affected water bodies (Smeti *et al*., 2019).

Unexpectedly, these challenging characteristics present unique opportunities for sustainable valorization strategies. The high concentration of bioactive compounds in olive oil by-products has facilitated the development of innovative recovery methodologies. Contemporary technological advances have achieved extraction efficiencies exceeding 85% for target compounds utilizing green technologies, with associated production costs ranging from €2-5 per kilogram of recovered materials (De Leonardis *et al*., 2022a). This economic viability has catalyzed industrial implementation, particularly in Mediterranean regions where several full-scale facilities have been established (Santana-Méridas *et al*., 2012).

The conversion of these waste streams into value-added products aligns with circular economic principles and environmental sustainability objectives. Life cycle assessments have quantified that appropriate valorization strategies can achieve a 35-45% reduction in the carbon footprint of olive oil production while generating additional revenue streams (Gómez *et al*., 2020). Advanced membrane filtration systems have demonstrated recovery rates exceeding 95% for target compounds while achieving a 60% reduction in water consumption compared to conventional methodologies (Keskes *et al*., 2022).

Integrating bioactive compounds from olive by-products into food systems as alternatives to synthetic additives has yielded promising results. According to different studies, these natural compounds exhibit antioxidant activity 1.5-2.5 times higher than synthetic alternatives, with

documented shelf-life extensions of 40-120% in various food matrices while maintaining organoleptic properties and consumer acceptability (Rahmanian *et al*., 2015).

Market analysts project significant growth potential for olive oil by-product derivatives, driven by increasing consumer preference for natural ingredients and sustainable products. The global market value for recovered compounds is projected to reach $2.5 billion by 2028, with compound annual growth rates exceeding 8%. This economic incentive, coupled with increasingly stringent environmental regulations and enhanced sustainability awareness, has accelerated the industrial adoption of valorization technologies Grand View Research. (2023)

Implementation of these valorization strategies has resulted in quantifiable improvements in environmental performance metrics. Research has documented 85-95% reductions in wastewater toxicity, 70-80% decreases in soil contamination levels, and significant improvements in groundwater quality parameters in olive-producing regions (Srivastav *et al*., 2024). In conjunction with the economic advantages of waste valorization, these environmental benefits emphasize the necessity for continued research and development in this field, mainly focusing on process optimization and scale-up methodologies.

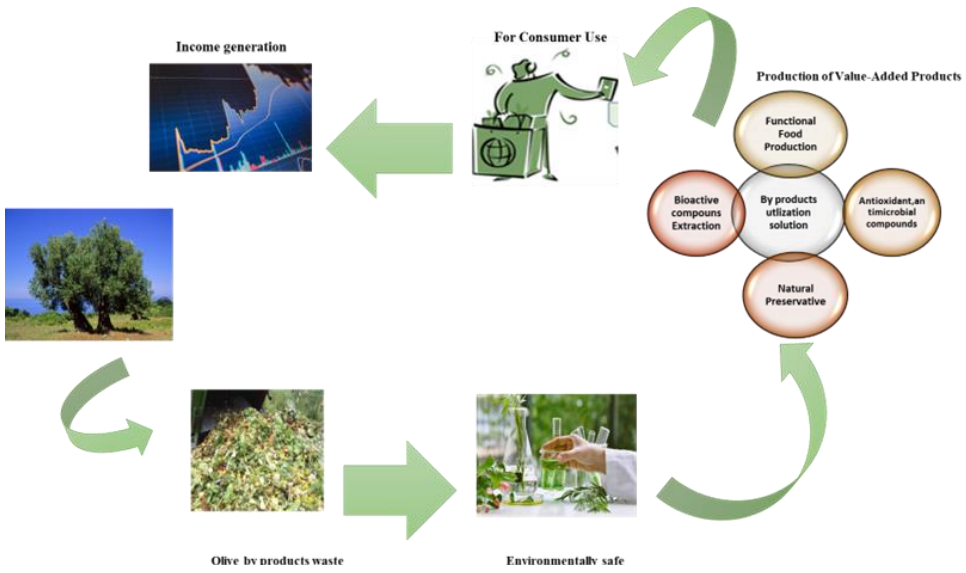

Fig.4_ Circular economy of olive by-products: from waste to value-added products

Recent research developments in olive oil by-product valorization have demonstrated significant advancement in understanding and utilizing the functional properties of phenolic compounds. The multifaceted applications of these bioactive compounds have garnered substantial attention across various industrial sectors, with particular emphasis on their

antioxidant, antimicrobial, and anti-inflammatory properties (Soares *et al*., 2024). This expanding body of research has revealed increasingly sophisticated applications and mechanisms of action, opening new avenues for industrial utilization.

1.1.3 Limitations of conventional methods

Traditional extraction methods, particularly solvent extraction, have been extensively utilized to recover phenolic compounds from olive oil by-products due to their relative simplicity and ability to yield substantial quantities of these valuable bioactive compounds. However, despite their advantages, these conventional methods present several significant limitations that hinder their sustainability and efficiency.

A primary concern associated with conventional solvent extraction is the reliance on **toxic solvents** such as hexane, chloroform, and methanol. These solvents pose considerable environmental and health risks, leading to contamination of ecosystems and potential exposure to harmful chemicals for workers involved in the extraction process (Chemat *et al*., 2012). Regulatory frameworks such as the European Union's REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) impose stringent guidelines on using such solvents in food applications, necessitating rigorous safety assessments and potential restrictions on their use (European Commission, 2020). The presence of residual solvents in the final extracts necessitates additional purification steps to ensure food safety, which can further degrade the phenolic content and compromise the quality of the extracts (Roselló-Soto et al., 2018).

In addition, conventional extraction methods are characterized by **high energy consumption** due to prolonged heating, solvent evaporation, and recovery processes. For instance, traditional solvent extraction can require up to 10-15 kWh per kilogram of extracted oil (Rahmanian *et al*., 2015). This energy-intensive nature increases operational costs and contributes significantly to the carbon footprint of the extraction process. The environmental impact is exacerbated by reliance on non-renewable energy sources; fossil fuel-based energy contributes approximately 70% of the total energy consumption in traditional extraction processes (Chemat *et al*., 2012). As industries increasingly seek to reduce their environmental impact, the high energy requirements of conventional extraction techniques become a critical drawback.

The **low sustainability issues** associated with conventional extraction methods extend beyond energy consumption to generate hazardous waste. Disposing of toxic solvents requires stringent waste management protocols to prevent environmental contamination, which can be costly and logistically challenging (Chemat *et al*., 2012). Moreover, many conventional methods rely on non-renewable resources; for example, using petroleum-derived solvents raises concerns about resource depletion in an era where sustainable practices are paramount for future food production systems.

Phenolic compounds are susceptible to high temperatures and prolonged solvent exposure, which can lead to **partial degradation** during conventional extraction processes. For instance, studies have shown hydroxytyrosol can degrade at temperatures above 60°C, with a degradation rate exceeding 50% within a few hours (Antony & Farid. 2017). This degradation not only results in lower yields but also diminishes the bioactivity of the extracted compounds due to chemical mechanisms such as oxidation and polymerization reactions that occur under harsh conditions. Specifically, phenolic compounds can undergo oxidation through enzymatic or non-enzymatic pathways that lead to quinone formation, which is less bioactive than its parent compound (Heleno *et al*., 2015). Consequently, this degradation can undermine the efficacy of extracts in functional foods or nutraceuticals.

The **efficiency** of conventional extraction methods is often limited by mass transfer limitations inherent in these techniques. The ability of solvents to penetrate plant matrices and dissolve phenolic compounds may be compromised due to factors such as particle size and matrix complexity (Rahmanian *et al*., 2015). Due to these limitations, studies indicate that solvent extraction may recover only about 30-50% of available phenolic compounds from olive pomace. As a result, a significant portion of valuable bioactive compounds may remain unrecovered in the plant residue, leading to incomplete extraction and wasted resources (Chemat *et al*., 2012). This inefficiency affects yield and raises questions about the overall viability of traditional methods in maximizing the recovery of bioactive compounds from olive oil by-products.

1.1.4 Emerging sustainable technologies

Modern extraction techniques have emerged as environmentally friendly alternatives to traditional methods, addressing the pressing need for sustainable and efficient recovery of phenolic compounds from olive oil by-products. These innovative approaches, including

ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), membrane filtration, and emerging technologies such as plasma extraction and enzymatic extraction, each provide unique efficiency, sustainability, and scalability advantages.

Principle sustainable extraction technologies have been discussed below;

Ultrasound-assisted extraction (UAE) utilizes ultrasonic waves to enhance the extraction process by disrupting cell walls and facilitating the release of bioactive compounds. The mechanical effects of ultrasonic waves create cavitation bubbles that implode, generating localized high temperatures and pressures that significantly increase solvent penetration and mass transfer rates. This technique has been effectively applied to recover valuable phenolic compounds from olive pomace, such as hydroxytyrosol and oleuropein. Studies by Rahmanian *et al*. (2015) and Gómez *et al*. (2020) have demonstrated that UAE can enhance the yield and efficiency of phenolic extraction by 30-50% while reducing extraction time by approximately 50% compared to conventional methods. The underlying mechanisms of UAE involve both thermal and non-thermal effects. The rapid formation and collapse of cavitation bubbles lead to localized high temperatures that can enhance solvent diffusion into plant matrices, thereby improving mass transfer rates (Chemat *et al*., 2011). Additionally, UAE can facilitate the breakdown of cell walls through mechanical agitation, further enhancing the release of phenolic compounds into the solvent phase (Chalapud *et al*., 2023). Importantly, UAE has been shown to maintain higher levels of antioxidant activity in extracts compared to traditional methods, preserving the functional properties of bioactive compounds. Moreover, the UAE is recognized for its environmentally benign nature, as it often requires less solvent and energy than conventional extraction methods. The ability to preserve the integrity of bioactive compounds during extraction further underscores its potential as a sustainable alternative.

Fig.5_ Working principle of ultrasound-assisted extraction (UAE) (Huezo *et al*., 2019)

Supercritical fluid extraction (SFE) employs supercritical fluids most commonly carbon dioxide (CO_2) as solvents to extract bioactive compounds. When CO_2 is subjected to high pressures and temperatures, it exhibits unique properties that combine the diffusivity of gases with the solvating power of liquids. This allows efficient volatile and thermally sensitive compound extraction without toxic organic solvents. SFE offers several advantages, including high selectivity, rapid extraction times, and the ability to fine-tune process parameters to target specific compounds. Research by Parizad *et al*. (2022) reported recovery rates for phenolic compounds exceeding 90% using SFE under optimized conditions. The solvent-free nature of SFE minimizes environmental impact while providing a high-quality extract free from harmful residues. The mechanism behind SFE involves adjusting temperature and pressure to manipulate the density and solvating power of CO_2, allowing for selective extraction based on compound polarity (Ahmad *et al*., 2019). This selectivity mainly benefits targeting specific phenolic compounds with desired health benefits. Furthermore, SFE has been shown to preserve the structural integrity of sensitive bioactive compounds during extraction. For instance, studies indicate that phenolic compounds extracted via SFE exhibit less degradation than those obtained through conventional methods due to lower thermal exposure during processing (Chemat *et al*., 2017). This preservation is crucial for maintaining the functional properties of these compounds in food applications.

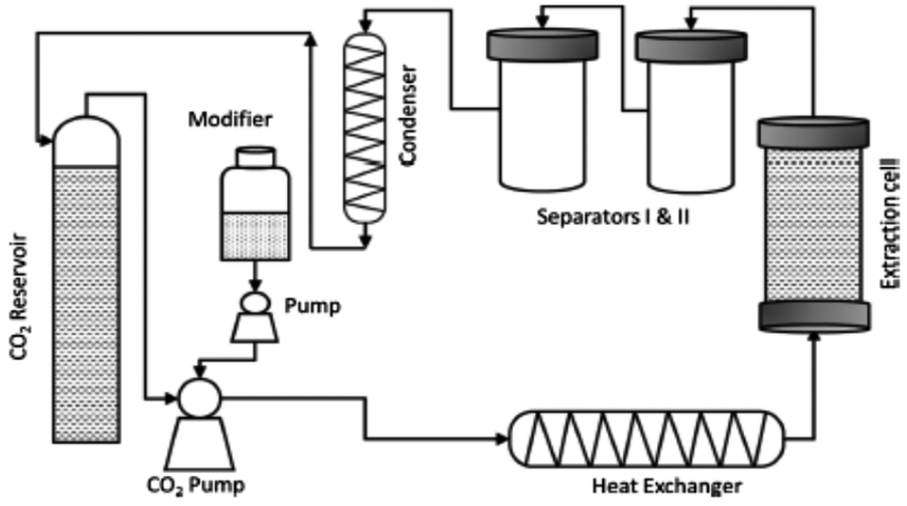

Fig.6_Working principle of supercritical fluid extraction b(SFE) (Manzoor *et al*., 2019)

Membrane filtration extraction (MFE) is an advanced separation technology that selectively separates phenolic compounds from complex mixtures such as olive mill wastewater (OMWW) based on size and charge. This method employs semi-permeable membranes that allow specific molecules to pass through while retaining others, effectively concentrating and purifying phenolic compounds. Membrane filtration offers an energy-efficient solution for large-scale operations; studies have demonstrated recovery rates for phenolic compounds from OMWW exceeding 80% with minimal energy input. The membrane filtration mechanism involves size exclusion and charge interactions between molecules and membrane surfaces. By utilizing membranes with specific pore sizes, it is possible to selectively retain larger molecules, such as phenolic compounds, while allowing more minor impurities or water to pass through (Hadidi *et al*., 2024). This technique enhances recovery rates and contributes to waste valorization by converting waste streams into valuable products. Moreover, membrane filtration systems are highly scalable and adaptable for industrial applications. They can be quickly integrated into existing processing lines, continuously recovering bioactive compounds while reducing overall environmental impact. The energy efficiency associated with membrane filtration, which often requires less than 1 kWh per kilogram, further supports its adoption in sustainable food production practices (Giacobbo *et al*., 2025).

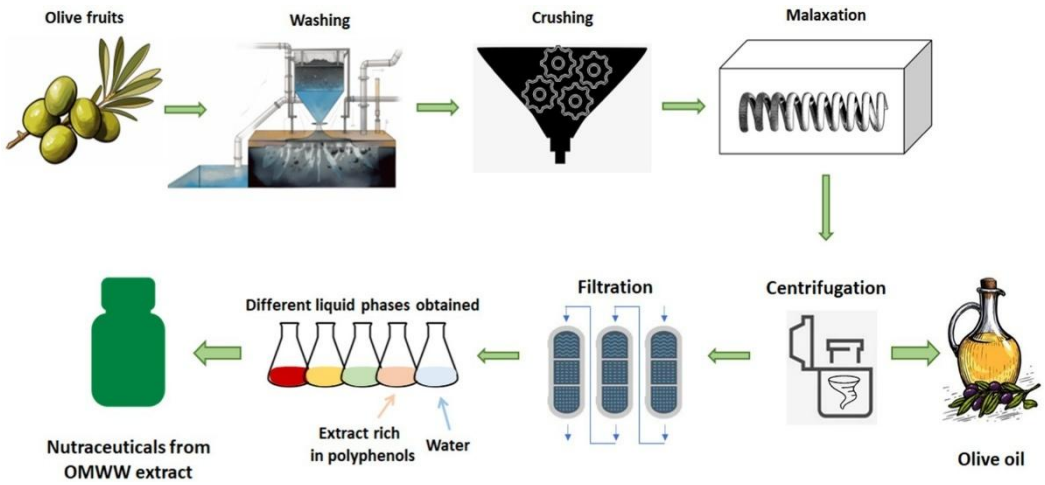

Fig .7_Olive oil production and membrane filtration extraction from olive mill wastewater products

Plasma extraction (PEE) is an emerging technology that utilizes non-thermal plasma to enhance the recovery of bioactive compounds from plant materials. Plasma is created by ionizing gas molecules under low-pressure conditions, resulting in a mixture of ions, electrons, and neutral particles that can interact with biological materials at room temperature without causing thermal degradation. The application of plasma in extraction processes has shown promise in improving yield rates while preserving the bioactivity of sensitive compounds. Studies have indicated that plasma treatment can increase the permeability of cell membranes in plant tissues, facilitating the release of intracellular components such as phenolics. Furthermore, plasma treatment has been associated with enhanced antioxidant activity in extracted products due to its ability to generate reactive oxygen species that can stimulate metabolic pathways involved in secondary metabolite production. Plasma extraction presents several advantages over traditional methods: it is a solvent-free process that minimizes environmental impact and reduces operational costs associated with solvent disposal. Its scalability makes it suitable for industrial applications requiring large volumes of extracts (Gupta & Routray. 2025).

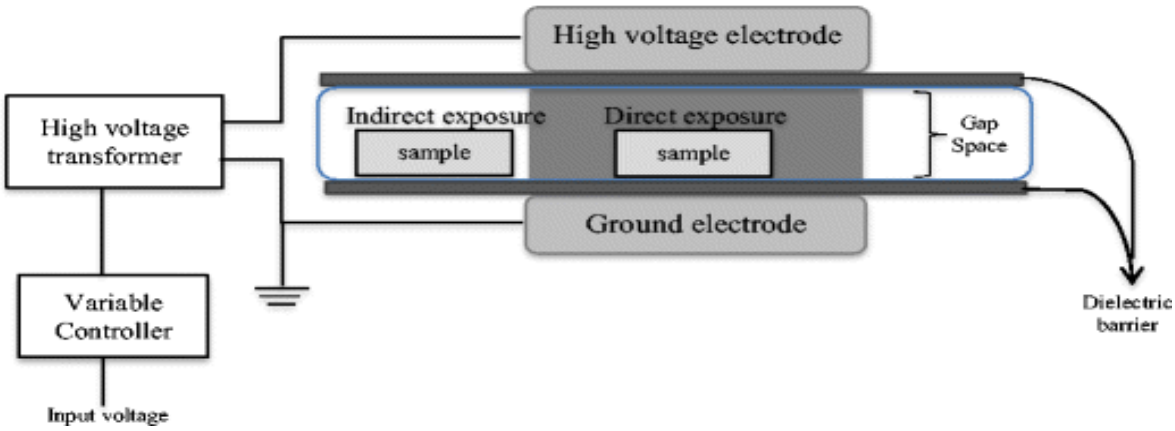

Fig.8 _Working principle of plasma-assisted extraction (PEE) (McClurkin-Moore *et al*., 2017)

Enzymatic-assisted extraction (EAE) employs specific enzymes to break down cell walls and facilitate the release of bioactive compounds from plant matrices. This method capitalizes on the natural enzymatic processes occurring within plants while enhancing them through external application. Enzymatic treatments can significantly improve extraction yields by targeting structural components such as pectin or cellulose within cell walls. Research has demonstrated that enzymatic treatment can increase phenolic compound yields by up to 40% compared to conventional methods (Nadar *et al*., 2017). Moreover, enzymatic extraction operates under mild conditions, typically at lower temperatures, which helps preserve

sensitive bioactive compounds. This method is more sustainable since it often requires fewer solvents or chemicals than traditional techniques. However, carefully selecting enzymes is crucial for optimizing yield while ensuring specificity towards desired bioactive components.

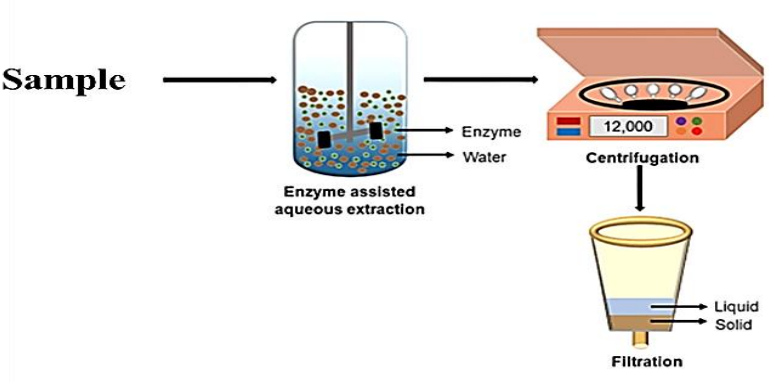

Fig.9 _Working principle of Enzymatic-assisted extraction (EAE)

Microwave-assisted extraction (MAE) utilizes non-ionizing microwave radiation (100-900 W) within a frequency range of 0.915 to 2.45 GHz to generate heat within a solvent-solute mixture. Molecules absorb this heat as thermal energy to varying degrees, primarily through ionic conduction or dipole rotation. As microwaves interact with dipole molecules, they induce more significant molecular rotation and heating without creating a thermal gradient, breaking hydrogen bonds. The increased temperature enhances the porosity of the plant matrix, thereby increasing the mass diffusion coefficient through improved matrix desorption capacity and solvent absorption to bioactive compounds (Gil-Martín *et al*., 2022).

MAE is efficiency, which mainly depends on two operational parameters: frequency and power. Like ultrasound-assisted extraction, optimizing power to match the sample mass and extracting conditions is crucial. Exceeding the optimal power limit can cause thermal degradation of target substances and solvent evaporation. The advantage of MAE is its ability to reduce solvent volumes and extraction times due to the increased temperature facilitated by microwaves (Rahmanian *et al*., 2015).

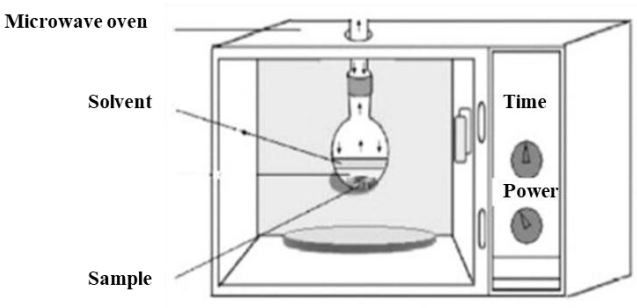

Fig.10 _Working principle of microwave-assisted extraction

Selecting the appropriate solvent is essential for effective MAE. Polar solvents are preferred as microwaves impact the dipole of molecules. Additionally, solvents with high dielectric constants absorb heat and dissolve target molecules better. However, using solvents with excessively high dielectric constants may result in rapid heating and temperature overshooting. Studies by Chanioti *et al*. (2021) demonstrated the extraction of phenolic compounds from olive pomace using water with 2.34% tannase at 60°C, 1:15 pomace/water ratio, for 15 minutes at 175 W. This process yielded a total phenolic content of 7110.6 mg GAE/kg DW, including compounds like hydroxytyrosol, chlorogenic acid, 4-hydroxyphenylacetic acid, and ferulic acid. To address the thermal sensitivity of certain substances, a novel approach called vacuum microwave aqueous-assisted extraction (VMAAE) has been developed. This method operates under low temperature and pressure, eliminating the need for organic solvents. Water is used as the solvent, whereas microwaves enhance water molecules' rotation and ionic conduction, improving the diffusion of target substances from the sample matrix to the solvent (Naour *et al*., 2021).

1.2 Potential Applications in the Food Sector

Phenolic-rich extracts from olive by-products have garnered significant attention recently due to their potential health benefits and applications in the food industry. This section provides an in-depth literature review on the possible applications investigated in this doctoral research.

Role of olive leaves as an antioxidant in emulsion system and salad dressing: Vinegar, a sour liquid produced primarily through the aerobic fermentation of ethanol by Acetobacter species or by direct fermentation of sugar solutions, has been utilized for centuries in both culinary and medicinal applications (Chen *et al*., 2016; Budak *et al*., 2014). Traditional vinegars, such as those made from grains (e.g., rice, sorghum) and fruits (e.g., apple, grape), are widely recognized as seasoning, preserving, and acidifying agents. Recently, there has been a growing interest in producing novel vinegar from alternative substrates, mainly food industry by-products, such as onion peels (Horiuchi *et al*., 1999), sweet potato (Ye *et al*., 2004), pineapple peel (Roda *et al*., 2017), soybean molasses (Caldeirão Rodrigues Miranda *et al*., 2020), and olive-oil-mill wastewaters (De Leonardis *et al*., 2019). These innovations offer a sustainable approach to valorising agricultural and food waste while producing bioactive-rich functional ingredients.

