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Development of innovative antioxidant food packaging systems based on natural extracts from food industry waste and *Moringa oleifera* leaves

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Abstract

Active packaging that prolongs food shelf life, maintaining its quality and safety, is an increasing industrial demand, especially if integrated in a circular economy model. In this study, the fabrication and characterization of sustainable cellulose-based active packaging using food-industry waste and natural extracts as antioxidant agents was assessed. Grape marc, olive pomace and moringa leaf extracts obtained by supercritical fluid, antisolvent and maceration extraction in different solvents were compared for their antioxidant power and phenolic content. Grape and moringa macerates in acetone and methanol, as the most efficient and cost-effective extracts, were incorporated in the packaging as coatings or in-between layers. Both systems showed significant free-radical protection

in vitro (antioxidant power ~50%) and more than 50% prevention of ground beef lipid peroxidation over 16 days by indirect TBARS and direct *in situ* Raman microspectroscopy measurements. Therefore, these systems are promising for industrial applications and more sustainable farm-to-fork food production systems.

Keywords: Active packaging, natural antioxidants, cellulose biopolymer, meat shelf life, food waste recovery, radical scavenging

1. Introduction

Food safety represents a global priority and one of the major concerns of consumers, administrations, and industry. The goal of food packaging is to satisfy customer and industry requirements, containing food in a cost-effective and appealing way while maintaining food safety and quality standards and minimizing the environmental impact (Marsh & Bugusu, 2007).

The demand for sustainable active packaging systems is increasing together with the compelling need for safe ready-to-eat products with long shelf lives. On this basis, the development of innovative food packaging materials represents a challenge of main interest in the food research field.

Nowadays, biopolymers are considered the best candidates to substitute nonbiodegradable and nonrenewable packaging materials due to their environmentally friendly characteristics (Rhim et al., 2013). All natural biopolymers, such as cellulose or starch derivatives, are biodegradable under open atmospheric conditions for definition (Karak, 2012).

Currently, environmental protection is one of the greatest challenges facing the food industry, including the management of huge amounts of waste. For instance, during the production of wine and olive oil, 10 to 20% of the total weight of plant material is discarded, leading to the necessity of managing millions of tons of dregs per year (Lafka et al., 2007). Most of the antioxidant active compounds, such as phenolic molecules or flavonoids, remain in the discarded parts of the plant, including the skins, seeds and leaves (Rodis et al., 2002). Thus, the employment of this kind of food-related industry waste as natural active compounds in biopolymeric food packaging material has generated great interest in the last few years in the context of a circular economy and pollution source reduction. This development goal follows the European Green Deal purpose to halve global food waste production per capita by 2030, ensuring an efficient and sustainable use of natural resources and moving toward healthier and more sustainable food systems (EU Green Deal Cluster 6 Food).

In addition, natural plant derivatives can be considered a safer and cost-effective alternative to synthetic antioxidant agents (Barbosa-Pereira et al., 2014; Realini & Marcos, 2014).

Many studies have already focused their attention on the development of active films containing natural antioxidants that efficiently enhance myoglobin stability and preserve fresh meat from oxidation (C. Nerín et al., 2008; Cristina Nerín et al., 2006). Natural antioxidant agents can be incorporated directly into meat packaging as scavengers of free radicals or as autoxidation protectors (Moudache et al., 2016). The main advantage of this strategy is that the agent does not need to be added to food, in compliance with European legislation (Regulation (EC) No. 1333/2008).

The presence of carboxylic groups on the conjugated ring structures of many compounds contained in plant derivatives allows the neutralization of free radicals or the decomposition of peroxides (Adedapo et al., 2008), leading to the inhibition of lipid peroxidation (Oyedemi et al., 2012), which is one of the main causes of food degradation during processing and storage. This process involves a chain reaction driven by the formation of secondary products and free radicals that enhance lipid autoxidation while negatively influencing the overall quality of foods, including their flavor, taste, and nutritional value, and promoting the production of toxic compounds. Thus, the shelf-life prolongation of fresh foods containing fatty acids, such as meat or fish, is strictly bound to a reduction or a slowdown of these reactions.

However, the real effectiveness of natural antioxidants in finished active packaging depends on many aspects, such as i) the extraction method, which can influence the final content of active compounds; ii) the kind of polymer, the physical, mechanical, optical and barrier properties of which might change upon the incorporation of active agents; and iii) the chemical interactions of the active compounds within the film, which may affect their antioxidant efficacy. Thus, the design of active food packaging polymers with natural extracts able to exploit their efficacy in each stage of production, from extraction to application to real food, while maintaining economic and environmental sustainability remains very challenging and needs to be further explored in depth.

The objective of the present study was to develop and characterize sustainable active food packaging employing industrial food processing waste and natural extracts as antioxidant agents to preserve foods from the oxidative damage remaining in the context of a circular economy and pollution source reduction. Analysis of the antioxidant properties of the active agents proposed here was performed at each crucial step of active film production from the extraction of the active compounds to their application on real food matrices by many different and complementary techniques, offering as comprehensive an evaluation of their efficacy as possible.

2. Material and Methods

2.1 Chemicals

DPPH powder, gallic acid (GA, 97.5-102.5%), 2,20-azobis(2-methylpropionamide)dihydrochloride (AAPH, 97%), fluorescein (3,60-dihydroxypirrol [isobenzofuran-1[3H],90[9H]-xanthen]-3one), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 98%), potassium phosphate dibasic heptahydrate, potassium phosphate monobasic dihydrate, Folin-Ciocalteu phenol reagent, sodium salicylate (>99.5%), 2,5-dihydroxybenzoic acid (>99%), hydrogen peroxide (>50%), and malondialdehyde tetrabutylammonium salt (MDA, >96%) were purchased from Sigma–Aldrich

(Milan, Italy).

Methanol (HPLC grade), ethanol absolute anhydrous, acetone, glacial acetic acid (>99%), sodium carbonate, trichloroacetic acid (TCA, >99%) and paraffin oil were obtained from Carlo Erba (Cornaredo, MI, Italy). Orthophosphoric acid (85%, reagent grade) and sodium hydroxide (0.01 mol/l) were obtained from Scharlab (Barcelona, Spain). 4,6-Dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid, TBA, 98%) was purchased from Acros Organics (Rodano, Milan, Italy).

For bacterial cultivation and assays, Muller Hilton (MH) broth and agar purchased from Lickson (Palermo, Italy), phosphate-buffered saline (PBS) tablets (200 ml) from PanReac AppliChem (Barcelona, Spain) and sodium chloride from Carlo Erba were used.

Standard polyphenols and reagents used for the UPLC-TQ-MS analysis included caffeine (CAS 58-08-2) [CAF], (+)catechin (>99.0%, 154-23-4) [C], (-)epicatechin (>95%, 490-46-0) [EC], (-)epicatechin gallate (>98%, 1257-08-5) [ECG], (-)catechin gallate (>98% 130405-40-2) [CG], (-)epigallocatechin (>95% 970-74-1) [EGC], (-)gallocatechin (>98% 3371-27-5) [GC], (-)gallocatechin gallate (>98%, 4233-96-9) [GCG] and (-)epigallocatechin gallate (>95%, 989-51-5) [ECG], all from Sigma Aldrich. Formic acid was purchased from Waters (Manchester, UK). Ultrapure water was obtained from a Wasserlab Ultramatic GR system (Barbatáin, Spain).

