MONITORING LIPID OXIDATION IN A PROCESSED MEAT PRODUCT PACKAGED WITH NANOCOMPOSITE POLY(LACTIC ACID) FILM

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Abstract

One of the most detrimental processes in fatty foodstuffs is lipid oxidation, which occurs during production and storage, and influences food composition and safety. Polylactic acid (PLA), a commercially available biopolymer, is biodegradable thermoplastic aliphatic polyester derived from renewable resources. Polymer layered silicate (PLS) nanocomposites have shown potential for enhancing physical, chemical, and mechanical properties of both conventional materials and biopolymers. In the present work nanocomposite films were prepared by incorporating unmodified montmorillonite clay (Cloisite® Na^+) in the PLA. Moreover, the lipid oxidation status of a processed meat product packaged with a film incorporating this nanocomposite was evaluated. In line with this, hexanal, Thiobarbituric Acid Reactive Substances (TBARS) and *p*-anisidine value were monitored after packaging salami during different storage times (15, 30, 60 and 90 days). The results of this study showed that the presence of montmorillonite (MMT) in the polymer film can reduce the lipid oxidation of processed meat products, extending their shelf life and, thus, suggesting that the new film is a potential good alternative to conventional bioplastics.

Introduction

In recent years, the concern to provide consumers with high quality foodstuffs has led to the adoption of measures to limit the oxidation phenomenon during the processing and storage of products [1]. The use of biodegradable materials for food packaging is considered an environmentally correct alternative, since it reduces the use of polyolefinic

plastics as packaging materials [2]. PLA, a commercially available biopolymer, is a biodegradable thermoplastic aliphatic polyester derived from renewable resources, such as corn starch (in the United States and Canada), tapioca roots, chips or starch (mostly in Asia), or sugarcane (in the rest of the world) [3]. Natural fibre reinforced PLA based biocomposites are widely investigated by the polymer scientists in the last decade to compete with non-renewable petroleum based products. [4].

During the last decades, the interest in polymer layered silicate (PLS) nanocomposites had an exponential growth since they have been shown to enhance physical, chemical, and mechanical properties of both conventional materials and biopolymers. Polymer nanocomposites are reinforced by nanometric fillers, one of the most used is montmorillonite (MMT) not only because it is an environmentally friendly material, occurring naturally, but also because is readily available in large quantities. It has been already used in the literature to overcome some weaknesses of PLA films such as poor barrier properties [5]. In this paper unmodified montmorillonite clay Cloisite® Na^+, was incorporated in PLA film and the obtained nanocomposite films were used to investigate their effect as packaging materials in the preservation of a model fatty food. In fact, one of the most detrimental processes in fatty foodstuffs is lipid oxidation. The lipid oxidation results from a spontaneous and inevitable event that the fatty foods undergo during the processing and storage and which has as main consequence the modification of the original flavour and the appearance of rancid odours and tastes. These changes represent for the consumers and for the food industry an important cause of depreciation or rejection. This phenomenon has a direct implication on the commercial value of the fatty substances and the products that are formulated from them (e.g. foods, cosmetics, medicines) [6]. Consequently, lipid oxidation has long been recognized as a leading cause

of quality deterioration of the food and is often the decisive factor in determining food shelf life [6]. Lipid oxidation is a complex process whereby unsaturated fatty acids react with molecular oxygen via a free radical mechanism [7]. As result of lipid oxidation, a complex mixture of lipid oxidation products is produced.