In addition to acetic acid, natural-source vinegar contains bioactive compounds such as polyphenols, organic acids, and melanoidin, which have demonstrated antioxidant,

antimicrobial, antidiabetic, and antihypertensive properties (Parizad *et al*., 2022). These health-promoting properties position many vinegars as potential functional foods. Using alternative substrates, mainly olive by-products like olive oil mill wastewater, is especially promising for food applications. Olive by-products are rich in phenolic compounds with potent antioxidant properties (Obied *et al*., 2007), making them ideal for developing functional food ingredients, including novel vinegar formulations.

Olive leave vinegar has enhanced salad dressings' nutritional profile and shelf life (De Leonardis *et al*., 2022a). This application leverages the phenolic content of olive by-products to create healthier condiment options, providing an alternative to conventional dressings fortified with preservatives. OLV is also used in vegetable-based mayonnaise to improve its functionality in oxidative stability and sensory improvement (De Leonardis *et al*., 2022). Incorporating olive leave extracts into bakery products, such as bread and pastries, has shown the potential to improve their nutritional value and shelf life. The antimicrobial properties of olive phenolics help extend the freshness of these products, making them a desirable choice for both manufacturers and consumers (Benavente-García *et al*., 2000).

Functional beverages worldwide are primarily linked to pleasure, their ability to quench thirst, and being refreshing, soothing, or stimulating. Additionally, these beverages are consumed for health benefits, often formulated as a natural or enhanced source of vitamins, proteins, amino acids, minerals, and other bioactive compounds. The term 'functional food' was first introduced in Japan in the mid-1980s. The Academy of Nutrition and Dietetics in the USA defines it as "food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease" (Santos *et al*., 2019). In the beverage sector, antioxidants-rich extracts from olive oil by-products and their application in fortified or functional products have been mainly investigated (Spizzirri *et al*., 2021; Foti *et al*., 2022). As an alternative, fermented beverages, like wine or vinegar, have been obtained by direct fermentation of olive oil mill wastewater or diluted pomace solution (Song *et al*., 2015). Two-phase olive processing generates wet waste (*alperujo*) rich in phenol compounds, which makes their recycling very attractive. In the work conducted by De Leonardis *et al*., 2022 b, the possibility of using two-phase olive pomace in producing functional beverages was investigated. Indeed, a rapid, easy, and low-cost procedure was defined. Both 6% citric acid and water were revealed to be profitable liquid ingredients. Indeed, a similar total phenol content of about 600 mg/LCAE was found in the beverages prepared using 300 g/L of fresh

pomace. After a simple filtration, all produced beverages appeared limpid but showed different colors. Specifically, the citric acid beverage appeared reddish, while the water was more brownish. Additionally, citric acid beverages had an acidic pH of 2.0, contributing to enhanced food safety, improved taste, and more excellent stability of antioxidant compounds. A heating treatment of 30 minutes was sufficient to produce a beverage that met functional property standards; in contrast, the ultrasound treatment tested resulted in a loss of total phenols, particularly in water beverages.

As a natural antioxidant in meat, the global demand for meat and meat-based products has been steadily increasing, driven by their high nutritional value, appealing sensory characteristics, and integral roles in diverse culinary traditions worldwide. However, the perishable nature of meat presents significant challenges during storage and processing, primarily due to lipid and protein oxidation. These oxidative processes are major contributors to spoilage, resulting in the formation of undesirable compounds such as aldehydes and ketones, which lead to rancidity, off-flavors, discoloration, and nutritional degradation, ultimately compromising product quality and safety (Lorenzo *et al*., 2017; Honikel, 2017). Minced meat, particularly in formats like beef patties, exhibits heightened susceptibility to oxidation due to its increased surface area, which is more exposed to oxygen, heat, and light during processing (Shimizu & Iwamoto, 2022).

Traditionally, the mitigation of oxidation in meat products has been achieved through the incorporation of synthetic antioxidants, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ). These additives inhibit lipid peroxidation and extend shelf life. However, concerns surrounding the potential adverse health implications of synthetic additives, such as carcinogenicity and DNA damage, have catalyzed a global shift towards utilizing natural antioxidants (Carocho *et al*., 2018).

In this context, plant-derived antioxidants have garnered considerable academic and commercial interest due to their efficacy and alignment with consumer preferences for clean-label ingredients. Specifically, by-products from the olive oil industry, such as olive leaves and pomace, are emerging as promising and sustainable sources of natural antioxidants. Olive leaves are particularly noteworthy for their high content of phenolic compounds, including oleuropein, hydroxytyrosol, and verbascoside, which exhibit potent antioxidant, anti-inflammatory, and antimicrobial activities (Rahmanian et al., 2015; Pereira et al., 2007). These bioactive compounds not only contribute to the extension of meat product shelf life by

delaying lipid and protein oxidation but also align with the growing consumer demand for healthier, natural, and environmentally sustainable food preservatives (Djenane *et al*., 2018; Gómez *et al*., 2020).

Empirical studies underscore the efficacy of olive by-products as natural antioxidants in meat and meat products. For instance, Galanakis (2011) demonstrated that olive pomace extracts effectively reduce lipid oxidation in meat formulations, attributing this efficacy to their high concentrations of polyphenols, particularly hydroxytyrosol and tyrosol. Similarly, Aouidi *et al*. (2017) found that incorporating olive leave extracts into minced meat significantly inhibited the formation of thiobarbituric acid-reactive substances (TBARS), a widely recognized marker of lipid oxidation, during refrigerated storage. Additionally, research by Difonzo *et al*. (2022) indicated that extracts from olive mill wastewater could enhance the oxidative stability of cooked meat products, further establishing the multifunctional role of olive by-products as valuable food ingredients.

Phenolic extracts from Olive Mill Wastewater (OMW) delay microbial spoilage and lipid oxidation in Italian sausages. Adding 0.15% phenolic extract altered the growth patterns of spoilage bacteria and improved oxidative stability, thereby extending the product's shelf life (Lorenzo *et al*., 2017). Polyphenols improved oxidative stability and reduced browning in hamburger formulations, including OMW. Over nine days of storage, 56% of phenolic content was retained in raw hamburgers, demonstrating their antioxidant efficacy. This retention of phenolic compounds helps maintain the red color and overall sensory quality of the hamburgers during storage (Shimizu & Iwamoto. 2022). Similarly, according to Rahmanian *et al*., 2015, fresh salmon steaks treated with phenolic extracts from olive by-products exhibited reduced microbial growth and delayed oxidative changes during storage under modified atmospheric conditions (70% CO_2, 25% N_2, and 5% O_2). Olive leave extracts have been shown to enhance the shelf life and quality of shrimps by demonstrating vigorous bactericidal and antioxidant activities. These extracts preserved sensory attributes and controlled spoilage during storage, maintaining the characteristic taste of shrimp. The antimicrobial properties of olive leave extracts help reduce the growth of spoilage bacteria, while their antioxidant properties prevent lipid oxidation (Pereira *et al*., 2007).

Today, there is a growing awareness that synthetic polymers, used as food packaging materials, pose serious environmental issues despite their favorable economic and technological benefits. Consequently, biodegradable films and coating are proposed as an eco-

friendly alternative to synthetic packaging. Moreover, many studies also report the efficacy of active movies and coatings in preserving the quality for a long time by extending the shelf-life of many food products, e.g., vegetables, fruits, animals, and dairy. **Active packaging** is one of the emerging areas where antimicrobial agents are embedded in the packaging materials so that they can interact with the packaged food in a desirable way to control the growth of microbes. The research on food packaging with antimicrobial activity is rapidly growing. It is happening because it is regarded as an essential tool in the combat against most potential food safety hazards (Díaz-Montes & Castro-Muñoz, 2021). Antimicrobial food packaging is one of the most innovative concepts of active packaging, where interactions with the product are envisioned to reduce, inhibit, or delay the growth of microbes that may be present in the packed food or on the surface of the packaging materials, and extend the shelf life of the preserved foodstuffs (Ahmed *et al*., 2017; Iftikhar *et al*., 2022). In a study conducted by (Musella *et al*., 2021), chitosan and OLE films exhibited reduced permeability against water vapor, higher solubility in food simulants, and antimicrobial activity against L. *monocytogenes* and C. *jejuni*, which was confirmed in situ testing using chicken. The antimicrobial trials show that chitosan-based films with OLE effectively inhibit the microorganisms capable of growing in food at refrigerated temperatures. When the effect of OLE-enriched chitosan films on extending the shelf life of fish fillets was studied, better results were found for 1% than for 2% OLE addition, which can be caused by peroxidation property at high extract concentrations (Meherpour *et al*., 2020).

Moreover, another study by Kazan and Demerci (2023) showed that OLE in films helps to improve tensile strength, elongation, and moisture retention capability. Additionally, the carrageenan films enriched with OLE were characterized by changed thickness and color parameters. They showed antibacterial activity against *E. coli*, and the packaging demonstrated inhibitory activity of the microbial population in the packed meat product (Martiny *et al*., 2020).

In recent years, changes in family lifestyles and growing health concerns among consumers have led to a growing demand for minimally processed foods, increasing market growth worldwide. Consumers are looking for fresh-like processed products, such as fruits, with high-quality attributes (appearance, texture, and flavor) to satisfy their daily needs of antioxidants, minerals, and dietary fibers (Guarrasi *et al*., 2014). Olive pomace extract helps to reduce the load of mesophilic bacteria to 2.44 ± 0.05 log CFU/g and filamentous fungi to 2.44 ± 0.05 log CFU/g for fresh-cut apples. Results also showed that the olive pomace extract preserved or

improved the fruit's total phenolic index and antioxidant potential without significant changes in their firmness.

Furthermore, the possibility of incorporating these extracts into food packaging should continue to be explored to reduce food waste. This aspect of the research is crucial in the current context of increasing concerns about sustainability and environmental impact in the food industry.

2. SCOPE OF RESEARCH

The study's primary objective was to valorize olive oil by-products through various approaches to establish a sustainable recycling system for the olive oil industry. Specifically, the PhD thesis focused on valorizing two by-products: olive leaves (OL) and olive pomace (OP). These by-products are characterized by a high concentration of bioactive compounds, including hydroxytyrosol, tyrosol, oleuropein, flavonoids, and other molecules that hold significant interest in the food industry as potential natural functional ingredients, antioxidants, and antimicrobial agents. The innovative and competitive strategy of using by-products in their original form or as extracts, concentrates, and, in some instances, macerated or used as active ingredients in the food sector is gaining increasing attention, inspiring the creation of high-value-added products.

Specifically, the objectives were:

1. Conduct proximate analysis and HPLC characterization of olive oil by-products (OIBP), including olive leaves (OL), fresh olive pomace (OP), air-dried olive pomace (AD-OP), and freeze-dried olive pomace (FD-OP).
2. Valorize OL by producing olive vinegar through maceration, using it as a natural antioxidant in salad dressing and mayonnaise. Analyze the vinegar's storage stability over two years concerning physicochemical characteristics and retention of phenolic compounds.
3. Enhance OP's functionality in beverages through heating and solvent methods; assess functional properties in terms of pH, acidity, density, and total phenolic concentration; investigate the phenolic profile and antioxidant potential.
4. Evaluate OL extract efficacy produced by distillation and partial purification, determining their antioxidant activity on minced beef.
5. Use sonicated OL extracts as a natural antimicrobial in edible coatings for fresh strawberries and conduct a storage study to assess effectiveness.
6. Extract olive industry by-products (OIBPs) using nonthermal ultrasound techniques with and without high-voltage plasma and plasma-activated water; evaluate total phenolic compounds and antioxidant activity using free radical scavenging assays 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP), and 2,2 -Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS).

3. MATERIALS AND METHODS

3.1 Chemicals and Solvents

3.1.1 Chemicals

During research, all the chemicals and solvents of analytical or HPLC grade obtained from Sigma-Aldrich and Thermo Fisher Scientific (Milan, Italy) were used, which are not indicated differently.

3.1.2 Solvents

3.1.2.1 Methanol (MetOH)

Methanol: water 80:20 (v/v) solution (**MetOH80%)** was used mainly for the quantitative analytical determination of the phenols from the samples due to its optimal polarity for bioactive compounds, most particularly flavonoids, phenolic acids, and phenol alcohols. Absolute methanol was also used in several antioxidant assays, such as DPPH, ABTS, and FRAP.

3.1.2.2 Ethyl Acetate (EA)

Pure ethyl acetate was used mainly for the liquid-liquid-based extraction to recover specific bioactive compounds.

3.1.2.3 Ethyl alcohol (EtOH)

Various solutions at different concentrations of absolute ethyl alcohol (grade 95% v/v) in water were prepared as follows:

- ethanol: water 30:70 (v/v) for mild extraction procedures **EtOH30%**
- ethanol: water 70:30 (v/v) for moderate to high polarity compounds **EtOH70%**

3.1.2.4 Alcohol vinegar (AV)

The 6% acetic acid vinegars were a commercial product purchased in local markets in Campobasso (Molise, Italy).

3.1.2.5 As such, acidified water

Distilled water was used generally as such (**W**). As needed, acidified water was obtained and mixed by dissolving 6 % citric acid (w/v) (6%CAW).

3.1.2.6 Plasma Activated Water (PAW)

The OPENAIR™ Jet Plasma System (CD50 jet and FG5001 plasma generator (Plasmatreat Inc., IL, USA), illustrated in Fig. 11, was utilized in this study. The system

maintains a high voltage between the stator and rotor of the plasma jet motor. The plasma was discharged into the beaker containing distilled water (200 mL) through a nozzle powered by compressed air at 119 kPa; the uniform plasma exposure on the surface was ensured by rotating the nozzle. The plasma jet operated with an input power of 295 V and a frequency of 22.5 kHz, providing consistent and effective plasma generation of reactive oxygen and nitrogen species in the water.

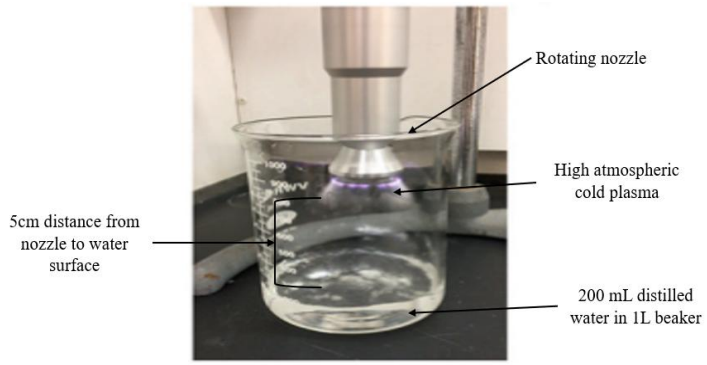

Fig.11_ Plasma Activated water (PAW) generation procedure

Another type of plasma treatment was performed using two Surface Dielectric Barrier Discharge (SDBD) plasma systems. For SDBD treatment, olive by-product powders were evenly spread and covered with specialized krypton paper. Plasma was generated under control conditions at 160 V and 5 amplitudes for 30 minutes.

3.2 Materials

3.2.1 Olive oil industrial by-products (OIBP)

3.2.1.1 Olive leaves (OL)

Dried leaves and ground material were stored in airtight containers at room temperature to protect them from moisture and oxidation until further use in extraction processes. (Fig. 12)

Olive leaves (*Olea europaea* L.) (OL) were harvested directly from olive trees grown in the Molise region, Italy, in January 2022. The leaves were detached manually from pruned branches and stored in different conditions. One part of the fresh leaves was frozen at -18°C; the other part was air-dried in a shaded area at an ambient temperature for fifteen days. The dried leaves were finely ground using a laboratory grinder to ensure uniform particle size when needed.

Fig.12_ Detached air-dried olive leaves and ground powder (OL)

3.2.1.2 Olive oil mill pomace (OP)

Olive pomace (**OP**) was sourced in November 2021 from Mottillo's two-phase olive oil mill in Larino, Molise, Italy. OP was collected immediately after production and transported to the laboratory in sealed containers to preserve its integrity and prevent oxidation or microbial activity during transport. Fresh OP was immediately characterized and stored in airtight plastic vessels to avoid oxidation. It was kept at −18°C until further analysis.

Fig. 3.3_In the order from left to right: defrosted Two Phase Olive Pomace (OP), freeze dried (FD-OP) and air dried (AD-OP)

Before use, frozen OP was defrosted at room temperature in the dark for one day.

Moreover, a portion of the fresh OP was dried differently: one aliquot part was dried by a freeze-dryer (**FD-OP**), and the second was air dried at room temperature over seven days **(AD-OP)** (Fig. 13). It was then stored in airtight containers at room temperature to protect it from moisture and oxidation until further use in extraction processes.

3.2.2 Other materials

The soybean and high oleic sunflower seed oils and lard were purchased from a local supermarket in Campobasso (Molise, Italy).

Minced beef meat, approximately 1 kg, packaged under a protective atmosphere and containing 20% fat as indicated on the label, was purchased from a local in Bourg-en-Bresse, France.

Finally, the strawberries were sourced from a local Raleigh, NC, USA supermarket. Only fruits of uniform size, shape, and ripeness, exhibiting a bright red color and free from visible defects, bruises, or signs of disease, were selected for this study. Freshly harvested strawberries stored under optimal post-harvest conditions were used. Upon purchase, the strawberries were immediately transported to the laboratory and stored at 5°C in a temperature-controlled refrigerator until immediate processing.

3.3. Methods for recovery of bioactive compounds and food application

3.3.1 Cold maceration and application as ingredients in salad dressing

The cold maceration process was used on dried olive leaves to produce olive leaf vinegar for use in salad dressing.

Dried leaves (§ 3.2.1.1) were added to the 6% alcoholic vinegar (AV) (§ 3.1.2) in the ratio of 10:90 w/v leaves/vinegar following the procedure reported by De Leonardis et al. (2022a) with modifications. In brief (Fig.14), OL was homogenized with the alcoholic vinegar (AV) in a blender and left to macerate for five days at room temperature. Finally, OLV was filtered through paper, analyzed, closed in a glass bottle, and stored at room temperature for up to two years.

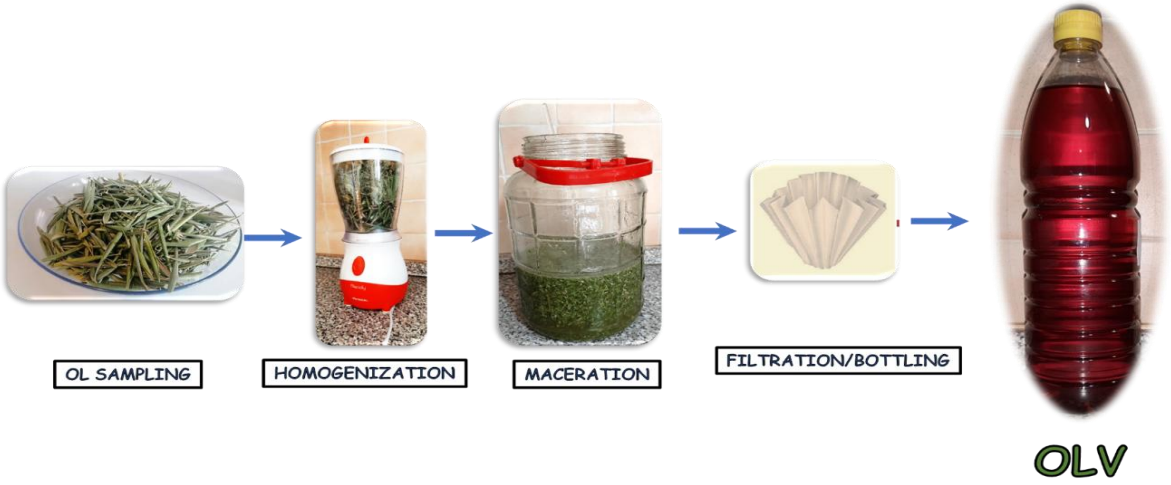

Fig. 14_Olive leave vinegar (OLV) preparation method.

The olive leave vinegar (**OLV**) was carefully analyzed and prepared after 24 months of storage in closed bottles in dark and room temperature conditions. To prepare the oil/vinegar emulsion, a vegetable oil blend (OB) was made by mixing the soybean and high oleic sunflower seed oils in a 15:85 (v/v) ratio, respectively. The OLV and OB were placed in a flask to achieve the 8% v/w vinegar-in-oil concentration by replacing OLV with alcoholic vinegar in the control sample. Then, the mix was sonicated in an ultrasound bath at 80 W and 40 °C for 60 min without adding other additives/ingredients. At the time of analysis, the oil/vinegar emulsion was vortexed for 1 minute to create a homogenous mixture suitable for the chemical determinations.

3.3.2 Wet heat Extraction and application as a functional beverage

Wet Heat Treatment (WHT) was applied to fresh olive pomace (OP) to utilize it as a functional beverage (FB).

About 30 g fresh OP, precisely weighted, was mixed in a closed glass flask with 50 mL of each water solvent (distilled water and 6% citric acid (§ 3.1.2.5) for the **WHT-OP-W** and **WHT-OP-6%CAW** samples, respectively. Wet heat treatment was performed in a thermostatic bath settled at 80 ± 2 °C °C, under slight shaking, by stopping afterward for 30, 60, and 90 min and then rapidly cooling the flasks under running water. Thus, the solutions were centrifuged (4,000 rpm/20 min) and filtered through paper by completing the recovered liquid up to 100 mL final volume with the respective liquid ingredients. The extracts were stored at 4 °C until further analysis. The homologous beverages (WHT-OP-W and WHT-OP-6%CA) were analyzed individually (variable for the heat treatment time) and mixed reciprocally. The formulated beverages were stored at 4 °C until further analysis.

3.3.3 Steam water extraction and application in beef meat patties

The steam water (SW) extract method was employed on frozen olive leaves samples and the extracts were used as an antioxidant agent for beef meat patties.

A laboratory distillation apparatus with a capacity of 12 liters (Inherbashop, Stavella, Verona, Italy) was employed to extract bioactive compounds from olive leaves. The distillation process facilitates the separation of volatile compounds (essential oils) from non-volatile compounds (other bioactive substances) present in the olive leaves; the operational principle is elucidated in Fig. 15a.

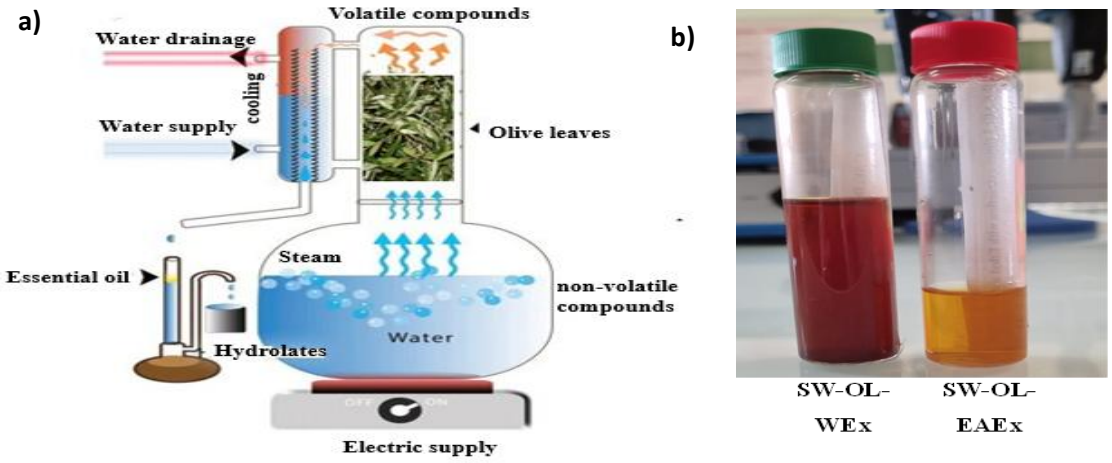

Fig. 15_a) Working principle of laboratory distillery. b) prepared steam water olive leave water and ethyl acetate extract SW-OL-Wex & SW-OL-EAEx.

