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PhD Thesis

**Antioxidant properties and applications of pecan**  
*(Carya illinoensis)*

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*I dedicate this thesis to my parents, for their constant support and unconditional love.*

*I love you all dearly.*

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## Abstract

At the present time, the environmental challenges are the second concern on a global level, and the first one in some countries. This is one of the main reasons behind the recent boost of new methods of production within the agri-food sector. Adopting a circular economy model, for example, means aiming at reducing, reusing and processing wastes. Wastes and food sub-products are a potential source of bioactive compounds, some of which can have antioxidant properties. In recent years, they have become more relevant in the food and drug industry, since they represent a good alternative in the formulation of functional foods, such as additives or even in the production of drugs.

The main focus of this thesis was on the pecan shell (*Carya illinoensis*) and its fruit. Secondly, the roselle flower (*Hibiscus sabdariffa*) and the cayenne pepper (*Capsicum annuum*) were also studied as well. These plants or their extract have antioxidant and, to a lesser extent, antibacterial properties on food.

Firstly, the total polyphenols content (TPC) and the antiradical activity were identified and analyzed through several methods. Secondly, the potential synergetic effects of these plants in real food environments, such as oil-in-water emulsions, fish and beef patties were evaluated.

On the other side, active packaging was also part of the study. Films were created from a basis of gelatine, with extracts of the pecan shell and the fruit. The film was characterized and evaluated both in its interaction with the food (hamburger), analyzing the delay in the oxidation, and for its mechanical and physic-chemical properties. Also, the film Poly ( $\alpha$ -dodecyl  $\gamma$ -glutamate) (PAAG-12) was formed by adding  $\alpha$ -tocopherol (one of the main compounds of the pecan shell) and was compared to the polylactic acid film in similar conditions.

The process of extrusion of the pecan shell was optimized. The extrudate shell was added as a new ingredient to the development of bread and tortilla production. The extruded pecan shell was encapsulated and its antioxidant activity was evaluated in patties of salmon. At the same time, the fermentation of the original pecan shell and extruded shell with *Aspergillus oryzae* fungus were studied.

Along with the antioxidant analysis, an in vitro evaluation was conducted on the antiproliferative activity of the extract from the fruit and shell of the pecan nut, in cultures of cells derived from tumors.

The results obtained from the pecan shell extract show high levels of TPC and good antiradical activity, similar to the values for the extract of green tea. At the same time, both the fruit and the pecan shell had a positive effect, slowing the formation of hydroperoxides in emulsions.

The synergy between the fruit of the nut and the roselle flower slowed down the fat oxidation in sardine burgers, thus decreasing the formation of reactive compounds to malondialdehyde and volatile compounds. Similar results were achieved in meat, by the addition of the mix of the pecan shell with the roselle flower and the cayenne pepper.

Concerning the two types of film developed, the result was positive both in terms of decrease of compounds, generated from the primary oxidation in emulsions, and the secondary oxidation in meat.

The extrusion of the pecan shell increased significantly the antioxidant properties and up to three times the content of soluble fiber versus the non-extruded pecan shell. The addition of this new ingredient in bread, tortillas and capsules showed favorable results. Moreover, the extracts of both the fruit and the pecan shell showed an anti-proliferative activity in cell cultures derived from tumors.

Taking advantage of residues like the pecan shell in order to obtain products with an added value represents an environmentally conscious option. Moreover, the synergies between different plants increased the antioxidant and antimicrobial activities, and potentially allowing the replacement of synthetic additives in food.

**Keywords:** Antioxidants, residues, food models, functional foods, cancer.

## Resumen

Los problemas relacionados con el medio ambiente constituyen actualmente la segunda preocupación a nivel mundial, y la primera en el caso de muchos países. Éste es uno de los principales motivos que está motivando la adaptación hacia nuevos sistemas de producción en el sector agroalimentario. Por ejemplo, adoptar un modelo de economía circular, que tiene por finalidades la reducción, reutilización y aprovechamiento de sus residuos. Los desechos y subproductos alimentarios constituyen fuentes potenciales de compuestos bioactivos, algunos de los cuales pueden proporcionar propiedades antioxidantes. Ello ha propiciado que en los últimos años adquieran relevancia para las industrias alimentarias y farmacéuticas, al representar una buena alternativa en la formulación de alimentos funcionales, como aditivos alimentarios o incluso en la producción de fármacos.

En esta tesis doctoral se ha analizado principalmente la cáscara de nuez pecana (*Carya illinoensis*), como también el fruto. Secundariamente se ha trabajado con la flor de hibisco (*Hibiscus sabdariffa*) y el pimiento (*Capsicum annuum*). Estos productos, directamente o sus extractos, poseen compuestos con propiedades antioxidantes, capaces de inhibir procesos de oxidación en alimentos, y también, aunque en menor medida, con propiedades bactericidas.

Inicialmente, se ha analizado e identificado el contenido de polifenoles totales (CPT) y la actividad antirradicalaria mediante distintos métodos. Posteriormente, se han evaluado posibles efectos sinérgicos de estas plantas en sistemas alimentarios reales, como emulsiones de aceite en agua, hamburguesas de carne y pescado.

Otro aspecto a destacar es el trabajo con envases activos. Se han elaborado films a base de gelatina con extractos del fruto y de la cáscara. Se caracterizó y evaluó tanto en la interacción con el alimento (hamburguesa), analizando el retraso en la oxidación, como por sus propiedades mecánicas y físico-químicas. También se ha sintetizado el film Poly ( $\alpha$ -dodecyl  $\gamma$ -glutamate) (PAAG-12) con adición de  $\alpha$ -tocoferol (uno de los principales compuestos del fruto de la nuez pecana) y se ha comparado con las características, en condiciones similares, del film con ácido poliláctico (PLA).

Se ha optimizado el proceso de extrusión de la cáscara de nuez. El extruido se ha añadido como nuevo ingrediente para el desarrollo de productos de panificación. Se ha llevado a cabo la encapsulación de dicho extruido y evaluado la actividad antioxidante en salmón. Así mismo, se ha estudiado la fermentación de la cáscara original y extruida con el hongo *Aspergillus oryzae*.

Además de los análisis antioxidantes comentados, también se ha evaluado *in vitro* la actividad antiproliferativa de extractos de fruto y cáscara de la nuez pecana en cultivos de líneas celulares derivadas de tumores.

Los resultados obtenidos con los extractos de cáscara muestran los niveles mayores de CPT y actividad antirradicalaria, de manera similar a los obtenidos con extractos de té verde. Así mismo, tanto el fruto como la cáscara tuvieron un gran efecto en la disminución de la formación de hidroperóxidos en emulsiones. Por su parte, la sinergia del fruto de la nuez con la flor de hibisco logró una reducción de la oxidación lipídica en hamburguesas de sardinas, disminuyendo la formación de

compuestos reactivos al malondialdehído y de compuestos volátiles. Resultados similares se obtuvieron en la carne con la incorporación de la mezcla de la cáscara con la flor de hibisco y el pimienta.

En cuanto a los dos tipos de films elaborados, el resultado fue positivo tanto en la disminución de compuestos formados por la oxidación primaria en emulsiones como de la oxidación secundaria en carne.

La extrusión de cáscara incrementó significativamente la capacidad antioxidante y hasta tres veces más el contenido de fibra soluble con respecto a la cáscara no extruida. La adición de este nuevo ingrediente en pan, tortilla y cápsulas han mostrado resultados muy favorables, como lo son el aumento de la capacidad antioxidante.

Adicionalmente, tanto los extractos del fruto como de la cáscara presentaron actividad antiproliferativa en líneas celulares derivadas de tumores.

Todo lo expuesto supone una alternativa respetuosa con el medio ambiente, permitiendo usar residuos como la cáscara de la nuez pecana para obtener productos con valor añadido. Además, las sinergias encontradas entre las plantas estudiadas han permitido potenciar la actividad antioxidante y antimicrobiana, mostrando un destacable uso potencial como sustitutos de aditivos sintéticos en alimentos.

**Palabras claves:** antioxidantes, residuos, modelos alimentarios, alimentos funcionales, cáncer.

## Abbreviations

AACC	Approved Methods of Analysis
ABTS	2,20 azino-bis(3-ethyl-benzothiazoline-6-sulfonicacid)
AMU	Atomic Mass Unit
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
b*	Yellowness
BA	Biogenic Amines
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
C*	Chrome
CAD	Cadaverine
CFU	Colony-Forming units
CNB	Control Bread
CNF	Control Flour
CNT	Control Tortilla
CTC	Condensed Tannin Contents
DAD	Diode-Array Detector
DHA	cis-4,7,10,13,16,19-docosahexaenoic Acid
DMSO	Dimethyl Sulfoxide
DO	Dopamine
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential Scanning Calorimeter
DW	Dry Weight
EDTA	Ethylene Diaminetetra Acetic Acid
EPA	cis-5,8,11,14,17-eicosapentaenoic Acid
EtOH	Ethanol

EU	European Union
FA	Fatty Acids
FAME	Fatty Acids Methyl Ester
FID	Flame Ionization Detector
FTIR	Fourier Transform Infrared Spectroscopy
Folin	Folin-Ciocalteu Analysis
FPC	Free Phenolic Compounds
FRAP	Ferric Reducing Antioxidant Power
FW	Fresh Weight
GAE	Gallic Acid Equivalents
GC	Gas Chromatography
h°	Hue angle
HAT	Hydrogen Atom Transfer
HepG2	Hepatocellular Carcinoma
HPLC	High Performance Liquid Chromatography
HIS	Histamine
L*	Lightness
LC-MS-TOF	Time-of-flight Mass Spectrometry
HDL	High Density Lipoprotein
IC <sub>50</sub>	Half maximal Inhibitory Concentration
IDF	Insoluble Dietary Fiber
ISO	International Organization for Standardization
LDL	Low Density Lipoprotein
MetMB	Metmyoglobin
MeOH	Methanol
MDA	Malonaldehyde
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
MUFA	Monounsaturated fatty acids
NMR	Nuclear Magnetic Resonance
NQ	No Quantification
n3	n3-polyunsaturated fatty acid
n6	n6-polyunsaturated fatty acid

OC	Octopamine
OAI	Oil Absorption Index
OPT	O-Ophtalaldehyde
PAAG-12	Poly ( $\alpha$ -dodecyl $\gamma$ -glutamate)
PLA	Polylactic Acid
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated Fatty Acids
PUT	Putrescine
PV	Peroxide value
PN	Pecan Nut
PW	Pecan Walnut
PWS	Pecan Walnut Shell
PWSE	Pecan Walnut Shell Extruded
RSA	Radical Scavenging Activity
ROS	Reactive Oxygen Species
RSM	Response Surface Methodology
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SET	Single Electron Transfer
SER	Serotonin
SDF	Soluble Dietary Fiber
SFA	Saturated Fatty Acids
SPD	Spermidine
SPM	Spermine
SW	Sample Weight
SFA	Saturate Fatty Acids
TBA	Thiobarbituric Acid
TBARs	Thiobarbituric Acid Reactive substances
TBHQ	Terbutil Hydroquinona
TDF	Total Dietary Fiber
TE	Trolox Equivalent
TPC	Total Polyphenol Content

TEAC	Trolox Equivalent Antioxidant Capacity
TGA	Thermal Gravimetric Analysis
TPA	Textural Profile Analysis
Trolox/TE	(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid equivalent
TRP	Tryptamine
UV	Ultraviolet
WAI	Water Absorption Index
WF	Wheat Flour
WHO	World Health Organization
WS	Pecan Walnut Shell
WSB	Pecan Walnut Shell Bread
WSEB	Pecan Walnut Shell Extruded Bread
WSF	Pecan Walnut Shell Flour
WSEF	Pecan Walnut Shell Extruded Flour
WSI	Water Solubility Index
WST	Pecan Walnut Shell Tortilla
WVP	Water Vapor Permeability



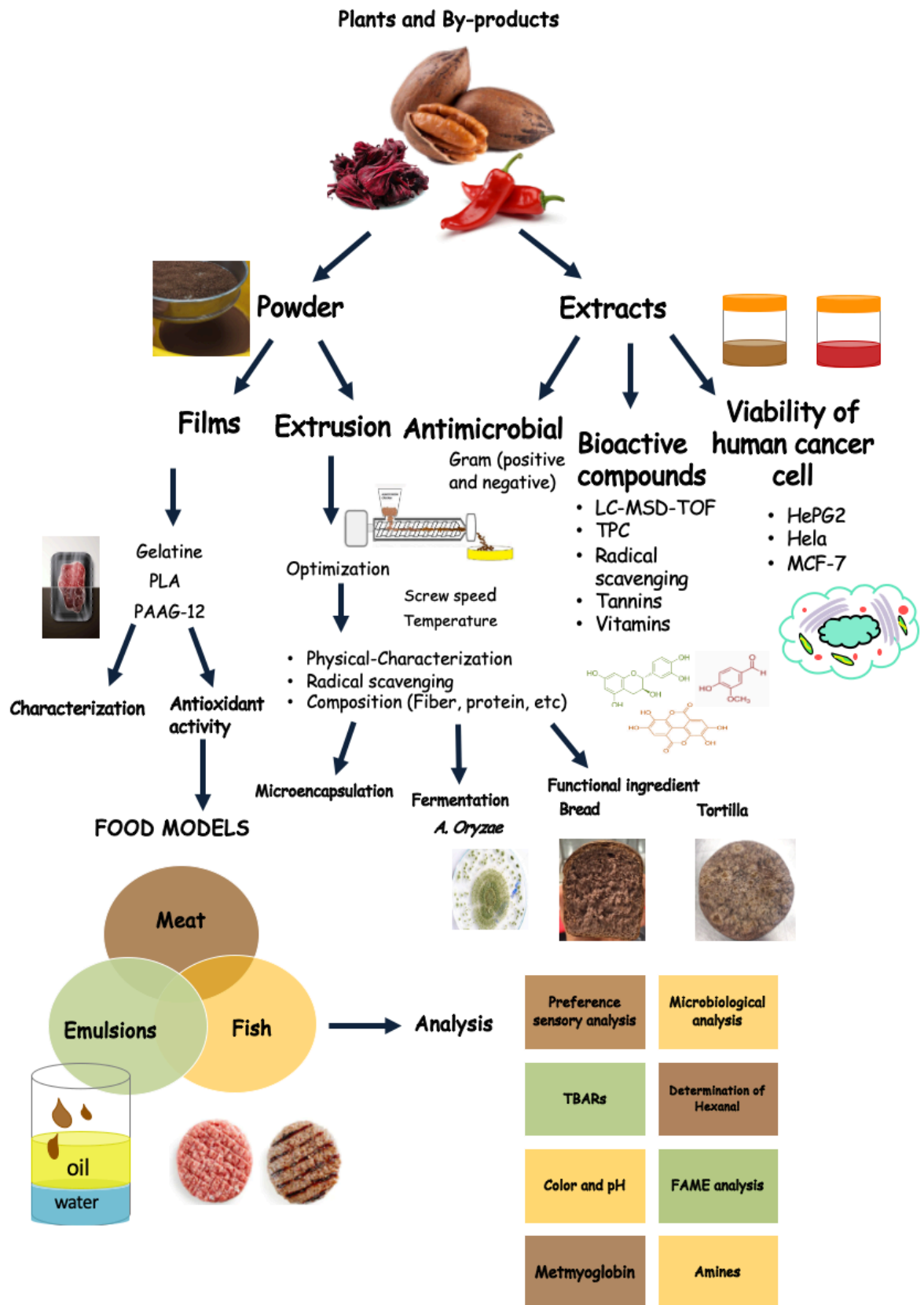
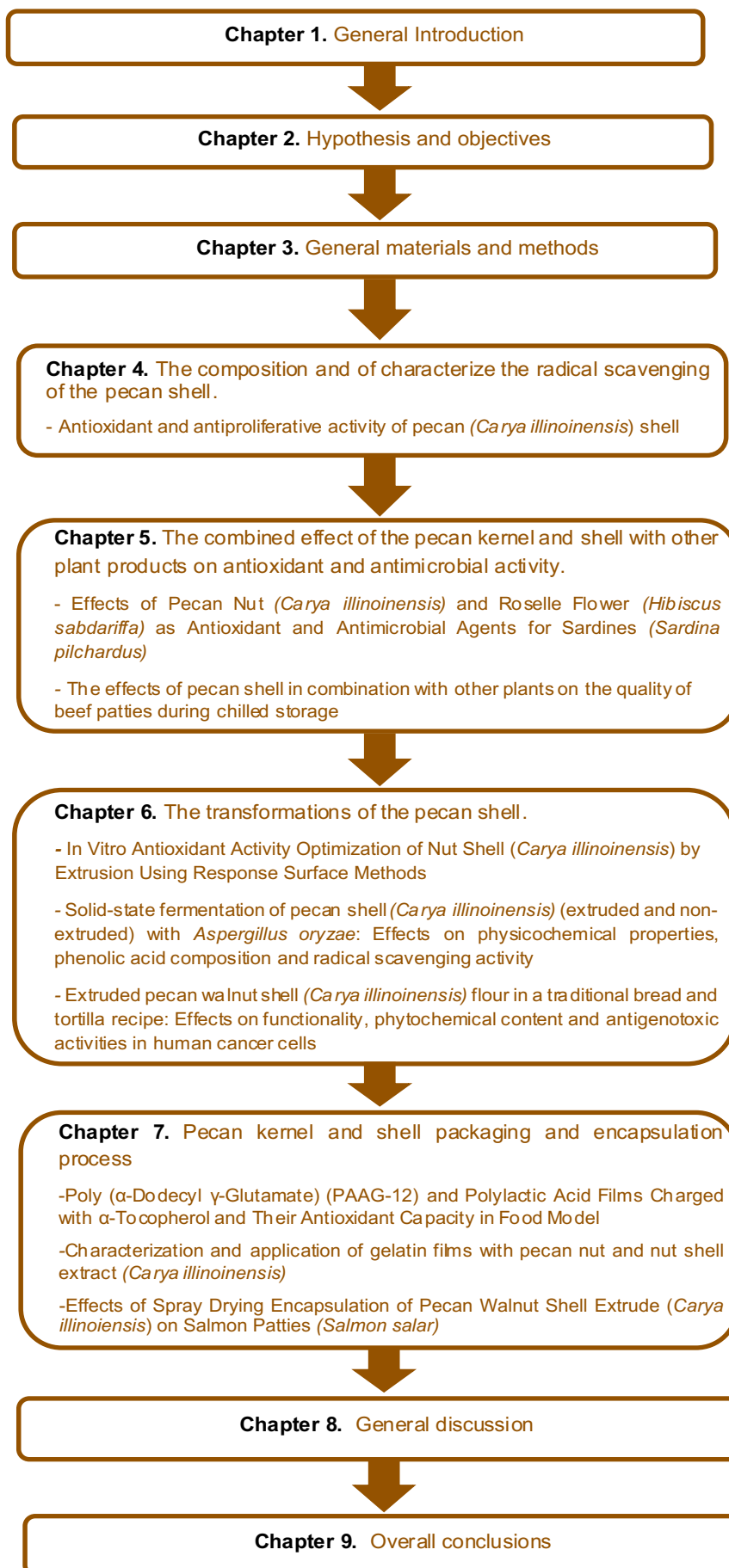


Figure 1. Document graphical abstract



**Figure 2.** The schematic outline of the dissertation

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## Chapter 1. General introduction

Food harvesting, processing and production generate high levels of waste and by-products. These types of residues have a significant environmental impact due to high organic content, and associated handling, transport, and storage costs [1]. Undernutrition and obesity coexist worldwide and both can be consequences of food insecurity. To manage nutritional concerns of today's society, more composite nutritional sources are required. Many of these residues can be a source of valuable macronutrients, micronutrients and bioactive compounds. Some of them can even have higher nutritional or functional value than the original product. Therefore, some of these compounds may have great potential for generating food additives or functional ingredients. Some examples of the use of these by-products and wastes are found in some fruits and vegetables. Oil, skin, pulp, and seeds from melons [2–5], mango, banana, lemon, and grape, are wasted. Such residues have a high content of polyphenols and fiber. Other studies have focused on the shells of chestnuts, almonds and walnuts, in which large amounts of tannins are found [6]. These by-products are currently studied as functional ingredients. Kaderides et al. incorporated pomegranate peel in cookies [7], Bedrníček et al. enriched bread with different onion by-products [8], and Cenobio-Galindo et al. added extracts of cactus pear on yogurt [9].

Antioxidants are molecules that inhibit free radical reactions and delay or inhibit cellular damage [10,11]. Antioxidant compounds in foods play an important role as a factor of health protection. Scientific research suggests that antioxidants reduce the risk of chronic diseases like cancer and Alzheimer [12]. Also, all foods containing fats or oils are susceptible to oxidation. As this process progresses, the sensorial characteristics and nutritional value of the food will deteriorate. To delay oxidation and extend shelf life of food, synthetic antioxidants are of common use in food industry. However, there is widespread agreement in replacing these with natural antioxidants due to their potential risks to health and the probable toxicity of some of them, such as butylhydroxytoluene (BHT) and butylated hydroxyanisole (BHA) [13]. Therefore, searching for natural antioxidants has received much attention, and efforts have been made to identify new natural active antioxidant compounds

### 1.1. *Plant antioxidants*

Fruits, vegetables, cereals, spices and traditional medicinal herbs are sessile organisms, and synthesize an array of secondary metabolites with important physiological and biological effects to defend themselves against exogenous biotic constraints [14]. The major classes of secondary metabolites are: phenols, terpenes and alkaloids. These phytochemicals, particularly phenol derivatives, frequently present an extensive range of biological effects in addition to antioxidant behavior, such as anti-inflammatory, antibacterial, antiviral, anti-aging, and anticancer activity [15,16]. Some principal terpenes include gibberellins, carotenoids and abscisic acid, which play an important role in plant pigmentation, growth and development. Alkaloids have been used as drugs to treat malaria and cancer [15].

Phenolic compounds play an important role in plant pigmentation, growth, reproduction, resistance to pathogens and predators, besides contributing to the nutritional properties of the plant

[17]. These compounds can be found as free forms or covalently bound to other macromolecules such as fatty acids, plant cell wall constituents (pectin, cellulose) and structural proteins [18].

Given the benefit of natural compounds in the human diet, the study of secondary metabolites has received great attention in recent years [18]. Different strategies have been studied with the objective of improving the production of secondary metabolites in plants, and also for optimizing their extraction. Santana-Gálvez et al. showed that postharvest (abiotic stresses) application of wounding stress in fruits and vegetables increases their nutraceutical content by inducing the biosynthesis and accumulation of phytochemicals. Abiotic stresses include plant submission to agents such as UV light, wounding, extreme temperatures, modified atmospheres (ozone, hyperoxia, hypoxia) salinity, nutrient (deficiency and excess), drought, phytohormones, and metals/metalloids [19]. Furthermore, the use of different technologies such as extrusion cooking [20], bioreactor [21], ultrasonication, solid-state fermentation [22], and high hydrostatic pressure [23] induces secondary metabolite production in plants.

For optimizing the extraction, there are no universal procedures for all plants. Procedures are highly dependent on the structures of the phytochemical compounds and the relative quantity present in the sample [24].

## 1.2. Pecan (*Carya illinoensis*)

Pecan (*Carya illinoensis*) walnut (PW) belongs to the family of Juglandaceae. The nut is known as pecan or paper-shell nut [25]. Pecans (*Carya illinoensis*) have been part of the human diet for years and they are commonly used in the bakery (41.8%), dairy (27.4%), snacks (23.8%), jellies and butters (5.6%), and drinks industry (1.4%) [26]. Mexico and the United States of America are responsible for 93% of the world's production of pecan nuts, with an average of nearly 60,000 and 40,000 metric tons per year, respectively [27]. 3

This type of walnut has beneficial nutritional components (Table 1), including polyunsaturated fatty acids, vitamins, such as E ( $\alpha$ -tocopherols), minerals, protein, and other functional compounds, such as polyphenols [28]. It has been shown to contain the highest concentration of phytochemicals of all nut types [29].

Nutrients	100 g of PW	Minerals	mg /100 g of PW	Vitamins	100 g of PW
Water (g)	3.52	Calcium	70	Riboflavin (mg)	0.130
Energy (Kcal)	691	Iron	2.53	Niacin (mg)	1.167
Protein (g)	9.17	Magnesium	121	Vitamin B6	0.210
Lipids (g)	71.97	Phosphorous	277	Vitamin A(IU)	56
Carbohydrates (g)	13.86	Potassium	410	Vitamin E (mg)	1.40
Fiber (g)	9.60	Sodium	0	Vitamin C (mg)	1.10

Sugar (g)	3.97	Zinc	4.53	Thiamin (mg)	0.66
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**Table 1.** Nutritional composition of pecan. Source: United States Department of Agriculture

In comparison with other nuts (brazil, pine, pistachio and cashew nuts), pecan nuts have the highest ratio of unsaturated/saturated fatty acids [30]. Venkatachalam et al. found that pecan nuts contain 66.73 g/100 g lipid of MUFAs (monounsaturated fatty acids) and 24.92 g/100 g lipid of PUFAs (polyunsaturated fatty acids) [31]. Oleic and linoleic acids are the predominant MUFA and PUFA, respectively, in the pecan [32].

Studies showed strong correlations between inclusion of pecan nut in the diet and reduction of blood triacylglycerols and LDL (low-density lipoprotein) cholesterol, while HDL (high-density lipoprotein) cholesterol increases [33].

Pecan nuts are also an important dietary source of  $\alpha$ -tocopherol ( $12.2 \pm 3.2$  mg/g) and  $\gamma$ -tocopherol ( $168.5 \pm 15.9$  mg/g) [32]. These tocopherols play an important role in the prevention of the oxidation of unsaturated fatty acids. Additionally, phenolic compounds in the nuts could be used as preservatives for the food industry. Phenolic compounds such as ellagic acid derivatives, catechin, epicatechin, gallic acid and tannins were identified and quantified in the pecan nuts by Medina-Juarez, et al [34], Lerma-Herrera et al. [34,35], Bouali et al. [36], and De la Rosa et al [37].

Previous research on the use of walnut as an antioxidant for meat showed its effectiveness for preventing food deterioration [38]. In addition, adding walnut paste to meat improved MUFA and PUFA content and altered the amino acid profile [38]. Orozco et al. concluded that the addition of pecan nut paste and oregano essential oil in the formulation of frankfurters can be a good strategy for increasing the UFA/SFA (unsaturated/saturated fatty acids) ratio without compromising the frankfurter quality [39]. Similar results were reported by Nieto et al. [40] and Colmenero et al [41]. However, pecan nuts are considered as allergenic foods. The consumption of food products containing pecan nut may cause severe allergy symptoms including life-threatening anaphylaxis in some individuals. Intensive research is underway to solve this problem [42,43].

**Green husk:** It is an outer pericarp which separates from the nut at maturity, acting as a protective cover from insects. It is used to make walnut liquor, which is rich in phenolic compounds and vitamins. Many phenolic compounds like chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, epicatechin, myricetin, and juglone were identified in green husk extracts [44].

**Pellicle and shell:** The pellicle is a special protective tan-brown skin that surrounds the kernel. It corresponds to 5% of the fruit weight. However, it contains the highest concentration of phenolic compounds compared to other parts of the nuts. The pellicle protects unsaturated fatty acids in the kernel against oxidation. Like the kernels, it also contains many phenolic acids such as chlorogenic acid, syringic acid, juglone, ellagic acid, ferulic and sinapic acid [45,46]. The shell is an inner thick pericarp which acts as a seal to protect the kernel from dust and insects. It can comprise 40-50% [47] of the total weight of the fruit and ranges from small hard-shelled wild types to those small paper-thin shells [45,48]. Pecan shell is an agricultural waste with little economic value or industrial usage, making it attractive as a potential source for natural phenolic extraction.

Prado et al. [49] found that pecan nutshell infusions have antibacterial activity. Müller et al. [50] found that the pecan shell infusion could be an economical agent to prevent liver diseases associated with ethanol consumption. Additionally, Reckziegel et al. [51] found that pecan shell tea prevents anxiety caused by cigarette abstinence, acting as a natural anxiolytic. On the other hand, in the industry, this by-product could be used as a biosorbent or as precursor of carbon for the removal of dyes and heavy metals from aqueous solutions [52,53].

The pecan shells are composed of around 70–80% fiber that is predominantly insoluble fiber: lignin, cellulose, and hemicellulose [37]. In addition, they contain proteins, various minerals, and also phenolic compounds and proanthocyanidins, including vanillic, caffeic, and gallic acid, catechin, and tannic acid, which could have an antioxidant effect on human and animals [54–56].

### **1.3. Roselle flower (*Hibiscus sabdariffa*)**

Roselle flower (*Hibiscus sabdariffa*), which is also known as “roselle”, “bissap” and “karkade”, is generally cultivated in countries with tropical and subtropical climate such as Malaysia, Nigeria, Sudan, India, Egypt, Mexico and Central America [57]. These flowers contain high, but variable amounts of total phenolics, flavonoids and anthocyanidins, depending on the cultivar, and were reported to possess antimicrobial activity for Gram-positive and Gram-negative bacteria [58]. Also, this crop has been reported to have therapeutic effects such as antihypertensive [59], anti-inflammatory [60], antihyperlipidemic [61], antifungal [62], and anti-carcinogenic [63].

Many studies, which were designed as applied research on food products, indicated the potential of *Hibiscus sabdariffa* extracts as food preservatives. In a meat model, Pinto et al. found that *H. sabdariffa* extracts maintain the ground beef microbiological stability during cold storage [64].

Incorporation of bioactive compounds from *H. sabdariffa* into emulsions (W/O) resulted in good oxidative stability during one month of storage at room temperature [65].

Additionally, Mata-Ramírez proposed to use roselle as a functional ingredient for the production of yeast-leavened bakery products, increasing levels of dietary fiber, phytochemicals and antioxidant activity [66]. On the other hand, Farias-Cervantes et al. [67] and Archaina et al. [68], used microencapsulation of the flower by spray drying for producing a stable functional product. In addition, Wihansah et al. [69] used roselle to improve the quality and the antioxidant activity of goat’s milk and cow’s milk probiotic yogurt. The authors studied the anti-diabetic potency of a probiotic yogurt supplemented with roselle extract, and found 36.70% inhibition.

### **1.4. Cayenne pepper (*Capsicum annuum*)**

Cayenne pepper (*Capsicum annuum*), belonging to the Solanaceae family, is a broadly diffused plant around the world. Cayenne pepper is commonly used for enriching food flavor [70]. However, *Capsicum annuum* contains phytochemical compounds, such as flavonoids, phenols, carotenoids, capsaicinoids, and vitamins [71]. Capsaicinoids are the compounds responsible for the spicy flavor of peppers [72], and reach almost 90% of hot chili peppers. Capsaicinoid properties include anticarcinogen [73], antioxidant [74], anti-diabetic [75], anti-inflammatory [76], protection of the gastric mucosa [75] and antimicrobial [70,77,78].



The high antioxidant and antimicrobial effect of *Capsicum annuum* make it a good option as a preservative in the meat industry. Carmona-Escutia et al. [78] used cayenne pepper in the preparation of chorizo. The authors concluded that Cayenne pepper not only contributed to both color and flavor, but also preserved the total content of biogenic amines after 19 days of ripening. Furthermore, Martinez et al. [79] added *Capsicum* peppers (sweet red and hot cayenne) and *Piper* peppers (black and white) powders to fresh pork sausages. The authors concluded that these types of peppers extended the shelf life of the fresh pork sausages.

### 1.5. Free radicals

Knowledge of the process of generation of free radicals is a pre-requisite to understand the mechanism of an antioxidant. A free radical is defined as any atom or molecule with one or more unpaired electrons [80]. The unpaired electrons tend to increase the chemical reactivity of an atom or molecule compared to the corresponding non-radical. These radicals are formed in the human body due to many factors, including electromagnetic radiation from the environment, smoke, breathing, etc. Some of these generated radicals can be reactive oxygen species (ROS) and nitric oxide (NO•). ROS include either oxygen radicals as the hydroxyl radical (•OH), peroxy radical (ROO•), the superoxide anion radical (O<sub>2</sub>•<sup>-</sup>) or non-radical compounds such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or singlet oxygen (O<sub>2</sub>) [10,80]. Some of the important radicals are listed in Table 2.

Hydroxyl radical (OH•) is generated in the body due to water split by gamma rays, whereas superoxide anion (O<sub>2</sub>•<sup>-</sup>) is formed by the interaction of various molecules present in the human body with oxygen. It has been established that approximately 1-3% of the oxygen consumed by the body is converted into ROS. Nitric oxide (NO•) is produced through a Ca<sup>2+</sup> sensitive mitochondrial NO synthase (mtNOS) [10,80].

Symbol	Name
<sup>1</sup> O <sub>2</sub>	Singlet oxygen
O <sub>2</sub> • <sup>-</sup>	Superoxide anion radical
•OH	Hydroxyl radical
RO•	Alkoxy radical
ROO•	Peroxy radical
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
LOOH	Lipid hydroperoxide

Table 2. List of ROS [81]

### 1.6. Types of antioxidants

Antioxidants can be classified by different criteria, such as molecular weight, solubility, mechanism of action and origin. In this study, the authors have chosen to classify them by their origin [82].

**Synthetic antioxidants:** These chemicals are synthesized to delay or inhibit lipid oxidation, making them of potential use in food industry, chemistry and medicine. The most popular synthetic antioxidants used are phenolic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG). However, BHA and BHT have been restricted by legislative rules to 1.0 and 0.25 mg/Kg/day, respectively. Nevertheless, a few studies have found that synthetic antioxidants may contribute to a variety of health concerns such as carcinogenic effects [83]. As a result, there is growing interest in discovering natural and safer antioxidants for food applications and an increasing trend in consumer preferences towards natural antioxidants.

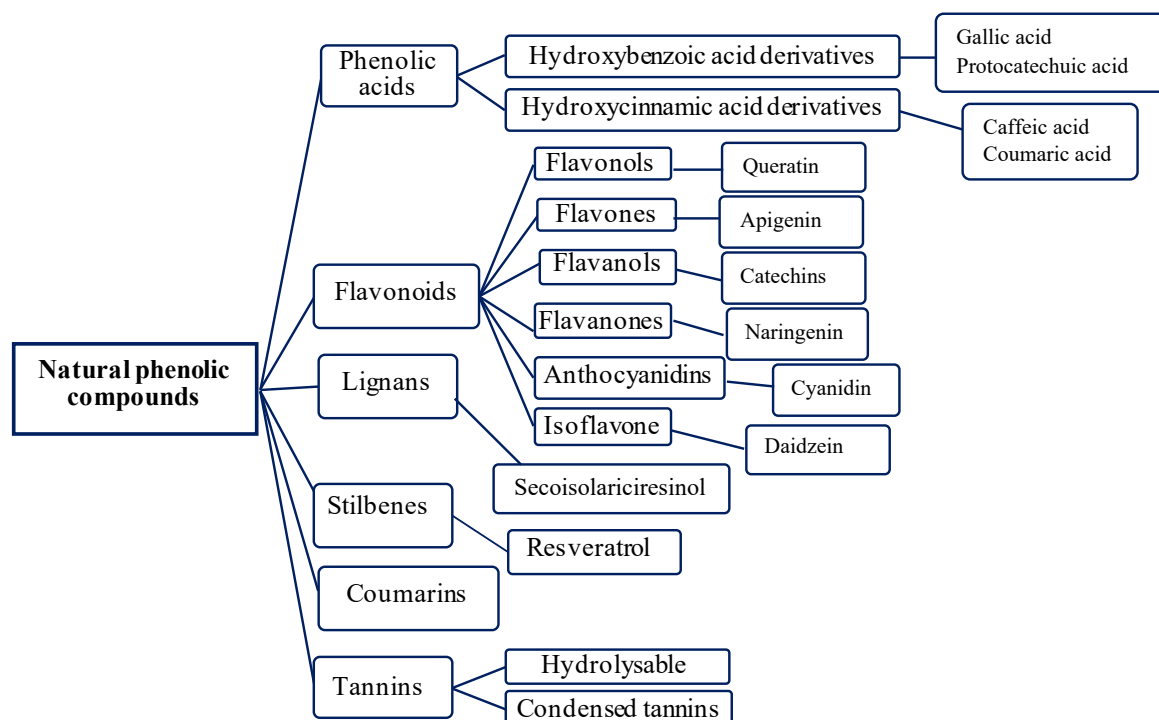
**Natural antioxidants:** As their name would suggest, natural antioxidants are derived from natural sources, most commonly plants. Unlike synthetic antioxidants, natural antioxidants are believed to be harmless to the consumers' health. Additionally, the natural antioxidants in food products provide health benefits such as reduction in the incidence of cardiovascular diseases and cancer [84]. As mentioned above, sources of natural antioxidants are primarily plant phenolic compounds that may occur in all parts of plants. They can be found in fruits, vegetables, nuts, seeds, leaves, flours, roots and barks.

Antioxidants can also be classified into two groups according to their mechanism of action [85]: Primary antioxidants and secondary antioxidants.

**Primary antioxidants** act by capturing free radicals directly and get destroyed during the induction period. Phenolic compounds are included in this category.

**Secondary antioxidants** do not capture free radicals directly but use other mechanisms to delay or inhibit oxidation, such as removing local oxygen, catalytic metal ions, quenching singlet oxygen or absorbing UV radiation. Normally, secondary antioxidants only work in the presence of another component. For example, citric acid displays antioxidant activity only in the presence of metal ions.

### 1.6.1. Phenolic compounds or polyphenols



**Figure 1.** Classification of phenolic compounds [13]

#### a. Phenolic acids

Phenolic acids (as shown in the Figure 1) are hydroxy derivatives of aromatic carboxylic acids, which can be divided into two categories: hydroxybenzoic acid derivatives and hydroxycinnamic acid derivatives. Hydroxybenzoic acid (Figure 2) derivatives come from the benzoic acid group such as gallic acid, whereas hydroxycinnamic acid derivatives belong to the cinnamic acid group, including coumaric, caffeic and ferulic acid [13].

Phenolic acids behave as antioxidants due to the reactivity of their hydroxyl substituent on the aromatic ring. The radical scavenging activity of phenolic acids and their derivatives depends on the number and position of the hydroxyl groups bound to the aromatic ring, the binding site and mutual position of hydroxyl groups (in the aromatic ring) and the type of substituents. The presence of electron-donating groups like hydroxyl in the ortho or para position increases the stabilizing effect by resonance on the phenoxyl radical; thus, enhancing the antioxidant activity of the aromatic ring [82]. For example, hydroxycinnamic acid derivatives have significantly higher radical scavenging activity than benzoic acid derivatives due to a stabilizing effect by resonance of the conjugated double bond in the side chain of hydroxycinnamic acid (Figure 3) [86]. Also, steric hindrance of the phenolic hydroxyls by a neighboring inert group, such as methoxyl groups, improves its antioxidant activity. The combination of two acid phenols increases the efficiency [82].

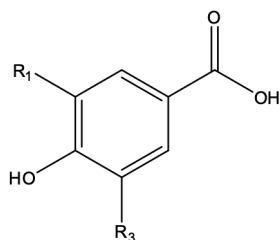


Figure 2. Hydroxybenzoic acid (PubChem)

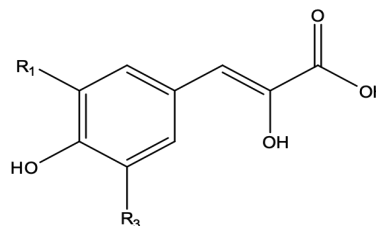


Figure 3. Hydroxycinnamic acid (PubChem)

## b. Flavonoids

Flavonoids are the most abundant plant phenolic compounds of low molecular weight present in plants, mostly found as glycoside derivatives. Flavonoids are cyclized diphenyl propanes that consist of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle as shown in Figure 4. The antioxidant ability of flavonoids depends on the following factors:

Metal-chelating potential (dependent on the arrangement of hydroxyls and carbonyl group around the molecule). The presence of hydrogen or electron-donating substituents are able to reduce free radicals.

The ability of the flavonoid to delocalize the unpaired electron leading to the formation of a stable phenoxyl radical.

For maximal radical scavenging activity (RSA), a flavonoid molecule needs to meet the following criteria: 3,4-dihydroxy structure in the B-ring, 2,3-double bond in conjunction with a 4-oxo group in the C-ring, presence of a 3-hydroxyl group in the C-ring and a 5-hydroxyl group in the A-ring (Figure 5) [82,85,87].

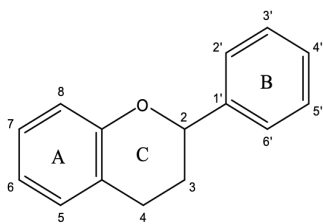


Figure 4. Flavonoid structure (PubChem)

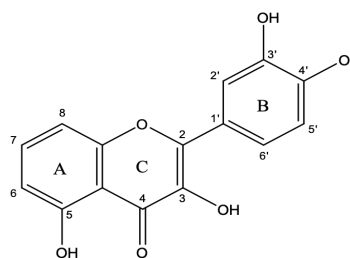


Figure 5. Flavonoid structure with maximal RSA (PubChem)

According to the oxidation state of the central C ring and different substituent positions, flavonoids are further divided into seven subgroups: flavonols, flavones, flavanols, flavanones, flavanonols, anthocyanins and isoflavones [88].

Flavonoids	Description	Structure
Flavonols	Flavonol can be detected in moringa, strawberry, spinach and cauliflower.	 Flavonol

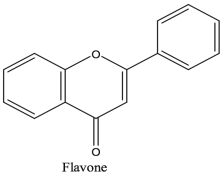
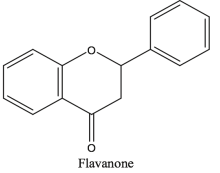
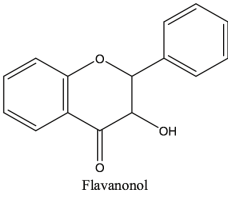
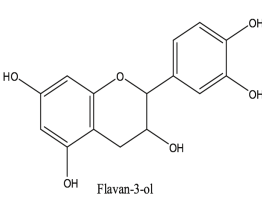
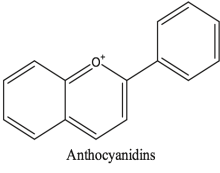
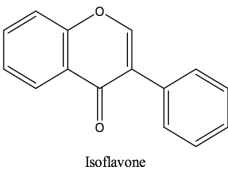
Flavones	Flavones can be found in celery, parsley and artichoke.	 Flavone
Flavanones	Flavanones are mostly found in citrus fruits in their glycosidic forms such as orange, lemons etc.	 Flavanone
Flavanonols	Flavanonols are present in white grapeskin, soyabean and some other fruits.	 Flavanonol
Flavanols (Catechin)	Flavanols are found abundantly in fruits such as apricots, sour cherries, grapes and blackberries. Catechin, a flavanol is also found in tea in significant amount.	 Flavan-3-ol
Anthocyanins	These are mostly found in berry fruits (black currant, raspberry, blackberry, etc.). Anthocyanins provide the orange, red, blue and purple colours of many fruits and vegetables such as apples, berries, beets and onions.	 Anthocyanidins
Isoflavonoids	Soybeans are almost the sole dietary source of isoflavones	 Isoflavone

Table 3. Classification of flavonoids [13] (Images from PubChem)

### c. Lignans

Lignans are stereospecific dimers of these cinnamic alcohols (monolignols) bonded at carbon 8 (C8-C8). In general, lignan content of foods is low and does not exceed 2 mg/100 g except for flaxseed and sesame seeds, which have 100 times higher lignin content than other sources. Lignans are also found in whole grains, nuts and legumes. In smaller amounts, lignans are also present in fruits and vegetables such as asparagus, grapes, kiwi fruit, lemons, oranges, pineapple, wine and even in coffee and tea [89,90].

### d. Stilbenes

Stilbenes are hydroxylated derivatives of stilbene with a C6-C2-C6 structure. It has a central double bond substituted with phenyl groups on each carbon atom of the double bond; the phenyl groups have several substituted hydroxyl groups (figure 6). Stilbenes, specifically; trans-resveratrol and its glucoside, have antioxidant, anticarcinogenic, and antitumor properties. Trans-resveratrol is produced by plants in response to damage and it is highly abundant in red wine and peanuts [13,91].

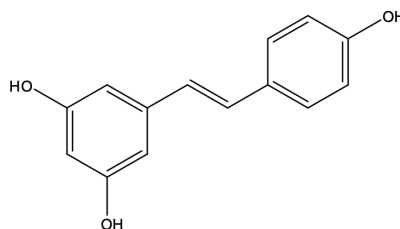


Figure 6. Resveratrol structure (PubChem)

#### e. Coumarins

Coumarins have the ability to inhibit lipid peroxidation and possess vasorelaxant, anti-inflammatory and anticoagulant activity. The recognition of key structural features within the coumarin family is crucial for the design and development of new analogues with improved activity and for the characterization of their mechanism of action and potential side effects. The coumarins are extremely variable in structure, due to the various types of substitutions in their basic structure, which can influence their biological activity [92].

#### f. Tannins

Tannins are compounds of intermediate to high molecular weight up to 30,000 Da. The phenolic groups of tannins can bind with the —NH groups of peptides and proteins, preventing their hydrolysis and digestion in the stomach. On the other hand, tannins can inhibit lipid peroxidation and scavenge radicals such as hydroxyl, superoxide, and peroxy. Also, tannins are toxic to bacteria, fungi and yeast [93]. Plant tannins can be subdivided into two major groups: hydrolyzable and condensed tannins [85].

**Hydrolyzable tannins:** Hydrolyzable tannins consist of a central core of glucose or another polyol esterified with GA, or with hexahydroxydiphenic acid. Hydrolysable tannins derived from GA are known as gallotannins and those derived from hexahydroxydiphenic acid are known as ellagitannins [94].

**Condensed tannins:** Condensed tannins are formed of flavan-3-ol linked through an interflavan carbon bond. Depending on the hydroxylation pattern of rings A and B of the flavan-3-ol units they are classified into: procyanidins, prodelfinidins and propelargonidins [95]. The structure diversity makes them rich in significant bioactivities such as anti- $\alpha$ -glucosidase, anti-cancer, antioxidant, and anti-tyrosinase activity [96].