It is well known that barrier properties of the packaging material play a major role in determining the shelf life of a food product. In particular, in order to control lipid oxidation, it is important to improve the barrier properties of packaging films, such as permeability of moisture and gases across the packaging material [8]. One of the most important control parameters for oils, fats and oilseeds is moisture because the stability of these foods decreases with increasing moisture content [9]. To this aim, in this paper low amount of layered nanoclays has been incorporated in polylactic acid in order to improve the water barrier of the packaging film. In fact, Sonjui and Jiratumnukul (2014) [10] reported a reduction by more than 14% in the water vapor transmission rate of PLA bionanocomposite coating films reinforced with 0.1% (w/w) of Cloisite 30B. Qiuhui Hu et al. (2011) [11] showed that a novel nanocomposite-based packaging prepared by blending polyethylene (PE) with montmorillonite significantly decreased the oxygen and water vapor permeability. In this paper the effect of PLA/MMT films in the packaging of sliced salami has been investigated. In particular, salami slices were packaged with PLA/MMT films and with a control film (PLA). After different storage times (0, 15, 30, 60 and 90 days), salami slices were analysed regarding their hexanal, TBARS and *p*-anisidine value and the obtained results were correlated with water barrier properties data of the nanocomposite films.

Statistical analysis

Results were expressed as mean ± standard deviations of at least three replicates. Differences among samples were tested using Kruskal Wallis followed by Mann Whitney. All statistical analyses were tested at 0.05 level of probability, using the SPSS® computer programme (SPSS Inc., Chicago, IL, USA).

Material and Methods

Preparation of the PLA/ Cloisite® Na^+ film

The pristine clay (Na^+-montmorillonite), hereafter named MMT, was purchased from Southern Clay Products, Inc., TX and used without further modification. NatureWorks™ Poly (L-lactide) polymer 2002D was supplied by Cargill Dow LLC (Minnetonka, MN). The density of the PLA was 1.25 g cm^{-3}. As an alpha hydroxyl ester, PLA tends to hydrolyze at elevated temperatures and high relative humidity. For this reason, PLA pellets were dried at 50 °C at least for 12 h in vacuum prior to use.

PLA/MMT (Cloisite® Na^+) composite films were prepared by direct melt processing. MMT (powder form) and PLA (pellet form) were first mixed at 180 °C and at 60 rpm for 5 min in an internal mixer (Rheomix® 600 Haake, Germany) with a volumetric capacity of 50 cm^3. After homogenization of PLA and MMT particles, the films with thickness of 100-150 μm were prepared by compression moulding using a Collin P300P press at 180 °C and at 5 MPa for 3 min, cooled down for 10 min at 10 °C min^{-1} and 1MPa. Films of pure PLA (as control) and films containing 5 wt. % of MMT were prepared and tested afterwards.

Packaging of salami

To test the effectiveness of the nanocomposite films, the selected model food was the salami due to its high fat content. Salami was purchased, already sliced, in a commercial area of Lisbon (Portugal). The selected salami presented the following nutritional composition per 100 g: protein 23.5 g, total lipids 26.5 g, carbohydrates 4.9 g and 3.9 g salt.

Salami slices (approximately 10 g each) with a thickness of approximately 2 mm each, were placed in direct contact with nanocomposites films containing 5% MMT (w/w) and a control film (PLA without nanoclay). Subsequently, they were packed in a vacuum to allow good contact between the films. All prepared samples were stored at 5 °C, protected from light. The samples were analyzed after 0, 15, 30, 60, 90 days of storage for evaluation of oxidation status of salami packaged with either the control films or films with nanoclays.

Effectiveness of the PLA-based films against lipid oxidation

The effectiveness of the PLA and PLA-MMT films against lipid oxidation was carried out by three different methods: *p*-anisidine value, TBARS assay and hexanal monitoring.

***p*-anidisine value**

p-anisidine value, a spectrophotometric analysis method measuring the absorbance at 350 nm, was used following the official method (AOCS Official Method Cd 18-90), [12]. The *p*-anisidine in acetic medium forms a yellow complex with the aldehydes having two conjugated double bonds, in particular with *trans*, *trans*-2,4-decadienal resulting from the degradation of linoleic acid.

In order to determine *p*-anisidine value, the fat was extracted from the salami slices packaged with either the control films or films with nanoclays. Ten grams of sample was shaken for 1 hour with 100 ml of petroleum ether. The solution was filtered into an evaporator flask with Whatman No. 4 filter to which anhydrous sodium sulphate was added to retain the sample water. The petroleum ether was evaporated at 40 °C. The fat was kept at 5 °C, protected from light, until the tests to obtain *p*-anisidine value were performed.