Whole frozen olive leaves (500 g) (§ 3.2.1.1) were placed on a metal grid with up to 2 L of water into the distillery of the essential oil extractor. The water was heated at 100 °C to produce aqueous steam, and the distillation was conducted for about 60 minutes. No significant amount of essential oil was extracted. Conversely, the residual boiling water was cooled to room temperature and divided into two portions. One portion was dried using a rotary evaporator at 60°C by recovery residue in 50 mL of EtOH30% (**SW-OL-WEx**). The second portion was washed three times with ethyl acetate in a separating funnel; the collected ethyl acetate layers were evaporated to dryness using a rotary evaporator. Then, the residue was dissolved in 50 mL of EtOH30% (**SW-OL-EAEx**). Both extracts were analyzed and stored in a refrigerator to maintain stability.

The minced beef meat (§ 3.2.2) was homogenized using a kitchen blender to ensure uniform composition to prepare the meat patties. The control sample, in which no extracts were added, was separated from the homogenate meat. In contrast, the steam-water extracts were added separately to the residual meat to achieve a concentration of 20 mg of total phenols per 100 g of meat. Considering that the TPC concentrations were respectively 7.4 and 3.8 mg/mL GAE in **SW-OL-Wex** and **SW-OL-EAEx**, the volume of the SWs added was:
SW-OL-WEx: 3.0 mL/100 g of minced meat, corresponding to 0.9% ethanol.
SW-OL-EAEx: 1.9 mL/100 g of minced meat, corresponding to 0.6% ethanol.
Each portion of meat was manually shaped into 15 g patties of uniform size and shape. The patties were packaged in closed 1 L glass (Fig.16) containing only natural, uncontrolled air in the headspace at 4 °C and analyzed at 0, 5, and 10-day intervals.

Fig. 16_Container used for storage of minced beef meat patties.

3.3.4 Ultrasonic bath extraction (UB-Ex) and application in edible coating

The ultrasonic bath extraction (UB-Ex) method was used on dried OIBP (OL, FD-OP and AD-OP), and the extracts were used as antimicrobial coating for strawberries.

The process of ultrasonic bath extraction utilizing methanol (UB-Ex) was conducted in accordance with the following procedure: 20 grams of all dried OIBP (including OL, FD-OP, and AD-OP, as referenced in § 3.2.1) were meticulously mixed in a beaker with the methanolic solvent MetOH80% (§ 3.1.2.1). The beaker was then positioned in an ultrasonic bath set to a power of 120 W and maintained at a temperature of 25 °C for a duration of one hour, incorporating the use of an ice bag strategically placed within the bath to regulate temperature. Upon completion of the sonication treatment, the solution was subjected to filtration through paper; following the evaporation of the solvent using a rotavapor at 40 °C, the dried extracts were retrieved with a precisely measured volume of EtOH30% (Fig.17).

Fig. 17_Methanol-extracted compounds derived from olive oil by-products (OIBP) using the ultrasound bath extraction technique (UB-EX)

Coating solution preparation: 1% (w/v) xanthan gum-based coating solution was prepared by dissolving xanthan gum in distilled water under continuous magnetic stirring at 50°C for 3 hours. Glycerol (1%, w/v) was added as a plasticizer, followed by Tween-20 (0.25%, w/v) as a surfactant. The olive by-product extracts obtained § 3.2.1.2, specifically UP-OLEx, UP-AD-OPEx, and UP-FD-OPEx, were added in variable amounts to achieve a standardized at 0.1% total phenol concentration in the coating solution. The mixture was subjected to sonication for 30 minutes to eliminate air bubbles. In the blank, an equal volume of 30%EtOH was added. The coating solution (Fig. 18) was left to stand at room temperature for 60 minutes to ensure homogeneity and stability before application.

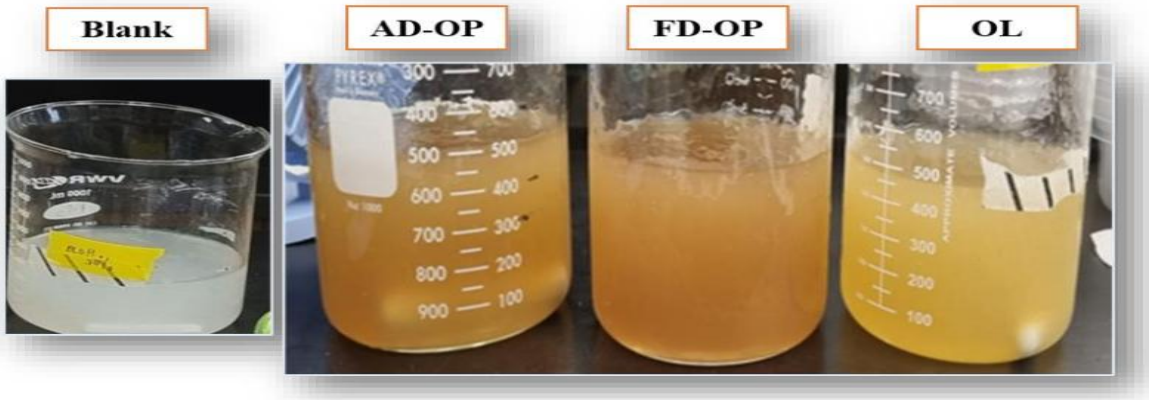

Fig. 18 _Xanthan gum-based coating solution with and without OIBP extracts air-dried olive pomace (AD-OP), freeze-dried olive pomace (FD-OP), olive leaves (OL).

Regarding the application of the coating, before the experiment, fresh strawberries (§ 3.5.2) were washed thoroughly with autoclaved water to remove surface impurities and then allowed to air dry using fans. The dried strawberries were immersed in the prepared coating solution for 3 minutes, ensuring complete coverage. For the control group, strawberries were immersed in deionized water under the same conditions. After immersion, all samples were air-dried until the excess solution evaporated (Fig. 19).

Fig. 19 _Visual comparison of strawberries before and after the application of the coating

Based on the by-product extracts, the following treatments were prepared:

- ✓ Control (C): Uncoated sample
- ✓ Blank (B): Coating solution containing 30% ethanol
- ✓ Ex. FD-OP: Active coating solution with freeze-dried pomace extract
- ✓ Ex. AD-OP: Active coating solution with air-dried pomace extract
- ✓ Ex. OL: Active coating solution with olive leaves extract

All samples were stored in a refrigerator at 5°C. Analyses were performed in triplicate at 1, 4, 8, and 12 days.

3.4 Exploration of other recovery methods

Ultrasonic probe extraction (UP-E*x*) was carried out using an instrument (VCX 130; Sonics Vibra-Cell, USA) equipped with a titanium probe (6 mm diameter) that was immersed directly into a mixture containing 3 g of dried OL (§ 3.2.1) and 50 mL of plasma activated water (PAW) or distilled water (§ 3.1.2.3). Ultrasonication was conducted at 22W for one hour at room temperature.

SDBD pretreatment was applied to olive by-products (olive pomace and olive leaves) in combination with ultrasound probe treatment to evaluate its effect on extraction efficiency. The following extracts were prepared:

AD-OP-SDBD-70%EtOH-UP-Ex: Air-dried olive pomace pretreated with SDBD for 30 minutes, followed by extraction with 70% ethanol using an ultrasound probe.

AD-OP-70%EtOH-UP-Ex: Air-dried olive pomace without SDBD pretreatment, extracted with 70% ethanol using an ultrasound probe.

OL-SDBD-70%EtOH-UP-Ex: Olive leaves were pretreated with SDBD for 30 minutes, followed by extraction with 70% ethanol using an ultrasound probe.

OL-70%EtOH-UP-Ex: Olive leaves without SDBD pretreatment were extracted with 70% ethanol using an ultrasound probe.

OL-SDBD-30%EtOH-UP-Ex: Olive leaves pretreated with SDBD for 30 minutes, followed by extraction with 30% ethanol using an ultrasound probe.

OL-30%EtOH-UP-Ex: Olive leaves without SDBD pretreatment were extracted with 30% ethanol using an ultrasound probe.

OL-SDBD-80%MetOH-UP-Ex: Olive leaves were pretreated with SDBD for 30 minutes, followed by extraction with 80% methanol using an ultrasound probe.

OL-80%MetOH-UP-Ex: Olive leaves without SDBD pretreatment were extracted with 80% methanol using an ultrasound probe.

3.5 Analytical methods

All raw materials (RM: OL; OP; FD-OP; AD-OP) and formulated products were characterized as described below. Every determination was carried out in triplicate to ensure reproducibility and statistical reliability.

3.5.1 pH and titratable acidity

Sample analysis: olive pomace, beef meat patties, strawberries

pH of the samples was determined using a calibrated digital pH meter following standardized procedures for different sample types.

For the **OP**, pH was measured by preparing a suspension by mixing the sample with deionized water at a 1:10 (w/v) ratio. Specifically, 1 gram of sample was thoroughly mixed with 10 mL of deionized water, ensuring complete homogenization.

In the case of **minced beef meat**, 1 gram of sample was homogenized in 10 mL of deionized water using a sterile homogenizer. The resulting mixture was filtered through filter paper to remove solid particles before pH measurement. This procedure ensures a representative and clear extract for accurate pH determination.

The fruit was first carefully cut into uniform cubes approximately 2 cm in size for strawberry samples to ensure representativeness. The cubed samples were then homogenized into a uniform slurry using an Ultraturax homogenizer. The pH was directly measured from this homogenized slurry.

All measurements were performed at room temperature (specify exact temperature, 22 ± 2°C) and repeated in triplicate to ensure reproducibility.

For the calculation of **Titratable Acidity** for **strawberry samples,** 5 g of content from the slurry prepared for pH measurement was mixed with 45 mL of deionized water and, after mixing, titrated with a 0.1 N solution of NaOH until the pH reached 8.1. The titratable acidity was measured using the following formula: (a).

(a) $$\text{Titratable acidity} = \frac{\text{V(NaOH) used} \times 0.1 \times 0.064}{\text{m(aliquot)}} \times 100$$

3.5.2 Moisture, lipid content, ashes, total soluble solids (TSS), peroxide value

Sample analysis: olive oil industrial by-products, oil blend, oil/vinegar emulsion, functional beverages, strawberries

The **moisture** was determined on OIBP (around 4.0–5.0 g) and precisely weighed into pre-dried and pre-weighed ceramic dishes. Samples were dried in a laboratory oven at 105°C until achieving constant weight (generally after about 4-6 hours). The percentage moisture content was calculated using the following formula (b):

$$\textbf{(b)}\quad \text{Moisture (\%) Fresh weight} = \left[\frac{(\text{RM Initial Weight} - \text{RM Final Dried Weight})}{(\text{RM Initial Weight})}\right] \times 100$$

A lot of the dried sample derived from moisture determination was used for lipid content or ash determination.

The dried OIBP was transferred to a cellulose thimble and subjected to continuous extraction using petroleum ether as the solvent to determine lipid content. The system cycles the solvent through heating, evaporation, condensation, and sample washing to dissolve the oil effectively. After several hours of extraction, the oil solvent evaporated, leaving the extracted oil residue (AOCS 2023). The oil content was calculated as a percentage of the initial sample weight using the formula (c):

$$\textbf{(c)}\quad \text{Lipid (\%)} = \left[\frac{(\text{Weight of Extracted Oil (g)})}{\text{Weight of Sample (g)}}\right] \times 100$$

For the **ash** content, dried RM was precisely weighed in pre-weighed ceramic dishes and placed in a muffle furnace at 550-600°C for the complete mineralization, reached when white or greyish-white ash without visible black carbon particles was obtained (generally after about 4-6 hours). Verified the constant weight of the samples. The ash content was calculated using the following formula (d).

$$\textbf{(d)}\quad \text{Ash (\%)} = [(\text{RM residual ash weight} / \text{RM initial weight})] \times 100$$

Finally, **total soluble solids (TSS),** expressed in Brix grade, were determined in functional beverages and strawberries samples using a refractometer calibrated to zero with distilled water.

3.5.3 Total phenol content

Sample analysis: olive oil industrial by-products (OIBP), OIBP extracts, strawberries

The total phenols (TPC) were extracted from OIBP using a generally 1 g (precisely weighted) ground-dried sample. Each RM sample (OL or OP) was accurately mixed with 7 mL of methanol: water 80:20 (v/v) solution (Met80%) by sonication for 15 minutes at room temperature, followed the centrifugation at 4,000 rpm for 15 minutes and the recovery of clear supernatant separately. The extraction was performed three times in the same way. Finally, the collected supernatant was brought to the final exact volume of 25 mL MetOH80%.

Total phenols (TPC) were determined using the Folin-Ciocalteu spectrophotometric method, modified from De Leonardis *et al*., 2022b.

In a 25 mL volumetric flask, a specific volume of RM methanolic extracts § 3.2.1 was first added, followed by a minimal volume of deionized water to ensure proper dispersion. Subsequently, 1.25 mL of Folin-Ciocalteu reagent (C. Erba, Rodano, Italy) was introduced and allowed to react for precisely 3 minutes at room temperature, initiating the initial color development process. Following this critical interval, 5 mL of a freshly prepared 15% sodium carbonate (Na_2CO_3) solution was added to the mixture, which triggered the complete chromogenic reaction. The reaction mixture was then meticulously brought to a final volume of 25 mL using ultrapure water. Every time, the blank sample (only reagents) and the referencing standard concentration curve points with gallic acid (mother concentration of 1 mg/mL in water) were also prepared under the same conditions mentioned above. All samples were left to stand in complete darkness for 1 hour, allowing full-color stabilization and maximum complex formation. Thus, the samples were analyzed using a UV-visible spectrophotometer (UV–Vis Spectrophotometer Evolution™ 201/220 (Thermo Fisher Scientific SpA, Rodano, MI, Italy), measuring the absorbance at 765 nm. Generally, total phenols were quantitatively determined using a gallic acid equivalent (GAE) curve.

To analyze the **total phenols** in **strawberries,** their extracts were prepared following the protocol described by De Bruno *et al*. (2023). Briefly, 5 g of strawberry homogenate was mixed with 10 mL of an acidified ethanol-water solution (EtOH/H2O, 70:30 v/v, pH three adjusted with HCl). The mixture was vortexed for 30 seconds and subjected to ultrasonic extraction in a bath operating at 40 kHz, 25 °C, and 50% power for one hour. After ultrasonication, the mixture was centrifuged at 9000 rpm for 10 minutes at 4 °C. The supernatant was then filtered using a syringe filter (regenerated cellulose, 0.45 µm pore size, 15 mm diameter) and collected for subsequent analyses. Finally, to calculate the TPC in the strawberries sample, the following method was employed: 35 µL of acidified ethanolic extract

was mixed in 150 µL of 10 times diluted Folin-Ciocalteu reagent directly in 96 healthy polystyrene plate and shaken for 30 s at 400 rpm. After five minutes stay, add 115 µL of 7.5% Na_2Co_3. After shaking for 30 seconds at 400 rpm, incubate it at 45 C for 30 minutes, and do an additional 1 hour of incubation at room temperature. The absorbance was read at 765 nm with a spectrophotometer.

Hydrolyses of combined phenols were performed by heat treatment with 2N HCl acid, 1:1 v/v, in a thermostatic water bath at 80 C for 1 h. In a separation funnel, 10 mL of fresh or acid-heat hydrolyzed FB was added with 0.5 mL of gallic acid standard (1 mg/mL) as recovery factor; after three-time extraction with 10 mL of ethyl acetate, the collected ethyl acetate clear phase was dried by rotavapor recovering the dry residue in 2 mL of water. All samples were filtered through a 0.45-m syringe filter before HPLC analysis.

3.5.4 Total flavonoid contents (TFC)

Sample analysis: Strawberries

TFC was evaluated in a 5 mL Volumatic flask by following the method described in (Letaief *et al*., 2016) with slight modification. Firstly, 300 µL of acidified ethanolic extracts of strawberries were diluted with 1000 µL of distilled water and 150 µL of 5% $NaNO_2$. This mixture was incubated in the dark for 6 minutes. Afterward, 150 µL of 10% $AlCl_3$ was added and incubated for 6 minutes in the dark at room temperature, followed by adding 2000 µL of NaOH and making the final volume of 5 mL. The absorbance was measured at 510 nm, and results were expressed as Milligrams of Quercetin (QC) per 100 grams of fresh weight (F.W).

3.5.5 HPLC analysis of phenol compounds

Sample analysis: All OIBP extracts

Phenolic analyses were performed using a ProStar 230 pump and a 330-photodiode array (PDA) detector (Varian Analytical Instruments, Palo Alto, CA, USA). Chromatographic separation was achieved on a Kinetex 5 µm C18 100 Å column (150 × 4.6 mm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of a mixture of 0.2% v/v phosphoric acid (eluent A), methanol (eluent B), and acetonitrile (eluent C). A gradient elution was applied with the following program:

- 0 minutes: 96% A, 2% B, 2% C
- 24 minutes: 50% A, 25% B, 25% C
- 27 minutes: 40% A, 30% B, 30% C

- 36 minutes: 0% A, 50% B, 50% C
- 49 minutes: 96% A, 2% B, 2% C

Detection was carried out at 280 and 240 nm to monitor phenolic compounds of interest. Quantitative analysis was performed using calibration curves prepared with independent hydroxytyrosol and oleuropein standards (Aldrich) over a linear range of 0.05–0.50 mg/mL. To determine phenolic compounds, the vinegars were first filtered through a 0.45 µm syringe filter to remove particulates. Before analysis, the extracts were diluted 50-fold to ensure concentrations were within the calibration range.

3.5.6 FRAP Assay

Sample analysis: SW-Wex and SW-EAE olive leave extracts (§ 3.3.2.2)

The ferric-reducing antioxidant power (FRAP) was measured following the procedure described by Pulido *et al*. (2000) with slight modifications. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPCTZ in 40 mM HCl, and 20 mM ferric chloride in a 10:1:1 (v/v) ratio. Microplate wells were filled with 300 µL of the FRAP reagent and incubated at 37°C for 15 minutes. Then, 10 µL of olive leave extracts or pure hydroxytyrosol solution were added and mixed. Absorbance was recorded at 593 nm after 4 minutes using a plate reader (BioTek Elx 800, Agilent, Les Ulis, France). The effective concentration (EC_{50}) was determined by plotting sample concentrations against FRAP values, with results expressed as mg Fe^{2+} equivalents per mL of extract.

Sample analysis: UB-OLEx, UB-AD-OPEx, UB-FD-OPExSW-Wex (§ 3.3.1.2 and § 3.3.1.3)

The extracts prepared for ultrasound and plasma treatment (§ 3.3.1) were evaluated using the FRAP Assay Kit (MAK509) from Sigma-Aldrich. The FRAP working reagent was freshly prepared by mixing 20 parts of Reagent A (acetate buffer, pH 3.6), 1 part of Reagent B (10 mM TPCTZ in 40 mM HCl), and 1 part of Reagent C (20 mM ferric chloride). For the assay, 50 µL of each standard or extract was added in duplicate to a 96-well transparent, flat-bottom plate. Next, 200 µL of the working reagent was added to each well, and the plate was gently mixed by tapping. Samples were incubated at room temperature for 40 minutes, after which absorbance was measured at 590 nm using a microplate reader. A standard curve was prepared using Fe^{2+} standards ranging from 0 to 180 µM, and the FRAP value of the samples was calculated in µM Fe^{2+} equivalents based on the standard curve. Further dilutions were

performed for samples with absorbance exceeding the highest standard, and the results were adjusted accordingly using the dilution factor.

3.5.7 DPPH assay

Sample analysis: SW-EXs (§ 3.2.2.2) and functional beverages

The DPPH free radical scavenging activity was determined using a modified method by Williams *et al*. (1995). **SW-EXs** and hydroxytyrosol (Hy) dilutions (7 µL) were added to 273 µL of 60 µM DPPH solution in methanol in 96-well flat-bottom microplates. Absorbance was measured at 515 nm using a plate reader (BioTek Elx 800, Agilent, Les Ulis, France) at 5-minute intervals until the reaction plateaued. The residual percentage of DPPH was calculated by comparing the sample values to the blank, and the effective concentration (EC_{50}) required to reduce the initial DPPH concentration by 50% was determined through concentration-response regression analysis.

To evaluate the antioxidant activity of functional beverages prepared by using MHT described in § 3.3.2.1 were evaluated by following the protocol as described for the analysis, a variable aliquot of either methanol (as a blank solution), a standard compound (0.01 mg/mL), or the sample was prepared and mixed with methanol to achieve a total reaction volume of 2.5 mL. Subsequently, 0.5 mL of a DPPH solution (0.5 mM in absolute methanol) was added to the mixture. The reaction was incubated in the dark at 25 °C for 40 minutes, after which the absorbance at 517 nm was measured using a spectrophotometer. The antioxidant activity was evaluated by determining the concentration of total phenols (expressed as mg/mL CAE) required to scavenge 50% of DPPH radicals (IC_{50}), calculated through linear regression of the inhibition values against total phenol concentration. This method enabled the quantitative assessment of the antioxidant capacity of the samples.

3.5.8 ABTS assay

Sample analysis: SDBD and ultrasonic extracts

For ABTS assay, ABTS radical cation ($ABTS^{\bullet+}$) is generated by incubating a 7 mM ABTS solution with 2.45 mM ammonium persulfate (APS) in the dark at room temperature for 12-16 hours. The resulting $ABTS^{\bullet+}$ stock solution is diluted with ultrapure water to absorb ~0.700 at 734 nm. Each sample (10 µL) is mixed with 190 µL of $ABTS^{\bullet+}$ solution in a 96-well microplate and incubated for 5 minutes in the dark. Absorbance at 734 nm is measured using

a microplate reader. Antioxidant capacity is calculated as a percentage of ABTS•+ decolorization by using the following formula

$$\text{(f) Decoloration \%} = \left[\frac{\text{Control Abs.} - \text{Sample Abs.}}{\text{Control Abs.}}\right] \times 100$$

3.5.9 TBARS assay

Sample analysis: beef patties (§ 3.4.3)

The oxidative stability role of SW EXs as antioxidants in beef meat was evaluated by TBARS assay, following the protocol by Nerín *et al*. (2006), with modifications. Approximately 2 g of meat sample was weighed into a 50 mL Falcon tube placed in crushed ice to maintain low temperatures during processing. To this, 8 mL of 10% trichloroacetic acid (TCA) was added for protein precipitation, along with 50 µL of a butylated hydroxytoluene (BHT) solution (1 mg/mL) to prevent oxidation during analysis. The sample was homogenized using an Ultra-Turrax homogenizer at 20,000 rpm for at least one minute.

The homogenized mixture was centrifuged at 1,500 × g for 30 minutes at 5 °C, and the resulting supernatant was filtered through filter paper. For the assay, 2 mL of the filtrate was combined with 2 mL of 20 mM 2-thiobarbituric acid (TBA) in a Pyrex glass test tube. The tubes were covered with aluminum foil, incubated in a water bath at 95 °C for 20 minutes to allow the reaction to occur, and then rapidly cooled under a stream of cold water. The absorbance of the final solution was measured at 531 nm using a spectrophotometer. A standard malondialdehyde (MDA) curve was prepared to quantify TBARS values, which were expressed as mg of MDA per kg of sample.

3.5.10 Accelerate lipid oxidation test: rancimat and oven test

Sample analysis: Salad dressing

The **Rancimat test** was performed using a Model 730 instrument (Metrohm AG, Herisau, Switzerland) at 120 °C under a constant airflow of 20 L/h. The induction time, representing the sample's oxidation resistance, was measured in hours.

The **oven test** was performed to evaluate the oxidative stability of the oil/vinegar dressings under accelerated storage conditions. For this purpose, 10 g of each sample (oil blend or

oil/vinegar dressing) was placed into 20 mL capped tubes, ensuring that approximately 50% of the tube volume was left as headspace. The tubes were then stored in an oven at 40°C for 28 days. Three tubes per dressing were withdrawn weekly to monitor oxidation, and the samples were analyzed for peroxide value (PV). The oxidative gradient (OTgr), which represents the daily increase in peroxide value, was calculated over the storage period to assess the oxidation rate. This method provided a controlled and accelerated environment for studying the oxidative stability of the samples, mimicking long-term storage conditions.