### 1.6.2. Vitamins as antioxidants

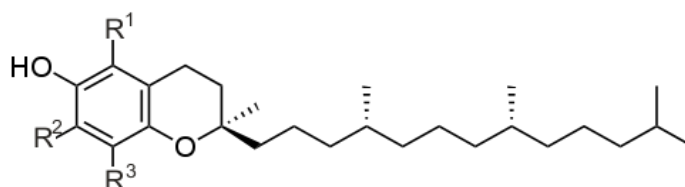
Vitamins are organic compounds and essential nutrients for the human body. Some vitamins have antioxidant properties and can be used as preservatives in the food industry through increasing the shelf life of products by preventing oxidation and consequent rancidity and discoloration [97].

### a. Vitamin C

Vitamin C (ascorbic acid, ascorbate) is an essential micronutrient that has to be supplied either with the diet or as a supplement. This hydrosoluble vitamin is found in plants (orange, broccoli, kiwi, etc.) and animals [98]. Vitamin C is reported to exert beneficial effects in the immune system and inflammatory response, which is crucial in fighting precancerous and other diseases [99]. Also, ascorbic acid has roles in the synthesis of collagen, hormones, neurotransmitters, and iron absorption [100].

### b. Vitamin E

Vitamin E is found mainly in vegetables, plants, nuts and vegetable oils. This liposoluble vitamin can be classified into tocopherols and tocotrienols [101]. Both class of compounds are subdivided into four lipophilic isomers:  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -Tocopherol, with  $\delta$ -Tocopherol being the most active antioxidant form of vitamin E, which acts as an antioxidant *in vivo* by reacting with free radicals solubilized in membrane lipids. Its active center is located at the hydroxyl group in position 6 of the aromatic ring. Vitamin E exerts important roles in reproduction, muscle function, red blood cell maintenance, and immune function [102].



**Figure 7.** Chemical structure of the four types of tocopherol.  $\alpha$ -Tocopherol:  $R^1$ ,  $R^2$  and  $R^3$  are  $\text{CH}_3$ .  $\gamma$ -Tocopherol:  $R^2$  and  $R^3$  are  $\text{CH}_3$  and  $R^1$  is H.  $\beta$ -Tocopherol:  $R^1$  and  $R^3$  are  $\text{CH}_3$  and  $R^2$  is H.  $\delta$ -Tocopherol:  $R^3$  are  $\text{CH}_3$  and  $R^1$   $R^2$  is H (Pub-Chem)

Food	Type of tocopherol	Study
Nuts: almond, hazelnut, peanut, pecan, walnut, apricot, pistachios, and cashew.	$\alpha$ , $\beta$ , $\gamma$ , and $\delta$	[103]
Sunflower and flaxseed oils	$\alpha$	[104]
Apple seed oil	$\alpha$ , $\beta$ , $\gamma$ , and $\delta$	[105]
Eggs	$\alpha$ , $\beta$ and $\gamma$	[106]
Avocado	$\alpha$ , $\beta$ , $\gamma$ , and $\delta$	[107]
Cocoa	$\alpha$	[108]
Cheese	$\alpha$ , $\beta$ and $\gamma$	[109]

**Table 4.** Examples of food with tocopherols

## 1.7. **Analysis and quantification of phenolic compounds and radical scavenging**

Generally, the methods used for the analysis of phenolic compounds measure the total phenolic content (Folin-Ciocalteu method), a specific group or class of phenolic compounds (flavonoids, anthocyanins, tannins etc.) or total radical scavenging capacity by using different radicals (AAPH, ABTS, DPPH etc.). Several factors can influence quantification of these compounds, such as the chemical nature of the analyte, as well as the assay method, selection of standards and presence of interfering substances.

There are no gold standard methods for quantifying phenolic compounds due to the heterogeneity of natural phenols and the possible interference from other readily oxidized substances present in plant materials. Therefore, different methods must be used for reaching this purpose.

Ideally a standardized method for antioxidant activity of a food component should meet the following requirements: (1) simple and reproducible; (2) use of a biologically relevant radical source; (3) having a clear endpoint and chemical mechanism; (4) commercially available chemicals and instrumentation; (5) adaptability for assaying both hydrophilic and lipophilic antioxidants and use of different radical sources; and (6) adaptability to high throughput analysis for routine quality control [110].

Depending on the mechanism, these methods can further be divided into two groups: methods involving HAT (hydrogen atom transfer) and methods involving SET (single electron transfer) [111,112]. The result of both mechanisms is similar, but the kinetics and potential for side reactions differ. However, sometimes it is difficult to distinguish these two reaction mechanisms.

**HAT based methods:** These methods measure the capacity of an antioxidant to quench free radicals by hydrogen atom donation. HAT reactions are quite fast and don't depend on solvent and pH. The presence of reducing agents, including metals, can lead to erroneous high results. Methods based on the HAT reaction include the ORAC (Oxygen Radical Absorbance Capacity) assay [110,113].

**SET based methods:** These methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls and radicals. As the oxidant is reduced, a change of color is observed. The reaction's end is determined when the color change stops. The degree of color change is proportional to the concentration of antioxidant. The SET reaction mechanism involves deprotonation and ionization potential of the reactive functional group, thus making the reaction pH dependent. SET reactions are usually slow, so radical scavenging capacity calculations are based on product percentage decrease rather than kinetics. SET methods are very sensitive to ascorbic acid, uric acid and metal ions, which cause high variability and poor reproducibility. The SET-based methods include the total phenolic assay by Folin-Ciocalteu reagent, Trolox Equivalence Antioxidant Capacity (TEAC) assay with ABTS as radical, Ferric ion Reducing Antioxidant Power (FRAP) assay and 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH<sup>•</sup>) scavenging [11,114].



**Table 5.** Methods: HEAT (hydrogen atom) and SET (single electron transfer)

Method	Assay	Principle	Disadvantages
HAT based method	ORAC	It is based on the inhibition of the peroxy-radical, oxidation initiated by the thermal decomposition of azo-compounds such as 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) [115]. The antioxidant competes with a fluorescent or pyrogallol red probe for quenching peroxy radicals generated from AAPH [11,110].	The low reactivity of fluorescein toward peroxy and hydroxyl radicals, also not against all reactive oxygen species [112].  The fluorescence intensity in a non-polar organic solvent is low [112].
SET based method	FRAP	It is based on the ability to reduce Fe (III) tripyridyltriazine (yellow) complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by electron-donating antioxidants [115].	The reaction is nonspecific, and any compound with a suitable redox potential will drive Fe III-TPTZ reduction.  Some antioxidants such as glutathione, can effectively reduce prooxidants but are not able to reduce Fe III.
	DPPH	A molecule with hydrogen/electron bond, reacts with the colored purple radical DPPH• (2,2-diphenyl-1-picrylhydrazyl) causing discoloration to a pale yellow, according to the number of electrons captured [11,110].	Depends on the reaction time, taken alone this parameter does not provide meaningful information of the actual reactivity of the antioxidant.  Some antioxidants have spectra that overlap with DPPH at 515 nm and interfere with the results.  DPPH radical can only be dissolved in organic solvents.
	TEAC	Based on the reaction with the colored (blue/green) and relatively persistent 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+•) radical cation (or monoanion, if the two sulfonate groups are considered) [110].	ABTS+• is a radical cation while the peroxy radical is neutral, so antioxidants react with ABTS+• by an electron transfer mechanism, whereas with peroxy radicals they react by formal H-atom transfer.
	Folin-Ciocalteu	Based on the transfer of single electrons (SET) in alkaline medium from phenolic compounds to molybdenum to form a blue complex [113].	It is not applicable for lipophilic antioxidants. Suffers from interference from sugar, aromatic amines, sulphur acids, Fe II.

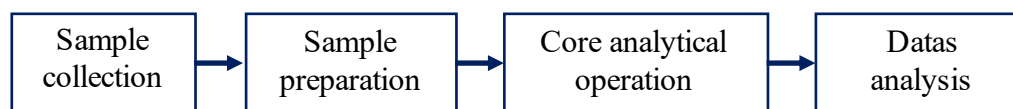
In conclusion, any currently available assay has limitations in reflecting the total antioxidant capacity of a specific sample. Therefore, it is convenient to use more than one assay to evaluate the antioxidant capacity of a natural compound. Assay selection depends on the type of sample, whether it is lipophilic or hydrophilic. Each assay has its own advantages and disadvantages.

### 1.8. **Methods for quantification and possible identification of antioxidant compounds**

An analytical method to separate, identify and quantify natural substances such as polyphenols in plants, must be sufficiently efficient, selective and sensitive [116]. Nowadays, different separation techniques, specific stationary phases and detectors are available.

The most common technique to use in many organic, biological, and environmental analyses is chromatography. For example, the use of a liquid chromatography (LC-MS/MS) method with MS detection *versus* high-performance liquid chromatography (HPLC) method with UV detection may offer higher sensitivity and positive identification of the analytes [116].

**High-performance liquid chromatography (HPLC)** is still the most popular used analytical method for the analysis of phenolic compounds (Figure 8). It is the most appropriate for quantitation purposes [117]. Some advantages of this technique are the relatively low cost involved in the analysis, high versatility and precision [118]. The separation of phenolic compounds was improved by using reversed-phase (RP) columns, with the most common being the C18 column. Lower molecular mass polyphenols can be analyzed by HPLC on normal or reversed-phase columns. However, HPLC methods provide insufficient resolving power and poor resolution when facing high molecular weight phenolic compounds and molecular mass distributions [119].

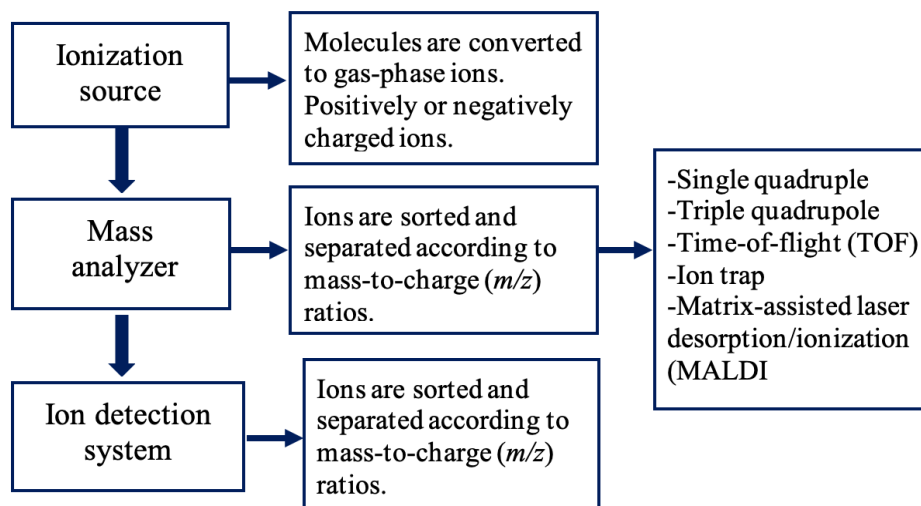


**Figure 8.** Simplified schematics for the path of a chemical analysis [117]

**Table 6.** Common HPLC detector attributes [120]

Detector	Key Attributes	Limitations
UV/Vis/Photodiode array (PDA)	Most widely used. Selective and Universal. Gradient compatible. Qualitative and Quantitative. PDA peak purity/homogeneity, spectral library searches. Nondestructive samples, low cost, very reliable and easy to use.	Must have a chromophore, solvents must be transparent, widely varying response for different solutes.
Light Scattering	Detects most nonvolatile analytes. Universal. Works well with gradient HPLC. Better sensitivity than RI detection.	Requires the use of volatile buffers. Optimization, limited dynamic range and reproducibility of methods.
Fluorescence (FL)	Very selective and sensitive. Works well with gradients.	Not all compounds fluoresce.
Refractive index	Original detector for HPLC in many methods. Universal. Excellent versatility, solvent compatibility, nondestructive, low cost, reliable and easy to operate.	Sensitivity. Gradient incompatible. Stability (temperature and flow).
Conductivity	Detector of choice for ion chromatography-inorganic ions and organic acids. Very selective and low cost.	Requires suppression of mobile phase background noise and electrode fouling.

**Mass Spectrometry (MS)** is an analytical technique useful for measuring the mass-to-charge ratio ( $m/z$ ) of one or more molecules present in a sample. It can be used to identify unknown compounds via molecular weight determination, to quantify known compounds, and to determine structure and chemical properties of molecules. The components of a mass spectrometer are shown in Figure 9.



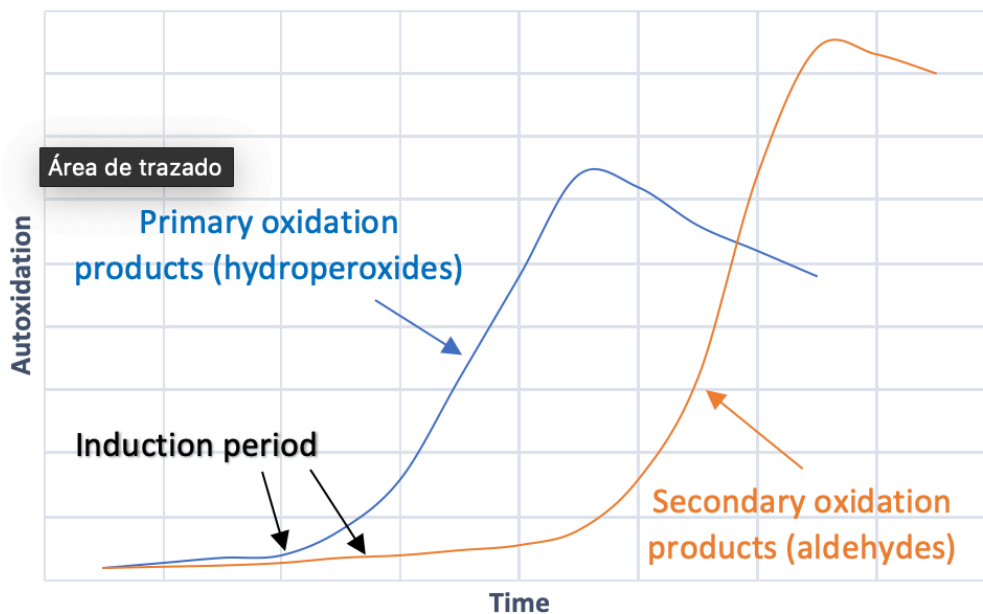
**Figure 9.** Components of mass spectrometer [121]

**Liquid Chromatography-Mass Spectrometry (LC-MS)** is an analytical technique that combines the physical separation of HPLC with the mass analysis capabilities of mass spectrometry. This technique is nowadays popular and considered the best analytical approach to study polyphenols. While HPLC separates the mix of different components, mass spectrometry separates them according to their specific mass-to-charge ratio ( $m/z$ ) and allows identification of the components with high molecular specificity and detection sensitivity [122].

### 1.9. Lipid oxidation

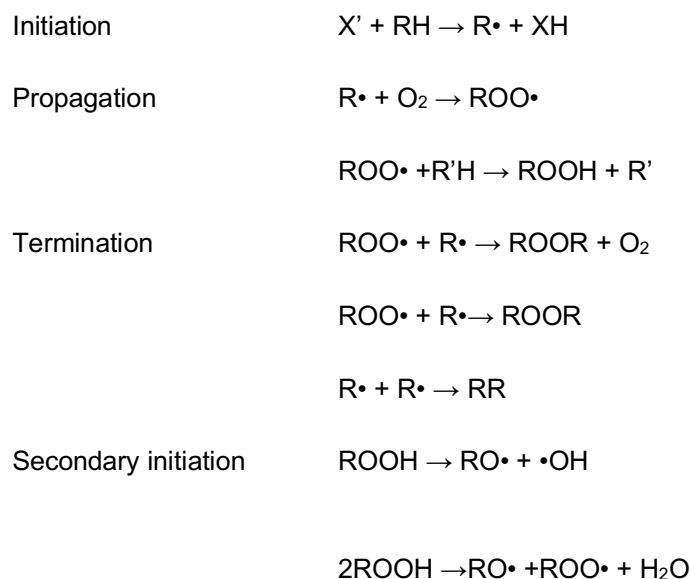
Lipid oxidation is a matter of great concern in the food industry as it causes food quality deterioration that occurs during manufacturing, storage, distribution and final preparation of foods. All products that contain lipids, even at a very low level, are susceptible to oxidation [123]. Due to the presence of fatty acids, which are electron deficient (due to the presence of carbonyl group ( $C=O$ ) and unsaturated carbon bonds ( $C=C$ )), lipids are more susceptible to oxidation and this can result in unwanted changes in color, texture, and appearance of rancid odor and flavor, which influences consumer acceptance [124]. In addition, changes in the nutrient value occur and multiple toxic compounds are produced during lipid oxidation [125].

Lipid oxidation is an autocatalytic free radical chain reaction, which can be divided into three stages (figure 11): initiation (generation of free radicals), propagation (sequence of free radical reaction) and termination (formation of non-radical products).



**Figure 10.** Theoretical development of primary and secondary lipid oxidation products as a function of time [87]

The autoxidation process in lipids starts with an induction period where little or no change occurs. After some time, primary oxidation products are formed. The process can be positively influenced by a source of energy (temperature or light), the presence of catalytic compounds including transition metals and increasing amounts of prooxidants and polyunsaturated fats. The main primary products of lipid oxidation are hydroperoxides, which accumulate during initiation and propagation steps. Hydroperoxides break down easily into highly reactive alkoxy ( $\text{RO}\cdot$ ) and hydroxyl ( $\cdot\text{OH}$ ) radicals by reactions catalyzed by transition metals or other prooxidants in highly unsaturated lipids, and reach maximum level of oxidation exponentially. After reaching the maximum oxidation level, a drop in hydroperoxide value is observed. This drop is caused by decomposition of hydroperoxides into secondary oxidation products i.e. aldehydes, alcohols, and other volatile and nonvolatile compounds. The carbon-carbon cleavage of alkoxy radical gives rise to the formation of aldehydes (Figures 10 and 11) [87,125,126].



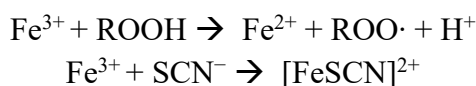
**Figure 11.** Mechanism of lipid oxidation [87]

### 1.9.1. Methods to determine lipid oxidation

Primary oxidation: Hydroperoxides are the main primary oxidation products, accumulating during the initiation and propagation step of the oxidation process. The Peroxide Value (PV) test is the most commonly used to measure primary oxidation products. Peroxide value directly measures the concentration of hydroperoxides formed in the initial stage of lipid oxidation.

In the presence of an initiator (light, heat or metal ions), unsaturated lipids generate carbon-centered alkyl radicals ( $L\cdot$ ), which in the presence of oxygen form unstable peroxy radicals. These peroxy radicals can abstract a hydrogen atom from a lipid molecule to form hydroperoxides.

Hydroperoxides can be measured by an official titration method based on their oxidation potential to oxidize iodide ( $I^-$ ) to iodine which is quantified using another titration against a standard solution of sodium thiosulphate. Their concentration can also be measured by using a colorimetric method based on their oxidation potential to oxidize iron (II) to iron (III) in an acidic medium. Iron (III) then forms a complex with thiocyanate to form a red-violet complex (ferric thiocyanate) which absorbs at 500-510 nm. This method is simple, reproducible, and more sensitive than the standard iodometric assay, and has been used to measure lipid oxidation in milk products, fats, oils, and liposome [127,128].



Secondary oxidation: A large variety of secondary oxidation products are produced through decomposition of fatty acid hydroperoxides. Analysis for determination of secondary oxidation products therefore tends to focus on a single compound or group of compounds.

The reacting secondary products are generally referred to as TBA-reacting substances (TBARs), because the reaction can involve several secondary oxidation products [129].

Lipid oxidation produces various volatile compounds including hydrocarbons, aldehydes, hexanals, dienals, ketones, and organic acids. As oxidation increases, these volatiles increase and can be measured by gas chromatography (GC).

## **1.10. Food models and real food**

Model food systems are based on the formulation and processing of foods, using laboratory and pilot plant facilities [130]. The goal of using these systems is determining how the ingredients and the process alter the characteristics of the final product. Emulsions are an example of this system. Factors such as initial composition can be more easily controlled in model food systems than using real food (meat, fish, etc).

### **1.10.1. Emulsions**

Radical scavenging assays are of great help to screen antioxidants, but they do not evaluate effectiveness of such antioxidants in the real food system, which largely depends on various factors including polarity, solubility, and metal-chelating activity [87]. Several dietary lipids are either water-in-oil (like butter and margarine) or oil-in-water emulsions (like mayonnaise, milk and cream). Therefore, the oil-in-water emulsion system is often used to study antioxidant effectiveness and shelf-life in food emulsions. Emulsion is a thermodynamically unstable system, which consists of two immiscible liquids (usually oil and water), with one being dispersed into the other in the form of small spherical droplets with the help of an emulsifier (0.1-100  $\mu\text{m}$ ). Emulsifiers are surface active molecules that stabilize the emulsion by reducing the surface tension [125].

Antioxidant effectiveness in emulsion food systems is affected by important factors determined by localization and orientation of antioxidants (between the aqueous phase and the lipophilic phase) and their interaction with emulsifiers [131]. In the food industry, the emulsifiers are classified in high-molecular weight molecules such as proteins (caseins,  $\beta$ -lactoglobulin, etc.) and low-molecular weight compounds such as phospholipids (soy and egg lecithin's), monoglycerides, sucrose and sorbitan esters (Span<sup>®</sup>), polysorbates (Tween<sup>®</sup> and polysorbate 80). An important concept for selecting an emulsifier or an optimal mixture, is the lipophilic-hydrophilic balance [132]. In oil-in-water emulsion systems, hydrophobic antioxidants are found in the oil and the oil-water interface, where they are more protective than hydrophilic antioxidants, which are partitioned into the aqueous phase and are not able to adequately protect lipids in the water-oil interface [125,126].

In the first stage of lipid oxidation, the free radicals react with the oxygen and produce hydroperoxides. The hydroperoxide value at which oxidation of oils can be detected as an off-flavor depends on the nature of the oil in the emulsion. Vegetable oils and fats with hydroperoxide values of <10 meq/kg are considered acceptable for human consumption. When the hydroperoxide values are above this value, the sample is considered rancid, which suggests change in color, taste and nutritional quality due to the deterioration of lipids [126,133].

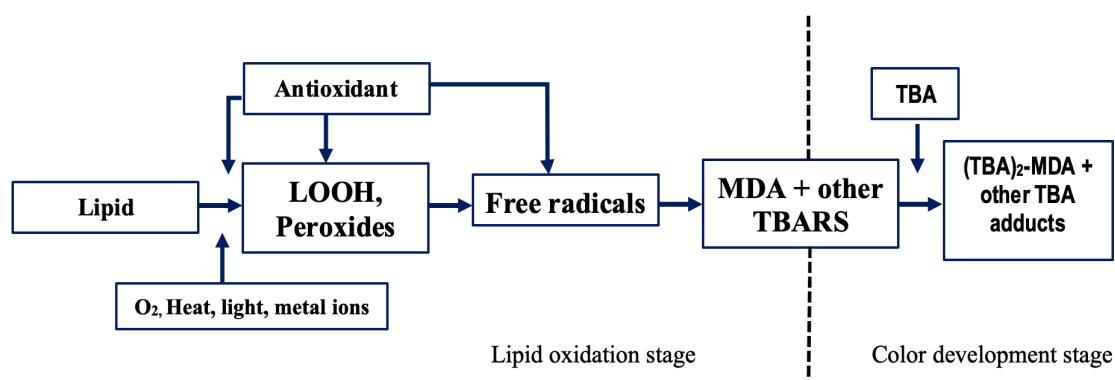
Costa et al. [134] studied the antioxidant capacity of an extract of *Thymus vulgaris* (thyme) by-products in simple oil-in-water emulsions in which the lipid phase was constituted by almonds or wheat germ oils (natural products). The authors found that the combination of higher concentrations of thyme waste extract (0.02%, 0.04%) and almond oil ( $\geq 50\%$ ) had the highest protective effect to

prevent primary oxidation of emulsion samples. Martillanes et al. evaluated the influence of antimicrobial and antioxidant activities of different bran extracts on a mayonnaise-type emulsion (O/W). The results of this experiment showed that the ethanolic extract was the most effective, preventing the oxidation and the growth of some microorganisms in the mayonnaise [135].

### 1.10.2. Meat

Lipid oxidation is a main concern in the meat industry, where oxidation gives rise to toxic compounds that change the meat quality: nutritional loss, color, aroma, flavor, etc [136]. Compounds that could be formed include aldehydes such as malondialdehyde (MDA) or 4-hydroxy-2-nonenal (4-HNE), which are identified as cytotoxic and genotoxic [137]. An efficient method to determine the oxidation grade in meat products during storage is the Thiobarbituric Acid Reactive Substances (TBARS) assay. This method consists of a reaction producing a pink-colored dimeric compound that is quantified using visible spectrometry at 531 nm [138]. The formation of metmyoglobin (which alters the color of the meat) is also associated with the oxidation of oxymyoglobin during storage [138].

Meat models have commonly been used to investigate lipid oxidation inhibition capacity of various natural antioxidants. Kim et al. found that meat treated with ethanol extracts of coffee residue after 12 days showed less than half MDA/kg of raw-meat in comparison with the control sample (without coffee residue) [139]. Souza et al. [140] worked with “banana inflorescences” residues and found that the addition of these by-products to a meat sausage formulation had a very positive effect on the control of lipid oxidation during storage. Furthermore, the addition of banana inflorescences (2%) did not affect the sensory acceptance of the products [140]. Figure 12 shows the stages of lipid oxidation and antioxidant action in the TBARS antioxidant activity assay.



**Figure 12.** Stages of lipid oxidation and antioxidant action in the TBARS antioxidant activity assay [136]

### 1.10.3. Fish

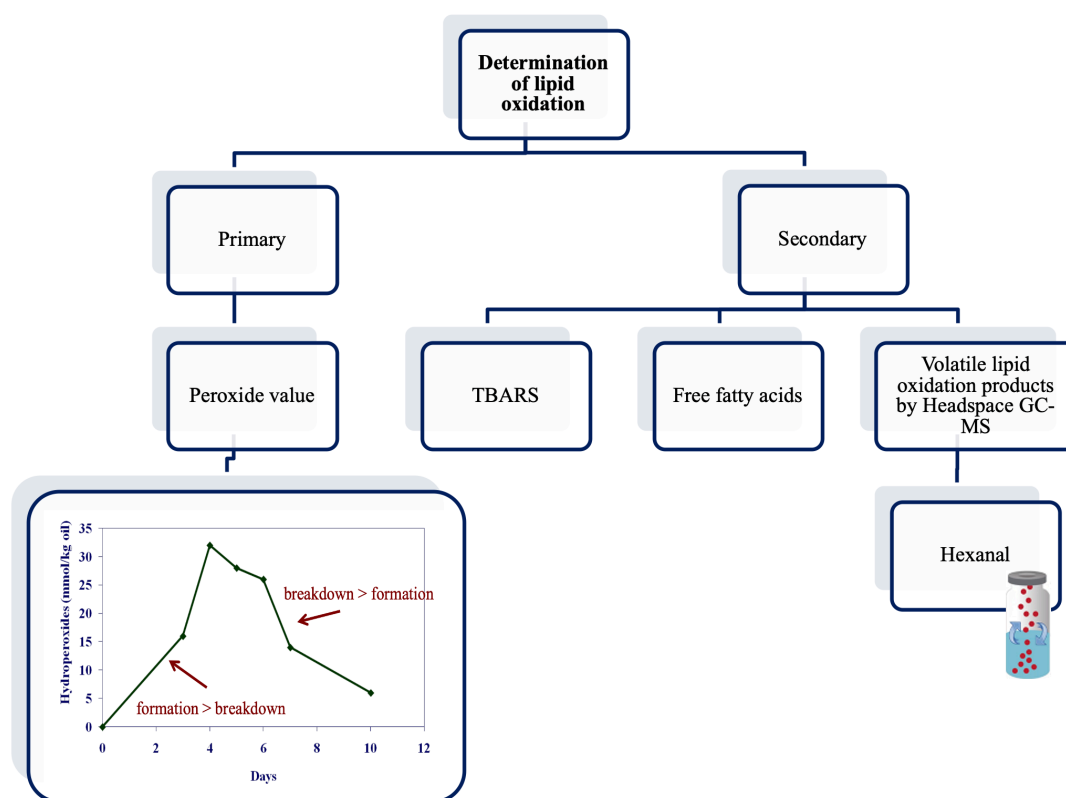
Similarly, to meat, fish flesh is subject to high chances of sensorial and nutritional quality loss, due to oxidation [141,142]. Fish products provide high contents of protein and polyunsaturated fatty acids (PUFA) [143,144], such as eicosapentaenoic acid (EPA or C20:5n3) and docosahexaenoic acid (DHA or C22:6n3). They offer remarkable nutritional health benefits, but are also considered amongst the most perishable foods [145]. The degradation process is carried out at first by muscle enzymes, eventually by microbial enzymes [146]. Moreover, similarly to red meat, oxidation in red and blue fish



like salmon and tuna, does not only cause deterioration of the lipids, but also changes in the color [143].

As explained in section 1.4. of this document, the oxidation process starts with the formation of lipid radicals that react with lipid peroxides and hydroperoxides. Like with meat products, the autoxidation of fish can be initiated by active oxygen species, heat, light, or by the presence of metal ions and radicals. In fish, a number of potential catalysts and mechanisms including non-enzymatic involving myoglobin, hemoglobin and cytochrome P450 and enzyme initiators such as lipoxygenase, could be involved in the activation reaction to generate active oxygen species [147]. At the end of the oxidation process, lipid peroxides will react freely and form products as aldehydes, alkanes, conjugated dienes and other low-molecular-weight volatile substances [148].

Along with the TBARs, there are other ways to measure the degree of oxidation in fish, as shown in Figure 13.



**Figure 13.** Methods for determining lipid oxidation in fish

Oxidation markers include flavor of rancidity or bad odor (secondary products) which can be correlated with sensory evaluation of oxidation.

Biogenic amines (BA) are basic nitrogenous compounds usually generated in foods and beverages by microbial decarboxylation of amino acids or amination and transamination of aldehydes and ketones [149]. In non-fermented foods the presence of BA above a certain level is considered to be indicative of undesired microbial activity. Therefore, the amine level could be used as an indicator of microbial spoilage. The best-known type of food poisoning caused by BA derives from consumption of high levels of histamine. It is also referred to as "scombrotoxic fish poisoning" because of the frequent

association of this illness with consumption of scrombroid fish such as tuna, mackerel, saury, bonito, seer fish and butterfly kingfish. Non-scrombroid fish such as sardine, anchovy, marline or herring have also been implicated in cases of histamine poisoning [150]. Putrescine and cadaverine, which are present in high levels in toxic fish, have been reported to potentiate the biological effects of histamine up to ten times [151]. In the European Union (EU) [152], the legal limit for histamine levels in raw fish is 100 mg/kg.

Several strategies are available to decrease the undesirable effects of lipid peroxidation and microbial activity. These include the use of freezing and the addition of antioxidants in the package or directly in the fish product. Essid et al. [153] studied the effects of pomegranate peel and artichoke leaf aqueous extracts on biochemical, microbiological and sensorial quality of sardine fillets during storage for 90 days. The authors observed high values of free fatty acids and histamine in control samples, whereas adding extracts of pomegranate peel or artichoke leaves significantly decreased total volatile basic nitrogen and trimethylamine during storage. Greater color and appearance scores were also found for samples marinated with pomegranate peel and artichoke leaf extracts than in control samples. Siqueira et al. found that skin and seed extracts of Muscadine grapes from viticulture waste reduced bacterial growth, change of color and oxidation activity on Atlantic salmon fillet [154].

### **1.11. Active packaging**

The European regulation defines active packaging as packaging systems that interact with the food to “deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food” [155]. The objective of packaging is intended to extend the shelf life and maintain or improve safety and sensory properties, while maintaining the quality of packaged food.

Active packaging systems can be divided into absorbers and emitters.

**Absorbers:** Absorbers constitute a barrier to protect from environmental influences and remove undesired compounds from the food such as carbon dioxide, oxygen, ethylene, moisture, volatiles, and both chemical and microbiological contamination [156].

Oxygen scavenging compounds react with oxygen to reduce its concentration inside the package. Ferrous oxide is the most widely used scavenger. For example, in the meat industry, oxygen scavenging technologies, alone or in combination with modified atmospheric packaging, have been successfully used [156]. The industry controls the humidity in packaged dried foods with sachets that contains desiccants (silica gel, calcium oxide, activated clays and minerals). Additionally, for the high-water activity products like meats, fish, fruit and vegetables, they use absorbent pads, sheets and blankets (plastic films like polyethylene or polypropylene between which a super absorbent polymer is contained) [157].

**Emitters:** Added compounds to the packaged food or into the headspace like antimicrobial compounds, antioxidants, carbon dioxide, flavors, ethylene or ethanol to prevent development of undesirable changes [158].

Antimicrobial films help to inhibit or retard the spread of pathogens and spoilage microorganisms to enhance food preservation and quality. Antimicrobial films can be produced by

incorporation of chemical preservatives or antimicrobial agents into a plastic film which can diffuse into the food to control the growth of microorganisms. Wieczynska et al. [35] demonstrated a significantly influenced quality of organic ready-to-eat iceberg lettuce, which was packed with a bio-based emitting sachet that had cellulose and polypropylene pillow packages containing eugenol, carvacrol and trans-anethole.

Natural antioxidants that are delivered in a gradual manner to maintain the properties of products have been demonstrated to improve the efficiency of the antioxidant activity. This progressive transmission of the additive is achieved by its loading on to a matrix or carrier, which is usually a polymer film to simplify the applicability to food packaging. Biopolymers for the food industry are characterized by non-toxicity and low environmental concern. Among the wide range of available biopolymers, some examples include:

Poly(lactic acid (PLA) is a renewable, biodegradable polyester, easily obtained from carbohydrate sources such as corn starch, sugar cane and biomass by-products [159]. It is used for multiple applications and exhibits good mechanical properties even when natural antioxidants are added [160]. Zeid et al. [161] reported that incorporation of essential oils from thyme, rosemary and oregano into a PLA resin at a concentration of 10% (w/w) may serve as effective antioxidant packaging materials for the preservation of fresh rainbow trout.

Poly ( $\alpha$ -dodecyl  $\gamma$ -glutamate) (PAAG-12) is obtained from poly( $\gamma$ -glutamic acid) (PGGA) by a two-step esterification process [161]. The latter is synthesized by bacteria of the genus *Bacillus*, which can be produced as a secretion product, either kept retained in the microorganism [162] capsule or liberated into the medium [163].

Gelatin possesses excellent film forming and barrier properties against oxygen and light [164]. Recent research on edible films has reported the incorporation of antioxidant and antibacterial compounds to improve the biological activity of films. Neira et al. evaluated the efficiency of edible fish gelatin films added with carvacrol on the quality retention of commercially available prefried breaded hake medallions [165]. Results showed migration of carvacrol as the active agent towards the packed food to induce antioxidant and antimicrobial protective action. Additionally, the authors demonstrated that the gelatin packed food can be directly cooked and consumed without removing the packaging with a good acceptance by the consumer.

Chitosan is a biopolymer formed from chitin that is isolated mainly from the exoskeleton of crustaceans [166]. It is the second most common biopolymer in nature. It is widely used in the development of films for its excellent barrier and antibacterial properties. The effect of plant extracts on the functional properties of chitosan films were reviewed by Xiaoyan et al. [167]. Addition of pine nut shell, peanut shell and jujube leaf in chitosan films, improves the antioxidant ability and gas permeability of the films. Similar effects were found by adding  $\alpha$ -tocopherol [168].

## **1.12. Encapsulation**

Encapsulation allows controlled release of active agents or ingredients at a desired specific rate and site of action. With the emergence of controlled-release technologies, some heat, temperature or pH sensitive additives can be used in food systems [169].

The encapsulation system can be classified for the size, shape or construction [170].

**Size:** Macrocapsules (>5,000  $\mu\text{m}$ ), microcapsules (0.2 to 5,000  $\mu\text{m}$ ) and nanocapsules (<0.2  $\mu\text{m}$ ).

**Shape or construction:** capsules and spheres. Capsules are particles consisting of a central core containing the active substance, which is covered with a polymer. Microspheres are matrix systems in which the active substance is uniformly dispersed and dissolved in a polymer network.

Microencapsulation is used in different areas such as the pharmaceutical, medical and food industries, being widely used in the encapsulation of essential oils, colorings, flavorings, sweeteners, etc. Additionally, it can minimize unpleasant tastes and odors.

Coating influences the encapsulation efficiency and stability of the microcapsule. The wall materials usually are polymers. The polymer acts as a protective film, isolating the core and protecting an active component from the adverse effects of moisture, acid, heat, oxygen, and ingredient interactions and degradation. Some polymers used are: alginate, chitosan, pectins, maltodextrin, carboxymethyl cellulose, or gums including Arabic gum [171].

To choose the encapsulation method, some requirements must be considered: size of the particle and site of application, the characteristics of the bioactive ingredient to be encapsulated, the release mechanism, and the properties of the wall material [172].

The main encapsulation methods are: spray drying, spray cooling or lyophilization, extrusion, coacervation, and emulsification [173].

**The spray drying method** is employed to obtain dry powders from a liquid that is sprayed through an atomizer in heated air. The encapsulation efficiency depends on the feed flow rate, air inlet/outlet temperature, feed temperature, and wall materials. This technology can be used for a diversity of applications in the pharmaceutical, cosmetic and food industries. Spray drying is the most frequently employed method for adding products in the food industry.

**The spray cooling or lyophilization method** resembles spray-drying. However, air temperature is lower than in spray drying. This method is one of the most efficient techniques to protect active compounds such as phenolics [174]. Freeze drying is considered one of the most suitable methods for drying biological materials and sensitive foods [175].

**Extrusion encapsulation**, the encapsulant mixture (a dual fluid stream of wall and core materials) is first pumped through concentric tubes forming a laminar jet, then droplets are formed under the influence of vibration. The main advantage of this methodology is that there is no use of either high/low temperatures or organic solvents [176].

**The coacervation process** involves three steps: emulsification, coacervation, and shell formation and hardening. Coacervation is the separation of a colloidal system into two liquid phases [176]. This technique offers advantages such as high encapsulation efficiency, integrity of wall material and a variety of biopolymers that can be used as wall materials [177].

**The emulsification technique** is a process of dispersing one liquid in a second immiscible liquid [178]. The process for preparing capsules by emulsion can be divided into four major steps: incorporation of the active phase, droplet formation, solvent removal, and harvest and drying (the

solidified microparticles are filtered and dried either at ambient or under reduced pressure conditions, by heating or lyophilization) [179].

Technique	Advantages	Disadvantages	Examples	Reference
Spray drying	Less molecular mobility, low costs, homogeneity of the components, low water activity	High temperatures, limited wall material to use (soluble in water)	Carotenoids, Anthocyanins	[180]
Spray cooling or lyophilization	Low temperatures, maintain nutritional factors, more resistant to oxidation and protected from heat-sensitive active compound	Long processing times, high energy, and high production cost	Lycopene Oleoresin Lactobacillus paracasei,	[181]
Extrusion	Long shelf life imparted to normally oxidation-prone flavor compounds, low water content	Larges particles formed, high production costs, limited wall material to use	Flavors and oils	[181]
Coacervation	Large number of wall material to use, room-temperature	Expensive technology and the crosslinking usually involve glutaraldehyde	Oils: Black pepper ( <i>Piper nigrum L.</i> ), Citronella, Microalgal, vitamins and nutrients	[177,182,183]
Emulsification	Lower energy input, control the final droplet size of the emulsion.		Oils, fish oil	[184]

**Table 7.** Encapsulations techniques

### 1.13. Functional food products

Functional foods are defined by the Functional Food Center as “natural or processed foods that contain known or unknown biologically active compounds; which in defined, effective non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease” [185]. Functional foods are dietary products with beneficial effects that go beyond traditional nutritional effects [186]. Functional foods differ from nutraceuticals, pharmafoods, designer food, and dietary supplements.

The concept of functional foods includes [186,187]:

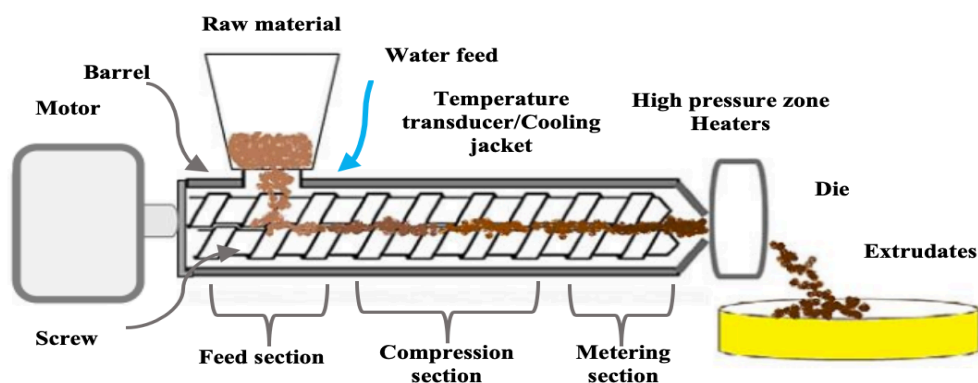
The development of new food components with traditional and novel raw materials to add or increase functionality. Examples include the use of by-products or unconventional natural sources

with high content of fiber, bioactive compounds including phenolics, vitamins, minerals, essential lipids as omega 3, etc [186].

An example of a natural source is inulin, which has a high content of dietary fiber. Inulin is currently used as a replacement for fat and sugar in the food industry. Milanivkov et al. evaluated the nutritional impact of inulin addition (20%) in spelt pasta. Inulin modified nutritional properties and reduced energy for consumers (27.2 %) [188]. Another example is the use of phenolics extracted from the industry by-products including apple pomace extracts, which are used as a source of fiber and phenolic compounds in baked products and a functional milk beverage fortified with phenolic compounds extracted from olive vegetation water [189]. Đurović et al. baked bread with the addition of stinging nettle leaves and its extract. The biological activity of the prepared bread samples showed significant antioxidant activity [190]. Subiria-Cueto et al. evaluated the impact of partial replacement of wheat flour with Ramón seed flour on the physicochemical, rheological and nutritional properties and antioxidant capacity of tortillas. Results indicated that tortillas with 25 % Ramón seed flour had higher dietary fiber (4.5 times), mineral content (8.8 %; potassium 42.8 %, copper 33 %), phenolic compounds and antioxidant capacity (11.7 times, 33 %–50 %, respectively) than wheat tortillas [191]. Mayo-Mayo et al. [192] analyzed the effect of the addition of mango peel and decocted Hibiscus flower powder on corn tortilla. Their results suggest that the use of mango peel or decocted Hibiscus flower may increase dietary fiber content to control the glycemic index and improve the functional properties of corn tortilla.

The optimization of functional components in raw materials and food facilitates maximal preservation of the components, modifies their function and increases their bioavailability. Conventional and emerging technologies allow molecular transformation and chemical reactions that modify functional properties, nutrient and phytochemical composition of the food. For instance:

Extrusion has been commonly used for the development of new products, including snacks, breakfast cereals, and pet and baby foods. It is a technology with low cost, high productivity, versatility, and also improved digestibility and nutritional bioavailability of the product for human and animal consumption [193]. Extrusion entails mechanical stress, which may alter physical and chemical characteristics [194]. Depending on the extruder (dry, wet, single, or twin) and the independent variables applied (barrel temperature, moisture, and screw speed) [193,195,196], we can obtain different functions by the processing of food. Some of these functions are: expansion, dehydration, gelatinization, pasteurization, protein denaturation, etc [197].



**Figure 14.** Sections of the extrusion process

The following sections are the ones most commonly used in food extrusion [198].

**-Feed section:** Feed section is usually performed with deep flight screws to carry the raw material immediately to the next section. The objective of the feeding section is to ensure that raw material is moving quickly to the barrel.

**-Compression section:** The main function of this section is to plasticize the raw material into a dough-like state. This section should have a gradual decrease in the screw flight depth in the direction of the die [199].

**-Metering section:** The principal function of this section is to increase the shear rate and cooking of the raw material. Therefore, this section should have a very shallow flight screw.

The use of extrusion in the processing of by-products derived from cereals, fruits, and vegetables is of great interest. Several studies increased the antioxidant activity, polyphenols composition and soluble fiber by using this technology in wastes such as soybean residue [35], shrimp shell [200], orange peel [201], blueberry press cake [202]. In addition to these improvements, extrusion has shown a benefit to the techno-functional characteristics of the products.

Food fermentation is a processing technology that applies the growth and metabolic activity of microorganisms for the stabilization and transformation of food materials [203]. In recent times there is growing interest in fermented foods containing probiotic organisms. These functional foods have beneficial health effects, such as improving intestinal tract health, enhancing the immune system, synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance, decreasing the prevalence of allergy in susceptible individuals, and reducing risk of certain cancers [204]. Indeed, various studies showed that fermentation with microorganisms improves the phenolic content and antioxidant activity [205,206].

The industrial fermentation processes use either liquid/submerged or solid-state methods.

**-Liquid or submerged-state fermentation (SF)** is based on microorganism cultivation in a liquid medium containing nutrients. It is the preferred technology for industrial enzyme production due to the ease of handling at a large scale [207]. Liquid fermentation is used in the production of yoghurt and other dairy-based beverages, alcoholic beverages, and food condiments such as vinegar [208–210].

**-Solid-state fermentation (SSF)** is carried out with microbes growing on nutrient impregnated solid substrate with little or no free water [211]. Solid-state fermentation has more technological advantages compared with the submerged-state fermentation, such as higher productivity and product stability. Additionally, the SSF has lower costs than SF [212].

Bread is a typical example of a procedure that implements this process, where the primary function of dough fermentation is to create the characteristic structure, texture, and sensorial profile of bread after the baking process [213].

Product	Type of fermentation	Microorganism/fungus	Reference
Citric acid from: Kumara (starch)	SSF	<i>Aspergillus niger</i>	[214]
Yogurt	SF	<i>Lactobacillus helveticus</i>	[215]
Citrus fruit peels for the Pectinases production	SF	<i>Aspergillus niger</i>	[216]
Orange bagasse and wheat bran for the Pectinolytic Enzyme Production	SSF	<i>Penicillium viridicatum</i>	[217]
Soy sauce	SF	Walnut meal and <i>Aspergillus oryzae</i>	[218]
Catechin-vinegars	SF	<i>S. cerevisiae</i> and <i>A. pasteurianus</i>	[210,219]
Industrial enzymes: $\alpha$ -amylase and protease from bread waste	SSF	<i>Rhizopus oryzae</i>	[220]

**Table 8.** Examples of fermented products by liquid or submerged-state fermentation (SF) or solid-state fermentation (SSF)

### 1.14. Sensory analysis

The acceptance of a food product by the consumer is highly dependent on the sensory perception. Sensory studies are based on food attributes such as gustatory, olfactory, textural, visual, thermal, and others. In the food industry, the sensory study has the same relevance as chemistry, physics and microbiological analysis. To provide credible and accurate results, the sensory evaluation should meet prerequisites, such as panelist (trained or not) (ISO 11132), sensory laboratory (ISO 8589), standard sensory tests, presentation of results, and evaluation after statistical processing [221].

