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# Pomegranate peel as a source of antioxidants for the control of lipid and protein oxidation during the ripening of Iberian dry uncured sausages

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ABSTRACT

This study aimed to evaluate a pomegranate peel extract (PPE), selected for its level of phytochemical compounds and antioxidant activities, as a nitrite substitute in dry sausages, as well as its effect on lipid and protein oxidative changes and instrumental colour during the drying period (28 days). Of the extraction solvents screened, water: acetone 3:7 (*v*/v) was the most effective extraction solvent, yielding extracts with the highest content of phenolic compounds, flavonoids and condensed tannins and antioxidant activities (ABTS, DPPH and FRAP). Four batches of dry sausages were produced with different incoming amounts of sodium nitrite ($NaNO_2$) and PPE: 1) 150 ppm $NaNO_2$; 2) 0 ppm $NaNO_2$; 3) 0 ppm $NaNO_2$ + 1% PPE (*v/w*) and 4) 0 ppm $NaNO_2$ + 2% PPE (v/w). Nitrite removal increased lipid oxidation in uncured dry sausages, while nitrite and PPE caused lower TBA-RS values in cured and PPE treated sausages. During drying, both nitrite and PPE addition significantly decreased carbonyl and thiol contents compared to the uncured dry sausages. A dose-response was found for PPE, with lower carbonyl and thiol concentrations, the higher the level of PPE added. PPE significantly modified instrumental colour coordinates *L*a*b** producing significant total colour changes compared to cured dry sausages.

## 1. Introduction

The addition of sodium nitrite ($NaNO_2$) to muscle foods prevents microbial spoilage, enhances food safety, produces an attractive colour and unique flavours, and retards lipid oxidation processes. Nitrite acts against lipid oxidation through several mechanisms related to its ability to prevent the release of catalytic iron from myoglobin by forming nitrosylmyoglobin, binds heme and non-heme iron inhibiting catalysis, chelates free radicals by nitric oxide, and stabilises lipids against oxidation (Skibsted, 2011). The impact of nitrite on protein oxidation in meat products is not yet fully understood due to the limited research available and conflicting results. Different authors have reported both antioxidant and pro-oxidant properties of nitrite in these products. As evidenced by the decreased generation of carbonyl compounds, sodium nitrite exhibits an antioxidant property towards protein oxidation. However, nitrite was also discovered to possess a pro-oxidant effect on protein oxidation by lowering the total sulfhydryl concentration and increasing disulphide bond formation (Berardo, Claeys, Vossen, Leroy, & De Smet, 2015; Bonifacie et al., 2021; Feng et al., 2016; Vossen & De Smet, 2015; Wang, Yu, He, Zhang, & Ma, 2022).

Lipid oxidation of meat products greatly influences their sensory properties, such as taste, odour, texture and colour, and their stability during processing and storage, and consequently shortens their shelf life (Shahamirian et al., 2019). The effect of oxidative processes on proteins is no less significant, causing a series of physicochemical and nutritional changes in meat proteins, changes in amino acid composition, a decrease in their bioavailability, a decrease in protein solubility, and a lack of proteolytic activity (Zhang, Xiao, & Ahn, 2013).

Although nitrites have been frequently used in meat products, today, consumers are demanding nitrite-free products due to health concerns associated with those related to the role of nitrite in the generation of carcinogenic nitrosamines during processing or digestion. The implications associated with limiting the use of nitrites in meat processing are not minor and related, among other things, to the lack of control over oxidation processes in the food. The adverse effects of lipid and protein oxidation on food quality have led food scientists to find effective antioxidant strategies to substitute synthetic preservatives with new natural additives, to extend the self-life and keep the quality attributes of their processed products (Gullón, Astray, Gullón, Tomasevic, & Lorenzo, 2020). Following this trend and the demand of the food industry for

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antioxidants from natural sources, plant powders, essential oils, fruits and extracts of their by-products have been extensively studied as substitutes for synthetic antioxidants to preserve and prolong the shelf life of meat and meat products (Calderón-Oliver & López-Hernández, 2022; Kaderides, Kyriakoudi, Mourtzinos, & Goula, 2021; Pateiro, Gómez-Salazar, Jaime-Patlán, Sosa-Morales, & Lorenzo, 2021).

When processing vegetable materials to produce extracts for use in foods, the characteristics of the extract obtained, such as yield, antioxidant compound content and antioxidant activity, depend mainly on the selection of a solvent system suitable for the solubility of the phytochemical compounds of interest. Other operating conditions and parameters, such as processing time, number of extractions, temperature, and solvent/sample ratio, play a determinant role in the efficiency of the process (Selvamuthukumaran & Shi, 2017).

Pomegranate (*Punica granatum* L.) fruit and its by-product contain considerable phytochemical compounds amounts, including phenolic and polyphenolic compounds, that function as antioxidants in meat and meat products. The use of its extracts has been shown to be an excellent alternative to conventional antioxidants that help slow down the lipid and protein oxidation phenomena, leading to healthier and more stable meat and meat products. Several studies have examined the impact of pomegranate, including its extracts, powders, and juices, on different meat types (pork, beef, chicken, lamb) and meat products (meatballs, marinated meat, steak, nuggets, hamburgers, cooked sausages, frankfurters) (Gullón et al., 2020; Kaderides et al., 2021; Kandylis & Kokkinomagoulos, 2020; Munekata et al., 2020). The inclusion of pomegranate extracts has been reported mainly in the inhibition of thiobarbituric acid reactive substances in fresh meat or minimally processed meat products leading to an improvement in oxidative stability (Das et al., 2021). In Tuscan sausages, Zago et al. (2020) reported that the incorporation of pomegranate peel lyophilized extract powder delayed lipid oxidation. Gutiérrez-Pacheco et al. (2021) incorporated pomegranate juice and peel powders into pork sausages and found a rise in the antioxidant capacity and phenolic content. Aliyari, Kazaj, Barzegar, and Gavlighi (2019) investigated a partial replacement of nitrite by pomegranate peel in cooked sausages and found a decrease in peroxide and thiobarbituric acid values after four weeks of refrigerated storage. Firuzi et al. (2019) evaluated the effect of pomegranate juice concentrate and rind powder extract on the oxidative stability of frankfurter during cold storage and reported an extension of the product shelf life.

Iberian dry-cured meat products elaboration comprises prolonged drying that favours the development of lipid peroxidation and protein carbonylation processes that contribute positively to the generation of sapid and aromatic substances, whereas their negative side triggers oxidative rancidity, alteration of texture, and loss of nutritional value (Cava, Ruiz, Ventanas, & Antequera, 1999; Ramírez & Cava, 2007). In contrast to other meat products, the application of pomegranate extracts in dry sausages has not received much attention, and the potential strategy for controlling lipid peroxidation and protein carbonylation in this type of meat product represents a knowledge gap. To the best of our knowledge, this aspect has not been addressed before and is the first work of its kind.

This study aimed to screen different extraction solvents to obtain a pomegranate peel extract rich in phenolic compounds, condensed tannins and flavonoids and high antioxidant activity, and to evaluate the incorporation of a selected pomegranate peel extract at two levels of addition (1% and 2%, *v/w*) on lipid and protein oxidation and instrumental colour in uncured Iberian dry sausages during the drying process.

## 2. Materials and methods

### 2.1. Preparation of pomegranate peel extract

Pomegranate (*Punica granatum, L),* cultivar "Mollar", fruits ($n = 20$) were harvested directly from pomegranate trees during the autumn season (Cáceres, Spain). The pomegranates were washed, and the peel was removed. Peels were chopped and dried at 60 °C. Dried pomegranate peel was crushed to powder using a knifemill Grindomix GM 200 (Retsch GmbH, Haan, Germany).

Extracts were carried out from dried pomegranate peel in a proportion of 1:10 (*w/v*). The extraction solvents used were: a. water ($H_2O$), b. ethanol (EtOH), c. acetone (ACE), d. water: ethanol (1:1 *v*/v) ($H_2O$: EtOH 1:1), e. water: ethanol (3:7 v/v) ($H_2O$: EtOH 3:7), f. water: acetone (1:1 v/v) ($H_2O$: ACE 1:1) and g. water: acetone (3:7 v/v) ($H_2O$: ACE 3:7).