3.5.11 Color analysis

Sample analysis: beef patties (§ 3.4.3) and strawberries

The color of the beef patty surface was evaluated using a colorimeter (CM-2300d, Konica Minolta, France) by assessing the L* (lightness), a* (redness), and b* (yellowness) values and calibrating the instrument with zero and white standard plates before analysis.

The surface color of **strawberries** was evaluated at three distinct points on each of the three replicates using a Minolta Spectrophotometer. The spectrophotometer measured the color parameters, including lightness (L^*), redness (a^*), and yellowness (b^*).

3.5.12 Texture analysis

Sample analysis: strawberries (§ 3.4.4)

The penetration test was conducted to assess the firmness of strawberries using a texture analyzer equipped with a cylindrical flat-head probe (P/5, 2 mm diameter). Strawberries were placed on the plate with the receptacle cavity positioned upright relative to the compression probe to ensure uniform testing conditions. The instrument was calibrated using a 2 kg loading weight and a 45 mm height standard. Instrumental settings were as follows: pre-test speed 10.00 mm/s, test speed 5.00 mm/s, post-test speed 10.00 mm/s, and trigger force 2.0 g. Three replicates were performed for each sample, with four batches analyzed to ensure robust statistical representation.

3.5.13 Microbiological analysis

Sample analysis: OIBP ultrasonic bath extracts

The antimicrobial activity of olive by-products was evaluated against *Listeria monocytogenes* and *Escherichia coli*. The experiment was designed to assess bacterial inactivation following

treatment with olive-derived extracts. All procedures were conducted under aseptic conditions to prevent contamination.

Materials and Media Preparation: Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB), and Buffer Peptone Water (BPW, 0.2%) were prepared as growth and dilution media. Olive by-product extracts were used as treatment agents. Additional materials included sterile Falcon tubes, microtubes, Petri plates, and a centrifuge. TSA was prepared by dissolving 24 g of TSA powder in 600 mL of deionized (DI) water. The mixture was heated on a hot plate at 100°C with continuous stirring at 1600 rpm until fully dissolved. The solution was autoclaved at 121°C for 20 minutes, cooled, and poured into sterile Petri plates in 20 mL aliquots, then solidified. TSB was prepared by dissolving 9 g of TSB powder in 300 mL of DI water, and BPW was prepared by dissolving 1.2 g of BPW powder in 600 mL of DI water. Both were autoclaved at 121°C before use.

Bacterial Inoculum Preparation: A single colony of Listeria monocytogenes or Escherichia coli was picked from a streaked plate and inoculated into 10 mL of TSB. A control tube containing TSB without bacterial culture was also prepared. The inoculated and control tubes were incubated at 37°C for 24 hours to allow the bacterial cultures to reach the logarithmic growth phase. Following incubation, 1 mL of the bacterial culture was transferred into a sterile microtube and centrifuged at 10,000 rpm for 2 minutes. The supernatant was discarded, and the bacterial pellet was resuspended in 1 mL of BPW. The washing step was repeated twice to ensure the complete removal of the residual growth medium.

Preparation of Treatment Solutions: Treatment solutions were prepared in Falcon tubes. For the control, 9990 µL of BPW was added to one tube. 9990 µL of olive by-product extracts at different concentrations were added to separate tubes for the test treatments. To each tube, 10 µL of the washed bacterial suspension was added and mixed thoroughly. The tubes were incubated at room temperature for 3 minutes to allow interaction between the bacterial cells and the treatment solutions.

Serial dilutions were performed to quantify bacterial viability. Six sterile microtubes, each containing 900 µL of BPW, were arranged. From the bacterial suspension (control and treatments), 100 µL was transferred to the first microtube and mixed thoroughly. Serial dilutions were continued by transferring 100 µL from one tube to the next until the 6th dilution was reached.

Following dilution, TSA plates were labeled according to the dilution factors. From each dilution, 30 µL (three 10 µL drops) of bacterial suspension was spotted on the plates. The

drops were allowed to solidify without smearing, and the plates were incubated at 37°C for 24 hours.

After incubation, bacterial colonies were enumerated on each plate. Colony-forming units (CFUs) per mL were calculated based on dilution factors. The reduction in CFUs for Listeria monocytogenes and Escherichia coli was compared between the control and olive by-product treatments to determine the antimicrobial efficacy of the extracts.

Sample analysis: strawberries (§ 3.4.4)

For the microbial analysis, the strawberry sample from each treatment was serially diluted (1:10) in 0.2 % buffer peptone water and homogenized using a stomacher for 1 minute. Thus, 1 mL from each dilution was transferred to ready-to-use 3M Petri films for aerobic plate count (APC) and yeast and mold (Y&M). For APC, the incubation temperature was 37 ± 1°C for 48 h, while Y&M were incubated at room temperature (around 25 °C) for 72h. The results are log 10 colony-forming units per gram of strawberries (CFU g^{-1}).

3.6 Statistical analysis

The experiment was conducted in a completely randomized design (CRD) with three replications. All the analyses were carried out in triplicate and the standard deviation (SD) was calculated. Data analyses were performed using variance analysis (ANOVA) using Statistix8.1 statistical software. Multiple comparisons among the treatments with significant differences tested with ANOVA were conducted using the least significant difference (LSD) at $P < 0.05$.

4. RESULTS AND DISCUSSION

4.1. Proximate analysis of olive industrial by-products (OIBP)

The proximate analysis of olive industrial by-products, namely olive leaves (OL) and two-phase olive pomace (OP), reveals significant differences in their composition, as shown in Table 2.

Table 2. Proximate analysis of raw olive products: olive leaves and pomace.

Parameters	Results	
	Olive leaves (OL)	Olive Pomace (OP)
Total phenols mg/g GAE	10.5 ± 0.5^{a}	7.5 ± 0.3^{b}
Fat %	2.7 ± 0.2^{a}	2.6 ± 0.4^{a}
Ash %	6.2 ± 0.4^{a}	1.5 ± 0.2^{b}
Humidity %	7.65 ± 0.5^{a}	63.4 ± 5.1^{b}
Protein (N X 6.25) %	5.1 ± 0.4^{a}	1.9 ± 0.3^{b}
pH	5.13 ± 0.5^{a}	5.9 ± 0.7^{b}

The focus is mainly on phenolic content, which is the main bioactive substance in this research. It is well known that the phenolic compounds present in olive plant derivatives show numerous health benefits (Visioli *et al*., 2002).

Olive leaves contain higher levels of total phenols at 10.5 mg/g GAE on wet, fresh raw material, compared to OP, which has 7.5 ± 0.3 mg/g GAE. On closer inspection, the water content in OL and OP was highly different. Specifically, the water content in OP was substantially higher (63.4 ± 5.1%) than in olive leaves (7.65 ± 0.5%). However, in both cases, this is physiological water rich in chemical and phenolic substances. The OP waters, known as vegetation water or olive oil mill wastewaters (OOMW), have been primarily investigated for profitable recycling (De Leonardis *et al*., 2023). The elevated moisture content is a critical factor that could significantly affect the storage stability and processing requirements of OP, and it warrants consideration by industry professionals when working with these products (Zbakh & El Abbassi, 2012).

The fat content in both samples is quite similar, with OL at 2.7 ± 0.2% and OP at 2.6 ± 0.4%. The fat in OP represents the residue of olive oil contained in the fruits at the end of the industrial process. However, the found value is consistent with relevant literature (Alburquerque *et al*.,2004).

The protein content, reported as N × 6.25, was higher in OL at 5.1 ± 0.4% than in OP at 1.9 ± 0.3%. In olive leaves, higher protein content contributes to their potential utilization as

nutritional supplements in functional beverages. Notably, the pH levels of both samples are relatively similar, with olive leaves at 5.13 ± 0.5 and OP at 5.9 ± 0.73. This slight acidity can consistently influence the sensory characteristics of the beverages, indicating a slightly acidic nature that can affect the beverages' sensory characteristics consistently. Olive leaves exhibited an ash content of 6.2 ± 0.4%, while the olive pomace had a lower ash content of 1.5 ± 0.2%. Ash content indicates the mineral composition, which impacts the final product's nutritional value product.

The chromatographic analysis of olive leaves revealed the presence of significant polyphenolic compounds, identified through absorbance peaks at 325 nm, 280 nm, and 240 nm (Fig.20).

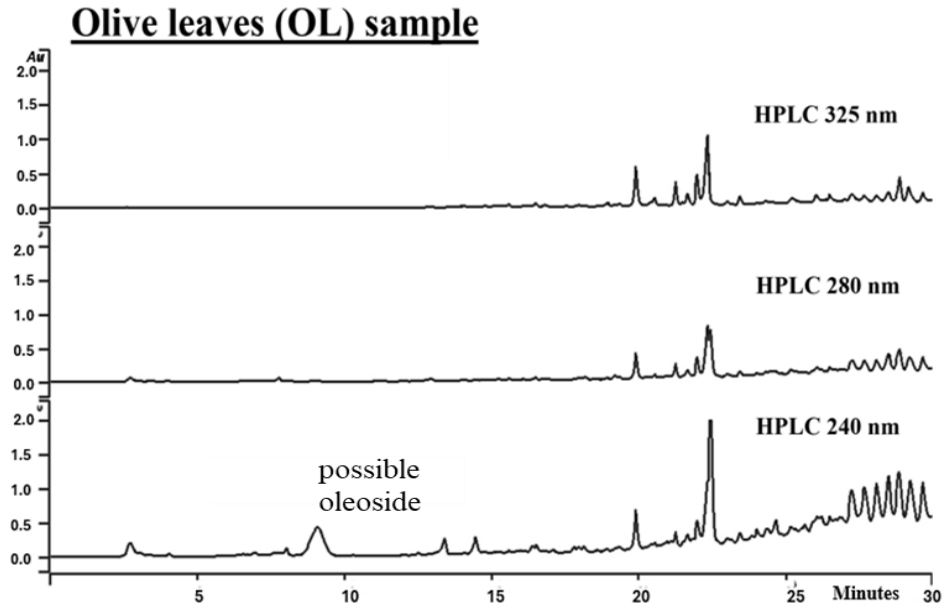

Fig. 20_ HPLC chromatogram of olive leaves (OL)

The 325 nm wavelength peaks indicated the presence of flavonoids and phenolic acids, including luteolin and apigenin glycosides, known for their potent antioxidant properties (Benavente-García *et al*., 2000). Additionally, strong absorbance at 280 nm confirmed the abundance of total phenolics, notably oleuropein and hydroxytyrosol, compounds well-documented for their antioxidant and anti-inflammatory activities (Visioli *et al*., 2002).

The strong absorbance at 280 nm confirmed the presence of total phenolics, including oleuropein and hydroxytyrosol, compounds well-documented for their antioxidant and anti-inflammatory activities (Visioli *et al*., 2002). Additionally, the 240 nm wavelength peaks

indicated the presence of oleosides and secoiridoid derivatives, particularly oleuropein, which support the antimicrobial and anti-inflammatory properties of the pomace (Cecchi *et al*., 2023).

Regarding the OP, the air-dried pomace exhibited a higher total phenolic content (21.2 mg/g GAE) than the freeze-dried pomace (14.3 mg/g GAE), suggesting that air-drying may be more effective in preserving phenolic compounds. These findings align with the established literature, underscoring the significant role of phenolic compounds in contributing to the therapeutic potential of olive pomace.

In Fig. 21, the phenolic profile of the freeze- and air-dried olive pomace samples.

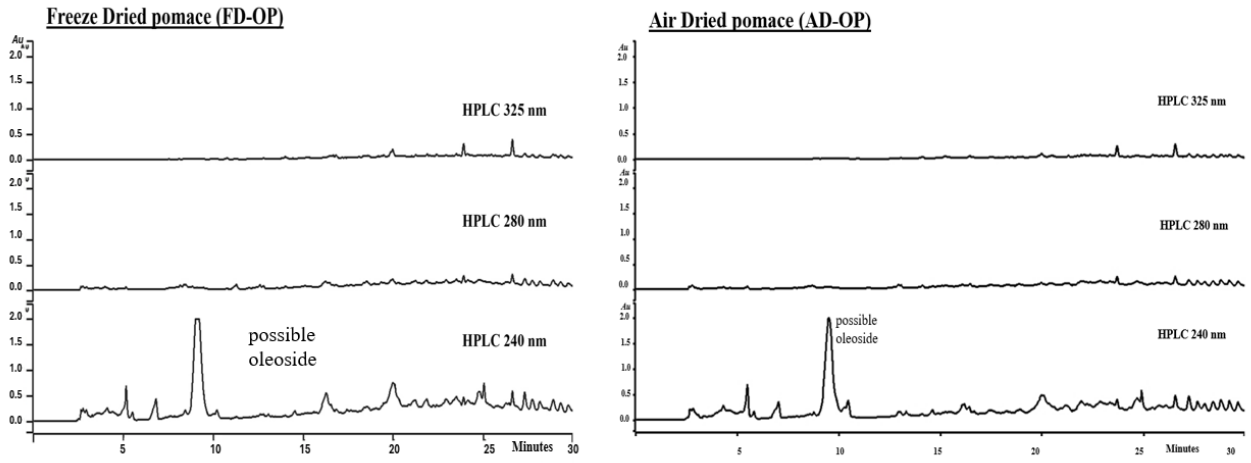

Fig. 21_ HPLC chromatograms of freeze-dried olive pomace and air-dried olive pomace

4.2 Recovery of bioactive compounds and possible food application

In this research, different recovery methods for bioactive compounds from raw materials have been investigated to define a sustainable protocol. In every case, all extraction procedures were based on liquid-liquid or solid-liquid extraction, privileging the choice of solvents with low impact on human health, such as water, ethanol, and acetic acid. Moreover, additional physical treatments, such as thermal, ultrasound, and activated plasma, have been investigated.

Thus, developing suitable extraction methods for the bioactive compounds from OIBP represents a significant part of the PhD course. However, not all obtained extracts were considered ideal for application in the food sector.

Table 3. summarizes the extraction procedures that have allowed the production of extracts potentially useful for food applications.

Table 3. Principle extracts prepared in this research with their possible food application.

Tags	Raw materials	Solvent	Food Application	Extraction methods
		Non-thermal technologies		
Olive leave vinegar **(OLV)**	dried olive leaves	6% alcohol vinegar	salad dressing	Cold maceration
UB-Ex *UB-OLEx* *UB-AD-OPEx* *UB-FD-OPEx*	all dried olive oil industry by-products	methanolic solvents	edible coatings	Ultrasonic bath extraction
		Thermal technologies		
WHT-OP *WHT-OP-W* *WHT-OP-6%CAW*	fresh olive pomace	water or 6% citric acid solution	functional beverage	Wet thermal extraction
SW-OL *SW-OL-WEx* *SW-OL-EAEx*	whole frozen olive leaves	only water steam or followed by ethyl acetate recovery	antioxidant in beef meat	Water Steam

4.2.1 Cold maceration to produce ingredients for salad dressing

This study aimed to explore the potential of olive leave vinegar (OLV) as an ingredient in emulsion-based dressings, focusing on its impact on oxidative stability.

The study, published in De Leonardis *et al*. 2022a and De Leonardis *et al*. 2022c, has produced interesting results, which are briefly mentioned here.

In De Leonardis *et al*. 2022a, the antioxidant effect of traditional and new kinds of vinegar on functional oil/vinegar dressing-based formulations has been investigated as reported in its published abstract: Vinegar and vegetable oil are frequently used in emulsion formulations, allowing the fabrication of new functional foods. In the present study, a potentially functional oil/vinegar dressing was formulated using an enriched omega-3 fatty acids oil blend (high-oleic sunflower and soybean oil) and several kinds of naturally occurring biophenols (white wine, red wine, pomegranate, apple, malt, alcohol, olive, and olive leave vinegar). In the present study, vinegar and oil were efficaciously emulsified with an ultrasound bath without adding emulsifiers. Accelerated oxidation tests have been carried out on the oil/vinegar dressing samples. Most vinegar (i.e., white and red wine, pomegranate, apple, malt, and alcohol) have been shown not to affect the oxidation processes of oil/vinegar dressing. Interestingly, olive vinegar, obtained by the fermentation of olive oil mill wastewater, and

olive leave vinegar, obtained by the maceration of leaves in alcohol vinegar, exhibited apparent antioxidant activity. Results obtained may help develop a range of natural and healthy ingredients for formulating novel and functional foods.

De Leonardis *et al*. 2022c results relative to characterization, sensory, and oxidative stability analysis of vegetable mayonnaise formulated with olive leave vinegar as an active ingredient, as described in its published abstract: Development of novel food products represents an essential meeting point for health and business requirements. Mayonnaise sauce is well-suited as a healthy and tasty dressing. In this study, mayonnaise was formulated by using unconventional ingredients, such as olive leave vinegar (OLV), soybean/high oleic sunflower oil blend, and soymilk (as an egg substitute). An 18% alcoholic vinegar was used as the control sample. OLV is a rich source of bioactive substances, especially polyphenols, and it represents a possible way to enhance the valorization of olive oil by-products. For this new typology of vinegar, a high level of phenolic compounds (7.2 mg/mL GAE), especially oleuropein (6.0 mg/mL oleuropein equivalent), was found. OLV mayonnaise had 57% fat, composed of 11%, 64%, and 23% saturated, monounsaturated, and polyunsaturated fatty acids, while linolenic acid was up to 1.7%. The phenol and oleuropein contents were 68 and 52 mg/100 g, respectively. Sensory panellists expressed moderate overall acceptability for both samples but attested to more distinctive and positive sensations for the color, odor, and taste attributes of OLV mayonnaise. Finally, oxidative stability and shelf life were better in OLV mayonnaise than in the control. Specifically, the peroxide value remained low (around 4.5 meqO2/kg) after 12 months of storage at room and low (4 ◦C) temperatures.

In this thesis, other unpublished data on olive leave vinegar (OLV) are presented and discussed. The principal novelty of the investigation conducted is the use of cold maceration to produce a new type of vinegar by using a 6% alcoholic vinegar at differences of the published data. OLV is a seasoning product. However, in this study, OLV was also tested as a possible ingredient in oil/vinegar dressing.

OLV was produced through maceration in alcoholic vinegar, as outlined in the Materials and Methods, precisely in § 3.3.1.1.

Vinegar is a sour-tasting liquid used since ancient times as a seasoning, acidifying, preserving agent, and folk medicine. Vinegar is commonly produced via a two-step process, generally called 'double fermentation' (Fig. 22).

AEROBIC PATHWAY FOR ACETIC ACID PRODUCTION

(1) Anaerobiosis - yeast *Saccharomyces ssp.*

$$1\ C_6H_{12}O_6 + 1\ H_2O \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$$

glucose — *ethanol*

(2) Aerobiosis – acetic acid bacteria (AAB) *Acetobacter ssp.*

$$2\ CH_3CH_2OH \xrightarrow[alcohol\ dehydrogenase]{O_2} 2\ CH_3CHO + 2\ H_2O \xrightarrow[aldehyde\ dehydrogenase]{O_2} 2\ CH_3COOH$$

ethanol — *acetaldehyde* — *acetic acid*

Fig. 22_Aerobic pathway for acetic acid production (De Leonardis *et al.*, 2018).

In the first step, fermentable sugars are converted to ethanol by yeasts, generally *Saccharomyces* species, under anaerobic conditions; successively acetic acid bacteria (*Acetobacter* species), under aerobic conditions, oxidize ethanol to acetaldehyde by the alcohol dehydrogenase and then, the acetaldehyde to acetic acid by the aldehyde dehydrogenase (De Leonardis *et al*., 2018)

However, it is well known that several types of bacterial species can produce acetic acid by operating directly on sugar-water solutions, as shown in Fig. 23.

ANAEROBIC PATHWAY FOR ACETIC ACID PRODUCTION

(1) Anaerobiosis - acetogenic bacteria (AB) *Clostridium*

$$1\ C_6H_{12}O_6 \xrightarrow{glycolysis} 2\ CH_3H_4O_3 \rightarrow 2\ CH_3COSCoA \rightarrow 2\ CH_3COOH + 2CO_2$$

glucose — *pyruvate* — *acetylCoA* — *acetic acid*

(2) Anaerobiosis - Wood Ljungdahl pathway (WLP) - acetogenic bacteria (AB) *Clostridium*

$$2CO_2 \xrightarrow{Wood\ Ljungdahl\ pathway\ (WLP)} 1\ CH_3COOH$$

acetic acid

Fig. 23_ Anaerobic pathway for acetic acid production (De Leonardis et al., 2018)

In this case, *Acetobacterium* and *Clostridium* species preliminary transform the glucose in acetyl-CoA and CO_2 through glycolysis; successively, acetyl-CoA is converted to acetyl phosphate by the phosphotransacetylase and to acetate by the acetate kinase. As a result, two moles of acetic acid and one mole of CO_2 are produced. The same bacteria species can successfully convert the CO_2 plus H_2 to acetate via the Wood–Ljungdahl pathway, forming the third mole of acetate. Therefore, three moles of acetic acid are generated from one mole of glucose.

Numerous traditional vinegars, which vary in raw materials, are commercially available. Grain vinegars are commonly obtained from fermented sorghum, rice, wheat, and other grains, whereas fruit vinegars are produced from fermented juices of grapes, apples, pomegranates, coconuts, and other fruits.

Apple and malt vinegar are traditionally produced and used in Anglo-Saxon countries. In contrast, white and red vinegar have been produced in Mediterranean countries since immemorial times (Tesfaye *et al*. 2002). Red wine vinegar is also appreciated as a prestigious condiment, such as the Italian balsamic vinegar of Modena and Reggio Emilia and the Spanish sherry vinegar from the Jerez region.

New vinegars obtained from alternative substrates are studied mainly, especially those produced using food industry by-products such as onion (Horiuchi *et al*., 1999), sweet potato, pineapple, and soybean molasses. Vinegar production from olive oil mill wastewater fermentation has also been demonstrated (De Leonardis *et al*., 2018; De Leonardis *et al*., 2019).

All the above-cited vinegars are classified as natural-source vinegars, and generally, they have an acetic acid concentration ranging from 4 to 8%. However, acetic acid can also be produced by fermentation of distilled alcohol, known as alcohol vinegar. Frequently, food manufacturers prefer alcohol vinegar because it is typically colorless and exclusively composed of acetic acid, which can reach a concentration of up to 18% (Yin et al., 2018).

Natural-source vinegar is commonly considered ‘functional food’ due to its scientifically proven antioxidative, antidiabetic, antimicrobial, antitumor, anti-obesity, antihypertensive, and cholesterol-lowering effects (Samad *et al*., 2016). Therapeutic effects are mainly attributed to acetic acid and other bioactive constituents such as organic acids, polyphenols, melanoidins, and minerals (Chen *et al*., 2016).

OLV was analyzed after production and after two years of storage in closed bottles in a dark room. As shown in the photo reported in Fig. 24, OLV appeared limpid and stable.

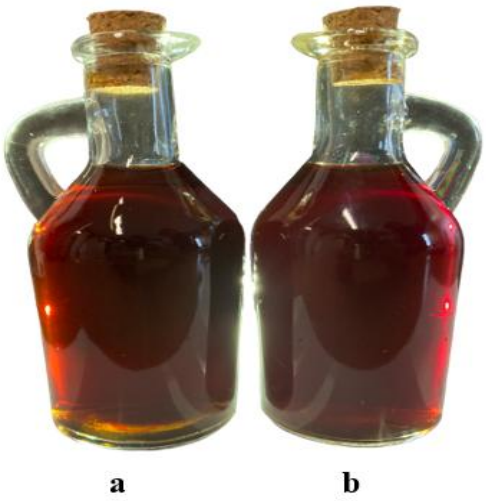

Fig. 24_Olive leave vinegar just produced (a) and after two years of storage (b).

In Table 4, the principal basic parameters are reported and determined on OLV.