There are three sensory evaluation techniques: affective, discrimination, and descriptive.

**-Affective studies** involve untrained panelists who evaluate their reactions to assess acceptance, liking, preference, or emotions for a stimulus or stimuli [222]. The most frequently used methods to directly measure preference and acceptance are the paired-comparison test and the nine-point hedonic scale, respectively [223]. The nine-point structured hedonic scale is probably the most widely used effective method, due to the reliability and validity of its results, as well as its ease of use for the tasters [224].

This test can be structured, unstructured, numerical, semantic, or graphic, as specified in ISO 412.



**-Discriminative testing** is used to determine if a difference exists between samples. Discrimination testing is easy to use and analyze, and for panelists (trained or untrained) to complete. In the food industry, this method is normally used to create or improve a product; in this case, consumer differentiation between the new product and preexisting ones is desirable.

Ideally, the consumer should not perceive any sensory variation when a reformulation of the ingredients or changes in the process, handling or packaging are carried out for cost reductions, quality control, and improvement of shelf life and nutritional value. Several tests derived from this method are listed in Table 9:

Test	Description	Legislation
Paired-comparison	To assess a perceptible sensory difference or similarity between two products concerning the intensity of a sensory attribute, based on defined criteria.	ISO 5495
Duo-trio	This is a forced-choice procedure test in which the reference sample is presented first. Two samples follow, one of which is identical to the reference sample and the judges are requested to identify it.	ISO 10399
Triangle	Three coded samples are presented simultaneously, two of them are identical. Testers are requested to select the different sample.	ISO 4120
Two-out-of-five	This is a discrimination test that uses five coded samples, two of them are of one type and three of another. Judges are asked to group them into the two sets of samples.	ISO 6558
Tetrad	This test uses four samples: two samples from one product and two from a second. The task of the assessor is to group the samples into two groups based on their similarity.	ASTM E3009-15
A-no A	An assessor is presented with a series of samples, some of them are composed of sample "A" while others are different "A-no"; for each sample, the tester has to determine whether the sample is identical to "A" or not.	ISO 8588

**Table 9.** Discriminative tests

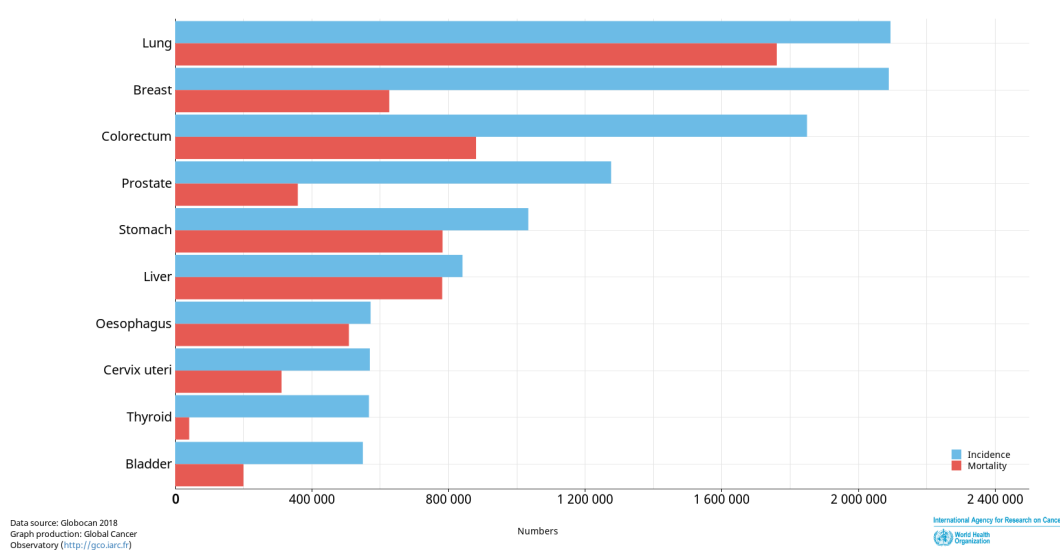
Finally, the **descriptive method** labels and quantitative sensory characteristics of stimuli by a trained panelist are specified in ISO 13299. These panelists are trained using detailed lexicons of physical and chemical descriptors including appearance, aroma, flavor and texture spectrum. The descriptive method is classified as quantitative and qualitative.

In the **quantitative profile**, the testers evaluate the samples using a checklist of attributes and rate their intensity. The results include intensity scores for each attribute that can be analyzed using univariate or multivariate techniques. In the qualitative sensory profile, the testers only assess the

presence or absence of the attribute from a checklist, without indicating the perceived intensity [225,226].

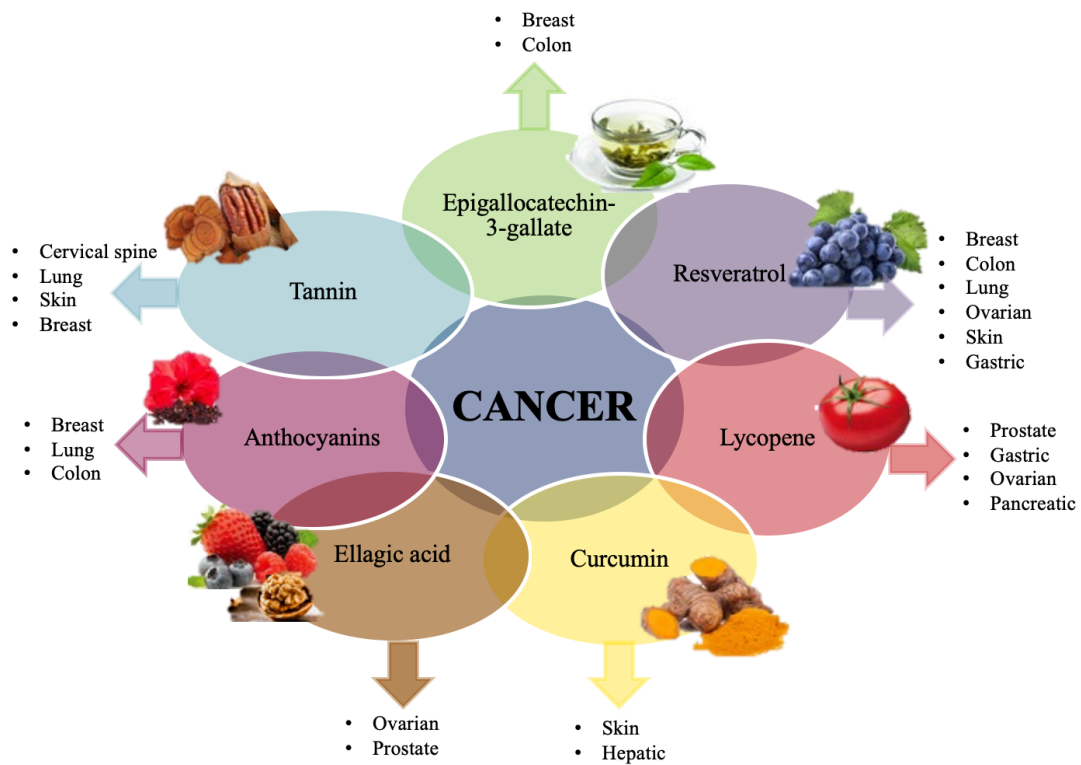
### 1.15. Viability-reducing activity of antioxidant extract against human cancer cell lines

The World Health Organization (WHO) estimates about 27 million new cases and 17.5 million cancer related deaths annually by 2050. Figure 15 shows an estimated number of incident cases and deaths from different cancer types worldwide in 2018 [227]. Around 85-90 % of cancers are produced by environmental and lifestyle factors. The lifestyle factors include high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, alcohol use, etc [228].



**Figure 15.** Estimated number of incident cases and deaths worldwide, both sexes and all ages (WHO, 2018) [227].

According to the International Institute of Cancer and the WHO, the term “cancer” refers to a group of diseases caused by abnormal and uncontrolled cellular division that can invade any tissue or organ; eventually, these cells invade adjoining parts of the body and spread to other organs [227,229]. Carcinogenesis is a multistep and multifactorial chronic process in which normal cells are transformed into cancer cells, characterized by genetic and epigenetic changes [230,231]. This process is divided into three stages: initiation, promotion, and progression. Chemoprevention is based on the use of compounds that can inhibit any stage of carcinogenesis and delay or prevent cancer development. Chemoprevention drugs can be natural or synthetic chemical agents. In the last decade, half of the pharmaceutical drugs developed against cancer come from natural sources. Fruits, vegetables, plants and their by-products are rich in bioactive phytochemicals as polyphenols. A wide range of evidence shows that these compounds have a potential role in protecting human health from chronic diseases such as cancer [232,233].



**Figure 16.** Examples of bioactive phytochemicals compounds in different types of cancer cells [234-248].

### 1.16. Literature review of pecan shell (*Carya illinoensis*)

Detection of phenolic compounds	Antimicrobial activity	Antiproliferative activity	Films and encapsulation	Other uses
-30 compounds (resveratrol, phenolic acids, and derivatives of gallic acid) [249]	-Staphylococcus aureus [249]	-HeLa gynecological cell (IC50 values of 153.72±2.03 and 174.19±1.05 µg/mL for pecan shell of Western and Wichita) [249]	-Polylactic acid (PLA) films containing pecan shell (antioxidant properties: DPPH reduction >60% with a 3% of pecan shell) [255]	-Pecan shell was use as solid substrate for the cultivation of the white-rot fungus Ganoderma lucidum (Curtis) P.Karst. (Transformation of biomass into mushrooms) [256].
-10 compounds (gallic, 4-hydroxybenzoic, chlorogenic, vanillic, caffeic and ellagic acid, and catechin, epicatechin, epigallocatechin and epicatechin gallate) [250]	-Listeria monocytogenes, staphylococcus aureus, vibrio parahaemolyticus and bacillus cereus [49].	-MCF-7 cells (IC50 values 74.11 µg/mL for pecan shell) [250]	-Pecan shell in Zein Microparticles [251]	
-3 compounds (catechin, gallic acid, ellagic acid, and condensed tannins) using extruded shell [35]	-Listeria monocytogenes, salmonella enteritidis, staphylococcus aureus, bacillus cereus, aeromonas hydrophila and pseudomonas aeruginosa [254].			
-4 compounds in Zein microparticles with pecan shell extract (Epigallocatechin, ellagic acid, epicatechin and gallic acid) [251]				
-5 compounds (ellagic, gallic, protocatechuic, and p-hydroxybenzoic acids and catechin [37]				
-4 compounds (gallic acid, ellagic acid, catechin and epicatechin) [252]				
-29 compounds (gallic acid, chlorogenic acid, p-hydroxybenzoic acid, epigallocatechin and epicatechin-gallate) using ultrasonic-assisted extraction [253]				

**Table 10.** Literature review of pecan shell (*Carya illinoensis*)

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## Chapter 2. Hypothesis and objectives

The hypothesis is that the pecan (*Carya illinoensis*), and especially the pecan shell, a commercial byproduct, could be used, alone or in combination with other products, to slow the deterioration of foods and provide them with functional properties. This is based on the fact that the pecan shell is a product rich in phenolic compounds and high fiber content.

The main objective of this project was to study the phenolic composition, radical scavenging activity and antioxidant activity of kernel and pecan shell, alone or in combination with other products (*hibiscus sabdariffa* and *capsicum annuum*).

This main objective is developed in the following blocks of specific objectives

1. **Analyze the composition** and characterize the radical scavenging and antioxidant properties of the pecan shell
  - a. To optimize the extraction conditions with response surface methodology with a three-level, two-factor central composite design and verify the model equation.
  - b. To quantify the phenolic content, and tannins and determine the radical scavenging capacity by ORAC, FRAP, DPPH and ABTS.
  - c. To identify the main phenolic compounds in ethanolic extracts using time-of-flight mass spectrometry liquid chromatography (LC-MS-TOF).
  - d. To evaluate the effectiveness of the extracts in the protection against oxidation of one food model: oil in water emulsion.
2. Carry out **transformations** in the pecan shell and analyze how the antiradical properties vary
  - e. To perform an extrusion process and optimize the extrusion conditions (by the response surface method) in order to maximize total polyphenols content, condensed tannins and radical scavenging capacity.
  - f. To ferment (direct shell and extruded shell) with *Aspergillus Oryzae* and characterize the new radical scavenging properties.
3. To analyze the **combined effect** with other plant products on antioxidant and antimicrobial activity.
  - g. To quantify the total polyphenols and radical scavenging capacity by DPPH and antimicrobial activity by the diffusion method with disks of roselle flower and cayenne pepper extracts.
  - h. To analyze antimicrobial activity by the diffusion method in discs prepared with pecan shell extracts.
  - i. To study the combination of the pecan shell extracts with roselle flower and cayenne pepper in the oxidation and stability of beef patties.
  - j. To evaluate the combination of pecan kernel extracts and roselle flower in the shelf life of small sardine patties.
4. To develop pecan kernel and shell **packaging and encapsulation** processes and analyze their possible application in food models
  - k. To prepare and characterize gelatin films and study the development of oxidation of beef patties protected by them.
  - l. To prepare PLA films with vitamin E, as the main non polyphenolic antioxidant of pecans, and assess its antioxidant effectiveness in emulsions

- m. To encapsulate with chitosan and optimize the encapsulation variables. To study the protection exerted by the pecan shell encapsulated in minced salmon
- 5. To **partially replace bakery flour** with flour from pecan shell (extruded and non-extruded) and make tortillas and bread at the pilot plant level
  - n. To analyze the rheological, physical and chemical properties of tortillas and bread produced with different proportions of pecan shell flour.
  - o. To assess the reduction of the viability of human cancer cell lines by extracts of the finished products.



## Chapter 3. General materials and methods

This chapter includes the samples, a schematic and overview of the general methods which were applied to the experiments of this project. All experiments were carried out in the laboratories at the Universitat Politècnica de Catalunya, the Tecnológico de Monterrey Campus Monterrey and the Universitat de Barcelona. The general procedures and analytical techniques, which were used in the present study, are mentioned in this chapter. Other techniques specific to particular trials and the description of each method are described in the relevant experimental chapters.

### 3.1. Samples

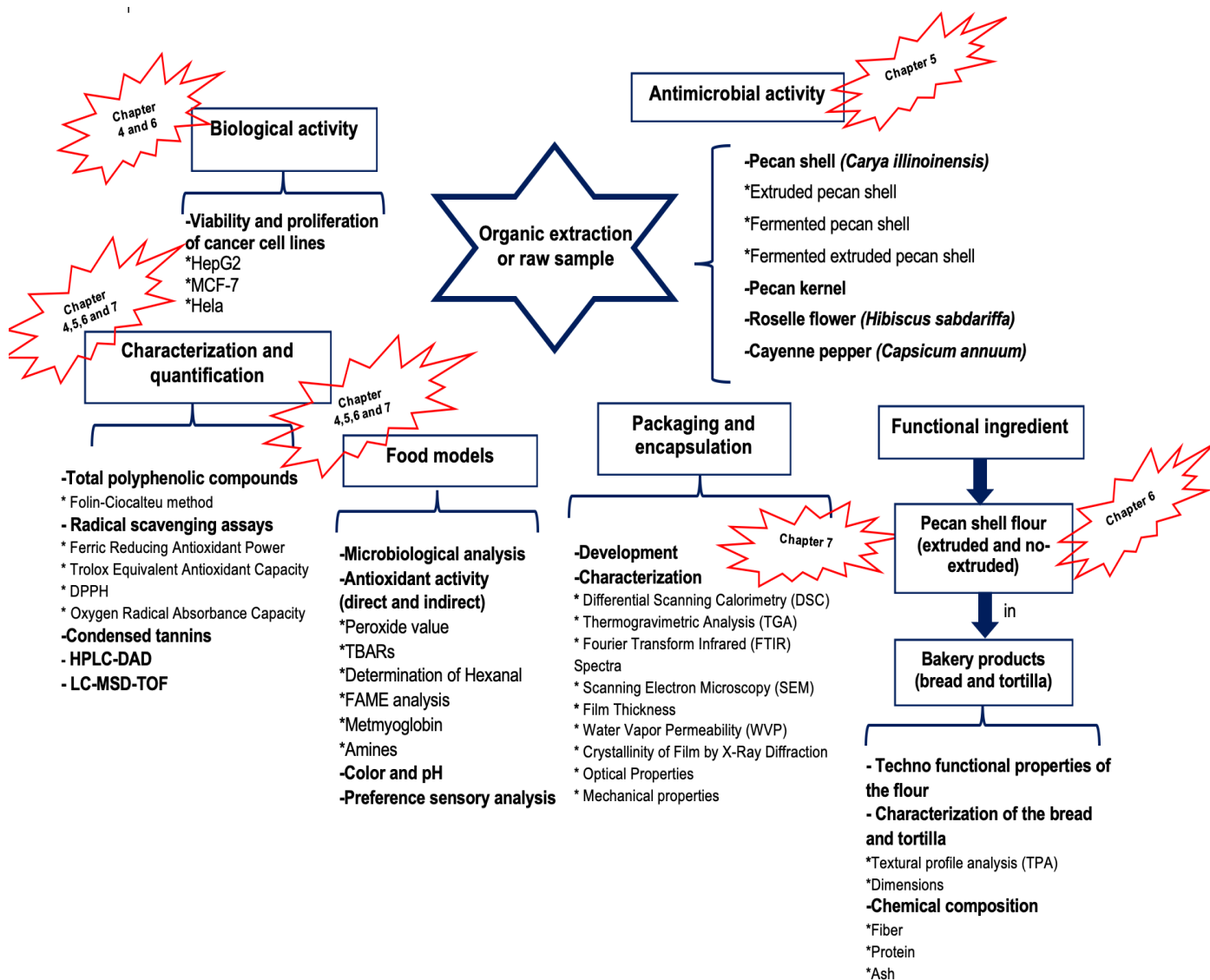
**Kernel:** Kernel (*Carya illinoensis*) purchased in a local market (Casa Ferris) in Barcelona, Spain, were frozen with liquid nitrogen and shredded with a mortar.

**Shell:** Shells (*Carya illinoensis*) were collected at 1090 m above sea level in San Pedro, Coahuila, which is located on the north of Mexico. The pecan nut shells were separated manually from the kernel, then were milled in a Wiley mill (Arthur Thomas, Philadelphia, PA, USA) equipped with a 2 mm screen.

***Hibiscus sabdariffa*:** Roselle flowers (*Hibiscus sabdariffa*) purchased in a local market (La Avenera) in Colima, a small state on the western of Mexico on the central Pacific coast. The dry flowers were frozen with liquid nitrogen and shredded with a mortar.

**Cayenne Pepper:** The dry powder of cayenne pepper (*Capsicum annuum*) was bought in a local shop (Mercadona) in Barcelona, Spain.

### 3.2. Methods



### **3.3. Overview of the methodology**

A large number of by-products are abundantly produced annually around the world from food processing and agriculture. This waste could potentially cause environmental damage and/ or unnecessary costs.

The food industry commonly uses synthetic antioxidants in order to prevent oxidative reactions in their products. The restrictions of the use of these antioxidants have been enforced due to the health risks and toxicity. Consumers are demanding clearly labelled products that guarantee the absence of synthetic additives.

The pecan is a tree nut native to North America. Its nutritional content has resulted in its growing worldwide consumption [1]. The global production of this nut from 2019 to 2020 is estimated to be 139,739 metric tons. Mexico is the top producer, followed by USA [2]. The shell of the pecan represents around 40-50% of its weight, which results in a big waste of the pecan industry. Hence, pecan shell may be further valorized for producing by-products such as an alternative biosorbent to remove Cu(II), Mn(II) and Pb(II) metallic ions from aqueous solutions [3], natural dye [4], and biodegradable composite material [5].

The main idea of this dissertation is to study and development a potential ingredient as a novel source of antioxidants and other important nutrients such as fiber.

The methodology to be implemented begins with an exhaustive review of the concept of antioxidants and their role in the health and food industry. The plants used in this research are presented, covering a brief explanation about their composition and their antioxidant activity. The lipid oxidation and how it affects the shelf life of some foods including emulsions, meat and fish is also included in the introduction.

Then, the generation process of free radicals is explained and a possible classification of antioxidants (incl. examples) is presented. Next, methods for the identification and quantification of phenolic compounds and radical scavenging are covered.

On the other hand, some techniques used for the development of active packaging and encapsulation are described and the concept of functional food (incl. examples that are currently used in the industry) are introduced.

Finally, an overview of the viability of reducing activity of antioxidant extracts against human cancer cell lines is presented.

According to Hussain et al. and Sultana et al., the quantity and quality of the extracts depend on the extraction methodology [6,7]. Hence, the first experiment conducted as part of this dissertation was a Response Surface Methodology (RSM) based on a central composite design, which was generated using MINITAB 18. RSM is a useful method to evaluate the effects of multiple factors and their interactions on one or more response variables. One of the advantages of this method is that it normally involves fewer experimental runs (thus the experimental error is less) than what is normally needed in traditional factorial designs [8]. The objective of the RSM was to optimize the conditions (solvent concentration and solute: solvent ratio) for the extraction of antioxidants compounds from the

pecan shell. This was followed by the determination of the Total Phenolic Contents (TPC) and the free radical scavenging capacity of obtained extracts. The solvent used for this experiment was a mix of water with ethanol. Most of the studies about the determination of TPC and antiradical scavenging capacity of the pecan shell were with aqueous-organic solvents [9–11].

By using these optimized extraction conditions, the analysis of the phenolic compounds was performed with liquid chromatography coupled to time-of-flight mass spectrometry (LC-MSD-TOF). The aim was to identify new compounds. The TOF-MS proved to be the most selective technique as it showed a high acquisition speed, rapid screening and provided accurate mass measurement. It is useful for the identification of unknown compounds and also for a greater differentiation of two different compounds with the same nominal mass but different elemental composition [12]. Some studies already used this technique to identify phenolic compounds [13,14]. In the same chapter, the antioxidant effectiveness of kernel and pecan shell extracts in food simulants as oil-in-water emulsions is presented. There are two ways to detect the first oxidation: UV (measurement of conjugated dienes and trienes) and Peroxide Values (PV) measurements. The latter is the preferred by the industry. In this dissertation, PV was used by the ferric thiocyanate method as it has shown to be simple, reproducible and considered more sensitive than other methods [15,16]. Finally, the complete study of the antiproliferative activity of the pecan kernel and pecan shell extracts on three human cancer-derived cell lines is presented: HeLa (derived from cervical cancer), HepG2 (hepatic) and MCF-7 (breast) [17,18]. Recently, the effect of *Carya illinoensis* shell cell culture extract was evaluated in the breast (MCF-7) by MTT assay. The study confirmed aforementioned activity. Moreover, the antiproliferative activity of extracts of *Carya illinoensis* shell has not been previously studied in HepG2 and MCF-7 cell lines.

Chapter five describes the effect of antioxidant and antimicrobial activity of the pecan shell and other Mexican traditional consumer plants (*hibiscus sabdariffa* and cayenne pepper) in beef patties. The study of the effect antioxidant of pecan kernel and *hibiscus sabdariffa* in sardine patties is also presented. Meat and fish were chosen to work with due to the yearly increase of its global consumption. According the Australian Bureau of Agricultural and Resource Economics and Sciences, the consumption increased by 58% over the last 20 years (2018), reaching 360 million tones around the world [19]. These types of products are a highly perishable foods to lipid oxidation. This oxidation is one of the main factors responsible of the reduction of the quality and acceptability of the consumers. Lipid oxidation in meat and fish generally implies the production of secondary decomposition products (malondialdehydes) and also the degradation of polyunsaturated fatty [20]. In this dissertation a TBARs test to measure the lipid oxidation in fish and meat was used for its simplicity and low cost [21,22].

On the other hand, the analysis of volatile lipid oxidation products as hexanal is used to measure the oxidative grade of foods rich in linoleic acid as the sardine [23]. Headspace GC-MS was used because it is a methodology with almost no sample treatment requirements that could potentially reduce the experimental error.

The production of biogenic amines is another indicator of oxidation. The creation of these compounds happen when organic bases produced during the decarboxylation of amino acids or the transamination of aldehydes and ketones [24]. Several methods are available for the analytical detection and quantification of these compounds. However, the high-performance liquid

chromatography (HPLC) has been one of the most used methods in fish products, and it has become an international standard analysis [23,25,26]. Fatty acid methyl ester analysis was used to assess the amount of fatty acids present in meat and sardine patties, aiming to monitor changes among samples containing pecan kernel, shell, hibiscus sabdariffa and cayenne pepper. Additionally, microbiological analysis was performed to assay sample contamination with mesophilic bacteria. Finally, a preference sensory analysis was performed in both experiments.

The extrusion technology is gaining increasing popularity in the global food processing. However, there are currently not studies on the extrusion of pecan shell. The use of this technology would present an opportunity to improve the nutrition quality of the pecan shell.

Chapter six shows a complete study of the effect of the extrusion temperature and screw speed on physical and chemical characteristics of the pecan shell. Some of these characteristics include: water and oil absorption index, water solubility index, color, phenolic compounds, condensed tannin compounds, antioxidant activity, fiber and protein composition.

Bread is the most widely consumed food in the world. The tortilla represents a very important source in the Mexican diet. They are both types of products that have a short life span thus, they are commonly preserved by chemical preservatives such benzoates and sorbates. Currently, various studies have been conducted on the use of by-products of fruits and vegetables as functional ingredients in bakery products [27–30]. Favorable results as high antioxidant potential, dietary fiber and protein content were obtained as well as low energy value, good physical characteristics (texture, color, volume, etc.), and acceptable sensory characteristics. For these reasons a new formulation to make bread and tortilla with the addition of extruded pecan shell is further explored in this chapter. This new ingredient on the bakery's products would represent an opportunity to preserve them without using benzoates and sorbates. The same methodology as previously presented was conducted to assess the total phenolic compounds and antiradical scavenging. The determination of total dietary fiber and crude protein content was determined by the official methods (AOAC 991.43 and AOAC 920.87). The antiproliferative activity of wheat flour fortification with shell was analyzed with a similar method as in chapter four.

Due to the composition rich in soluble fiber and sugars, some by-products are easily assimilated by the microorganisms in a fermentation process. However, the fermentation process may be split up into two systems: submerged (SF) and solid-state (SSF). Rodriguez et al. concluded that commercially available SSF produced enzymes were richer in side activities compared to submerged fermentation [31]. Additionally, several studies concluded that SSF improves the phenolic release and antioxidant activities [32,33]. That explains the use of SSF in this dissertation. Extruded and no-extruded pecan shell could be used as support-substrate in solid-state fermentation (SSF) for the production of industrially relevant compounds with great financial benefits. After the extrusion process of the pecan shell, the effect of SSF with *Aspergillus oryzae* on the phenolic content and antioxidant activity was explored.

Chapter seven presents the development of biodegradable films with the addition of derivatives of the pecan, their characterization and the evaluation in food models as emulsions and meat patties. The main objective of the development of these films based on natural wastes and biopolymers was not only to reduce the use of non-biodegradable packaging, but also to improve the antioxidant potential, mechanical, thermal and dimensional stability of the films.

Recent studies have been developed on the reinforcement of PLA (polylactic acid) with organic wastes, such as the shells from pistachio, almond, and walnut [34,35].

In this dissertation, PAAG-12 ((Poly ( $\alpha$ -dodecyl  $\gamma$ -glutamate)) films enriched with  $\alpha$ -tocopherol (a pecan compound) were developed. PLA films with  $\alpha$ -tocopherol were developed as well. Thermogravimetric Analysis (TGA) was determined to get to know the thermal stability of the films. Water 50% ethanol (EtOH) and 95% EtOH were used as food simulants and HPLC analyses were performed to determine diffusion of  $\alpha$ -tocopherol and the partition coefficients of PAAG-12 and PLA films. Finally, the films were put in an oil-in-water emulsions and the primary oxidation was measured with a peroxide value as in chapter four.

Additionally, gelatin films with pecan kernel and shell were designed and analyzed. Similar as presented in chapter five, the TBARs methodology was used to determine how films affect the oxidation of beef burgers when they are brought into contact with the meat. Physical analyses of these films were performed, such film thickness, water vapor permeability, morphology of the surface and the transversal section by SEM, crystallinity by x-ray diffraction, optical, and mechanical properties.

Finally, the development of encapsulation of extruded pecan shell and the antioxidant effect of them in salmon patties is covered. Encapsulation was conducted in order to protect the bioactive substances of the pecan. These compounds are susceptible to losing its antioxidant capacity by oxidation when exposed to oxygen, moisture, light, and high temperatures [36]. The method used in this research was spray drying. This system is usually used for encapsulation of bioactive compounds. The advantages of this technique involve low cost, high flexibility, high speed and the possibility of applying different carriers [34].

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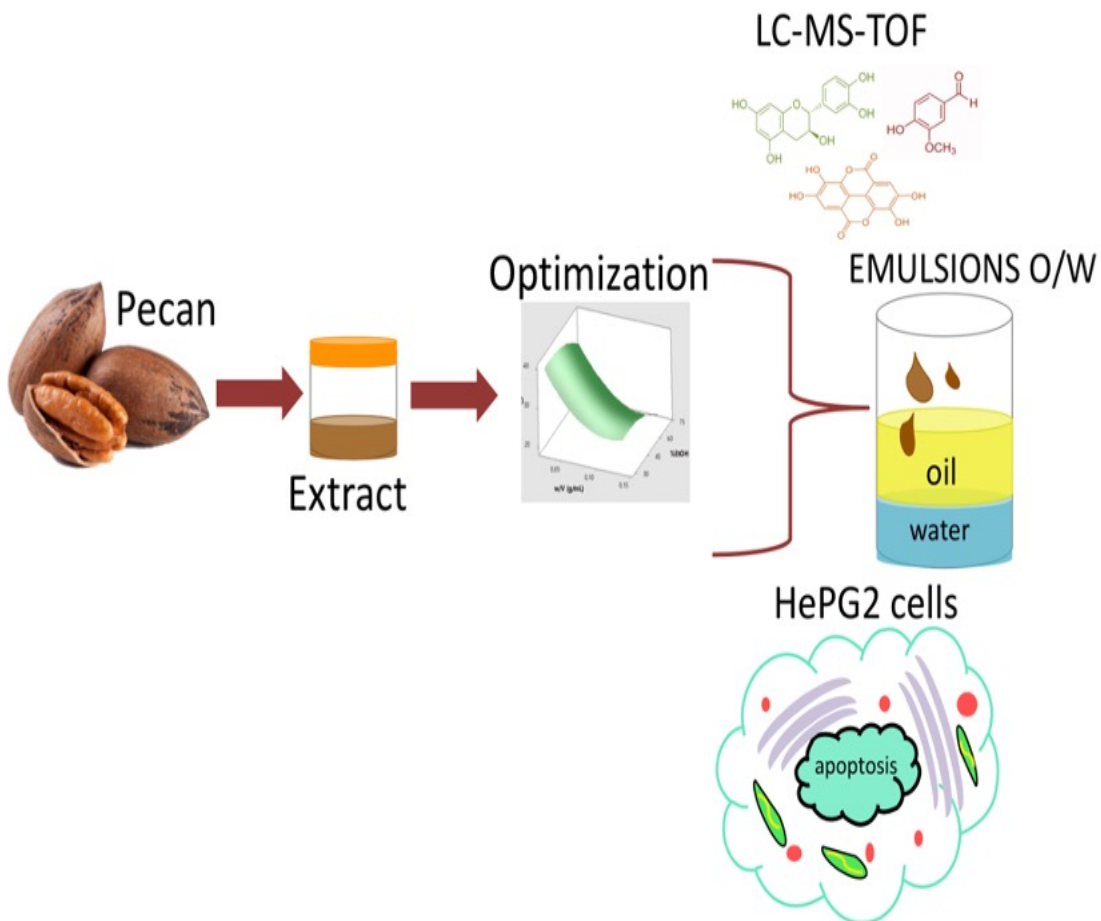
**Chapter 4. The composition and characterize of the radical scavenging of the pecan shell.**

#### 4.1. Antioxidant and antiproliferative activity of pecan (*Carya illinoensis*) shell

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# Antioxidant and antiproliferative activity of *Carya illinoensis* shell

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## Abstract

**Purpose-** Herein, the potential use of pecan (*Carya illinoensis*) walnut shell (WS) in the food and pharmacological industry was addressed. **Design/methodology/approach-** Using response surface methodology, sample weight to solvent ratio (w/v) and ethanol concentration were optimized to improve total phenolic content (TPC) and oxygen radical absorbance capacity in WS extracts. Extraction with 0.0234 (w/v) gives the maximum value for total phenolic content (TPC) and oxygen radical absorbance capacity. Such extraction condition and 44 % of ethanol were applied to determine TPC and radical scavenging activity (SA) in WS extracts. **Findings-** Sixteen polyphenolic compounds were identified in WS extracts by liquid chromatography coupled to time-of-flight mass spectrometry such as quercetin, vanillic and ellagic acid. Both pecan walnut kernel (WK) and WS extracts delayed lipid oxidation in oil-in-water emulsions, with WS extracts having the highest antioxidant activity. WK and WS exhibited antiproliferative activity on three human cancer-derived cell lines: HeLa, HepG2 and MCF-7. The highest reduction was found in HepG2 cells incubated with WS extracts, while WK extracts were more effective for HeLa cells. **Originality/value-** Our findings support the potential use of WS extracts as a natural preservative in the food industry and pharmacotherapy of hepatocellular carcinoma.

## Introduction

Pecan (*Carya illinoensis*) walnuts, commonly present in the human diet, are produced at huge scale in USA and the north of Mexico (De la Rosa *et al.*, 2014). The beneficial effects of pecan walnuts on human health have stimulated its production and the development of new pecan walnut-derived products (Bada *et al.*, 2010). Pecan walnuts can be divided into four parts: kernel, pellicle, shell and green husk. The pecan walnut shell (WS) represents approximately 50 % of the nut and an agricultural waste with little economic value. Around 1.5 million tons of WS are left behind yearly worldwide (Pirayesh *et al.*, 2012). The industry started to use WS as a filtration media to remove crude oil from water (Aversa *et al.*, 2016). Besides, WS is a by-product that can be used also for blast cleaning, tumble and polish metals, wood, plastics, and other materials such as jewelry (Fernández-Agulló *et al.*, 2013; Srinivasan and Viraraghavan, 2008). Recently, Dolan *et al.* proposed that high levels of WS could be safely consumed by humans and animals (Dolan *et al.*, 2016). WS contains fiber, various minerals and phenolic compounds, such as, ellagic acid, gallic acid, vanillic acid, catechin, caffeic

acid, chlorogenic acid, epicatechin, and 4-hydroxybenzoic acid (Kureck *et al.*, 2018; Villasante *et al.*, 2019). The phenolic composition of pecan walnut shell makes this a by-product of interest as a functional food ingredient. The Institute of Medicine's Food and Nutrition Board defined functional foods as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (Noomhorm *et al.*, 2014). Antioxidant compounds play an important role as a natural preservative, particularly in the food industry because consumers prefer natural preservatives rather than synthetic ones. To evaluate antioxidant effectiveness, food models such as oil-water emulsions (O/W) can be used. Antioxidants can react with peroxide radicals at the O/W interface of emulsions, preventing or slowing down lipid oxidation, and therefore increasing the oxidative stability of the emulsion (Narkiewicz-Michalek *et al.*, 2019). In this context, it is known that there are a large number of products, remarkably plant-derived products (extracts of some edible plants and aromatic plants, fruits, vegetables and even by-products obtained from the industrial manufacture of juices and prepared meals), that have been proposed as a convenient source to inhibit or diminish lipid oxidation in O/W (Celus *et al.*, 2020; Velderrain-Rodríguez *et al.*, 2019).

It is well known that the risk resulting from oxidative stress is involved in the pathogenesis of many diseases, including cancer (Martinon, 2010). In this regard, anticarcinogenic effects of phenolic compounds from different raw materials have been widely reported (Liu *et al.*, 2019; Wang *et al.*, 2018). Indeed, a study of the antiproliferative activity of walnut polyphenols, such as ellagic acid, showed promising results (Sánchez-González *et al.*, 2015).

The aim of the present study was to optimize the extraction procedure of pecan walnut kernel (WK) and shell by using response surface methodology (RSM) and determine total phenolic content (TPC) and antioxidant capacity (by ORAC value) as response variables. The main phenolic compounds were determined by means of liquid chromatography coupled to time-of-flight mass spectrometry (LC-MSD-TOF). In addition, we addressed the suitability of pecan walnut kernel and shell extracts to avoid oxidation within O/W emulsions. Finally, viability of human cancer-derived cell lines exposed to pecan walnut kernel and shell extracts was evaluated.

## **Materials and Methods**

**Plant materials and powder preparation.** Pecan (*Carya illinoensis*) walnuts were harvested throughout the region of Chihuahua, Mexico. The hull, shell, pellicle (brown skin) and WK were separated. WK and WS were reduced to a fine powder with a Thermomix (Vorwerk 3300) and dried at 50 °C for 1 hour to reduce moisture content. The powdered samples were stored at -18 °C until analysis.

### **2.1. Response Surface Methodology (RSM)**

#### Experimental design

Factors studied included sample weight to solvent ratio (w/V; ranging from 0.023 to 0.14) and solvent effect of different concentrations of ethanol in water (v/v %; ranging from 29 to 71). Taking into consideration edible safety and green manufacturing, ethanol was selected as the solvent to perform the study. To identify the factors that significantly affected extraction of phenolic compounds, a three level, two factors central composite design was generated using MINITAB-18 with -1, 0, +1 as level coded values, and considering  $\alpha = 1.414$  (Table 1). A 22 run experiment was designed,

including duplicates of factorial and axial points and 6 replicates of the central point. All experiments were performed on the same day. Experimental runs were randomized.

**Table 1.** Independent variables, their coded and real values used in central composite rotatable design.

Independent variables	Coded values				
	-1.414	-1	0	+1	+1.414
	Real values				
m/V (g/mL)	0.023	0.040	0.080	0.12	0.137
Ethanol (%)	29	35	50	65	71

#### Extraction of polyphenolic compounds for RSM

For extracting the phenolic compounds, fine powdered WS was weighed in vials and 10 mL of solvent (different concentrations of ethanol in water) were added. The mixture was stirred for 90 minutes at 200 rpm at room temperature (circa 20 °C) and then centrifuged at 2400 rpm for 10 minutes. Following removal of the supernatant, the residue was washed with 2.5 mL of solvent, centrifuged again at 14000 rpm for 5 minutes and the solution was combined with the previous extract solution. Extracts were stored at -18 °C until used for analysis.

#### Determination of total phenolic content

Total phenolic compounds (TPC) were quantified following the Folin-Ciocalteu method as described by (Villasante *et al.*, 2019). The absorbance was measured in triplicate at 765 nm using a spectrophotometer Fluostar OMEGA (Perkin-Elmer, Paris France). TPC was expressed as mg of Gallic Acid Equivalent (GAE) per gram of dry weight (DW) sample. The standard curve was obtained from measurements the absorbance against different concentrations of GA from 0.12 to 1.70 mM.

#### Oxygen Radical Absorbance Capacity assay (ORAC)

The radical scavenging activity of pecan walnut shell extracts obtained by RSM was determined with the ORAC assay at 37 °C, as previously described by (Segovia *et al.*, 2018). An initial reading was recorded with excitation and emission wavelengths at 485 nm and 520 nm, respectively. Then, 40 µL of 0.3 M, 2'-azobis (2-amidinoprone) dihydrochloride (AAPH) was added and fluorescence was recorded every 2 min for 2 h using a fluorescence spectrometer (Fluostar OMEGA, Perkin-Elmer, Paris France). ORAC values were expressed as µM Trolox Equivalents (TE)/g of dry weight (DW) ± standard deviation (SD). Each sample was measured in triplicate. Area under the curve (AUC) and net AUC were calculated using the following equations (1):

$$AUC = \left( \frac{0.5 + \sum_i^n f}{f_0} \right) t; \quad Net\ AUC = AUC - AUC_{blank} \quad (1)$$

Where f = fluorescence at a given time; fo = initial maximum fluorescence; n = number of cycles; and t = time of each cycle (in this study t = 2 min).

### Statistical analysis for RSM values

Two responses were measured for each set of variable combinations. MINITAB 18.0 statistical software was used. Raw experimental data was fitted to a second-order polynomial model. Regression coefficients were obtained with significance level ( $\alpha$ ) at 0.05. The generalized second-order polynomial model used in the response surface analysis is shown in (equation 2):

$$Y = \beta_0 + \sum_i^j \beta_i X_i + \sum_i^j \beta_{ii} X_i^2 + \sum_i^j \beta_{ij} X_i X_j \quad (2)$$

Where  $Y$  is the response variable;  $X_i$  and  $X_j$  are independent variables;  $\beta_0$  is the model constant coefficient;  $\beta_i$  is the linear coefficient;  $\beta_{ii}$  is the quadratic coefficient; and  $\beta_{ij}$  is the interaction coefficient of variables  $i$  and  $j$ .

### 2.2. Other scavenging activity assays

#### Extraction of polyphenolic compounds for radical scavenging assays

The extraction procedure was the same as that used for RSM (section 2.1.2). The ratio of sample and solvent used was the one that gave the best result in RSM (3.1): 0.0234 g/mL. In the case of the solvent, the % of ethanol in water was 44 % (the average value calculated from those generating optimal polyphenol concentrations according to TPC and ORAC assays)

#### Determination of radical scavenging capacity

Radical scavenging ability of shell pecan walnut samples was determined using a previously described 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay by (Villasante et al., 2019). Initial absorbance measurement was determined at 517 nm using an UV-spectrophotometer (Fluostar OMEGA, Perkin-Elmer, Paris France). Thereafter, 20  $\mu$ L of appropriate diluted samples were added and 75 min later absorbance was measured. The standard curve was obtained by plotting inhibition against different concentrations of Trolox (ranging from 0.02 to 0.5 mM). Results were expressed in  $\mu$ mol TE/g of dry weight (DW)  $\pm$  standard deviation. The measurements were done in triplicate for each sample.

The inhibition percentage for each sample was calculated using the equation (3):

$$\% \text{ inhibition of sample} = \left( \frac{A_0 - A_{\text{of sample}}}{A_0} \right) \times 100 \quad (3)$$

Where  $A_0$  = initial absorbance of DPPH solution;  $A$  of sample = sample absorbance at the end of the reaction.

#### Ferric Reducing Antioxidant Power assay (FRAP)

Ferric reducing power of pecan walnut shell samples was determined following the FRAP method, as described by (Segovia et al., 2018). Initial absorbance reading of FRAP reagent was determined at 593 nm using a UV-spectrophotometer (Fluostar OMEGA, Perkin-Elmer, Paris France). Thereafter, 150  $\mu$ L of sample were added and after 15 min final absorbance was measured at the same wavelength. Results are expressed in  $\mu$ mol TE/ g of DW  $\pm$  SD. The measurements were done in triplicate for each sample.

### Trolox Equivalent Antioxidant Capacity (TEAC)

The radical scavenging ability of pecan walnut shell samples against ABTS radical was determined using a modified TEAC method, proposed by (Segovia *et al.*, 2018). At 30 °C, initial absorbance reading of ABTS reagent was determined at 734 nm using a UV-Vis absorbance spectrophotometer (Fluostar OMEGA, Perkin-Elmer, Paris France). Walnut shell extract was added and after 10 min of reaction, final absorbance was measured at 734 nm, and percentage of inhibition was plotted against different concentrations of Trolox, and calculated using the equation (4).

$$\% \text{ inhibition of sample} = \left( \frac{A_0 - A_{10}}{A_0} - \frac{A_{0 \text{ of blank}} - A_{10 \text{ of blank}}}{A_{0 \text{ of blank}}} \right) \times 100 \quad (4)$$

Where A0 = initial absorbance of ABTS+ solution; A10 = absorbance after 10 min of reaction. Measurements were done in triplicate for each sample. Results are expressed in  $\mu\text{mol TE} / \text{g of DW} \pm \text{SD}$ . The standard curve was obtained by plotting inhibition against different concentration of Trolox (0.021 to 0.55).

### 2.3. Identification of polyphenolic compounds by LC-MSD-TOF

#### Extraction of polyphenolic compounds

The polyphenolic compounds extraction was performed with the best conditions according to radical scavenging assays (section 2.3.1.). The mixture was centrifuged for 10 min at 3000x g. Ethanol was evaporated with nitrogen at room temperature, thereafter the extracts were lyophilized. After lyophilization, the samples were dissolved in 1 mL of methanol, filtered through 0.45  $\mu\text{m}$  nylon filters (Millipore Express®), and stored at -20 °C in darkness until analysis.

#### LC-MSD-TOF

The phenolic compounds were identified according to (Acosta-Estrada *et al.*, 2014), with few modifications. The lyophilized samples were dissolved in 1 mL of methanol and then filtered through 0.45  $\mu\text{m}$  nylon filter. Identification was performed using liquid chromatography coupled to time-of-flight mass spectrometry (LC-MSD-TOF) (1100 Series, Agilent Technologies, Santa Clara, CA), with a reverse-phase column (Zorbax SB-Aq) 4.6 mm ID x 150 mm (3.5  $\mu\text{m}$ ), and scanning samples at different wavelengths (254 nm, 280 nm, and 320 nm). Chromatographic separations were performed using a mixed mobile phase composed of A: water acidified (pH=2) with formic acid and B: acetonitrile. The flow rate was 0.6 mL/min at 25 °C. The gradient was as follows: 0-10 min 15 % B, 10-14 min 58 % B, 14-20 min 80 % B, and 20-30 min 100 % B. It was operated with electrospray ionization (ESI) with the following parameters: nitrogen gas temperature, 300 °C; drying gas flow rate, 8 L/min; curtain gas, 50 psig; capillary voltage, 4000 V; and fragment voltage, 70 V. MS spectra were recorded in the range of m/z 100–1000. Spectral data was collected using Mass Hunter workstation software (Agilent's, California, USA).