2.2 Analysis of the plant extracts

2.2.1 Plant materials and extraction methods

Moringa desiccated leaves (*Moringa oleifera* Lam.) were purchased from Alesframa (Málaga, Spain). Olive pomace (*Olea europaea* fruits harvested in December 2020) was supplied by a local olive oil mill in Ayerbe (Spain, 42° 18 '12' N, 1° 57' 53' W). Grape marc (skin and seeds) obtained from the "Garnacha" variety for red wine production was collected after pressing fruits harvested in the zone of Rioja Baja, near Calahorra (Spain, 42° 16 '36' N, 0° 41 '21' W), in October 2020.

The three plant materials were extracted at the University of Zaragoza (Spain) using three techniques. The first technique was supercritical fluid extraction (SFE) using CO₂ in three steps. The solid residues were collected manually from each of the two collectors. Then, hexane was employed to dissolve and collect the remaining residues in another two fractions.

In the second technique, a portion of the solid residues collected from the SFE extractor was macerated in ethanol to solubilize them and processed in a second extractor to perform supercritical antisolvent extraction (SAE) employing CO₂ in two steps. First, from the extractor, two fractions were collected, one manually and another after its dissolution in ethanol. Then, the remaining residues after another step of pressure regulation were collected manually from the collector. The scheme of the extractions and the collected fractions is presented in the Supplementary Information (SI) (Figure S1).

All the extract fractions were solubilized in ethanol or water and ethanol 1:1 at final concentrations of 1400 ± 5 mg/l for the moringa and grape extracts and of 5000 ± 13 mg/l for the olive cake extracts.

In the third technique, maceration (MAC) was applied at room temperature (25 °C) to plant residues and leaves using methanol, ethanol, acetone and DCM (100 g of plant sample per liter of solvent). Cold and hot water was also considered. Samples were kept in the dark for 10 and 30 days in closed glass bottles and shaken every two days. After maceration, samples were filtered (Whatman qualitative filter paper grade 1), concentrated in a Büchi R-124 rotary evaporator (Flawil, Switzerland) until a volume of approximately 5 ml was achieved, and finally air-dried in glass Petri dishes for 48 h.

All solutions were placed in glass tubes, stirred and sonicated for at least 1 h at 300 W (Q-Sonica Q700 sonicator, 20 kHz, Newtown, CT, USA). Then, the tubes were covered with aluminum foil, plugged and sealed with Parafilm® M and stored at 4 °C until analysis.

A legend of all the fractions analyzed is reported in SI (Table S1).

2.2.2 Total phenol content determination by the Folin-Ciocalteu assay

The protocol for total phenol content determination was adapted by the official method described by Kupina et al. (Kupina et al., 2018).

Briefly, five dilutions of gallic acid (40 mg/l – 200 mg/l) were obtained in Milli-Q® water to construct a standard calibration curve. A series of test tubes, each containing 3 ml of water and 200 µl of Folin-Ciocalteu reagent, were prepared, and in each tube, one of the following was added: 200 µl of sample test solution, calibration standard solution or water as a blank. The tubes were vortexed and allowed to settle for 6 min.

Then, 200 µl of a 20% (w/v) sodium carbonate solution was added to each tube and mixed well. All test tubes were incubated at 37 ± 1 °C for 30 min, and then 200 µl of each sample was placed in a well of a 96-well plate in triplicate. The absorbance was measured at 765 nm in a multiwell reader by subtracting the absorbance of the blank. A linear fit was calculated from the gallic acid calibration curve, and the total phenolic content % (w/w) of each sample was calculated by interpolating their average absorbance subtracted from the blank from the fit, normalizing the results according to the sample concentration (mg/l).

2.2.3 UPLC-TQ-MS analysis of polyphenols

UPLC-TQ-MS is a sensitive and reliable technique that is already used for the determination of polyphenols in plants, such as mint (Krzyzanowska et al., 2011) or sweet tea tree (Ning et al., 2019). All the plant extracts solubilized in ethanol were analyzed by UPLC-TQ-MS targeted for polyphenolic compounds using the standards listed in the chemicals. Semiquantitative analysis was carried out by injecting 10 µl of each sample in an Acquity™ UPLC coupled to an electrospray ionization (ESI) interface used in both positive and negative modes with a capillary voltage of 3.5 kV and a Xevo TQ-S microdetector (Waters, Manchester, UK). A UPLC BEH C18 column with a 1.7 µm particle size (2.1 × 100 mm) was used at a flow rate of 0.3 ml/min at 35 °C. The mobile phases were water (phase A) and methanol (phase B), both with 0.1% formic acid. The gradient was 95% A/5% B (6 min); 5% A/95% B (10 min). The sampling cone voltage was optimized for each

compound by using standards and was set at 40 V. The source and desolvation gas (400 l/h) temperatures were 150 and 350 °C, respectively. Selected ion recording (SIR) mode was used. MassLynx v.4.1 software was used for data acquisition and processing. Analyses were performed in duplicate. For each phenolic compound, the result is expressed as a normalized area by dividing its area by the sum of the areas of all compounds of interest and multiplying by 100. For instance, in the case of GA:

$$A_{GA}\% = A_{GA}/[A_{GA} + A_{CAF} + A_{EC} + A_{CG} + A_{ECG} + A_{GC} + A_{EGC} + A_{GCG} + A_{EGCG}] \times 100 \quad (\text{Eq. 1})$$

2.2.4 Antibacterial activity evaluation

Escherichia coli ATCC 8739 and *Staphylococcus aureus* ATCC 75380 were used for this work as Gram- and Gram+ bacterial models, respectively. The assay performed in this work is described in the SI.

2.2.5 Antioxidant activity evaluation

2.2.5.1 DPPH radical scavenging activity

The reduction of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical signal at 516 nm was measured using a Varioskan LUX multiplate reader (Thermo Scientific, Rodano, Milan, Italy) following the method previously described by Prieto (Prieto, 2012). The details can be found in the SI. The radical scavenging activity or the antioxidant power (AP%) after one hour of incubation with DPPH for each extract was expressed as the percentage of reduction of the free radical by the sample and calculated as follows:

$$AP\% = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100 \quad (\text{Eq. 2})$$

where A_{control} is the mean of the methanolic DPPH control (DPPH in methanol) absorbance values subtracted from the methanol absorbance and A_{sample} is the mean of each sample replicate absorbance value (DPPH with extracts) subtracted from their methanolic blank absorbance values (extracts in methanol). The AP% of GA at 8 different concentrations from 0.06 mg/l to 2.5 mg/l was measured as well as an internal control. The extracts that did not reach almost 50% AP% were excluded from further analysis. For each sample, five concentrations (AP% between 20% and 70%) were chosen, and a linear fit was calculated. The half maximal effective concentration (EC_{50}) was calculated from the linear range of each sample.

2.2.5.2 ORAC assay

Working solutions were prepared following the Association of Official Agricultural Chemists (AOAC) standard method described by Ou et al. (Ou et al., 2013). Details can be found in the SI. The net area under the curve (AUC) was calculated for each sample, including the Trolox dilutions, by summing all the fluorescence measurements over time and normalizing them to their initial fluorescence values. Then, a Trolox calibration curve was obtained by plotting the calculated net AUC over Trolox tested moieties (μmol). Each sample average AUC was interpolated in the

calculated linear fit to obtain the μmol of relative Trolox equivalents (TE). The ORAC values were calculated as μmol of TE per gram of sample.