The determination of the *p*-anisidine value was performed according to British Standard method BS 684-2.24-1998 (British Standard Method 1998) [13]. First the *p*-anisidine solution is prepared by weighing 50 mg of *p*-anisidine into a 20 ml flask and make up the volume with acetic acid. To 0.5 g of fat (previously extracted), 25 ml of *n*-hexane was added and the solution was placed 5 to 10 minutes in the ultrasonic bath at room temperature until the fat dissolves. The absorbance of the solution is measured at 350 nm against n-hexane. One ml of the solution of *p*-anisidine in acetic acid was added to 5 ml of the sample solution and it was stored in the dark for 10 minutes at room temperature. For the control test, 1 ml of the solution of *p*-anisidine was added to 5 ml of n-hexane. Finally, the absorbance of the samples against the control test was measured. All analyzes were performed in triplicate.

The *p*-anisidine value was calculated according to the following equation:

$$AV = 25\ (1.2\ Abs_2 - Abs_1) / m \qquad \text{(Equation 1)}$$

Where:

AV - value of *p*-anisidine;

Abs_2 - absorbance of the sample after 10 minutes of reaction;

Abs_1 - initial sample absorbance;

m - amount of fat used in the test (in g).

Thiobarbituric Acid Reactive Substances (TBARS) assay

TBARS assay was based on the spectrophotometric measurement of a complex formed by the reaction between Thiobarbituric acid (TBA) and malondialdehyde (MDA) according to the method of Miller (1998) [14]. Malondialdehyde (MDA) is formed as a result of the degradation of polyunsaturated fatty acids, therefore this test is a measure of the oxidative status of a sample.

About 5 g of packaged salami was homogenized with 50 mL of trichloroacetic acid (10 %) in 0.02 M of orthophosphoric acid using an Ultra-Turrax (IKA DI 25 basic, Werke GmbH & Co, Germany). Then, the solution was filtered through filter paper (Whatman n° 1) and then 5 mL of this solution was homogenized with 5 mL of TBA aqueous solution 0.02 M and heated at 100 °C for 40 min. Afterwards, solutions were cooled down for 10 min and the concentration of the substances that have reacted with TBA after heat treatment was calculated by measuring the absorbance at 530 nm. A standard curve of 1,1,3,3-tetraethoxypropane (TEP) was prepared with rate concentration ranging from 0 to 5 μg/mL. Results were expressed as mg malondialdehyde (MDA) per kg of salami (mg MDA/Kg).

Hexanal determination

The extraction of hexanal was carried out according to the method of Wen *et al*. (1997) [15], while the hexanal quantification was performed with an Ultra High Performance Liquid Chromatography coupled with a Diode-Array Detection (UHPLC-DAD), using a

method adapted from Sanches-Silva et al. (2004) [16]. In order to carry out the hexanal extraction, 1 g of salami was homogenized using an Ultra-Turrax (IKA DI 25 basic, Werke GmbH & Co, Germany) with 5 mL of solution of 2,4-dinitrophenylhydrazine (2,4-DNPH) in sulfuric acid. The mixture was left to stand in the dark for 4 hours to complete the reaction of derivatization. Then, an extraction with *n*-hexane (10 mL) was performed for three times and the pooled hexane phase was evaporated to dryness. The residue was reconstituted with methanol (10 mL), filtered and analyzed by UHPLC-DAD. The analysis was conducted in triplicate. For determination of hexanal by UHPLC-DAD an ACQUITY^{TM} UPLC$^{®}$ BEH C18 pre-column (2.1 × 5 mm, 1.7 μm particle size) and an AQUITY UPLC$^{®}$ BEH C18 column (2.1 mm x 50 mm, 1.7 μm particle size) were used. The mobile phase comprised acetonitrile and ultra-pure water (75:25, v/v). The column was maintained at 20 ° C, the mobile phase flow was 0.5 ml / min and the injection volume was 10 μl. The hexanal was quantified at the wavelength of 365 nm. The run time was 5 min. The method was previously validated regarding the different parameters (linear range, linearity, limit of detection and limit of quantification, precision and accuracy). Hexanal identification was carried out by comparing UHPLC retention time and spectrum with those of an analytical pure standard from Sigma-Aldrich® (Madrid, Spain). To determine the concentration of hexanal, a standard calibration curve was constructed by plotting hexanal area of different standard solutions *versus* hexanal concentration.

