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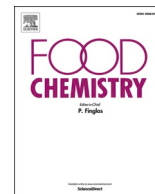
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Novel antioxidant-active food packaging based on olive oil byproducts extracts

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ABSTRACT

Food waste reduction remains a major challenge in the European Union, where annual losses exceed 88 million tons. Active food packaging offers a sustainable strategy to extend shelf-life and maintain product quality. This study evaluated the antioxidant performance of natural extracts from olive by-products stone, leaf and pomace obtained through a green ethanol-based extraction. Polyphenolic profiles were determined by UHPLC-DAD, and antioxidant capacity was measured using DPPH assays, with olive stone extract showing the best activity ($EC_{50} = (63.6 \pm 4.6) \text{ mg/L}$). Total biophenol contents ranged from 10 g/kg to 130 g/kg, with high levels of gallic acid and related polyphenols. Among all, olive stone extracts exhibited the strongest performance in terms of antioxidant power percentage ($(84.4 \pm 1.6) \%AP$). Packaging films incorporating these extracts significantly delayed lipid oxidation in minced beef meat with 50% fat, reducing it to 18.4% after 10 days versus 46.6% in the control, demonstrating strong potential for sustainable industrial application.

1. Introduction

In the framework of the circular economy action plan, food waste reduction is one of the most challenging goals. The European Union has made reducing food waste a key priority to achieve the objectives of its European Green Deal and in line with its Farm to Fork strategy (Scherhauer et al., 2018). To counter waste of food and dietary products, industries and the scientific community are moving forward to create new and sustainable strategies, including the use of active food packaging systems (EU Commission, 2019, Korte et al., 2021, Lockrey et al., 2019). Sharing, renting, reusing, repairing, refurbishing, and recycling existing resources and products for as long as possible are all part of the circular economy model of production and consumption (European Parliament, 2023). Packaging preserves the advantages of food processing after it is finished, allowing foods to be transported securely over large distances while being healthy when consumed (Marsh & Bugusu, 2007). As technology and modern living improve, there is an increasing demand for healthy, high-quality food products that are easy to transport and, most importantly, have a long shelf life; this highlights the necessity of food packaging innovation (Ahari & Soufiani, 2021). New, clever, and intelligent or active packaging that

can sense and convey information from the packed food product has been created in this regard (Asiri et al., 2024). The primary goals of this packaging innovation are to employ additives on the basic package to achieve antibacterial (AB) and antioxidant (AO) properties (Aziz et al., 2024), the ability to absorb moisture, regulate the atmosphere, and be a gas scavenger (Versino et al., 2023). According to the definition of the Official Journal of the European Union, food additives “are substances that are not normally consumed as food itself but are added to food intentionally for a technological purpose”, so the use of active packaging allows to achieve comparable results to additives without breaking any regulation (European Parliament and Council of the European Union, 2008).

Along with microbial growth, lipid oxidation is the primary cause of food spoilage for a wide range of foods, including nuts, seafood, meats, whole milk powders, sauces, and oils (Gómez-Estaca et al., 2014). By gradually releasing antioxidants and antimicrobials over time to restore the original active ingredients that were consumed, controlled-release packaging presents a substantial opportunity to increase the shelf life of foods (Mastromatteo et al., 2010). Active food packaging materials represent a major breakthrough in food preservation technology and offer potential answers for prolonging shelf life, guaranteeing safety,

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and cutting waste. The use of these materials in regular food packaging is set to grow in popularity as the sector innovates further, which will help both customers and the environment.

This work was mainly focused on the use of natural extracts that were obtained from industry wastes. Specifically, waste from the olive supply chain was considered. Olives are consumed all around the world, particularly as olive oil. The amount of olive oil consumed worldwide was approximately 2.6 million metric tons in 2022–2023 and is predicted to decrease to 2.39 million metric tons in 2023–2024, while estimated world production (in thousands of tons) for the 2024–2025 season is almost 3100, of which 5% comes from Italian production (Olivenews.gr, 2024). Twelve million tons of non-environmentally friendly wastes (olive peels, pulp, stones, and wastewater) are produced annually by the global olive oil business (SCI, 2024). The extracts came from various parts of the plant: the stone, leaves, and pomace (waste from processing), which have already shown potential usage in food packaging development, achieving both antimicrobial and antioxidant effects (Crizel et al., 2018; Grabska-Zielińska, 2024; Spizzirri et al., 2011). It's worth noting that a “green” extraction method was used, employing non-toxic solvents like ethanol. The materials and solvents used for the active packaging development were chosen in order to be food grade and already coherent with the current regulatory requirements of national and continental legislation (Regulation (EC) 1935/2004). This choice was meant to be already compliant with a possible scale-up of the process to the industrial level.

The extracts present the molecular pattern obtained from the ethanol extraction polarity range, and a point that will be faced in the next studies will be to focus also on the addition of some percentage of water in order to help the extraction of highly polar active compounds. The extracted by-products were then evaluated to determine their antioxidant and antibacterial activity using a colorimetric test, the so-called 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Baliyan et al., 2022) test, to examine the ability of compounds within the extracts to reduce free radicals, while the antibacterial activity of the extracts was assessed using a disk diffusion test against *Listeria innocua* (Gram +) and *Escherichia coli* (Gram-). The antibacterial effect was supposed to be explained by the olive by-product due to the possible presence of molecules such as luteolin, oleuropein (coming from olive leaf), and hydroxytyrosol acetate (Sousa et al., 2006; Techathuvanan et al., 2017; Wei et al., 2018). Once characterized, the extracts with the most promising AO properties were added to food packaging materials in two ways (Barzan et al., 2023; Gemili et al., 2010; Vasile & Baican, 2021): one-layer coating and two-layer water-based adhesive. The packaging films were tested both for their antibacterial and antioxidant properties. Antibacterial activity was assessed using a well diffusion plate test against the same organisms. Regarding the antioxidant qualities, the film was examined using the DPPH test as well as a second test to provide insight into the lipid oxidation that produces aldehydes in minced beef meat with 50% fat.