2.2.5.3 Uncertainty budget evaluation

For both the DPPH and ORAC results, the relative uncertainty budget was calculated for each value considering a linear fit, and all the uncertainties were related both to the x- and y-axes. For the first uncertainty, the sample purity of each dilution performed (considering for the final volume uncertainty the propagation of those related to each specific pipette used) and the weight of the sample were considered. For the second uncertainty, a propagation of the calculated standard uncertainty values, derived from the three replicates of absorbance (DPPH) or the AUC measurements (ORAC), was performed.

2.3 Active film production

The developed film was composed of 45- μm -thick cellulose-based biopolymer NatureFlex™ NK by Futamura UK Ltd. (Burgos, Spain). The optimum conditions corresponded to a 5% (w/w) concentration of moringa leaves and olive extracts, which were mixed in a food contact-safe methyl ethyl ketone-based varnish, ADCOTE™ 17-3, by DOW Chemical Ibérica (Madrid, Spain). They were manually coated as homogeneous layers on the cellulose biopolymer employing a wire close-wound bar (bar number: 7; color code: brown; wire diameter: 1.02 mm; wet film deposit: 80 μm). Samples with 5% (w/w) GA and with varnish were prepared as well as positive controls and blanks, respectively (SI Figure S2).

The grape marc macerates were included at the same concentration as the other samples in a water-based biodegradable adhesive (Flex Tack 4M35) for food packaging applications from Samtack (Barcelona, Spain). The active adhesive was spread on a cellulose sheet using a K control coating machine from RK print coat instruments (Litlington, UK) using the same bar. A cellulose sheet with dry adhesive was covered with another layer of cellulose. The developed double-layer biomaterial was placed in a BiO 330 A3 Heavy Duty Laminator (South Korea) and pressed at 40 °C with a speed of 5. Positive and negative controls with 5% Trolox and with pure adhesive, respectively, were also prepared.

2.4 Characterization of antioxidant activity

2.4.1 DPPH radical scavenging activity of active films

The DPPH inhibition assay applied directly on active films was previously described (Benbettaïeb et al., 2018; Busolo & Lagaron, 2015). Briefly, squares of 2 × 2 cm were cut in triplicate from each active film, including the controls. These squares were placed in sterile Falcon tubes and covered with aluminum foil, and then 12 ml of DPPH radical solution (50 mg/l) was placed in each tube, including one tube without any film as a control. Aliquots of 200 μl of each replicate of each sample were collected immediately after the addition of DPPH and after 24 h, and their absorbance at 516 nm was measured. Then, the AP% was calculated for all the samples, including the blank, at the two time points.

2.4.2 Free radical scavenging assay of active films

The antioxidant capacity of all cellulose-based active films was measured using a method developed by Pezo et al. (Pezo et al., 2006) (SI Figure S3) which consists of the hydroxylation of salicylic acid to 2,5-dihydroxybenzoic acid (2,5-DHB) by the free OH· radicals generated from an aqueous H₂O₂ aerosol under UV-light irradiation. Samples of 1 dm² of each active cellulose film were placed inside PE bags of 150 × 150 mm. A PFS-200 impulse sealer from Zhejiang Dongfeng Packing Machine Co (Wenzhou, Zhejiang, China) was used to thermoseal the bags, which were placed in triplicate in a holder and connected to the OH· radical generator. The assessment of sample oxidation was carried out for 24 h. As a result, the percentage of hydroxylation (H%) was obtained according to the formula where 100% is the peak area of 2,5-DHB in the blank sample (control) and x% is the area of the 2,5-DHB peak in a sample with antioxidants.

$$H\% = [\text{Area}_{2,5\text{DHB (active film)}}/\text{Area}_{2,5\text{DHB (blank)}}] \times 100 \quad (\text{Eq. 3})$$

2.4.3 TBARS assay to assess lipid oxidation reduction in ground beef

Twelve grams of fresh beef meat from a local butchery, minced twice and with a content of approximately 50% fat, was weighed for each of the tested film samples and packaged individually in two film squares of 6 × 6 cm over and above the meat piece, flattening it to ensure that all the active area was in full contact with the meat.

Each sample was inserted into a PET thermosealable 12 cm × 14 cm bag, sealed aerobically with nonmodified atmospheric air and stored in a refrigerator at 4 °C. Samples were prepared in triplicate for each kind of film, including the blank, and for each time of analysis (0, 3, 6, 9 and 16 days) to follow the meat lipid peroxidation over time. The thiobarbituric acid index (TBARS value) was determined by adapting previously described protocols (Ahn et al., 1998; Henriott et al., 2020; Nerin, 2010; Ribeiro et al., 2021) (details in SI). The concentration of MDA in the samples was calculated by interpolating the values in the linear fit obtained from the calibration curve. The TBARS values are expressed as mg of MDA per kg of meat. All measurements were made in triplicate. The statistical significance of the difference between the average of the measurements on the blank and the active samples was analyzed by performing a t test, and the relative p values were calculated.

2.4.4 Raman evaluation of meat oxidation

Just before the TBARS assay, each meat sample was analyzed by Raman spectroscopy immediately after opening the packaging and flattening the meat as much as possible. Before data collection, the alignment and calibration of the Raman DXR™ Thermo Fisher Scientific microscope were performed using the instrument calibration tool. A 10× dry objective (0.25 NA) with a 50 μm slit confocal aperture was employed. The analyzed spectral range was 3500–200 cm⁻¹. All spectra were acquired using an excitation wavelength of 780 nm, a power of 5.0 mW and exposure time of 5 s for 24 scans. At least five spectra were collected for each sample, and they were manually corrected for the baseline and normalized for the intensity of the peak at 1000 cm⁻¹ corresponding to the stretching vibrational mode of aromatic rings (Sócrates, 2001), which do not vary their Raman intensity from

one sample to another, for the comparison between the initial and final times (Days 0 and 16 were considered). For the analysis of the reduction of the Raman signal at 1450 cm^{-1} at each time point (Days 0, 3, 6, 9 and 16), at least 5 spectra were collected for each triplicate group, and both the average and the standard deviation were calculated. The spectra were corrected for the baseline and normalized for the intensity of the signal at 1450 cm^{-1} , corresponding to the $\delta(\text{CH}_2/\text{CH}_3)$ of fatty acids, of the starting point (Day 0).

2.4.5 Statistical analysis

All the experiments were repeated three times on three different meat samples for each film and at each time point, and the reported data represent the mean values. The relative uncertainty budget calculated as described in paragraph 2.2.5.3 has also been provided for each measurement. To determine if there were significant differences between the results obtained, Student's t test was employed at a probability level of $p < 0.05$ or $p < 0.01$ (single or double stars in the histograms, respectively). The null hypothesis stated that the samples being analyzed were identical. However, if the calculated t values exceeded the tabulated values, the differences between the samples were considered significant, and the null hypothesis was rejected. Furthermore, the calibration plot for chemical analysis was adjusted using the least squares method.