Each solution was injected in duplicate in the chromatographic system under optimized conditions.

Water permeability tests

Water vapour permeability of PLA and PLA/MMT films was determined using the infrared sensor technique by means of a PermatranW3/31 (Mocon, Germany). Samples with a surface area of 5 cm^2 were tested at 25 °C. Permeation tests were performed by setting the relative humidity at the downstream and upstream sides of the film to 0% and to 50% respectively. A flow rate of 100 ml/min of a nitrogen stream was used. Each test was carried out in duplicate.

Results and discussion

Usually the measurement of lipid oxidation in foods is made using a multiple analytical methods, which means that there is no standard method for detecting all oxidative changes in all food systems. The accessible methods to monitor lipid oxidation in foods, can be classified based on what they measure (the absorption of oxygen, the loss of initial substrates, the formation of free radicals, and the formation of primary and secondary oxidation products) [17]. A great variety of methodologies have been developed and implemented so far [18, 19], depending if primary lipid oxidation products or secondary lipid oxidation products must be measured.

In the present study three tests were used to monitor the lipid oxidation state of salami packaged with a PLA/MMT film and a control film (neat PLA) and tested after 0, 15, 30, 60 and 90 days of contact.

The *p*-anisidine value measures the secondary oxidation products like aldehydes, carbonyls, trienes, ketons. This chemical analysis method determines principally 2-alkenals and 2,4-dienals in fats by reaction of these compounds with the *p*-anisidine.

Figure 1 shows the results of the *p*-anisidine value of salami samples packaged with a PLA and PLA/MMT films. From the data it is possible to infer that there are no differences between the values of salami packaged with both films, thus showing that the use of nanocomposite film has no influence on this oxidation parameter.

So far, we have had great difficulty in finding documents that report studies of *p*-anisidine values in meat packaged with flexible films containing clays and/or active compounds, so it is not possible to compare results with those from similar studies.