Comprehensive identification and structural characterization of molecular patterns in olive by-products were enabled by the performed Ultra-High-Performance Liquid Chromatography coupled with Diode Array Detection and High-Resolution Mass Spectrometry (UHPLC-DAD-HRMS) technique, especially data-dependent MS/MS for accurate mass and elemental composition. This technique enabled the characterization of different groups of molecules, such as phenolic acids and alcohols, flavonoids, lignans, and secoiridoids. While the presence of aglycones was constant and relevant, the occurrence of glycosylated polyphenols was lower.

While the antioxidant capacity of olive stone extract (i.e., suggested usage as a natural food additive or pharmaceutical ingredient) (Nakilcioglu-Taş & Ötleş, 2020) and its application in developing materials for food packaging are well-established (Khwalidia et al., 2022), its direct efficacy as a food preservative to prolong shelf life has not been fully investigated. A different situation has been reported for olive leaf extract, which has been successfully shown in the literature to inhibit

lipid peroxidation in food systems like meat (Djenane et al., 2019). The oxidation of meat delayed by active packaging was also investigated by means of different techniques (Khan et al., 2024).

The present study addresses this research gap by demonstrating that active films incorporating olive stone extract, a valorized agri-food by-product, possess significant antioxidant properties capable of extending food shelf life by mitigating meat oxidation, and it was analyzed and confirmed by means of the Thiobarbituric Acid Reactive Substances (TBARS) test. The release of unwanted molecules from the packaging in the food will be applied in future studies due to an untargeted screening approach developed with the HRMS technique.

2. Material and methods

2.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH) powder, malondialdehyde tetrabutylammonium salt (MDA, > 96%), and gallic acid (GA, 97.5–102.5%) were obtained from Sigma-Aldrich. Methanol (HPLC grade), absolute anhydrous ethanol, acetone, glacial acetic acid (>99%), trichloroacetic acid (TCA) (> 99%), sodium chloride (NaCl), and paraffin oil were supplied by Carlo Erba. 4,6-Dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid, TBA, 98%) was purchased from Acros Organics (Geel, Belgium). Müller-Hinton (MH) broth and agar were provided by Lickson. Phosphate-buffered saline (PBS) tablets were obtained from PanReac AppliChem.

2.2. Olive byproducts: Extraction and characterization

2.2.1. Olive by-products extractive method

The natural by-products extracted with an ethanol green approach were olive stone (STO), olive leaf (LEA), and olive pomace (POM).

The extraction of compounds with possible AO properties (i.e., the phenolic fraction) was performed using a liquid-solid extraction technique with ethanol as a solvent, based on previous studies with some changes (Bucić-Kojić et al., 2009). (25.0 ± 0.1) g of the natural by-products was accurately weighed into a round-bottom flask. To this, 150 mL of ethanol was added. The mixture was then agitated using a magnetic stirrer at 40 °C for 2 h. Following the extraction period, the resulting suspension was centrifuged at 2500 rpm by a “REMI bench top centrifuge XS R-10M” for 10 min to separate the solid residue. The supernatant, containing the extracted compounds, was removed from the flask with a pipette and subsequently filtered by gravity through filter paper (12 mm diameter, 0.45 µm pore size). The filtrate was collected in a clean round-bottom flask.

The extraction procedure was repeated on the remaining solid residue with an additional 100 mL of ethanol under identical conditions. The resulting supernatant from this second extraction was combined with the first filtrate in the same flask.

The combined extracts were then concentrated to dryness using a rotary evaporator under reduced pressure at 40 °C. The dried extract was subsequently reconstituted in 10 mL of ethanol. The resulting solution was transferred to a pre-weighed plastic Falcon tube, and the weight of the solution was recorded. Finally, the ethanol was completely removed under a gentle stream of nitrogen gas, yielding the dried extract for further analysis. This green extraction process, involving mainly only ethanol, is optimal and suitable for a scale-up to industrial application.

2.2.2. Evaluation of the antioxidant activity of the extracts

The AO capacity of the samples was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, based on the decrease in absorbance at 516 nm resulting from the reduction of the DPPH radical (Fig. 1). Measurements were conducted using a Thermo Scientific™ Varioskan™ LUX multimode microplate reader operating in kinetic mode. Over a 60-min incubation period, absorbance values were recorded at 10-min intervals, resulting in seven time points per sample.

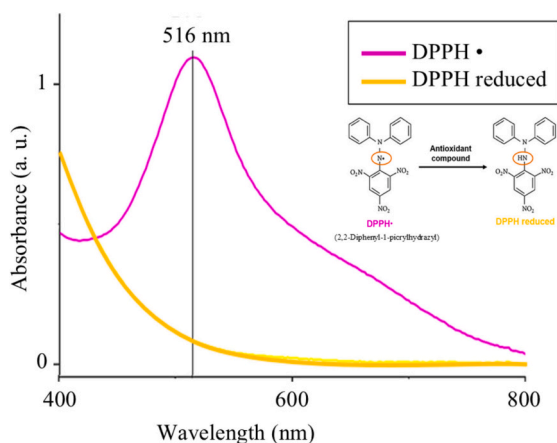


Fig. 1. Absorption spectra of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and its reduced form.

During the incubation, the samples were gently agitated at 120 rpm to ensure homogeneity; agitation was maintained after the heating module was deactivated.