For the extraction process, a tube roller mixer (Cole-Parmer, Staffordshire, UK) was used, operating at 60 rpm, 21 °C for 120 min in the dark. After extraction, samples were centrifuged (4700 x*g* for 5 min), and the supernatant was filtered and collected. The extraction process was performed twice, and the supernatants were combined. Extracts were stored at −70 °C in amber bottles. Extractions for each solvent were performed in triplicate.

Based on antioxidant activities measured in the extracts (see sections 2.4. Analysis of antioxidant activities in sausages and pomegranate peel extract and 3.1 Characterization of the obtained extracts), the pomegranate peel extract (PPE) was produced using water: acetone (3:7, *v*/v) to manufacture the sausages in the experiment. Thus, one hundred grams of dried pomegranate peel was extracted using 900 mL of water: acetone (3:7) extraction solvent and were left to stir in the dark for 120 min at room temperature. The obtained extract was then centrifuged (4700 x*g* for 5 min), filtered through filter paper, and acetone evaporated with a rotary vacuum evaporator (Heidolph, Schwabach, Germany). Several extractions were carried out to obtain the volume of extract necessary to produce the experimental batches of dry sausages. The aqueous PPEs obtained (~250 mL/extraction) from the different extractions were combined and stored (−20 °C) in amber bottles until use.

### 2.2. Dry-sausage manufacture, drying process, and sampling

Minced shoulder collar (*m. Triceps brachii, m. Infraspinatus, m. Supraspinatus, and m. Trapezius*) from Iberian x Duroc pigs fed on concentrate were used for the manufacture of dry-cured sausages. Four experimental batches of Iberian dry-cured sausages were manufactured with different ingoing amounts of $NaNO_2$ and PPE (Table 1): 1) 150 mg/kg $NaNO_2$ + 0% PPE (150_Nitr); 2) 0 mg/kg $NaNO_2$ + 0% PPE (0_Nitr); 3) 0 mg/kg $NaNO_2$ + 1% (*v/w*) PPE (PPE_Low); and 4) 0 mg/kg $NaNO_2$ + 2% (v/w) PPE (PE_High). The formulation and mixing process for each formulation was replicated. For each replicate, 25 kg of sausage batter were prepared. In each of the replicates, three sausages per experimental batch, replica and day of processing were sampled ($n = 72$).

To counterbalance the volume of water added with the pomegranate peel extract, an equal volume of water was added to the remaining batches. Batters were stuffed into 34 mm diameter collagen casings and

**Table 1**
Formulation of Iberian dry cured sausages with different ingoing amounts of nitrite and PPE incorporated.

| | 150 mg/kg $NaNO_2$ (150_Nitr) | 0 mg/kg $NaNO_2$ | | |
|---|---|---|---|---|
| | | + 0% PPE (0_Nitr) | + 1% PPE PPE_Low | + 2% PPE PPE_High |
| $NaNO_2$[1] | 150 | – | – | – |
| PPE[2] | – | – | 1.0 | 2.0 |
| $H_2O$[2] | 0.5 | 0.5 | 0.25 | – |
| Dextrin[3] | 11.85 | 11.85 | 11.85 | 11.85 |
| Dextrose[3] | 3.4 | 3.4 | 3.4 | 3.4 |
| NaCl[3] | 21.5 | 21.5 | 21.5 | 21.5 |

PPE: Pomegranate peel extract.
[1] : mg/kg;
[2] : % v/w;
[3] : g/Kg.

dried for 28 days under the thermohygrometric conditions shown in Fig. 1. Samples (3 samples/batch/replica/day of sampling) were taken on day 0 (initial), 14 (half of the drying period) and 28 (end of the drying process) of the drying process. Samples were vacuum packed and stored at −80 °C until analysis. The samples were minced and homogenized before analysis, except for the instrumental colour measurements.

### 2.3. Analysis of phytochemical compounds in pomegranate peel extract

#### 2.3.1. Total phenolic content (TPC)

Total phenolic content was quantified according to the procedure described by Medina (2011) using the Folin-Ciocalteau reagent. In a test tube containing 50 μL of water and 20 μL of Folin-Ciocalteau reagent, 50 μL of the extract was added. Subsequently, 50 μL of $Na_2CO_3$ (20% *w/v*) and 450 μL of water were added, and the sample was incubated for 1 h at 25 °C. The absorbance was measured at 765 nm. Quantification was performed using a gallic acid (GA) calibration curve (500–10 μg/mL). Results were expressed as mg of GA equivalents (GAE)/mL extract. The analyses were carried out in triplicate.

#### 2.3.2. Condensed tannins content

Determination of condensed tannins was performed using the method described by Waterman and Mole (1994). Twenty-five microliters of extract were added to 250 μL vanillin reagent (1% vanillin in MeOH: 10% HCl in MeOH), incubated for 30 min, and the absorbance at 500 nm was recorded. For reporting the condensed tannin content, a calibration curve of (+) catechin (10–0.16 mg/mL) was built. Results were expressed as mg catechin equivalents/mL extract. Assays were done in triplicate.

#### 2.3.3. Flavonoid content

Total flavonoid content was assayed as described by Zhishen, Mengcheng, and Jianming (1999). Fifty microliters of sample, 400 μL $H_2O$ and 15 μL 10% sodium nitrite were added to a 2 mL test tube, mixed and incubated (5 min, 20 °C). After incubation, 15 μL 20% aluminium chloride, 200 μL 1 M sodium hydroxide, and 320 μL of deionized water were added. Absorbance was measured at 510 nm. The standard used was (+) catechin (1.0–0.1 mg/mL) and the results were expressed in mg catechin equivalents/mL extract. Assays were performed in triplicate.

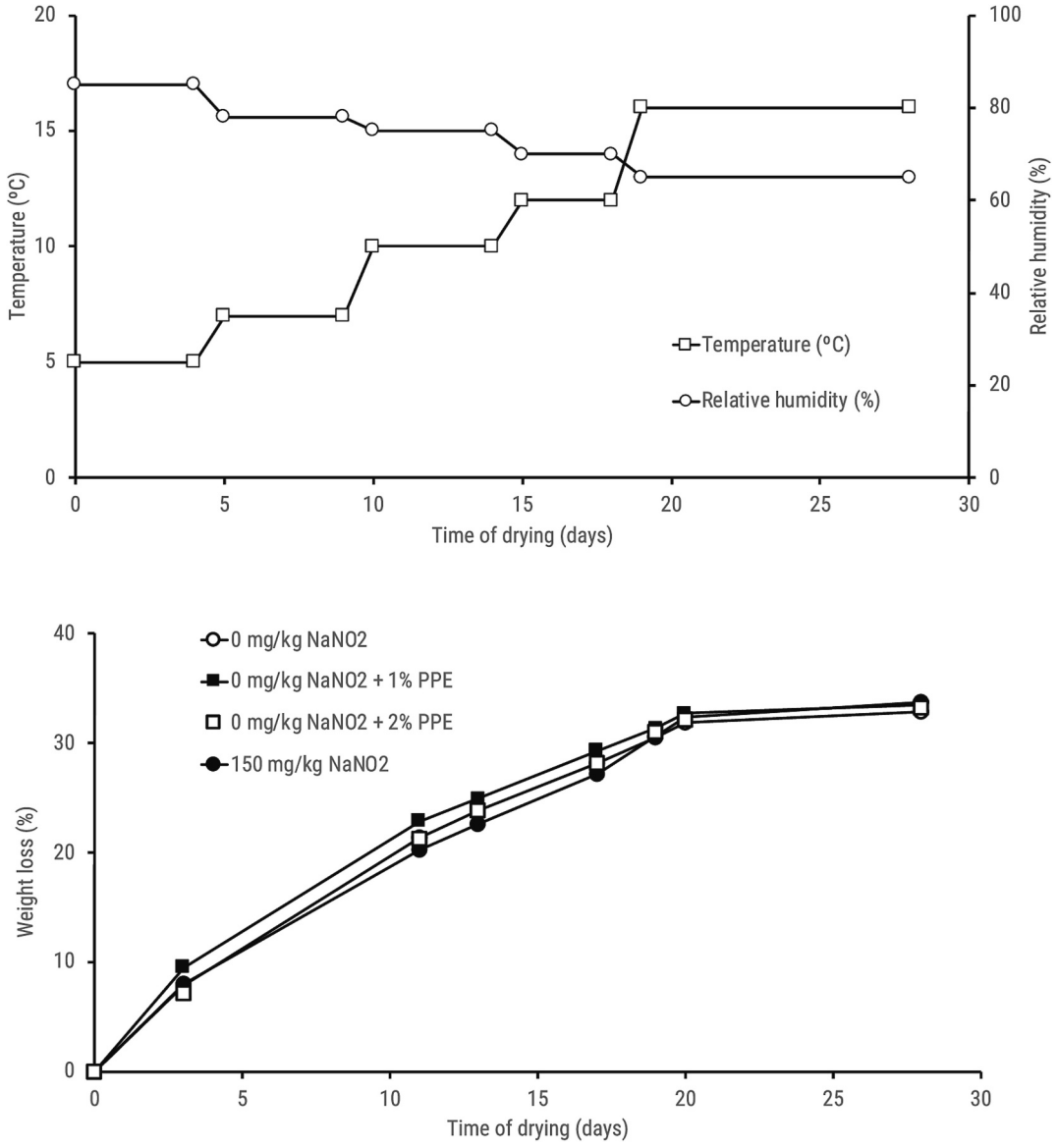


**Fig. 1.** Drying conditions (temperature and relative humidity) and weight loss (%) of experimental dry sausages.
For each experimental group, error bar represents the standard error.