### 2.4. Determination of antioxidant activity in emulsions

#### Preparation of emulsions

Oil-in-water emulsions were prepared by dissolving Tween-20 (1% of final concentration) in Milli-Q water and purified sunflower oil (10 % of final concentration), previously treated with aluminum oxide to remove tocopherols. The emulsion was prepared using a modified method proposed by



(Gallego, Skowrya, *et al.*, 2017). Oil was added dropwise to a mixture of water and Tween-20, using ultrasonic mixing for 3 min on ice. Twenty mL of each emulsion were poured into bottles and added to each sample: two negative controls (water and ethanol 50 %), three positive controls corresponding to different concentrations of gallic acid, and three different concentrations of shell extract in water (1:20 g/mL). The emulsion for each sample was prepared in triplicate. The emulsions were stored at 30 °C for 32 days.

#### Determination of peroxide value

Primary oxidation products were measured periodically, every 2-3 days. An aliquot of 0.05 g of each sample was used to determine the peroxide value (PV) by the ferric thiocyanate method (Gallego, Skowrya, *et al.*, 2017). The results were expressed as meq hydroperoxides/kg of emulsion.

### **2.5. Biological assays**

#### Preparation of walnut kernel and shell extracts for cell viability assay

To extract the phenolic compounds, solvent extraction was carried out using 44 % ethanol and 0.0234g/mL of powdered WK or WS. The mixture was stirred at 20 °C for 90 min at 200 rpm, and then centrifuged at 2400 rpm for 10 min. The supernatant was recovered. Ethanol in the extracts was removed using a rotary evaporator (Buchi Labortechnik AG, Switzerland). The samples were frozen in liquid nitrogen and lyophilized for 48 hours. Dried WS extracts were weighed and resuspended in 2 mL of PBS. All samples were filtered (0.22 µm; Tecknochrome).

#### Cell culture and viability

Human tumor-derived HeLa (derived from cervical cancer cells; ATCC no. CCL-2), HepG2 (derived from liver hepatocarcinoma; ATCC no. HB-8065) and MCF-7 (derived from breast adenocarcinoma; ATCC no. HTB-22) cell lines were cultured at 37 °C in 5 % CO<sub>2</sub> using Dulbecco's Modified Eagle's (DMEM) medium supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin and 10 % (v/v) of fetal bovine serum. Cell viability in the presence of 2 % and 7.5 % of pecan walnut kernel and shell extracts was determined in 24-well plates by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described by Gallego *et al.* (2017a). In brief, the cells were incubated in the presence of 0.63 mM MTT and 18.4 mM of sodium succinate for 3 h at 37 °C. Following removal of the medium, formazan was re-suspended with dimethyl sulfoxide (DMSO) supplemented with 0.57 % CH<sub>3</sub>COOH and 10 % sodium dodecyl sulphate. Spectrophotometric determinations were performed at 570 nm in a Cobas Mira S analyzer (Hoffman-La Roche, Basel, Switzerland).

### **2.6. Statistical analysis**

Experimental data was analyzed using Minitab 18 software. Significant differences were determined by ANOVA and mean comparisons were evaluated using Tukey's test at 95 % significance level.

## Results

### 2.7. Response Surface Methodology (RSM)

**Table 2.** TPC and ORAC response values for each set of variables combination.

Weight/volume (g/mL)	Ethanol concentration (%)	n	TPC (mg GAE/g DW)	ORAC ( $\mu\text{mol TE/g DW}$ )
0.04	35	2	34 $\pm$ 0.4 <sup>b</sup>	732 $\pm$ 5 <sup>c</sup>
0.04	65	2	29 $\pm$ 0.3 <sup>c</sup>	914 $\pm$ 6 <sup>b</sup>
0.08	50	6	27 $\pm$ 0.4 <sup>de</sup>	567 $\pm$ 29 <sup>d</sup>
0.12	35	2	26 $\pm$ 0.0 <sup>ef</sup>	430 $\pm$ 5 <sup>ef</sup>
0.12	65	2	21 $\pm$ 0.1 <sup>h</sup>	496 $\pm$ 14 <sup>de</sup>
0.023	50	2	40 $\pm$ 0.5 <sup>a</sup>	1315 $\pm$ 65 <sup>a</sup>
0.08	29	2	28 $\pm$ 0.8 <sup>cd</sup>	496 $\pm$ 11 <sup>de</sup>
0.08	71	2	23 $\pm$ 0.5 <sup>g</sup>	402 $\pm$ 3 <sup>ef</sup>
0.14	50	2	25 $\pm$ 0.01 <sup>f</sup>	380 $\pm$ 10 <sup>f</sup>

Average  $\pm$  standard deviation; different letters in columns represent significant ( $P < 0.05$ ) differences according to Tukey's test.

RSM of solid–liquid extraction was performed to maximize phenolic compounds (TPC) and compounds with radical scavenging activity according to reactivity towards AAPH radical. TPC and ORAC were measured for all variable combinations (Table 2), and the quadratic equation was obtained (Table 3). For TPC normalized response, linear and quadratic terms of sample weight to solvent ratio and ethanol concentration were significant. In contrast, two-way interaction between these factors was not significant.

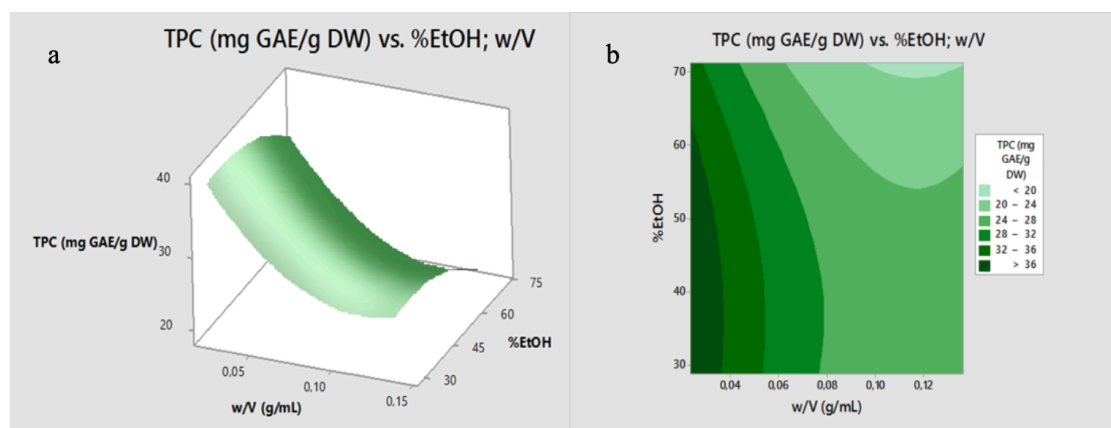
**Table 3.** p-values for each term and respective response variable ( $p < 0.05$ ).

Terms	p-values	
	Response	
	TPC normalized	ORAC normalized
w/v	0.000	0.000
% EtOH	0.000	0.623
(w/v) <sup>2</sup>	0.000	0.000
(% EtOH) <sup>2</sup>	0.000	0.044
w/v x % EtOH	0.651	0.262

As expected, sample weight to solvent ratio was the factor that showed the greatest effect in both: TPC and ORAC response. For TPC, normalized, linear and quadratic terms of sample weight to solvent ratio and ethanol concentration were significant ( $p < 0.05$ ). However, the interaction between w/v and % of ethanol was insignificant ( $p > 0.05$ ). The extraction of phenolic content was increased with increase of ethanol concentration until a maximum at 36% concentration. However, over 36 % of ethanol, the phenolic content started to decline slightly (Figure 1). The surface plot for TPC response indicates a strong decline when sample dry weight to solvent ratio increases to a level,

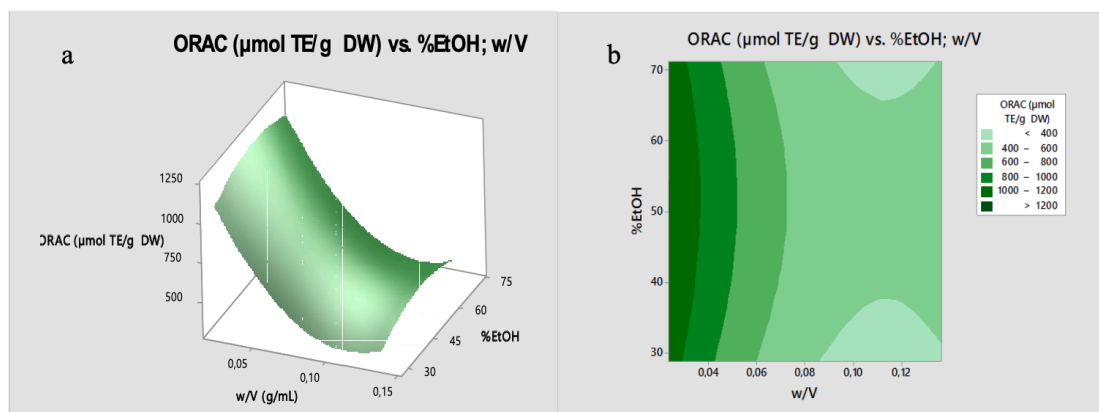
of 0.1g/ml where it no longer significantly varies. Considering that this value is not a direct, absolute term, and that by increasing the mass, the TPC would increase (corrected per unit of mass), the extraction was quantitatively more efficient when more solvent was added (Alberti et al., 2014).

The surface plot for TPC response indicates a strong decline when sample dry weight to solvent ratio increases to a level of 0.1g/ml where



**Figure 1.** Response surface (a) and contour model plot (b) for TPC normalized response.

In contrast, for ORAC, normalized, linear and quadratic terms of sample weight to solvent ratio were significant ( $p < 0.05$ ). Figure 2 shows the effect of sample weight to solvent ratio and ethanol concentration on the radical scavenging activity measured by ORAC.



**Figure 2.** Response surface (a) and contour model plot (b) for ORAC normalized response.

Optimal conditions in the selected range of parameters were: for TPC response, 0.0234 sample weight to solvent ratio and 36 % of ethanol concentration; for ORAC response, 0.0234 sample weight to solvent ratio and 51 % of ethanol concentration; and for the combination of TPC and ORAC responses the best-fitting experimental conditions were 0.0234 sample weight to solvent ratio and 44 % of ethanol concentration.

The high  $R^2$  value of the reduced model indicates that it can be useful for predicting responses to changes in sample weight to solvent ratio and ethanol concentration, at least in the range of conditions used for the experimental (Table 4).

**Table 4.** Quadratic equations for each response variable.

Response variable	Equation	p-value	R <sup>2</sup>	Predicted R <sup>2</sup>
TPC normalized	41.18 – 382.3 w/V + 0.3762 %EtOH + 1637 w/V*w/V - 0,005202 %EtOH*%EtOH	0.000	97.89%	95.75%
ORAC normalized	1062 - 21252 w/V + 22,8 %EtOH + 93434 w/V*w/V - 0,221 %EtOH*%EtOH	0.000	88.38%	80.31%

### 2.8. Total phenolic compounds, antioxidant and radical scavenging activity equations

Various assays have been described to estimate overall radical scavenging of plant extracts. However, there are no specific methods allowing accurate quantification of phenolic compounds due to their heterogeneity and interference from other readily oxidized substances in plant extracts. Thus, each assay may reflect different aspects with regards to the antioxidant behavior of extracts. A few factors can influence quantification of phenolic compounds, such as chemical nature of the analyte, the assay method, standard selection and presence of interfering substances. In the present study, we assayed different methods for this purpose: TPC, ORAC, FRAP, DPPH and ABTS, using the best extraction condition described in section of extraction of polyphenolic compounds for radical scavenging assays.

Table 5. TPC (as gallic acid equivalent), ORAC, FRAP, DPPH and ABTS (as Trolox equivalent) of ethanol and aqueous extracts of shell walnut.

Sample	Solvent	TPC (mg/g DW)	ORAC (μmol TE/g DW)	FRAP (μmol TE/g DW)	DPPH (μmol TE/g DW)	ABTS (μmol TE/g DW)
Walnut	Water	20 ± 0.2	210 ± 15	74 ± 5	145 ± 1	36 ± 3
shell	44%Ethanol	41 ± 0.8	1190 ± 10	114 ± 3	205 ± 3	60 ± 5

Average ± standard deviation; all the samples by column are significant ( $P < 0.05$ ) differences according to Tukey's test. The measured for each sample was in triplicate ( $n=3$ ).

Table 5 shows the results of TPC and the antiradical capacity of shell extracts. Extraction with 44 % ethanol provided with the best TPC content. TPC values correlated with those in the equation shown in Table 4. A similar pattern was observed by (Einali *et al.*, 2018); the authors worked with Persian walnut, and the total phenolic content for ethanol extracts was nearly 4.5-fold higher than water extracts. This behavior can be explained by the low polarity effect of phenolic component in pecan walnut shell, which cannot be extracted with water (Samaranayaka *et al.*, 2008).

ORAC values obtained represents ROO<sup>-</sup> (peroxyl radical) scavenging capacity. In this study, the results were 210 and 1190 μmol TE /g DW for aqueous and ethanolic extracts, respectively. DPPH is a stable free radical with purple color that changes to light yellow when antioxidant molecules scavenge free radicals by hydrogen donation (Rahmani *et al.*, 2018). The value from the DPPH assay for ethanolic extracts was higher than that for aqueous shell extracts (205 and 145 μmol/g DW, respectively). The DPPH scavenging values were lower values than those presented by (Villarreal-Lozoya *et al.*, 2007) in pecan walnut shell from Texas, USA. FRAP and ABTS values for ethanolic extracts were 1.5 times greater than for aqueous extracts.

**Table 6.** Phenolic profile of pecan walnut shell extrudate ethanolic extracts obtained with LC-MSD-TOF

Peak	Tentative Identification	t <sub>R</sub>	Molecular formula	Expected mass (m/z)	Observed mass [M-H] <sup>-</sup> (m/z)	Fragments ions (m/z)	Mass error (ppm)
1	Caffeic acid 3-glucoside	0.86	C <sub>15</sub> H <sub>18</sub> O <sub>9</sub>	342.0951	341.0881	133.01	-2.4920
2	3-p-Coumaroylquinic acid	2.94	C <sub>16</sub> H <sub>18</sub> O <sub>8</sub>	338.1002	337.0907	153.02, 164.16, 192.17	4.8651
3	Protocatechuic acid	3.17	C <sub>7</sub> H <sub>6</sub> O <sub>4</sub>	154.0266	153.0201	109,137,123,95	-5.0713
4	(+)-Gallocatechin	3.90	C <sub>15</sub> H <sub>14</sub> O <sub>7</sub>	306.0740	305.0700	261.01, 153.02	-12.7186
5	Quercetin	6.10	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	302.0427	301.0359	175.06	-3.5876
6	Vanillic acid	6.12	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	166.0266	165.0188	150, 136, 122	-0.1212
7	Procyanidin C2	7.47	C <sub>45</sub> H <sub>38</sub> O <sub>18</sub>	866.2058	865.1965	165.02, 289.07, 319.12, 475.14, 584.12, 785.08	1.7106
8	(+)-Catechin	7.48	C <sub>15</sub> H <sub>14</sub> O <sub>6</sub>	290.0790	289.0709	165.01	1.0724
9	Dactylorhin E	14.40	C <sub>27</sub> H <sub>40</sub> O <sub>16</sub>	620.2316	619.2229	579.26, 481.13, 451.12, 300.99, 187.09	2.2997
10	Ellagic acid	14.47	C <sub>14</sub> H <sub>6</sub> O <sub>8</sub>	302.0063	300.9989	187.09, 212.07	0.4120
11	Phloridzinyl glucoside	15.32	C <sub>27</sub> H <sub>34</sub> O <sub>15</sub>	598.1898	597.1811	491.08, 317.03, 273.04	2.3845
12	Myricetin	15.42	C <sub>17</sub> H <sub>10</sub> O <sub>8</sub>	318.0376	317.0292	200.12, 273.03	3.5454
13	(-)- Epigallocatechin gallate	17.36	C <sub>22</sub> H <sub>18</sub> O <sub>11</sub>	458.0849	457.0791	331.01, 212.08	-4.4194
14	Syringetin-3-o-glucoside	17.39	C <sub>23</sub> H <sub>24</sub> O <sub>13</sub>	508.1217	507.1129	457.17, 331.01	3.0052
15	Ferulic acid	29.27	C <sub>10</sub> H <sub>10</sub> O <sub>4</sub>	195.0657	194.0577	136, 150, 164, 178	1.0306
16	1-O-Galloyl Castalagin	32.93	C <sub>48</sub> H <sub>30</sub> O <sub>30</sub>	1086.0822	1085.0784	893.19, 701.15, 401.19, 311.20, 249.15	-3.7232

Table 6 show phenolic compounds identified in pecan walnut shell extrudate ethanolic extracts after LC-MSD-TOF. Component analysis of pecan shell extracts indicated the presence of 16 major phenolic species. Identification of phenolic compounds was based on spectral MS/MS information, previous literature data and high-resolution MS/MS databases (e.g. Metlin database and PubChem). Retention times provided in Table 6 correspond to the start and the end of the whole chromatographic band. Some of the compounds identified in the raw material (epigallocatechin gallate, ellagic acid, catechin, vanillic acid, and caffeic acid 3-glucoside) were also reported in previous studies (Hilbig et al., 2018; Do Prado et al., 2014). The first peak of the MS/MS spectra corresponded to negative molecular ion [M - H]<sup>-</sup> at m/z 341.11 and was identified as caffeic acid 3-glucoside. Caffeic acid 3-glucoside is a hydroxycinnamic acid very commonly found in woody plants, biomass and by-products, being an intermediate molecule in lignin biosynthesis (Boerjan et al., 2003). (Pinto et al., 2020) also reported the presence of caffeic acid 3-glucoside in chestnut shells (15.4 µg/mg of dry chestnut shell). Peak 2 displayed [M - H]<sup>-</sup> at m/z 337.11 and was identified as a 3-p-coumaroylquinic acid. Similarly, (Jiang et al., 2019) found 3-p-coumaroylquinic acid in bamboo shoot shell. The present study identifies for the first time this compound in pecan shell. Peak 3 showed a [M - H]<sup>-</sup> at m/z 153.02, which corresponded to protocatechuic acid, also named 3,4-dihydroxybenzoic acid. Benzoic acid was previously reported in pine nuts by (Zulfqar et al., 2020), and in pecan shell by (Kureck et al., 2017). Protocatechuic acid exhibited potential chemotherapeutic activity against human cancer-derived cell lines (Yin et al., 2009). Peak 4 corresponded to molecular ion [M - H]<sup>-</sup> at m/z 305.07 and was identified as (+)-gallocatechin. (+)-Gallocatechin is a flavan, catechin found in pecan shell (Do Prado et al.,

2014), brazil nuts (Hu et al., 2016), and chestnut (Sorice et al., 2016). Peak 5 had a mass spectrum with  $[M - H]^-$  at  $m/z$  301.00 and corresponded to the negative molecular ion of quercetin. Quercetin is a polyphenolic flavonoid widely distributed in many plants and fruits including chia seed (Lako et al., 2007) and almonds, macadamia, pine nuts and walnuts (Harnly et al., 2006). Previous in vitro studies demonstrated that quercetin impedes the growth of several tumors, such as breast (Li et al., 2018), and ovary (Vafadar et al., 2020). (Yi et al., 2017) evaluated the antioxidant properties of quercetin and rutin in O/W emulsions under metal catalysis and riboflavin photosensitization. The authors found that 0.5 mM quercetin and 1.0 mM rutin exerted antioxidative effects in O/W emulsions under riboflavin photosensitization (Yi et al., 2017). Peak 6  $[M - H]^-$  at  $m/z$  165.01 was identified as vanillic acid. Peak 7 showed parent ions at  $[M - H]^-$  at  $m/z$  865.19, which were tentatively identified as isomers of procyanidin dimers. Procyanidin C2 is a proanthocyanidin a type of condensed tannin. It is found in red wine. Previous works showed health benefits of this compound. (Touriño et al., 2005) found that procyanidin fractions from pine (*Pinus pinaster*) bark inhibited SK-Mel-28 human melanoma cell proliferation. Furthermore, the authors studied the effect of the same compound on lipid peroxidation in an oil-in-water emulsion. Peak 8, with a mass spectrum of  $[M - H]^-$  at  $m/z$  289.07, corresponded to catechin, which is a flavan-3-ol highly present in some nuts as Brazilian nut, and almond hull (Gomes et al., 2019). (Turkoglu and Mansuroglu, 2020) evaluated the cytotoxic effect of catechin loaded nanoparticles on breast cancer cell lines. After 48 h, MCF-7 cells exposed to catechin nanoparticles decreased cell viability with an  $IC_{50}$  22.59  $\mu\text{g/mL}$ . (Rathore and Wang, 2012) investigated the activity of green tea catechin extract on MCF10A cells (breast cancer). Green tea catechin extract at 100 mg/mL, but not at 0.5, 2.5, 10, and 40 mg/mL, reduced cell viability by inhibiting cell proliferation or inducing apoptotic cell death (Rathore and Wang, 2012). Peak 9 was identified as dactylorhin E, which is found in some medicinal plants such as *Dactylorhiza hatagirea* (Parimelazhagan Thangaraj, 2018). Peak 10 corresponded to negative molecular ion  $[M - H]^-$  at  $m/z$  300.99 and was identified as ellagic acid. Ellagic acid derives from gallic acid and is found in numerous fruits and vegetables, including: grapes, raspberries, strawberries, cranberries, almonds, hazelnuts, walnuts, pecans, and other plant-derived foods (Gorji et al., 2018). Several studies provides evidence that ellagic acid possesses antimutagenic, anti-inflammatory, and antiproliferative properties (Baradaran Rahimi et al., 2020). (Cheshomi et al., 2020) found the mechanism of action of ellagic acid on various types of human malignancies. Peak 12, identified as myricetin ( $[M - H]^-$  at  $m/z$  317.02) is a hexa hydroxy flavone found in almonds, hazelnuts, walnuts, cranberries, and more fruits (Dormán et al., 2016; Gorji et al., 2018). Peak 13 corresponded to epigallocatechin gallate ( $[M - H]^-$  at  $m/z$  457.17), a phenolic antioxidant found in green and black tea, apple skin, plums, onions, hazelnuts, and pecans (Bhagwat et al., 2011). Peak 14 had a mass spectrum with  $[M - H]^-$  at  $m/z$  ,507.11, and corresponded to syringetin-3-o-glucoside, a member of the flavonoid family found in the chichá pellicle (De Britto Policarpi et al., 2018). Peak 15 was identified as ferulic acid ( $[M - H]^-$  at  $m/z$  194.08), which is a hydroxycinnamic acid found in plant cell walls. A lot of by-products contain this compound, such as the shell and pellicle of the chichá and pecan walnut (De Britto Policarpi et al., 2018; De la Rosa et al., 2014). Oh et al. (2015) worked with the addition of roasted barley in O/W emulsions. Oh et al. (2015) previously analyzed by HPLC-MS major phenols contained in the barley, which were p-coumaric, ferulic, protocatechuic, chlorogenic, 4-hydroxybenzoic, and vanillic acids (Oh et al., 2015). The last peak had a mass spectrum with  $[M - H]^-$  at  $m/z$  1085.22, and corresponded to 1-O-galloyl castalagin, a hydrolysable tannin firstly described in chestnut bark samples and that might originate from the esterification of castalagin or vescalagin with a gallic acid residue (Comandini et al., 2014).

Given the phenolic compounds determined in the extract of pecan walnut shell, this by-product was subsequently applied to an emulsion (o/w) food model. In addition, based on the bibliography and the results obtained (Table 6), antiproliferative activity of pecan walnut shell extracts was determined by assaying the effect on cell viability of three human tumor-derived cell lines.

### 2.9. Oil-in-water emulsion

**Table 7.** Changes in peroxide value (PV) over storage time at  $30 \pm 1$  °C in O/W emulsions prepared with different concentrations of kernel and shell extract (1.09, 4.35 and 6.52 g/L) and gallic acid (0.21, 0.39 and 0.78 g/L).

Treatments	Time to reach 10 PV (hours) <sup>(a)</sup>	Time to reach 50 PV (hours) <sup>(b)</sup>
Water control	< 64 <sup>f</sup>	176 ± 16 <sup>e</sup>
Gallic acid (0.21 g/L)	72 ± 9 <sup>f</sup>	266 ± 15 <sup>d</sup>
Gallic acid (0.39 g/L)	245 ± 18 <sup>d</sup>	594 ± 23 <sup>c</sup>
Gallic acid (0.78 g/L)	420 ± 13 <sup>c</sup>	Not reached
Kernel (1.09 g/L)	138 ± 2 <sup>e</sup>	302 ± 10 <sup>d</sup>
Kernel (4.35 g/L)	537 ± 7 <sup>b</sup>	757 ± 10 <sup>b</sup>
Kernel (6.52 g/L)	539 ± 16 <sup>b</sup>	722 ± 3 <sup>b</sup>
Shell (1.09 g/L)	206 ± 2 <sup>d</sup>	325 ± 5 <sup>d</sup>
Shell (4.35 g/L)	680 ± 14 <sup>a</sup>	Not reached
Shell (6.52 g/L)	695 ± 5 <sup>a</sup>	Not reached

Different letters by line represent significant differences by Tukey's test ( $p < 0.05$ ). Average ± standard deviation. The emulsion for each sample was prepared in triplicate ( $n=3$ ).

Oil-in-water emulsion was chosen as a model food system to assay the antioxidant effectiveness capacity of pecan walnuts samples. The formation of hydroperoxides (Table 7) was significantly faster in control samples (without the presence of antioxidants). Control samples reached 10 meq of hydroperoxides/kg in less than 65 hours, whereas samples containing gallic acid at 0.21 g/L reached this value in 72 hours, and those containing gallic acid at 0.39 g/L reached it in 245 hours. The lowest concentration of kernel and shell extracts assayed (1.09 g/L) prevented oxidation at levels similar to gallic acid concentrations between 0.21 g/L and 0.39 g/L. Higher concentrations of shell extract were more stable and started oxidation towards the end of the experiment (after 768 hours), offering more protection than gallic acid at 0.78 g/L. The oxidation of samples (reaching the hydroperoxide value of 10 meq of hydroperoxides/kg emulsion) followed this order: control (without any walnut or gallic acid extract) > gallic acid 0.21 g/L > walnut at 1.09 g/L > shell 1.09 g/L > gallic acid at 0.39 g/L > gallic acid at 0.78 g/L > walnut at 4.35 g/L > walnut at 6.52 g/L > shell at 4.35 g/L > shell at 6.52 g/L.

According to the results of the present study, both pecan walnut kernel and shell presented high contents of polyphenols and radical scavenging activity, with shells extracts having the highest antioxidant activity. Similarly, De la Rosa *et al.* reported higher polyphenol content and antioxidant activity in walnut shell than in the kernel (De la Rosa *et al.*, 2014). These authors reported that increased levels of  $\alpha$ -tocopherol in water-in-walnut oil emulsions decreased antioxidant ability.

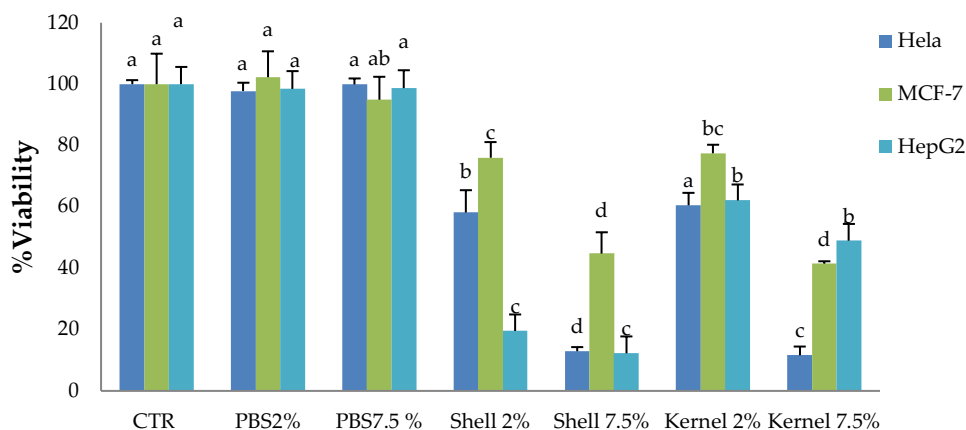
Furthermore, Jianhua Yi *et al.* (2015) observed similar effects that this work. The application of pecan walnut extracts as antioxidants in oil-in-water emulsions could be a good alternative to synthetic antioxidants. These results provide evidence supporting the potential use of pecan walnut kernel and shell extracts as natural preservatives in the food industry.

#### **2.10. Viability-reducing activity of antioxidant extract against human-derived cancer cell lines.**

Major compounds from the ellagitannin family in pecan walnut kernel extracts have been reported as cytotoxic for human prostate cancer and breast adenocarcinoma-derived cell lines (Le *et al.*, 2014; Sánchez-González *et al.*, 2014). Given that WK are an important source of polyphenols for the human diet, and that polyphenolic compounds such as ellagitannin may contribute to the prevention and reduced progression of cancer (Sánchez-González *et al.*, 2015), the viability of three human-derived cancer cell lines derived from cervical cells (HeLa), liver hepatocarcinoma (HepG2) and breast adenocarcinoma (MCF-7) was assayed in the presence of 2 % and 7.5 % of walnut WK and WS extracts (solvent with 44 % ethanol and sample weight to solvent ratio of 0.0234g/mL). As shown in Figure 3, both WK and WS significantly reduced in a dose-dependent manner viability of the three cell lines. Hilbig *et al.* previously reported cytotoxicity of WK on MCF-7 cells and the authors attributed the cytotoxic effect to the phenolic profile of the extract, which contained compounds such as gallic, 4-hydroxybenzoic, chlorogenic, vanillic, caffeic acid, ellagic acid, catechin, epicatechin, epigallocatechin and epicatechin gallate (Hilbig *et al.*, 2018). As mentioned above, some of these compounds were identified in pecan walnut shell extract (Table 6). The results of the present study confirmed the antiproliferative properties of WS extracts on MCF-7 cells. Furthermore, no significant differences were found when using WK or WS extracts. Both WK and WS extracts also affected similarly viability of HeLa cells. In this regard, the strong reduction of viability observed for HeLa cells (up to 87-88 % of reduction using 7.5 % kernel and shell extracts) emphasizes the potential use of pecan walnut extracts for the prevention and treatment of cervical cancer. Interestingly, shell extracts had a remarkably higher reducing effect on the viability of HepG2 cells (81 and 88 % reduction using 2 % and 7.5 % shell extracts, respectively) than WK extracts (38 and 51 % reduction using 2 % and 7.5 % kernel extracts, respectively). Our results suggest that WS may thus contain bioactive compounds that can potentially be good targets to explore their effectiveness in the pharmacotherapy of hepatocellular carcinoma.



**Figure 3.** Effect of walnut kernel and shell extracts on cell viability of HeLa, MCF-7 and HepG2 cells. Viability of HeLa, MCF-7 and HepG2 cells was assayed 48 h after treatment with 2 % and 7.5 % kernel and shell walnut extracts, using PBS as solvent.



Different letters by line cell represent significant differences by Tukey's test ( $p < 0.05$ ). The experiment was performed in triplicate ( $n=3$ ). Average  $\pm$  standard deviation.

## Conclusion

The results of the present study indicate that the amount of polyphenols extracted from pecan walnut shell and their radical scavenging capacity are highly dependent on the solvent. A mixture of ethanol in water (44 %) was found to be more effective in extracting phenolic compounds than water. Also, the phenolics compounds present in the shell extract were identified by LC-MSD-TOF. It was demonstrated that addition of 6.52 g/L of shell and kernel extract to the emulsion with 10 % oil provided better protection against oxidation compared to gallic acid at 0.78 g/L. Both WK and WS at 7.5 % significantly reduced in a dose-dependent manner viability of the three cell lines. The highest cell viability reduction was observed in HeLa cells (up to 87-88 %), followed by HepG2 cells (81 and 88 %). These results provide evidence supporting the potential use of pecan walnut kernel and shell extracts as natural preservatives in the food industry.

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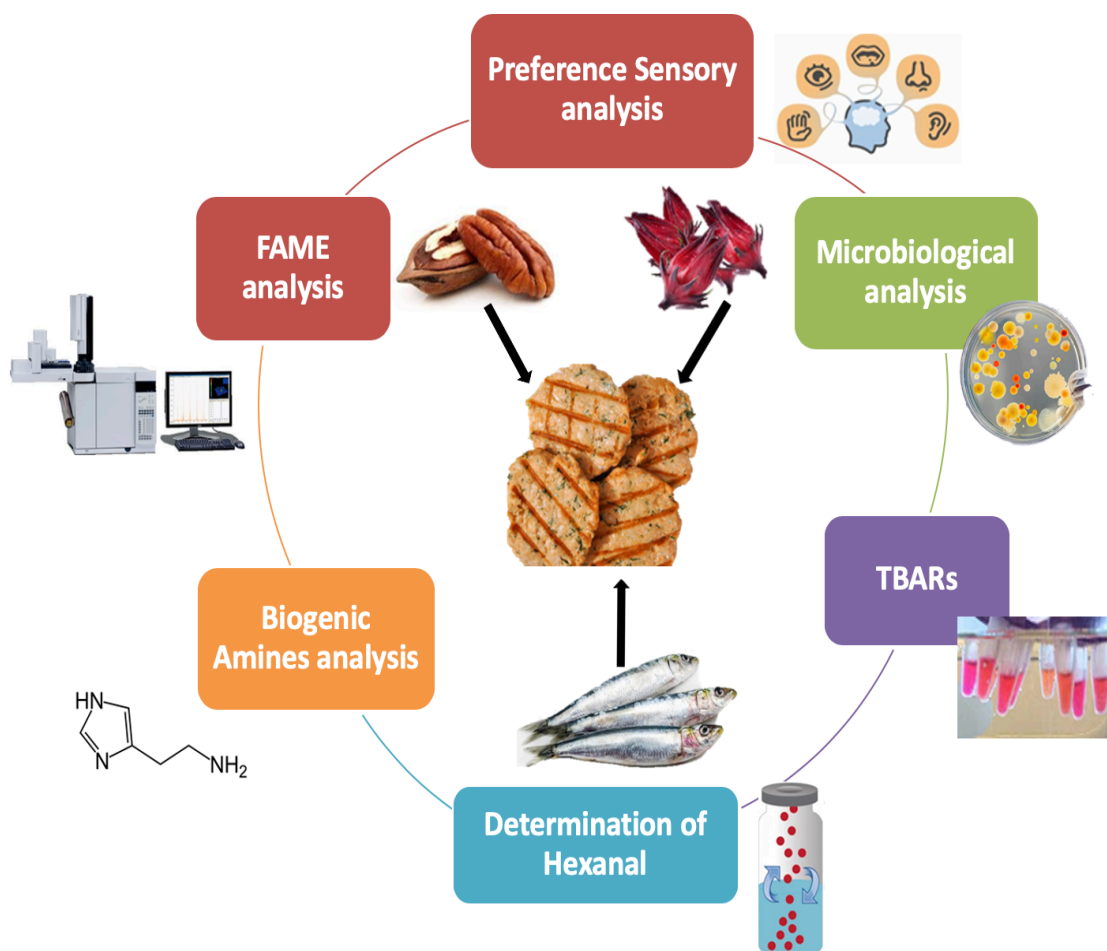
**Chapter 5. The combined effect of the pecan kernel and shell with other plant products on antioxidant and antimicrobial activity.**

## 5.1. Effects of Pecan Nut (*Carya illinoensis*) and Roselle Flower (*Hibiscus sabdariffa*) as Antioxidant and Antimicrobial Agents for Sardines (*Sardina pilchardus*)

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Article

# Effects of Pecan Nut (*Carya illinoensis*) and Roselle Flower (*Hibiscus sabdariffa*) as Antioxidant and Antimicrobial Agents for Sardines (*Sardina pilchardus*)

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**Abstract:** The effects of pecan nut (*Carya illinoensis*) and roselle flower (*Hibiscus sabdariffa*) as antioxidant and antimicrobial agents on shelf life extension of sardines (*Sardina pilchardus*) were evaluated over a period of 5 days at  $7 \pm 1$  °C. Treatments consisted of the addition of 5% and 10% w/w pecan nut, 5% w/w roselle flower and a combination of 5% of each. Physicochemical (lipid oxidation, fatty acids, hexanal and biogenic amines), sensory and microbiological characteristics of fish samples were periodically analyzed. All treatments effectively improved physicochemical quality parameters, with 10% w/w pecan nut having the highest effectiveness. The presence of roselle flower reduced microbial growth. Our findings suggest that addition of a natural preservative combining pecan nut and roselle flower may extend the shelf life of fresh sardines during chilled storage while maintaining quality indexes.

**Keywords:** pecan nut; roselle flower; *Sardina pilchardus*; health benefits; oxidation; biogenic amines; hexanal

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## 1. Introduction

Sardine (*Sardina pilchardus*) fisheries have an important economic impact on Europe and North Africa. Its production in Europe reached 175 thousand tonnes in 2014, with a value of 161 million euro [1]. Due to its high content of protein and polyunsaturated fatty acids (PUFA) the sardine is a highly perishable fish with remarkable nutritional benefits for human health [2]. Lipid oxidation during storage causes rancidity, off-taste, off-odor and color changes, which can affect consumer's perception. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) [3] are used to extend its shelf life, but health problems derived from excessive consumption of these antioxidants have been reported [4]. Therefore, there is increasing concern

from consumers and the food industry to avoid the use of synthetic antioxidants and find healthier alternatives. Research into the use of natural antioxidants present in spices, herbs, fruits, plants or teas containing high levels of phenols, anthocyanins and ascorbic acid (among other compounds, which can act as radical scavengers and prevent the reaction of lipid peroxidation), has increased [5].

Moreover, consumption of plants containing natural phenolic compounds, has been recommended to prevent free radical formation and reduce the risk of developing cancer, diabetes, cardiovascular disease and Alzheimer's disease, among others pathologies [6].

Pecan nut (*Carya illinoensis*) has been shown to contain the highest amount of phytochemicals of all nut types [7]. Studies show strong correlations between inclusion of pecan nut in the diet and reduction of triacylglycerols, LDL cholesterol and increase of HDL cholesterol [8]. This beneficial effect on health comes from the high amounts of phenolics, flavonoids, proanthocyanidins, monounsaturated fatty acids and tocopherols present in pecan nuts [9].

Previous research on the use of walnut as an antioxidant for meat showed its effectiveness on preventing food deterioration. In addition, adding walnut paste to meat improved MUFA and PUFA contents and amino acid profiles [10].

Roselle (*Hibiscus sabdariffa*) flowers contain high, but variable amounts, depending on the cultivar, of total phenolics, flavonoids and anthocyanidins, and are reported to possess antimicrobial activity for Gram-positive and Gram-negative bacteria [11].

The aim of this research is to test the effectiveness of pecan nut as a natural preservative to delay the oxidation of naturally occurring fish lipids. To this effect, sardine was chosen because it is a fish known to contain high amounts of fat, therefore it can be deduced that if pecan nut works as a preservative for sardines it would also work for other fish species with lower amounts of fat. Also, another objective was to assess the antimicrobial effect of roselle flowers, to be used alone or combined with other natural antioxidants or preservatives for the food industry.

## 2. Results and Discussion

### 2.1. Determination of Total Phenolic Content and DPPH Radical Scavenging Activity

The total phenolic contents (TPC) of defatted pecan nut and roselle flower were measured on the corresponding ethanol extracts (50%) and no significant difference was found, while the radical scavenging activity (RSA) was significantly higher for defatted pecan kernels than for roselle flower extracts.

TPC content and RSA for defatted pecan nut kernel and roselle flower ethanol extracts varied significantly in previous research. This variability could result from the origin and cultivar of each sample. Table 1 displays the results obtained in this work.

**Table 1.** Total phenolic compounds and radical scavenging activity.

Sample	TPC (mg GAE/g FW)	RSA (mg TE/g DW)
Defatted Pecan Nut kernel	22.95 ± 0.04	37.63 ± 1.08
Roselle flower	22.40 ± 0.02	19.69 ± 0.42

Results were expressed as milligrams gallic acid equivalents (GAE) per gram of fresh sample weight (FW) and milligrams of Trolox equivalents (TE) per gram of dry sample.

The total phenolic content (TPC) of defatted pecan nut and roselle presented no significant difference (22.95 mg GAE/g FW  $\pm$  0.04 and 22.40 mg GAE/g FW  $\pm$  0.02), but the radical scavenging activity (RSA) was significantly higher in defatted pecan kernels (37.63 mg TE/g FW  $\pm$  1.08 and 19.69 mg TE/g FW  $\pm$  0.42 for defatted pecan kernels and roselle, respectively). Results are expressed as mean  $\pm$  SD ( $n = 3$ ).

Values for TPC content and RSA for defatted pecan nut kernel vary significantly in the literature: Alasalvar and Shahidi [12] reported similar results for TPC in pecan; Villarreal-Lozoya, Lombardini and Cisneros-Zevallos [13] obtained around double TPC and triple RSA mean values. A review on tree nut phytochemicals by Bolling et al. [7] described a smaller value for TPC and higher for RSA. De la Rosa et al. [8] reported similar but lower RSA pecan nut kernel mean values. Mak et al. [14] obtained a two-fold higher TPC and very similar RSA value for roselle flower ethanol extract and Afify and Hassan [15] described much lower TPC and RSA values. Possible reasons for the great variability found in literature values could be related to the origin and cultivar of each sample.

## 2.2. Microbiological Analysis

Presence of colony-forming units was evaluated in the treatments (control, 5% w/w PN, 10% w/w PN, 5% w/w R, 5% w/w PN + 5% w/w R, and 0.1% w/w BHA) with one and three days of incubation after treatment (Table 2). The main goal of this analysis was to assess the antimicrobial properties of roselle flower and pecan nut as well as checking initial contamination of the samples, with a qualitative analysis. The amount of bacteria present in all samples at day one of incubation was less than 10 CFU/g sample. Three days' post-treatment showed that roselle flower and BHA acted as antimicrobial agents. Our findings are consistent with previous reports in which roselle flower was successfully used to disinfect carrots, tomatoes [16] and Hass avocado [17]. The results of the present study suggest that roselle flower can be used to supplement the antioxidant activity of pecan nut, hence obtaining a food preservative with both antioxidant and antimicrobial properties.

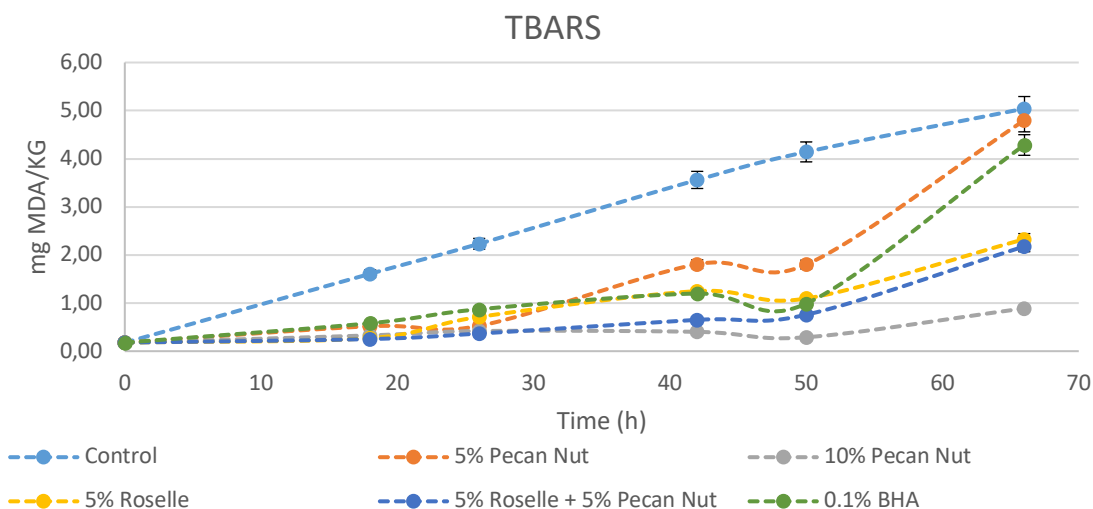
**Table 2.** Presence of mesophilic bacteria in the samples.

Treatment	Day 1	Day 3
Control	-	+
5%Pecan Nut	-	+
10% Pecan Nut	-	+
5% Roselle	-	-
5% Roselle + 5% Pecan Nut	-	-
0.1% BHA	-	-

- indicates there is no presence; + an amount between 30 and 100 CFU/g.

### 2.3. Thiobarbituric Acid Reactive Substances (TBARS)

The TBARS method is widely used for determining the oxidation of fats and oils in foods. Malondialdehyde and related compounds are formed when the concentration of hydroperoxides is appreciable in oil or fat. Hydroperoxides decompose to form secondary oxidation products [18,19]. Results shown in Figure 1 and Table 3 indicate that after 66 h of incubation in the fridge, samples containing 10% w/w of pecan nut were significantly less oxidized than in all other samples, including incubation with the artificial antioxidant BHA. Until 50 h all treatments, except the control, follow a similar trend; after this time, there is a noticeable increase of oxidation rate in samples containing 5% w/w pecan nut and 0.1% BHA. Samples with 5% w/w roselle flower and 5% of both pecan nut and roselle flower were also effective in relation to the control. Özogul et al. [20] studied the effects of rosemary and sage tea extracts in preventing lipid oxidation of sardine at 3 °C after six days. Erkan et al. [21] reported TBARS values of sardines after storage at 2 °C for five days using thyme and laurel essential oils as additives. Results in both studies and in the present work follow a similar tendency and together support the idea that the addition of natural compounds with antioxidant and antimicrobial activity provides an effective methodology to extend shelf life of fresh sardines.



**Figure 1.** Evolution of TBARS value 1 ( $7 \pm 1$  °C) in *Sardina pilchardus* samples with different treatments in a period of 66 h. 1 Results are expressed in mg MDA/kg.

**Table 3.** Evolution of TBARS values (mg MDA/kg) in *Sardina pilchardus* samples at 18, 50 and 66 h of incubation at  $7 \pm 1$  °C.