To correct for background interference, sample-specific blanks were subtracted from the raw absorbance values. A fresh 0.2 mM DPPH solution in methanol was prepared daily to ensure consistency across measurements. A direct linear relationship between antioxidant concentration and DPPH radical reduction was observed.

The Antioxidant Power percentage (AP%) was calculated using Eq. 1:

$$AP\% = (\Delta Abs \cdot 100) / Abs_{max} \quad (1)$$

where:

$$\Delta Abs = Abs_0 - Abs_m$$

Abs_0 is the mean absorbance of eight replicates of 0.1 mM DPPH solution (control);

Abs_m is the absorbance of the sample at the final time point;

Abs_{max} is the maximum absorbance value recorded for the DPPH radical before reaction (plateau).

The effective concentration at which 50% of the DPPH radicals were scavenged (EC_{50}) was determined by interpolating the absorbance value corresponding to 50% of Abs_{max} (i.e., $Abs_{max}/2$) on the calibration curve obtained for each antioxidant compound. EC_{50} values were used as comparative indicators of antioxidant efficacy among the tested samples.

2.2.3. Characterization of antioxidant compounds in complex natural matrices

Ultra-High-Performance Liquid Chromatography (UHPLC-DAD-HRMS) was employed for the separation, identification, and quantification of the phenolic fraction of the extracts. This analytical platform enables detailed profiling of compound classes and facilitates structural elucidation. The Diode array detector (DAD) records analyte absorbance across a wide spectral range, leveraging the characteristic UV-visible absorption patterns of specific compound classes. This feature is particularly advantageous for the characterization of phenolic compounds, including flavonoids and other UV-active phytochemicals compounds associated with antioxidant activity. Analyses were performed using a Thermo Vanquish UHPLC system coupled with a Thermo Orbitrap Exploris 120 mass spectrometer, equipped with a Vanquish Diode Array Detector. The optimized chromatographic conditions are detailed in Table S1. A gradient elution method (Fig. S1) was optimized to ensure adequate resolution of bioactive constituents in the by-product extracts. Polyphenolic compounds, including phenolic acids and flavonoids, were quantified via integration, in the retention time range of 1

min to 70 min, of the area under the curve (AUC) of chromatographic peaks, monitored at a wavelength of 280 nm, commonly used for polyphenol detection (International Olive Council, 2022). Quantitative results were expressed as g/kg, using tyrosol as an external calibration standard. For compound identification, UHPLC was interfaced with the Orbitrap Exploris 120 mass spectrometer via a Heated Electrospray Ionization (HESI) source. An untargeted metabolomics approach was applied using Full-MS data acquisition followed by data-dependent MS/MS (dd-MS²). High-resolution fragmentation was performed in the higher-energy collisional dissociation (HCD) cell. The acquisition method, designed to alternate between positive and negative ionization, is described in Table S2. The five most intense precursor ions per scan cycle were automatically selected for MS/MS fragmentation. Preliminary annotation of compounds was carried out using MS-DIAL software (version 5.5.241113) (de Oliveira Costa et al., 2024; Shah et al., 2023), which provides automated feature detection and tentative compound identification. Each proposed identification was subsequently verified manually using Thermo Scientific FreeStyle™ software, enabling inspection of raw data and validation of fragmentation patterns and retention times.

2.3. Characterization of active film

Briefly, active films containing solubilized extracts at 5% (w/w) were produced, as described in Fig. 2, using two different approaches: 1) a single-layer coated (solvent-based—SB) packaging material, i.e., a cellulose-based biopolymer film coated with a solvent-based resin incorporating active compounds; 2) a double-layer (water-based—WB) packaging material, i.e., two layers of the same cellulose-based biopolymer film joined by a water-based adhesive containing the active compounds (Crizel et al., 2018).

The effective functionalization of the packaging was initially evaluated by Fourier transformation infrared spectroscopy FTIR analysis, comparing the spectra of (i) the cellulose acetate film, (ii) the film containing the resin (BLK), and (iii) the film containing 5% (w/w) olive stone extract in the resin (STO). However, the 5% (w/w) olive stone loading in the resin was not sufficient to be clearly detected by FTIR measurements either in transmittance or attenuated total reflectance (ATR) mode. The ATR method and spectra are summarized in Table S3 and Fig. S2. However, the successful functionalization of the olive stone-based packaging and its antioxidant capacity were confirmed by the DPPH assay performed on the films, which will be better discussed in section 3.2.

The industrial application of active food packaging based on natural extracts from agri-food by-products remains limited by the variability of bioactive compounds, the lack of standardized extraction procedures, and the suboptimal mechanical and barrier properties of biopolymer matrices. Concerns related to compound stability, controlled release, and regulatory compliance further hinder large-scale adoption. This packaging process could nevertheless be suitable for industrial scaling, as it is recognized as food-safe and compliant with EU frameworks such as Regulation (EC) 1935/2004; however, its main limitation remains the higher production cost compared with conventional plastics, as highlighted by material cost analyses. Advancing research in this field is therefore essential to overcome these barriers and fully exploit its potential for sustainable and safer food packaging solutions. (Kumari et al., 2022).