### 2.4. Analysis of antioxidant activities in sausages and pomegranate peel extract

For determinations of the residual antioxidant activities in sausages, 1 g of sample and 5 mL of water were stirred 10 min at 4 °C. Samples were homogenized using a lysing matrix type A (MP Biomedicals Inc., Santa Ana, CA, USA) employing a bead-beating benchtop homogenizer Fast Prep-24TM 5G (MP Biomedicals Inc., Santa Ana, California, USA) for 30 s at 4 m/s. Homogenized samples were centrifuged (2000 rpm/2 min) and the resulting supernatant was filtered through glass wool. Sausage extracts and pomegranate peel extract (PPE) were used for the antioxidant activity assays. Assays were carried out in quadruplicate.

#### 2.4.1. 2,2-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

To evaluate the DPPH activity the method described by Brand-Williams, Cuvelier, and Berset (1995) was adopted. $DPPH^{\bullet}$ radical (100 μM) was diluted in 80% MeOH and the absorbance was measured at 515 nm. An aliquot (5 μL) of SE/PPE was mixed with 195 μL of 0.1 mM $DPPH^{\bullet}$ reagent, incubated (25 °C, 30 min), and the absorbance at 515 nm recorded. A calibration curve of Trolox ranged 1.28–0.08 mM was used for quantification. The DPPH antioxidant activity was expressed as mM Trolox eq/mL and mM Trolox eq/g.

#### 2.4.2. 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay

For the ABTS assay, the procedure followed the method of Re et al. (1999). The day before the assay the $ABTS^{\bullet+}$ radical (2.45 mM $K_2S_2O_8$: 7 mM ABTS; 1:1 *v*/v) was prepared and diluted with ethanol to reach an absorbance value of 0.7 ± 0.1 at 734 nm. Five microliters of SE/PPE were mixed with 245 μL $ABTS^{\bullet+}$ radical and the absorbance was measured for 7 min at 60 s intervals. To determine ABTS activity a standard curve of Trolox (4.0–0.25 mM) was used. Results were expressed as mg of Trolox equivalents/mL and mM Trolox eq/g.

#### 2.4.3. Ferric reducing activity power (FRAP) assay

The FRAP assay of SE/PPE was measured according to the procedure described by Pulido, Bravo, and Saura-Calixto (2000). A daily prepared FRAP reagent (2.5 mL of 10 mM tri-pyridyl triazine (TPTZ) in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$-$6H_2O$, and 25 mL of 0.3 mM acetate buffer, pH 3.0) was used. In a 96-well plate, 200 μL of FRAP reagent were dispensed and the absorbance at 593 nm was recorded. Sequentially, 7 μL of SE/PPE and 20 μL $H_2O$ were added. After incubation (25 °C, 30 min), the absorbance at 593 nm was recorded. A calibration curve of $FeSO_4 \bullet 7H_2O$ (1.5–0.0234 mM) was used for quantification. FRAP was expressed as mM $Fe^{2+}$ eq/mL and mM $Fe^{2+}$ eq/g

### 2.5. Proximate composition of the sausages

#### 2.5.1. Moisture, protein, and total fat content

A FoodScan TM optical NIR analyser (FOSS Iberia S.A., Barcelona, Spain) was used for the determination of moisture, protein, and total fat content of dry sausages using artificial neural network prediction models. Moisture content is expressed as g/100 g and protein and total fat contents are expressed in g/100 g of dry matter (d.m.). Analyses were carried out in triplicate.

#### 2.5.2. Water activity

The water activity (Aw) was measured using a LabMASTER-aw water activity measuring device (Novasina, Axair Ltd., Pfaeffikon, Switzerland).

#### 2.5.3. pH analysis

For pH determination, 1 g of the sample was homogenized in 10 mL of deionized water at 21 °C. After 10 min, the pH was determined using a pH-meter with automatic temperature compensation Accumet AB15 (Cole-Parmer, Illinois, USA). For calibration of the pH-meter buffers pH 4.01 and 7.00 at 21 °C were used.

#### 2.5.4. Sodium chloride content

Sodium chloride content was assayed according to the procedure described in ISO 1841-2: 1996 (AENOR, 2017), titrated with 0.1 N $AgNO_2$ solution using a T70 Titrator (Mettler-Toledo Inc., Greifensee, Switzerland). Titration was measured in duplicate. Results are expressed as g NaCl/100 g d.m.

#### 2.5.5. Nitrite content

The nitrite content was measured according to ISO 2918: 1975 (ISO, 2018). The colourimetric reagent was prepared by mixing equal volumes of 0.35 mM sulfamide solution and 2 mM N-(1-Naphthyl) ethylenediamine dihydrochloride (NED). An equal volume of sample and the colourimetric reagent were mixed and incubated (15 min, room temperature in the dark), after which the absorbance at 538 nm was recorded. For quantification, a $NaNO_2$ standard curve (0.16–0.001 mM) was used. The results are expressed as mg nitrite/100 g d.m.

### 2.6. Lipid and protein oxidation marker compounds

#### 2.6.1. Thiobarbituric acid reactive substances quantification (TBA-RS)

The method described by Salih, Smith, Price, and Dawson (1987) was used to assess the TBA-RS. The sample (2.5 g) was mixed with 7.5 mL 3.86% $HClO_4$ and 250 μL 4.2% BHT in EtOH, homogenized (12,000 rpm, 45 s), centrifuged (750 *x*g for 2 min) and the supernatant filtered through filter paper. In a polypropylene tube, equal amounts of homogenate and thiobarbituric acid (20 mM) were mixed and heated at 90 °C for 30 min. After cooling, tubes were centrifuged (5000 xg, 5 min) and the absorbance of the supernatants was measured at 532 nm. A standard curve of malondialdehyde (MDA) with 1,1,3,3 tetraethoxypropane (0.16–0.001 mM) was prepared for TBA-RS estimation. All the results are expressed in mg MDA/kg.

#### 2.6.2. Carbonyls quantification

Protein carbonyl content was assessed using the methodology described by Soglia, Petracci, and Ertbjerg (2016). The sample was homogenized (15,000 rpm for 30 s) in 0.15 M KCl (1:10 *w/v*). The homogenate (100 μL) was dispensed into test tubes (2 per sample) containing 1 mL 10% trichloroacetic acid (TCA) and vortexed. After centrifuging (5000 *x*g for 5 min., 4 °C) and removing the supernatant, 400 μL 5% sodium dodecyl sulphate were added and incubated at 100 °C for 10 min and then sonicated in an ultrasonic bath for 30 min. Then, 0.8 mL 3 M HCl + 0.3% 2,4-dinitrophenylhydrazine (DNPH) was added to one tube (carbonyl determination), while 0.8 mL of 3 M HCl (blank) was added to the other replicate. Tubes were incubated at room temperature for 30 min. After incubation, 400 μL of 40% TCA were added and centrifuged (2000 *x*g for 20 min) and the supernatant was discarded. The pellet was washed (x3) with EtOH: ethyl acetate (1:1) and evaporated to dryness. Finally, the pellet was resuspended in 1.5 mL of 6 M guanidine, and it was incubated overnight in refrigeration. Both carbonyls and blanks were done in duplicate for each sample and read at 280 and 370 nm. Carbonyl concentration was calculated using the formula (1):

$$Carbonyls = \frac{(DNPH_{370nm} - Blank_{370nm})}{(2200 \times (Blank_{280nm} - (DNPH_{370nm} - Blank_{370nm})) \times 0.43)} \times 10^6 \quad (1)$$

where $DNPH_{370}$ refers to carbonyl reads at 370 nm; $Blank_{370nm}$ refers to blank reads at 370 nm and $Blank_{280nm}$ refers to blank reads at 280 nm. Results are expressed as nmol carbonyls/mg protein.