Table 4. Basic parameters of olive leave vinegar

Determination	Time zero at bottling	Two years of storage
Ph	2.42 ± 0.03^{a}	2.53 ± 0.08^{a}
Total acidity (% acetic acid)	6.1 ± 0.5^{a}	5.8 ± 0.8^{a}
Total phenol (g/L GAE)	4.2 ± 0.2^{a}	2.7 ± 0.4^{b}

Letters indicate a significant difference ($P \leq 0.05$) on the line.

The pH and total acidity remained constant during the storage period. Conversely, total phenols decreased significantly, going from 4.2 to 2.7 g/L.

HPLC analyses revealed evident modification of the phenolic profile of the OLV during the storage (Fig. 25). It was apparent that depletion of complex phenols, given from the oleuropein and its homologs, and the releasing of hydroxytyrosol.

However, the total phenols' values were very high, attesting to the functional potential of the OLV. Thus, OLV has been shown to have high potential for the following reasons: The raw materials used to produce it (byproducts of the olive oil industry) meet the global New Green Deal for sustainable agricultural production. In addition, OLV must be considered an experimental new vinegar, which is being produced exclusively in our laboratory.

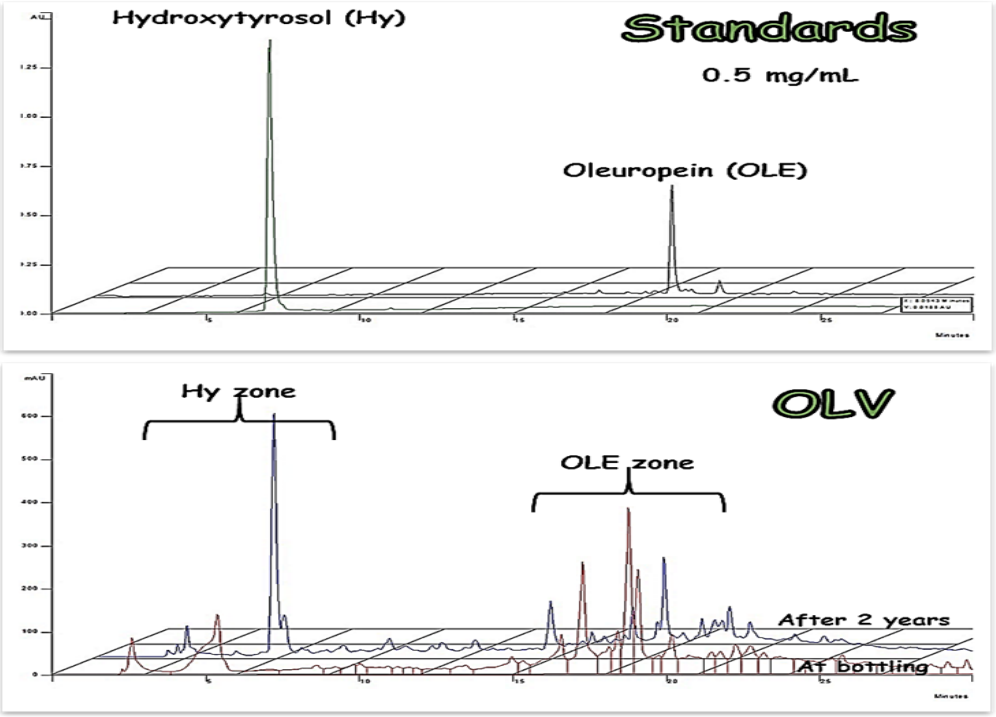

Fig. 25_Changes of phenolic profile of olive leave vinegar during the storage

Vinegar and vegetable oil are frequently used as main ingredients in emulsion formulations, such as creams, mayonnaise, sauces, salad dressings, ketchup, and others, and even to produce new functional foods.

In the current study, OLV was also tested as an ingredient of emulsion-based dressing. Emulsions are colloid-based systems in which two immiscible liquids are transformed into a stable mixture in which one phase, in the form of tiny droplets (dispersed phase), is closely dispersed in the other (continuous phase) phase. Generally, emulsifying agents are added to prevent coalescence and the consequent separation of phases.

Emulsions are highly encouraged in the development of novel functional food by allowing the combination of usually non-mixable ingredients. Nevertheless, emulsions are susceptible to the autoxidation of the unsaturated fatty acids contained in the oil, which consequently form potentially toxic products and undesirable off-flavors. Lipid types, aqueous phase components, oxygen concentrations, presence of pro-oxidants and/or antioxidants, droplet conditions, and interfacial interactions can affect the oxidative stability of emulsions. The addition of antioxidants, preferably of natural origin, can delay the emulsion of oil oxidation (De Leonardis *et al*., 2022c).

Table 5. Basic characteristics of oil blend (OB) sample. Mean ± SD of three independent determinations.

Determinations	Sunflower/soybean blend (85:15, v/v) *OB*
Free acidity (% oleic acid)	0.3 ± 0.0
Peroxide Value ($meqO_2/kg$)	3.1 ± 0.5
Fatty acid composition (%)	
Palmitic acid	6.01 ± 0.94
Stearic acid	3.23 ± 0.48
Oleic acid	73.05 ± 3.68
Linoleic acid	14.61 ± 0.75
Linolenic acid	0.91 ± 0.05
Others	2.19 ± 0.08
Total mono- and di-acylglycerols (%)	3.43 ± 0.18
Induction time Rancimat 120 °C (hours)	6.26 ± 0.18

Similarly to the above-mentioned manuscripts, the presented study designed the oil/OLV dressing keeping in mind making a potential functional food. Thus, an enriched omega-3 fatty acid oil blend (§ 3.2.2), made with 85% high oleic sunflower (HOSO) and soybean (SO) oils, was chosen. Table 5 gives the essential quality parameters, fatty acid composition, and Rancimat induction time of the single oils and OB.

Analytical determinations performed immediately after the preparation showed no significant change in pH, VP, and TPC due to the ultrasound treatment at 80 W and 40 °C for 60 min (§ 3.4.1). Otherwise, ultrasound mixing is confirmed to be an effective method of stabilizing emulsions without adding any additives. Immediately after the preparation, the oil/OLV emulsion (E) appeared as a turbid fluid with a whitish-to-reddish color (Fig. 26).

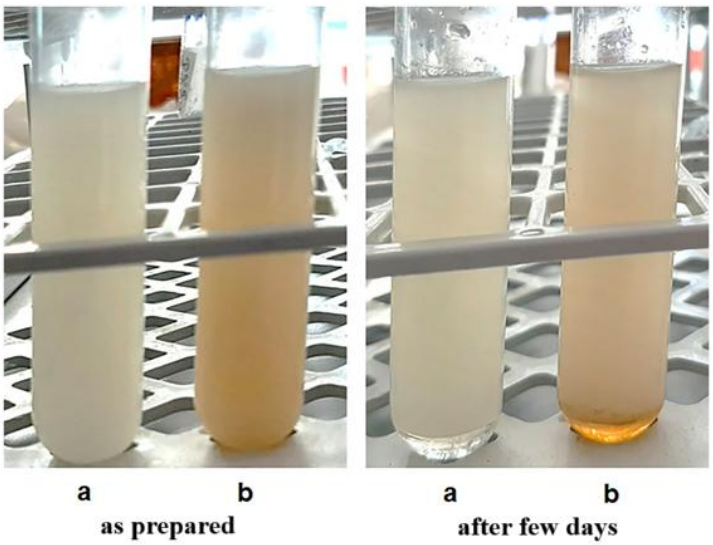

Fig. 26_Oil/olive leave vinegar emulsion (E). a (E with alcohol vinegar) and b (E with olive leave vinegar) as prepared and after few days.

The emulsified phase remained dispersed, avoiding coalescence for at least 3–4 days. After this period of visual observation, a three-layer system was visible, including (i) a lower aqueous layer, (ii) a sizeable intermediate emulsion layer, and (iii) a little upper oily layer. However, the emulsion layer was more abundant. At the same time, the aqueous phase was found to be about 20% of OLV added (Fig. 26). This three-layer system remained stable even after storage and after centrifugation at 4000 rpm for 30 min.

Emulsion stability is undoubtedly a crucial requirement of oil/vinegar-based dressings.

It is reasonable to suppose that the emulsifying effect of OLV was also due to an esterification reaction between the acetic acid and the mono- and di-glycerides of fatty acids present in the oil, otherwise known as ACETEM. The ACETEM (Fig. 27) are food additive approved into the type-E472 emulsifiers often added to several food products to improve their emulsion state, viscosity, and foaming stability.

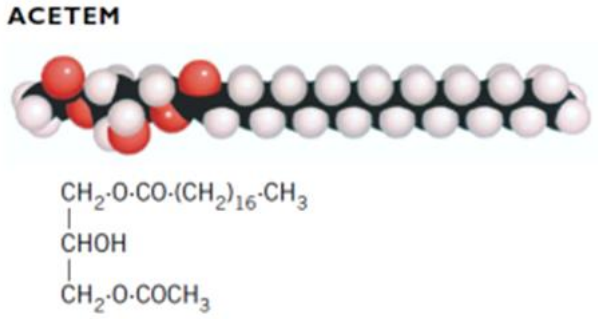

Fig. 27_Chemical structure of ACETEM

However, further investigation is needed to confirm the formation of ACETEM in the oil/OLV dressing model proposed in the present study.

In addition to the emulsifier effect, OLV evidenced a significant antioxidant rule during the accelerated oxidation tests in the oil/vinegar dressing (§ 3.5.11).

The results of the oven test are given in Fig. 28.

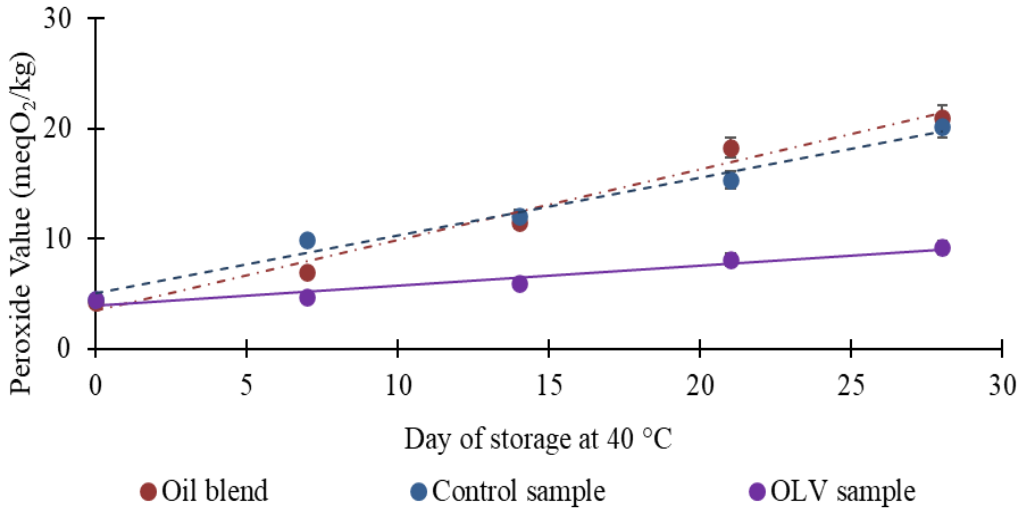

Fig. 28 _Evolution of the peroxide value in the oil/vinegar dressing during storage in the oven at 40 °C up to 28 days. Means of three independent measurements.

The peroxide value (PV), a crucial indicator of oxidative stability, was meticulously analyzed to evaluate the oil blend (OB), oil blend with only the alcoholic vinegar (Control sample), and oil blend with olive leave vinegar (OLV sample) during the storage at 40°C for 28 days. The distinct oxidative behaviors observed in the graph among the samples, attributed to the presence or absence of antioxidant compounds, underscore the significance of this study's findings.

Both the oil blend and control samples, devoid of any added antioxidant compounds, showed the highest susceptibility to oxidation. For both these samples, the PV gradient, calculated as the slope of the regression curve, was 0.8, corresponding to a daily increase of 0.8 $mEqO_2/kg$ of peroxides. This is consistent with the findings in the literature, where pure oil blends, especially those lacking natural antioxidants, are prone to oxidative degradation because of

their unsaturated fatty acid content, which is more reactive to oxygen and heat (Nehdi *et al*., 2019).

Including in the oil blend the alcoholic vinegar (OLV sample), a halved daily oxidative gradient of 0.4 $mEqO_2/kg$ of peroxides was found. This striking difference can be attributed to the high concentration of phenolic compounds in olive leave vinegar, such as oleuropein, hydroxytyrosol, and verbascoside, which are well-documented for their potent antioxidant properties. These compounds inhibit lipid oxidation by neutralizing free radicals and interrupting chain propagation reactions, thereby delaying hydroperoxide formation. Several studies have demonstrated the effectiveness of olive leave extracts in enhancing the oxidative stability of oils and lipid-based products (Benavente-García *et al*., 2000).

The Rancimat test, carried out at 120 °C, substantially confirmed OLV's antioxidant potential. Fig. 29 shows the induction time of the oil blend (OB), control, and OLV samples, respectively.

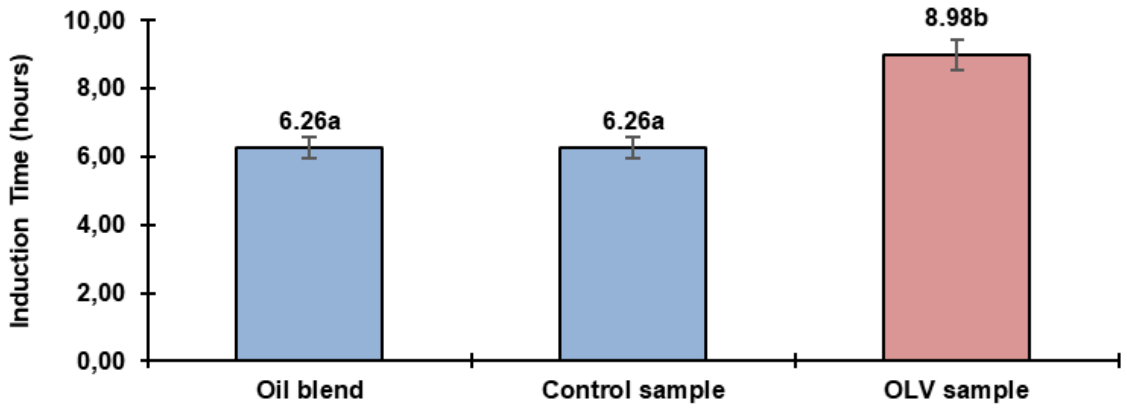

Fig. 29 _ Induction time determined with rancimat test on the oil blend and oil/vinegar emulsions. Means ± SD of three independent measurements. Superscript letters indicate significant differences at $p < 0.05$.

The oil blend and control sample exhibited an induction time (IT) of 6.26 ± 0.18 hours, indicating moderate oxidative stability. Conversely, the induction time of OLV was 8.98 hours, which demonstrates a significant antioxidant effect of the olive leave phenols.

These findings clearly demonstrate the protective effects of OLV, which delay oxidation and reduce the rate of hydroperoxide formation. This gives hope for their ability to delay oxidation and extend shelf life.

4.2.2. Wet heat treatment to produce functional beverages (FB)

Olive leave-based beverages are already a consolidated commercial reality. Alternatively, during the doctoral course, the production of a functional beverage from fresh olive pomace as raw material has been investigated. The central part of the obtained results has already been published in the manuscript of De Leonardis *et al*. (2022b), of which the abstract is reported here. Demand for functional beverages is increasing worldwide. This study used a rapid, easy, low-cost procedure to directly prepare a functional beverage (FB) using two-phase olive pomace (OP). The liquid ingredients (water and 6% citric acid), extraction system (heat treatment), and treatment times (30, 60, and 90 min) were studied. Conversely, no substantial differences were found in FB, neither for liquid ingredients nor for the treatment or time adopted. Both 6% citric acid and water were profitable liquid ingredients. A 30-minute heating treatment was sufficient to produce a satisfactory beverage, whereas ultrasound treatment caused a loss of total phenols, especially in the water FB. All FBs appeared limpid after simple filtration; the citric acid beverage was reddish, while the water was brownish. Finally, the prepared FBs had an average total phenol content of approximately 600 mg/L CAE (using 300 g/L fresh pomace), with hydroxytyrosol and related compounds well represented, confirming their potential functionality.

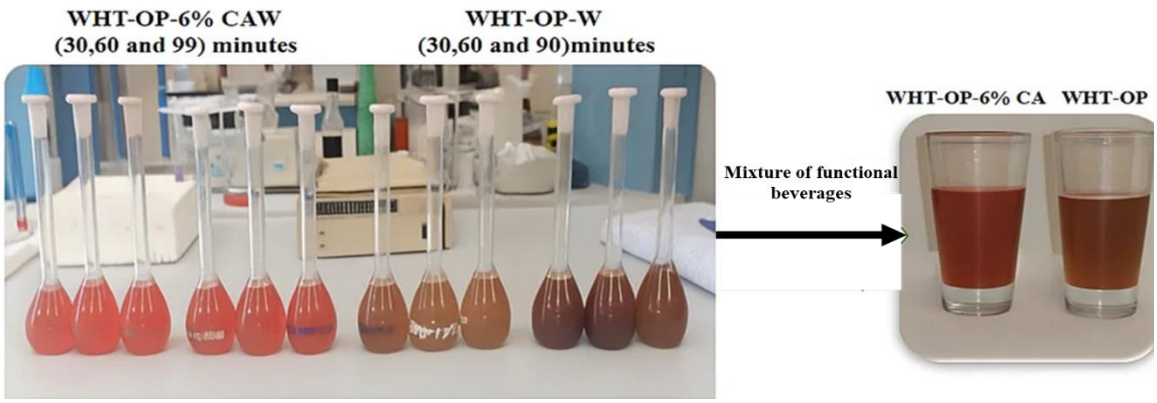

Fig. 30 _Wet heat extraction of fresh olive pomace with 6 % citric acid (6% CAW) and water (W) for different time intervals (30, 60, 90 minutes) and a mixture of homologs beverage.

This chapter focuses on the effectiveness of using only water and 6% citric water to obtain a possible functional beverage. Wet heat treatment, described in § 3.4.2 and shown in Fig. 30, helped the extraction process.

Table 6. Determination was conducted on the obtained functional beverages. Mean ± standard deviation of three replicates.

Sample Code	pH	TSS (°Brix)	Total Phenols (mg CAE/g OP)	o-Diphenols mgCAE/gTPOP
WHT-OP-W30	5.10 ± 0.12^{a}	0.7 ± 0.1^{a}	2.0 ± 0.1^{a}	2.5 ± 0.1^{a}
WHT-OP-W60	5.16 ± 0.11^{a}	0.7 ± 0.1^{a}	1.7 ± 0.1^{b}	2.3 ± 0.1^{a}
WHT-OP-W 90	5.10 ± 0.10^{a}	0.6 ± 0.1^{b}	1.9 ± 0.2^{a}	2.5 ± 0.2^{a}
WHT-OP-W (Mixed)	5.12 ± 0.09^{a}	0.7 ± 0.1^{a}	1.7 ± 0.2^{b}	2.5 ± 0.2^{a}
WHT-OP-6% CAW30	1.98 ± 0.11^{b}	5.8 ± 0.4^{b}	2.1 ± 0.2^{b}	2.5 ± 0.2^{a}
WHT-OP-6% CAW 60	2.02 ± 0.10^{b}	2.02 ± 0.10^{b}	5.6 ± 0.3^{b}	2.5 ± 0.2^{a}
WHT-OP-6% CAW 90	1.99 ± 0.12^{b}	1.99 ± 0.12^{b}	5.6 ± 0.2^{b}	2.2 ± 0.1^{a}
WHT-OP-6% CAW (Mixed)	1.99 ± 0.08^{b}	1.99 ± 0.12^{b}	5.9 ± 0.3^{b}	2.4 ± 0.2^{a}

Small letters indicate significant differences in the column at $p < 0.05$

Table 6. shows the physicochemical analysis of prepared functional beverages. The study found that the W-HT samples had mildly acidic pH levels around 5.10 to 5.16, whereas the CA-HT samples were much more acidic, with pH levels around 1.98 to 2.02.

The Total Soluble Solids (TSS) values were significantly higher in the CA-HT samples (5.6 °to 5.9 °Brix) than in the W-HT samples (0.6 to 0.7 °Brix). Findings indicate that WHT-OP-6% CAW beverages are sweeter, potentially due to added sugars or other soluble ingredients that enhance flavor and increase consumer appeal.

WHT-OP-W and WHT-OP-6% CAW samples showed substantial phenolic compounds, with total phenols ranging from 1.7 to 2.2 mg CAE/g TPCOP. These high levels of phenolics highlight the beverages' antioxidant capacity, which is beneficial for health and can help reduce oxidative stress. The results suggest that WHT-OP-6% CAW beverages have a more pronounced tangy flavor, which can be a key factor in taste preference and overall acceptability. WHT-OP-6% CAW beverages appear more acidic and sweeter than WHT-OP-W beverages, which might influence consumer preferences and marketability.

The phenolic profile of functional beverages (FB) derived from WHT-OP-W (water heat treatment) and WHT-OP-6% CAW (citric acid heat treatment) highlights the differential influence of the treatment medium on the recovery and stability of phenolic compounds shown in Fig.31.

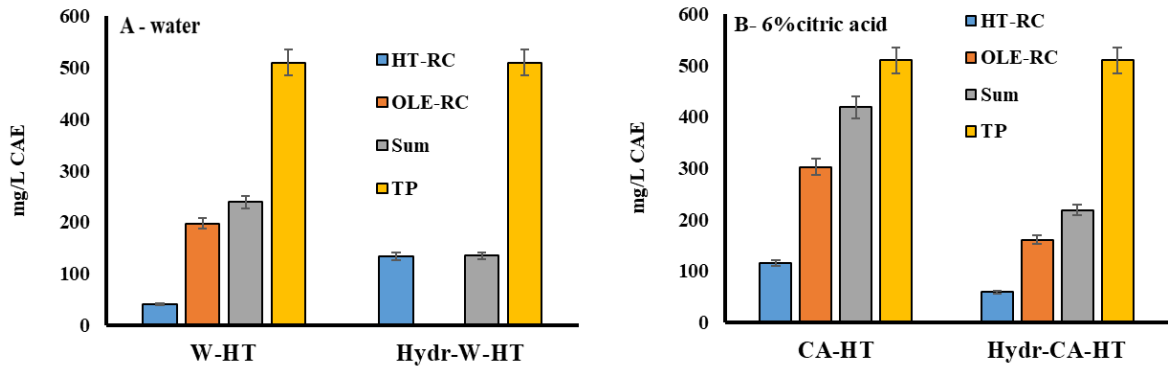

Fig. 31 _ Phenolic profile of fresh and HCl-heat hydrolyzed (Hydr-) functional beverages determined by HPLC analyses (HY-RC, hydroxytyrosol related compounds; OLE-RC, oleuropein related compounds; SUM of HY-RC and OLE-RC) and Folin Ciocalteu's method (TPC, Total phenols)

The observed differences indicate the critical role of the extraction medium in increasing the solubility and stability of phenolic compounds during heat treatment. Citric acid is essential as an extraction medium and stabilizer in WHT-OP-6% CAW. Its acidic nature will inhibit the activity of polyphenol oxidase (PPO), thus reducing the enzymatic browning and oxidative degradation of phenolic compounds. In addition, the chelating properties of citric acid may help to preserve hydroxytyrosol-related compounds (HY-RC) and oleuropein-related compounds (OLE-RC), which are now more abundant in CA-HT than in W-HT. Aliakbarian *et al*. (2009) showed that organic acids play a role in maintaining the stability of phenolic compounds under thermal and oxidative stress. Similarly, Rodríguez *et al*. (2007) reported high-temperature values of olive-derived phenolic compounds, supporting the results found in this study. When these results were compared with previous studies, the recovery of TPC in WHT-OP-6% CAW (2.1 mg/g) was consistent with the findings of De Leonardis *et al*. (2009), who stated that an acidic environment can reduce the degradation of phenols while increasing their yield.