Treatment	Hours		
	18	50	66
Control	1.61 <sup>a</sup> ± 0.05	4.14 <sup>a</sup> ± 0.25	5.04 <sup>a</sup> ± 0.01
5% Pecan Nut	0.52 <sup>bc</sup> ± 0.13	1.80 <sup>b</sup> ± 0.35	4.80 <sup>a</sup> ± 0.01
10% Pecan Nut	0.33 <sup>cd</sup> ± 0.03	0.29 <sup>d</sup> ± 0.01	0.89 <sup>c</sup> ± 0.15
5% Roselle	0.27 <sup>d</sup> ± 0.02	1.10 <sup>c</sup> ± 0.08	2.33 <sup>b</sup> ± 0.23
5% Roselle + 5% Pecan Nut	0.25 <sup>d</sup> ± 0.02	0.76 <sup>cd</sup> ± 0.03	2.18 <sup>b</sup> ± 0.43
0.1% BHA	0.58 <sup>b</sup> ± 0.06	0.99 <sup>b</sup> ± 0.08	4.29 <sup>a</sup> ± 0.02

Results are expressed as mean ± SD (n = 3). <sup>a-d</sup> Different letters in the same column indicate significant differences (p < 0.05).

#### 2.4. FA Analysis

FAME analysis was used to assess the amount of FA present in sardine loins and monitor changes among samples containing pecan nut and roselle flower. Results are displayed in Table 4.

Major FA present in the control and samples treated with 0.1% BHA are myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1), oleic acid (C18:1n), erucic acid (C22:1n9), linoleic acid (C18:2n6c),  $\alpha$ -linolenic acid (C18:3n3) and cis-4,7,10,13,16,19-docosahexaenoic Acid (DHA, C22:6n3). The FA composition ranged from 33.89 to 38.39% of saturated FA (SFA), 23.44 to 27.04% of monounsaturated FA (MUFAs) and 25.04 to 31.89% of polyunsaturated FA (PUFAs). Even though FA amounts in sardine may vary significantly depending on the origin and season, the results of the present study are in agreement with those obtained previously for sardine muscle [22]. FA content in samples incubated with 5% w/w and 10% w/w pecan nut vary significantly in relation to the control due to the presence in pecan nuts of amounts four-fold higher of oleic acid (C18:1n9) and linoleic acid (C18:2n6c) [23]. As a consequence, total SFA is halved, MUFA doubled and PUFA content remains similar compared to control samples. Therefore, the use of pecan nuts would have a beneficial side effect by increasing the total amount of the healthier types of FA (MUFA and PUFA).

Incubation with 5% w/w roselle flower did not significantly change FA concentrations compared with control samples. Palmitic acid, oleic acid and DHA are the acids with a higher percentage within control samples. These results are similar to those previous studies [24] reporting the presence of myristic (2.1%), palmitic (35.2%), palmitoleic (2.0%), stearic (3.4%), oleic (34.0%) and linoleic (14.4%) acids in roselle seeds.

All treatments promoted low levels of arachidonic acid (C20:4n6, 0.03–0.58%), which may have antagonistic effects to the health benefits of the n-3 FA [25]. Moreover, the UK Department of Health recommends a maximum ratio of n6/n3 of 4.0, which is much higher than in any of the present treatments (0.04–0.07%) [26]. Indeed, a minimum value of PUFA/SFA ratio recommended is 0.45 [26] which is lower than those obtained for all fish treatments (0.65–2.86%).

Palmitic acid was the primary saturated FA, contributing 9.33–26.44% of total SFA in all treatments. Oleic acid was the major MUFA, accounting for 15.03–52.62% of total MUFAs and linoleic acid as well as DHA were the major FA identified as PUFAs, accounting for 6.22–31.30% and 1.04–22.98%, respectively.

**Table 4.** Fatty acids profiles of *Sardina pilchardus* muscle with different treatments.

Fatty Acids (%)	Control	5% Pecan Nut	10% Pecan Nut	5% Roselle	5% Roselle + 5% Pecan Nut	0.1% BHA
C6:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C8:0	0.20 ± 0.28	0.02 ± 0.02	0.01 ± 0.01	0.17 ± 0.23	0.02 ± 0.03	0.00 ± 0.00
C10:0	0.18 ± 0.26	0.02 ± 0.02	0.02 ± 0.02	0.13 ± 0.19	0.02 ± 0.03	0.06 ± 0.09
C11:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C12:0	0.00 ± 0.00	0.01 ± 0.02	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.02	0.06 ± 0.08
C13:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C14:0	2.54 <sup>a</sup> ± 0.10	0.63 <sup>b</sup> ± 0.14	0.29 <sup>b</sup> ± 0.06	2.49 <sup>a</sup> ± 0.24	0.71 <sup>b</sup> ± 0.05	3.22 <sup>a</sup> ± 0.78
C15:0	0.54 ± 0.01	0.12 ± 0.02	0.06 ± 0.01	0.54 ± 0.06	0.14 ± 0.01	0.72 ± 0.15
C16:0	24.29 <sup>a</sup> ± 0.71	11.40 <sup>bc</sup> ± 0.76	9.33 <sup>c</sup> ± 0.59	24.75 <sup>a</sup> ± 0.71	12.04 <sup>b</sup> ± 0.38	26.01 <sup>a</sup> ± 0.58
C17:0	0.45 ± 0.03	0.14 ± 0.01	0.10 ± 0.00	0.47 ± 0.05	0.17 ± 0.00	0.64 ± 0.20
C18:0	3.82 <sup>a</sup> ± 0.19	2.52 <sup>b</sup> ± 0.08	2.35 <sup>b</sup> ± 0.07	3.35 <sup>a</sup> ± 0.19	2.54 <sup>b</sup> ± 0.05	3.95 <sup>a</sup> ± 0.38
C20:0	0.82 ± 0.06	0.14 ± 0.04	0.07 ± 0.00	0.20 ± 0.10	0.19 ± 0.01	0.57 ± 0.81
C21:0	0.07 ± 0.10	0.01 ± 0.02	0.01 ± 0.01	0.12 ± 0.01	0.01 ± 0.02	0.19 ± 0.04
C22:0	0.67 ± 0.04	0.13 ± 0.05	0.05 ± 0.01	0.68 ± 0.11	0.16 ± 0.00	0.83 ± 0.23
C23:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C24:0	0.69 ± 0.10	0.10 ± 0.01	0.04 ± 0.04	0.80 ± 0.15	0.16 ± 0.04	0.67 ± 0.01
ΣSFA <sup>2</sup>	33.89	15.19	12.30	33.40	16.13	36.87
C14:1	0.00 ± 0.00	0.01 ± 0.02	0.00 ± 0.00	0.05 ± 0.08	0.01 ± 0.02	0.08 ± 0.11
C15:1	0.07 ± 0.10	0.01 ± 0.02	0.00 ± 0.00	0.06 ± 0.08	0.01 ± 0.02	0.07 ± 0.10
C16:1	1.72 <sup>a</sup> ± 0.04	0.39 <sup>b</sup> ± 0.09	0.20 <sup>b</sup> ± 0.03	1.65 <sup>a</sup> ± 0.19	0.49 <sup>b</sup> ± 0.01	2.12 <sup>a</sup> ± 0.42
C17:1	0.18 ± 0.06	0.05 ± 0.04	0.07 ± 0.01	0.14 ± 0.04	0.03 ± 0.00	0.10 ± 0.14
C18:1n	15.03 <sup>b</sup> ± 1.19	48.49 <sup>a</sup> ± 2.02	52.62 <sup>a</sup> ± 1.83	16.24 <sup>b</sup> ± 0.35	46.1 <sup>a</sup> ± 1.37	15.62 <sup>b</sup> ± 3.44
C20:1n9	0.23 ± 0.10	0.22 ± 0.00	0.21 ± 0.02	0.45 ± 0.41	0.22 ± 0.01	0.67 ± 0.21

C22:1n9	5.76 <sup>a</sup> ± 0.06	0.96 <sup>b</sup> ± 0.20	0.46 <sup>b</sup> ± 0.12	5.70 <sup>a</sup> ± 0.33	1.34 <sup>b</sup> ± 0.04	6.39 <sup>a</sup> ± 0.53
C24:1	0.46 ± 0.23	0.09 ± 0.06	0.03 ± 0.05	0.54 ± 0.25	0.11 ± 0.05	0.56 ± 0.37
ΣMUFA <sup>2</sup>	23.44	50.23	53.60	24.82	48.32	25.61
C18:2n6t	0.10 ± 0.14	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.12 ± 0.04
C18:2n6c	6.57 <sup>b</sup> ± 0.56	28.15 <sup>a</sup> ± 0.40	29.32 <sup>a</sup> ± 0.70	7.25 <sup>b</sup> ± 0.10	27.55 <sup>a</sup> ± 0.57	6.22 <sup>b</sup> ± 2.18
C18:3n3	0.17 <sup>a</sup> ± 0.02	0.67 <sup>a</sup> ± 0.92	1.19 <sup>a</sup> ± 0.02	0.39 <sup>a</sup> ± 0.39	1.27 <sup>a</sup> ± 0.02	0.53 <sup>a</sup> ± 0.41
C18:3n6	0.64 ± 0.02	0.66 ± 0.78	0.10 ± 0.01	0.71 ± 0.02	0.11 ± 0.00	0.44 ± 0.39
C20:2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:3n6	0.31 ± 0.26	0.05 ± 0.08	0.00 ± 0.00	0.06 ± 0.08	0.00 ± 0.00	0.07 ± 0.10
C20:4n6	0.24 ± 0.34	0.04 ± 0.06	0.04 ± 0.01	0.58 ± 0.15	0.12 ± 0.01	0.51 ± 0.00
C20:3n6	0.00 ± 0.00	0.01 ± 0.02	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:5n3	0.87 ± 0.45	0.13 ± 0.08	0.07 ± 0.03	0.51 ± 0.17	0.09 ± 0.02	0.58 ± 0.49
C22:2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C22:6n3	22.98 <sup>a</sup> ± 0.27	3.59 <sup>bc</sup> ± 0.35	1.75 <sup>c</sup> ± 0.57	23.79 <sup>a</sup> ± 1.11	4.87 <sup>b</sup> ± 0.44	22.68 <sup>a</sup> ± 0.31
ΣPUFA <sup>2</sup>	31.89	33.29	32.50	33.28	34.01	31.15
PUFA/SFA <sup>3</sup>	0.94	2.19	2.64	1.00	2.11	0.84
Σn6	1.29	0.76	0.16	1.35	0.24	1.14
Σn3	24.03	4.39	3.02	24.68	6.22	23.78
n6/n3 <sup>4</sup>	0.05	0.17	0.05	0.05	0.04	0.05
DHA/EPA <sup>5</sup>	26.38	27.60	25.08	47.02	55.97	39.33
Unidentified	10.40	1.26	1.58	8.20	1.50	6.31

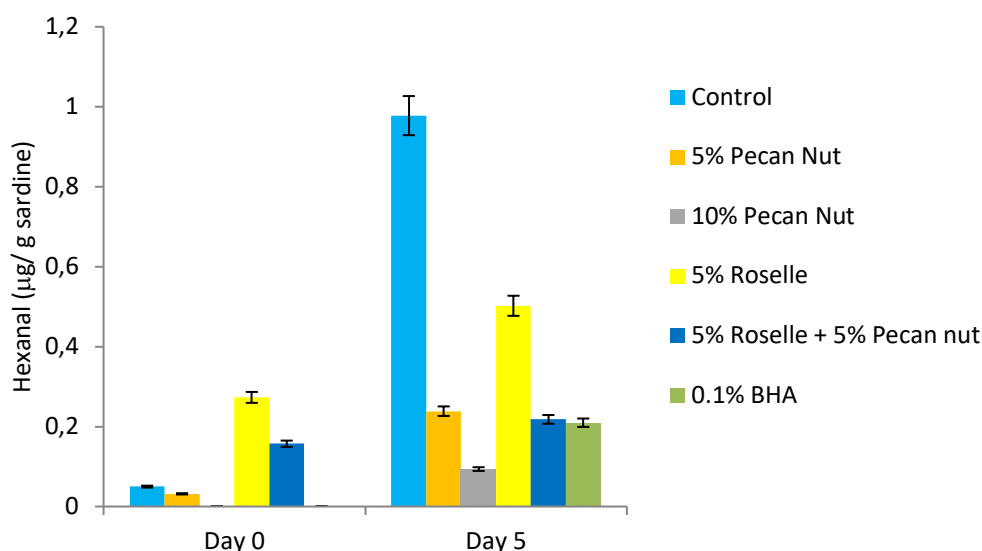
Results expressed as percentage of total FAME. The values are means ± S.D. of the samples analyzed in duplicate. <sup>a,b,c</sup> The means followed by different letters in the same row indicate significant differences ( $p < 0.05$ ). Saturated, monounsaturated and polyunsaturated fatty acids. <sup>3</sup> Ratio of polyunsaturated to saturated fatty acids. <sup>4</sup> Ratio of Σn6 to Σn3. <sup>5</sup> Ratio of *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA, C22:6n3) to *cis*-5,8,11,14,17-icosapentaenoic acid (EPA, C20:5n3).

## 2.5. Determination of Hexanal by HS-GC-MS

Oxidation of unsaturated fatty acids generates hydroperoxides, highly reactive substances which rapidly decompose into volatile and non-volatile compounds such as hydrocarbons, alcohols, acids, aldehydes and ketones [27]. These are called secondary lipid oxidation products and contribute to flavor and taste deterioration. Among these products, hexanal is a by-product of lipid oxidation that

is mainly generated by oxidation of  $\omega$ -6 fatty acid peroxides, mostly from linoleic acid through 13-hydroperoxide [28].

The content of hexanal in samples greatly changed during the treatment (Figure 2). At day 5 post-treatment, control samples exhibited the highest levels, which were 20-fold greater than control values at the beginning of the experiment (day 0). Lower levels of hexanal were found in samples containing 10% *w/w* of pecan nut: with barely detectable levels at day 0 and slightly increased values at day 5 of incubation. These results suggest that 10% *w/w* pecan nut was the most effective treatment for preventing hexanal formation as a by-product of lipid oxidation. Samples with 5% *w/w* pecan nut and 0.1% *w/w* BHA presented similar increased levels of hexanal after 5 days of treatment, which indicated that 5% *w/w* of pecan nut is enough to equal the effects of currently used synthetic antioxidants. Our findings also suggest that roselle flower can retard hexanal formation.



**Figure 2.** Hexanal content at days 0 and 5 of the experiment in samples of *Sardina pilchardus* with different treatments. Results are expressed in  $\mu\text{g}$  hexanal/g sardine. At day 0 the amount of hexanal in the samples with treatments of 10% *w/w* pecan nut and 0.1% *w/w* BHA are under the limit of detection.

## 2.6. Biogenic Amine (BA) Analysis

BAs are basic nitrogenous compounds usually generated in foods and beverages by microbial decarboxylation of amino acids or amination and transamination of aldehydes and ketones [29]. In non-fermented foods the presence of BA above a certain level is considered to be indicative of undesired microbial activity. Therefore, the amine level could be used as an indicator of microbial spoilage [29]. The best-known type of food poisoning caused by BA derives from consumption of high levels of histamine. It is also referred to as “scombrotoxic fish poisoning” because of the frequent association of this illness with consumption of scombrotoxic fish such as tuna, mackerel, saury, bonito, seer fish and butterfly kingfish. Non-scombrotoxic fish like sardine, anchovy, marline or herring have also been implicated in cases of histamine poisoning [30]. Putrescine and cadaverine, which are present in high levels in toxic fish, have been reported to potentiate the biological effects of histamine up to ten times [31]. In the European Union (EU) the legal limit for histamine levels is 100 mg/kg in raw fish.



In the present study, the amount of BA in sardine flesh significantly varied depending on the treatment (Table 5). Other authors have reported similar results for the quantification of BA in sardine in a period between 3–6 days while stored with refrigeration [20,32]. As for the effectiveness of the treatments, after 5 days of incubation, samples containing 10% w/w of pecan nut had much lower amounts of BA than the control and any other treatment. Since BA result from protein decomposition, the results obtained indicate that pecan nut may be more effective in preserving sardine meat than the synthetic food preservative BHA. Samples containing 5% w/w pecan nut showed also better results in preventing the formation of most BA than BHA (in the used concentration). Most BA levels in samples treated with the antimicrobial material roselle flower were similar or higher than in control samples. Karabacak and Bozkurt [33] reported histamine, putrescine and tyramine concentrations in sucuk batters during the ripening period. In this study, the BA content in samples incubated with roselle flower were similar or smaller than the control, although samples containing roselle flower doubled the amount of tyramine present in controls at day 4, which follows the same tendency shown in this study (Table 5).

**Table 5.** Biogenic amines present in *Sardina pilchardus* meat samples with different treatments at day 5 1.

	Control	5% Pecan Nut	10% Pecan Nut	5% Roselle	5% Roselle + 5% Pecan Nut	0.1% BHA
OC	8.02 <sup>a</sup> ± 7.83	5.93 <sup>a</sup> ± 1.91	5.94 <sup>a</sup> ± 3.68	4.48 <sup>a</sup> ± 4.57	9.12 <sup>a</sup> ± 7.59	6.76 <sup>a</sup> ± 8.74
DO	4.73 <sup>a</sup> ± 3.10	0.68 <sup>a</sup> ± 0.50	1.36 <sup>a</sup> ± 0.00	8.27 <sup>a</sup> ± 0.98	10.26 <sup>a</sup> ± 3.62	7.57 <sup>a</sup> ± 6.25
PUT	3.34 <sup>b</sup> ± 0.83	3.19 <sup>b</sup> ± 1.86	1.26 <sup>b</sup> ± 0.36	22.76 <sup>a</sup> ± 5.10	19.92 <sup>a</sup> ± 0.20	5.97 <sup>b</sup> ± 4.00
TYR	4.61 <sup>a,b,c</sup> ± 0.76	4.97 <sup>a,b</sup> ± 0.88	1.14 <sup>c</sup> ± 0.55	7.92 <sup>a</sup> ± 1.24	8.24 <sup>a</sup> ± 0.39	2.13 <sup>b,c</sup> ± 1.30
CAD	1.03 <sup>a</sup> ± 0.20	0.78 <sup>a</sup> ± 0.18	0.10 <sup>a</sup> ± 0.03	0.99 <sup>a</sup> ± 0.76	1.08 <sup>a</sup> ± 0.73	0.81 <sup>a</sup> ± 0.37
SER	11.80 <sup>a</sup> ± 4.45	12.41 <sup>a</sup> ± 1.57	4.51 <sup>a</sup> ± 3.33	30.39 <sup>a</sup> ± 5.71	21.91 <sup>a</sup> ± 17.55	15.78 <sup>a</sup> ± 2.08
HIS	1.62 <sup>b</sup> ± 1.11	1.53 <sup>b</sup> ± 0.12	0.90 <sup>b</sup> ± 0.58	14.08 <sup>a</sup> ± 1.23	11.69 <sup>a</sup> ± 2.88	4.49 <sup>b</sup>
SPD	7.28 <sup>a</sup>	6.14 <sup>a</sup>	6.55 <sup>a</sup> ± 2.47	ND	ND	7.17 <sup>a</sup>
TRP	6.64 <sup>a</sup>	5.42 <sup>a</sup>	2.01 <sup>a</sup> ± 1.62	ND	ND	10.34 <sup>a</sup>
SPM	ND	ND	6.48	ND	ND	ND

Results are expressed as mean ± standard deviation in mg/100 g sardine. ND = not detected. <sup>a,b,c</sup> The means followed by different letters in the same row indicate significant differences ( $p < 0.05$ ) on amine levels in treatments.

## 2.7. Sensory Analysis

In order to know the acceptability of this new product, sensory analysis was performed, using Basker's tables, establishing a significant difference for 28.5 points. Given that no such difference was found for any of the fish patties included in the preference sensory analysis, no treatment could be established as the preferred one by participants. The results for 5% pecan nut + 5% roselle, 5%

pecan nut, control, 10% pecan nut were a total rank of 98, 91, 99 and 80, respectively. General assessor's comments pointed out that patties with fish incubated with roselle flower had an acid shade and boosted fish taste. The other three treatments were considered very similar, although pecan nut seemed to soften fishy flavor and even make the sample taste like meat. In fact, lower scores are considered to indicate higher taster's acceptability and the lowest value was attributed to samples with 10% w/w pecan nut.

### **3. Materials and Methods**

#### **3.1. Natural Products**

Pecan nuts and roselle flowers purchased in a local market in Mexico were frozen with liquid nitrogen and shredded with a mortar.

#### **3.2. Preparation of Extracts for Determination of Total Phenolic Content and DPPH radical Activity scavenging**

Extracts were prepared in order to assay radical scavenging activity and total phenolic content. Defatted pecan nut kernels and roselle flowers were weighed (1 g) and extracted with 20 mL of 50:50 (v/v) ethanol-water at and 10 mL of 70:30 (v/v) ethanol-water with 0.1% (v/v) HCl 37%, respectively. Pecan nut extract was stirred for 90 min at room temperature and roselle flower extract at 60 °C. Both extracts were centrifuged and the supernatants were stored at -20 °C in darkness until analysis.

#### **3.3. Antioxidant Analyses of Extracts of Pecan Nut and Roselle**

##### **3.3.1. DPPH Radical Scavenging Activity**

Radical scavenging potential of pecan nut and roselle was evaluated using the DPPH method described by Gallego et al. [34]. Results are expressed in  $\mu\text{mol Trolox Equivalents (TE)}/\text{g}$  of sample weight (SW)  $\pm$  SD. Measurements were done in triplicate for each sample.

##### **3.3.2. Determination of Total Phenolic Content**

The Folin-Ciocalteu (Folin) method was used to measure the total polyphenol content of the extract [35]. Measurements were done in triplicate for each sample. Results are expressed as mg Gallic Acid Equivalents (GAE)/g of SW  $\pm$  SD.

#### **3.4. Fish Sample Preparation**

Fresh sardines with an average weight and length of  $27.2 \pm 7.5$  g and  $14.8 \pm 1.25$  cm respectively, results expressed as mean  $\pm$  SD (standard deviation) and with  $n = 10$ , were purchased from a local market in Barcelona, and transported under refrigeration to the laboratory. Fish were gutted and head, tail and spine were removed, keeping only the flesh.

#### **3.5. Preparation of Samples**

Sardine patties were prepared in order to measure the evolution of a range of parameters throughout a period of time. Burgers were prepared by shredding sardine flesh and adding direct and minced pecan nut and roselle with the following treatments: control (1% w/w salt), 5% PN (5% w/w pecan nut, 1% salt), 10% PN (10% w/w pecan nut, 1% salt), 5%R (5% w/w roselle, 1% salt), 5%PN

+ 5%R (5% w/w pecan nut, 5% w/w roselle, 1% salt), 0.1% BHA (0.1% w/w BHA, 1% salt). Samples were kept at  $7 \pm 1$  °C until analysis.

### **3.6. Microbiological Analysis**

Microbiological analysis was performed to assay sample contamination with mesophilic bacteria. To perform the analysis, 10 g of sample were added to 90 mL of Ringer solution and homogenized with a Stomacher for 5 min. Triptone Soya Agar (TSA) was used as the growth media and plaques were incubated at 35 °C. The recount was performed after 24 h and 48 h.

### **3.7. Thiobarbituric Acid Reactive Substances (TBARS)**

Determination of TBARS value was performed following the method described by Gallego et al. [35] with some modification. Triplicates of 0.5 g for each sample were weighed, added 0.5 mL 0.3% EDTA solution and 2.5 mL TBARS reagent and homogenized with an Ultra-Turrax blender (Ika-Werke, GmbH & Co, Staufen, Germany) for 1 min. During all procedure's tubes were kept in an ice bath to prevent sample deterioration. They were then filtered through Whatman filters no. 1 and the reaction was activated through insertion of the tubes in a water bath at  $95 \pm 1$  °C for 10 min. After cooling to RT, absorbance was measured at 531 nm in a UV/VIS microplate reader spectrophotometer Fluostar Omega (Paris, France). Results were expressed as mg of malondialdehyde (MDA)/kg sample.

### **3.8. Fatty Acid Methyl Ester (FAME) Analysis**

Fatty acids (FA) analysis was performed according to the method described by Viegas et al. [36] with modifications. Duplicates of 200 mg of sample were weighed into glass tubes and 750  $\mu$ L of methanol:water solution 2:1 (v/v), followed by 500  $\mu$ L of chloroform and 250  $\mu$ L Milli-Q water were added, vortexing for 1 min after each addition. Thereafter, the samples were centrifuged at 2000 g, 4 °C for 20 min. The lower layer was transferred to opaque vials and evaporated at 25 °C, with a nitrogen stream until only oil residue was present. Following addition of 2 mL hexane, the samples were vortexed for 30 s and left to rest for 5 min, to ensure fat dilution in hexane. Afterwards, 200  $\mu$ L 2 M potassium hydroxide in methanol solution were added and the samples centrifuged for 10 min at 2000 g. The upper phase was transferred into a 2 mL tube and kept at  $-80$  °C until gas chromatography analysis was performed.

FA composition was analyzed using a GC-2025 with autosampler (Shimadzu, Tokyo, Japan), equipped with a flame ionization detector (FID) and a BPX-70 (SGE) column (L  $\times$  I.D. 30 m  $\times$  0.25 mm,  $d_f$  0.25  $\mu$ m). Temperature in the oven was 60 °C for 1 min and then it was raised to 260 °C at the rate of 6 °C/min, while the injector and the detector temperatures were set at 260 and 280 °C, respectively. Sample volume was 1 mL and the carrier gas were helium. The split used was 1:20. FAME were identified by comparing the retention times with those of a standard 37 component FAME mixture. Two replicate GC analyses were performed and the results were expressed in GC area % as mean values  $\pm$  range.

### **3.9. Determination of Hexanal by HS-GC/MS**

0.5 g of minced sample was added to 1.5 mL milli-Q water in a headspace vial, which was then sealed air-tight with a PTFE septum. The standard curve was prepared using hexanal with concentrations ranging from 0.005 to 0.450 ppm. Results were expressed in mg hexanal/g sardine.

The vials were incubated at 80 °C during 30 min. The analysis was performed by HS-GC/MS, by injecting 1 mL of vapor phase through a special syringe kept at 85 °C. Equipment used consisted of a Trace GC gas chromatograph with a Head Space Triplus autosampler coupled to a DSQII mass spectrometer (Thermo Fisher Scientific, Austin, Texas, USA) with TRB-624 (60 m × 0.32 mm × 1.8 mm) column, 1.8 mL/min helium flow. The injector temperature was 220 °C with split mode injection (split flow 20 mL/min). Temperature program was 60 °C held for 2 min and then raised to 220 °C at the rate of 8 °C/min (5 min). Interface temperature was 260 °C and ionization source temperature 230 °C. Ionization mode: electron ionization, SCAN mode (29–250 amu).

### **3.10. Biogenic amines (BA) Analysis**

Samples were prepared according to the method of Komprda et al. [37] with some modifications. 1 g of sample was extracted with 2 mL of HCl 0.1 M and homogenized using an Ultra-Turrax blender (Ika-Werke, GmbH & Co, Staufen, Germany) for 1 min. Homogenized samples were centrifuged for 15 min at 4 °C and 2000 g. The supernatant was separated and the solid residue was repeatedly extracted with 2 mL of HCl 0.01 M, vortexed for 30 s and centrifuged for 15 min with the same conditions. The supernatant was separated again and the combined extracts were made up to 10 mL. The samples were filtered through 0.45 µm filter prior to liquid chromatography analysis.

BA analysis was performed following the method of Hernández-Jover et al. [38]. As biogenic amine standards histamine (HIS), tyramine (TYR), serotonin (SER), tryptamine (TRP), octopamine (OC) hydrochloride, dopamine (DO) 3-hydroxytyramine hydrochloride, cadaverine (CAD), putrescine (PUT), spermine (SPM), and spermidine (SPD) were used. A concentrated 1000 mg/L stock solution as a free base for each biogenic amine in 0.1 M HCl was prepared. A 50 mg/L intermediate solution was prepared in 0.1 M HCl from the stock solution. Calibration standards of 0.25 mg/L for all amines and 2 mg/L for spermine were prepared in 0.1 M HCl from the intermediate standard solution, stored at 4 °C and protected from light. The HPLC analysis parameters, mobile phase, gradient program, postcolumn derivatizing reagent and other procedures were as described in Hernández-Jover et al. [38].

### **3.11. Preference Sensory Analysis**

Sensory analysis was conducted by a taste panel consisting of 37 semi-trained judges (21 males and 16 females) with age ranging from 17 to 60. All participants declared that they do not suffer from dried fruits allergy. Participants tasted four fish patties, each corresponding to 18 h of incubation of sardine flesh with the following additions: 5% w/w pecan nut, 10% w/w pecan nut, 5% w/w pecan nut + 5% w/w roselle flower and control (nothing added). The samples were distributed in plates and coded with a random three-digit number. The subjects were instructed to taste each sample and grade them from 1 (most preferred) to 4 (least preferred). Results were analyzed using the tables developed by Basker [39].

### **3.12. Statistical Analysis**

The mean value and standard deviation were calculated from the data obtained from the three samples for each treatment. Where significant differences were detected by one-way Anova, means were compared using Tukey's test  $p < 0.05$ . All statistics were performed using Minitab-16 for Windows software (Pennsylvania State University, Pennsylvania, USA).

#### 4. Conclusions

The study showed that pecan nut and roselle flowers are highly effective as a fish preservative, thus opening the way to perform other experiments varying the fish type or even switching to pecan nut byproducts such as leaves or shell. It can be considered as a preliminary study to assess the interaction between pecan nut and fish lipids. All analyses showed that samples treated with pecan nut and roselle flower had better quality parameters than the control.

Adding pecan nut to sardine, creates a functional product which is useful. Firstly, because it enhances the shelf-life of sardine and secondly because it has an increased percentage of healthy monounsaturated fatty acids and a smaller percentage of saturated fatty acids. This research shows that when adding pecan nut to sardine the amount of linoleic acid (Omega-6 fatty acid family) is more than tripled (from an average percentage of 15% to 50% of the total amount of fatty acids). Linoleic acid is one of the two essential fatty acids that need to be ingested through food since the body cannot synthesize them. The other most notable change when adding pecan nut to sardine is the increase of the average percentage of oleic acid ( $\Omega$ -9 family) from 6.5% to 28% (more than four times greater). This monounsaturated fatty acid is also known for its beneficial effects on health such as reducing blood pressure. Pecan nut alone or in combination with roselle flower has potential to be used as a natural food preservative for the food industry.

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**Abbreviations used:** TPC, Total Polyphenol Content; ABTS, 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid); Trolox, ( $\pm$ )-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; GAE, mg of gallic acid equivalents; DW, dry weight; HPLC, High Performance Liquid Chromatography; TEAC, Trolox Equivalent Antioxidant Capacity; TE, Trolox Equivalent; TBARs, Thiobarbituric Acid Reactive substances; BHA, Butylated hydroxyanisole; BHT, Butylated hydroxytoluene; r.t., room temperature; AMU, atomic mass unit; BA, Biogenic amines; CAD, Cadaverine; CFU, Colony-forming units; DHA, cis-4,7,10,13,16,19-docosahexaenoic acid; DO, Dopamine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDTA, Ethylenediaminetetraacetic acid; EPA, cis-5,8,11,14,17-eicosapentaenoic acid; EU, European Union; FA, Fatty Acids; FAME, Fatty Acids Methyl Ester; FID, flame ionization detector; Folin, Folin-Ciocalteu Analysis; FRAP, Ferric reducing antioxidant power; FW, Fresh weight; GC, Gas chromatography; HIS, Histamine; HS-GC/MS, Headspace gas chromatography mass spectrometry; MDA, Malonaldehyde; MUFA, monounsaturated fatty acids; n3, n3-polyunsaturated fatty acid; n6, n6-polyunsaturated fatty acid; OC, Octopamine; OPT, o-Ophtalaldehyde; PN, Pecan Nut; PTFE, Polytetrafluoroethylene; PUFA, polyunsaturated fatty acids; PUT, putrescine; RSA, Radical scavenging activity; SD, Standard deviation; SER, Serotonine; SFA, Saturated fatty acids; SPD, Spermidine; SPM, Spermine; SW,

Sample weight; TRP, Tryptamine; TSA, Tryptone soya agar; TYR, Tyramine; UV/VIS, Ultraviolet-visible spectroscopy; v/v, volume volume; w/w, weight weight. RT, Room Temperature

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Sample Availability: Samples of the compounds are not available from the authors.



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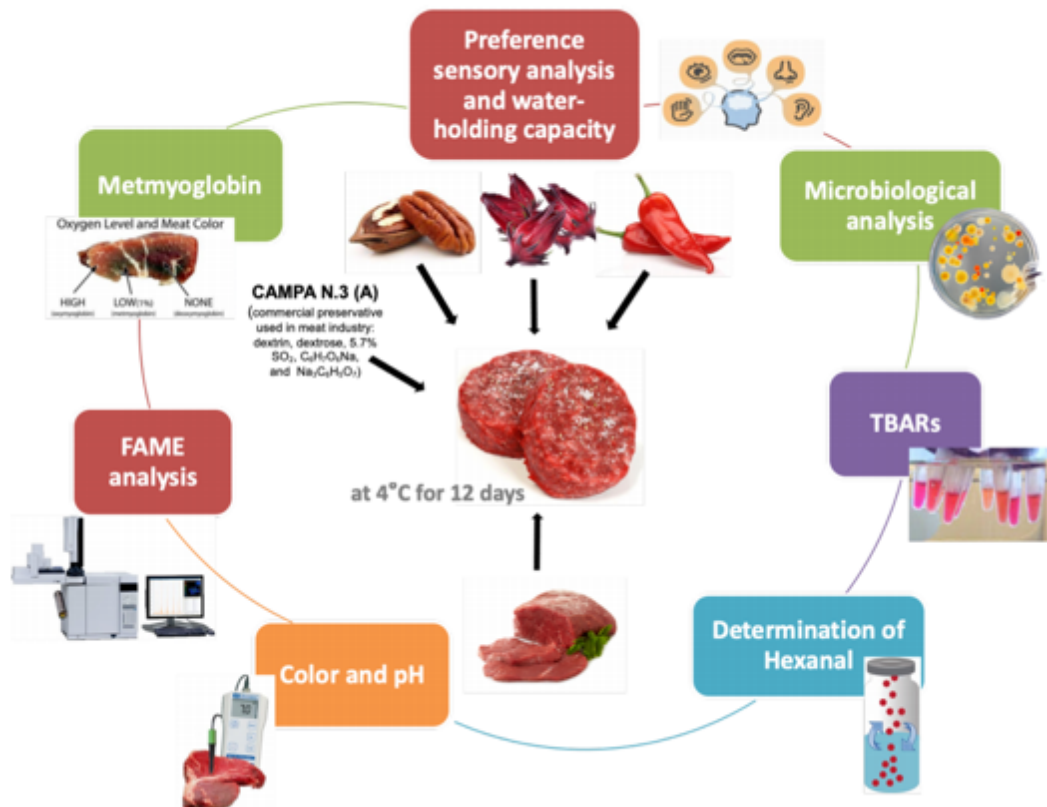


## 5.2. The effects of pecan shell in combination with other plants on the quality of beef patties during chilled storage.

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# The effects of pecan shell in combination with other plants on the quality of beef patties during chilled storage

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**Abstract:** The antioxidant and antimicrobial effects of pecan shell (PSW), combined with roselle flower (RS) and cayenne pepper (CA) were analyzed in raw beef patties by several methods during chilled storage for 13 days. Also, the antioxidant and antimicrobial activities of PSW, RS and CA extracts were determined. The PSW extract exhibited a higher radical scavenging activity (by DPPH method) and total phenolic compounds than RS and CA. RS presented the best antimicrobial capacity. Nine formulations of beef patties were prepared, including a control (CM), a synthetic preservative (CAMPA N.3 (A)) and different combinations of PSW, RS and CA. The bacterial counts of the beef patties with RS (4-5 log colony-forming unit (CFU)/g meat)) were significantly lower than those of the control sample (CM) (6-7 CFU/g meat) at day 6. The Thiobarbituric Acid Reactive Substances (TBARS) values at day 7 of all treatments were similar to the values of samples containing the synthetic antioxidant and significantly lower than the CM. The order of stability assessed by the TBARS values were in agreement with the hexanal content. Thus, these results support the hypothesis that the combination of PWS, RS and CA could represent a good natural food preservative.

**Keywords:** *Carya illinoensis*; *Capsicum annuum*; *Hibiscus sabdariffa*; meat lipid oxidation; antimicrobial activity.

## 1. Introduction

Antioxidant compounds in food play an important role in health protection [1]. Beef meat for hamburgers contains a high amount of polyunsaturated and unsaturated fatty acids, proteins, minerals and shows a high-water activity that makes the meat susceptible to oxidation and to microbiological damage. On the other hand, the bovine meat has a higher proportion of red fibers, containing iron and phospholipids, which are more sensitive to oxidation compared to white fibers. Also, minced meat experiences greater lipid oxidation than whole cuts, because the grinding process incorporates oxygen [2,3]. Therefore, as this process evolves, the sensory characteristics such as color, texture, flavor, odor, etc., and nutritional value of the beef meat deteriorate [4]. Lipid oxidation

produces many end products including aldehydes, especially malondialdehyde (MDA). The variation of the color of the meat, from pink-red to greyish-red, is unacceptable for consumers. This change is the result of the conversion of oxymyoglobin to metmyoglobin [3].

Synthetic antioxidants, such as BHA (Butylate Hydroxyanisole) and BHT (Butylate Hydroxytoluene), are commonly used in the meat industry to delay oxidation and extend the shelf life of food. However, due to their potential risks to health and potential toxicity, there is a widespread agreement of the desirability of replacing synthetic with natural antioxidants [5].

Pecans (*Carya illinoensis*), are a common dietary component in many countries around the world. Their shell is the by-product with the highest amount of polyphenols in its nut [6]. Previous studies reported that the pecan shell contains bioactive compounds including ellagic acid, gallic acid, chlorogenic acid, p-hydroxybenzoic acid, epigallocatechin and epicatechin-gallate and tannins [7–9].

Cayenne pepper (*Capsicum annuum*) (CA) is commonly used to enrich food's flavors. However, CA contains phytochemical compounds, including flavonoids, phenols, carotenoids, capsaicinoids and vitamins, thus making this type of pepper a good source of antioxidant, antimicrobial, antiviral, anti-inflammatory, and even anticancer nutrients [10].

Roselle (*Hibiscus Sabdariffa*) (RS) flower is native to Asia and also widely cultivated in tropical and subtropical areas such as Central America and Africa [11]. This flower presents high contents of anthocyanins and flavonoids including quercetin, delphinidin-3-sambubioside, delphinidin-3-glucoside, cyanidin-3-glucoside, cyanidin-3-sambubioside, and kaempferol [12]. These compounds possess antioxidant and antimicrobial properties, and are also responsible for the prevention of chronic health pathologies [13,14].

Several studies have focused on different natural antioxidants with the aim of protecting beef meat against oxidation. Olive leaf extracts were effective against the oxidation of lipids and myoglobin during storage of raw and cooked meat [15]. Furthermore, Villasante et al. studied the combination of pecan with RS (*Hibiscus sabdariffa*) as an antioxidant and antimicrobial agent in sardine burgers [16]. They found that the combination of these plants is effective in extending the freshness and safety of sardines.

Therefore, the purpose of this study was to determine the total phenolic compounds, antiradical scavenging and antimicrobial activities of pecan shell, cayenne pepper and roselle flower. Subsequently, the effects of these plants on surface color, pH, lipid oxidation, and microbial growth of raw beef patties during refrigerated storage at  $4 \pm 1^\circ\text{C}$  were evaluated. Finally, sensory analysis was conducted.

## **2. Materials and Methods**

### **2.1 Plant Material**

The nuts were collected in the north of Mexico (coordinates:  $25^\circ45'32''\text{N}$ ,  $102^\circ58'58''\text{W}$ ). The shells (PSW) were milled in a Wiley mill (Arthur Thomas, Philadelphia, PA) equipped with a 2 mm screen. The Cayenne pepper (*Capsicum annuum*) (CA) was bought in a local market in Barcelona, Spain. The roselle flower (*Hibiscus sabdariffa*) (RF) was collected in Colima, Mexico ( $19^\circ14'36''\text{N}$ ,  $103^\circ43'30''\text{W}$ ) and dried at room temperature to obtain a final moisture of 12%.

Beef meat for the patties was collected seven days after slaughter, to allow it to mature. The meat was purchased from a local fresh food market in Barcelona, Spain, brought to the laboratory under refrigeration ( $4 \pm 1^\circ\text{C}$ ) and prepared in beef patties for testing during the same day.

## **2.2 Chemicals**

All of the reagents were of analytical grade, Ethanol (EtOH), 2-thiobarbituric acid (TBARS), diphenyl-1-picrylhydrazyl (DPPH), Ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu reagent, Ferric reducing antioxidant (FRAP), gallic acid (GA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and penicillin; purchased at Sigma-Aldrich S.A. (Madrid, Spain). Methanol (MeOH), hydrogen chloride, Phosphate Buffered Saline (PBS), and ferrous chloride ( $\text{FeCl}_2$ ); acquired at Panreac Química S.L.U. (Barcelona, Spain). Miller Hinton agar and Tryptone glucose yeast agar were bought from Thermo-Fisher Scientific (Barcelona, Spain). Distilled water and Milli-Q water (Millipore, Barcelona, Spain). Synthetic antioxidant CAMPA N.3 (A) (dextrin, dextrose, 5.7%  $\text{SO}_2$ , E-224, E-301, E-331) commonly used in the meat industry (Barcelona, Spain).

## **2.3 Extract preparation**

Extracts were prepared in order to determine the total phenolic content and to assay the radical scavenging activity (DPPH). Extraction was carried out using 1 g of PSW or CA in 10 mL of solvent (ethanol-water 50:50 v/v). One g of RF was weighed and extracted with 10 mL of 70:30 (v/v) ethanol-water. The PSW extract was stirred for 90 min at room temperature and RS extract at  $60^\circ\text{C}$ . CA extract was kept under ultrasound for 30 min at  $50^\circ\text{C}$ . All the extracts were centrifuged, and the supernatants were stored at  $-80^\circ\text{C}$  until used for analysis.

## **2.4 DPPH radical scavenging activity**

The assay was conducted according to the report of Gallego et al. [17]. Results were expressed in  $\mu\text{mol}$  Trolox Equivalents (TE)/g of dry weight (DW)  $\pm$  standard deviation (SD). Measurements were done in triplicate for each sample.

## **2.5 Total Polyphenol Content (TPC)**

The TPC assay was carried out according to the procedure described by Segovia et al. [18]. The total phenolic content was expressed as mg of Gallic Acid Equivalents (GAE)/g (DW)  $\pm$  (SD). All measurements were taken in triplicate.

## **2.6 Antimicrobial capacity**

Microorganisms were obtained from the collection of the Microbiology department of the University of Barcelona. Gram+ (*B. cereus* ATCC 11778, *S. aureus* ATCC 25923, *Listeria monocytogenes* ATCC 15313, and *Micrococcus luteus* ATCC 4698) and Gram- (*E. coli* ATCC 25922 and *Salmonella enterica* ATCC 14028). Before using them experimentally, the microorganisms were cultured aerobically for 24 h at  $37^\circ\text{C}$  in nutrient agar medium. The antimicrobial activity screening against Gram+ and Gram- bacteria and the determination of the inhibitory effect of the different compounds was achieved by agar disk-diffusion testing based on the method described by Balouiri et al. [19]. Filter paper discs with 6 mm of diameter, containing each one 80  $\mu\text{L}$  of PSW, CA and RS extracts, ampicillin (100  $\mu\text{g}/\text{ml}$ ) as a positive control and Mili-Q water as a negative control, were placed on the agar surface. The Petri dishes were incubated at  $35^\circ\text{C}$  for 24 h; eventually, the disc

diameter of the inhibition zone around the extract was measured. The antimicrobial activity was determined in duplicate for each sample

## 2.7. Formulations

**Table 1.** Formulations for the beef patties

Experiment	
Treatments	CM (control)
	2% PSW
	4% PSW
	0.35% CA
	2% RS
	2%PSW + 0.35% CA
	2% PSW + 0.35% CA + 2% RS
	4% PSW + 0.35% CA + 2% RS
	0.7% CAMPA N.3 (A)

\*CAMPA N.3 (A): (dextrin, dextrose, 5.7% SO<sub>2</sub>, E-224, E-301, E-331)

### 2.7.1. Preparation of the beef patties

Beef patties were prepared (Table 1) in order to measure the evolution of oxidation by Thiobarbituric Acid Reactive Substances method (TBARS), fatty acids, production of hexanal, % metmyoglobin, microbial quality, and other physical characteristics namely color and pH, throughout a period of 13 days under refrigeration at  $4 \pm 1$  °C. Different sets of ground beef were used, each one taken from the round part of different cuts, and minced three times through 8 mm industrial plates. The meat was mixed with salt (1.5% w/w) and assigned randomly to each treatment (Table 1). All batches were mixed for 3 min to attain an even distribution of powders throughout the meat. Afterwards, each part was flattened and cut by a round cutter into small patties; the patties used to determine fat oxidation weighed approximately 8 g, while the samples used for microbiological analysis weighed 10 g. The patties were placed in plastic trays and covered with transparent film. The samples were stored in the dark at  $4 \pm 1$  °C. All the preparation was done aseptically and in triplicated.

### 2.7.2. TBARS assay

The determination of TBARS of raw beef patties was assessed by the method described by Azman et al. [20]. The absorbance of each sample was measured at 531 nm in a UV/VIS microplate reader spectrophotometer Fluostar Omega (Paris, France), and the results were expressed as mg malondialdehyde MDA/kg meat sample. Eventually, 0.5 g of the sample was weighed and treated with 0.5 mL aqueous EDTA. Afterwards, it was mixed at 32,000 rpm speed for 1 min with 2.5 mL of

thiobarbituric acid reagent using an Ultra-Turrax (IKA, Staufen, Germany). Then, it was filtered through a Whatman filter no. 1 to obtain the solution. All procedures were performed with cooling in ice and carried out in the dark. The mixture was incubated at  $95 \pm 1$  °C in hot water for 10 min. Then it was cooled for 10 min and subsequently the absorbance measured. Percentage of inhibition was determined using the formula (1):

$$\% I = \frac{C - T}{C} \quad \dots$$

% I: Percentage of inhibition, C: value of TBARS (mg MDA/kg sample) of the control sample (CM) and T: value of TBARS (mg MDA/kg sample) of the sample.