#### 2.6.3. Thiols quantification

The thiol content in the sausages was assessed using the procedure described by Martínez, Jongberg, Ros, Skibsted and Nieto, (2020). Samples (1.5 g) were homogenized in 12.5 mL of 0.05 M 2-(N-

morpholino) ethanesulfonic acid (MES) buffer (pH = 5.8) at 11600 rpm for 30 s on ice. Five hundred microliter of the homogenate was dispensed (in duplicate) in a 2 mL tube containing 1.5 mL 5% SDS in 0.10 M TRIS buffer (pH = 8.0), mixed and heated at 80 °C for 30 min. Subsequently, tubes were centrifuged at 3000 xg for 20 min at 21 °C. The supernatant was collected and filtered through a 0.45 μm nylon syringe filter. To 50 μL of the supernatant 200 μL 5% SDS in 0.10 M TRIS buffer (pH = 8.0) was added and the absorbance read at 412 nm. After reading, 50 μL of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) 0.01 M in 0.10 M TRIS buffer (pH = 8.0) were added and incubated in the dark for 30 min, after which the absorbance at 412 nm was recorded. Thiol content was obtained using a standard curve of cysteine (Cys) (1000–3.25 μM). The absorbance corresponding to thiol concentration was calculated as follows (2):

$$Abs_{corr\ 412nm} = Abs_{412nm\ after} - Abs_{412nm\ before} - Abs_{412nm\ Blank} \quad (2)$$

Pierce Rapid Gold BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for the quantification of protein. Absorbance was measured at 480 nm. Bovine serum albumin (2.5–0.3125 mg/mL) standard curve was used for determining protein concentration. The results are expressed as nM Cys eq/mg protein.

### 2.7. Instrumental colour

Instrumental colour coordinates (CIE $L^*a^*b^*$) measurements were performed following the AMSA meat colour measurement guidelines (AMSA, 2012). A Minolta CM-600d spectrophotometer (Minolta Camera Co., Osaka, Japan) was used for colour determination under the following conditions: a 0.8 cm port/viewing area, a $10^o$ viewing angle, and a D65 illuminant. Nine measurements were taken in randomly selected areas on the surface of the sausages and averaged to obtain the CIE $L^*$, $a^*$, and $b^*$ values. The hue (3) and chroma (4) values were calculated as follows:

$$H^o = arc\tan\left(\frac{b^*}{a^*}\right) \times \left(\frac{360}{2\pi}\right) \quad (3)$$

$$C* = \sqrt{a^{*2} + b^{*2}} \quad (4)$$

The total colour difference (ΔE) was calculated using the formula (5):

$$\Delta E = \sqrt{\left(L^{*2} - L^*{}_{ref}{}^2\right) + \left(a^{*2} - a^*{}_{ref}{}^2\right) + \left(b^{*2} - b^*{}_{ref}{}^2\right)} \quad (5)$$

Where $L^*_{ref}$, $a^*_{ref}$ and $b^*_{ref}$ correspond to the values of batch 150_Nitr.

Reflectance spectra were measured using a Minolta CM-600d spectrophotometer operating in the 360 to 740 nm spectral range following AMSA recommendations with previously described conditions. The cured index (CI) was calculated using the formula (6)

$$CI = \frac{R_{650nm}}{R_{570nm}} \quad (6)$$

### 2.8. Statistical analysis

Extracts of each solvent were obtained in triplicate. Characterization of each replicate extract, in terms of phytochemical compounds and antioxidant activities, was performed in triplicate to determine reproducibility. Means were statistically compared by one-way analysis of variance (ANOVA) and when ANOVA determined significant differences ($P < .05$) between mean values, a Tukey's honest significant difference (HSD) test was used to assess the significance of differences between pairs of group means.

The formulation and processing of dry sausages were performed in duplicate. For proximate composition, TBA-RS, carbonyl, and thiol contents, data were analyzed using the Mixed Linear Model procedure (GAMLj module. https://gamlj.github.io/) of the statistical software Jamovi (https://www.jamovi.org) (Version 2.0.0.0). Data was evaluated using a mixed model analysis that included sausage formulation and time of drying as fixed effects and the sausage sample as the random effect. When significant effect was detected ($P < .05$), means were compared using Tukey's test. When significant interaction was present, data were analyzed using a general linear model, considering the sausage formulation, and drying time as fixed effects and replicates were considered as a random effect. Means were compared using Tukey's HSD test. Relationships among lipid and protein oxidation markers and residual antioxidant activities were calculated using Pearson's correlation coefficients.

In the case of instrumental colour parameters and antioxidant residual activities in fully dried sausages, the analysis only included one factor, sausage formulation, as a fixed effect, and the replicates as a random effect. In all cases, the significant differences were analyzed by Tukey's test at the 5% level of significance.

All physicochemical parameters were carried out in duplicate, except for instrumental colour, that was determined by nine repetitions, using 3 sausages of each treatment, replica, and sampling time. Results are reported as mean and standard error (SE).

## 3. Results and discussion

### 3.1. Characterization of the obtained extracts

Results revealed a statistically significant effect of the extraction solvent on the content of total phenolic compounds, condensed tannins, and flavonoids (Fig. 2).

Overall, 30% water binary mixtures ($H_2O$: ACE 3:7 and $H_2O$: EtOH 3:7) were shown to be the most effective extraction solvents for any of the three phytochemicals families. In contrast, acetone produced extracts with the lowest concentrations of TPC, TAN and FLAV. The concentration of phenolic compounds increased significantly in the order ACE < EtOH = $H_2O$: EtOH 1:1 < $H_2O$ = $H_2O$: ACE 1:1 < $H_2O$: EtOH 3:7 < $H_2O$: ACE 3:7. The concentration of phenolic compounds was significantly higher in the $H_2O$: ACE 3:7 extract, followed by the $H_2O$: EtOH 3:7 extract than in the counterparts. The contents of total phenolic compounds were ~1.4, ~5, ~8, and ~ 24-fold higher than those obtained with $H_2O$: EtOH 3:7, $H_2O$ and $H_2O$: ACE 1:1, $H_2O$: EtOH 1:1 and ACE, respectively. For flavonoids, solvents with higher yields increased in the order ACE < EtOH = $H_2O$ = $H_2O$: EtOH 1:1 = $H_2O$: ACE 1:1 < $H_2O$: ACE 3:7 < $H_2O$: EtOH 3:7. In this case, the concentration of phenolic compounds was significantly higher in the $H_2O$: EtOH 3:7 extract followed by the $H_2O$: ACE 3:7 extract than in the other extracts. Finally, the concentrations of condensed tannins increased in the order ACE > $H_2O$ = EtOH = $H_2O$: EtOH 1:1 = $H_2O$: ACE 1:1 < $H_2O$: EtOH 3:7 = $H_2O$: ACE 3:7. The extracts with the highest condensed tannin content were found in 3:7 water binary mixtures ($H_2O$: ACE 3:7 and $H_2O$: EtOH 3:7).

As described above, results revealed a statistically significant effect of the extraction solvent on the *in vitro* antioxidant activities of the extracts (Fig. 2). The *in vitro* antioxidant activities reflected the reported differences in the composition of phytochemical compounds. The extracts obtained with binary mixtures $H_2O$: ACE 3:7 and $H_2O$: EtOH 3:7 showed a higher antioxidant activity than the other experimental extracts. In this sense, the extract obtained with $H_2O$: ACE 3:7 showed significantly and consistently higher ABTS, DPPH and FRAP activities than the counterpart $H_2O$: EtOH 3:7 extract.