Furthermore, the amount of TPC in WHT-OP-6% CAW meets the antioxidant activity recognized by the European Food Safety Authority (EFSA), according to which the phenolic content of olive oil should exceed 5 mg hydroxytyrosol and its derivatives per serving. Although the actual concentrations in FBs vary depending on the nature of the matrix, the relative recovery of citric acid suggests that it is beneficial. The scientific implications of these findings can be extended to the production of functional beverages. The higher phenolic

stability in WHT-OP-6% CAW indicates its ability to act as an antioxidant, essential for developing health information on hydroxytyrosol and other compounds. More importantly, the amount of HY-RC and OLE-RC in WHT-OP-6% CAW showed that these bioactive substances significantly affect the total phenolic content. These properties confirm the findings of Rodríguez *et al*. (2009) on the significant antioxidant activities of hydroxytyrosol derivatives. This shows the importance of citric acid in preserving and improving the bioactivity of phenolic compounds during processing.

In contrast, the low phenolic retention in WHT-OP-W reflects the limitations of using water as an extraction medium. However, water is cost-effective and widely used in heavy industry; it does not reduce oxidation and enzymatic activity during heat treatment, thus providing good recovery of phenolic compounds. These results resonate with the observations of Servili *et al*. (2011), who reported the effects of water-extracted phenolics on degradation during processing.

The above results highly emphasize the importance of optimizing extraction media to improve phenolic recovery and stability; the performance of WHT-OP-6% CAW showed its potential for industrial use in producing healthy drinking water.

The antioxidant activity of functional beverages (FB) prepared using heat treatment (HT) with 6% citric acid (WHT-OP-6% CAW) and distilled water (WHT-OP-W) was evaluated using the DPPH radical scavenging assay. Ascorbic acid was used as the standard reference for comparison due to its well-documented radical-scavenging properties described in Table 7.

Table 7. Linear regression (percentage DPPH radical inhibition versus mg/mL antioxidant concentration) and IC_{50} value of the functional beverage and ascorbic acid. IC_{50} of samples was expressed as mg/mL CAE

Samples	Linear equation	R^2	IC_{50} (mg/mL)
WHT-OP-6% CAW	y = 1.85x ± 4.83	0.999	24.4 ± 1.5^{a}
W-HT	y = 5.43x ± 8.10	0.967	7.7 ± 1.6^{b}
Ascorbic acid	y = 6.94x ± 3.42	0.996	6.7 ± 1.6^{b}

Small letters indicate significant differences in the column at $p < 0.05$

The IC_{50} values, indicative of the concentration required to inhibit 50% of DPPH radicals, demonstrated notable differences between the treatments. The IC_{50} for WHT-OP-W was 7.7 ± 1.6 mg/mL CAE, closely approximating that of ascorbic acid (6.7 ± 1.6 mg/mL CAE), suggesting robust antioxidant activity. Conversely, WHT-OP-6% CAW exhibited a higher IC_{50} value of 24.4 ± 1.5 mg/mL CAE, reflecting comparatively moderate activity.

The observed difference in antioxidant activity is mainly due to the phenolic composition of the two beverages. The high antioxidant capacity of WHT-OP-W is consistent with the higher content of hydroxytyrosol-related compounds (HY-RC), known for their excellent free radical scavenging efficiency. In comparison, WHT-OP-6% CAW, despite its phenolic richness, contains more oleuropein-related compounds (OLE-RC), which were reported to have low antioxidant activity in the DPPH effect assessment. This finding is consistent with previous studies, such as Rodríguez *et al*. (2009), whose studies highlighted the superior antioxidant capacity of hydroxytyrosol over oleuropein in similar experimental contexts. While it provides stability to phenolic compounds by preventing enzymatic oxidation during processing, its acidic nature (pH ~ 2) will affect the reactivity and accessibility of some phenols, especially in DPPH analysis. Similar results were found by Aliakbarian *et al*. (2009), who showed that organic acids can alter antioxidant activity by changing the solubility and integrity of phenolic extracts.

Furthermore, the antioxidant activity of WHT-OP-6% CAW is lower compared to WHT-OP-W, which may be due to the interaction of citric acid and oleuropein, which may cause adverse effects of free radical neutralization. As suggested by Adiamo *et al*. (2017). This result demonstrates the potential of WHT-OP-W as a natural and effective antioxidant source, in agreement with the findings of Fernández-Bolaños *et al*. (2004), who revealed that hydroxytyrosol-rich extracts from olives showed high bioactivity from the product. Such comparisons confirmed the suitability of WHT-OP-W for food applications, especially where intense radical scavenging activity is required. However, they also highlight their wider operational benefits. The use of citric acid, in addition to improving the beneficial nutritional properties and microbial stability of the beverage, also preserves phenolic compounds during storage and processing. This feature of citric acid was also noted in the study by Foti *et al*. (2022), where citric acid-containing systems showed improved phenolic stability and product shelf life compared to neutral pH systems. The oxidation potential is greatly affected by solvent selection and processing. WHT-OP-W exhibits antioxidant activity compared to synthetic samples, making it a candidate for functional beverages where the ability to scavenge free radicals is more critical. At the same time, WHT-OP-6% CAW has a balanced profile, combining antioxidant activity with additional benefits in terms of stability and palatability. These findings are consistent with the growing literature suggesting that agro-industrial by-products are a stable source of bioactive compounds in food.

4.2.3 Steam water extraction and application in beef meat patties

Innovative olive leaf extracts were obtained utilizing solvent steam water, employing a novel technology developed during the PhD program. The extracts were characterized and evaluated as potential antioxidants for minced meat. This study was conducted in collaboration with the Université Claude Bernard Lyon 1 (UCBL) as part of the doctoral research mobility program funded by the University of Molise, Italy.

Part of the results have been published in Iftikhar *et al*., 2024 and the abstract of the manuscript is reported here: In this work, by using water steam only, two antioxidant extracts were obtained from olive leaves (*Olea europaea* L.), a by-product of olive oil chain. Olive leave extracts (OLEs) were tested as such (water extract: WE) and partially purified with ethyl acetate (ethyl acetate [EA] extract). Total phenols were 7.4 mg/mL and 3.8 mg/mL in WE and EA final solutions, respectively, evidencing a different composition by high-performance liquid chromatography analysis. Both extracts were evaluated in vitro compared to pure hydroxytyrosol (Hy). A 2,2-diphenyl-1-picrylhydrazyl (DPPH) EC_{50} of 57.6, 76.5, and 39.7 µg/mL and a ferric reducing antioxidant power EC_{50} of 84.8, 69.9, 41.2 µg/mL were determined for WE, EA, and Hy solution, respectively. The Rancimat induction time determined at 120°C in a lard sample with 200-ppm total phenol equivalent addition of WE, EA, and Hy was 8.92 h, 12.74 h, and 7.27 h, respectively (vs. 2.24 h for lard only). Extracts were added at the same dose (200 ppm) to minced beef patties that were put in closed containers under controlled air headspace composition (NA, natural air; HOA, modified air with 80% O_2; and 20% N_2) and stored at 4°C up to 10 days. Extracts showed significant effectiveness in contrasting the decrease of O_2 level in containers and the patties' pH and color changes. A significant increase (expressed as mg of malondialdehyde MDA/kg; thiobarbituric acid-reactive substance (TBARS) assay was conducted in control samples at an interval of 0–10 days (from 0.52 to 0.78 mg of MDA/kg in NA samples; and from 0.55 to 1.31 mg of MDA/kg in HOA samples), while minimal changes were observed in treated samples. These findings suggest using OLEs to maintain beef patties' quality and oxidative stability during storage.

Antioxidant extracts from olive leaves (*Olea europaea* L.) were prepared using water steam, resulting in a water extract (SW-OL-WEx) and an ethyl acetate-purified extract (SW-OL-EAEx). The extracts showed different total phenolic contents: 7.4 mg/mL for WE and 3.8 mg/mL for EA, with distinct compositional profiles revealed by HPLC analysis.

The **HPLC analysis** (Fig. 4.13) revealed the unique and distinct phenolic profiles of SW-EAEx (ethyl acetate extract) and SW-WEx (water extract).

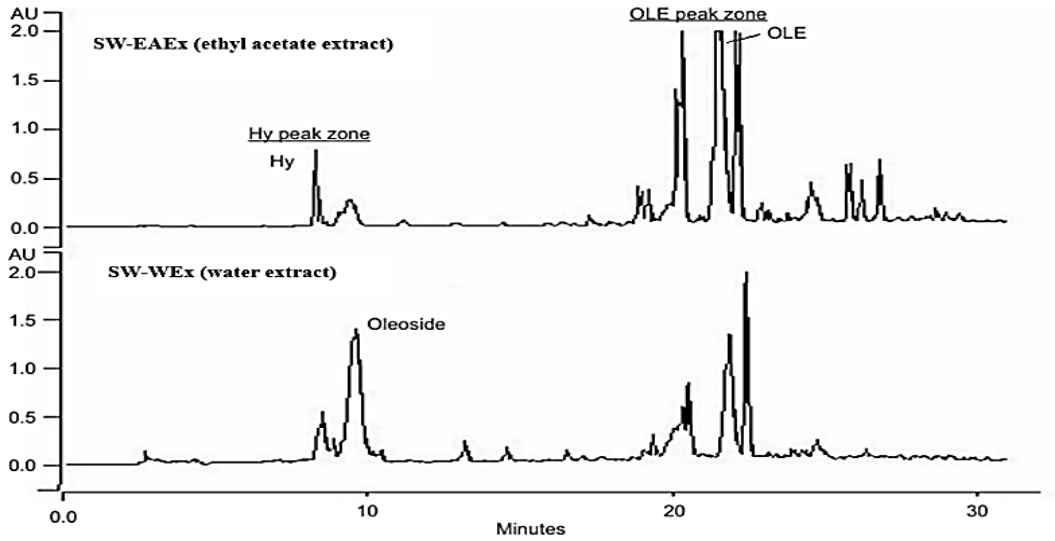

Fig.32 _ HPLC analysis at 240nm of steam water olive leaves extracts; ethyl acetate (SW_EAE) & water (SW-WEx)

With a one-of-a-kind UV absorption spectrum, SW-WEx exhibited a dominant peak in the hydroxytyrosol region. This suggests the presence of an oleoside-like compound (Fig. 32), possibly derived from oleuropein decomposition during extraction. The SW-EAEx chromatogram displayed multiple peaks corresponding to phenolic acids, oleoside and secoiridoids, indicating a selective affinity for less polar compounds.

The observed differences underscore the solvent polarity's significant effect on the extracts' phenolic composition. The unique ability of water to solubilize polar and oxidized phenolic compounds has contributed to the distinctive profile of SW-WEx. At the same time, the role of ethyl acetate in facilitating the extraction of less polar phenolics in SW-EAEx is a key factor in understanding the compositional variations and the potential functional applications of the two extracts potentially favouring antioxidant-rich formulations due to their unique phenolic structure.

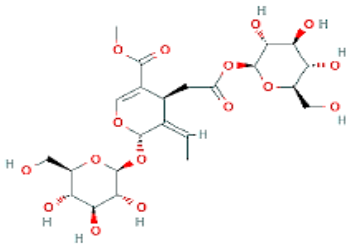

Fig.33 Formula of oleoside

The antioxidant potential of olive leave extracts (OLEs) produced through steam distillation was evaluated using two standard assays, **DPPH and FRAP**, to determine their efficacy as natural antioxidants in food. The comparison between SW-WEx (water-extracted olive leave extract) and SW-EAEx (ethyl acetate-extracted olive leave extract) demonstrated distinctive antioxidant capacities. The DPPH scavenging assay measures the ability of a substance to scavenge free radicals, whereas the FRAP assay assesses the reducing power of a substance. The calculated EC_{50} through the linear line equation was very low for SW-WEx (57.6 µg/mL) compared to SW-EAEx (76.5 µg/mL), as shown in Fig.34, indicating its superior radical scavenging ability.

This finding can be attributed to polar phenolic compounds, which may exhibit synergistic effects, enhancing the capacity of the extract to neutralize free radicals efficiently. Furthermore, the rapid kinetics of DPPH radical scavenging were observed in SW-WEx, achieving stability within 80 min compared with 180 min for SW-EAEx, further supporting its efficacy in mitigating oxidative stress in aqueous systems. In contrast, the FRAP assay demonstrated a higher reducing power in SW-EAEx, with an EC_{50} of 69.9 µg/mL compared to 84.8 µg/mL for SW-WEx.

This outcome suggests that the ethyl acetate extraction process enriches SW-EAEx with compounds better suited for electron transfer reactions.

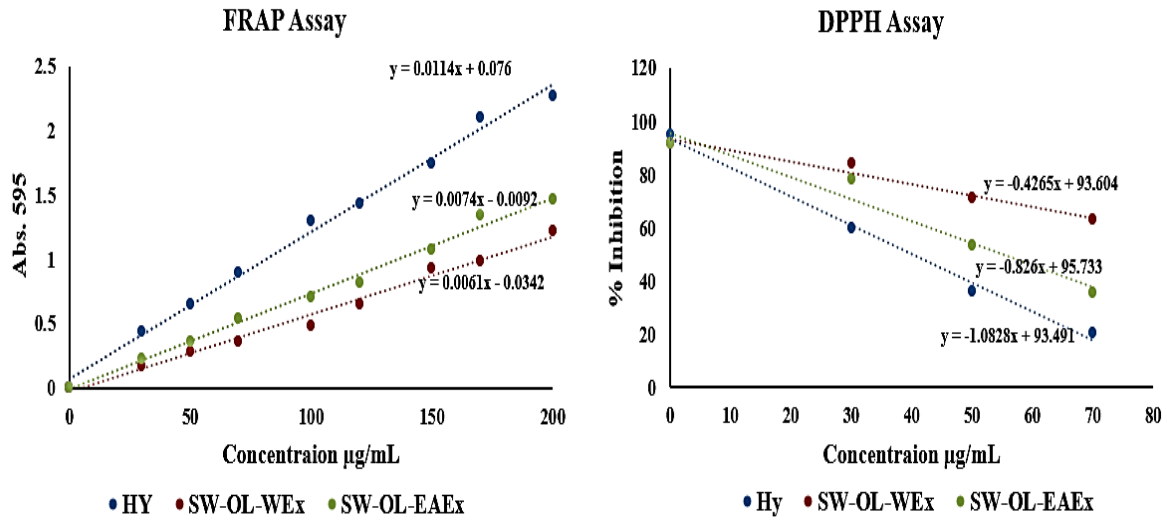

Fig. 34 _ Antioxidant assays (DPPH & FRAP) for steam water extracts ethyl acetate (SW_EAE) & water (SW-Wex) compared to standard of hydroxytyrosol (Hy)

These findings indicated that the antioxidant efficacy of OLEs is significantly influenced by the extraction solvent, which alters the phenolic composition and functionality of the extracts. SW-WEx, enriched with water-soluble phenolics, demonstrated superior radical scavenging performance, making it suitable for applications in aqueous systems.

These results are consistent with those of previous studies, such as those by De Leonardis et al. (2015), who emphasized solvent polarity's crucial role in determining extracts' phenolic profile and antioxidant performance. Furthermore, the performance of both SW-WEx and SW-EAEx, comparable to standard antioxidants such as gallic acid and hydroxytyrosol, underscores their potential as natural alternatives for oxidative stability in food systems. These findings provide a strong basis for applying OLEs as multifunctional natural antioxidants in diverse food and industrial applications.

The steam extracts were applied to **minced beef patties** at 200 ppm and stored under natural air conditioning at 4°C for 10 days. During storage, the extracts effectively maintained pH stability and color attributes throughout storage. TBARS analysis of control samples showed an increase in lipid oxidation from 0.52 to 0.78 mg MDA/kg over the 10 days, while treated samples exhibited minimal oxidative changes. These results demonstrate the potential of olive leave extracts as natural antioxidants for maintaining beef patty quality during refrigerated storage.

The **pH values** of patties samples, including control (C), water extract-treated (SW-OL-Wex), and ethyl acetate extract-treated (SW-OL-EAEx) beef meat patties, were stored in the same manner for 10 days shown in Table 8.

Table 8. pH determination in beef meat patties during storage

Storage Days	C	SW-OL-Wex	SW-OL-EAEx
0	5.3 ± 0.2^{a}	5.3 ± 0.2^{a}	5.3 ± 0.2^{a}
5	5.2 ± 0.1^{a}	5.2 ± 0.1^{a}	5.2 ± 0.1^{a}
10	5.5 ± 0.2^{b}	5.5 ± 0.2^{b}	5.5 ± 0.2^{b}

Small letters indicate significant differences in the column at $p < 0.05$

The pH value of all treatments initially showed a slight decrease from 5.3 ± 0.2 on day 0 to 5.2 ± 0.1 on day 5 and then slightly increased to 5.5 ± 0.2 on day 10 evidencing that the microorganism growth was limited. More importantly, there is no significant difference between the control and treatment samples, indicating that adding the extract (SW-OL-Wex and SW-OL-EAEx) did not affect the pH. The stability of the pH value for all samples suggests the effectiveness of the refrigerator in preserving product quality in the short term.

Although the extract did not affect the pH, its antioxidant and antimicrobial properties may cause other adverse effects, such as slow lipid oxidation or inhibition of microbial growth. These findings suggest that low-temperature natural conditioning is suitable for short-term meat storage.

Table. 9 Color characteristics of beef patties formulated with olive leaves steam water extracts (SW) stored at 4°C. Different letters show significant differences in the same column for L*, a*, and b* ($p < 0.05$).

	Days	C	SW-OL-Wex	SW-OL-EAEx
L*				
	0	46.2 ± 0.7^{a}	47.4 ± 0.9^{a}	47.7 ± 0.8^{a}
	5	41.3 ± 0.7^{b}	54.9 ± 1.6^{b}	50.7 ± 1.5^{b}
	10	39.5 ± 0.5^{b}	43.5 ± 2.4^{c}	45.6 ± 0.4^{c}
a*				
	0	13.9 ± 0.5^{a}	13.2 ± 0.6^{a}	13.9 ± 0.5^{a}
	5	9.9 ± 0.7^{b}	11.3 ± 0.5^{b}	10.6 ± 0.7^{b}
	10	8.3 ± 0.3^{b}	9.1 ± 1.9^{c}	8.9 ± 0.7^{c}
b*				
	0	11.9 ± 1.6^{a}	10.5 ± 2.4^{a}	9.1 ± 0.7^{a}
	5	13.6 ± 0.4^{b}	$11.2 \pm 1.0^{a, b}$	$11.3 \pm 0.9^{a, b}$
	10	16.0 ± 0.4^{c}	13.9 ± 2.3^{b}	10.5 ± 4.9^{b}

Small letters indicate significant differences in the column at $p < 0.05$

In consideration of meat quality, **color parameters** L* (lightness), a* (redness), and b* (yellowness) are critical indicators influencing consumer perception and acceptance. In this study, we observed a decline in all three parameters across control patties (C), ethyl acetate-treated patties (SW-OL-EAEx), and water extract-treated patties (SW-OL-Wex) over a 10-day storage period, as shown in Table 9.

All samples exhibited a decrease in lightness (L*), indicating a darkening of the meat. For instance, the L value for control patties (C) decreased from 46.2 ± 0.7 on day 0 to 39.5 ± 0.5 on day 10. This trend is consistent with the oxidation of myoglobin to metmyoglobin, which imparts a brownish hue to beef during storage. Such oxidation is a common phenomenon in meat products stored under aerobic conditions.

The observed decline in redness (a) across all samples indicates a loss of the fresh meat color, likely due to the oxidation of oxymyoglobin to metmyoglobin. For C decreased from 13.9 ± 0.5 on day 0 to 8.3 ± 0.3 on day 10. This change is typical in stored meat and can influence consumer perception, as a bright red color is often associated with freshness.

Yellowness (b) increases during storage, which may be attributed to the degradation of pigments and lipid oxidation. Lipid oxidation can lead to the formation of compounds that affect color stability, contributing to the observed increase in b values. For instance, the b* value for C increased from 11.9 ± 1.6 on day 0 to 16.0 ± 0.4 on day 10, indicating a complex interaction of factors affecting yellowness.

Comparing the treatments, ethyl acetate-treated patties (SW-OL-EAEx) and water extract-treated patties (SW-OL-Wex) did not exhibit significant differences in the rate of color degradation compared to control patties (C). For example, the L* value for SW-OL-EAEx decreased from 47.7 ± 0.8 on day 0 to 45.6 ± 0.4 on day 10, and the L* value for SW-OL-Wex decreased from 47.4 ± 0.9 on day 0 to 43.5 ± 2.4 on day 10.

Overall, the reduction in L, a, and increment in b* values over the 10-day storage period reflects the natural progression of meat discoloration due to oxidative processes. These findings align with previous studies indicating that meat color deteriorates over time during storage, affecting its visual appeal and marketability (Petracci & Fletcher, 2002; Lopez *et al*., 2004).

The thiobarbituric acid reactive substances (TBARS) assay, a widely accepted indicator of lipid oxidation, shows that control (C), SW-OL-Wex, and SW-OL-EAEx cured patties kept under control conditions show significant differences in lipid oxidative stability, as shown in Fig.35.

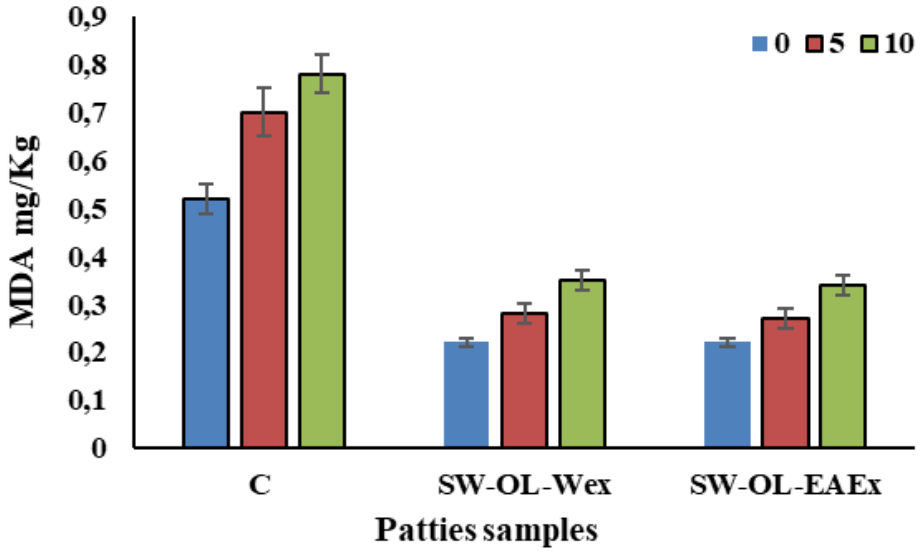

Fig. 35_Lipid oxidation determination for TBARS assay during storage.

Patties treated with OL-EAEx showed significantly decreased TBARS values during storage. Both treatments started with an initial MDA value of 0.22 on day 0, followed by a slight increase to 0.35 and 0.34 on day 10, respectively. These findings suggest that water and ethyl acetate extract effectively prevent lipid peroxidation, thereby preserving the oxidative stability of the patties ongoing research on antioxidant power (OLE). Kurt *et al*. (2017) demonstrated the ability of OLE to reduce oxidative stress in meat products and attributed its effect to bioactive phenolic compounds. It was shown that hydrophilic phenols in aqueous extracts and lipophilic phenols in ethyl acetate extracts can scavenge free radicals and inhibit lipid peroxidation. The comparable lipid oxidation trends observed between SW-OL-Wex and SW-OL-EAEx treated samples in this study further support the findings of North *et al*. (2019), who reported that various solvent-extracted OLEs had similar antioxidant activity in meat preservation.

The lower TBARS values associated with OLE-treated meat patties demonstrate the potential of these natural products as effective antioxidants in meat preservation. OLE extends the shelf life of meat products by preventing lipid oxidation and helps maintain sensory and nutritional quality. These findings argue for olive leave extract as an alternative to synthetic products to meet the increasing consumer demand for clean-label and sustainable foods.