2.3.1. DPPH test of the film

Differently from the analysis of the pure extracts, the DPPH test was adapted to be performed in active film as described in literature (Barzan et al., 2023). Based on the results of the DPPH test summarized in Table 1, olive stone and olive leaf extracts were selected for the active film prototype. For the characterization of the antioxidant power, a 2 cm × 2 cm film was soaked into a 12 mL DPPH solution prepared at a concentration of 0.13 mM. After one day, the solution was treated as

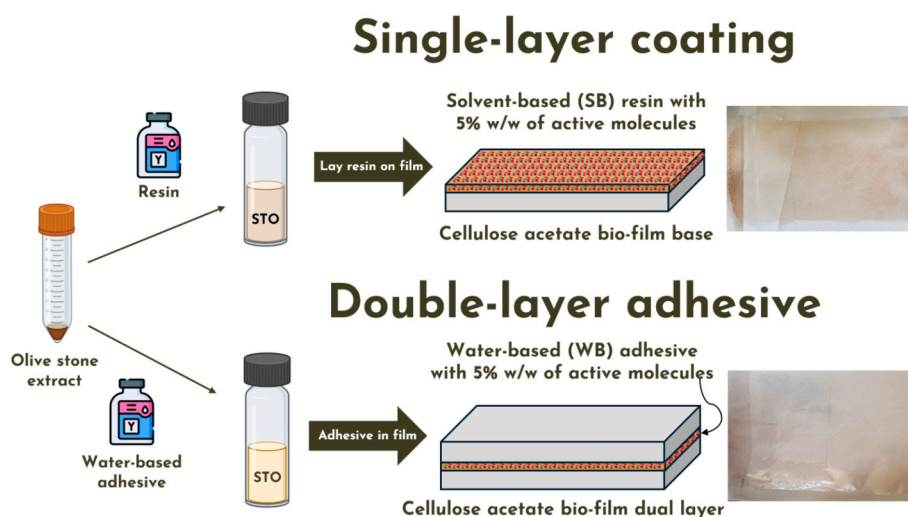


Fig. 2. Packaging fabrication workflow for olive stone (STO) extract.

Table 1

EC₅₀ in mg/L obtained for positive GA control and all olive by-products.

Sample	EC ₅₀ (mg/L)
Gallic acid (GA)	1.6 ± 0.1
Olive stones (STO)	63.6 ± 4.6
Olive leaves (LEA)	291 ± 44
Olive pomace oil (POM)	779 ± 35

well as the normal DPPH test in order to quantify antioxidant activity.

2.3.2. TBARS test on packed minced beef meat with 50% fat

For each tested film, (12 ± 0.1) g of fresh minced beef (with 50% fat content) was packaged with a 6 × 6 cm film in direct contact with the meat (Barzan et al., 2023). The samples were sealed under vacuum in PET bags and stored at 4 °C. The meat samples, prepared in duplicate for each film type (including a blank), were analyzed at 0, 4, 7, and 10 days to monitor lipid peroxidation using the TBARS assay. To determine the TBARS value, which is expressed as mg of malondialdehyde (MDA) per kg of meat, MDA standards were prepared and mixed with TBA to build a calibration curve. Meat samples were homogenized in 20 mL of 10% TCA by means of Polytron PT 10–35 Homogeniser Stirrer with Kinematica PCU 8 S and subsequently centrifuged. The supernatant was collected and used to perform the reaction with TBA in a heated oil bath (97 °C for 20 min). After cooling, the samples were centrifuged again, and the absorbance at 532 nm was measured. MDA concentrations were calculated using the calibration curve.

3. Results and discussion

3.1. Characterization of olive by-products extracts

3.1.1. Antibacterial activity of olive by-products extract

An antibacterial study on pure extracts and produced film was performed due to the findings in literature and the established antimicrobial effect shown by similar byproduct extracts and compounds identified.

Escherichia coli ATCC 8739 and *Listeria innocua* were used for this work as a Gram- and a Gram+ bacterial model, respectively. Initial disk diffusion (Fig. S3) tests on olive extracts, using *E. coli* and *L. innocua*, showed no significant antibacterial activity. Despite testing various concentrations, bacterial growth was not inhibited, also with packaging developed by means of the well diffusion test, Fig. S4 and Table S4. The

lack of antimicrobial activity could be explained by the molecular pattern found with the extraction performed, better shown in section 3.1.4. Some water content in the extraction solvent could have led to more presence of glycosylated polyphenols, which could be capable of inhibiting antimicrobial activity and growth compared to aglycones. (De Rossi et al., 2025; Wang et al., 2024) These results are detailed in the supplementary information, shifting the focus to the antioxidant properties.

3.1.2. Antioxidant DPPH test on extract result

Calibration curves were constructed after an initial screening across 4 to 5 orders of magnitude to determine a feasible concentration range, ensuring a linear response around 50% of the DPPH radical reduction. Based on this evaluation, at least four data points were selected within the identified range. For example, for olive stone, the chosen concentrations were 25 mg/L, 37.5 mg/L, 50 mg/L, 62.5 mg/L, and 75 mg/L, with each value representing the mean of triplicate measurements in a 96-well plate.

The calibration curves confirmed a linear response between %AP and mg/L within the selected concentration range, making the DPPH assay suitable for quantification through linear regression. Typically, the linear range falls between 20%AP and 80%AP. Linear regression equations, including slope (m), intercept (q), and R² values, all >0.99, for each sample are reported in Table S5.

Once the equations were established, EC₅₀ values were interpolated, and uncertainty was assessed using error propagation. The results are summarized in Table 1.

The DPPH assay on pure extracts indicated that, besides GA as the positive control, olive stone exhibited the best EC₅₀ values, while olive leaf and olive pomace showed lower antioxidant activity within the olive by-product chain, with results comparable to previous works in the literature (Hayes et al., 2011). Although useful for screening, this test alone does not determine the most effective extract for final packaging, as antioxidant activity may vary once incorporated into the material.

3.1.3. Quantification of antioxidant in the extracts

An example of the chromatographic separation and the corresponding chromatogram recorded at 280 nm for the olive stone extract is shown in Fig. 3.

As expected from the use of an organic solvent-based extraction, the initial portion of the chromatogram is relatively sparse. More lipophilic compounds were eluted between 18 and 30 min, consistent with the chemical nature of the extracted matrix.