The results demonstrated that the solvent used for the extraction was decisive for the phytochemical composition of the extract, in accordance with previous reports by several authors (Elfalleh, 2012; Feng et al., 2022; Singh et al., 2014). The lower phenolic content observed in acetone might be due to its polarity, which led to a lower extraction of polar phenolic compounds because of the lower ability to break covalent molecules into ions, in contrast to more polar solvents (water and

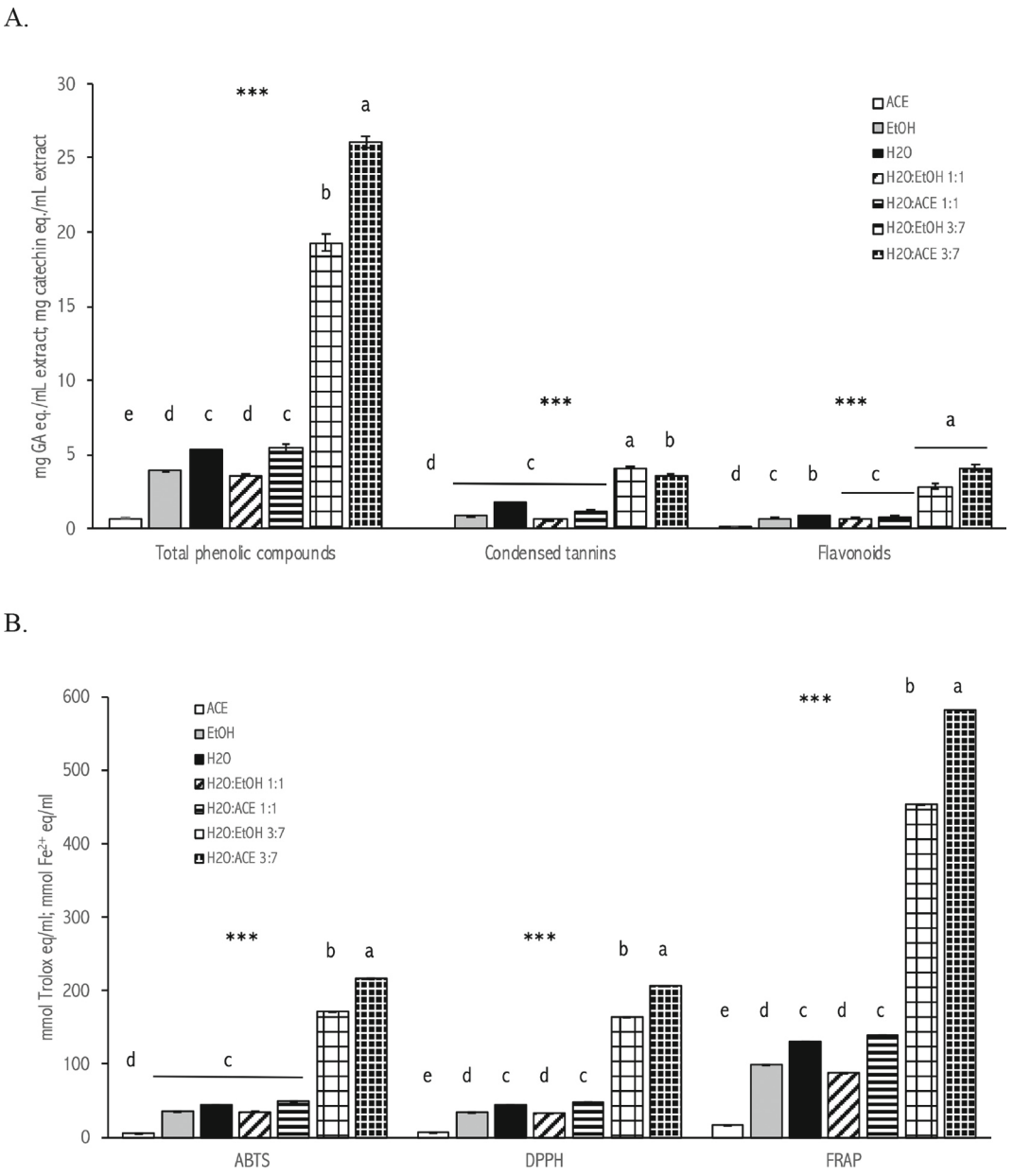


**Fig. 2.** Effect of the extraction solvent on the (A) phytochemicals contents and (B) antioxidant activities of pomegranate peel extracts.
Total phenolic compounds: mg GA eq/mL extract; condensed tannins: mg catechin eq/mL extract; flavonoids: mg catechin eq/mL extract. ABTS: mmol Trolox eq/mL extract; DPPH: mmol Trolox eq/mL extract; FRAP: mmol $Fe^{2+}$ eq/mL extract. For each solvent of extraction bar, error bar represents the standard error. ***: $P < .001$. a,b,c,d,e: Bars within the same parameter with different letters differ significantly (Tukey's test, $P < .05$).

ethanol) because of the better solvation of phenolic compounds due to interactions (hydrogen bonds) between the polar sites of the polyphenol molecules and the solvent (Boeing, Barizão, & Visentainer, 2014). In this manner, our findings are consistent with those of Calderón-Oliver and López-Hernández (2022), who reported that pomegranate peel extracts rich in total phenolic compounds and flavonoids had higher *in vitro* antioxidant activities when solvents richer in ethanol (50–75%) were used, in comparison with water or ethanol as extraction solvents. These authors attributed the increase in the concentration of phenolic compounds in binary ethanol-water extracts to the increase in the polarity of the medium and the increase in the extraction of less polar compounds. This behavior might be similar to that found in the present study in the extractions carried out with water: acetone (3:7), in accordance with Boeing et al. (2014), who reported that the water: acetone solvent mixture was a highly efficient mixture for the extraction of phenolic compounds. Owing to the polarity of the phenolic compounds in pomegranate peels, water or its mixtures with organic solvents (ethanol or acetone) are the most efficient solvents for extracting phytochemicals from pomegranate peels. It could be the reason for the higher yield of phenolic compounds in combinations of water with organic solvents (ethanol or acetone) as extraction solvent systems.

For the manufacture of the sausages, the extract $H_2O$: ACE (3:7) was produced. After acetone evaporation, the resulting aqueous extract was characterized for phytochemical compound content and antioxidant activity. The obtained PPE extract was rich in these compounds and had a high antioxidant capacity. The extract contained 102, 18, and 43 mg/mL of total phenolic compound, flavonoids, and condensed tannins, respectively. The radical scavenging activities, expressed as mmol Trolox eq/mL, were 794 and 190 for ABTS and DPPH, respectively. The FRAP activity was 2483 mmol $Fe^{2+}$ eq/mL. The content of phytochemicals and the antioxidant activity of the extract obtained are in agreement with the available literature dealing with the effect of the

extraction solvent and the raw material (pomegranate peel) on the characteristics of the extracts (Elfalleh, 2012; Li et al., 2006; Marchi et al., 2015; Russo et al., 2018).

### 3.2. Physicochemical composition of dry sausages

Tables 2 and 3 summarize the physicochemical composition of dry sausages throughout the drying.

A formulation x time of drying interaction was identified for pH, moisture, protein, and residual nitrite contents (Table 2). No other interactions were significant ($P > .05$); therefore, main effects were considered for Aw, fat and NaCl contents. Drying caused a sustained loss of water leading to a decrease in moisture content and a concomitant reduction in Aw, without these parameters being affected by the nitrite content and added PPE. At the end of the drying process, moisture content and Aw were not significantly different among experimental batches (Table 3). Results agree with those reported in the literature for dry sausages with similar drying processes and processing characteristics (Pateiro, Bermúdez, Lorenzo, & Franco, 2015; Perea-Sanz, Montero, Belloch, & Flores, 2018; Prado, Sampayo, González, Lombó, & Díaz, 2019).

In all groups, the pH decreased from the beginning to the end of the drying process. On day 28, the uncured sausages (0_Nitr) had significantly lower pH values than their equivalents with nitrite or PPE (Table 3). These results could be due to an increase in the population of acidifying bacteria, which would be favoured in the absence of nitrite. These differences do not seem to be technologically significant for these types of products. The residual nitrite content decreased throughout the drying process, with significantly higher residual amounts of nitrite in the 150_Nitr group than in the other experimental groups.

### 3.3. Lipid and protein oxidation

Statistical analysis revealed a significant effect of sausage formulation, drying time and the interaction of both factors on TBA-RS, carbonyl and thiol contents (Table 4). Lipid peroxidation and protein carbonylation tended to increase during drying in the uncured dry sausages, while no significant changes occurred in the batches with added nitrite or PPE (Table 5). The significant interaction of the formulation (nitrite and PPE) and the drying time suggests a different rate and intensity on the formation of MDA and carbonyls throughout the drying time of the dried sausages.