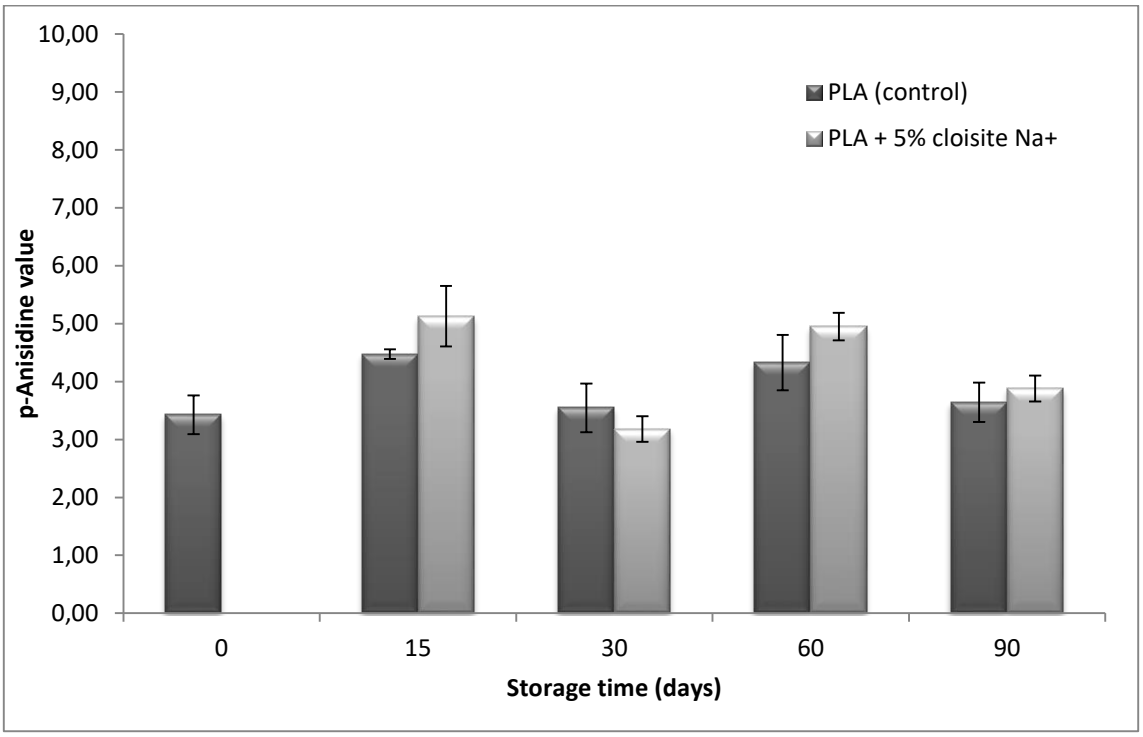

Figure 1 Results of the *p*-anisidine value in salami samples packaged during 90 days with PLA and PLA/MMT films.

TBARS are formed as by-products of lipid peroxidation (i.e. as degradation products of fats) which can be detected by the TBARS assay using thiobarbituric acid as a reagent. TBARS is the most commonly used method for assessing lipid oxidation in foods, since

it allows determining their oxidative state [20]. This method is based on the spectrophotometric determination of extracted malonaldehyde, a minor product of oxidation [21]. The TBARS reaction is not specific for malonaldehyde, so other lipid oxidation compounds or compounds not related to lipid oxidation, may react with thiobarbituric acid, thereby the extent of lipid oxidation can be overestimated. This is the major disadvantage of the TBARS reaction, and the reason why TBARS procedure should be used to assess the overall extent of lipid oxidation [19].

In this work, TBARS test results showed that salami packaged with the PLA/MMT presented lower amount of MDA with respect to salami packaged with pristine PLA film during all contact periods, thus evidencing the influence of nanocomposite film to reduce the extent of lipid oxidation (Figure 2). However, we must remember that there are no significant differences between the values obtained. So far very few papers report studies on lipid oxidation of salami and/or meat packaged with flexible films containing clays and/or active compounds. Yu-Yue Qin *et al.* (2013) [22] investigated the effect of chitosan (CH) film incorporated with tea polyphenol (TP) on quality and shelf life of pork meat patties stored at 4 ± 1 ◦C for 12 days. Their results showed that wrapping with CH-TP composite film tended to retard the increases in TBARS values when compared to the control group. More recently Nalçabasmaz et al. 2017 [23] reported lipid oxidation results of ready-to-eat salami packaged with polypropylene loaded with 1% nanoclay and 5% polybetapinene under vacuum, modified atmosphere packaging under 50% CO_2 and 50% N_2 and air, and stored at 4 °C for 90 days. They showed that TBARS gradually increased at all applications during increased storage. The highest increase in TBARS values was observed when salami was packaged under air and 50% CO_2 /50% N_2. They concluded that higher TBARS values were possibly due to high oxygen concentration of modified

atmosphere packaging applications leading to increased lipid oxidation. Nagarajan et al. 2015 [24] showed that, mackerel meat powder covered with tilapia and squid skin, covered with nanocomposite film incorporated with ethanolic extract from coconut husk (EECH) at 0.4% (w/w) generally had the lower moisture content than those covered with other gelatin films (without covering) ($P < 0.05$) during storage for 30 days at 28–30 ºC. They also reported that substances with lower peroxide value (PV) and TBARS were observed for the samples covered with EECH compared to the samples covered with PE (polyethylene) and the control samples ($P < 0.05$). They concluded that nanocomposite film incorporated with both EECH and nanoclay could be an alternative to synthetic commercial film to maintain the quality and extend the shelf-life of mackerel meat powder.