3. Results and discussion

Extracts were obtained from grape marc and olive pomace from Spanish industries by i) supercritical fluid extraction (SFE) by CO_2 , which allows the separation of mainly nonpolar fractions that are not expected to contain high amounts of active molecules (this type of extraction has been successfully used to extract nonpolar fats from Parmesan grated cheese, without significant changes in sensory tests (Yee et al., 2007)); ii) subsequent supercritical antisolvent extraction (SAE) of the solid residues redissolved in ethanol, which should extract the highest amounts of active agents; and iii) maceration (MAC), as a more cost-effective procedure, in five solvents that differ in polarity, and for two incubations. Various fractions were collected at different steps of the processes, characterized and compared for their bactericidal properties and antioxidant power by both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) assays and comparing the results with the contents of active compounds obtained by Folin-Ciocalteu and ultra-performance liquid chromatography-triple quadrupole-mass spectrometry (UPLC-TQ-MS). *Moringa oleifera* leaf extracts were also included in the study. Indeed, many studies have demonstrated that this tropical plant possesses valuable antioxidant, therapeutic and nutritional properties (Vergara-Jimenez et al., 2017).

The extracts that showed the highest antioxidant properties were included in the biopolymer by coating them on the film surface using a food-safe resin or in a double-layer system incorporating them in the adhesive between two films of the polymer. Then, the two materials were characterized for their radical scavenging capacity in both liquid by DPPH and the atmosphere by the free radical scavenging assay. The two systems that showed the highest efficacy in both tests were used to pack ground beef meat to test their protective action against lipid oxidation in comparison to conventional cellulose films. Meat analysis was performed both indirectly by thiobarbituric acid reactive substance

(TBARS) assay and directly *in situ* by Raman microspectroscopy as a rapid and nondestructive technique.

3.1 Characterization of the antibacterial and antioxidant properties of the natural extracts

Regarding antibacterial properties, no significant antibacterial effects were observed for any of the fractions tested (SI Figure S4) against either *E. coli* or *S. aureus*. To determine the antioxidant properties, the AP% was calculated for each using Equation (2) and plotted against their concentration (Figure 1).

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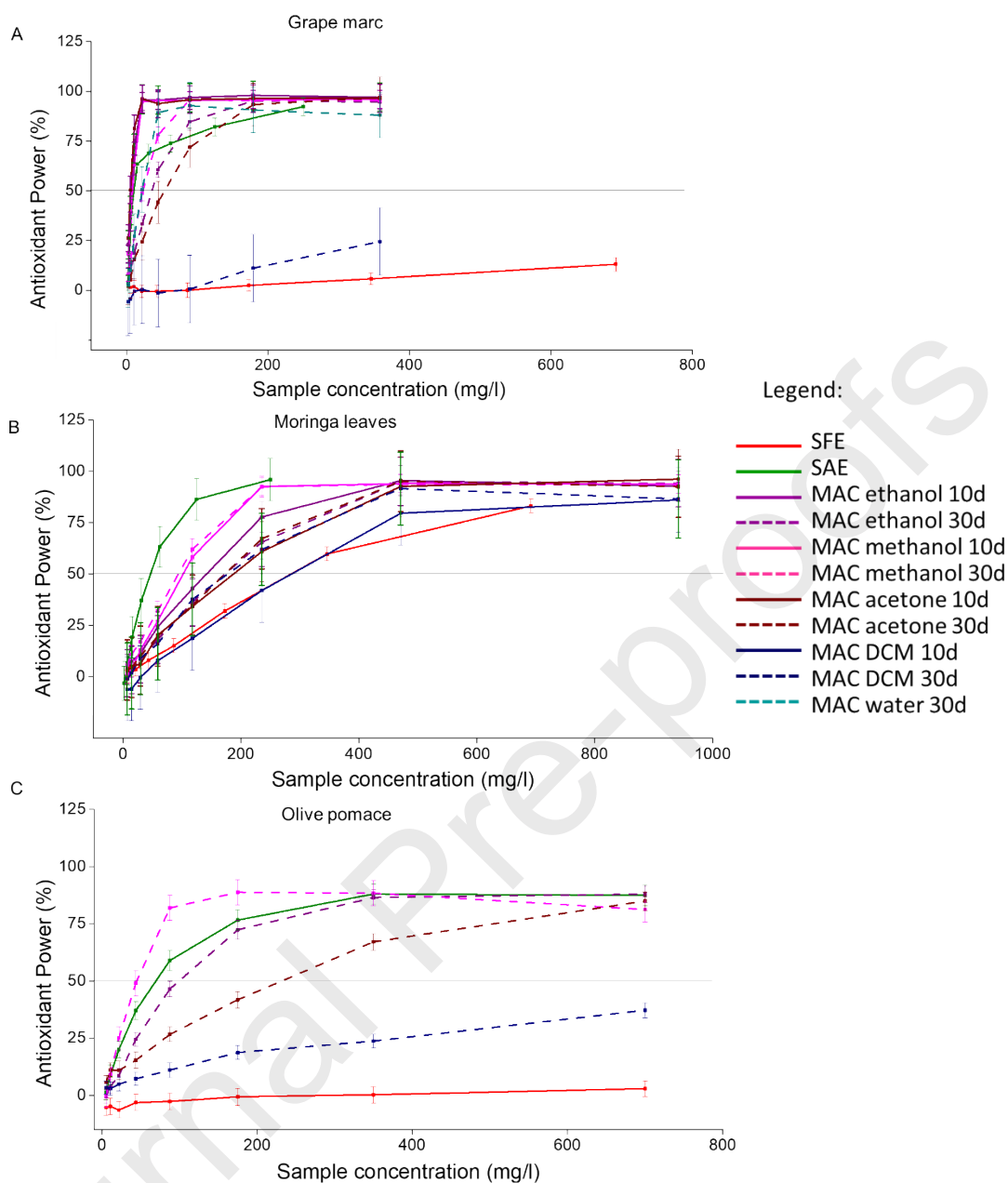


Figure 1: DPPH assay results expressed as antioxidant power percentage (AP%).

AP% was plotted against sample concentrations for each extraction fraction by SFE (red lines), SAE (green lines) and MAC for 10 days (solid lines) or 30 days (dashed lines) in the four different solvents (ethanol, purple; methanol, pink; acetone, carmine; DCM, blue; and water, light blue) of A) grape marc extracts, B) moringa leaf extracts and C) olive pomace extracts.

As expected, the fractions obtained by SFE and MAC in DCM showed the lowest AP% values, which did not even reach 50% in most cases. This result was due to the mostly nonpolar composition of these extracts, which do not contain active compounds such as polyphenols, flavonoids and tannins, which are usually extracted by polar solvents such as water, ethanol or methanol. Conversely, SAE fractions showed very high antioxidant efficacy (green lines in the plot), especially those collected immediately after passage in the extractor (scheme in SI, Figure S1). Additionally, the macerates, principally those obtained after 30 days with ethanol or methanol, showed good capability to reduce the DPPH radical due to their higher polarity. The slope of the curves linearly reflects the antioxidant efficacy of the samples, as the higher the slope is, the lower the sample concentration needed to reduce the same amount of DPPH. Consequently, the highest efficacy was clearly obtained by grape marc extracts, excluding the SFE and MAC in DCM fractions, followed by moringa and olive pomace extracts, possibly due to the more lipophilic composition of olive extracts, which could partially affect their antioxidant efficacy in liquid assays under hydrophilic conditions.

From these results, the half-maximal effective concentration (EC_{50}) was interpolated for each sample, excluding those that did not reach an AP% of 50%, analyzing a linear range of at least 4 concentrations in the surrounding 50% for each one (SI Table S2). The EC_{50} values were compared with the ORAC assay, and the percentage of the total polyphenol content (w/w) was determined by the Folin-Ciocalteu assay (Table 1). A higher polyphenol content is often related to a higher antioxidant efficacy due to their capability to donate H^+ , thus reducing radical compounds. Indeed, as expected, the samples with lower EC_{50} values displayed higher polyphenol contents.