Relative quantification of total biophenol content was carried out,

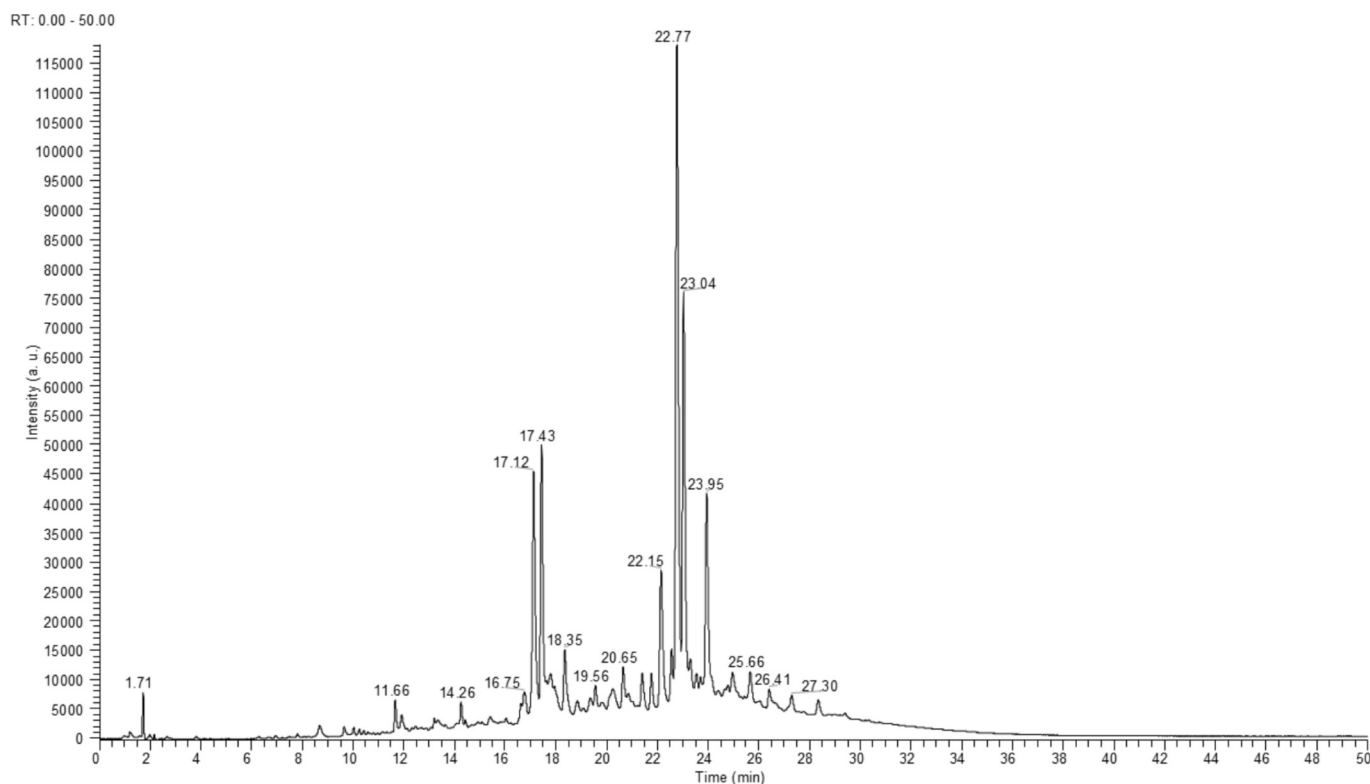


Fig. 3. Representative HPLC chromatogram recorded at 280 nm for the olive stone extract.

with an integration range between 1 min and 70 min, according to analytical conditions present in the International Olive Council (IOC) method (International Olive Council, 2022) using HPLC-DAD, and the results for the various olive oil by-product extracts are summarized and compared to the DPPH test in Table 2. The olive stone extract exhibited the highest concentration of total biophenols, with an average value of 130 g/kg. This result is consistent with the antioxidant capacity data obtained from the DPPH assay, in which the olive stone extract showed the lowest EC_{50} value, indicating the strongest radical scavenging activity among the analyzed by-products.

These findings highlight the potential of olive stones as a valuable source of natural antioxidants for applications such as active food packaging and are in the range of content already found in previous scientific works (Abbattista et al., 2021).

3.1.4. Identification of antioxidant compounds in olive stone extracts

A comprehensive identification and structural characterization of antioxidant compounds present in the olive stone extract was carried out by UHPLC-HRMS. The detailed results are provided in Tables S6, S7, and S8. For each compound, the following information is reported: retention time (min), compound name, molecular formula, high-resolution precursor ion (m/z), major fragment ions (m/z), and the corresponding neutral losses. Compound assignments were made based on MS/MS fragmentation patterns and corroborated through comparison with literature data (Celano et al., 2018; de la Torre-Carbot et al., 2005; Kalogiouri et al., 2016; Kanakis et al., 2013; Malapert et al., 2018;

Peralbo-Molina et al., 2012). The analysis revealed a diverse range of antioxidant and polyphenolic constituents distributed across various chemical classes, including phenolic alcohols, phenolic acids, flavonoids, lignans, secoiridoids, triterpenes, and their glycosylated derivatives (Fig. 4.a). An illustrative example of spectral interpretation is provided in Fig. 4.b, which shows the fragmentation pattern and structural assignment of syringaresinol, a lignan identified in the extract (Hanhineva et al., 2012). This characterization highlights the complex phytochemical composition of olive stone extracts and supports their potential application as a natural source of bioactive compounds for antioxidant-enriched formulations.