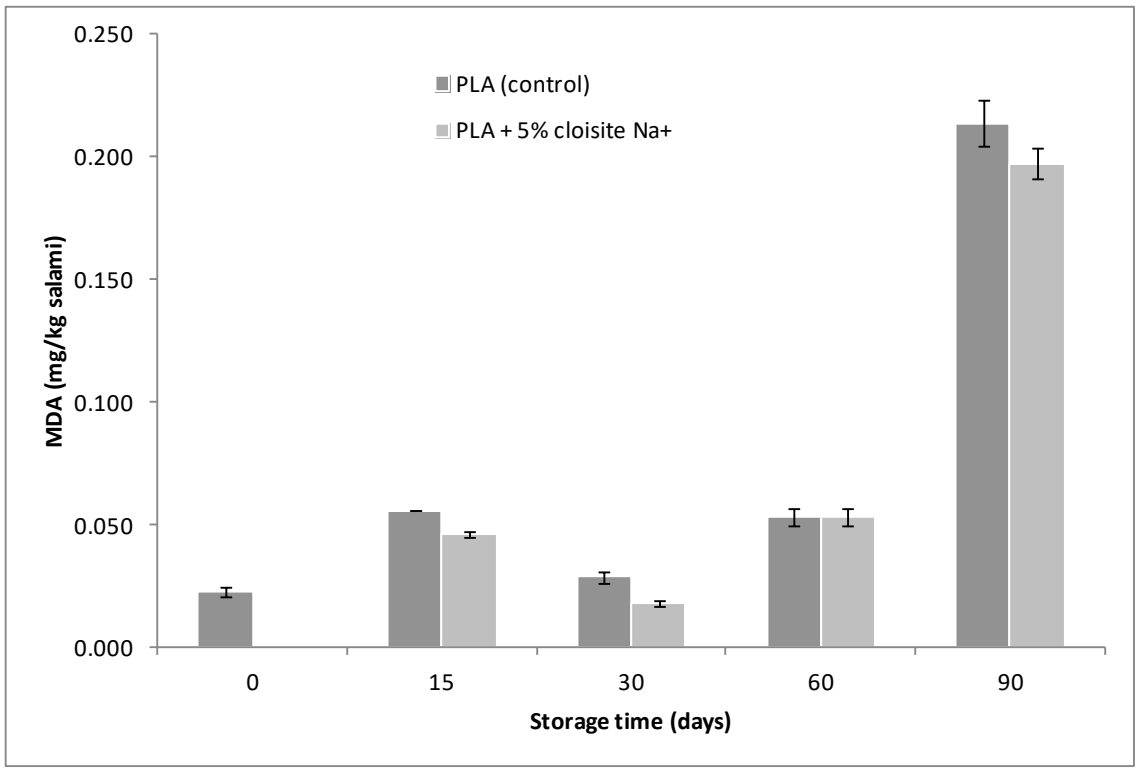

Figure 2 Results of the thiobarbituric acid reactive substances (TBARS) assay in salami samples packaged during 90 days with PLA and PLA/MMT films.

Volatile compounds, hydrocarbons (pentane, *n*-hexane, ethane), aldehydes (pentanal, hexanal, hexenal, 2-octenal, 2-nonenal), ketones (1,5-octadien-3-one, 1-octen-3-ona) or acids (formic acid), result from the decomposition of the primary products of the oxidative process (peroxides). The measurement of volatile compounds may indicate the state of lipid oxidation. They appear at a very early stage of the evolutionary cycle and are the source of rancidity [25, 26]. Aldehydes are one of the most abundant volatiles, they increase initial free radical reactions, once are highly reactive and regarded as second toxic messengers [27]. Hexanal is the compound usually determined, since it comes from the degradation of linoleic and arachidonic acid, which form an integral part of a great variety of products. Researchers have suggested that hexanal indicates lipid oxidation of meat more effectively than any other volatile component [28]. Most of the authors use High Performance Liquid Chromatography (HPLC) to allow determination of peroxides, hydroperoxides and by-products of oxidation. They usually propose reverse phase HPLC and use ultraviolet or diode array detector [6]. In the present paper, hexanal was determined in packaged salami by UHPLC-DAD (Ultra High Performance Liquid Chromatography coupled to Diode Array Detection).