Furthermore, the SAE fractions presented the highest polyphenol contents, especially the two fractions collected from the first extractor. These samples were immediately followed by methanolic macerates, representing the solvent with the highest extractive capacity of active compounds. These findings are in line with literature data (Babbar et al., 2014).

Additionally, grape marc extracts demonstrated the highest polyphenol contents with a relatively very high antioxidant activity, consistent with the fact that the peel and seeds of red grapes contain large amounts of antioxidants (Lapornik et al., 2005).

For the maceration extracts, the time of maceration seems to influence the extraction of active compounds. Indeed, for both moringa and olive, the best antioxidant efficacy and polyphenolic content were obtained by maceration for 30 days. In contrast, for grapes, the best results corresponded to 10 days of maceration. These findings were also confirmed by the redder color observed in the macerates after 10 days in all 4 solvents, while after 30 days, the ethanolic macerates showed a loss of the red color, indicating their lower contents of phenolic compounds. This phenomenon has been confirmed in the literature (S. Meyer et al., 1997) because excessive extraction times could cause the oxidation of phenols or their polymerization into insoluble species. Furthermore, it is known that the changes in the solid to liquid ratio occurring over time could influence the mass transfer of active compounds. Consequently, the optimal extraction conditions, including solvent, temperature and time of extraction, could be different between the plant materials due to their different compositions.

Even if the ORAC assay substantially confirmed the DPPH results, demonstrating that the SAE fractions were more active in fluorescein protection from decay, it represents a more standard parameter to quantify and compare the total antioxidant capacity (TAC) of these food derivatives representing a unit of measurement for antioxidant content and was originally developed by the National Institute on Aging (NIA) at the National Institutes of Health (NIH). This assay confirmed that among the MAC, the moringa and olive samples extracted in methanol for 30 days and the grape sample extracted in acetone for 10 days retained the highest antioxidant efficacy, showing high ORAC values. Furthermore, this technique also evidenced the high extractive potential of water with regard to grape marc, confirming that complementary techniques are needed to better evaluate the antioxidant capacity of each extract when comparing different extractive methods and solvents.

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Table 1. DPPH, ORAC and Folin-Ciocalteu assay results for all the natural extracts.

Extract	Grape marc			Moringa			Olive pomace		
	Fraction	EC ₅₀ (mg/l)	Total polyphenols % (w/w)	ORAC values (μ mol TE/g)	EC ₅₀ (mg/l)	Total polyphenols % (w/w)	ORAC values (μ mol TE/g)	EC ₅₀ (mg/l)	Total polyphenols % (w/w)
SFE 1	1544 \pm 148	9.9 \pm 0.3	18730 \pm 540	223 \pm 87	2.931 \pm 0.052	21790 \pm 610	>5000	0.543 \pm 0.062	2480 \pm 130
SFE 2	2547 \pm 508	8.4 \pm 0.1	23960 \pm 760	198 \pm 53	2.83 \pm 0.10	22990 \pm 660	>5000	0.502 \pm 0.058	3000 \pm 80
SFE 3	4194 \pm 1074	8.4 \pm 0.1	19530 \pm 570	115 \pm 24	3.086 \pm 0.057	24190 \pm 670	>5000	0.689 \pm 0.051	3340 \pm 150
SFE 4	2985 \pm 262	8.6 \pm 0.2	31400 \pm 860	738 \pm 76	3.20 \pm 0.08	22880 \pm 640	>5000	0.577 \pm 0.056	3530 \pm 90
SAE 1	6.45 \pm 0.91	45.0 \pm 4.9	127700 \pm 3700	69 \pm 25	10.693 \pm 0.067	x	46 \pm 16	5.05 \pm 0.11	4060 \pm 110
SAE 2	6.0 \pm 1.7	38.23 \pm 0.74	91600 \pm 2600	91 \pm 17	10.217 \pm 0.081	46900 \pm 1400	66 \pm 33	3.614 \pm 0.081	2950 \pm 140
SAE 3	352 \pm 50	10.34 \pm 0.12	80900 \pm 2400	139 \pm 35	3.479 \pm 0.068	43500 \pm 1300	139 \pm 35	3.41 \pm 0.15	3400 \pm 180
MAC Met 10 d	6.7 \pm 1.2	44.8 \pm 1.9	74600 \pm 2300	78 \pm 13	7.476 \pm 0.050	26040 \pm 750	x	x	x
MAC Ac 10 d	3.80 \pm 0.42	47.34 \pm 0.71	78000 \pm 2300	92 \pm 20	4.69 \pm 0.20	33900 \pm 990	x	x	x

MAC Et 10 d	8.3 ± 1.9	31.78 ± 0.91	69000 ± 2000	93 ± 20	3.643 ± 0.029	42900 ± 1200	x	x	x
MAC DCM 10 d	657 ± 12	9.70 ± 0.11	72300 ± 2000	149 ± 55	3.688 ± 0.082	30160 ± 890	x	x	x
MAC Met 30 d	31.0 ± 7.6	16.35 ± 0.12	75100 ± 2100	54 ± 10	7.60 ± 0.49	42600 ± 1200	46 ± 16	3.78 ± 0.15	4300 ± 110
MAC Et 30 d	29.8 ± 4.7	14.67 ± 0.21	60600 ± 1800	164 ± 30	5.652 ± 0.088	26240 ± 910	108 ± 34	3.309 ± 0.073	3420 ± 90
MAC Ac 30 d	51 ± 12	14.74 ± 0.59	75300 ± 2200	108 ± 20	4.085 ± 0.046	28300 ± 840	214 ± 71	2.133 ± 0.039	3340 ± 170
MAC DCM 30 d	x	x	x	177 ± 71	3.968 ± 0.029	31670± 950	>5000	1.240 ± 0.031	3010 ± 170
MAC water 30 d	21 ± 12	15.40 ± 0.21	89400 ± 3500	x	x	x	x	x	x

To better elucidate the relationship between higher antioxidant properties and higher content of phenolic compounds, a semiquantitative analysis of the contents of the principal polyphenolic molecules was performed by targeted UPLC-TQ-MS, with the results expressed as relative peak ratio% following Equation (1) (Figure 2 and Tables S3-S4-S5 SI).