A distinctive feature of one of the lignans identified lies in the loss of a methoxy functional group, which is evident in the mass spectrum as a characteristic neutral loss of 15.024 m/z (highlighted by yellow arrows in Fig. 4.b). The associated molecular fragments, including both precursor and product ions, are indicated by red squares and correspond to annotated positions in the molecular structure. Comprehensive profiling of the phenolic composition revealed the presence of several key classes of antioxidant compounds all of which are well-known for their potent radical-scavenging activity. In addition, a variety of phenolic acids were detected, (i.e., vanillic acid) each contributing to the overall antioxidant potential through their hydroxyl group-mediated activity. Within the flavonoid subclass, apigenin, luteolin, and methoxyluteolin (also known as diosmetin) were identified. These compounds are widely recognized for their antioxidant and anti-inflammatory effects and are commonly reported in olive-derived matrices. It is important to underline that the analysis also confirmed the presence of secoiridoids, a unique class of phenolic compounds characteristic of olive-based products. These molecules are known for their multifunctional bioactivities, including antioxidant, anti-inflammatory, and antimicrobial properties. Moreover lignans (such as syringaresinol) illustrated in Fig. 4.b) were detected. These compounds, categorized as phytoestrogens, are of increasing interest due to their antioxidant capacity and potential anticancer effects. Triterpenoids also identified are associated with anti-inflammatory, hepatoprotective, and antimicrobial activities; however, in the present

Table 2

Total biophenol content (g/kg) in olive oil by-product extracts as determined by HPLC-DAD (mean $\pm$ SD, $n = 3$) and comparison with reported DPPH results.

Sample	Total Biophenols (g/kg)	EC_{50} (mg/L)
Olive stones (STO)	130 $\pm$ 14	63.6 $\pm$ 4.6
Olive leaves (LEA)	36.5 $\pm$ 5.2	291 $\pm$ 44
Olive pomace oil (POM)	10.8 $\pm$ 1.1	779 $\pm$ 35

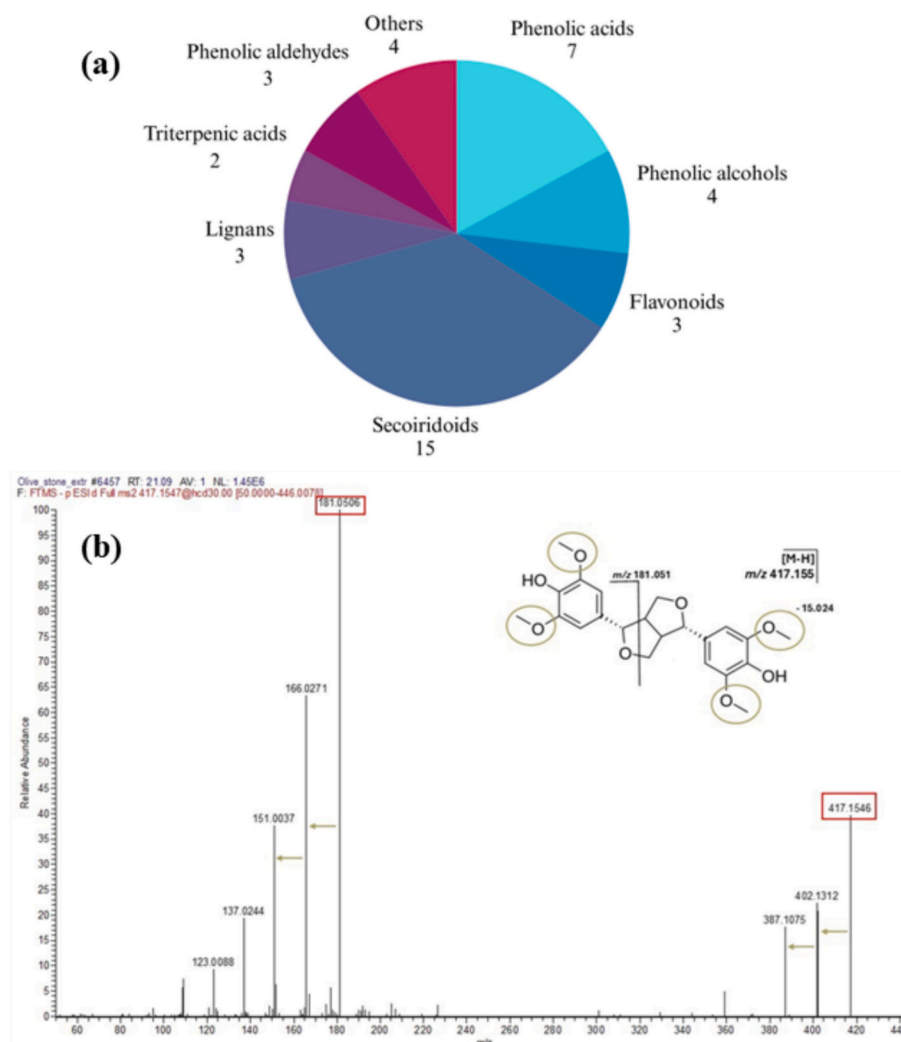


Fig. 4. Cake diagram of olive stone MS characterization divided by compound class (a); mass spectrum, molecular structure, and fragmentation pattern of syringaresinol, a lignan (b). In (b), yellow circles and arrows are showing methoxy losses, while red squares show the parent ion and most abundant fragment ion in the mass spectrum acquired. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