4.2.4 Ultrasonic bath extraction (UB-Ex) and application in edible coating

Edible coatings have emerged as a promising method to prolong the shelf life of perishable foods, such as fruits and leavey vegetables. Incorporating natural bioactive extracts into biopolymer-based coatings has recently gained considerable attention due to their potential to preserve quality and inhibit microbial activity. This doctoral research focused on utilizing olive oil by-products in edible coatings to assess their impact on fruit preservation. However, other significant findings related to edible coatings are published in peer-reviewed manuscripts, summarized below.

In Iftikhar *et al*., 2022, the application of guar gum and chitosan coatings enriched with lemon peel essential oil (LPEO) was investigated to enhance the quality of pears during storage. Pear is a typically climacteric fruit and highly perishable with a low shelf life owing to extreme metabolic activity after harvesting the present study aimed to reduce weight loss and improve the firmness of pear during storage. The lemon peel essential oil (LPEO) has gained considerable attention due to being the richest source of bioactive compounds, behaving as a natural antioxidant agent, cost-effective, and generally recognized as safe (GRAS). Edible

coatings equipped with a natural antioxidant agent and renewable biopolymers have gained more research fame due to their involvement in biodegradability and food safety.

In this work, edible skin coating materials (ESCMs) embedded by chitosan (1 %) and guar gum (2 %) were fabricated, and afterward, five concentrations of LPEO (1, 1.5, 2, 2.5, and 3.0 %) were incorporated individually into the ESCMs. Findings revealed that LPEO-ESCMs significantly reduced weight loss and improved the firmness of pear up to 45 days of storage at 4 ± 2 °C. Further, the LPEO–ESCMs have enhanced the antioxidant capacity, antibacterial efficiency, and malondialdehyde level of pear during storage time. It was concluded that 3 % of LPEO–ESCMs improved the overall acceptability of pear fruits. Taken together, the novel insights of guar gum and chitosan-based ESCMs entrapped with LPEO will remain a subject of research interest for researchers in the future.

According to Cofelice *et al*., 2024, minimally processed fresh-cut leafy vegetables are becoming one of the fastest-growing segments in the food industry. In this context, new strategies to extend the preservation, such as applying edible coatings, represent a key issue for the research community. This study investigated the effects of emulsion-based edible coatings on the quality parameters of ready-to-eat Salanova lettuce. The coatings, sprayed on the fresh-cut lettuce, were composed of lemongrass essential oil (0.1%) dispersed in different polymers, such as alginate (ALG), chia mucilage (CM), and chitosan (CHIT). After storage at 4 °C for 14 days, the coatings showed similar visual appearance and water loss performance. Instead, using the spectroscopic and HPLC analyses, the ALG coating was the most effective in preserving bioactive pigments (chlorophyll and carotenoids) and the phenolic compounds. Precisely, concerning the initial content of hydroxycinnamic compounds (2.53 mg/g chlorogenic acid equivalents), their depletion was estimated to be 27%, 62%, 79%, and 84% in the ALG, CTRL, CM, and CHIT samples, respectively.

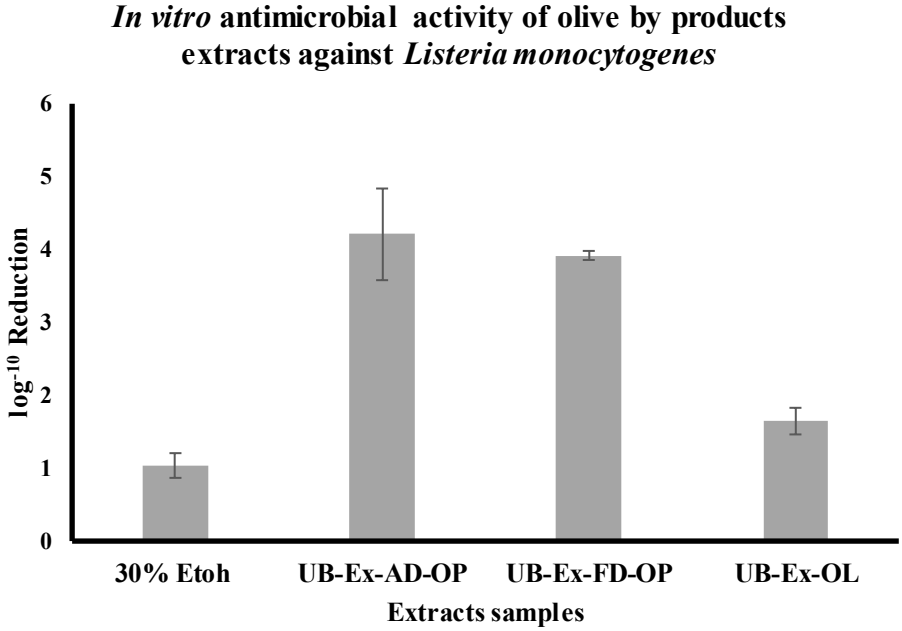

Fig. 36_In vitro analysis of water bath extracts from olive by-products against *Listeria monocytogenes*

In vitro experiments demonstrated the significant antimicrobial potential of olive by-product extracts, even when diluted tenfold from their initial concentration. These extracts exhibited complete inhibition against *Escherichia coli*, confirming their strong antibacterial properties. Additionally, their effectiveness against *Listeria monocytogenes* is illustrated in the histogram Fig.36, showing a notable reduction in bacterial growth. Notably, olive pomace (OP) extracts outperformed olive leaf (OL) extracts, reinforcing their superior antimicrobial activity. Building on these findings, this chapter prepares and applies edible coatings incorporating olive oil by-products, specifically pomace and leave extracts, into xanthan gum-based formulations. The study evaluates their effects on strawberries' shelf life and quality during cold storage. Analytical and microbiological assessments are discussed in detail to highlight the potential of these coatings as sustainable and effective solutions for post-harvest preservation. This study was conducted in collaboration with North Carolina State University (NCSU) as part of the doctoral research mobility program funded by the University of Molise, Italy.

The **physical appearance of strawberries** significantly changed during the 12-day storage period, as illustrated in Fig. 37.

Both uncoated control samples (C) and the blank-coated samples (B) displayed visible signs of spoilage by the 12th day. These signs included surface deterioration, visible mold growth, and loss of structural integrity. The rapid spoilage of these samples can be attributed to the

high moisture content and delicate epidermal structure of strawberries, which make them highly susceptible to microbial growth and physical degradation when stored under ambient or low-temperature conditions (Ali *et al*., 2019; Nair *et al*., 2018).

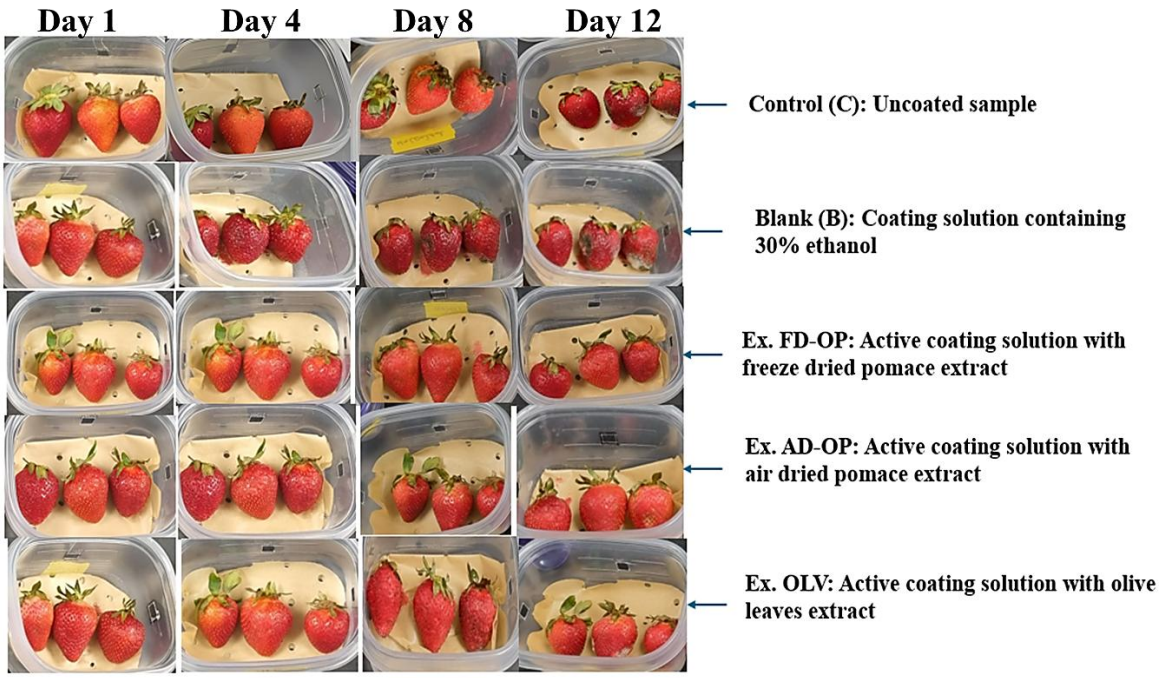

Fig. 37_Visual appearance of strawberries during storage for 12 days.

The strawberries with OL extracts significantly better preserved their physical characteristics. However, the effectiveness of the coating varies depending on the treatment used. By day 12, visible discoloration appeared, showing that even Ex.OL helped delay the spoilage process but did not work well to preserve the visual quality compared to other treatments. The discoloration may be attributed to oxidative stress or pigment degradation, frequently noted in studies involving plant-based edible coatings (Chitarra *et al*., 2020).

In contrast, strawberries coated with olive pomace (OP) extracts, specifically freeze-dried olive pomace (Ex.FD-OP) and air-dried olive pomace (Ex.AD-OP), displayed superior preservation of color, firmness, and overall physical integrity. These samples retained their vibrant red color and structural quality throughout the 12-day storage period, with no visible mold growth or spoilage.

The **physicochemical properties** of strawberries, including pH, titratable acidity (TA), total soluble solids (TSS), and texture, were significantly affected by the coating treatments over the 12-day storage period. As shown in Table 10, uncoated control samples demonstrated notable changes in quality parameters, indicative of accelerated ripening and spoilage. The

pH of the control samples increased slightly from 3.43 to 3.47, while titratable acidity increased marginally from 0.82% to 0.83%. These changes align with previous studies that reported metabolic activity in uncoated strawberries, where organic acids are consumed as substrates during respiration, leading to alterations in pH and acidity levels (Ali *et al*., 2019; Holcroft and Kader, 1992).

Table 10. Quality parameter analysis of strawberries coated with xanthan gum-based edible coating containing olive by-product extracts (OIBP) at 1 and 12 days of storage.

Storage (Days)	Samples	pH	Titratable Acidity (g citric acid/100g)	TSS	Texture (N)
1	C	3.43 ±0.12^{a}	0.82±0.01^{a}	9.00±0.00^{a}	2.07±0.06^{c}
	B	3.52±0.02^{b}	0.81±0.00^{a}	8.67±0.58^{a}	2.03±0.01^{c}
	Ex. FD-OP	3.51±0.06^{b}	0.82±0.05^{a}	9.00±0.50^{a}	2.33±0.23^{b}
	Ex. AD-OP	3.55±0.03^{b}	0.80±0.00^{a}	8.50±0.50^{a}	2.63±0.24^{a}
	Ex. OL	3.47±0.03^{ab}	0.80±0.00^{a}	8.83±0.29^{a}	2.23±0.40^{b}
12	C	3.47±0.02^{a}	0.83±0.02^{a}	7.83±0.58^{a}	1.41±0.10^{c}
	B	3.46±0.04^{a}	0.84±0.04^{a}	7.67±0.76^{a}	1.52±0.35^{bc}
	Ex. FD-OP	3.44±0.01^{a}	0.84±0.02^{a}	8.17±0.29^{a}	1.89±0.15^{ab}
	Ex. AD-OP	3.47±0.01^{a}	0.80±0.01^{a}	7.17±0.58^{a}	1.92±0.05^{a}
	Ex. OL	3.42±0.04^{a}	0.87±0.04^{a}	7.67±0.29^{a}	1.88±0.15^{ab}

Different letters show significant differences in the same column ($p < 0.05$) for each parameter: uncoated (C), blank (B), coated with freeze-dried olive pomace extract (Ex. FD-OP), air-dried olive pomace extract (AD-OP), and olive leaves extract (Ex-OL)

In addition to **pH** and **acidity**, a significant decline in TSS was observed in the control samples, which dropped from 9.00 to 7.83 °Brix by day 12. This reduction in TSS can be attributed to the degradation of sugars during respiration, which is accelerated in unprotected fruits due to increased metabolic activity and microbial spoilage (Silva *et al*., 2017). Furthermore, the **texture** of control strawberries deteriorated significantly, with firmness decreasing from 2.07 N to 1.41 N. This loss of firmness is a key indicator of softening caused by the enzymatic breakdown of pectic substances and cellular structure during storage, which is more pronounced in uncoated samples lacking protective barriers (Kader, 1992). In contrast, strawberries treated with olive by-product extracts exhibited improved stability in their physicochemical characteristics throughout the storage period. Among the coated fruits, those treated with freeze-dried olive pomace (Ex. FD-OP) extract demonstrated the best preservation of fruit quality.

The **TSS** in Ex. FD-OP-coated strawberries decreased more slowly, reaching 8.17 °Brix after 12 days. This outcome highlights the ability of Ex. FD-OP coatings decelerate sugar metabolism, likely due to the antioxidant and antimicrobial effects of the polyphenolic compounds found in the olive pomace extract. Comparable results were noted by Xu *et al*.

(2019), who found that bioactive coating enhanced with polyphenols significantly reduced metabolic processes and delayed sugar loss in strawberries.

The effectiveness of Ex. FD-OP coating was further confirmed through texture analysis, which showed a minimal decrease in firmness that showed minimal firmness decrease during storage. The final firmness value of 1.92 N was significantly higher than that of the control (1.41 N), indicating that Ex. FD-OP coatings effectively inhibited the enzymatic activity responsible for fruit softening. This effect can be attributed to the protective layer formed on the fruit's surface, which restricts moisture loss and oxygen exposure, thereby maintaining cellular integrity and texture (Jiménez-Munguía *et al*., 2022; Oliveira *et al*., 2020).

Strawberries coated with air-dried olive pomace (Ex.AD-OP) extract also showed promising outcomes, particularly in maintaining texture. By day 12, the firmness of Ex. AD-OP-coated strawberries decreased to 1.92 N, nearly matching the effectiveness of Ex. FD-OP. Although Ex. AD-OP coatings were slightly less efficient in preserving TSS levels (ending at 8.00 °Brix), they still outperformed the control significantly. The difference between Ex. FD-OP and Ex.AD-OP coatings can be attributed to their drying techniques, as freeze-drying is known to better preserve bioactive compounds and functional properties in plant extracts compared to air-drying (Wang *et al*., 2021).

This study demonstrates that Ex. FD-OP coatings exhibited the highest effectiveness in slowing down ripening and maintaining the physicochemical properties of strawberries during storage, followed closely by Ex. AD-OP, and to a lesser extent, Ex. OL coatings. These findings reinforce previous research emphasizing the importance of polyphenolic compounds in edible coatings, which provide antioxidant and antimicrobial protection, thereby reducing respiration rates, sugar loss, and enzymatic activity in perishable fruits (Rojas-Graü *et al*., 2009; Aziz *et al*., 2021) the superior performance of Ex. FD-OP coatings can be attributed to the enhanced retention of bioactive compounds during freeze-drying, which improves their functional properties and protective effects.

Surface color determines the freshness of strawberries and directly affects consumer satisfaction and market value. More than 80% of the initial judgment for fresh strawberries will be based on visual appearance regarding consistency and color intensity. As seen in Table 11, the first application layer directly affects the L*. FD-OP maintained its brightness throughout storage, especially on the eighth day (35.1 ± 2.3).

Table 11. Surface color of strawberries coated with xanthan gum-based edible coating containing olive by-product extracts (OIBP) during storage.

			L*		
Days	**C**	**B**	**Ex. FD-OP**	**Ex. AD-OP**	**Ex. OL**
1	31.3 ± 2.3^{a}	32.700 ± 3.0^{a}	33.367 ± 3.0^{a}	32.167 ± 3.0^{a}	31.633 ± 3.0^{a}
4	34.5 ± 1.4^{a}	33.567 ± 2.2^{a}	32.300 ± 2.1^{a}	32.167 ± 2.1^{a}	35.167 ± 2.1^{b}
8	29.3 ± 1.5^{c}	29.467 ± 2.8^{b}	31.067 ± 2.3^{a}	32.200 ± 2.3^{b}	35.100 ± 2.3^{b}
12	28.6 ± 2.2^{b}	27.200 ± 3.8^{c}	32.100 ± 2.3^{a}	30.533 ± 2.3^{b}	30.667 ± 2.3^{a}
			a*		
	C	**B**	**Ex. FD-OP**	**Ex. AD-OP**	**Ex. OL**
1	28.067 ± 3.0^{a}	31.967 ± 3.6^{a}	29.533 ± 3.6^{a}	29.733 ± 3.6^{a}	30.000 ± 3.6^{a}
4	28.767 ± 2.6^{a}	29.100 ± 3.8a^{b}	29.400 ± 3.9^{a}	28.467 ± 3.9^{b}	32.067 ± 3.9^{b}
8	30.133 ± 1.0^{b}	28.767 ± 2.1^{b}	31.833 ± 1.0^{b}	30.833 ± 1.0^{a}	27.900 ± 1.0^{c}
12	31.800 ± 2.1^{b}	28.800 ± 4.6^{b}	32.033 ± 2.5^{b}	30.700 ± 2.5^{a}	32.867 ± 2.5^{b}
			b*		
	C	**B**	**Ex. FD-OP**	**Ex. AD-OP**	**Ex. OL**
1	14.200 ± 3.0^{a}	17.200 ± 2.6^{a}	17.400 ± 2.6^{a}	14.833 ± 2.6^{a}	16.533 ± 2.6^{a}
4	16.000 ± 2.1^{b}	17.233 ± 2.4^{a}	14.467 ± 2.4^{b}	14.067 ± 2.4^{a}	18.267 ± 2.4^{b}
8	16.667 ± 2.3^{b}	15.933 ± 3.4^{b}	16.800 ± 2.7^{a}	16.033 ± 2.7^{b}	13.433 ± 2.7^{c}
12	18.033 ± 4.4^{c}	14.833 ± 4.2^{b}	17.300 ± 3.8^{a}	18.267 ± 3.8^{c}	17.900 ± 3.8^{b}

Different letters show significant differences in the same column ($p < 0.05$) for each parameter

These results are consistent with the findings of Khaliq *et al*. (2019); according to their research, the freshness of fresh fruits can be preserved by adding antioxidants. Martinez *et al*. (2023) stated that a* value above 32 indicates good strawberry quality. Table 4.10 shows a significant difference ($p < 0.05$) among the treatments on day 12; Ex. OL showed the highest a* value of 32.87 ± 2.5, indicating that the red color was improved. This difference differs from studies conducted with traditional methods, where color development is generally slow (Hassan *et al*., 2018). It also indicates that the ripening process will be faster; visible color changes may be because of olive leaves and fruit metabolism, changes in internal climate, and ripening dynamics. The highest value was in uncoated strawberries (C), 18.03 ± 4.4, and the lowest was found in blank strawberries (B), 14.83 ± 4.2. The decreasing pattern in L* values of coated strawberries is like that mentioned in the study of Gol *et al*. (2023). The data presented in table 4.10 show that there are significant differences ($p < 0.05$) among the treatments on the 12th day; OL shows the highest a* value (32.87 ± 2.5), shown in red. Unlike traditional method studies that often delay color development (Hassan *et al*., 2018), these

models also show that the ripening process can be faster. One reason for this color development may be the interaction between olive leaves and fruit metabolism, changes in internal climate, and ripening dynamics. Yellowness (b*) values increased on day 12 for all treatments, with the highest value observed in uncoated strawberries (18.03±4.4) and the lowest value observed in Blank strawberries (14.83±4.2).

Table 12 illustrates the variations in **Total Phenol Content (TPCC)** and **Total Flavonoid Content (TFC)** of fresh strawberries stored for 12 days under different coating treatments. These bioactive molecules are crucial for strawberries' antioxidant properties, nutritional quality, and overall appeal. Coating treatments incorporating olive leave extract (Ex. OL) and olive powder extracts (Ex. FD-OP and Ex. AD-OP) demonstrated notable efficacy in preserving these compounds, aligning with earlier findings in food preservation studies.

Table. 12 Total bioactive compounds retention during storage of strawberries coated with xanthan gum-based edible coating containing OIBP extracts

			TPCC (mg GAE/100 g F.W.)		
Days	**Control**	**Blank**	**Ex. FD-OP**	**Ex. AD-OP**	**Ex. OL**
1	124.8±14.1^{a}	113.4±2.2^{a}	114.9±1.8^{a}	112.9±3.4^{a}	115.0±1.7^{a}
4	111.3±0.6^{a}	111.6±2.0^{a}	110.5±0.5^{a}	111.6±1.1^{a}	110.8±0.8^{a}
8	92.4±1.5^{b}	78.7±11.5^{b}	95.9±9.7^{b}	90.7±10.0^{b}	84.6±1.8^{b}
12	87.5±7.9^{b}	98.9±2.7^{c}	98.4±6.7^{b}	105.9±3.4^{a}	100.4±2.0^{b}
			TFC (mg QC / 100 g F.W.)		
	Control	**Blank**	**Ex. FD-OP**	**Ex. AD-OP**	**Ex. OL**
1	32.5±2.8^{a}	31.3±1.5^{a}	33.5±1.2^{a}	31.7±0.4^{a}	33.0±1.9^{a}
4	25.4±4.9^{a}	19.9±0.9^{b}	18.9±0.4^{b}	16.7±1.7^{b}	18.4±0.9^{b}
8	48.1±0.2^{b}	30.4±3.0^{a}	30.2±5.2^{b}	27.3±2.2^{c}	40.5±0.1^{c}
12	36.9±1.3^{c}	29.3±1.7^{a}	35.0±1.6^{b}	45.4±1.0^{d}	33.7±1.3^{a}

Different letters show significant differences in the same column ($p < 0.05$) for each parameter.

The TPC of uncoated strawberries significantly declined over the storage period, dropping from 124.8±14.1 mg/100g F.W on day 1 to 87.5±7.9 mg/100g F.W by day 12. This decline is consistent with the oxidative degradation of phenolic compounds during storage, as previously reported by Khaliq et al. (2019), who observed a similar decline in uncoated fruits. Among coated strawberries, Ex. OL and Ex. AD-OP treatments were the most effective in maintaining TPCC levels. By day 12, TPCC values for Ex. OL and Ex. AD-OP coated strawberries were 100.4±2.0 and 105.9±3.4 mg/100g F.W, respectively. The antioxidant properties of olive leaves and powder extracts likely contributed to this effect by scavenging free radicals and inhibiting oxidative enzymes. Talebi *et al*. (2021) also highlighted the role of natural antioxidants in coatings to preserve phenolics in fruits during storage. In comparison, the Blank treatment showed a moderate decline, with TPCC values reaching 98.9±2.7 mg/100g F.W on day 12, reinforcing the need for bioactive components in coatings to protect phenolics effectively.

The TFC of strawberries remained relatively stable across all treatments during the storage period. For instance, in the Blank treatment, TFC values slightly decreased from 31.3±1.5 mg/100g F.W on day 1 to 29.3±1.7 mg/100g F.W on day 12, indicating that flavonoid content was not significantly affected by the storage conditions. Similar trends were observed in other treatments: Ex. OL strawberries retained TFC values of 33.7±1.3 mg/100g F.W on day 12, compared to 33.0±1.9 mg/100g F.W on day 1. Ex. AD-OP strawberries showed TFC values of 32.9±1.4 mg/100g F.W on day 12, compared to 33.1±1.6 mg/100g F.W on day 1. These minimal changes suggest that flavonoids are relatively stable under the tested storage conditions. The slight preservation effect observed with Ex. OL and Ex. AD-OP treatments can be attributed to incorporating olive leaves and powder extracts, which likely stabilized flavonoids by reducing their exposure to oxidation and enzymatic degradation.