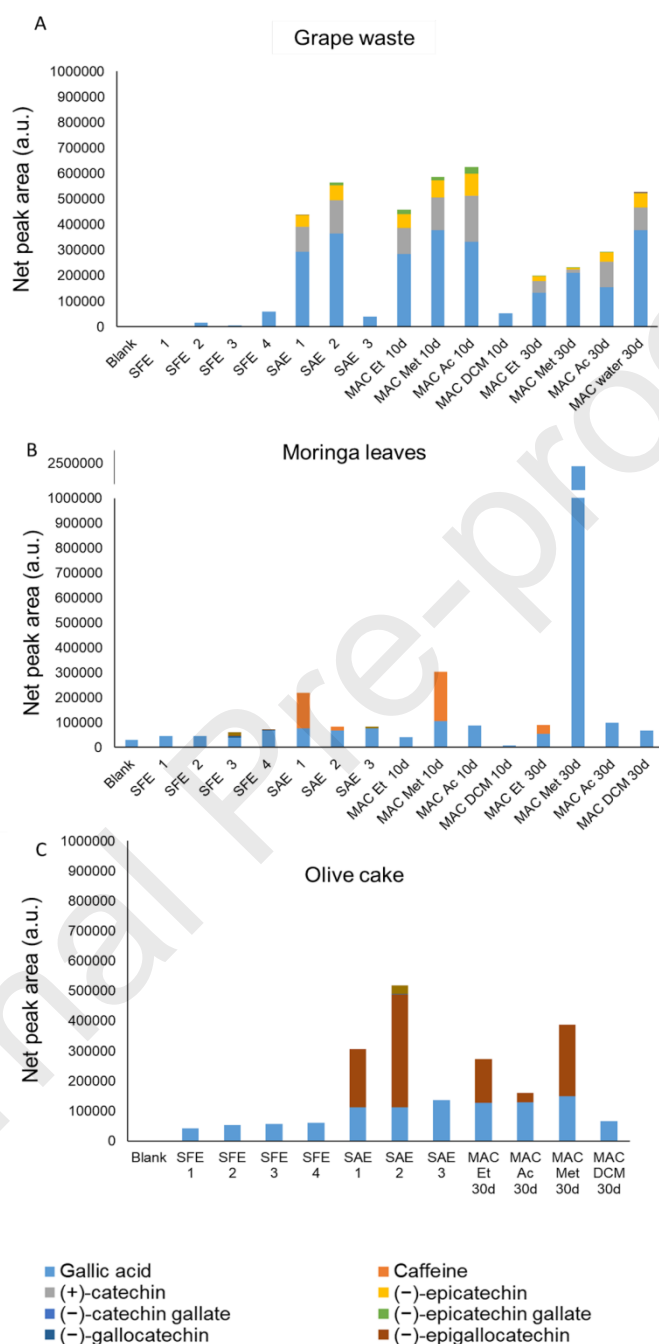


Figure 2: UPLC-TQ-MS results for each polyphenolic standard listed in the legend below the plots obtained by each extract.

A) Grape marc, B) moringa leaves, and C) olive pomace samples.

The phenolic contents of all three plant extracts were mostly composed of GA, which was more abundant in samples with high polyphenolic content according to the Folin-Ciocalteu assay, such as the MAC and SAE fractions. In particular, grape extracts (except for the moringa MAC in methanol for 30 days, which had the highest amount of GA) had the highest contents of polyphenolic compounds, including catechin, epicatechin and epicatechin gallate. However, the moringa extracts showed the presence of caffeine, especially in the MAC in methanol and in the first SAE fraction. Even if the olive pomace extracts contained lower amounts of polyphenols, they exhibited quite high amounts of epigallocatechin and epigallocatechin gallate, especially in the first two SAE fractions and in the MAC in ethanol and methanol, suggesting the potential for antioxidant effects for these extracts. These results are perfectly in line with the DPPH, ORAC and Folin-Ciocalteu results, confirming that the grape extracts have the highest content of active polyphenols, antioxidant effects and GA content. For all three plants, the most efficient solvent for extraction was methanol.

3.2 Active film production and antioxidant efficacy characterization

According to the obtained results, the extracts of grape MAC in acetone and methanol for 10 days, moringa MAC in methanol for 10 and 30 days, and olive pomace MAC in methanol and ethanol for 30 days were selected to be incorporated into a biodegradable polymer. The extracts were coated on a film of cellulose-based biopolymer or incorporated into an adhesive between two layers of the same material. The criterion was based on their solubility in varnish for the coating or in food-safe adhesive. In both cases, the extract concentration was 5% w/w. MAC was chosen instead of SAE as a more sustainable and less expensive extraction process (Vongsak et al., 2013).

The AP% values were calculated accordingly, as shown in Table 2. The DPPH assay showed that the two most effective DPPH radical scavengers, apart from the two standards, GA and Trolox, were the films coated with 5% moringa MAC in methanol for 30 days and grape MAC in acetone for 10 days. AP% values of $50.1 \pm 7.8\%$ and $48 \pm 11\%$, respectively, were obtained, suggesting very promising antioxidant efficacy.

The results of the ability to scavenge $\text{OH}\cdot$ radicals by the developed active films are presented in Table 2.

Table 2: DPPH and free OH· radical scavenging results of the active films.

Sample	Antioxidant Power %	Mean H%*
Blank (varnish)	0.1 ± 5.9	100
Gallic acid	89.8 ± 3.0	60.53 ± 0.16
Moringa MAC methanol 10 d	37.4 ± 4.9	57.92 ± 0.10
Moringa MAC methanol 30 d	50.1 ± 7.8	52.13 ± 0.17
Olive MAC ethanol 30 d	13.3 ± 2.4	74.01 ± 0.32
Olive MAC methanol 30 d	13.0 ± 2.9	55.11 ± 0.19
Blank (adhesive)	5.7 ± 7.0	100
Trolox	71.6 ± 6.7	65.81 ± 0.13
Grape MAC acetone 10 d	48 ± 11	58.10 ± 0.18
Grape MAC methanol 10 d	28.6 ± 6.7	66.84 ± 0.10

*Calculated by Equation (3)

The best radical scavenging capacity was obtained from the film coated with 5% moringa MAC in methanol for 30 days with an H% of 52.13 ± 0.17 , which was even lower than that of GA. This test also revealed the good radical scavenging capacity of the films coated with olive MAC, which did not show satisfactory results in the DPPH assay.

This difference in performance could be due to the different lipophilic contents between the extracts: olive pomace extracts may contain more lipidic fractions derived from the oils of the seeds that cannot be dissolved in the methanolic DPPH solution, resulting in a reduction in the total antioxidant efficacy. However, this assay highlighted the ability of these films to scavenge atmospheric free radicals, suggesting that the more volatile parts of these extracts could have some potential as noncontact materials active on the atmosphere around food.

Furthermore, the Trolox and grape extracts also showed a good OH· radical scavenging capacity, comparable to those of GA and moringa, even in the case of adhesive, where their availability could be mitigated by the presence of the layer of cellulose-based biopolymer. Consequently, different tests are needed to accurately characterize all the antioxidant effects of the active materials produced. Some of these materials are more indicated for direct contact use, while others would be better suited for noncontact packaging due to their higher scavenging activity or their behavior against lipophilic substances.

Finally, the two most active films of all those tested (moringa MAC Met for 30 d and grape MAC Ac for 10 d) were chosen to be applied on a real food matrix. Two different packaging systems, direct contact (coating) and indirect noncontact radical scavenging activity (double layer + adhesive), were considered.

For these film thicknesses, the transmission rates of oxygen (OTR) and water vapor (WVTR), as well as the permeability properties, were studied. The data are summarized in SI (Table S6). Two independent films and ten determinations per film were considered in all cases.

3.3 Active film application for the protection of ground beef meat from oxidative damage

As lipid peroxidation is one of the main causes of meat degradation, during transportation or storage, meat oxidation over 16 days of storage in the refrigerator at 4 °C was compared with the same meat packed in blank controls (cellulose polymer with only varnish or adhesive without adding any active agent) and in films with 5% concentrations of the two standards (GA and Trolox) as negative and positive controls, respectively (Figure 3 A-D).