study, maslinic acid was not subjected to further bioactivity testing. A range of glycosylated derivatives was observed which are known to enhance the solubility, stability, and bioavailability of their aglycone counterparts, further contributing to the overall antioxidant efficacy. Furthermore, hydroxy acids and other compounds were detected which are intermediates in key metabolic pathways and contribute to the antioxidant capacity of the extract. When assessing antibacterial activity, the olive-derived compounds most widely recognized for their antimicrobial effects are primarily polar and glycosylated phenolic molecules, such as oleuropein, oleacein, and oleocanthal (Bisignano et al., 1999). However, these compounds were not detected in the UHPLC-HRMS analysis, except for the presence of more lipophilic derivatives, such as the dialdehydic form of oleuropein, or were identified only in negligible quantities. Similarly, tyrosol, hydroxytyrosol, and luteolin were detected only at trace levels (0.090 g/kg, 0.84 g/kg, and 0.00032 g/kg, respectively). When compared with antimicrobial threshold concentrations reported in the literature (approximately 1 g/kg for hydroxytyrosol, >1 g/kg for tyrosol, and 0.3 g/kg for luteolin against *E. coli*; and 0.032 g/kg for luteolin against *L. innocua*) (Medina-Martínez et al., 2016; Xi et al., 2022), the concentrations detected in this study are below those required to exert antibacterial activity. Overall, these results indicate that the potential antimicrobial activity of the ethanolic extract may be constrained by the low abundance of key bioactive phenols typically associated with antibacterial properties in

olive tissues. Nevertheless, this diverse array of bioactive constituents underscores the potential of olive stones as a valuable source of natural antioxidants and health-promoting phytochemicals. These findings support the utilization of olive stone extracts in functional food applications and active packaging systems aimed at enhancing oxidative stability.

3.2. Characterization of antioxidant properties of packaging layers

In order to decide the active layer packaging to use in the real food experiment, the DPPH assay was performed (Barzan et al., 2023). After the solution was collected from the Falcon tube, the DPPH assay was performed to determine the %AP of each layer. The results, with the image of different SB packaging tested, are shown in Fig. 5 below:

A higher %AP indicates a stronger antioxidant effect from the natural by-product incorporated into the layers. Olive stone SB demonstrated the highest %AP of 84.4 ± 1.6 , showing the best antioxidant effect.

These results confirmed the suitability of STO SB active packaging for the real food experiment, as it exhibited the most promising antioxidant potential compared to other materials due to the better scavenging properties of olive stone extract also once dispersed in the resin applied on the cellulose acetate film.

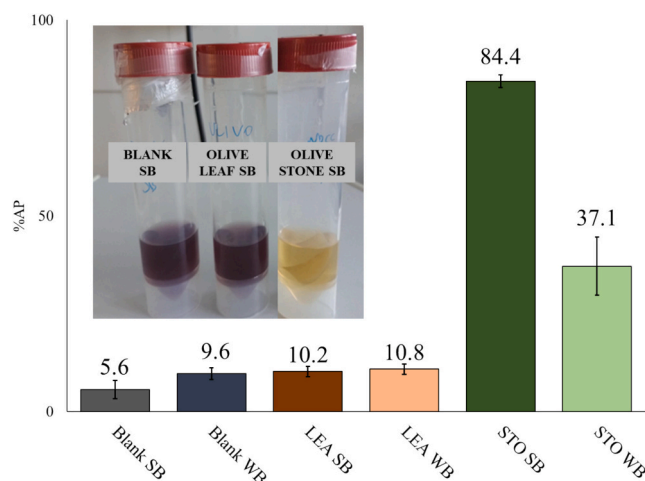


Fig. 5. The DPPH test and antioxidant power (%AP) were assessed for olive stone (STO), olive leaf (LEA), and blank (control) samples. Respective uncertainties for each solvent-based (SB) and water-based (WB) extract are presented.

3.3. Testing of active packaging under real food conditions

Using the single-layer packaging with olive stone SB as the active layer, a test on a real food sample was performed to evaluate the protection from the lipid peroxidation using the TBARS method. The MDA concentrations were derived using the calibration curve shown in Fig. S5.

For each packaging type at each measurement interval, two distinct minced beef meat with 50% fat samples were analyzed, with uncertainties calculated through the propagation of standard uncertainty.

The mg of MDA per kg of meat were determined by interpolating TCA extracts from the TBARS test, and the results are shown in Fig. 6:

The variability in the measurements can be attributed to the natural biological differences in the samples, which, despite undergoing mincing and homogenization, still exhibit variation in fat content. Upon reviewing the data, it is clear that the olive stone film performed better than the blank packaging in preserving meat, as well as the gallic acid

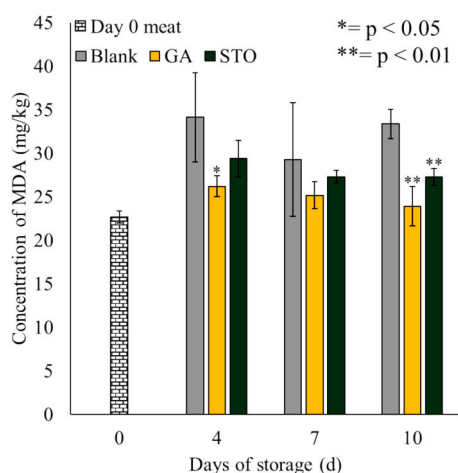


Fig. 6. TBARS assay results showing lipid oxidation in minced beef meat with 50% fat over time for meat at day 0 (blank with black points), olive stone (STO) 5% (green), gallic acid (GA) 5% (yellow), and blank (B) (grey). Results are presented as mg of malondialdehyde (MDA) per kg of meat. Absorbance measurements of lipid extracts from each sample were taken on days 0, 4, 7, and 10. A *p*-value less than 0.05 was marked with “*”, while “**” was assigned for a *p*-value less than 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(GA) one. This conclusion is based on observations from the 10th day of the experiment, where the mg/kg of MDA in olive stone and gallic acid were significantly lower than the blank ones. A robust statistical significance (*p*-value < 0.01) from the Student's *t*-test further confirms these findings. All the calculated *p*-values are shown in Table S9.