Analyzing the **microbiological quality** of highly perishable fresh produce, particularly strawberries, is essential for ensuring food safety and extending shelf life. During storage, the growth of spoilage microorganisms, mainly aerobic bacteria, yeasts, and molds, can lead to accelerated deterioration, reducing nutritional value and posing potential health risks to consumers.

The results shown in Fig. 38 illustrate the impact of xanthan gum-based edible coatings enriched with various olive byproduct extracts on the aerobic plate count (APC) and yeast and mold (Y&M) levels of fresh strawberries over a 12-day storage period. The APC values reflect the overall microbial load on the strawberries.

On day 1, uncoated strawberries (C) exhibited the highest initial APC load at 2.43±0.12 log10 CFU/mL, followed by coated treatments with loads of 2.20±0.10 (blank), 1.67±0.15 (Ex. FD-OP), 1.30±0.26 (Ex.AD-OP), and 1.60±0.35 (Ex. OL) log10 CFU/mL. By day 12, FD-OP and AD-OP showed significantly lower APC values at 1.47±0.25 log10 CFU/mL compared to C (3.33±0.12) and B APC (3.20±0.10) log10 CFU/mL.

The reduction in microbial load can be attributed to the antimicrobial compounds in the olive pomace extract, which worked synergistically with the xanthan gum matrix to inhibit aerobic bacteria growth. Yang *et al*. (2022) also observed decreased bacterial counts in strawberries treated with xanthan gum and green tea extract coatings.

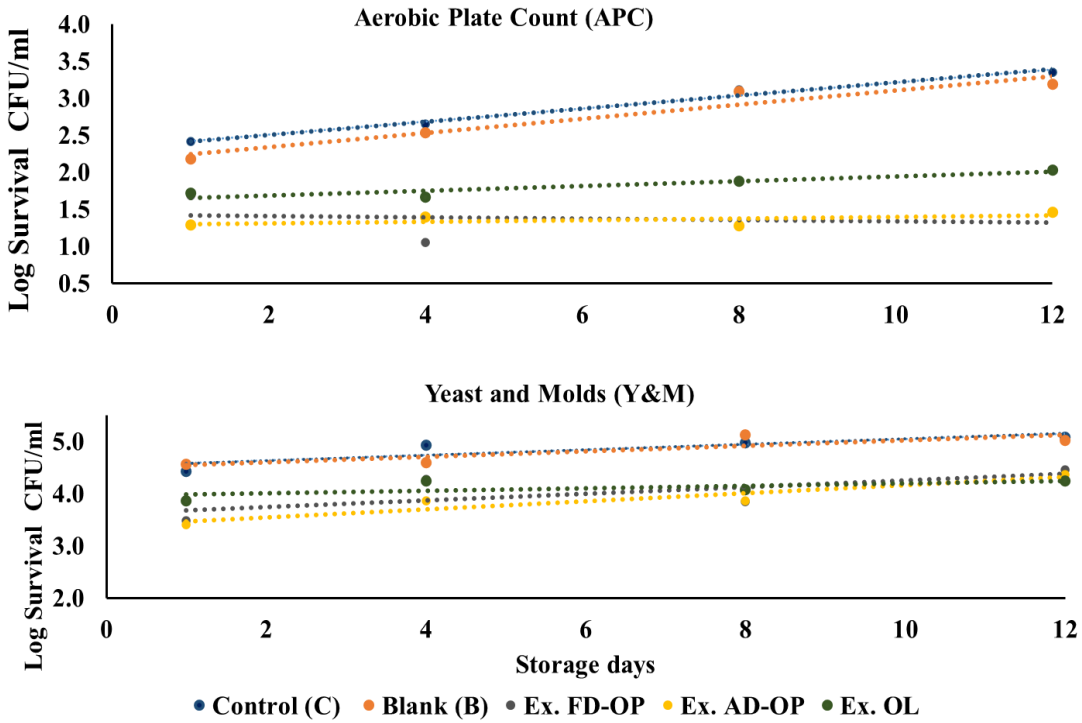

Fig. 38_ Antimicrobial activity of xanthan gum-based edible coating enriched with olive oil by-product (OIBP) extracts prepared via ultrasound bath extraction, assessed against aerobic plate count (APC) and yeast and mold (Y&M) on strawberries during storage.

The results for Y&M counts demonstrated the effectiveness of the xanthan gum-based edible coating on fresh strawberries, especially those enriched with olive byproduct extracts. Initially, on day 1, Control had 4.40±0.36 log10 CFU/mL, while the coated treatment Ex. OL showed a significantly lower value of 3.83±0.29 log10 CFU/mL. As storage progressed, Y&M counts increased across all treatments, but the coated strawberries, particularly Ex. OL maintained considerably lower levels than the control. By day 12, the Y&M count for Ex. OL was 4.23±0.06 log10 CFU/mL, notably lower than the control at 5.07±0.12 log10 CFU/mL. In contrast, blank-coated strawberries showed a higher Y&M count of 4.57±0.12 log10 CFU/mL at day 12, indicating less effective control of yeast and mold growth over the storage period. For instance, Mohammadi *et al*. (2024) reported similar yeast and mold count reductions for lime coated with xanthan gum and pomegranate peel extract.

These findings suggest that incorporating olive byproducts, mainly olive leave extract, into the xanthan gum matrix enhances the antimicrobial properties of the coatings, effectively limiting the growth of yeasts and molds on strawberries throughout the storage period. The less pronounced effect observed in the blank indicates that olive-derived compounds played a

crucial role in improving the antifungal efficacy of the xanthan gum-based coatings. This improvement in microbiological quality is vital for preserving fresh strawberries' overall postharvest life and market acceptability. Analyzing the microbiological quality of highly perishable fresh produce, particularly strawberries, is essential for ensuring food safety and extending shelf life. During storage, the growth of spoilage microorganisms, mainly aerobic bacteria, yeasts, and molds, leads to accelerated deterioration, reducing nutritional value and posing potential health risks to consumers.

4.3 Exploration of other recovery methods

During the PhD course, other methods were investigated at the laboratory scale to create an alternative and sustainable protocol for recovering bioactive compounds from olive industrial byproducts Table 13. Unfortunately, controversial results were obtained; consequently, the final extracts were considered unsuitable for food applications.

Table 13. Principle extracts obtained by the non-thermal investigation in this research.

Tags	Raw materials	Solvent	Food Application	Extraction methods
Non-thermal technologies				
Comparative Analysis of Ultrasonic Probe Extraction (UP-Ex) with and without Surface Dielectric Barrier Discharge (SDBD) Pretreatment				
AD-OP-SDBD-70%EtOH-UP-Ex *AD-OP-70%EtOH-UP-Ex* *OL-SDBD-70%EtOH-UP-Ex* *OL-70%EtOH-UP-Ex* *OL-SDBD-30%EtOH-UP-Ex* *OL-30%EtOH-UP-Ex* *OL-SDBD-80%MetOH-UP-Ex* *OL-80%MetOH-UP-Ex*	all dried olive oil industry by-products (OIBP)	organic solvents (30% & 70% EtOH and 80% MetOH)	None	Ultrasonic probe extraction
Plasma active water (PAW) vs Distilled water as solvent				
OL-W-UP-Ex OL-PAW-UP-Ex	dried olive leaves	plasma-activated water (PAW) and distilled water	None	Cold atmospheric plasma + Ultrasonic bath extraction

This chapter discusses the applied extraction methods considered more innovative than those typically reported in the literature. Specifically, it reports a comparative study to evaluate the combinate effect of non-thermal emerging technologies, such as the Ultrasonic Probe (UP-Ex) and the Cold Atmospheric Plasma Extraction, tested singularly or in combination. In addition, suitable solvents have been chosen, such as water, water-alcoholic solution, and

plasma active water. The operative conditions are described in § 3.3.5 and summarized in Table 13.

Plasma Activated Water (PAW) technology involves the treatment of water with plasma, a state of matter composed of highly reactive particles such as electrons, ions, and reactive oxygen and nitrogen species (RONS). This treatment modifies the chemical composition of the water, enhancing its efficacy for various applications (Thirumdas *et al*., 2018). Plasma Activated Water is recognized for its environmental safety, high efficiency, and lack of residual harmful substances, making it a compelling alternative to traditional chemical solvents.

The use of PAW as a solvent has been documented since the 1970s, owing to its ability to generate an acidic environment and produce reactive species capable of microbial disinfection and surface modification. Our decision to utilize PAW in this research was driven by its superior disinfection capabilities and versatility in various applications. Unlike conventional chemical disinfectants, PAW does not leave toxic residues, thereby mitigating environmental concerns and ensuring safety in its applications (Soni *et al*., 2021).
In the context of agriculture and food safety, PAW has demonstrated significant potential. It has been employed to enhance seed germination, promote plant growth, and control plant diseases and pests. In food safety, PAW has been used to disinfect food products, extending their shelf life. Its application in surface disinfection in medical and industrial settings has also been documented, highlighting its efficacy in maintaining hygiene standards. Additionally, PAW has found use in improving the efficiency of nitrate fertilizer production, showcasing its broad utility across different sectors (Guo *et al*., 2021).

Surface Dielectric Barrier Discharge (SDBD) is another type of plasma technology used for surface disinfection. SDBD involves the application of plasma to a surface through a dielectric barrier, which insulates at least one of the electrodes. This configuration generates plasma microdischarges that kill bacteria and other pathogens on surfaces. SDBD has been widely studied for its efficacy in microbial disinfection, with studies demonstrating its effectiveness against bacteria such as *Pseudomonas aeruginosa* in both in vitro and in vivo settings (Mohsenimehr & von Keudell, 2023).

In this research, SDBD and PAW technologies were explored to improve the extraction rate and facilitate the extraction of phenolic compounds. Despite their potential to enhance extraction processes, the results indicated that SDBD and PAW did not significantly improve the extraction and antioxidant activity of the extracts when applied to olive by-product (OIBP) powders. This finding suggests that while SDBD and PAW are effective in other contexts, their application in this specific extraction process may require further optimization or alternative approaches.

The adoption of PAW and SDBD technologies in this study was motivated by the need to explore sustainable and effective extraction methods. Previous research has highlighted the benefits of PAW and SDBD in various applications, underscoring their potential as environmentally friendly and efficient technologies. However, the findings of this study indicate that further investigation is necessary to fully harness these technologies' potential in extracting phenolic compounds from olive by-product powders.

Table. 14 Total phenolic compounds and DPPH assay measured on the extracts obtained using distilled water and plasma-activated water (PAW) combined with probe ultrasound treatment.

Extracts	TPC (mg/g GAE)	DPPH (% inhibition)
OL-W-UP-Ex	3.52 ± 0.02^{a}	50.01 ± 0.44^{a}
OL-PAW-UP-Ex	2.28 ± 0.01^{b}	18.74 ± 0.52^{b}

Different letters show significant differences in the same column ($p < 0.05$) for each parameter.

Ultrasound produces solvent cavitation that causes an efficient cell rupture. As a result, solvent penetration is improved, and compound diffusion is optimized, achieving a higher yield of bioactive compounds.

A probe ultrasound treatment was applied to the dried olive leave powder by testing as solvent distilled water or plasma-activated water (PAW) obtained the following samples:

OL-W-UP-Ex: olive leave extract using distilled water with ultrasonic probe treatment.

- OL-PAW-UP-Ex: Olive leaves extract using plasma-activated water with ultrasonic probe treatment.

When comparing the extraction methods results of Table 14, the ultrasonic probe technique showed interesting trends. The water-based ultrasonic probe extraction consistently demonstrated higher extraction efficiency, with a TPCC value of 3.52 mg/g GAE, compared to the plasma-activated water ultrasonic probe extraction, which yielded a TPCC value of 2.28 mg/g GAE. These findings align with previous research by Ahmad-Qasem *et al*. (2013), who

reported similar TPCC ranges of 2.5-4.0 mg/g GAE using conventional water extraction methods.

The superior performance of regular water extraction maybe attributed to water's optimal solvent properties for extracting phenolic compounds from olive leaves. These observations support the findings of Şahin and Şamlı (2013), who suggested that ultrasonic probe extraction often provides more consistent results due to more uniform energy distribution throughout the extraction medium. The consistently lower efficiency observed in plasma-activated water extracts might be attributed to several factors, including potential degradation of phenolic compounds due to reactive oxygen species present in plasma-activated water, altered pH conditions affecting compound solubility, and modified water structure potentially impacting the overall extraction dynamics. These results provide valuable insights into optimizing olive leave extraction methods and suggest that conventional water-based ultrasonic probe extraction might be more effective for maximizing phenolic content and antioxidant activity.

Similarly, the antioxidant activity assessment revealed that OL-W-UP-Ex exhibited notably stronger DPPH radical scavenging activity (50.01 ± 0.44%) than OL-PAW-UP-Ex (18.74 ± 0.52%). This substantial difference in antioxidant activity suggests that plasma activation might significantly alter the extraction dynamics of bioactive compounds. These results correspond with Xie et al. (2015), who documented DPPH inhibition values between 40-60% for conventional water extracts of olive leaves.

The extraction efficiency and antioxidant properties of olive by-products were evaluated using various extraction conditions, incorporating Surface Dielectric Barrier Discharge (SDBD) plasma pretreatment and different solvent systems.

The samples' results, listed in Table 15, revealed interesting patterns across different extraction parameters and sample types.

The extracts presented different colors at a preliminary visual examination, as shown in Fig.39.

Fig. 39_Color difference in extracts prepared b Ultrasonic probe extraction with and without Surface Dielectric Barrier Discharge (SDBD) Pretreatment

Table. 15 Total phenolic compounds and antioxidant assays (DPPH, FRAP, and ABTS) of extracts obtained with and without Surface Dielectric Barrier Discharge (SDBD) plasma as pretreatment on OIBP in combination with different solvents (70 and 30% EtOH&80% MetOH) and probe extraction.

Combination of extraction	TPCC mg/g GAE	DPPH Assay (% inhibition	FRAP assay (μM Fe3+)	ABTS Assay (% inhibition)
AD-OP-SDBD-70%EtOH-UP-Ex	6.40 ± 0.09^{a}	69.3 ± 4.1^{a}	324.0 ± 18.3^{a}	74.6 ± 0.4^{a}
AD-OP-70%EtOH-UP-Ex	6.50 ± 0.04^{a}	82.8 ± 2.1^{b}	337.2 ± 33.3^{a}	78.1 ± 1.7^{a}
OL-SDBD-70%EtOH-UP-Ex	4.34 ± 0.02^{b}	50.2 ± 5.1^{c}	368.9 ± 11.5^{b}	57.6 ± 4.4^{b}
OL-70%EtOH-UP-Ex	4.74 ± 0.07^{b}	60.5 ± 8.1^{d}	378.8 ± 20.2^{b}	61.8 ± 4.0^{b}
OL-SDBD-30%EtOH-UP-Ex	4.68 ± 0.16^{b}	61.5 ± 7.1^{d}	306.4 ± 13.1^{a}	66.7 ± 2.6^{c}
OL-30%EtOH-UP-Ex	5.29 ± 0.10^{b}	85.6 ± 2.6^{b}	320.2 ± 15.9^{a}	78.9 ± 3.4^{a}
OL-SDBD-80%MetOH-UP-Ex	4.71 ± 0.05^{b}	63.2 ± 1.8^{d}	305.0 ± 11.3^{a}	65.5 ± 5.0^{b}
OL-80%MetOH-UP-Ex	6.36 ± 0.08^{a}	88.1 ± 0.2^{e}	345.9 ± 12.2^{b}	79.1 ± 2.9^{a}

Different letters show significant differences in the same column ($p < 0.05$) for each assay.

As expected, the OP extracts were supposed to be reddish, while those from the OL were green.As is known, chlorophyll is modified by oxidation reactions; consequently, the green color disappears, while orange-red colors appear. The 70% EtOH solvent evidenced the ability to conserve the original color of the OIBP with an evident lower intensity in the samples AD-OP-SDBD-70%EtOH-UP-Ex and OL-SDBD-70%EtOH-UP-Ex. Conversely, a significant loss of color occurred in the samples, which had 30%EtOH and 80%MetOH. It was reasonable to suppose a possible correlation between the loss of color and content and/or modification of the phenolic compounds and antioxidant activity. The total phenolic content and the antioxidant activity of the samples are given in Table 15.

For olive pomace extracts, AD-OP-70%EtOH-UP-Ex showed slightly higher total phenolic content (6.50 ± 0.04 mg/g GAE) compared to its SDBD-pretreated counterpart (6.40 ± 0.09 mg/g GAE). However, the DPPH radical scavenging activity demonstrated an opposite trend, with the non-pretreated sample exhibiting higher inhibition (82.8 ± 2.1%) compared to the SDBD-pretreated sample (69.3 ± 4.1%). The result suggests that SDBD pretreatment might not significantly enhance the total phenolic extraction but could affect the specific composition of extracted compounds.

In the case of olive leaves extracts, varying ethanol concentrations produced notably different results. The 80% methanol extraction without SDBD pretreatment (OL-80%MetOH-UP-Ex) yielded the highest TPCC (6.36 ± 0.08 mg/g GAE) among all olive leave extracts, accompanied by superior DPPH inhibition (88.1 ± 0.2%), FRAP activity (345.9 ± 12.2 μM Fe3+), and ABTS inhibition (79.1 ± 2.9%). Interestingly, SDBD pretreatment consistently resulted in lower extraction efficiency across all solvent systems for olive leaves. For instance, with 70% ethanol, the non-pretreated sample (OL-70%EtOH-UP-Ex) showed higher TPCC (4.74 ± 0.07 mg/g GAE) compared to its SDBD-pretreated counterpart (4.34 ± 0.02 mg/g GAE). This trend was consistent across different antioxidant assays, suggesting that SDBD pretreatment might alter the matrix structure or compound stability in olive leaves.

The ethanolic solvent concentration also played a selectively important role in extraction efficiency. For olive leave extracts, 30% ethanol without SDBD pretreatment (OL-30%EtOH-UP-Ex) showed remarkably high DPPH inhibition (85.6 ± 2.6%) and ABTS inhibition (78.9 ± 3.4%), despite having a moderate TPCC value (5.29 ± 0.10 mg/g GAE); indicating that lower ethanol concentrations might selectively extract compounds with higher antioxidant activity. The FRAP assay results showed relatively consistent values across different extraction conditions, with slight variations ranging from 305.0 to 378.8 μM Fe3+, suggesting that the reducing power of the extracts was less affected by the extraction conditions compared to other antioxidant properties.

The reduced efficiency of SDBD pretreatment could be attributed to several factors. First, exposure to plasma-generated reactive species might lead to degradation or chemical modification of phenolic compounds, as suggested by Ellera *et al*. (2019), who observed that prolonged plasma exposure could cause degradation of sensitive bioactive compounds in plant materials.

Additionally, the high voltage (160 V) and treatment duration (30 minutes) used in the SDBD process might have induced thermal stress or structural changes in the plant matrix that impaired the subsequent extraction process. These findings align with Kovačević *et al*. (2016), who reported that excessive plasma treatment parameters could lead to degradation of phenolic compounds and reduced antioxidant activity in plant extracts. The consistent trend of lower antioxidant potential assayed by (DPPH, FRAP, and ABTS) in SDBD-pretreated samples supported the hypothesis that plasma treatment might have altered the chemical structure or stability of the target compounds in olive by-products powders, thereby affecting their biological activities.

5. CONCLUSION

Using olive oil by-products (OIBP) has shown great potential for enhancing food formulations with functional and antioxidant properties.

The macerated olive leaf vinegar stands out as a novel ingredient, capable of being used alone or in salad dressings, significantly boosting antioxidant activity. When incorporated into oil/vinegar dressings, it extended the oxidative stability of emulsions by up to 41%, showcasing its unique effectiveness. This stabilization effect correlated closely with its high phenolic content of 5228 mg/L of total phenols. Another study conducted during the doctoral period did not add that the thesis explored the formulation of vegetable mayonnaise using olive leave vinegar (OLV) to enhance its nutritional and sensory qualities. The OLV mayonnaise contained 57% fat, with a favorable fatty acid profile, including 11% saturated, 64% monounsaturated, and 23% polyunsaturated fats. It was rich in bioactive compounds, showing 68 mg/100 g phenolic content and 52 mg/100 g oleuropein. Sensory evaluations indicated that panellists preferred the OLV mayonnaise over the control sample, particularly for its color, odor, and taste. Furthermore, the OLV mayonnaise exhibited superior oxidative stability, maintaining a low peroxide value of around 4.5 meqO_2/kg after 12 months of storage at various temperatures. These findings suggest that incorporating olive leave vinegar improves the health profile of mayonnaise and enhances its shelf life and sensory appeal, supporting the valorization of olive by-products in food applications.

Similarly, olive pomace (OP) has been innovatively utilized as a novel, direct ingredient in the development of functional beverages. This groundbreaking approach leverages the pomace's rich phenolic content, reaching up to 7.5 mg/g CAE (caffeic acid equivalent), with hydroxytyrosol and related compounds being the dominant antioxidants. Functional beverages crafted with OP showcased an impressive total phenolic content of approximately 600 mg/L CAE, meeting nutraceutical standards for health-promoting effects. Notably, heat treatments did not diminish these valuable compounds; instead, they preserved the phenolic content, yielding stable, antioxidant-rich beverages. This highlights the unique potential of olive pomace as a direct, innovative ingredient in beverage formulations, opening new avenues for health-oriented products.

This research applied various extraction techniques to recover enriched bioactive compounds in extracts. The water steam-based method used for extracting olive leave compounds in this study has proven to be the most economical and sustainable compared to other extraction

technologies explored in the research. This green extraction process yielded olive leave extracts (OLEs) with a total phenol content of up to 7.4 mg/mL HyE, demonstrating superior antioxidant capacity while minimizing environmental impact through the exclusive use of water as a clean solvent. As a natural antioxidant agent of steam water, olive leave extracts (SW-OLEs) demonstrated significant antioxidant potential in vitro and effectively maintained the quality of beef patties during refrigerated storage. In antioxidant assays, WE and EA extracts exhibited intense radical scavenging activities and extended lipid stability, with induction times of 8.92 and 12.74 hours, respectively, compared to 2.24 hours for the control. Storage studies indicated that OLE-treated beef patties showed lower lipid oxidation, stable oxygen levels, minimal pH variations, and improved color retention over 10 days. These results emphasize the potential of OLEs as natural antioxidants to enhance the shelf life of meat products.

Exploring xanthan gum-based edible coatings utilizing olive oil by-products for fresh strawberries revealed significant shelf life and microbial stability improvements. The study demonstrates that these coatings maintain critical quality parameters, including pH, titratable acidity, total soluble solids (TSS), and texture throughout storage periods. Notably, strawberries coated with freeze-dried olive pomace (FD-OP) exhibited the best retention of TSS, while air-dried olive pomace (AD-OP) showed superior texture preservation. Regarding microbial safety, the coatings significantly inhibited microbial growth compared to uncoated and blank samples. The antimicrobial properties of the coatings were particularly pronounced with FD-OP and AD-OP extracts, which effectively suppressed microbial proliferation during storage. This suggests that incorporating olive by-products enhances the physical quality of strawberries and contributes to their safety for consumption.

Lastly, investigating nonthermal emerging technologies reaffirmed the importance of innovative extraction methods, further enhancing the value derived from olive oil by-products. These findings collectively advocate for the sustainable recovery and utilization of agricultural by-products, underscoring their role in reducing environmental impact while improving food quality.

This research contributes to the understanding of sustainable practices within the food sector and underscores the importance of advancing the valorization of agricultural by-products. The implications of this work extend beyond the immediate findings, presenting opportunities for future research and development aimed at enhancing food sustainability and quality.

Opportunities exist in the pharmaceutical and nutraceutical sectors to develop dietary supplements and cosmeceuticals with OIBP's bioactive compounds. Continued research will focus on optimizing green extraction methods and exploring innovative applications, ultimately promoting environmental sustainability and reducing agricultural waste. These advancements highlight the significant potential of OIBP in promoting health, sustainability, and quality in food and beyond.

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