#### 2.7.3. Determination of hexanal by HS-GC-MS

The hexanal content was determined according to Villasante et al. [16]. The meat sample (0.5 g) was mixed with 1.5 mL milli-Q water in a headspace vial. Then, it was sealed air-tight with a PTFE septum. The analysis was executed by HS-GC-MS. A Trace GC gas chromatograph with a Head Space Triplus autosampler coupled to a DSQII mass spectrometer (Thermo Fisher Scientific, Austin, Texas, USA) with TRB-624 (60 m x 0.32 mm x 1.8 mm) column, 1.8 mL/min helium flow was used. The temperature of the injector was 220 °C with split mode injection (split flow 20 mL/min). Ionization mode: electron ionization, SCAN mode (29–250 amu). The standard curve was prepared using hexanal (0.006 to 0.250 ppm). Results were expressed in mg hexanal/kg meat.

#### 2.7.4. Fatty acid methyl ester (FAME) analysis

Fatty acids (FA) analysis was performed according to the method described by Mosca et al. [22]. The samples of meat were weighed (200 mg) and vortexed for 1 min after the addition of 750 µL of methanol: water solution 2:1 (v/v), then 500 µL of chloroform and finally 250 µL Milli-Q water. Subsequently, the samples were centrifuged at 4 °C, 2000 g for 15 min. The lower phase, containing lipids, was transferred to amber glass vials and dried under a nitrogen stream with ice. The extracted lipids were dissolved in 2 mL hexane, the samples were vortexed for 30 sec. After, 200 µL 2 M potassium hydroxide in methanol solution was added and the samples centrifuged for 5 min at 3000 g. The upper phase was analyzed by a Gas Chromatograph coupled to Flame Ionization Detector (GC-2025, Shimadzu, Tokyo, Japan) and a BPX-70 (SGE) capillary column (L x I.D. 30 m x 0.25 mm, df 0.25 µm). An automatic injector (AOC20i) was employed. The temperature in the oven was 60°C for 1 min, then it was raised to 260 °C for 30 min, and the detector set at 280 °C. Sample volume was 1 mL and the carrier gas was helium. Extracts were introduced with a 1:10 dilution. Analyses were performed in duplicate. FAMES were identified by comparing the retention times of a standard 37 component FAME mixture. The results were expressed in area % as mean values  $\pm$  SD.

#### 2.7.5. Microbiological analysis

Samples (10 g) were diluted with 90 ml of Ringer solution and were placed in sterile 18 x 30 cm 400 ml Fisherbrand stomacher bags (Fisher Scientific SL, Madrid, Spain) and homogenized for 1 min using a Seward stomacher 400 (Seward Medical UAC House, London, United Kingdom). One hundred microliter of dilution were spread onto the surface of pour plates with agar (PCA). Afterwards, the plates were incubated at  $35 \pm 1$  °C for 48 h. After incubation, colony forming units (CFU) were

counted and reported as log colony forming units per gram (log CFU/g). The experiment was conducted in duplicate for each sample at day 0 and 6 of the study.

#### 2.7.6. pH measurements

The pH of the samples was measured as a parameter since some hydroperoxide decomposition products are acidic. The pH value of six grams of beef patties was determined in triplicate using an electronic pH meter (Crison Instruments, GLP 21 pH METER, Barcelona, Spain).

#### 2.7.7. Determination of Metmyoglobin (MetMb)

The determination of metmyoglobin was carried out according to the procedure described by Gallego et al. [24]. One g of sample was homogenized with 5 mL of 0.04 M phosphate buffer (pH 6.8) for 30 s, using an Ultra-Turrax (IKA, Staufen, Germany). The mixture was refrigerated at 4 °C for 1 h. Then, it was centrifuged at 4000 g for 10 min at 4 °C. The absorbance of the upper phase was read at 572, 565, 545, and 525 nm using a UV/VIS microplate reader spectrophotometer Fluostar (Paris, France). The percentage of metmyoglobin was determined using the formula (2):

$$MetMb (\%) = [1.395 ((A572 - A700) / (A525 - A700))] \times 100 \quad (2)$$

#### 2.7.8. Color measurements

The color of the hamburgers was evaluated in triplicate at different points of the surface by using a Minolta Chromameter CR-300. The luminosity determined by  $L^*$ , red-green by  $a^*$ , and yellow-blue by  $b^*$ . The chrome ( $c^*$ ) and Hue angle ( $h^\circ$ ) were calculated, with the formulas (3) and (4). The equipment was calibrated using a black-and-white glass tile.

$$c^* = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

$$h^\circ = \tan^{-1} (b^*/a^*) \quad (4)$$

#### 2.7.9. Sensory analysis

Sensory analysis was performed for 5 types of samples: CM, 0.7% CAMPA N.3 (A), 2% PSW + 0.35% CA + 2% RS and 4% PSW + 0.35% CA + 2% RS. Thirty-one untrained panelists (19 males and 12 females, age from 18 and 60 years) students and workers from the University Polytechnic of Catalunya, evaluated beef patties (10 g) of each formulation studied. The samples were presented to each panelist randomly on a white plate at  $37 \pm 1$  °C, accompanied with an unsalted cookie and a glass of water to clean the palate. Each sample was coded with a randomly selected three-digit number. The panelists evaluated the following attributes: aroma, color, flavor, texture and overall acceptability, using hedonic scale (10= extremely like, 1 = extremely dislike). The purchase intention was also evaluated using a scale (10 = certainly would buy the product and 1 = certainly would not buy the product).

#### 2.7.10. Water holding capacity

The water holding capacity (%) was determined according to the amount of water that was lost when cooking the beef patties. The weight percentage difference between uncooked (UNC) and

cooked samples (C) is calculated using the formula (4) [15]. Beef patties were cooked at 80 °C during 5 min. The experiment was done in triplicate for each sample.

$$\% \text{ Water – holding capacity: } [(UNC(g) - C(g)) / UNC(g)] \times 100 \quad (4)$$

## 2.7 Statistical Analysis

Statistical analyses were conducted by using Minitab statistical software, version 18 (Minitab Inc., State College, PA, USA). The data was reported as means  $\pm$  standard deviation. The results were subjected to the analysis of variance (ANOVA), and then post hoc Tukey's test was applied to determine significant differences among formulations ( $P < 0.05$ ).

## 3. Results

### 3.1. Determination of DPPH and TPC

Several studies demonstrated that natural compounds with antioxidant properties are capable of inhibiting oxidation processes in food models and provide health benefits [25,26]. Between the three samples studied, there were significant differences in the total amount of polyphenols (Table 2). The extract that contained more TPC was PSW, followed by RS and finally, CA, with a low value. Similar results in Mexican PSW were obtained by De La Rosa et al. (92.5 $\pm$ 9.00 mg GAE /g DW). On the other hand, Kureck et al. [6] determined in a shell aqueous extract (186.02 $\pm$ 2.31 mg AGE /g DW) almost two times more polyphenol content than that reported by De La Rosa et al. [27]. The difference in the results could be due to the origin of the sample and to the method used to make the extracts. In previous studies, Villasante et al. [16] and Borrás-Linares et al. [28] reported similar values of the total amount of polyphenols in RS extracts 22.40 $\pm$ 0.02 and 28.0 $\pm$ 4.00 mg GAE/g DW respectively; in both cases, the RS samples used were from Colima, Mexico. CA was the sample containing the lowest TPC compared to the other plants. According to Hallmann & Rembiałkowska [29], the TPC of different sweet bell pepper samples (*Capsicum annuum*) grown under organic and conventional growing systems, was between 953.6 $\pm$ 141.0 to 724.1 $\pm$ 115.5 mg GAE /Kg DW: this is lower than the value reported in this study. Farhoudi et al. [30] reported 3.38 $\pm$ 0.03 mg GAE/g DW for a Jalapeno pepper.

The radical scavenging activity of PSW, RS, and CA extracts was determined (Table 2). The radical scavenging activity was significantly higher in PSW than in the CA and the RS. The result obtained for the PSW was four times lower than that found in Wichita variety (467.54 $\pm$  $\mu$ mol TE/g DW) by Flores-Córdova et al. [31]. In the case of the RS, it presented a similar antiradical scavenging activity to that reported by Villasante et al. [16] (78.66  $\pm$   $\mu$ mol TE/ g DW). The CA extract shows the lowest DPPH activity (73.06 $\pm$ 0.01  $\mu$ mol TE/ g DW).



Table 2. TPC and DPPH

Sample	TPC (mg GAE/ g DW)	DPPH ( $\mu$ mol TE/ g DW)
PSW	96.81 $\pm$ 0.08 <sup>a</sup>	104.63 $\pm$ 0.01 <sup>a</sup>
RS	15.02 $\pm$ 0.01 <sup>b</sup>	98.29 $\pm$ 0.04 <sup>b</sup>
CA	7.03 $\pm$ 0.0 <sup>c</sup>	73.06 $\pm$ 0.01 <sup>c</sup>

Results were expressed as milligrams gallic acid equivalents (GAE) per gram of dry sample weight (DW) and micromoles of Trolox equivalents (TE) per gram of DW. Results are expressed as mean  $\pm$  SD (n=3). Different superscripts in the same column indicate significant differences (P < 0.05)

### 3.2. Antimicrobial capacity

The meat industry aims to control lipid oxidation, but also to develop safe products. The antimicrobial activity of the PSW, RS and CA was determined with the objective to use them as antimicrobial agents. The results show (Table 3) that the RS was the sample that exhibited more antimicrobial activity in relation to all the microorganisms studied. In the case of the *E. coli* and *S. enterica*, penicillin showed less resistance than the RS. On the other hand, the PSW also showed antimicrobial capacity towards five out of the six microorganisms studied. Previous studies of CA determined a high antimicrobial activity [10,32] but in the present study the CA extract shown a poor activity; in fact, the CA studied showed less antimicrobial efficacy than the PSW and RS, and only showed a moderate effect against *L. monocytogenes* and *M. luteus* microorganisms. This result confirmed the finding reported by Zhang et al. [33], who found that spice extracts are more active against gram-positive than gram-negative bacteria.

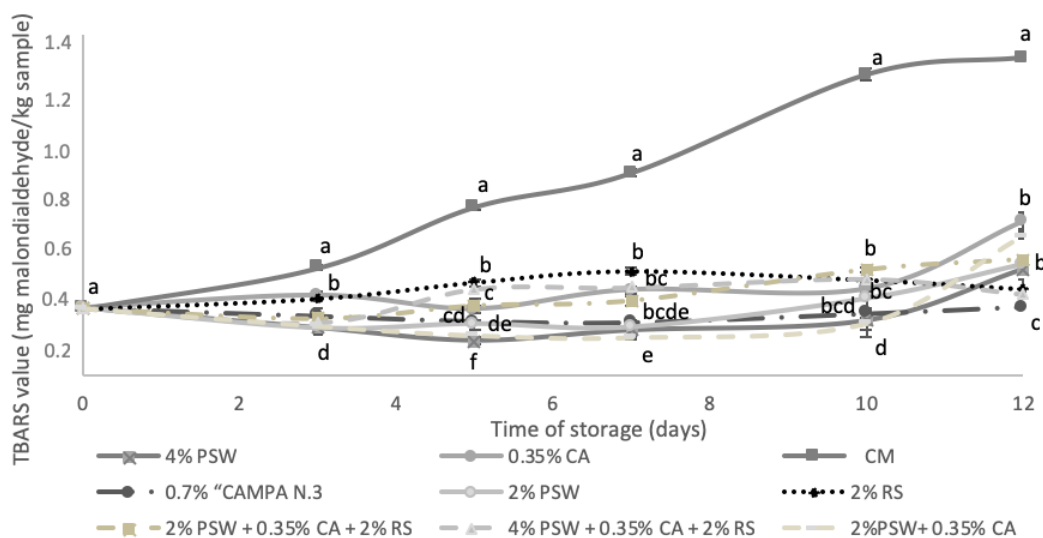
**Table 3.** Antimicrobial activity of PSW, CA, RS and controls (+ and -)

Sample	<i>B. cereus</i>	<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>M. luteus</i>	<i>E. coli</i>	<i>S. enterica</i>
CA	-	-	++	++	-	-
PSW	++	++	++	+++	-	++
RS	+++	+++	+++	++++	+++	++
Control +	++	+++	++++	-	++++	++++
Control -	-	-	-	-	-	-

No antimicrobial activity (-), inhibition zone <1 mm. Slight antimicrobial activity (+), inhibition zone 2–8 mm. Moderate antimicrobial activity (+ +), inhibition zone 9–15 mm. High antimicrobial activity (+ + +), inhibition zone 16–22 mm. Strong antimicrobial activity (+ + + +), inhibition zone >23 mm. Control +: water, Control-: Ampicillin.

### 3.3. TBARS assay

Meat products, when stored for a long time, suffer changes in their physical and chemical characteristics. These changes include formation of free radicals, which are the cause of lipid oxidation [34]. Figure 1. shows the evolution of the TBARS value (mg MDA/kg sample), and consequently, the degree of oxidation of the samples during the experiment. The CM had a significant difference compared to the other samples. This fact demonstrated the antioxidant activity of the CAMPA N. 3 (A), PSW, CA, RS, and their synergies. After 7 days at  $4\pm 1^\circ\text{C}$ , significant differences ( $P < 0.05$ ) were already observed between samples. The CM sample contained almost double the level of oxidation products assessed by mg of MDA per kg of meat than the 2% RS sample. The 2% PSW + 0.35%CA had lower TBARS values compared to other samples at day 7. On the last day of the experiment, three groups of samples with similar degrees of oxidation were observed. The most effective treatments were the commercial preservative CAMPA N.3 (A), RS 2% and PSW 4% + CA 0.35% + RS 2%, with a percent inhibition of 79, 73 and 75 respectively, compared with the control. Recently it was reported that lipid oxidation was significantly inhibited ( $p < 0.05$ ) in pork patties treated with black currant extract, and TBARS values (% inhibition) decreased up to 91.7% compared with the control at day 9 of the experiment [35]. Also, in meat, Rodríguez-Carpena et al. [37] investigated the effects of the waste (seed and peel) of two classes of avocado (Hass and Fuerte), and the percentage of inhibition ranged from 72.36 to 91.54 after 15 days. So, the potential radical scavenging activity and the high TPC results exhibited by PSW, CA and RS extracts in vitro, demonstrated that they can be used in a food model such as beef patties.



**Figure 1.** TBARS values (mg MDA/kg) in beef patties during 12 days of refrigerated storage at  $4\pm 1^\circ\text{C}$ . Different letters in the same day indicate significant differences between samples.

### 3.4. Determination of hexanal by HS-GC-MS

Hexanal is a volatile secondary oxidation product that increases during the oxidation of meat and fish products. This byproduct of lipid oxidation is generated mainly by the oxidation of polyunsaturated fatty acids [24]. At the beginning of the experiment, the amount of hexanal in the

samples was below the detection limit (Table 4). At day 6, the concentration of hexanal in the CM was significantly ( $P < 0.05$ ) higher than in the other samples presented. However, the patties with CAMPA N.3 (A) and 2% RS showed no hexanal formation. The other samples with PSW, CA and RS showed significantly ( $P < 0.05$ ) lower quantities of hexanal compared to the control sample. There is a relation between the PSW percent increment in the sample and the amount of hexanal, but the values were not significantly different ( $P < 0.05$ ) from those of the other samples. In a study reported by García-Lomillo et al. [35] with seasoning derived from wine pomace in refrigerated beef patties, no hexanal formation was found, except for some low levels at the end of the experiment (day 15). They concluded that the seasonings were more effective than sulfites.

TBARS assay is one of the most widely methods for measuring secondary oxidation, but the determination of the hexanal content also supports the conclusions from MDA values presented in Fig 1. The use of PSW, CA, RS and CAMPA N.3 (A) could be effective in preventing flavor and taste deterioration in beef patties.

**Table 4.** Values of hexanal content at day 6 of experiment.

Sample	Hexanal ( $\mu\text{g}$ hexanal/ kg meat)
CM	609.3 $\pm$ 21.7 <sup>a</sup>
0.7% CAMPA N.3 (A)	0.0 $\pm$ 0.0 <sup>b</sup>
2% PSW	21.4 $\pm$ 21.0 <sup>b</sup>
4% PSW	43.0 $\pm$ 10.0 <sup>b</sup>
0.35% CA	4.0 $\pm$ 0.0 <sup>b</sup>
2% RS	0.0 $\pm$ 0.0 <sup>b</sup>
2% PSW + 0.35% CA + 2% RS	5.1 $\pm$ 0.4 <sup>b</sup>
4% PSW + 0.35% CA + 2% RS	9.2 $\pm$ 7.1 <sup>b</sup>
2% PSW + 0.35% CA	5.0 $\pm$ 0.4 <sup>b</sup>

Results expressed as mean  $\pm$  SD (n=3). Different superscripts in the same column indicate samples that differ significantly ( $P < 0.05$ ).

### 3.5. FA analysis

The analysis of fatty acids was used to determine the concentration of fatty acids in the beef patties and to monitor changes in samples that contained PSW, CA and RS. Table 5 shows the results obtained. The values were presented as the total percentage of fatty acids in each of the samples studied. Additionally, their evolution during 13 days (analyzed on days 0, 6 and 13), grouped into SFA (saturated fatty acids), MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids) and ND (unspecified fatty acids) was analyzed. Previous studies on the composition of fatty acids in beef meat [38] showed that MUFA and SFA were present at the highest concentrations with 49.82 % of MUFA and 41.03% of SFA. PUFAs were present at lower concentrations. Higher concentrations of PUFA in are claimed to be associated with healthier meat [38].

The results from this study demonstrated changes in the percentage of saturated fatty acids (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) in all samples during the experiment. The general tendency was a decrease in the concentration of polyunsaturated fatty acids (PUFA) and, consequently, an increase in monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA). The decrease in the amount of PUFA was due to the oxidation they suffer, also observed in the results presented during the determination of hexanal. The oxidation of the  $\omega$ -6 fatty acids (PUFA) was principally a consequence of the degradation of linoleic acid [24], resulting in the increase of monounsaturated (MUFA) and saturated fatty acids (SFA).

**Table 5.** Fatty acid composition (%) of raw beef patties.

Fatty acids (%)	CM	0.7% CAMPA N.3 (A)	2% PSW	4% PSW	0.35% CA	2% RS	2% PSW + 0.35% CA + 2% RS	2%PSW+ 0.35% CA	4% PSW + 0.35% CA + 2% RS
Day 0 $\Sigma$ SFA	25.30 $\pm$ 1.60 <sup>c</sup>	25.30 $\pm$ 1.60 <sup>c</sup>	25.30 $\pm$ 1.60 <sup>c</sup>	25.30 $\pm$ 1.60 <sup>c</sup>	25.30 $\pm$ 1.60 <sup>c</sup>	25.30 $\pm$ 1.60 <sup>c</sup>	25.30 $\pm$ 1.60 <sup>b</sup>	25.30 $\pm$ 1.60 <sup>c</sup>	25.30 $\pm$ 1.60 <sup>b</sup>
Day 6 $\Sigma$ SFA	37.01 $\pm$ 0.38 <sup>bE</sup>	40.58 $\pm$ 2.12 <sup>bC</sup>	44.79 $\pm$ 1.32 <sup>bB</sup>	32.54 $\pm$ 2.12 <sup>bF</sup>	50.23 $\pm$ 1.04 <sup>aA</sup>	38.52 $\pm$ 1.24 <sup>bD</sup>	20.32 $\pm$ 1.54 <sup>dI</sup>	27.09 $\pm$ 0.12 <sup>bG</sup>	24.53 $\pm$ 0.45 <sup>dH</sup>
Day 13 $\Sigma$ SFA	42.30 $\pm$ 0.25 <sup>aG</sup>	43.23 <sup>F</sup> $\pm$ 1.25 <sup>a</sup>	48.17 $\pm$ 1.04 <sup>aB</sup>	44.84 $\pm$ 2.18 <sup>aE</sup>	48.80 $\pm$ 2.03 <sup>aA</sup>	47.36 $\pm$ 2.07 <sup>aD</sup>	48.13 $\pm$ 1.32 <sup>aB</sup>	47.59 $\pm$ 2.12 <sup>aC</sup>	47.52 $\pm$ 1.32 <sup>aC</sup>
Day 0 $\Sigma$ MUFA	34.54 $\pm$ 1.02 <sup>c</sup>	34.54 $\pm$ 1.02 <sup>b</sup>	34.54 $\pm$ 1.02 <sup>c</sup>	34.54 $\pm$ 1.02 <sup>b</sup>	34.54 $\pm$ 1.02 <sup>c</sup>	34.54 $\pm$ 1.02 <sup>c</sup>	34.54 $\pm$ 1.02 <sup>b</sup>	34.54 $\pm$ 1.02 <sup>c</sup>	34.54 $\pm$ 1.02 <sup>b</sup>
Day 6 $\Sigma$ MUFA	49.30 $\pm$ 0.12 <sup>aA</sup>	20.75 $\pm$ 0.15 <sup>chI</sup>	44.25 $\pm$ 0.12 <sup>bB</sup>	19.57 $\pm$ 1.20 <sup>dI</sup>	39.47 $\pm$ 0.12 <sup>bC</sup>	39.35 $\pm$ 0.89 <sup>bD</sup>	21.81 $\pm$ 0.56 <sup>cG</sup>	36.91 $\pm$ 0.78 <sup>bE</sup>	25.66 $\pm$ 1.34 <sup>dF</sup>
Day 13 $\Sigma$ MUFA	48.38 $\pm$ 0.14 <sup>bC</sup>	54.03 $\pm$ 2.10 <sup>aA</sup>	45.22 $\pm$ 1.67 <sup>aD</sup>	49.18 $\pm$ 1.23 <sup>aB</sup>	41.27 $\pm$ 1.76 <sup>aH</sup>	40.94 $\pm$ 2.12 <sup>aI</sup>	41.46 $\pm$ 1.12 <sup>aG</sup>	41.77 $\pm$ 0.89 <sup>aE</sup>	41.44 $\pm$ 0.65 <sup>aF</sup>
Day 0 $\Sigma$ PUFA <sup>2</sup>	34.26 $\pm$ 1.20 <sup>a</sup>	34.26 $\pm$ 1.20 <sup>a</sup>	34.26 $\pm$ 1.20 <sup>a</sup>	34.26 $\pm$ 1.20 <sup>a</sup>	34.26 $\pm$ 1.20 <sup>a</sup>	34.26 <sup>a</sup> $\pm$ 1.20	34.26 $\pm$ 1.20 <sup>a</sup>	34.26 $\pm$ 1.20 <sup>a</sup>	34.26 $\pm$ 1.20 <sup>a</sup>
Day 6 $\Sigma$ PUFA <sup>2</sup>	8.90 $\pm$ 0.56 <sup>bC</sup>	2.22 $\pm$ 0.12 <sup>bH</sup>	5.58 $\pm$ 0.32 <sup>dF</sup>	22.63 $\pm$ 0.78 <sup>bA</sup>	1.49 $\pm$ 0.32 <sup>dI</sup>	8.58 $\pm$ 0.87 <sup>bD</sup>	12.47 $\pm$ 0.23 <sup>bB</sup>	2.50 $\pm$ 0.21 <sup>cG</sup>	7.61 $\pm$ 0.23 <sup>bE</sup>
Day 13 $\Sigma$ PUFA <sup>2</sup>	1.84 $\pm$ 0.02 <sup>dH</sup>	1.34 $\pm$ 0.08 <sup>dI</sup>	2.42 $\pm$ 0.03 <sup>bE</sup>	2.19 $\pm$ 0.04 <sup>cG</sup>	2.56 $\pm$ 0.21 <sup>bD</sup>	2.38 $\pm$ 0.32 <sup>cF</sup>	3.32 $\pm$ 0.04 <sup>cA</sup>	3.02 $\pm$ 0.05 <sup>bB</sup>	2.75 $\pm$ 0.06 <sup>cC</sup>
Day 0 ND	6.30 $\pm$ 0.78 <sup>b</sup>	6.30 $\pm$ 0.78 <sup>b</sup>	6.30 $\pm$ 0.78 <sup>a</sup>	6.30 $\pm$ 0.78 <sup>b</sup>	6.30 $\pm$ 0.78 <sup>c</sup>	6.30 $\pm$ 0.78 <sup>c</sup>	6.30 $\pm$ 0.78 <sup>c</sup>	6.30 $\pm$ 0.78 <sup>c</sup>	6.30 $\pm$ 0.78 <sup>c</sup>
Day 6 ND	4.71 $\pm$ 0.89 <sup>dI</sup>	36.45 $\pm$ 2.13 <sup>aC</sup>	5.38 $\pm$ 0.56 <sup>bE</sup>	25.26 $\pm$ 1.21 <sup>aG</sup>	8.81 $\pm$ 0.97 <sup>aF</sup>	13.55 $\pm$ 0.45 <sup>aA</sup>	45.40 $\pm$ 0.32 <sup>aD</sup>	33.5 $\pm$ 0.34 <sup>aB</sup>	42.37 $\pm$ 2.11 <sup>aB</sup>
Day 13 ND	8.01 $\pm$ 0.67 <sup>aC</sup>	1.40 $\pm$ 0.12 <sup>dI</sup>	4.19 $\pm$ 0.34 <sup>cG</sup>	3.79 $\pm$ 0.21 <sup>chI</sup>	7.37 $\pm$ 0.33 <sup>bE</sup>	9.32 $\pm$ 0.86 <sup>bA</sup>	7.09 $\pm$ 0.45 <sup>bF</sup>	7.62 $\pm$ 1.00 <sup>bD</sup>	8.23 $\pm$ 0.32 <sup>bB</sup>

The values are means  $\pm$ S.D. of the samples analyzed in duplicate. <sup>a,b,c, ...</sup>The means followed by different letters in the same sample but different day indicate significant differences ( $P < 0.05$ ). <sup>A,B,C ...</sup> The means followed by different letters in the same day but different sample indicate significant differences ( $P < 0.05$ ).

Saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids.

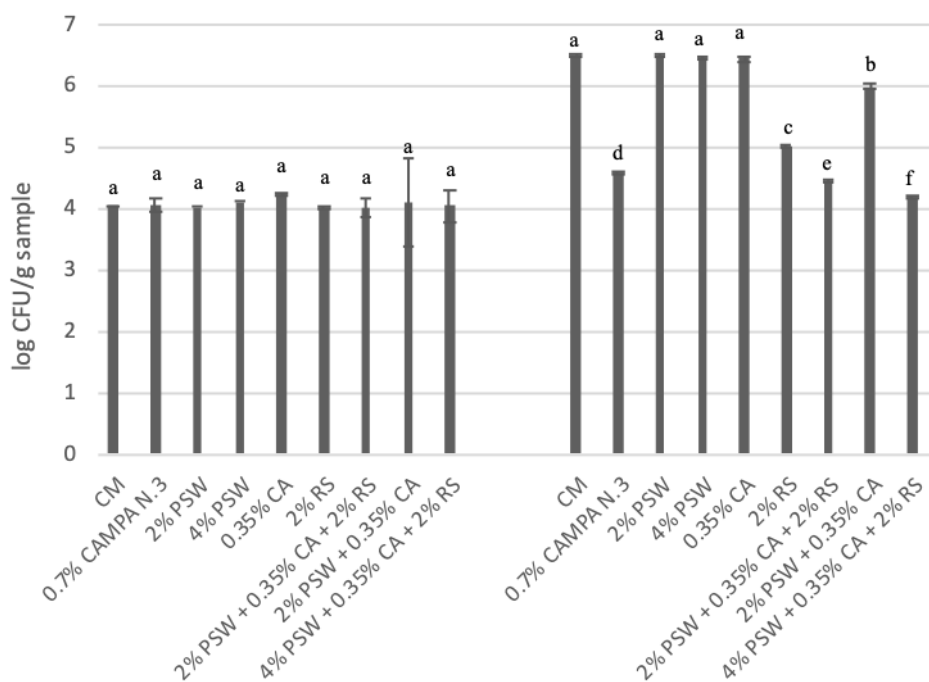
### 3.6. Microbiological analysis

The high-water content and nutrients present in the beef meat, made the patties extremely prone to bacterial growth. Figure 2 shows the level of microbial contamination of the different samples on the first and sixth day of the experiment. The results show that the samples which contained RS had a similar behavior to the patties with synthetic preservative (0.7% CAMPA N.3) after 6 days of storage, with values between 4 and 5 log CFU/g meat. These values were consistent with the results presented in assessment of the antimicrobial activity.

However, for Cayenne pepper, the low antimicrobial activity of the extract is reflected in the results of Figure 2. The value of microbial contamination was similar to the CM, between 6 and 7 log CFU / g meat and did not have an inhibitory effect on microbial growth in meat.

The PSW also had moderate antimicrobial activity (Table 3). In the microbiological results (Figure 2.), the 2% PSW + 0.35% CA + 2% RS and 4% PSW + 0.35% CA + 2% RS samples presented lower microbial growth than the 2% PSW + 0.35% CA and 2% RS samples. On the other hand, for the 2% PSW and 4% PSW samples, no decrease was observed in the microbial growth, and the values obtained were similar to the CM. So, the results suggested that there is a synergy between the PSW and the RS in the prevention of microbiological contamination in raw meat. Similar results

were reported in a study on the effect of the RS and pecans (*Carya illinoensis*) on antioxidant and antimicrobial activity in raw sardines [16].



**Figure 2.** Microbiological analysis at days 0 and 6 in beef patties on refrigerated storage at 4 °C. Results are given with standard error. Different letters in the same day indicate significant differences between samples.

### 3.7. pH

Table 6 shows the pH values of the samples at different days of the experiment. In the majority of the samples, the pH increased significantly ( $P < 0.05$ ) over time. Nevertheless, in the case of the treatments with 0.7% CAMPA N.3 (A) and all the samples with RS, the pH remained around 5 during the whole experiment (Table 6). The hibiscus flower showed a strong antimicrobial activity against different Gram + and Gram- bacteria. This could be directly related to the low pH values of the RS samples.

A previous study investigating *Agave angustifolia* [4] extract in pork patties, stored under an aerobic environment at refrigerator temperatures for several days, found an increase of pH ( $P < 0.05$ ). These results could be due to the degradation of nitrogenous components in meat during the storage period. This process was directly influenced by the action of endogenous or microbial enzymes such as proteases or lipases [4,39].

**Table 6.** pH values of the samples at different days of experiment.

Day	CM	0.7% CAMPA N.3	2% PSW	4% PSW	0.35% CA	2% RS	2% PSW+ 0.35CA	2%PSW+ 0.35%CA+ 2% RS	4%PSW+ 0.35% CA+ 2% RS
0	5.66±0.05 <sup>aF</sup>	5.71±0.03 <sup>aBC</sup>	5.64±0.05 <sup>aF</sup>	5.64±0.23 <sup>aF</sup>	5.70±0.02 <sup>aC</sup>	5.42±0.04 <sup>aAB</sup>	5.71±0.02 <sup>bCB</sup>	5.28±0.08 <sup>aD</sup>	5.20±0.10 <sup>aBC</sup>
3	5.88±0.02 <sup>aDE</sup>	5.89±0.03 <sup>aA</sup>	5.84±0.03 <sup>aDE</sup>	5.85±0.00 <sup>aD</sup>	5.89±0.02 <sup>aC</sup>	5.18±0.13 <sup>bB</sup>	5.82±0.03 <sup>bB</sup>	5.26±0.06 <sup>aCD</sup>	5.29±0.05 <sup>bB</sup>
4	5.78±0.01 <sup>aE</sup>	5.69±0.07 <sup>aC</sup>	5.70±0.04 <sup>bDE</sup>	5.67±0.01 <sup>aEF</sup>	5.73±0.05 <sup>aC</sup>	5.11±0.11 <sup>bB</sup>	5.72±0.04 <sup>bB</sup>	5.22±0.06 <sup>aD</sup>	5.16±0.01 <sup>bC</sup>
5	5.89±0.03 <sup>aDE</sup>	5.85±0.05 <sup>abA</sup>	5.82±0.02 <sup>abEF</sup>	5.81±0.00 <sup>abDE</sup>	5.82±0.05 <sup>abC</sup>	5.17±0.03 <sup>cb</sup>	5.77±0.03 <sup>bB</sup>	5.23±0.04 <sup>cd</sup>	5.22±0.02 <sup>cbC</sup>
6	5.87±0.02 <sup>aDE</sup>	5.82±0.06 <sup>aAC</sup>	5.77±0.03 <sup>aDEF</sup>	5.79±0.01 <sup>cdE</sup>	5.82±0.01 <sup>bcC</sup>	5.20±0.05 <sup>dAB</sup>	5.77±0.04 <sup>dB</sup>	5.27±0.03 <sup>abCD</sup>	5.24±0.01 <sup>cbC</sup>
7	5.97±0.03 <sup>aD</sup>	5.87±0.05 <sup>bcA</sup>	5.88±0.06 <sup>abCD</sup>	5.80±0.03 <sup>aDE</sup>	5.85±0.05 <sup>aC</sup>	5.20±0.02 <sup>baB</sup>	5.91±0.05 <sup>cb</sup>	5.28±0.01 <sup>cdC</sup>	5.21±0.02 <sup>bBC</sup>
10	7.15±0.04 <sup>aC</sup>	5.81±0.04 <sup>dB</sup>	6.73±0.08 <sup>cC</sup>	6.80±0.11 <sup>bcDE</sup>	7.06±0.05 <sup>abB</sup>	5.30±0.22 <sup>aAB</sup>	7.10±0.04 <sup>ab</sup>	5.24±0.04 <sup>ab</sup>	5.22±0.01 <sup>abC</sup>
11	7.36±0.08 <sup>ab</sup>	5.85±0.06 <sup>caB</sup>	6.93±0.04 <sup>bB</sup>	7.01±0.06 <sup>bB</sup>	7.37±0.17 <sup>aA</sup>	5.23±0.11 <sup>dAB</sup>	7.26±0.02 <sup>dB</sup>	5.29±0.03 <sup>ab</sup>	5.18±0.04 <sup>abC</sup>
13	7.54±0.01 <sup>aA</sup>	5.93±0.07 <sup>ba</sup>	7.31±0.13 <sup>aA</sup>	7.33±0.06 <sup>aA</sup>	7.53±0.08 <sup>aA</sup>	5.59±0.28 <sup>aA</sup>	7.45±0.01 <sup>aA</sup>	5.47±0.01 <sup>aA</sup>	5.46±0.01 <sup>aA</sup>

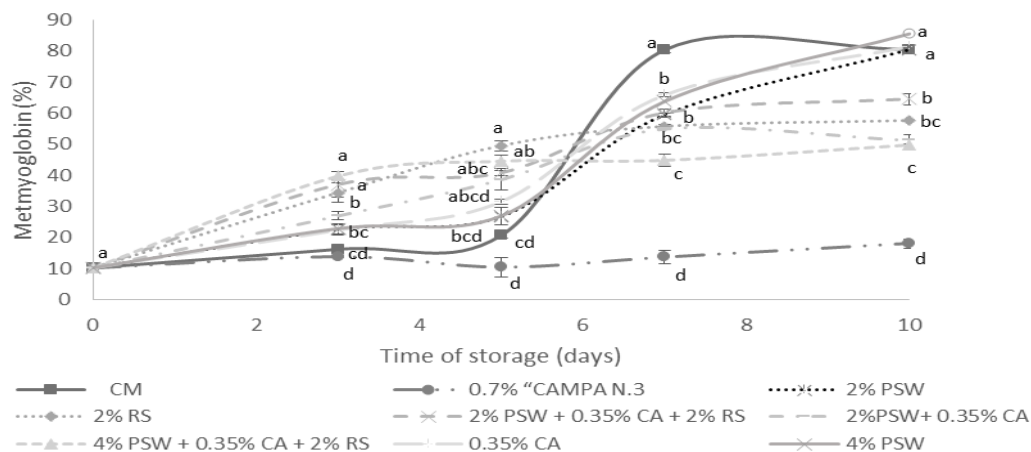
The values are means  $\pm$ S.D. of the samples analyzed in duplicate. <sup>a,b,c...</sup> The means followed by different letters in the same sample, but different day indicate significant differences ( $P < 0.05$ ). <sup>A,B,C...</sup> The means followed by different letters in the same day, but different sample indicate significant differences ( $P < 0.05$ ).

### 3.8. Percentage of metmyoglobin

The evolution of the percentage of metmyoglobin in the samples, studied for 10 days storage in a refrigerator, is shown in Figure 3. Many factors could influence the color of the meat products, but the susceptibility of self-oxidation of myoglobin was the predominant factor. The change of color from red to brown during storage is associated with the oxidation of red oxymyoglobin (OxyMb) to brownish metmyoglobin (MetMb) [25,40]. The sample that exhibited least reduction in the percentage of metmyoglobin during the experiment was the commercial preservative (CAMPA No.(A)), which remained stable ( $P < 0.05$ ) during the 10 days of storage at  $4 \pm 1$  °C. However, the rest of the samples retained a high percentage of metmyoglobin for 10 days. The samples with least metmyoglobin, apart from the CAMPA N.3 (A), were the 4% PSW + 0.35% CA + 2% RS and 2% PSW + 0.35% CA, which had very similar values (49.85% and 51.35%, respectively). The samples with 2% RS and 2% PSW + 0.35% CA + 2% RS had higher values of 57.61% and 64.49% respectively. Finally, the samples with the highest percentage of metmyoglobin were: 2% PSW, CTR, 0.35% CA and 4% PSW.

In general, the samples with RS contained less % of metmyoglobin than the rest and a stabilization of metmyoglobin formation was observed between days 7 and 10 of the experiment (no significant differences existed ( $P < 0.05$ )).

In agreement with the present results the literature reported [41], after day 6 of storage, a stabilization of the % of metmyoglobin in beef patties treated with carnosine.



**Figure 3.** Percent of Metmyoglobin in beef patties during 12 days of refrigerated storage at  $4\pm 1^{\circ}\text{C}$ . Different letters in the same day indicate significant differences between samples.

### 3.9. Color

The color of the raw beef meat, that could be affected by bacterial contamination and the formation of metmyoglobin, was dependent on low oxygen partial pressure, lipid oxidation and other parameters such as diet, genetics and the breed of the animal [42,43]. The color values of the beef patties were characterized as  $L^*$ ,  $c^*$ , and  $h^{\circ}$  during every day of the experiment (Table 7). There was no significant difference in  $L^*$  values between the control and the other groups. Similar results were reported by Araújo do Prado et al. [44], who did not find any differences in  $L^*$  values for beef burgers with added tannins. However, the different treatments produced significant changes in values of both  $c^*$  and  $h^{\circ}$ , even in the same sample but in different days. The  $c^*$  value of the control and the other samples decreased as storage time progressed, and higher  $c^*$  values were associated with increases in the  $a^*$  (red-green) and  $b^*$  (yellow-blue) values. These findings were consistent with the results shown in Table 7. Where a reduction in color values  $a^*$  and  $b^*$  over time indicates a change of the meat color, from red (oxymyoglobin) to brown (metmyoglobin) [45].

Nevertheless,  $c^*$  values over time decreased more in the samples containing natural additives than the CM or the CAMPA N.3 (A) samples, probably due to the composition of the PSW, CA and RS. In the case of the PSW, the high content of fiber affected the values where findings agreed with a literature report [46], which supports the hypothesis of gel formation between added fibers and the meat proteins, with a reduction of red color.



**Table 7.** Effect of antioxidants on L\*, c\* and h° parameters of raw beef patties during refrigerated storage

Days	CM	0.7% CAMPA N.3 (A)	2% PSW	4% PSW	0.35% CA	2% RS	2% PSW+ 0.35% CA	2%PSW+ 0.35%CA+ 2% RS	4% PSW+0.35% CA+ 2% RS		
Lightness (L*)	0	48.32±1.79 <sup>abcA</sup>	50.54±3.30 <sup>aA</sup>	44.40±3.03 <sup>abcdA</sup>	42.16±3.14 <sup>bcdA</sup>	50.32±1.68 <sup>aA</sup>	49.03±1.08 <sup>abAB</sup>	43.38±4.83 <sup>abcdA</sup>	40.87±2.38 <sup>cdA</sup>	38.67±1.82 <sup>dA</sup>	
	3	49.10±3.50 <sup>aA</sup>	44.08±4.13 <sup>aA</sup>	40.77±4.56 <sup>aA</sup>	37.42±4.70 <sup>aA</sup>	47.56±5.92 <sup>aA</sup>	44.64±3.85 <sup>ab</sup>	43.49±4.23 <sup>aA</sup>	37.47±2.34 <sup>aA</sup>	42.25±2.18 <sup>aA</sup>	
	4	48.57±3.37 <sup>aA</sup>	43.17±4.95 <sup>abA</sup>	40.88±0.67 <sup>abA</sup>	36.13±2.06 <sup>aA</sup>	47.16±4.32 <sup>aA</sup>	49.01±2.06 <sup>abAB</sup>	42.64±0.61 <sup>abA</sup>	37.87±1.17 <sup>ba</sup>	37.10±3.77 <sup>ba</sup>	
	5	45.39±2.32 <sup>abcA</sup>	42.59±3.82 <sup>abcdA</sup>	40.06±0.44 <sup>cdeA</sup>	34.76±1.92 <sup>aA</sup>	48.39±2.03 <sup>abA</sup>	49.45±2.84 <sup>abAB</sup>	40.95±3.02 <sup>bcddeA</sup>	37.36±2.98 <sup>deA</sup>	37.25±2.85 <sup>deA</sup>	
	6	51.76±3.77 <sup>aA</sup>	50.03±1.89 <sup>aA</sup>	44.10±5.31 <sup>abA</sup>	38.35±2.39 <sup>ba</sup>	50.55±1.35 <sup>aA</sup>	53.02±3.26 <sup>abAB</sup>	49.77±5.67 <sup>aA</sup>	42.11±5.17 <sup>abA</sup>	43.12±3.80 <sup>abA</sup>	
	7	49.32±4.99 <sup>aA</sup>	46.50±4.08 <sup>abA</sup>	43.32±2.60 <sup>aA</sup>	42.83±7.90 <sup>aA</sup>	43.18±2.30 <sup>aA</sup>	52.15±3.09 <sup>abAB</sup>	45.05±1.56 <sup>aA</sup>	40.30±3.97 <sup>aA</sup>	42.33±7.21 <sup>aA</sup>	
	10	48.18±2.51 <sup>abA</sup>	42.14±2.82 <sup>abA</sup>	43.68±5.15 <sup>abA</sup>	40.84±3.03 <sup>ba</sup>	46.70±1.31 <sup>abA</sup>	53.57±5.33 <sup>abAB</sup>	43.23±1.36 <sup>abA</sup>	37.61±6.64 <sup>ba</sup>	40.15±5.09 <sup>ba</sup>	
	11	47.71±3.89 <sup>abA</sup>	47.31±3.47 <sup>abA</sup>	45.50±0.98 <sup>abcA</sup>	34.28±4.44 <sup>aA</sup>	48.96±5.44 <sup>abA</sup>	54.44±2.67 <sup>aA</sup>	47.03±5.09 <sup>abA</sup>	37.44±2.71 <sup>bcA</sup>	41.67±2.06 <sup>bcA</sup>	
	13	44.04±3.83 <sup>abA</sup>	44.42±5.93 <sup>abA</sup>	46.95±2.96 <sup>abA</sup>	41.14±1.37 <sup>abA</sup>	45.45±1.61 <sup>abA</sup>	49.53±3.52 <sup>abAB</sup>	46.65±2.14 <sup>abA</sup>	41.84±4.73 <sup>abA</sup>	38.45±1.09 <sup>ba</sup>	
	Chroma (c*)	0	47.27±1.23 <sup>abA</sup>	54.93±4.86 <sup>aA</sup>	39.73±3.44 <sup>bca</sup>	27.07±1.20 <sup>dA</sup>	52.87±0.70 <sup>aA</sup>	39.30±1.51 <sup>ca</sup>	35.37±2.70 <sup>ca</sup>	32.37±4.12 <sup>cdA</sup>	26.03±2.04 <sup>dA</sup>
		3	43.53±6.26 <sup>abAB</sup>	45.05±3.19 <sup>abAB</sup>	31.35±2.78 <sup>bab</sup>	17.24±1.35 <sup>cbC</sup>	44.96±1.91 <sup>ab</sup>	31.28±4.51 <sup>baB</sup>	22.39±2.14 <sup>bcB</sup>	28.25±1.32 <sup>baB</sup>	14.74±2.92 <sup>cb</sup>
		4	41.01±2.05 <sup>abAB</sup>	47.92±3.51 <sup>abAB</sup>	26.66±4.24 <sup>bBC</sup>	19.87±0.69 <sup>baB</sup>	39.54±2.99 <sup>abCD</sup>	24.52±3.91 <sup>bcB</sup>	20.55±2.25 <sup>bcB</sup>	23.82±3.55 <sup>baBC</sup>	10.43±1.99 <sup>cbC</sup>
		5	40.47±3.41 <sup>abAB</sup>	47.10±2.90 <sup>abAB</sup>	29.68±0.37 <sup>baB</sup>	17.61±2.60 <sup>dbBC</sup>	40.60±0.44 <sup>bc</sup>	25.34±3.32 <sup>bcBC</sup>	19.74±3.87 <sup>cddeBCD</sup>	24.40±0.85 <sup>bcdAB</sup>	14.84±1.20 <sup>cb</sup>
6		31.14±5.19 <sup>bCD</sup>	45.02±7.50 <sup>abAB</sup>	24.63±2.29 <sup>bcBC</sup>	11.22±0.61 <sup>dbC</sup>	32.77±2.26 <sup>bDE</sup>	19.31±3.28 <sup>cdCD</sup>	16.29±3.13 <sup>cdBCD</sup>	19.41±2.71 <sup>cdBC</sup>	11.20±0.97 <sup>dbC</sup>	
7		24.30±5.07 <sup>bcD</sup>	47.69±4.04 <sup>abAB</sup>	23.04±1.76 <sup>bcBC</sup>	14.81±0.76 <sup>cdBC</sup>	28.87±4.36 <sup>bc</sup>	18.56±7.18 <sup>bcdCD</sup>	13.67±2.90 <sup>cdDE</sup>	15.48±1.70 <sup>cdC</sup>	10.27±2.25 <sup>dbC</sup>	
10		35.13±2.66 <sup>abCD</sup>	41.01±2.57 <sup>abBC</sup>	20.63±5.75 <sup>bBC</sup>	12.88±7.30 <sup>bcBC</sup>	31.79±0.84 <sup>bc</sup>	10.20±1.79 <sup>bcD</sup>	13.50±0.48 <sup>bcDE</sup>	17.19±5.02 <sup>bCC</sup>	7.18±0.47 <sup>cb</sup>	
11		31.93±3.21 <sup>bCD</sup>	41.01±4.45 <sup>abBC</sup>	23.68±2.95 <sup>cbBC</sup>	13.24±0.50 <sup>bcBC</sup>	29.02±1.51 <sup>bcE</sup>	11.28±2.66 <sup>cd</sup>	14.46±1.61 <sup>cdE</sup>	17.60±4.33 <sup>deC</sup>	10.17±1.33 <sup>cbC</sup>	
13		33.86±1.98 <sup>abBCD</sup>	34.68±6.08 <sup>abBC</sup>	17.47±5.96 <sup>abBC</sup>	9.62±4.14 <sup>abBC</sup>	34.22±3.19 <sup>abCDE</sup>	11.18±4.41 <sup>ad</sup>	9.16±1.32 <sup>abE</sup>	16.19±2.63 <sup>abBC</sup>	7.55±0.54 <sup>bc</sup>	
Hue angle (h°)		0	19.02±3.87 <sup>abBC</sup>	25.17±4.60 <sup>abBC</sup>	19.03±0.38 <sup>abBCD</sup>	11.94±7.21 <sup>ba</sup>	24.16±2.17 <sup>abBC</sup>	24.89±2.23 <sup>abAB</sup>	17.69±1.41 <sup>abAB</sup>	16.51±2.80 <sup>abDE</sup>	17.17±6.20 <sup>abA</sup>
		3	24.23±5.30 <sup>abBC</sup>	25.76±1.24 <sup>abBC</sup>	27.78±2.30 <sup>abBCD</sup>	26.80±5.76 <sup>aA</sup>	31.66±2.81 <sup>abBC</sup>	32.54±4.72 <sup>abAB</sup>	20.66±4.34 <sup>abAB</sup>	27.54±1.76 <sup>abCDE</sup>	26.75±6.52 <sup>aA</sup>
		4	22.32±1.30 <sup>abBC</sup>	26.58±1.31 <sup>abBC</sup>	19.40±5.82 <sup>abBCD</sup>	19.16±3.31 <sup>aA</sup>	24.41±4.97 <sup>abBC</sup>	26.97±1.47 <sup>abAB</sup>	17.96±3.59 <sup>abAB</sup>	20.42±3.29 <sup>abCDE</sup>	-7.06±5.02 <sup>bb</sup>
		5	32.83±4.13 <sup>abBC</sup>	32.31±1.82 <sup>ba</sup>	33.90±1.98 <sup>baB</sup>	33.34±4.16 <sup>ba</sup>	34.43±0.60 <sup>abAB</sup>	44.09±3.12 <sup>aA</sup>	32.58±1.11 <sup>baB</sup>	35.59±6.52 <sup>aABC</sup>	31.71±1.65 <sup>ba</sup>
	6	33.02±7.22 <sup>abBC</sup>	29.99±1.22 <sup>abAB</sup>	32.58±3.79 <sup>abBC</sup>	25.19±6.11 <sup>aA</sup>	38.02±4.71 <sup>aA</sup>	45.51±6.06 <sup>aA</sup>	38.79±4.12 <sup>aA</sup>	37.60±5.98 <sup>abAB</sup>	28.90±2.57 <sup>aA</sup>	
	7	40.66±6.99 <sup>abCA</sup>	31.27±4.04 <sup>bcA</sup>	34.57±1.76 <sup>bcA</sup>	32.78±0.76 <sup>bcA</sup>	38.22±4.36 <sup>abCA</sup>	44.92±7.18 <sup>abA</sup>	30.22±2.90 <sup>bcAB</sup>	49.45±1.70 <sup>aA</sup>	27.34±2.56 <sup>ca</sup>	
	10	34.88±3.45 <sup>abAB</sup>	23.38±0.92 <sup>abBC</sup>	31.63±5.20 <sup>abBCD</sup>	41.15±22.64 <sup>aA</sup>	37.79±1.62 <sup>aA</sup>	43.36±4.39 <sup>aA</sup>	14.50±4.98 <sup>abAB</sup>	32.26±6.60 <sup>abBCD</sup>	4.67±5.50 <sup>bb</sup>	
	11	22.96±0.98 <sup>ac</sup>	20.15±4.66 <sup>bc</sup>	17.14±4.08 <sup>bd</sup>	13.09±6.02 <sup>aA</sup>	21.66±2.20 <sup>bc</sup>	45.69±0.89 <sup>aA</sup>	7.87±2.21 <sup>ab</sup>	13.07±4.10 <sup>de</sup>	-33.58±7.23 <sup>bc</sup>	
	13	24.09±6.85 <sup>abBC</sup>	26.11±2.13 <sup>abBC</sup>	25.03±6.10 <sup>abBCD</sup>	11.21±2.11 <sup>aA</sup>	20.97±6.23 <sup>bc</sup>	38.54±2.30 <sup>abAB</sup>	8.24±2.10 <sup>ab</sup>	32.86±3.74 <sup>abABC</sup>	-24.29±6.77 <sup>bc</sup>	

The values are means ±S.D. of the samples analyzed in duplicate. a,b,c... The means followed by different letters in the same sample but different day indicate significant differences (P < 0.05). A,B,C... The means followed by different letters in the same day but different sample indicate significant differences (P < 0.05).