Initial lipid oxidation rates (day 0), measured as TBA-RS, were equivalent for all experimental dry sausages, with no changes induced by the dose of nitrite or PPE incorporated in the sausages. During the drying process, TBA-RS levels remained unchanged in cured dry sausages (150_Nitr) and in dry sausages with added PPE (PPE_Low and PPE_High). However, in uncured dry sausages (0_Nitr), removal of nitrite led to an increase in lipid oxidation, resulting in higher TBA-RS values. The PPE addition counteracted nitrite removal, and it was an effective way to control lipid oxidation during drying. On days 14 and 28 of drying, uncured samples had the highest TBA-RS values while no differences were found among nitrite-cured (150_Nitr) and PPE-added (PPE_Low and PPE_High) sausages.

The higher oxidation in the uncured sausages could be associated with the lack of antioxidant mechanisms capable of inhibiting lipid peroxidation reactions, as nitrite did. The mechanisms by which nitrite inhibits lipid oxidation are clearly defined, including the formation of nitrosylmyoglobin and nitric oxide ferrous complexes, the synthesis of S-nitrosocysteine, and the inhibition of the Fenton reaction due to the neutralization of the release of $Fe^{2+}$ from myoglobin (Kanner, 1994). Therefore, in dry sausages without sodium nitrite, there is no system capable of controlling the large amounts of endogenous/exogenous catalysts, such as free iron ions and sodium chloride, which enhanced lipid oxidation. The results of this study are in line with the findings described in previous works, which demonstrated the negative effect of

**Table 3**
Interaction of nitrite/PPE addition and time of drying on pH, moisture, protein, and residual nitrite content.

| Drying time (days) | Batch | pH | Moisture[1] | Protein[2] | Nitrite[2] |
|---|---|---|---|---|---|
| 0 | 0 mg/kg $NaNO_2$ | 6.4 a | 53.3 a | 31.3 bcd | 5.7 d |
| | 150 mg/kg $NaNO_2$ | 6.4 a | 53.9 a | 29.9 d | 17.6 b |
| | + 1% PPE (PPE_Low) | 6.4 a | 54.2 a | 32.6 ab | 1.4 e |
| | + 2% PPE (PPE_High) | 6.3 a | 53.8 a | 32.4 ab | 1.7 e |
| 14 | 0 mg/kg $NaNO_2$ | 6.1 b | 40.8 b | 30.2 cd | 0.3 e |
| | 150 mg/kg $NaNO_2$ | 6.2 b | 41.1 b | 31.6 bc | 24.9 a |
| | + 1% PPE (PPE_Low) | 6.1 b | 40.6 b | 31.9 b | 0.1 e |
| | + 2% PPE (PPE_High) | 6.1 b | 38.9 b | 32.3 ab | 0.7 e |
| 28 | 0 mg/kg $NaNO_2$ | 5.9 c | 30.2 c | 32.4 ab | 0.4 e |
| | 150 mg/kg $NaNO_2$ | 6.2b | 28.8 c | 32.8 ab | 10.8 c |
| | + 1% PPE (PPE_Low) | 6.1 b | 29.7 c | 33.8 a | 0.2 e |
| | + 2% PPE (PPE_High) | 6.2 b | 29.4 c | 33.8 a | 1.1 e |
| | Standard error | 0.02 | 1.19 | 0.16 | 0.95 |
| | Sig. | *** | *** | *** | *** |

Sig: Statistical significance. ***: $P < .001$.
a,b,c,d,e: Means within the same parameter with different letters differ significantly (Tukey's test, $P < .05$).
[1] : g/100 g;
[2] : mg/kg of dry matter.

**Table 2**
Estimated marginal means for moisture, protein, fat, Aw, pH, NaCl and NO2 for nitrite/PPE addition and drying time.

| Main factor | | Aw | pH | Moisture[1] | Protein[2] | Fat[2] | NaCl[2] | $NO_2^3$ |
|---|---|---|---|---|---|---|---|---|
| $NaNO_2$/PPE | 0 mg/kg $NO_2$ | 0.92 | 6.11 b | 41.42 | 31.31 b | 60.79 a | 4.30 | 2.12 b |
| | 150 mg/kg $NO_2$ | 0.92 | 6.26 a | 41.23 | 31.42 b | 59.55 b | 4.39 | 17.77 a |
| | + 1% PPE (PPE_Low) | 0.92 | 6.22 a | 41.48 | 32.75 a | 58.53 b | 4.43 | 0.54 b |
| | + 2% PPE (PPE_High) | 0.92 | 6.20 a | 40.74 | 32.85 a | 57.76 bc | 4.47 | 1.15 b |
| Drying time | Day 0 | 0.96 x | 6.39 x | 53.80 x | 31.54 y | 59.51 | 4.40 x | 6.59 x |
| | Day 14 | 0.93 y | 6.13 y | 40.34 y | 31.50 y | 59.29 | 4.33 y | 6.49 x |
| | Day 28 | 0.88 z | 6.08 z | 29.51 z | 33.22 x | 58.68 | 4.47 x | 3.11 y |
| | Standard error | 0.00 | 0.02 | 1.19 | 0.16 | 0.20 | 0.02 | 0.95 |
| Sig. | $NaNO_2$/PPE | n.s. | *** | n.s. | *** | *** | *** | *** |
| | Drying | *** | *** | *** | *** | n.s. | *** | *** |
| | Interaction | n.s. | *** | * | ** | n.s. | n.s. | *** |

Sig: Statistical significance. *: $P < .05$; **: $P < .01$; ***: $P < .001$; n.s.: not significant.
a,b,c: for each parameter, means with different letters differ significantly (Tukey's test, $P < .05$).
x, y, z: for each parameter, means with different letters differ significantly (Tukey's test, $P < .05$).
[1] : g/100 g;
[2] : mg/kg of dry matter.

**Table 4**
Estimated marginal means for TBA-RS, carbonyls and thiols for each ingoing amounts of nitrite and levels of PPE and drying time.

| Main effect | | TBA-RS[1] | Carbonyls[2] | Thiols[3] |
|---|---|---|---|---|
| $NaNO_2$/PPE | 0 mg/kg $NO_2$ | 8.63 a | 14.96 a | 39.69 a |
| | 150 mg/kg $NO_2$ | 0.89 b | 11.84 b | 34.71 b |
| | + 1% PPE (PPE_Low) | 1.28 b | 12.16 b | 25.82 c |
| | + 2% PPE (PPE_High) | 1.23 b | 9.86 c | 15.44 d |
| | Day 0 | 2.09 z | 11.42 x | 32.25 x |
| | Day 14 | 4.04 x | 12.37 x | 27.99 y |
| Drying time | Day 28 | 2.89 y | 12.83 y | 26.50 z |
| | Standard error | 0.51 | 0.32 | 1.17 |
| | $NaNO_2$/PPE | *** | *** | *** |
| | Drying | *** | * | *** |
| *Sig.* | Interaction | *** | *** | *** |

Sig: Statistical significance. *: $P < .05$; ***: $P < .001$.
a,b,c: for each parameter, means with different letters differ significantly (Tukey's test, $P < .05$).
x, y, z: for each parameter, means with different letters differ significantly (Tukey's test, $P < .05$).
[1] : mg MDA/kg;
[2] : nmol/mg protein;
[3] : nmol Cys eq/mg protein.