To fully evaluate the test's efficacy, lipid peroxidation data across the ten-day period was considered. The percentages of lipid peroxidation were calculated using Eq. 2 (applied following Eq. 4 (Barzan et al., 2023)):

$$\text{Lipid peroxidation\%} = \left(\frac{C_{\text{day10}} - C_{\text{day0}}}{C_{\text{day0}}} \right) \cdot 100 \quad (2)$$

where C_{day0} is the concentration of MDA in mg per kg of meat on day 0, and C_{day10} is the concentration on day 10.

The lipid peroxidation results after 10 days of minced beef meat with 50% fat storage resulted in a lipid oxidation percentage of 46.6 ± 2.9 for blank, 2.2 ± 0.2 for gallic acid, and 18.4 ± 1.0 for olive stone. Especially the olive stone packaging, while not as effective as the gallic acid film, prevented meat degradation nearly 2.5 times better than the blank packaging material, confirming the efficacy of those materials in prolonging shelf life already shown in the literature (Barzan et al., 2023).

A comprehensive comparison of results achieved, including the and shelf-life extension and the estimated production costs based on extraction and coating steps, is shown in Table S10.

4. Conclusions

This study evaluated the antioxidant effectiveness of natural extracts sourced from olive stone, olive leaf, and olive pomace, taking into account the entire olive oil by-product chain.

The employed extraction technique is based on ethanol, a solvent that efficiently recovered active compounds due to its polarity and compatibility with phenolic molecules, while on the other hand, it could not be able to extract a high quantity of compound with antibacterial effect.

The assessment was conducted using DPPH assays, and the extracts' polyphenolic content was characterized via the UHPLC-DAD technique. In particular, the extracts exhibited substantial antioxidant capacity, largely attributed to their elevated levels of bioactive compounds, such as gallic acid and other polyphenols, which, expressed as tyrosol equivalent, ranged from 10 g/kg to 130 g/kg.

Extracts derived from defatted olive stones demonstrated impressive antioxidant activity after being analyzed across different techniques, exploiting the best DPPH reducing capacity once included in an active packaging system (84.4 ± 1.6) %AP and the highest total biophenol content (130 ± 14) g/kg.

The best antioxidant matrix (olive stone) underwent thorough characterization using HRMS, which confirmed the presence and stability of over 40 active polyphenolic compounds within the matrix, verified through pattern fragmentation and literature references.

Active cellulose films incorporating 5% (w/w) olive stone extracts were fabricated using coating methodologies. Our approach valorizes a high-volume agro-industrial by-product and achieves effective inhibition of lipid oxidation in meat, despite the absence of antimicrobial activity due to the extract's polarity-dependent phenolic profile.

Compared with other antioxidant packaging concepts reported in the literature, our system differs substantially in structure and application. Asiri et al. (2024) developed fully edible sodium alginate films enriched with cactus pear extract, which combine antioxidant and antimicrobial properties but require more complex formulation steps and exhibit the typical moisture sensitivity of hydrocolloid-based films. Aziz et al. (2024) highlighted bacterial exopolysaccharides as multifunctional biopolymers capable of improving film sustainability, though these materials often demand tailored processing conditions and may have limited mechanical strength depending on the EPS type.

In contrast, our packaging provides a practical advantage by integrating the active components into a ready-to-use cellulose-based film without altering its mechanical performance or requiring additional structuring agents. This enables straightforward industrial adoption while maintaining regulatory compliance and leveraging a circular-economy resource.

Furthermore, the active packaging prototype, containing the olive stone extract, was proven to possess antioxidant properties that markedly retarded the progression of lipid peroxidation in minced beef meat with 50% fat under refrigerated conditions.

The TBARS assay has proven the effectiveness of the active film in significantly reducing lipid peroxidation after a 10-day period, offering superior protective advantages over blank films, which preserve lipids from oxidation by $46.6 \pm 2.9\%$, while the olive stone film improved the protection to only $18.4 \pm 1.0\%$.

Furthermore, the gallic acid used as a positive control has proven the efficiency of the method developed, preserving the sample and achieving only $2.2 \pm 0.2\%$ of protection.

This underscores the potential application of olive stone extract-based films in prolonging the shelf life of perishable food commodities.

In summary, the findings of this research point out the promising antioxidant efficacy of natural extracts from byproducts of the olive industry in active packaging applications. These insights pave the way for developing innovative, sustainable, and secure food packaging solutions, while at the same time elevating agricultural and industrial waste to high-value materials.

In future work, we will test different green extraction protocols and conduct an exhaustive investigation of the leakage of NIAS under realistic storage and use conditions in order to better characterize potential exposure and related risks, with the ultimate aim of scaling up the packaging development to the industrial level. The migration test will be performed in accordance with EU frameworks, specifically Commission Regulation (EU) No 1935/2004, implemented by Commission Regulation (EU) No 10/2011 for plastic materials.

CRediT authorship contribution statement

Consolato Schiavone: Writing – original draft, Data curation. **Francesco Romaniello:** Writing – review & editing, Investigation. **Paola Fusari:** Methodology, Data curation. **Antonio Perna:** Investigation, Data curation. **Pierangela Rovellini:** Methodology, Conceptualization. **Andrea Mario Giovannozzi:** Writing – review & editing, Investigation, Conceptualization. **Andrea Mario Rossi:** Writing – review & editing, Methodology. **Chiara Portesi:** Writing – review & editing, Investigation, Data curation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Francesco Romaniello, given his role as reviewer, had no involvement in the peer review of this article and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to another journal editor. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2026.147951>.

Data availability

Data will be made available on request.

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