Figure 3 shows the monitoring of hexanal, an indicator of the lipid oxidation, in salami during 90 days. Hexanal tends to decrease until 60 days of storage. In this period of time the hexanal content of the salami packaged with the PLA/MMT films was lower than the salami packaged with control film, thus showing an interesting effect in the use of nanocomposite films. This is likely due to the presence of nanoclay which is known to improve the barrier properties of polymers by creating a tortuous path for the molecule diffusion. In order to prove this, water permeability (WP) tests were performed, the

obtained data are reported in Figure 4. It is worth noting that the presence of MMT in the film brings a WP reduction of about 33% with respect to pristine PLA. The presence of silicate platelets in nanocomposite films, in fact, leads to a reduction of water permeability, because they create a tortuous path which hinders the diffusion of the permeant.

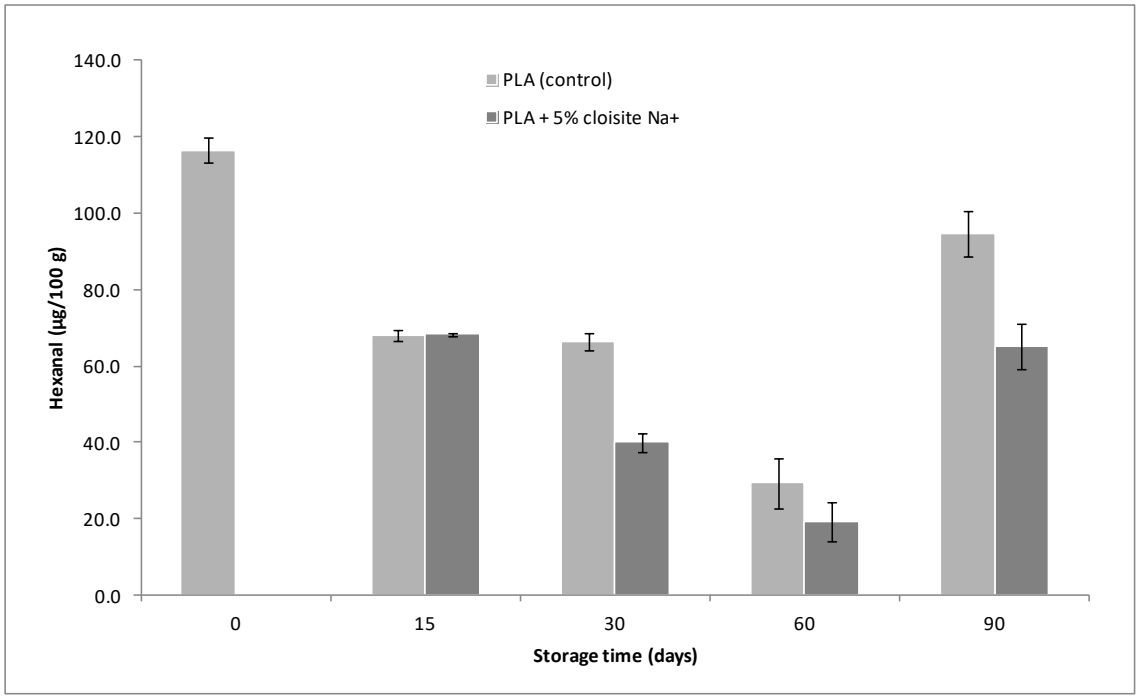

Figure 3 Results of the hexanal determination in salami samples packaged during 90 days with PLA and PLA/MMT films

After 90 days of storage, the amount of hexanal in the samples increased, but, again, it was higher in the samples packaged with PLA (94.7 ± 6.02 µg/100g salami) than salami packaged with PLA/MMT films (65.1 ± 6.12 µg/100g salami). This result shows that the use of nanocomposite films allowed a reduction of about 30% of hexanal, being effective in reducing lipid oxidation. This effect is more evident after long storage period because it has been proven that water permeability of both films, particularly the one of

nanocomposites further decrease after long storage periods. Permeability tests, in fact, were also performed on the films which had been in contact with salami slides for 90 days. It can be noticed that after exposure to the packaged model food for 90 days, a WP reduction of 33% and 30% has been registered, for PLA and PLA/5MMT respectively (Figure 4). This is likely due to the diffusion of fatty components from the salami to the polymeric film, which enhances the hydrophobicity of the film, thus playing a role in the improvement of water barrier properties by repulsing water molecules as also reported by in other papers [29, 30, 31].