**Table 5**
Interactions of nitrite/PPE addition and time of drying on TBA-RS, carbonyl, and thiol contents.

| Drying time (days) | Batch | TBA-RS[1] | Carbonyls[2] | Thiols[3] |
|---|---|---|---|---|
| 0 | 0 mg/kg $NaNO_2$ | 2.5 c | 11.7cde | 40.4 ab |
| | 150 mg/kg $NaNO_2$ | 1.5 cde | 13.1 cd | 36.4 c |
| | + 1% PPE (PPE_Low) | 2.1 cd | 11.1 cde | 31.4 e |
| | + 2% PPE (PPE_High) | 2.2 c | 9.8 e | 20.8 f |
| 14 | 0 mg/kg $NaNO_2$ | 15.3 a | 16.1 ab | 41.2 a |
| | 150 mg/kg $NaNO_2$ | 0.1 e | 12.7 cd | 35.4 cd |
| | + 1% PPE (PPE_Low) | 0.7 de | 11.4 cde | 22.9 f |
| | + 2% PPE (PPE_High) | 0.1 e | 9.3 e | 12.6 g |
| 28 | 0 mg/kg $NaNO_2$ | 8.1 b | 17.1 a | 37.5 bc |
| | 150 mg/kg $NaNO_2$ | 1.1 cde | 9.7 e | 32.3 de |
| | + 1% PPE (PPE_Low) | 1.1 cde | 14.0 bc | 23.2 f |
| | + 2% PPE (PPE_High) | 1.4 cde | 10.5 de | 12.9 g |
| | Standard error | 0.51 | 0.37 | 0.17 |
| | Sig. | *** | *** | *** |

Sig: Statistical significance. ***: $P < .001$.
a,b,c,d,e: Means within the same column with different letters differ significantly (Tukey's test, $P < .05$).
[1] : mg MDA/kg;
[2] : nmol/mg protein;
[3] : nmol Cys eq/mg protein.

removing sodium nitrite from various meat products by producing an increase in lipid oxidation due to the lack of the antioxidant effect provided by nitrite (Bonifacie et al., 2021; Higuero, Moreno, Lavado, Vidal-Aragón, & Cava, 2020; Van Hecke et al., 2014; Vossen & De Smet, 2015).

The inhibition of lipid oxidation in dry sausages by PPE confirms the high *in vitro* antioxidant activity assayed in PPE extract and residual antioxidant activity found in the sausages. The high polyphenol content is behind the powerful antioxidant effect of PPE. Li et al. (2006) reported pomegranate peel as a source of extracts with a high antioxidant activity mainly mediated by phenolic compounds. The elevated antioxidant activity of the PPE on experimental dry sausages agrees with various studies previously demonstrated that extracts obtained from pomegranate (juice) and pomegranate by-products significantly decreased the TBA-RS values by inhibiting lipid oxidation and production of secondary oxidation products in Tuscan sausage (Zago et al., 2020), beef (El-Nashi, Abdel Fattah, Abdel Rahman, & Abd El-Razik, 2015), and pork sausages (Gutiérrez-Pacheco et al., 2021) and in meat models (Dua, Bhat, & Kumar, 2016) and other muscle food products (Das et al., 2021).

Both carbonyls and free thiol content were evaluated as chemical markers of protein oxidation. Analogously for lipid oxidation, the formation of carbonyls from protein oxidation was significantly affected by the presence of nitrite (150_Nitr) and the PPE addition. Our results showed a significant increase in protein carbonylation in uncured sausages (150_Nitr) and those added with 2% PPE (PPE_High) but not in the cured ones (0_Nitr). On day 0, PPE_High dry sausages presented significantly lower carbonyl contents than the cured sausages, which were not different to the other experimental groups. On days 14 and 28, protein carbonylation was more intense in the uncured sausages, with higher carbonyl values. Adding nitrite protein carbonylation lowered, as did the addition of PPE. On day 28, results revealed a dose-response relationship in the PPE-treated samples with lower carbonyl concentrations the higher the level of PPE added. PPE at 2% diminished protein oxidation of the same extent as nitrite added at 150 ppm.

The effect of nitrite on protein oxidation is unclear, and there are contradictory results regarding its impact on protein carbonylation. Depending on the concentration, nitrite has both anti- and pro-oxidant outcomes on protein oxidation (Feng et al., 2016). Our results contrast the findings reported by Bonifacie et al. (2021) on fermented cured sausages. Contrary to the increase in nitrite-mediated carbonyl formation described by these authors, our results show a lower content of carbonyls from protein oxidation in cured sausages than in uncured ones. Thus, the results of the present study are in line with those reported by several authors (Feng et al., 2016; Vossen & De Smet, 2015; Wang et al., 2022), who showed that in sausages, salted meat and meat models added with sodium nitrite, the carbonyl content decreased significantly with increasing nitrite concentration compared to that of the uncured counterparts, demonstrating that nitrite can prevent such oxidative changes.

Several studies have confirmed that the incorporation of pomegranate juice or its by-products has been found to prevent protein oxidation and thus diminish carbonyl accumulation in muscle foods (Horbańczuk, Kurek, Atanasov, Brnčić, & Brnčić, 2019). The results of the present study agree with the research described in the literature that demonstrates the antioxidant effect of PPE in meat and meat products. Fourati et al. (2020) described that a pomegranate peel ethanolic extract (1%, *w*/w) reduced the carbonyl group accumulation in refrigerated minced beef. Turgut, Soyer, & Işıkçı, (2016); Turgut, Işıkçı, & Soyer, (2017) found that the extracts of pomegranate peel were effective in retarding carbonyl formation in meatballs during refrigerated and frozen storage. In another study, Smaoui et al. (2019) in minced beef reported an inhibitory effect of pomegranate peel extract in decreasing the carbonyl contents.

Statistical analysis revealed a significant interaction between nitrite/PPE added and time of drying on free thiol contents (Table 5). At the beginning of drying (d 0), our results already showed statistically significant differences in the content of free thiols between the experimental groups which were maintained throughout the drying process. Results revealed a significant diminution of free -SH during drying, with the lowest contents on day 28. The drop in thiol group contents during the drying process is in agreement with the findings of Berardo et al. (2016). At any stage of the drying process, the uncured sausages (0_Nitr) had significantly higher thiol content than their counterparts (150_Nitr, PPE_Low, and PPE_High). The addition of both nitrite and PPE resulted in a significant decrease in the -SH content of the dry sausages. In addition, a dosage effect of PPE was observed, and the higher the addition of PPE, the greater the loss of thiols. Our results contrast with those reported by Bonifacie et al. (2021) and Sullivan and Sebranek (2012) regarding the lack of effect or the loss of -SH groups in cured sausages, while they agree with those of Rysman, Van Hecke, De Smet, and Van Royen (2016) regarding the decay in the amounts of -SH groups in the presence of phenolic compounds.

In meat products, the level of free thiols depends on a complex relationship between the phenomena of oxidation of the -SH group, its reduction, and in the presence of nitrite, its nitrosation/nitrosylation to

form nitrosothiol compounds (Bonifacie et al., 2021; Domínguez et al., 2022). Rysman et al. (2016) proposed a mechanism for the loss of thiol groups in the presence of phenolic compounds. In this case, the cysteine in the protein chain can bind to a quinone molecule derived from the oxidation of the phenolic compounds, resulting in protein-phenolic adducts, which can be re-oxidised and rejoint another cysteine side chain, leading to loss of -SH groups.

Assuming that in both instances, nitrite and PPE, the oxidation of cysteine took place, a different mechanism could be involved in the loss of free thiols in cured and PPE-added sausages. As reported previously, based on a nitrite-mediated inhibition of lipid and protein oxidation processes in the experimental group 150_Nitr, it is logical to consider the formation of nitrosylcysteine as the factor responsible for the lower concentration of free thiols. Similarly, in the groups of sausages with added PPE, the higher loss of -SH groups than in the control batch could be assigned to the formation of cysteine-polyphenolic compound adducts, even more so with the observed dose effect. As several mechanisms occur at the same time, it is difficult to assess the share and impact of each of them in the evolution of free thiol content. In both cases, the role played by oxidation phenomena of the thiol group of cysteine in causing decreases in free thiol contents, cannot be discarded.

### 3.4. Residual antioxidant activities in dry sausages

Table 6 shows the residual antioxidant activity in the dry sausages at the end of drying. Significant differences were obtained between experimental groups for ABTS and FRAP residual activities. However, DPPH activity did not show differences. There were no changes in ABTS, DPPH, and FRAP residual activities between uncured (0_Nitr) and cured (150_Nitr) dry sausages. In dry sausages with PPE added (PPE_Low and PPE_High) were found significantly higher DPPH and FRAP residual activities were than in their uncured (0_Nitr) and cured (150_Nitr) equivalents. In PPE dry sausages, the residual DPPH and FRAP activities were affected by the added dose of PPE, being significantly higher in the PPE_High group than in the PPE_Low equivalents. In line with our results, Gutiérrez-Pacheco et al. (2021) found a rise in the antioxidant capacity and phenolic content in pork sausages formulated with pomegranate juice and peel powders. Similarly, Jin, Choi, Lee, Lee, and Hur (2016) detected residual antioxidant activities in meat products enriched with essential oils. The residual activity recovered in PPE sausages could be involved in the control of oxidative processes during subsequent storage, thus contributing to enhancing the shelf-life of the product.