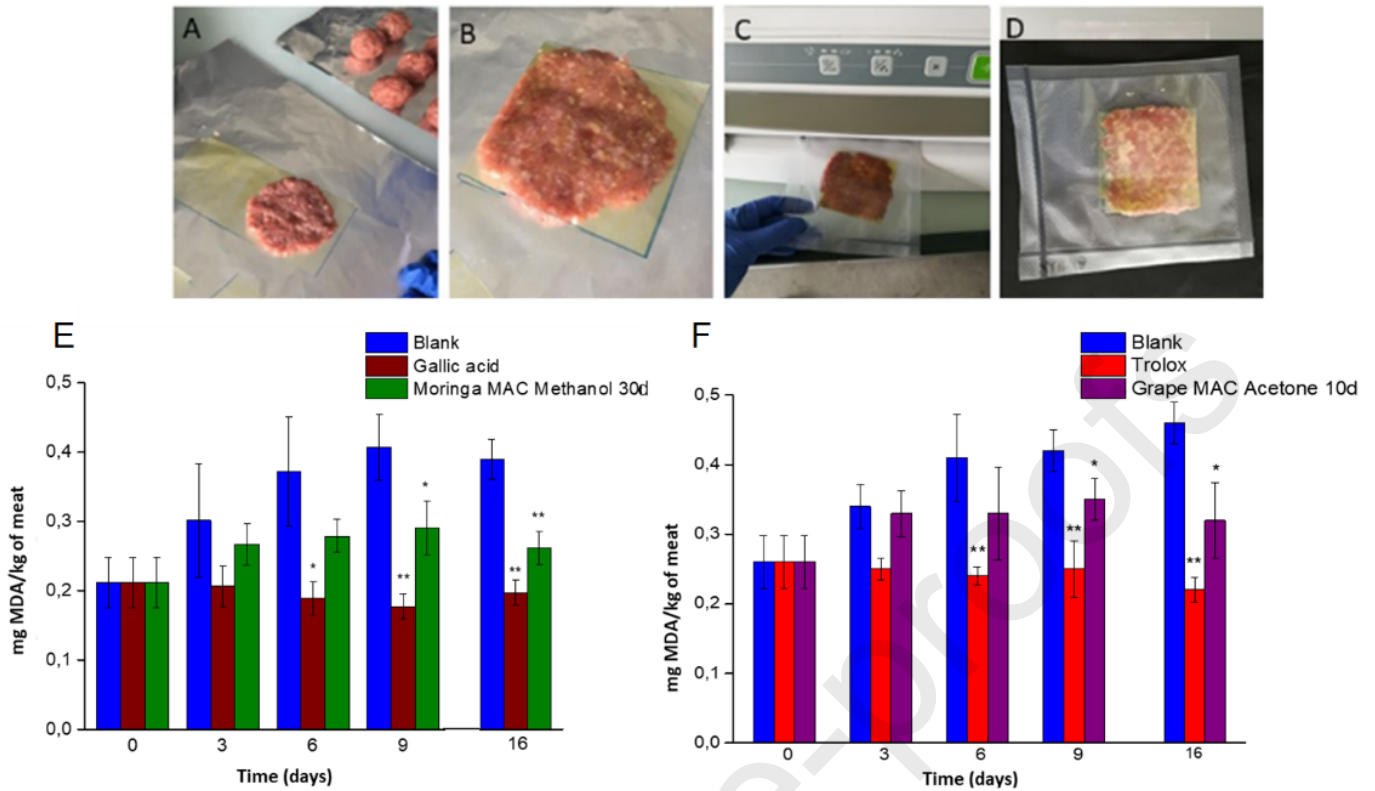


Figure 3: Ground beef meat packaging preparation and TBARS results.

A) Weighing and flattening of meat samples. B) Packing of the meat samples in rectangles of the active packaging. C) Thermosealing of the PET bags outside the test sample, leaving the same volume of nonmodified atmosphere inside. D) Final sample to be stored in the refrigerator. E-F) TBARS assay results of meat samples packed in blank controls (blue bars), in film coated with 5% GA (carmine bars) or moringa MAC Met for 30 d (green bars) and in film with 5% Trolox (red bars) or grape MAC Ac for 10 d (purple bars) in adhesive between two layers of cellulose. Asterisks: *: p value < 0.05. **: p value < 0.01.

The TBARS results are represented in Figure 3E and 3F and Table S7 (SI). The higher the MDA/kg measured, the more lipid peroxidation took place on the analyzed piece of meat.

The relatively high uncertainty values among the three replicates could be due to the differences in the total lipid contents among the samples. These results showed significant differences in lipid oxidation between the active and blank samples. Considering the moringa and grape films, statistically significant protection was already detectable from Day 9 ($p < 0.05$ for moringa and $p < 0.01$ for grape). The results of the two controls indicated that the test had been developed appropriately. Meat packed in the blanks showed increased oxidation over time, whereas the samples packed in GA and Trolox maintained their levels of meat integrity over all the storage times tested. Significant differences compared to the blank controls ($p < 0.05$) were observed after only 6 days. After 16 days, the lipid oxidation of the blank controls reached approximately 84%, whereas the oxidation levels of the corresponding samples of moringa and grape active packaging were only 24% and 34%, respectively. Furthermore, the oxidation levels of the samples stored in the packaging with GA and Trolox were 7% and 26%, respectively. The percentages of oxidation were calculated using the following equation:

$$\text{OX}\% = [(A_{d0} - A_{d16})/A_{d0}] \times 100 \quad (\text{Eq. 4})$$

where A_{d0} and A_{d16} are the absorbances of the sample measured at Days 0 and 16, respectively.

Therefore, cellulose biopolymer with 5% moringa MAC Met for 30 d and that in 5% grape MAC Ac for 10 d were demonstrated to be able to protect ground beef meat from lipid peroxidation by at least 60% and 50%, respectively, after 16 days of storage in the refrigerator.

3.4 Raman spectroscopy as a nondestructive and rapid alternative to TBARS assay

To confirm the observed protective action of these films against lipid oxidation of the meat, ground beef meat samples were also analyzed by Raman microspectroscopy as a more direct measurement method.

In particular, Raman spectroscopy was already reported to follow the lipid oxidation of meat over time (Moudache et al., 2017; Olsen et al., 2007), but these studies eventually involved a lipid extraction step or the use of a special sample holder with silver nanoparticles employing the surface-enhanced Raman spectroscopy (SERS) configuration to detect the Raman signals.

However, in this work, meat was analyzed directly by nonenhanced Raman spectroscopy without the use of any preparative steps or extractions.

The Raman spectra of ground beef meat were collected at each time point for all the samples before the TBARS assay.

Complete average Raman spectra of ground beef meat are provided in the SI (Figure S5). All the spectra were preprocessed for baseline correction to eliminate fluorescence contribution and normalized by the intensity of the signal at 1000 cm^{-1} corresponding to aromatic ring stretching modes

(Sócrates, 2001), which do not vary in their Raman intensity from one sample to another. The average ($n \geq 5$) of the normalized intensity of the peak at 1450 cm^{-1} , due to $\delta(\text{CH}_2/\text{CH}_3)$ of fatty acids, was plotted over time (Figure 4).