### 3.10. Sensory analysis

Sensory analysis was performed on samples CM, 0.7% CAMPA No. 3 (A), 2% PSW + 0.35% CA, 2% PSW + 0.35% CA + 2% RS, and 4% PSW + 0.35% CA + 2% RS, in order to evaluate the final acceptability of the product by consumers.

Figure 4. shows the change of appearance, color, flavor, texture, spicy flavor, acidity, acceptability, and purchase intention for the different samples. CM and 0.7% CAMPA N.3 (A) were the most acceptable, thanks to a better aroma and flavor, probably because they were comparable in taste to commercial hamburgers.

The consumer intention to purchase beef patties with 2% PSW + 0.35% CA, 2% PSW + 0.35% CA + 2% RS and 4% PSW + 0.35% CA + 2% RS was the lowest; this might be due to the spicy taste due to the addition of CA powder. Regarding the pH results, it was previously observed that the formulations with RS had the lowest pH; this factor made the beef acquire a characteristic acidic flavor. Appearance and color could also be influenced by the addition of hibiscus flower.

Generally, the CM and 0.7% CAMPA N.3 (A) samples presented the best results. Out of the treatments where a natural powder was added, the highest acceptability and intention of purchase was obtained for the 4% PSW + 0.35% CA + 2% RS, possibly because the panelists did not have a habit of eating spicy products.



**Figure 4.** Sensorial results from 40 untrained panelists. Sensorial attributes and purchase intention of beef patties.

### 3.11. Water-holding capacity (WHC)

The water-holding capacity of the meat is an important quality parameter in the food industry, due to its economic implications [15]. Table 8 shows the results of the WHC in percentage of the samples: CM, CAMPA N. 3 (A), 2% PSW + 0.35% CA + 2% RS, 4% + 0.35% CA + 2% RS, 2% PSW + 0.35% CA. All the samples showed significantly higher percentages ( $P < 0.05$ ) than the control

sample. The sample that exhibited the highest WHC contained the most PSW (4% PSW + 0.35% CA + 2% RS). This can be related to the nature of the shell, which contained more than 58.90% fiber [31]. Previous studies reported an increased WHC with the addition of dietary fiber into beef patties, probably due to the moisture and fat in the fiber matrix [47–49]. Gibis & Weiss reported the effect of marinades of RS extract in beef patties and showed that after 120 seconds of broiling time the weight loss was 31%; this value is similar to the results obtained in this study [50]. However, according to Jung & Joo, the addition of hibiscus flower didn't affect the water retention [51].

#### 4. Conclusion

The present study revealed the efficacy of the combination of PSW, CA and RS to prevent lipid oxidation, with the lowest formation of MDA and hexanal in beef patties stored at  $4\pm 1^\circ\text{C}$  for 13 days. Furthermore, the mix was effective in reducing the microbial activity, % metmyoglobin and pH. Based on the above data, PSW, CA and RS represent a natural, environmentally friendly and cost-effective alternative in the control of oxidative processes and microbial activity in meat products.

**Abbreviations:** PSW: Pecan shell (*Carya illinoensis*), RS: roselle flower (*Hibiscus sabdariffa*), CA: Cayenne pepper (*Capsicum annum*), TE: Trolox equivalent, DW: Dry weight, GAE: Gallic acid equivalent, TPC: Total phenolic compounds, CM: Control, CFU: Colony-forming unit, TBARS: Thiobarbituric acid reactive substances, MDA: Malondialdehyde, BHA: Butylate Hidroxianilose, BHT: Butylate Hidroxitoluene, PUFA: Polyunsaturated fatty acids, MUFA: Monounsaturated fatty acids, and SFA: Saturated fatty acids.

#### Declarations

**Compliance with ethics requirements:** This article does not contain any studies with human or animal subjects.

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**Conflict of interest:** All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Chapter 6. The transformations of the pecan shell.**



## 6.1. In Vitro Antioxidant Activity Optimization of Nut Shell (*Carya illinoensis*) by Extrusion Using Response Surface Methods

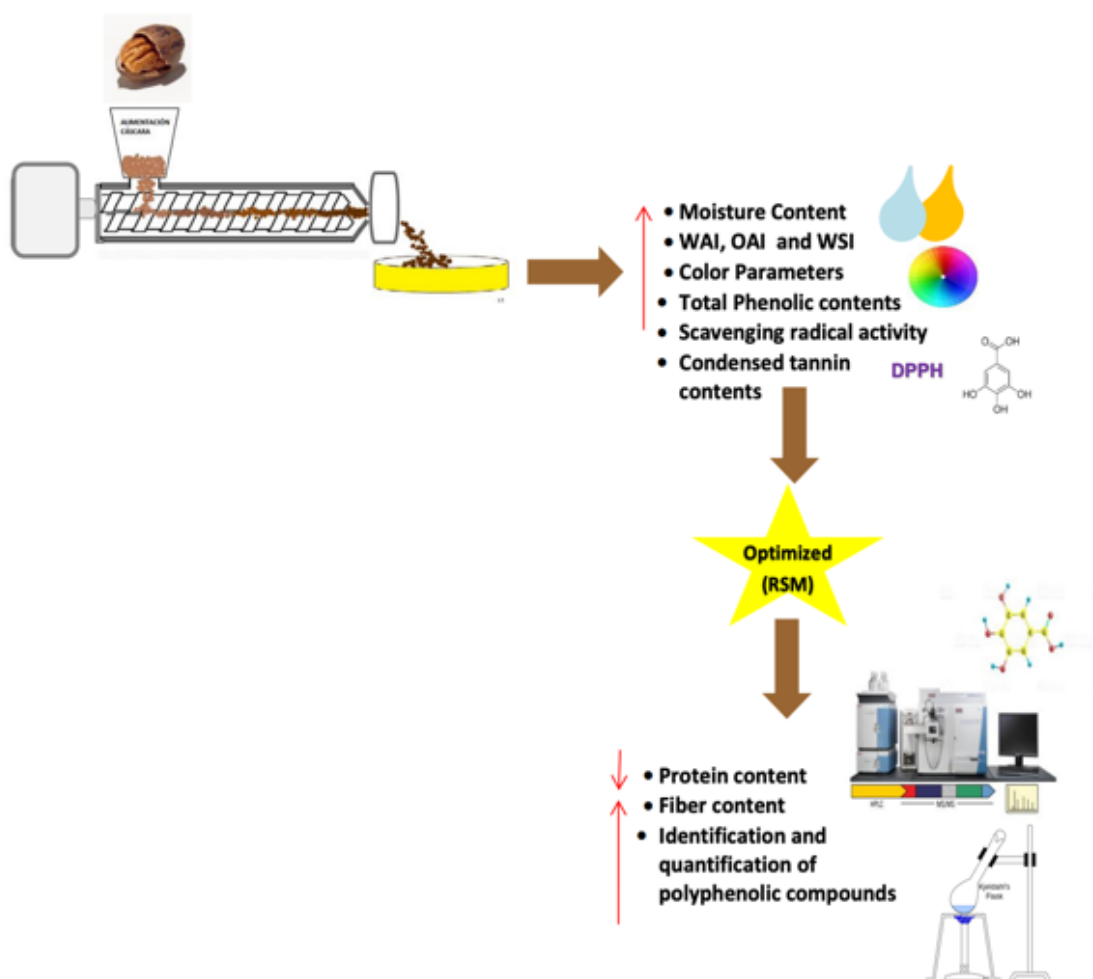
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Article

# In Vitro Antioxidant Activity Optimization of Nut Shell (*Carya illinoensis*) by Extrusion Using Response Surface Methods

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**Abstract:** The pecan (*Carya illinoensis*) nut shell is an important byproduct of the food processing industry that has not been previously explored as a material with antioxidant properties. This work aims to study the effect of the extrusion temperature and screw speed on the moisture content, water and oil absorption index, water solubility index, color, phenolic compounds, condensed tannin compounds, and antioxidant activity of pecan nut shell extrudates. Extrusion variables were adjusted using a response surface methodology. Extrusion, performed at 70 °C and 150 rpm, almost doubled the concentration of polyphenols in the non-extruded shell and significantly increased radical scavenging activity. Compounds in extrudates, prepared at 70 °C and 150 rpm, were quantified by high-performance liquid chromatography (HPLC) with a diode-array detector (DAD) and identified by liquid chromatography coupled with time-of-flight mass spectrometry (LC-MSD-TOF). Extrusion significantly increased most phenolic acid compounds, including gallic acid, ellagic acid pentose, ellagic acid, dimethyl ellagic acid rhamnoside, and dimethyl ellagic acid. The soluble fiber in extrudates was more than three-fold higher than in the control. Therefore, extrusion at 70 °C and 150 rpm increased the concentration of phenolic compounds, antioxidant activity, and total dietary and soluble fiber. Our findings support the notion that extruded pecan nut shell can be used in clean-label products and improve their nutraceutical value.

**Keywords:** extrusion; pecan nut shell (*Carya illinoensis*); physicochemical properties; antioxidants; total dietary fiber

## 1. Introduction

Pecan nuts (*Carya illinoensis*) have been part of the human diet for hundreds of years and they are commonly used in the bakery and snack industry [1]. This type of nut has beneficial nutritional components, including polyunsaturated fatty acids, vitamins, minerals, protein, and other functional compounds, such as polyphenols [2]. Mexico and the United States are responsible for 93% of the world's production of pecan nuts, with an average of nearly 60,000 and 40,000 metric tons per year, respectively [3]. However, between 40 and 50% of the original nut is wasted [4].

Prado et al. found that a pecan nutshell infusion presents antibacterial activity [5]. Müller et al. found that the shell infusion could be an economical agent in the prevention of liver diseases associated with ethanol consumption [6], additionally Reckziegel et al. found that the shell tea prevents anxiety caused by cigarette abstinence, acting as a natural anxiolytic [7]. On the other hand, in the industry, this by-product could be used as a biosorbent or as precursor of carbon for the removal of dyes and heavy metals from aqueous solutions [8].

In recent years, consumers have demonstrated an interest in increasing the consumption of foods with a high content of dietary fiber, with the idea to reduce the development of chronic diseases [9]. The by-products of nut shells are composed of around 70–80% fiber that is predominantly insoluble fiber: lignin, cellulose, and hemicellulose [10]. In addition, they contain protein, various minerals, and also phenolic compounds and proanthocyanidins, including vanillic, caffeic, and gallic acids, catechin, and tannic acid, which could have an antioxidant effect on human and animal organisms [11–13].

Extrusion cooking has been commonly used for the development of new products, including snacks, breakfast cereals, and pet and baby foods. It is a technology that has a low cost, high productivity, versatility, and also improves the digestibility and nutritional bioavailability of the product for human and animal consumption [14]. Extrusion represents a mechanical stress, which may alter physical and chemical characteristics [15]. It can be applied by controlling three independent variables, namely the barrel temperature, moisture, and screw speed [14,16,17]. The use of extrusion in the processing of by-products derived from cereals, fruits, and vegetables is of great interest. Several studies have focused on increasing the antioxidant activity and the polyphenols and soluble fiber using this technology in wastes like soybean residue [18], shrimp shell [19], orange peel [20], bilberry press cake [21], and so forth. In addition to these improvements, extrusion has shown a benefit to the techno-functional characteristics of the byproducts. In the residues mentioned above, the water and oil absorption increased.

However, there have not been relevant reports about using twin-screw extruders in shells of nuts. The aim of this work is to study the effects of extrusion process variables, including the barrel temperature and screw speed, on the chemical characteristics of pecan nut shells. The objective is to employ response surface methodology to optimize the extrusion process conditions on the total phenol content (TPC), condensed tannin contents (CTC), and antiradical activity (1,1-Diphenyl-2-Picrylhydrazyl; DPPH). The work includes the quantification of polyphenol compounds by HPLC, with a diode-array detector and the identification by LC-MSD-TOF (Liquid Chromatography time-of-flight mass spectrometry), and also the changes of fiber and protein contents in the samples under optimal experimental conditions (70 °C and 150 rpm).

## **2. Materials and Methods**

### **2.1. Chemicals and Reagents**

All the chemical reagents were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). The sunflower oil was from a local supermarket in Monterrey, Mexico. The total dietary fiber kit (K-TDFR-100A/) was purchased from Megazyme™ (Bray, Ireland).

### **2.2. Pecan Nut Shell Samples**

The raw material used for non-extruded (control) and the extrusion process was pecan nut shell (*Carya illinoensis*). The pecan nut shells were separated manually from the kernel and collected at 1090 m above sea level (coordinates 25°45'32" N, 102°58'58" W) in San Pedro, Coahuila, which is located on the north of Mexico. The pecan nut shells collected were milled in a Wiley mill (Arthur Thomas, Philadelphia, PA, USA) equipped with a 2 mm screen.

### **2.3. Extrusion Processing**

The shell powder was processed in a twin-screw co-rotating extruder (BCTM-30 Bühler, Uzwil, Switzerland) with an 800 mm length, 30 mm diameter, and L/D = 20 screws, with a configuration similar to that described by Cortés-Ceballos et al. [22] with some changes. The initial section comprised only conveying elements, the second included both conveying and mixing elements, and the last one, the shear section contained conveying, one reverse element and transport elements. A TT-137N water heater (Tool-temp, Sulgen, Switzerland) controlled the temperature at the final section of the extruding chamber. The shell was processed at a fixed feed rate of 7.79 kg/h dry matter. The extrudates and the control (non-extruded shell) were dried at 60 °C in an air convection oven (Edel Ingenieros, Monterrey, Mexico). Finally, all samples were stored at room temperature until analysis.

### **2.4. Experimental Design and Extrusion Conditions**

A face-centered central composite RSM (Response Surface Methodology) was used to determine the effects of extrusion on total phenols, condensed tannins and DPPH radical scavenging activity. Two different factors were evaluated: screw speed and barrel temperature in the range: temperature (minimum = 33.25 °C, maximum = 106.75 °C) and screw speed (minimum = 88.75 rpm, maximum = 211.75 rpm). The experimental design to obtain extruded nut shells with two factors is found in Table 1.

**Table 1.** Experimental design to obtain extruded nut shells with two factors.

Treatment	Order Run	Replicates	Factors	
			Temperature (°C)	Screw Speed (rpm)
1	2	2	33.25	150
2	11	2	40	100
3	4	2	40	200
4	8	2	70	150
5	10	2	70	88.75
6	9	2	70	150
7	7	2	70	211.25
8	1	2	70	150
9	3	2	100	100
10	6	2	100	200
11	5	2	106.75	150

### 2.5. Techno-Functional Characterization

The moisture of the extrudates and control (non-extruded shell) samples was determined by the method of AOAC 925.10 [23] just after the extrusion process. The color measurements of shell extrudates and the control (non-extruded shell) were performed using a Konica Minolta CM-600d colorimeter, calibrated with a standard series ( $X = 94.9$ ,  $y = 0.32$ , and  $x = 0.31$ ). The luminosity was determined by  $L^*$ , red-green by  $a^*$ , and yellow-blue by  $b^*$ . Parameters  $a^*$  and  $b^*$  were used to calculate chrome ( $C^*$ ) and Hue angle ( $h^\circ$ ) according to Equations (1) and (2):

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (1)$$

$$h^\circ = \tan^{-1}(b^*/a^*) \quad (2)$$

The water absorption index (WAI) was determined according to the method described by Ruiz-Gutiérrez et al. [24]. The extruded shell or the control (non-extruded shell) ( $2.5 \pm 0.05$  g) was suspended in distilled water (30 mL) at 25 °C and then centrifuged at  $3000\times$  g for 10 min. The supernatant was removed. The WAI was calculated as the weight of sediment per weight of dry solid shell. The water solubility index (WSI) was the weight of dry solids in the supernatant from the WAI test after decantation and evaporation at 105 °C. The oil absorption index (OAI) was determined by the Ruiz-Gutiérrez et al. [24] method with few modifications. The extruded shell and the control (non-extruded shell) ( $2.5 \pm 0.05$  g) were suspended in sunflower oil (10 mL) at 25 °C, then stirred for 30 s, and then centrifuged at  $3000\times$  g for 10 min. The supernatant was removed. The OAI was calculated as the weight of sediment per weight of dry solid shell.

## **2.6. Phenolic Determination**

### 2.6.1. Extraction for Total Phenolic Compounds

Dried extruded shells and the control (non-extruded shell) were weighed ( $1 \pm 0.05$  g) and extracted with 20 mL of an ethanol-water mixture at 50:50 (v/v). The mixture was stirred for 90 min at 25 °C. All samples were centrifuged (Sigma 6K10, Osterode am Harz, Germany) for 15 min at  $3000\times$  g.

The Folin–Ciocalteu method was used to determine the TPC, as reported by Singleton et al. [25], using a ultraviolet (UV)-Vis microplate reader spectrophotometer Fluostar Omega (Paris, France) at 25 °C; the results were expressed in mg gallic acid equivalents/g dry weight (mg GAE/g DW). The standard curve was obtained by plotting the absorbance against different concentrations of gallic acid (ranging from 0.12 to 1.73 mM).

### 2.6.2. Condensed Tannin Content

The vanillin assay method, according to Flores-Córdova et al. [26], was followed with some modifications to quantify the CTC. Hereby, 50  $\mu$ L of the shell extract (1:20 (w/v) in ethanol-water at 50:50 (v/v)) was added to 4 mL of 37% HCl solution with 8% MeOH (v/v) and a vanillin solution prepared in 4% MeOH (v/v) at a proportion of 50:50 (v/v).

Following 20 min of incubation in the dark, the solution was measured at 500 nm in a UV-Vis microplate reader spectrophotometer Fluostar Omega (Paris, France). The CTC was expressed as mg equivalents of catechin/dry weight of the sample (mg EC/g DW). The standard curve was obtained by plotting the absorbance against different concentrations of catechin (ranging from 0.3 to 1.5 mg/mL).

### 2.6.3. Determination of 1,1-Diphenyl-2-Picrylhydrazyl Radical Scavenging Activity

The DPPH free radical scavenging activity of the control (non-extruded shell) and extruded shell treatments was measured according to the method described by Villasante et al. [27]. An initial absorbance measurement of the DPPH reagent was recorded ( $A_0$ ). The shell extracts (same extraction used for TPC 2.6.1.) in five different dilutions reacted with 200  $\mu$ L of DPPH in methanol for a short period of time. The absorbance ( $A_1$ ) was measured at 517 nm after a period of 75 min using a UV-Vis microplate reader spectrophotometer Fluostar Omega (Paris, France) at 37 °C. The

inhibition percentage of radical scavenging activity of each treatment was calculated by the following equation (Equation (3)).

$$\%inhibitionofsample = \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \quad (3)$$

where A<sub>0</sub> is the absorbance of DPPH without sample and A<sub>1</sub> is the absorbance of the extracts at time 75 min. All analyses were conducted in triplicate. The results are presented as means ± standard deviation (SD) based on the three field replicate samples. Half inhibitory concentration (IC<sub>50</sub>) of DPPH radical was calculated based on the liner regression of the percentage of the remaining DPPH radical against the sample concentration.

## **2.7. Optimized Treatment Characterization**

An optimum treatment was produced by applying the regression obtained from the RSM in which the DPPH capacity was maximized. This treatment was characterized

### **2.7.1. Free Phenolic Compounds Extraction and Identification and Quantification**

From optimized treatment, FPC (Free Phenolic Compounds) were extracted using the method described by Adom and Liu [28] with some modifications. One g of the extruded shell and the control (non-extruded shell) were mixed with 10 mL of ethanol-water 50:50 (v/v) and vortexed for 10 min. The mixture was centrifuged for 10 min at 3000× g. The ethanol was evaporated with nitrogen at room temperature, then the extracts were lyophilized and stored at -20 °C in the darkness until analysis. The phenolic compounds in control and optimized treatment were identified and quantified according to Acosta-Estrada et al. [29], with few modifications. The lyophilized samples were dissolved in 1 mL of methanol and then filtered through 0.45 µm nylon filter. The compounds were quantified by high-performance liquid chromatography with a diode-array detector (HPLC-DAD) (1200 Series, Agilent Technologies, Santa Clara, CA, USA) using a reverse-phase column (Zorbax SB-Aq, Santa Clara, CA, USA) 4.6 mm ID × 150 mm (3.5 µm) scanning at different wavelengths of 254, 280, and 320 (obtained from an sample scanning). Chromatographic separations were performed using a mixed mobile phase composed of (A): water acidified (pH = 2) with formic acid and (B): acetonitrile. The flow rate was 0.6 mL/min at 25 °C. The gradient was as follows: 0–10 min 15% B, 10–14 min 58% B, 14–20 min 80% B, and 20–30 min 100% B. Identification was performed using liquid chromatography coupled to time-of-flight mass spectrometry (LC-MSD-TOF) (1100 Series, Agilent Technologies, Santa Clara, CA, USA) under the same chromatographic conditions described for the HPLC-DAD analysis, with an electrospray source in positive ion mode (ESI+) with the following parameters: nitrogen gas temperature, 300 °C; drying gas flow rate, 8 L/min; curtain gas, 50 psig; capillary voltage, 4000 V; and fragment voltage, 70 V. MS spectra were recorded in the range of m/z 100–1000. The quantification of phenolic compounds, for which standards were available, was carried out using appropriate calibration curves. Spectral data was collected using Mass Hunter (Santa Clara, A.02.01(B730), Agilent Technologies, CA, USA) workstation software.

### 2.7.2. Protein and Dietary Fiber Determination

The crude protein content was determined by the Kjeldahl digestion and distillation methods (No. 920.87 AOAC) [23]. A conversion factor of 6.25 was selected to calculate the protein content. The in vitro protein digestibility assay was used for analyzing the digestibility of protein samples [30]. The analysis was carried out by a multi-enzyme solution: trypsin, chymotrypsin, and peptidase, that was added to the solution of the sample with pH = 8. The sample and enzyme solution were mixed and stirred for 10 min at 37 °C. The resulting pH was recorded for 10 min and the in vitro protein digestibility was calculated with Equation (4) (where the  $\Delta\text{pH}_{10\text{min}}$  is the change in pH in 10 min from the initial pH of 8.0):

$$\text{IVDP}\% = 65.66 + 18.10 \cdot \Delta\text{pH}_{10\text{min}}. \quad (4)$$

The standardized enzymatic-gravimetric method (AOAC 991.43) was used for the determination of insoluble (IDF) and soluble dietary fiber (SDF) content. Total dietary fiber (TDF) was calculated as the sum of SDF and IDF [9].

### 2.8. Statistical Analysis

For the techno-functional characterization, each experiment was performed in triplicate and data were reported as means  $\pm$  standard deviations. Results were subjected to analysis of variance with ANOVA and differences among means were compared by the Tukey test ( $p < 0.05$ ). Statistical analysis was performed with software MINITAB 18<sup>®</sup>-0.

The RSM data was submitted to the MINITAB 18<sup>®</sup>-0 and fitted to a second-order polynomial model, and regression coefficients were obtained with a significance level ( $\alpha$ ) of 0.05. The generalized second-order polynomial model used in the response surface analysis followed the Equation (5)

$$Y = \beta_0 + \sum_i^j \beta_i X_i + \sum_i^j \beta_{ii} X_i^2 + \sum_i^j \beta_{ij} X_i X_j \quad (5)$$

where  $\beta_0$  is the model constant coefficient,  $\beta_i$  is the linear coefficients,  $\beta_{ii}$  is the quadratic coefficient and  $\beta_{ij}$  is the interaction coefficient of variables  $i$  and  $j$ .

The statistical analysis for the optimized treatment characterization was the same as that used for the techno-functional characterization.

## 3. Results and Discussion

### 3.1. Physical Characterization

Although in the extrusion process the water content remained constant, the moisture content of the extruded shell was significantly affected ( $p < 0.05$ ) by the extrusion treatment (Table 2). Compared to control samples (non-extruded shell), extrusion significantly decreased the moisture content 1.2- to 1.4-fold. The screw speed and the temperature affected the moisture percentage. This difference in moisture content could be explained by the effect of the temperature and the speed of the screw on the material structure. In a study presented by Borchani et al. it has been found that in



a by-product from dates with the incorporation of a temperature treatment, the dry matter increased as well as the content of TDF [31]. Several authors suggest that products with high dry matter contents contribute to an easier auto conservation of fiber [32,33].

In the case of WAI and OAI, only samples T2 and T7 were significantly higher than control values (Table 2). However, in the case of WSI, most of the samples were significantly different from the control. Condition T11 (106.75 °C, 150 rpm) produced the highest WSI (%) value:  $4.47 \pm 0.10$  versus  $3.00 \pm 0.14$  g in the control (non-extruded shell). Consistently, similar results were obtained in agro-industrial by-products extrudates like rice bran and paddy husk, the WSI increased with the increment of temperature up to 110 °C [34]. Extruders will provide high shear, rapid heat transfer, and effective mixing in a short residence time. The physical and chemical structure of the material will be disturbed and changed during the passage through the extruder barrel, resulting in a large specific area. Jan et al. and Zheng and Rehmann support the results with the thermal degradation during the extrusion process (high temperatures), as this could be the reason for the physical and chemical change on the material structure, resulting in an increment of the accessibility of cellulose for enzymatic action [34,35].

The results in luminosity ( $L^*$ ), red-green ( $a^*$ ) and yellow-blue ( $b^*$ ) parameters, the chroma (C) and hue angle ( $h^\circ$ ) of shell extrudates, and the control (non-extruded shell) are presented in Table 2. According to the results, the extrusion conditions evaluated had an important effect on color parameters. A significant difference was detected when comparing the  $L^*$  value of the control (non-extruded) and all the extrusion treatments, within the range of 10.92–23.37, respectively. Similar results were obtained with carrot pomace-based extrudates, where the increase of the L value was observed with the increment of temperature. This effect shows the relation with the increase in brightness and rise in air cells [36].

The screw speed and the temperature also had a significant effect on the values of  $a^*$  and  $b^*$ . The maximum  $a^*$  (10.58) and  $b^*$  (14.63) values were reported with treatment 11 (150 rpm, 106.75 °C) while a minimum  $a^*$  (6.51) and  $b^*$  (7.88) values were revealed for the control. Increase of red-green and yellow-blue could be related to the changed structure due to higher extruder temperature. A maximum change was produced at a screw speed of 150 and temperature of 106.75 °C on red-green and yellow-blue in comparison with the control:  $a^*$  (10.58–6.51) and  $b^*$  (14.63–7.88). Likewise, for the chroma and hue angle the values increased with the extrusion process. Regarding  $C^*$  and  $h^\circ$ , non-extruded samples showed the lowest values 10.23 and 50.39 respectively. Treatment 11 (150 rpm and 106 °C) showed the higher value for  $C^*$  with 18.06 and for  $h^\circ$  the treatment 9 (100 rpm and 100 °C) with a value of 54.17.

The values for the extrudate samples ( $L^*$ ,  $C^*$ ,  $h^\circ$ ) may be affected by the destruction of thermosensitive pigments. Jan et al. [34,37] observed similar results in agro-industrial wastes, and the authors found that the extruder variables had an effect on the color. Thermal processing of food produces reactions, such as Maillard, caramelization, or hydrolysis, which can affect the color in extruded products. Melanoidins are dark color pigments [38–40]. Until now, the effect of the extrusion process on the color of fibrous materials, such as the shell, has not been researched.

**Table 2.** Moisture, color, and techno-functional properties of pecan nut shell without extrusion and extruded at different temperatures (33.25, 40, 70, 100, or 106.5 °C) and velocities (88.75, 100, 150, 200, and 211.25 rpm).

Treatment	Moisture (%)	WAI (Water absorption index) (g)	OAI (Oil absorption index) (g)	WSI (Water solubility index) (%)	L*	a*	b*	C* (Chroma)	h° (Hue Angle)
Control	5.97 ± 0.06 <sup>a</sup>	1.96 ± 0.03 <sup>c</sup>	2.40 ± 0.02 <sup>cde</sup>	3.00 ± 0.14 <sup>f</sup>	10.92 ± 0.68 <sup>g</sup>	6.51 ± 0.42 <sup>f</sup>	7.88 ± 0.75 <sup>g</sup>	10.23 ± 0.47 <sup>f</sup>	50.39 ± 0.00 <sup>b</sup>
T1 (33.25 °C, 150 rpm)	4.74 ± 0.09 <sup>bc</sup>	2.88 ± 0.10 <sup>abc</sup>	2.29 ± 0.01 <sup>e</sup>	3.70 ± 0.03 <sup>de</sup>	14.83 ± 0.05 <sup>f</sup>	8.81 ± 0.22 <sup>de</sup>	11.93 ± 0.07 <sup>f</sup>	14.83 ± 0.06 <sup>de</sup>	53.60 ± 0.68 <sup>a</sup>
T2 (40 °C, 100 rpm)	4.21 ± 0.04 <sup>d</sup>	3.33 ± 0.10 <sup>ab</sup>	3.02 ± 0.01 <sup>a</sup>	3.44 ± 0.01 <sup>e</sup>	14.40 ± 0.09 <sup>f</sup>	9.95 ± 0.01 <sup>b</sup>	13.73 ± 0.15 <sup>b</sup>	16.96 ± 0.00 <sup>b</sup>	54.10 ± 0.05 <sup>a</sup>
T3 (40 °C, 200 rpm)	4.88 ± 0.05 <sup>b</sup>	2.26 ± 0.08 <sup>c</sup>	2.54 ± 0.01 <sup>bc</sup>	4.360 ± 0.09 <sup>b</sup>	22.42 ± 0.08 <sup>b</sup>	10.74 ± 0.06 <sup>a</sup>	14.49 ± 0.02 <sup>a</sup>	18.04 ± 0.09 <sup>a</sup>	54.15 ± 0.41 <sup>a</sup>
T4 (70 °C, 150 rpm)	4.40 ± 0.06 <sup>cd</sup>	2.55 ± 0.31 <sup>abc</sup>	2.42 ± 0.01 <sup>cde</sup>	3.50 ± 0.01 <sup>e</sup>	16.66 ± 0.03 <sup>e</sup>	9.23 ± 0.14 <sup>c</sup>	12.24 ± 0.14 <sup>ef</sup>	15.33 ± 0.02 <sup>cd</sup>	53.01 ± 0.05 <sup>ab</sup>
T5 (70 °C, 88.75 rpm)	4.68 ± 0.06 <sup>bc</sup>	2.43 ± 0.22 <sup>bc</sup>	2.37 ± 0.02 <sup>cde</sup>	4.36 ± 0.06 <sup>b</sup>	19.64 ± 0.03 <sup>c</sup>	10.13 ± 0.01 <sup>b</sup>	13.26 ± 0.01 <sup>bc</sup>	16.68 ± 0.02 <sup>b</sup>	52.64 ± 0.03 <sup>ab</sup>
T6 (70 °C, 150 rpm)	4.39 ± 0.02 <sup>cd</sup>	2.55 ± 0.32 <sup>abc</sup>	2.39 ± 0.01 <sup>cde</sup>	3.59 ± 0.14 <sup>e</sup>	16.22 ± 0.10 <sup>e</sup>	9.19 ± 0.09 <sup>cd</sup>	12.38 ± 0.15 <sup>ef</sup>	15.41 ± 0.15 <sup>c</sup>	53.41 ± 0.05 <sup>ab</sup>
T7 (70 °C, 211.25 rpm)	4.60 ± 0.01 <sup>bc</sup>	3.55 ± 0.67 <sup>a</sup>	2.74 ± 0.00 <sup>b</sup>	4.14 ± 0.06 <sup>bc</sup>	19.55 ± 0.00 <sup>c</sup>	10.02 ± 0.01 <sup>b</sup>	13.06 ± 0.06 <sup>cd</sup>	16.46 ± 0.00 <sup>b</sup>	52.52 ± 0.03 <sup>ab</sup>
T8 (70 °C, 150 rpm)	4.42 ± 0.09 <sup>cd</sup>	2.50 ± 0.24 <sup>bc</sup>	2.27 ± 0.18 <sup>e</sup>	3.58 ± 0.15 <sup>e</sup>	16.38 ± 0.12 <sup>e</sup>	9.19 ± 0.04 <sup>cd</sup>	12.46 ± 0.21 <sup>def</sup>	15.48 ± 0.05 <sup>c</sup>	53.59 ± 0.03 <sup>a</sup>
T9 (100 °C, 100 rpm)	4.15 ± 0.14 <sup>d</sup>	2.56 ± 0.41 <sup>abc</sup>	2.33 ± 0.01 <sup>cde</sup>	3.53 ± 0.01 <sup>cd</sup>	16.66 ± 0.14 <sup>e</sup>	8.66 ± 0.15 <sup>e</sup>	11.90 ± 0.01 <sup>f</sup>	15.33 ± 0.02 <sup>e</sup>	54.17 ± 0.05 <sup>a</sup>
T10 (100 °C, 200 rpm)	4.95 ± 0.01 <sup>b</sup>	2.43 ± 0.22 <sup>bc</sup>	2.31 ± 0.01 <sup>de</sup>	2.87 ± 0.14 <sup>f</sup>	17.78 ± 0.14 <sup>d</sup>	9.27 ± 0.14 <sup>c</sup>	12.69 ± 0.01 <sup>cde</sup>	15.72 ± 0.01 <sup>c</sup>	53.86 ± 0.07 <sup>a</sup>
T11 (106.75 °C, 150 rpm)	4.59 ± 0.02 <sup>bc</sup>	2.42 ± 0.22 <sup>bc</sup>	2.52 ± 0.01 <sup>cd</sup>	4.47 ± 0.10 <sup>a</sup>	23.37 ± 0.01 <sup>a</sup>	10.58 ± 0.02 <sup>a</sup>	14.63 ± 0.03 <sup>a</sup>	18.06 ± 0.01 <sup>a</sup>	53.48 ± 0.02 <sup>ab</sup>

Average ± standard deviation (SD) (n = 3); different letters by column represent significant differences by Tukey's test (p < 0.05).

### 3.2. Chemical Characterization

Table S1 (Total phenolic contents (TPC), condensed tannins, and DPPH assay (IC50) after 50 % ethanol extraction) and Table S2 (Coefficients of extrusion conditions variables (B temperature and A screw speed) of the predictive quadratic model for Total Phenolic Content (TPC), Radical Scavenging Activity (DPPH) and Condensed Tannin Contents (CTC)) are in the supplementary material.

Estimated regression coefficients for TPC, DPPH assay (IC50), and CTC were expressed as coded units in second order polynomial equations (Equations (6)–(8)) as follows (A: screw speed (rpm); B: temperature (°C)):

$$TPC \left( mg \frac{GAE}{gDW} \right) = -185.2 + 1.780A + 3.187B - 0.005900A * A - 0.02210B * B \quad (6)$$

$$CTC \left( mg \frac{GAE}{gDW} \right) = 208.0 - 0.425B + 3.238A + 0.00236B * B - 0.00540A * B \quad (7)$$

$$DPPH(IC50) \left( \mu g \frac{DW}{ml} \right) = 2547 - 15.74B - 25.25A + 0.0491B * B + 0.1651A * A. \quad (8)$$

### 3.2.1. Total Phenolic Content

The coefficient of determination ( $R^2$ ) of the model was 0.8854, which indicated that 11.46% of the total variability was not explained by the model. A second order polynomial equation (Equation (6)) was obtained by depicting the correlation between the temperature and screw speed with TPC. A negative quadratic effect of the screw speed and the temperature exists. Figure 1a shows that the temperature, screw speed, and the interaction between both of them show no significant variables in the process. The smaller the p-value was, the more significant the corresponding coefficient was [18].

Previous studies showed that the extrusion-cooking process increased the phenolic content and the antioxidant activity. Brennan et al. [14] assumed that increased levels of certain phenolic components in extruded products are frequently due to their release from the cell wall matrix. However, in a study by Mahungu et al. [41], it was found that the barrel temperature in the extrusion process plays a significant role in the stability of phenolic compounds, such as isoflavones in soy products. The increase in phenolic content with barrel temperature has been ascribed to the formation of Maillard reaction products [42]. The effect of extrusion on bioactive compounds depends on the physical and chemical characteristics of the product and the extrusion conditions [43,44].

### 3.2.2. Condensed Tannin Contents

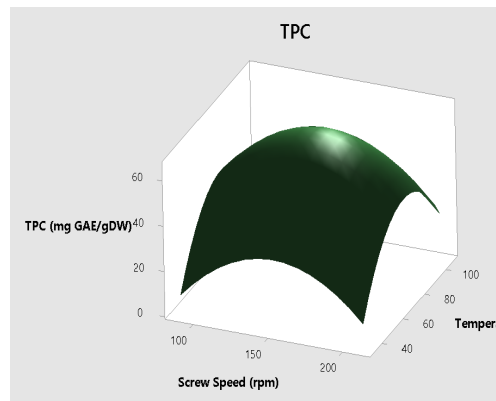
Pecan nut shell contains high concentrations of condensed tannins that make pecans an interesting source of phytochemicals [13]. Similar results reported by Adarkwah-Yiadom and Duodu [45] showed that the extrusion cooking of whole grain sorghum (type II and type III) significantly reduced condensed tannins (Figure 1b). The authors concluded that this might be due to tannin interaction with protein and other macromolecules, which reduces their extractability [45]. Unlike the TPC, the CTC has a negative correlation with the temperature and the interaction between speed screw and temperature (Equation (7)). A positive correlation was presented by the quadratic of the temperature.

The temperature (p-value 0.024) and the interactions between temperature–screw speed (p-value 0.021) were statistically significant, but the screw speed was not significant (p-value 0.064).

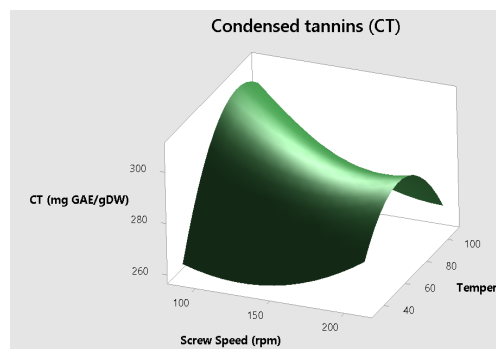
### 3.2.3. Radical Scavenging Activity

There are some variables to consider in order to improve the recovery of polyphenols and antioxidants [25]. The radical scavenging activity (RSA) of extruded products depends not only on the phenolic compounds but also on the interactions between these bioactive compounds and the food matrix [46]. The antioxidant capacity of extrudates and non-extruded control samples was determined using the DPPH assay, a sensitive electron-transfer reaction [47]. Figure 1c shows that the highest RSA (lowest  $IC_{50}$ ) was obtained for samples processed at 70 °C and 150 rpm. The second order polynomial equation (Equation (8)) was obtained by depicting the correlation between the temperature and screw speed with the DPPH assay ( $IC_{50}$ ). The modeling was fitted as a linear regression ( $R^2$  87.06%).

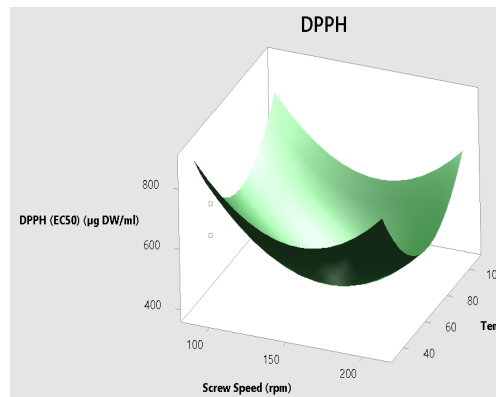
The extrusion process increased antioxidant activity in most of the treatments. The same tendency was previously observed by Ramos-Enríquez et al. [48], who, by means of RSM showed that high temperatures increased the RSA of wheat bran. However, it was shown that the antioxidant capacity of the lentil-orange (raw sample) was reduced from 95.6 to 60.6% by extrusion [49].



(a)



(b)



(c)

**Figure 1.** Responses surface for Total phenolic content (TPC) (a), condensed tannins (b), and DPPH (1,1-Diphenyl-2-picrylhydrazyl) (c) in pecan nut shell extruded at different temperatures (33.25 °C, 40 °C, 70 °C, 100 °C, or 106.5 °C) and velocities (88.75, 100, 150, 200, and 211.25 rpm).

### 3.3. Polyphenolic Compounds, Dietary Fiber, and Protein Composition

The identification and quantification of polyphenolic compounds and total dietary fiber and protein contents were determined for samples obtained with the treatment that produced the highest amount of total polyphenols and the lowest IC<sub>50</sub> (the highest radical scavenging activity (DPPH)): 70 °C and 150 rpm.