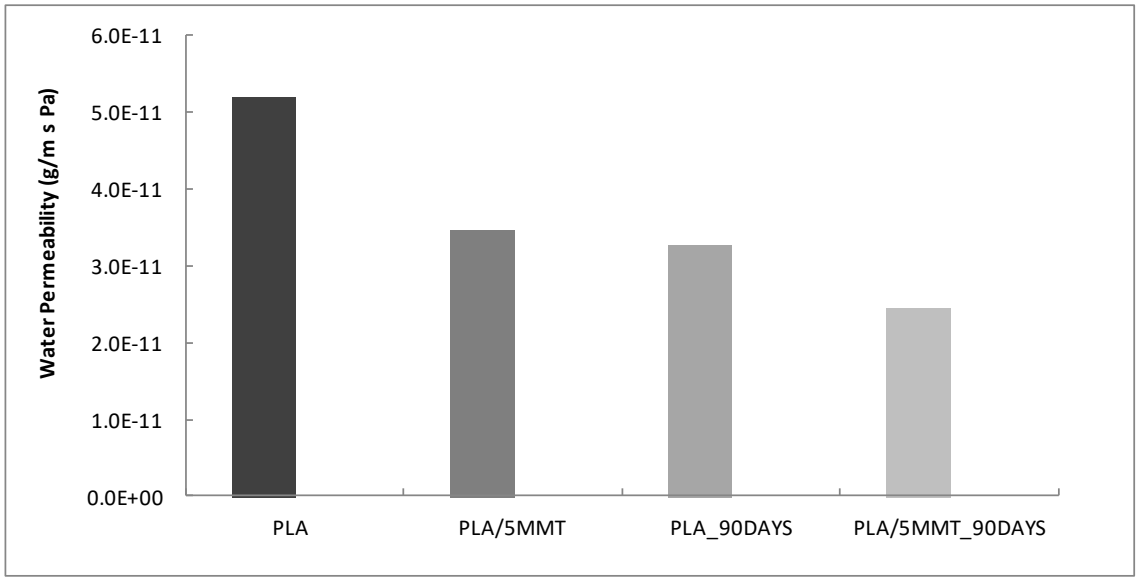

Figure 4 Results of permeability tests performed on the pristine PLA and PLA/5%MMT which had not been in contact with salami slices and pristine PLA and PLA/5%MMT which had been in contact with salami slides for 90 days

Conclusion

PLA nanocomposite films obtained embedding unmodified MMT clay into polylactic acid was developed and evaluated regarding their effectiveness in retarding lipid oxidation of a packaged fatty food such as sliced salami. Three different methods: *p*-

anisidine value, Thiobarbituric acid reactive substances (TBARS) assay and hexanal monitoring, were used to evaluate the oxidation status of a fatty model food. Results indicated that the nanocomposite films, mainly due to their enhanced water barrier properties, tend to reduce lipid oxidation of the packaged fatty food. Therefore, the presence of MMT in the polymer film can reduce the lipid oxidation of processed meat products, extending their shelf life, suggesting that this nanocomposite film is a good alternative to conventional bioplastics. However, it is worth noting that the use of engineered nanosized particles in food packaging materials is not permitted by EU legislation and should be assessed on a case-by-case basis. For this reason, a further investigation could be represented by the study of lipid oxidation of fatty food packaged with a PLA/MMT films in presence of a functional barrier which could act as a physical barrier to the migration of nanoparticles.

Acknowledgements

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