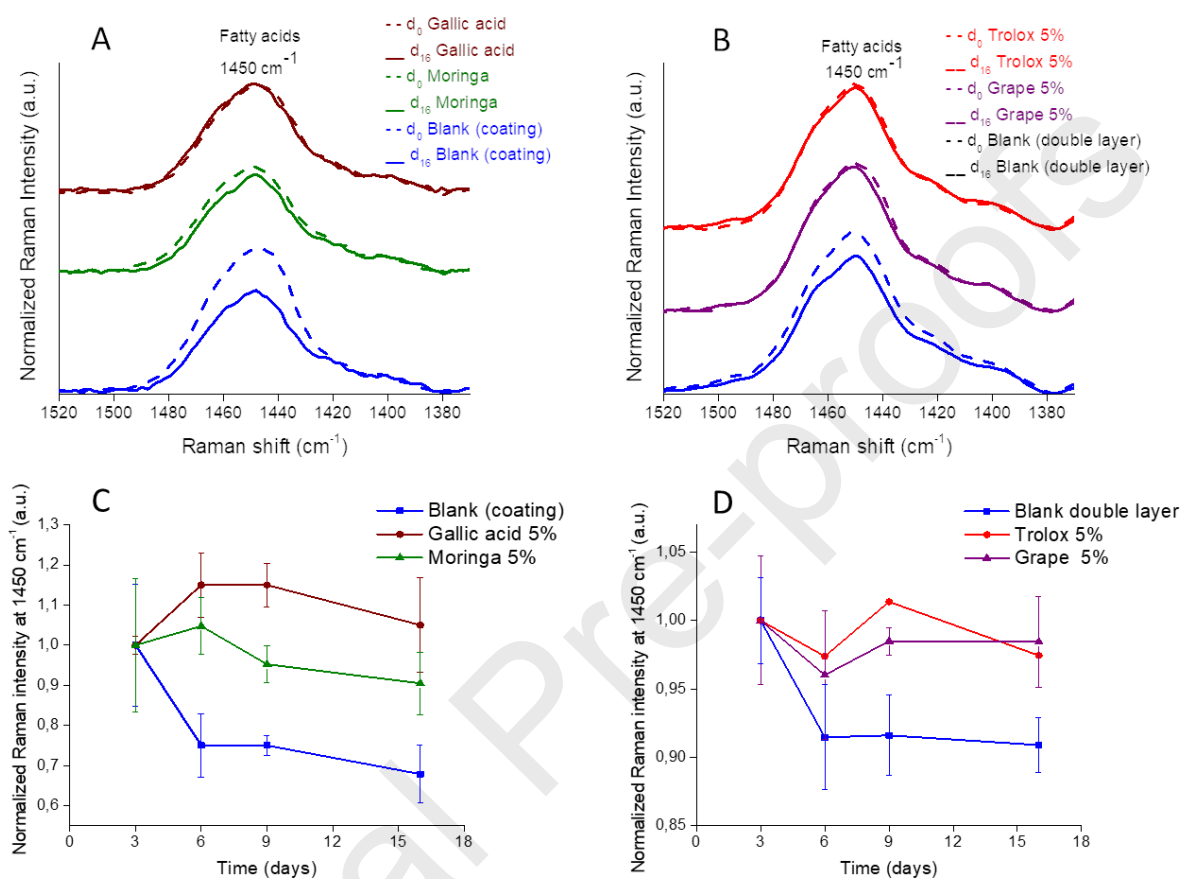


Figure 4: Raman *in situ* analysis of ground meat oxidation over time.

A-B) Reductions of the intensity of the typical Raman peak assigned to lipids (δCH_2) at 1450 cm^{-1} during meat oxidation for 0 (dashed lines) and 16 (solid lines) days are shown. C-D) Measured Raman intensities of the peak at 1450 cm^{-1} of meat over time. The samples in triplicate correspond to meat packed in cellulose biopolymer with 5% moringa MAC Met for 30 d (green line), grape MAC Ac for 10 d (purple line), GA (carmine line), Trolox (red line) or varnish or adhesive without any active agent as blank controls (blue lines).

The Raman analysis of meat revealed that the intensity of the band at 1450 cm^{-1} significantly decreased over time in the blank samples, indicating the progressive desaturation of the fatty acids (Moudache et al., 2017). This decrease was significantly lower in the samples packed in cellulose with 5% of both the moringa and grape extracts and was even lower in the samples with GA or Trolox. These results agree with those obtained by the TBARS assay and confirm the protective action of the active films tested against meat oxidation.

Furthermore, these results demonstrated, for the first time to the authors' knowledge, that nonenhanced Raman microspectroscopy, if applied in these conditions of acquisition, is sensitive enough to follow the peroxidation of meat lipids directly *in situ* without the need for any sample preparation, specific support or particular training for users. Thus, with respect to TBARS or other analytical assays, the time of analysis can be reduced to a few minutes, and the samples remain intact. This could be very interesting in terms of translating this type of food oxidation analysis to an industrial scale, greatly reducing the costs of analysis.

4. Conclusions

Many extracts obtained from *Moringa oleifera* Lam. desiccated leaves, olive oil and red wine industrial waste using different procedures were characterized to determine their composition and antioxidant efficacy. The samples obtained by SAE and MAC were determined to have the highest antioxidant efficacy by both DPPH and ORAC assays, as they had the highest contents of polyphenolic compounds and other active molecules, especially GA. Among all macerates, methanol provided the highest extraction yield of active compounds, followed by ethanol and acetone due to their higher polarity and affinity for phenolic molecules. For grape marc, water also resulted in extract fractions with high antioxidant efficacy.

The importance of comparing the results obtained by different assays was emphasized in this study, as the different properties of the natural extracts were considered, resulting in a more global evaluation of their antioxidant activity.

Two types of cellulose-based biopolymer packaging were developed containing 5% concentrations of the highest antioxidant macerates of moringa and grape extracts, one obtained by coating and one by mixing the agent into adhesive layered between two films. Both demonstrated high radical scavenging ability in different *in vitro* assays and when applied to real food matrices. Specifically, both packaging systems protected ground beef meat from lipid peroxidation by at least 50% over 16 days. This protective effect was confirmed by direct *in situ* Raman spectroscopy, avoiding any extractive steps and strengthening the reliability of the results.

Hence, although further analyses should be performed on these materials before exploring their possible commercialization, such as permeability and migration tests, this work is valuable in the field of food packaging, as it proposes the use of sustainable and degradable materials together with an innovative method to recover food industry waste to prolong the shelf life of food.

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Highlights

- Olive pomace, grape marc, moringa leaves were used as natural active agents;
- Solvent, anti-solvent and maceration extractive capacity was compared;
- The extracts' antioxidant properties were analyzed by DPPH, ORAC, UPLC-TQ-MS;
- The best grape and moringa macerates were included in a cellulose biopolymer;
- The packages' ability to preserve lipid peroxidation of meat was demonstrated.

Giulia Barzan: Conceptualization, Metodology, Investigation, Writing original draft, Writing review & editing, Data curation; **Alessio Sacco:** Formal analysis, Validation, Investigation; **Andrea Mario Giovannozzi:** Conceptualization, Data curation, Writing review & editing, Supervision, **Chiara Portesi:** Conceptualization, Resources; **Consolato Schiavone:** Investigation, Data curation; **Jesús Salafranca:** Conceptualization, Metodology, Investigation, Resources, Supervision, Writing review & editing; **Magdalena Wrona:** Data Curation, Investigation, Metodology, Writing review & editing; **Cristina Nerín:** Metodology, Resources; **Andrea Mario**

Rossi: Conceptualization, Resources, Metodology, Writing review & editing, Supervision.

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