These findings are related to the concentrations of oxidation markers in dried sausages. Higher residual activities matched to lower development of oxidation processes, particularly lipid oxidation. This association was evidenced by the correlation values obtained between the residual antioxidant activity values and MDA, carbonyls, and thiols. Thus, TBA-RS negatively correlated with ABTS ($\rho = -0.602$, $P = .002$) and FRAP ($\rho = -0.480$, $P = .018$). Thiol contents were negatively correlated with ABTS ($\rho = -0.770$, $P < .001$), DPPH ($\rho = -0.576$, $P = .003$) and FRAP ($\rho = -0.950$, $P < .001$). Contrary to the significant correlations found for TBA-RS and thiols, no significant correlations were found between carbonyls and residual antioxidant activities. The results suggest that lipid oxidation is highly affected by the antioxidant activity of added PPE and nitrite to a greater extent than protein carbonylation. Similarly, the loss of thiols is associated with the inclusion of PPE because of the more than likely interaction between SH groups and extract phenolic compounds, as evidenced by the significant correlation values between thiol content and ABTS, DPPH and FRAP activities.

**Table 6**
Effect of different ingoing amounts of nitrite and levels of PPE incorporated on the residual antioxidant activities of Iberian dry-cured sausages at the end of the drying process (day 28).

| | 150 mg/kg $NaNO_2$ (150_Nitr) | 0 mg/kg $NaNO_2$ | | | SE | Sig |
|---|---|---|---|---|---|---|
| | | + 0% PPE (0_Nitr) | + 1% PPE (PPE_Low) | + 2% PPE (PPE_High) | | |
| ABTS[1] | 5.00 b | 4.64 b | 6.11 a | 6.07 a | 0.163 | *** |
| DPPH[1] | 0.04 c | 0.00 c | 0.30 b | 0.73 a | 0.106 | * |
| FRAP[2] | 3.20 c | 3.15 c | 6.44 b | 9.77 a | 0.572 | *** |

Sig: Statistical significance. *: $P < .05$; ***: $P < .001$.
a, b, c: Means within the same row with different letters differ significantly (Tukey's test, $P < .05$).
[1] : mM Trolox eq/g;
[2] : mM $Fe^{2+}$ eq/g.

### 3.5. Instrumental colour, total colour changes (ΔE) and cured index of dry sausages

The instrumental colour coordinates, total colour changes and cured index were evaluated at the end of the drying process (28 days). Overall, nitrite and PPE produced noticeable changes in the instrumental colour parameters of the dried sausages (Table 7). Compared to uncured sausages (0_Nitr), nitrite significantly decreased the $L^*$ value, while it did not show differences between PPE-added sausages and their 150_Nitr and 0_Nitr counterparts.

Regarding CIE $a^*$-value, nitrite significantly increased the $a^*$-value which was significantly higher than in the other experimental groups, which did not show differences. Cured (150_Nitr) and uncured (0_Nitr) sausages did not differ in CIE $b^*$-values, which were significantly lower than those in PPE-added dry sausages. The addition of PPE increased the $b^*$ value, and this increase was greater at higher doses of added PPE. The orange-yellowish dye from the pomegranate peel extract, primarily due to carotenoids instead of anthocyanins (Borochov-Neori et al., 2009), had a significant impact on the CIE b*-value of the sausages treated with the pomegranate peel extract. With a higher incorporation of the PPE, there was a greater increase in the CIE b* value, indicating that the colorant molecules from the extract were responsible for the changes in the colour of the sausages. Variations in instrumental colour coordinates caused changes in °H-values. Thus, PPE had significantly higher °H-values than uncured and cured sausages. Several studies have reported the effects of pomegranate on colour stabilization, as well as the effects of inhibiting surface discolouration in various muscle food treated with pomegranate juice or extracts (reviewed by Das et al., 2021). In our experiment, the addition of PPE gave an orange tint to the processed products, with higher °H-values due to the enhanced CIE-$b^*$ values. This variation in colour causes changes in the characteristic colour of this type of meat product that could affect consumer acceptance.

**Table 7**
Effect of different ingoing amounts of nitrite and levels of PPE incorporated on instrumental colour parameters of Iberian dry-cured sausages at the end of the drying process (day 28).

| | Dry-sausages | | | | | |
|---|---|---|---|---|---|---|
| | 150 mg/kg $NaNO_2$ (150_Nitr) | 0 mg/kg $NaNO_2$ | | | SE | Sig. |
| | | + 0% PPE (0_Nitr) | + 1% PPE (PPE_Low) | + 2% PPE (PPE_High) | | |
| $L^*$ | 42.4 b | 50.6 a | 47.2 ab | 44.1 ab | 1.05 | * |
| $a^*$ | 11.7 a | 8.4 b | 7.4 b | 7.5 b | 0.44 | *** |
| $b^*$ | 10.4 c | 11.0 c | 13.4 b | 16.6 a | 0.57 | *** |
| Hue | 41.8 c | 52.4 b | 61.3 a | 65.7 a | 2.06 | *** |
| Chroma | 15.7 b | 13.8 b | 15.4 b | 18.2 a | 0.44 | ** |
| Cured index | 3.0 | 2.4 | 2.4 | 2.5 | | |
| ΔE | | 8.8 | 7.1 | 7.6 | | |

Sig: Statistical significance. *: $P < .05$; **: $P < .01$; ***: $P < .001$.
a, b: Means within the same row with different letters differ significantly (Tukey's test, $P < .05$).

Total colour change (ΔE) values (8.8, 7.1 and 7.8 for 0_Nitr, PPE_Low and PPE_High, respectively) reflected noticeable colour changes concerning the cured sausages (150_Nitr), indicating that the consumer could differentiate uncured and PPE-added sausages from cured ones.

The cured index (reflectance ratio 650 nm/570 nm) was significantly higher in 150_Nitr than in uncured sausages, regardless of the addition of PPE. This finding is consistent with the well-established relationship between nitrite and the formation of nitrosylmyoglobin, which is responsible for the desired red colour in dry cured sausages (Adamsen, Møller, Laursen, Olsen, & Skibsted, 2006; Wakamatsu, Hayashi, Nishimura, & Hattori, 2010). The use of pomegranate peel extract as a substitute for nitrite did not affect the cured index, and the values were similar to those of uncured sausages. Therefore, in terms of pigment formation responsible for curing, PPE did not have any significant effect.

## 4. Conclusions

The use of different extraction solvents provides pomegranate peel extracts with a wide range of contents in phenolic compounds, flavonoids, and condensed tannins with different antioxidant activities. The use of water: acetone 3:7 provides extracts with the highest content of phytochemical compounds and high ABTS, DPPH and FRAP activities. To the best of our knowledge, this study was the first to study the changes in lipid and protein oxidation phenomena in uncured Iberian dry sausages added with a pomegranate peel extract. Our results indicate that nitrite removal enhanced lipid peroxidation and protein carbonylation during the drying of sausages. Pomegranate peel extract counteracted this prooxidant scenario; thus, pomegranate peel extract added at 1 or 2% (*v/w*) to dry sausages improved oxidative stability lowering the formation of malondialdehyde from lipid peroxidation and carbonyls from protein carbonylation during drying. In contrast, its addition causes the loss of thiol because of the potential formation of cysteine-polyphenol adducts. Changes produced by the incorporation of PPE are dose-dependent, and the effect on the parameters analyzed is greater the higher the dose used. Although, the colour changes that PPE could cause could minimise the expectations for its use as a substitute for nitrite in dry sausages. The use of lower doses (< 1% v/w) of PPE could restrain the intense oxidative processes in dry sausages without added nitrite, causing a less marked effect on the colour characteristics. Other traits could be affected by PPE, such as taste, odour, flavour, and texture, that need further studies to be evaluated.

## CRediT authorship contribution statement

**Ramón Cava:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition, Investigation, Formal analysis, Writing – review & editing. **Luis Ladero:** Investigation, Formal analysis, Writing – review & editing.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ramon Cava Lopez reports financial support and administrative support were provided by FEDER and JUNTA DE EXTREMADURA.

## Data availability

Data will be made available on request.

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