#### 3.3.1. Identification and Quantification of Polyphenolic Compounds by LC-MSD-TOF

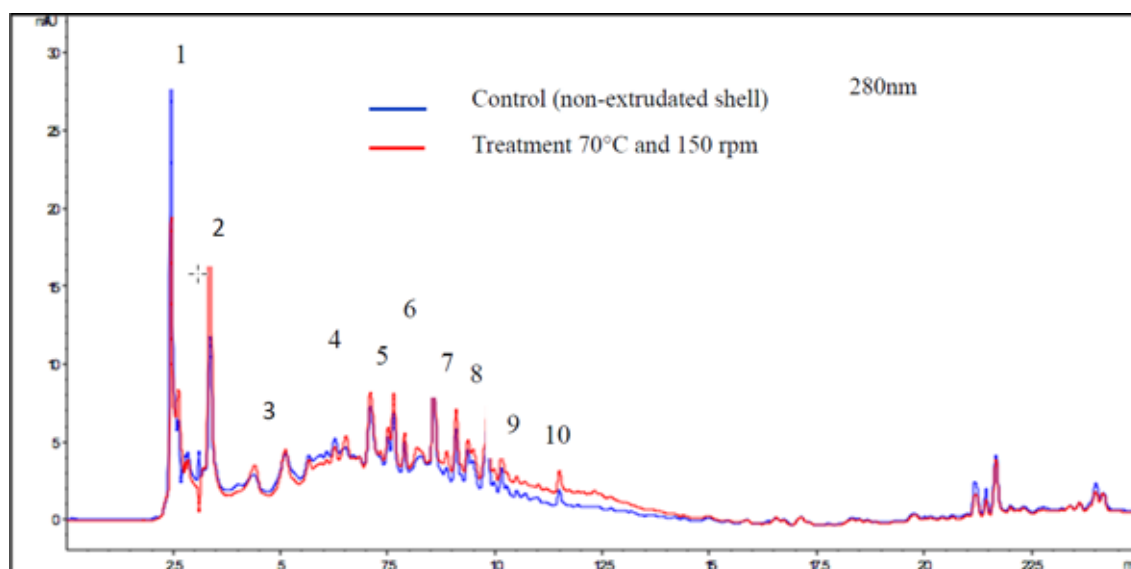
Table 3 shows the parameters of identification and quantification of each phenolic compound obtained for the control (non-extruded shell) and the extruded shell after treatment at 70 °C and 150 rpm. The samples contained several phenolic acids including gallic, ellagic, p-hydroxybenzoic, protocatechuic, and also pentose, methyl ellagic pentoside, epigallocatechin gallate, dimethyl ellagic rhamnoside, and dimethyl ellagic acid. Figure 2 shows an HPLC chromatogram measured at 280 nm. For both the control and treatment (70 °C and 150 rpm) samples, the major phenolic acid was ellagic acid (1.56 and 1.74 µg/g DS, respectively). However, in the control extract (non-extruded shell), dimethyl ellagic acid rhamnoside was not identified. After the treatment, the samples presented significantly higher concentrations of phenolic compounds than the control (non-extruded shell): gallic acid, ellagic acid pentose, ellagic acid, dimethyl ellagic acid rhamnoside, and dimethyl ellagic acid.

A study by Hilbig et al., which evaluated the effects of different conditions involving ultrasound extraction of the total phenolic content from pecan nut shells, found that catechin was the most concentrated compound in shell extracts, followed by gallic acid [50]. The concentrations reported by the authors [51] were higher than those observed in the present study (all the treatments). This could be attributed to the extraction process and the origin of the nuts. Moreover, Gulati et al. found that *Panicum miliaceum L.* flour roasted for 10 min at 110 °C increased the content of secondary compounds and the antioxidant properties. HPLC identified a higher concentration of ferulic acid, among other acids [52].

**Table 3.** Phenolic profile ( $\mu\text{g/g}$  dry shell) of pecan nut shell extrudate ethanolic extracts, with HPLC-DAD and LC-MSD-TOF, detected at 280 nm.

Peak	UV/MAX	Phenolic Compounds	[M - H] <sup>-</sup> m/z		Molecular Weight Da	t <sub>R</sub> (min)	Content in Control (Non-Extruded Shell) ( $\mu\text{g/g}$ DS)	Content in 70 °C and 150 rpm ( $\mu\text{g/g}$ DS)
			Experimental Mass (m/z)	Theoretical Mass (m/z)				
1	215, 270	p-hydroxybenzoic acid	137.01	137.02	138.12	2.49	1.46 ± 0.17 <sup>a</sup>	0.88 ± 0.10 <sup>b</sup>
2	213, 271	Gallic acid	169.02	169.08	170.12	3.35	0.90 ± 0.08 <sup>a</sup>	1.55 ± 0.09 <sup>b</sup>
3	231, 259	Protocatechuic acid	153.02	153.02	154.12	5.11	NQ	NQ
4	252, 360	Ellagic acid pentose	433.05	433.05	434.31	7.87	0.32 ± 0.06 <sup>a</sup>	0.41 ± 0.09 <sup>b</sup>
5	255, 368	Ellagic acid	301.01	301.00	302.19	8.56	1.56 ± 0.23 <sup>a</sup>	1.74 ± 0.17 <sup>b</sup>
6	254, 286	Methyl ellagic acid pentoside	477.08	477.08	448.33	9.06	NQ	NQ
7	223, 289	Epigallocatechin gallate	457.18	457.18	458.37	9.74	NQ	NQ
8	221, 251, 365	Di-methyl ellagic acid rhamnoside	475.10	475.10	476.39	10.13	NQ	0.32 ± 0.02 <sup>a</sup>
9		Di methyl ellagic acid	329.04	329.04	330.25	10.68	0.32 ± 0.05 <sup>a</sup>	0.33 ± 0.04 <sup>a</sup>

Average ± SD; different letters by column represents significant differences by Tukey's test ( $p < 0.05$ ). NQ—the samples were detected but not quantified because the levels were under the quantification limit.



**Figure 2.** HPLC-DAD (high pressure liquid chromatography-diode-array detector) chromatograms of compounds in control (non-extruded shell) and milled shells extruded at 70 °C and 150 rpm with detection at 280 nm. Blue: control. Red: treatment 70 °C and 150 rpm. Retention time (t<sub>R</sub>, min).

### 3.3.2. Protein and Fiber Content

High temperature and pressure may alter protein structure, leading to protein denaturation and the formation of cross-linking reactions [53]. Therefore, it is likely that in the present study, pressure and temperature affected protein structure during the extrusion process, decreasing protein extractability. In fact, the protein content in the extruded sample was lower than in the control (non-extruded shell). Although, the *in vitro* digestibility of protein (IVDP%) increased significantly with the extrusion process (Table 4).

Dietary fiber is usually divided into SDF and IDF. SDF is composed of pectin and gums, and IDF by cellulose and lignin [54]. SDF has an important role in the food industry thanks to its beneficial physiological functions in the organism [54]. Table 4 shows the content of the total soluble and insoluble dietary fiber in non-extruded and extruded shell at 70 °C and 150 rpm. TDF in the extruded sample was significantly higher than in the control with levels, 75.41 and 79.10 % , respectively. Likewise, under the same extrusion conditions (70 °C and 150 rpm), SDF was significantly higher than in the control. A recent study reported by Ge et al. [55] showed that the extrusion process applied to bamboo shoot flours caused a similar tendency, and the results led them to conclude that the process of extrusion may break glycosidic linkages in TDF, causing the conversion of IDF into SDF. Another study with banana peel reported that temperature and screw speed conditions in the extrusion process can enhance transformation of IDF to SDF [56]. This could result from the release of oligosaccharides and polysaccharides (cellulose, hemicellulose, and lignin). Similar results are presented in a work about the extrusion of soybean residues, they found that with extrusion temperature of 115 °C; feed moisture, 31%; and screw speed, 180 rpm, the SDF content of soybean residue increased 10.60% compared with the un-extruded soybean residue [18].

In this regard, Hu et al. reported that samples with higher soluble fiber have greater water and oil absorption [16]. We observed that extrusion produced, in general, higher concentrations of SDF, which were associated with an increased solubility in water [57]. Table 2 shows that the treatments with 150 rpm and 70 °C (4, 6 and 8) present similar results with respect to WAI, OAI, and WSI. Furthermore, there exists a significant increment of the WAI and WSI values compared to the control.

**Table 4.** Protein and dietary fiber content in the control (non-extruded pecan nut shell) and optimized extrudate of pecan nut shell.

Treatment	Protein %	IVDP %	TDF %	SDF %	IDF %
Control (non-extruded)	2.56 ± 0.25 <sup>a</sup>	85.07 ± 0.11 <sup>b</sup>	75.41 <sup>b</sup>	0 ± 0 <sup>b</sup>	75.41 ± 1.70 <sup>a</sup>
Optimized extruded (70 °C and 150 rpm)	2.41 ± 0.21 <sup>b</sup>	87.70 ± 0.20 <sup>a</sup>	79.1 <sup>a</sup>	3.07 ± 0.21 <sup>a</sup>	76.03 ± 1.59 <sup>b</sup>

Average ± standard deviation; different letters by rows represent significant differences by Tukey's test ( $p < 0.05$ ).

#### 4. Conclusions

This research uses a response surface method to determine the effect of extrusion techno-functional properties and antioxidant activity. The extrusion process affected significantly the techno-functional properties of pecan nut shell, such as the moisture and color. When extrusion was performed at 70 °C and 150 rpm, the extruded nut shell contained more total polyphenols and soluble dietary fiber and presented higher radical scavenging activity in comparison to the non-extruded nut shell control. In summary, these results demonstrate that twin-screw extrusion could be used as a tool to generate novel food ingredients with modified functionality, using industry wastes as raw materials. Moreover, the nut shell could be used in food formulations as both a fiber ingredient and additive, due to its antioxidant capacity.

**Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Table S1: Total phenolic contents (TPC), condensed tannins, and DPPH assay (IC50) after 50 % ethanol extraction; Table S2: Coefficients of extrusion conditions variables (B temperature and A screw speed) of the predictive quadratic model for Total Phenolic Content (TPC), Radical Scavenging Activity (DPPH) and Condensed Tannin Contents (CTC).

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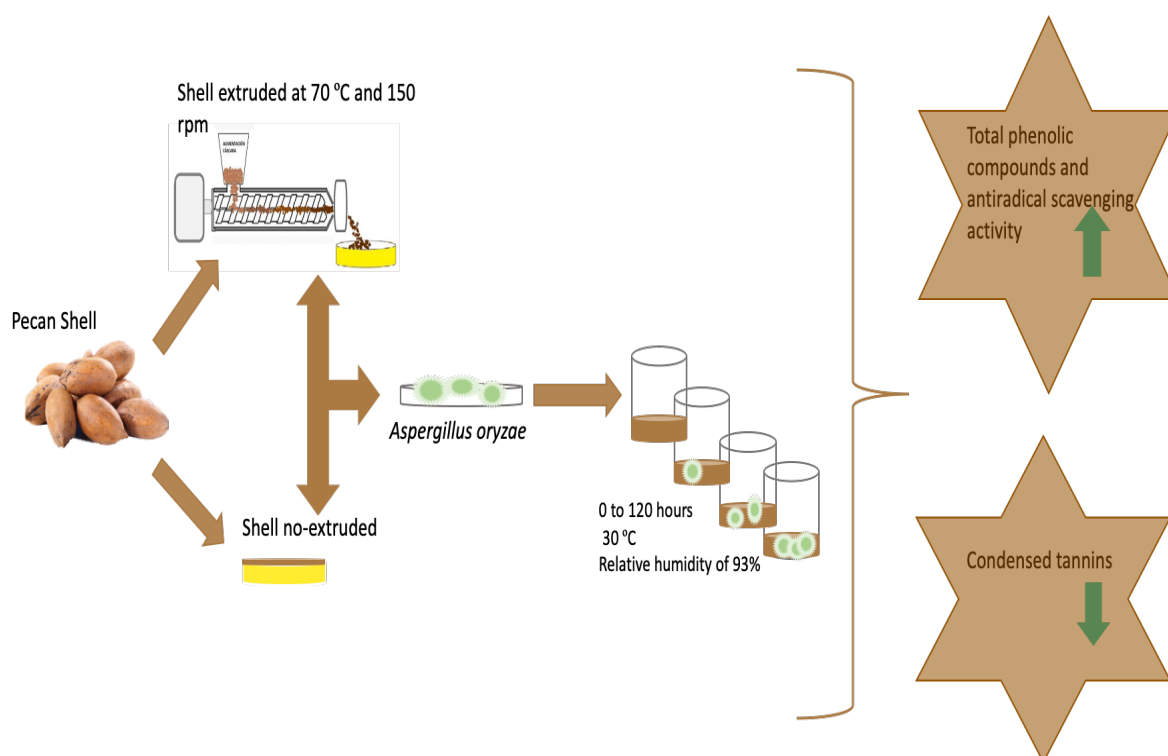


## 6.2. Impact of extrusion and solid-state fermentation with *Aspergillus oryzae* on the phenolic compounds and radical scavenging activity of pecan walnut (*Carya illinoensis*) shell

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# Impact of extrusion and solid-state fermentation with *Aspergillus oryzae* on the phenolic compounds and radical scavenging activity of pecan walnut (*Carya illinoensis*) shell

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## Abstract

Purpose- Solid state fermentation (SSF) has been highlighted as an alternative to obtain valuable compounds using agro-industrial wastes as substrate. The present study evaluated the impact of extrusion combined with SSF on the production of phenolic compounds and their antioxidant activity using pecan walnut shell (PWS) as substrate. Design/methodology/approach- PWS and extruded pecan walnut shell (PWSE) were fermented for 120 hours at 30°C using *Aspergillus oryzae*. Samples were withdrawn from incubator at 6 h, 12 h and then every 12 h until 120 h fermentation. PWS and PWSE samples were extracted. The total phenolic content and radical scavenging activity extracts were characterized from the resulting extracts. Findings- The use of PWSE yielded higher *A. oryzae* biomass and at a higher rate after 120 h of fermentation (PWS 75.74% vs PWSE 87.50%). In general, the polyphenol content (TPC) and the radical scavenging activity (RSA) increased with fermentation time. However, the PWSE yielded significantly higher ( $p < 0.05$ ) TPC and RSA values after SSF in comparison to the non-extruded PWS. Condensed tannins showed different trends depending on the fermented substrate. Overall, results showed that the extrusion pretreatment joint with the SSF represents a good alternative to raise the phenolic content and antiradical activity of lignocellulosic materials such as pecan walnut shells. Practical/social implications- This study offers valuable information that may be used by the pecan walnut industry to valorize the shell co-product as a substrate to produce functional ingredients or fungal enzymes.

## Introduction

Pecan walnut shell (PWS) from *Carya illinoensis* is an agro-industrial waste which main component is fiber (70-80%) including lignin, cellulose, and hemicellulose fractions (Liu *et al.*, 2016). PWS also contain a significant amount of phytochemicals comprising phenolic acids such as gallic and chlorogenic acids, epigallocatechin and epicatechin gallate and a high content of tannins (Kureck *et al.*, 2018; Villasante *et al.*, 2019). These compounds have antioxidant properties which are associated with the prevention of anti-cardiovascular disease and anti-cancer effects (Hugo *et al.*, 2012; Olchowik-Grabarek *et al.*, 2017). Moreover, these compounds may also be used to improve the quality of foods based on their ability to reduce the oxidation of lipids (Bhat *et al.*, 2019).

Due to the great interest to revalorize agro-industry wastes to achieve more sustainable processes, traditional and emergent technologies have been evaluated to improve their nutritional profile and to increase their content and recovery of valuable compounds (Longo, M.A; Deive F. and Domínguez A., 2007). One of the processes that is gaining attention to achieve this goal is fermentation, which may be performed under submerged conditions or in solid state. Solid-state fermentation (SSF) has more technological advantages compared with the submerged fermentation (SF) including higher fermentation efficiency and lower operational costs (Reyes I, Cruz-Sosa F, Hernandez-Jaimes C, 2017). Fungi are ideal microorganisms to perform SSFs since the culture conditions (temperature and humidity) in this type of fermentation are similar to those that these microorganisms require in their natural environment (Handa *et al.*, 2019). One aspect to take care is that if the fermented product will be used for human consumption, GRAS (Generally Recognized as Safe) microorganisms must be used. In this sense, an adequate GRAS fungal strain that has the capacity to produce high quantities of enzymes and beneficial secondary metabolites is *Aspergillus oryzae* (Kawauchi and Iwashita, 2014).

Studies that used agro-industrial wastes as substrates for fungal SSF were initially focused in the production of enzymes of industrial importance (Medina-Morales *et al.*, 2011; Rodrigues *et al.*, 2008). However, recently these materials have gained attention for the production and recovery of phytochemicals such as phenolic compounds (Shin *et al.*, 2019), leading to materials with improved antioxidant activity (Lee *et al.*, 2019; Shin *et al.*, 2018). The increment of phenolic compounds after fermentation processes has been explained by the cell wall degrading enzymes produced by the microorganisms which cause the extraction/release of these compounds from the matrix used as substrate (Bei *et al.*, 2018; Handa *et al.*, 2019) and also, by the synthesis of new compounds as a result of the secondary metabolism (Bhanja Dey *et al.*, 2016).

The use of combined treatments to modify the properties of lignocellulosic materials that will be submitted to fermentation processes has also been recommended, being extrusion a good alternative due to its versatility and low operational cost (Marone *et al.*, 2018; Medina-Morales *et al.*, 2011). Extrusion is a process that utilizes a combination of temperature, feed moisture, screw speed and screw configuration, endorsing mechanical stress that results in physical and chemical changes in the extruded material. These changes include protein denaturation, starch gelatinization, increment of the bioavailability of bioactive compounds, changes in texture, among others (Janić Hajnal *et al.*, 2016). Extrusion has been studied as a pretreatment of fiber-rich materials to improve the fermentation yields during bioethanol production (Muthukumarappan and Swamy, 2020) and also to improve the conversion of these type of materials into carboxylates (Marone *et al.*, 2018). A recent study that evaluated the effect of extrusion on PWS demonstrated that this treatment increased its soluble fiber content and the antioxidant capacity (Villasante *et al.*, 2019). However, the use of extrusion as a pretreatment of agro-industrial waste materials to achieve higher yields of phenolic compounds during SSF, has not yet been evaluated. Therefore, the aim of the present study was to investigate the effect of extrusion and solid-state fermentation with *A. oryzae* on the total phenolic compounds and radical scavenging activity of pecan walnut shell.

## Materials and Methods

## Plant material and chemicals

Pecan walnut shells (*Carya illinoensis*) were collected at 1,090 above sea level (coordinates 25°45'32"N, 102°58'58"W) in San Pedro, Coahuila located on the north of Mexico. The shells were ground in a Wiley mill (Arthur Thomas, Philadelphia, PA) equipped with a 2 mm screen. *Aspergillus oryzae* 22788 was acquired from the American Type Culture Collection (ATCC).

Potassium hydroxide, gallic acid (GA), Folin-Ciocalteu reagent, phosphate buffered saline tablets (PBS), fluorescein sodium salt, Trolox<sup>®</sup> (6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonsaeure), potassium peroxodisulfate, DPPH (1,1-Diphenyl-2-picrylhydrazyl (2,2-Diphenyl-1-picrylhydrazyl), catechin, vanillin, ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt), trifluoroacetic acid, and the analytical standards: gallic and ellagic acids were acquired from Sigma-Aldrich Quimica S.A. (Madrid, Spain). Pure and distilled water were obtained after filtration through a Millipore-Q system (Barcelona, Spain). Acetonitrile were from VWR International, Radnor, PA, USA.

## Extrusion of pecan walnut shell

The PWS powder was processed according to the method described by (Villasante *et al.*, 2019) in a co-rotating twin-screw extruder (BTSM-30 Bühler, Uzwil, Switzerland). The extrusion system was composed of two screws with 800 mm length, 30 mm diameter and L/D=20, and was operated at 150 rpm and a temperature of 70°C in the last barrel. The extruded PWS (PWSE) was dried at 60°C in an air convection oven (Edel Ingenieros, Monterrey, Mexico) and milled using a Knife Mill Wiley (Arthur Thomas Co., Philadelphia, PA, USA). The ground PWSE was sieved through a 177 µm mesh (US No. 80) during 5 min in a Rotap (Duratap Model DT 168, Advantech Mfg., New Berlin, WI, USA). Sample was stored at room temperature until analyzed.

## Preparation of fungal inoculum

Fungal *Aspergillus oryzae* (ATCC 22788) was inoculated in potato dextrose agar (PDA) and incubated at 30°C for 7 days. Spores were collected by suspension in 10 mL of distilled water containing 0.1% Tween 80 and counted using a hemocytometer. Spores were diluted to reach a concentration of  $1 \times 10^6$  spores/mL and this suspension was used for further inoculation of PWS and PWSE.

## Solid state fermentation of PWS and PWSE

For SSF, 10 g of the sample (PWS or PWSE) were moistened until 50% moisture, selected to reach water activity values around 0.98-0.99 for optimum fungal growth. Samples were autoclaved in a 250 mL glass flask at 121 °C for 15 min. After cooling under sterile conditions, 1.0 mL of the spore inoculum was added to reach a spore concentration of  $1 \times 10^5$  spores/g of PWS and PWSE. SSF was then conducted at 30°C with a relative humidity > 92% for 120 hours. Samples were withdrawn from incubator at 6 h, 12 h and in intervals of 12 h until 120 h fermentation, and were immediately frozen for further lyophilization. The experiments were carried out in duplicate.



### Chitin quantification

Chitin was assayed in the lyophilized fermented samples to estimate the growth of fungus during SSF, following the method reported by (González *et al.*, 2019). Briefly, the lyophilized materials were treated with 1 M HCl at 85–90 °C for 50 min under constant stirring. The ratio of raw sample to acid solution during the extraction was 1/20 (w/v). Then, the suspension was centrifuged at 3000g for 5 min and washed with distilled water to remove the excess of HCl. Sediment from this step was suspended at a ratio of 1/20 (w/v) in alkali (1 M NaOH) and kept at 85–90 °C for 35 min under constant stirring to remove proteins completely. The mixture was vacuum filtrated in a Buchner funnel with filter paper (pore size 20–25 µm), washed several times with deionized water to remove the excess of NaOH, and then dried in an oven at 100 °C overnight. The residue obtained was designated as purified chitin in the form of a very light brown powder. The chitin content was translated to fungal dry weight using the conversion factors presented by (Hu *et al.*, 2004; Ride and Drysdale, 1972), and expressed as percentage (%).

### Extraction of phenolic compounds

For the determination of total phenolic content, phenolic acid profile and radical scavenging activity, the PWS or PWSE samples were extracted in 50% aqueous ethanol (1:50 w/v) with stirring at room temperature for 90 min. Then, all samples were centrifuged (Sigma 6K10, Osterode am Harz, Germany) for 15 min at 2500 g, and the supernatants were recuperated and used for analysis.

### Determination of total phenolic content (TPC)

The total phenolic content (TPC) of the fermented PWS and PWSE extracts was determined by the Folin-Ciocalteu method described by (Mosca *et al.*, 2018; Singleton *et al.*, 1998). Measurements were done in triplicate for each sample. Results were expressed as mg Gallic Acid Equivalents (GAE)/g of dry walnut shell (extruded and non-extruded).

### Determination of condensed tannins content

For the extraction of condensed tannins, 1 mL of the extract (Extraction of phenolic compounds) was mixed with 1 mL of 4% v/v vanillin solution in methanol and 4 mL 8% v/v HCl in methanol. The condensed tannins method was carried out as described by (Flores-Córdova *et al.*, 2017; Price *et al.*, 1978). The results were expressed as mg equivalents of catechin/g dry walnut shell (extruded and non-extruded).

### Radical scavenging activity

For the radical scavenging activity, the methods DPPH (Gallego *et al.*, 2013; Villasante *et al.*, 2018) and ABTS (Almajano *et al.*, 2008; Skowrya *et al.*, 2014) were used. Results were expressed as µmol equivalents of Trolox (ET)/g dry walnut shell (extruded and non-extruded).

### Phenolic acid profile

For the determination and quantification of the phenolic acid profile, the extraction was carried out as described in “extraction of phenolic compounds”. After centrifugation, the ethanol was evaporated with nitrogen, the extract was lyophilized and diluted in 80% methanol. The phenolic acid compounds were quantified by HPLC-DAD (1200 Series, Agilent Technologies, Santa Clara, CA,

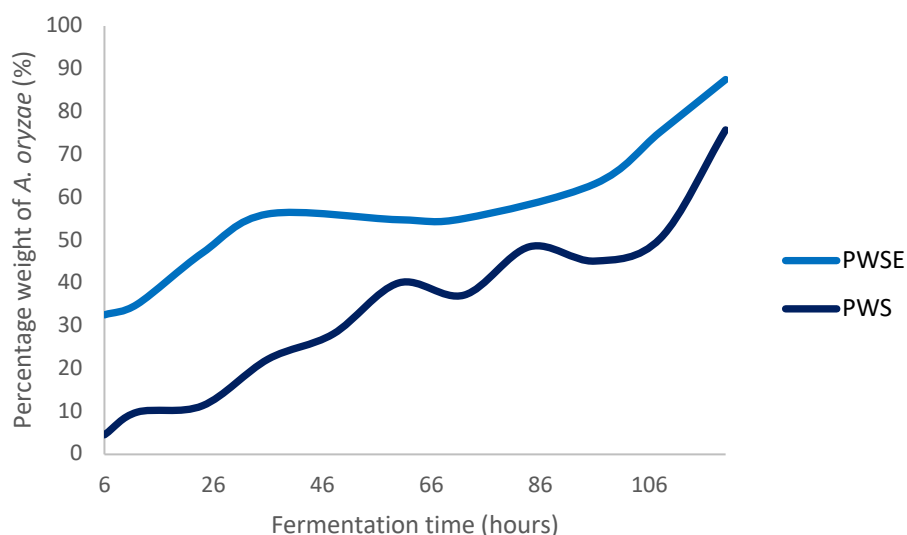
USA). 10  $\mu\text{L}$  aliquots were analyzed with a C-18 column (Zorbax SB-Aq, Santa Clara, CA, USA) 4.6 mm ID x 150 mm (3.5  $\mu\text{m}$ ), under a gradient condition with 0.5% water/formic acid (A) and 0.5% acetonitrile/formic acid (B). The solvent gradient was programmed as follows: 0–15 min, 95% A–5% B; 15–18 min, 65% A–35% B; 18–21 min, 5% A–90% B; 21–27 min, 95% A–5% B. The solvent flow rate was 1.0 mL/min. Gallic acid was measured at 270.8 nm and ellagic acid at 253.1 nm.

### Statistical Analysis

SSF experiments were performed in duplicate and the analyses performed to characterize the fermented PWS and PWSE samples were assayed in triplicate. Experimental data was analyzed using the Minitab-18 software. Significant differences among treatments were determined by analysis of variance (ANOVA) and mean comparisons were evaluated by Tukey's test at 95% significance level.

## Results

### Determination of *A. oryzae* growth



**Figure 1.** Percentage weight of *A. oryzae* in the fermented pecan walnut shell (PWS) and extruded pecan walnut shell (PWSE) samples.

The fungal biomass is difficult to quantify in SSFs since fungal cells may not be easily separated from the fermented substrate (Shin et al., 2019). Previous reports have related the fungal growth with the chitin content in solid state fermented samples since it is a component of the fungal cell walls (Aidoo et al., 1981). Therefore, in the present study the *A. oryzae* growth was estimated through chitin quantification in fermented sample. The fungal dry weight was represented in Figure 1. In general, the growth profile was similar to that reported by (Shin et al., 2019) for *A. oryzae* and *A. awamori* using black rice bran as substrate. (Medina et al., 2010) also observed similar results in their study with pecan walnut shell and *Aspergillus niger*. However, differences were observed in the amount of *A. oryzae* recovered from fermented PWS or PWSE at different times, representing different fungal growth rate depending on the substrate (Figure 1). For PWS, the fungus percentage was 4.57% after

6 h of fermentation, and 75.74% at the end of fermentation (120 h), while for PWSE, it was 32.52% at 6 h and 87.50% after 120 h. Moreover, PWSE led to a significantly higher growth of *A. oryzae* in the first 48 h when compared to PWS (Figure 1). These results showed the capacity of using pecan walnut shell especially after an extrusion treatment, as a substrate to grow *Aspergillus oryzae* under SSF conditions. The extrusion treatment before the fermentation of the PWS could be a good strategy to reduce time and costs. This could be valuable to improve the fungal growth for the generation of high-value metabolites. For instance, the use of SSF with *A. oryzae*, has been used to generate different enzymes including catalases, glucanase, proteases, cellulases. (Melnichuk *et al.*, 2020) showed the bioconversion of two agro-industrial wastes (soybean husk and flour mill waste) by SSFs employing the fungus *A. oryzae* to produce high quantities of alpha-amylase. (Raza *et al.*, 2011) used SSF for the production of  $\beta$ -glucosidase enzyme by co-culture of *A. niger* and *A. oryzae*. Additionally, these authors worked with different agricultural by-products and concluded that the nutrients contained in each substrate had relation with the fungal growth and enzyme production.

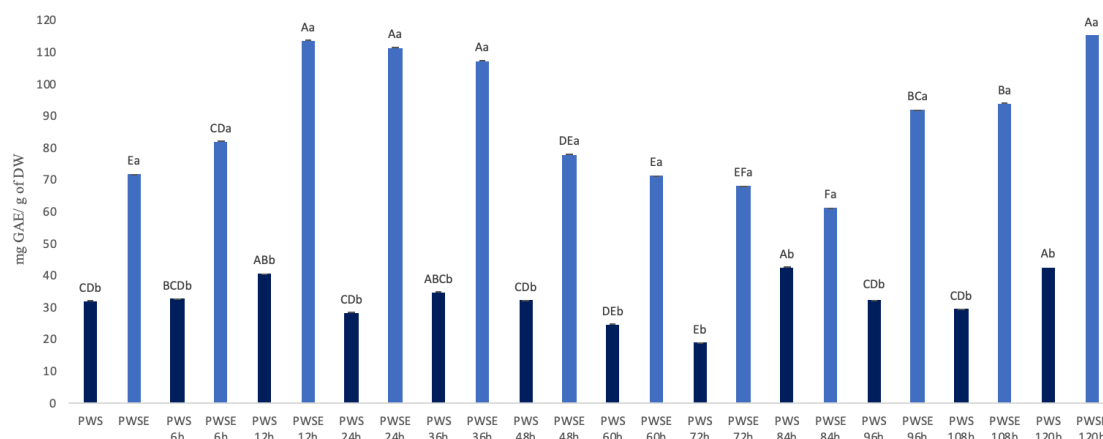
#### Total phenolic content

Fermentation processes have been gaining attention to produce phenolic compounds (Lee *et al.*, 2019). The development of TPC in the PWS and PWSE samples during 120 h of SSF was represented in Figure 2. PWS used as raw material contained 31.99 mg GAE/g while the extruded counterpart had a higher initial concentration of TPC (71.83 mg GAE/g). These values were expected since (Villasante *et al.*, 2019) reported 41 mg GAE/g in pecan walnut shell and also found and increased content in the TPC as a result of the extrusion process. During SSF, the TPC increased significantly, which was consistent with previous studies (Puértolas *et al.*, 2010; Queiroz Santos *et al.*, 2018). According to (Queiroz Santos *et al.*, 2018), phenolic compounds in non-fermented by-products are combined or bound with sugars, but during fermentation, enzymes from the starter cultures hydrolyses phenolic complexes into soluble-free phenolic compounds. Interestingly, although for both substrates the final TPC concentration was higher compared to the initial value, fermented PWS and PWSE presented the lowest TPC values after fermentation for 72 h and 84 h, respectively (Figure 2). This behavior may be associated to the fungal growth since a similar trend was observed in Figure 1.

The effect of the fermented substrate (PWS or PWSE) in the production of TPC was also evaluated. Along the fermentation, the TPC was significantly higher ( $p < 0.05$ ) in PWSE in comparison to PWS fermented samples. After 120 hours of fermentation TPC expressed like mg of GAE were 2.7 times higher in PWSE, compared to PWS.

The changes in TPC during fermentation were also different when PWS and PWSE were compared. In PWSE, the mg of GAE significantly increased during the first 12 h and remained in high levels up to 36 h of the SSF process. However, GAE decreased in the fermentation times between 48 h and 84 h. (Rodrigues *et al.*, 2008) investigated the use of cashew apple bagasse supplemented with carbon sources (glucose, starch, sucrose, maltose, etc.) in a SSF with *A. oryzae*. They found greatest concentrations of GAE at 24 h of fermentation and after that time, the gallic acid production declined. This decrement could be for the addition of carbon sources as maltose and glycerol that inhibited the tannase synthesis which help in the generation of phenolic compounds (Rodrigues *et al.*, 2008).

However, in our study the mg of GAE increased again significantly ( $p < 0.05$ ) during the last hours of the SSF of PWSE. On the other hand, for PWS the highest TPC were found at 12 h, 36 h, 84 h and 120 h. The insoluble fraction of the TPC is covalently bound to the cell wall structural components such as cellulose, hemicellulose, arabinoxylans, lignin, pectin and rod-shape structural proteins (Alberti *et al.*, 2014). Extrusion may have caused modifications in the three-dimensional structure of the fiber molecules and the phenolic compounds that are bound in the structure (Ramos-enrriquez *et al.*, 2018). Therefore, the differences found among the fermented PWS and PWSE could be related to structural changes that occurred in the fiber fraction during extrusion, which may have led to a different releasing behavior of TPC along the fermentation.



**Figure 2.** Total phenolic content developed during the solid-state fermentation of pecan walnut shell (PWS) and extruded pecan walnut shell (PWSE).

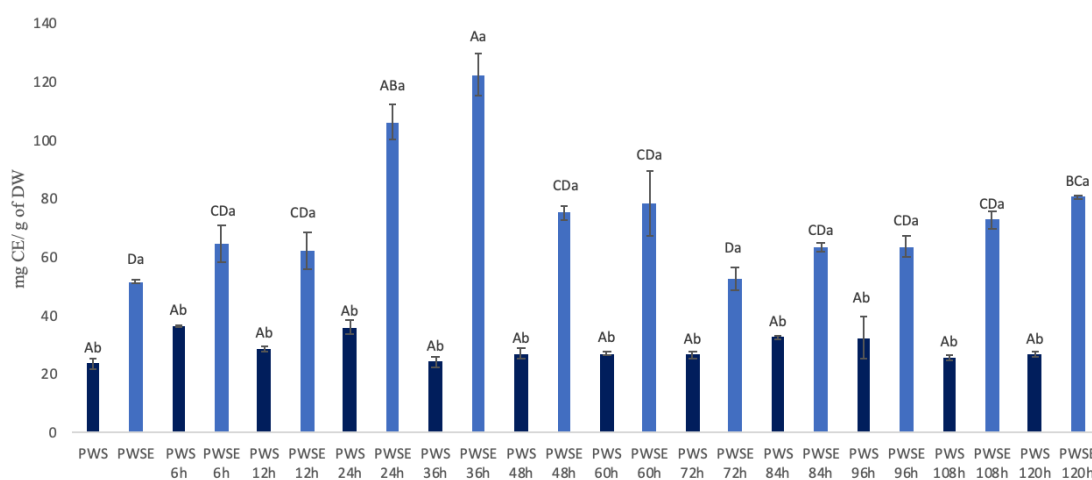
Each bar represents the mean  $\pm$  SD of three replicates. Different capital letters (A,B, etc.) indicate significant differences between bars of the same treatment but different times ( $p < 0.05$ ). Different lowercase letters (a and b) indicate significant differences between treatments (PWS and PWSE) but at the same time ( $p < 0.05$ ). The results are presented in mg acid gallic equivalent (GAE)/gram of pecan walnut shell non-extruded or extruded, in dry weight (DW).

### Condensed tannins

When the content of condensed tannins was evaluated, no significant differences ( $p < 0.05$ ) were found in fermented PWS despite the fermentation time (Figure 3). On the other hand, during the SSF of PWSE, the mg of catechin equivalents reached a maximum value at 36 h, increasing almost 4 times compared to the non-fermented feedstock and afterwards, a decline was observed. In a study by (Lin *et al.*, 2016) on litchi pericarp with *Aspergillus awamori*, authors observed an increment in total tannins due to fermentation. They suggested that the SSF with *A. awamori* releases the non-extractable condensed tannin from cell walls of the plant.

Conversely, (Soetan and Oyewole, 2009) found that the content of different antinutritional factors like tannins are considerably reduced through fermentation processes. Accordingly, (Bhat *et al.*, 1998) found that some *Aspergillus* and *Penicillium* strains are capable of degrading both, hydrolysable and condensed tannins. In this way, (Medina-Morales *et al.*, 2011) fermented pecan shell with *Aspergillus niger* and found that this fungus was capable to degrade condensed tannins, which are one of the mayor bioactive components of the pecan walnut shell. Likewise, these authors

concluded that in the presence of tannin acid, this fungus can produce microbial enzymes such as tannin acyl hydrolase (tannase), monooxygenase, gallic acid decarboxylase, and other phenoloxidases (Chavez-Gonzalez et al. 2018). According to (Chávez-González *et al.*, 2012), these enzymes as tannase (occurs in the presence of tannic acid) could generate molecules as glucose, gallic and ellagic acid during SSF. The production of these enzymes during the SSF performed in our study, could explain the diminishing of condensed tannins observed during PWSE fermentation. Furthermore, Fang et al. (2019), observed that the efficiency of biotransformation of green tea catequins by *Aspergillus niger* was affected by the carbon or nitrogen sources. The effect of extrusion on the PWS carbohydrates and nitrogen fractions could have affected the bioavailability of nutrients for *Aspergillus oryzae*, explaining the different behavior in the biotransformation of the condensed tannins from PWS and PWSE samples.



**Figure 3.** Condensed tannins developed during the solid-state fermentation of pecan walnut shell (PWS) and extruded pecan walnut shell (PWSE).

Each bar represents the mean  $\pm$  SD of three replicates. Different capital letters (A,B, etc.) indicate significant differences between bars of the same treatment but at different time ( $p < 0.05$ ). Different lowercase letters (a and b) indicate significant differences between treatments (PWS and PWSE) but at the same time ( $p < 0.05$ ).

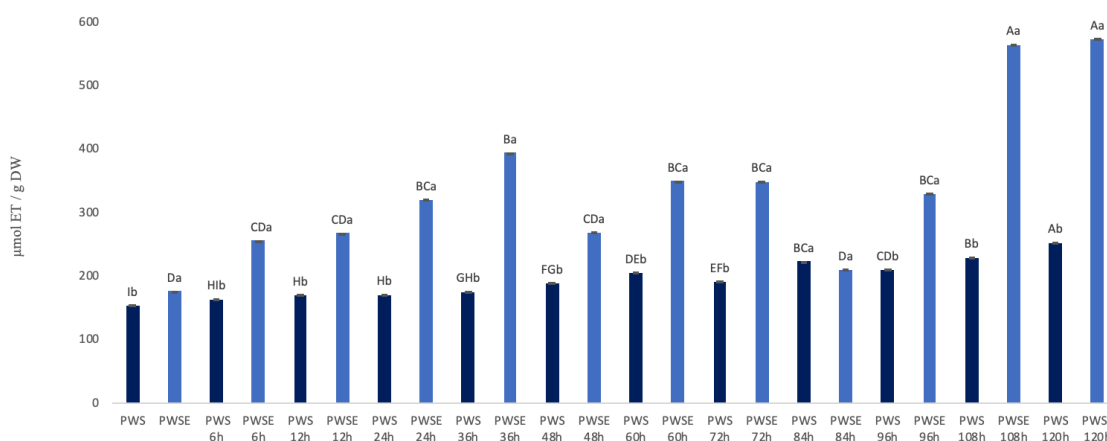
The results are presented in mg of catechin equivalents (CE)/gram of pecan walnut shell non-extruded or extruded, in dry weight (DW).

#### Radical scavenging activity

Figure 4 shows the effect of SSF with *A. oryzae* on the DPPH radical scavenging activity of PWS and PWSE fermented at different times. For PSW, no significant increments in the amount of  $\mu\text{mol}$  equivalents of Trolox were observed among 6 h and 36 h of fermentation. However, between 48 h and 120 h of SSF, the antiradical activity increased slightly but significantly ( $p < 0.05$ ). The highest amount of  $\mu\text{mol}$  equivalents of Trolox in fermented PWS was obtained at 120 h. On the other hand, the SSF promoted greater increments in the DPPH radical scavenging activity when the PWSE was fermented, resulting in the highest activity values when fermentation was carried out for 108-120 hours (564.18 and 572.60  $\mu\text{mol}$  equivalents of Trolox, respectively). Similar to the TPC, fermented PWSE presented higher values in comparison with the PWS counterpart. A similar trend was observed in the DPPH radical scavenging activity of black rice bran extracts after fermentation with

*A. awamori* or *A. oryzae* (Shin *et al.*, 2019). This result demonstrates that the extrusion of by-products could be an excellent pretreatment for the increment of the radical scavenging activity.

ABTS radical scavenging activity showed similar results to those found with DPPH (Figure 5). As the fermentation time increased, the amount of  $\mu\text{mol}$  equivalents of Trolox augmented significantly in most of the samples. In both cases (DPPH and ABTS) the radical scavenging activity increased significantly ( $p < 0.05$ ) to maximum levels during the last fermentation times. Again, the fermented PWSE presented the higher values.



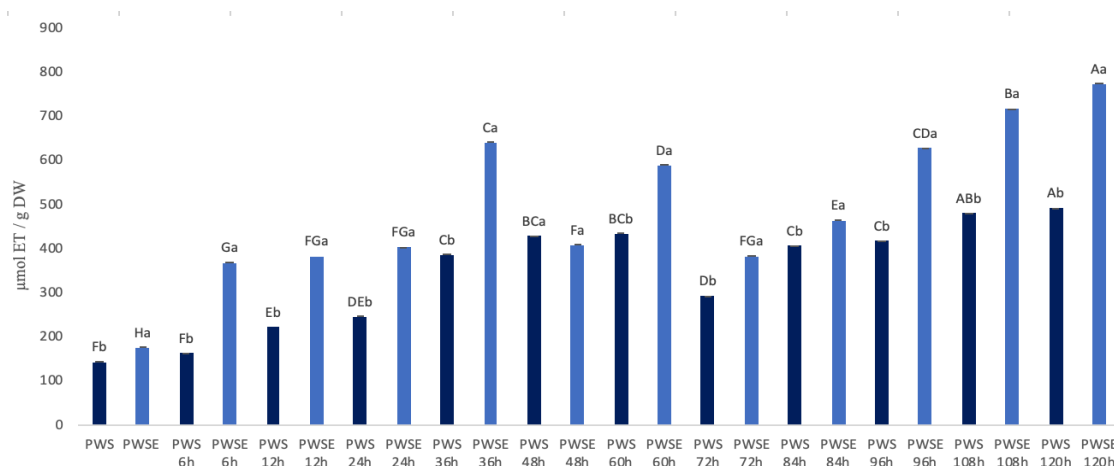
**Figure 4.** DPPH radical scavenging activity developed during the solid-state fermentation of pecan walnut shell (PWS) and extruded pecan walnut shell (PWSE).

Each bar represents the mean  $\pm$  SD of three replicates. Different capital letters (A,B, etc.) indicate significant differences between bars of the same treatment but at different time ( $p < 0.05$ ). Different lowercase letters (a and b) indicate significant differences between treatments (PWS and PWSE) but at the same time ( $p < 0.05$ ). The results are presented as  $\mu\text{mol}$  equivalents of Trolox (ET)/gram of pecan shell non-extruded or extruded, in dry weight (DW).

#### Phenolic acid profile

Table 1 shows the concentration of the two phenolic compounds (gallic and ellagic acids) identified in the PWS and PWSE samples without fermentation (WF) and fermented during 6 h and 120 h with *A. oryzae* under SSF conditions. Both phenolic acids were reported in pecan shell samples in previous studies (Hilbig, Alves, et al., 2018; Hilbig, Policarpi, et al., 2018; Do Prado et al., 2014). For the PWS, the major phenolic compound was the ellagic acid (EA). After 120 hours of SSF, the EA increased 3.8 times in PWS and the gallic acid (GA), 20 times. For PWSE, the major phenolic compound after 120 hours of fermentation was GA with a significant ( $p < 0.05$ ) increment of almost 15 times higher compared to the sample without fermentation. On the other hand, the EA did not show significant differences in PWSE samples fermented during 6 h or 120 h and only increased 0.4 mg/g compared to the non-fermented PWSE (Table 1). The initial concentration of GA (0.02 mg/g of PWS) and EA ( $0.18 \pm 0.01$  mg/ g of PWS) in non-fermented PWS samples were lower than those reported by Hilbig et al. (2018) in their work with pecan shell where the authors found GA and EA contents around 0.17 mg/ g of dry sample and 1.11 mg/g of dry sample, respectively (Hilbig, Alves, et al., 2018; Hilbig, Policarpi, et al., 2018). Differences with previous reports may be attributed to the

sample preparation, the extraction method, or the HPLC-DAD method that was used. On the other hand, (Villasante et al., 2019) found that the extrusion process increased the content of some phenolic compounds in the pecan shell. Similar than results shown in Figure 2, data in Table 1 demonstrated that after 120 h of fermentation, the amount of phenolic compounds significantly increased ( $p < 0.05$ ). The increase of phenolic compounds in other fiber-rich materials fermented with *A. oryzae* under SSF conditions, has been previously associated to the activation of fungal hydrolyzing enzymes that assist the release of phenolic acids from insoluble cell wall matrices (Shin et al., 2019). On the other hand, the high content of tannic acid in the pecan shell promotes the production of the tannase enzyme (Jing et al., 2019; Lerma-Herrera et al., 2017). According to (Chávez-González et al., 2018), the degradation of tannic acid during SSF promotes the production of ellagic acid. This could be the reason of the increment in phenolic compounds after SSF of pecan walnut shell substrates (extruded and non-extruded).



**Figure 5.** ABTS radical scavenging activity developed during the solid-state fermentation of pecan walnut shell (PWS) and extruded pecan walnut shell (PWSE).

Each bar represents the mean  $\pm$  SD of three replicates. Different capital letters (A,B, etc.) indicate significant differences between bars of the same treatment but at different time ( $p < 0.05$ ). Different lowercase letters (a and b) indicate significant differences between treatments (PWS and PWSE) but at the same time ( $p < 0.05$ ). The results are presented as  $\mu\text{mol}$  equivalents of Trolox (ET)/gram of pecan shell non-extruded or extruded, in dry weight (DW).

**Table 1.** GA and EA ( $\mu\text{g/g}$  dry walnut shell) of pecan walnut shell (PWS) and extruded pecan walnut shell (PWSE) extracts (without fermentation, at 6 h and 120 h of fermentation) obtained with HPLC-DAD.

Sample	Content of GA in PSW and PWSE (mg/g shell (no-extruded and extruded))	Content of EA in PSW and PWSE (mg/g shell (no-extruded and extruded))
PWS (WF)	0.02 $\pm$ 0.00 <sup>cB</sup>	0.18 $\pm$ 0.00 <sup>cB</sup>
PWSE (WF)	0.30 $\pm$ 0.03 <sup>cA</sup>	1.01 $\pm$ 0.00 <sup>bA</sup>
PWS (6 h)	0.20 $\pm$ 0.01 <sup>bB</sup>	0.70 $\pm$ 0.14 <sup>bB</sup>
PWSE (6 h)	1.12 $\pm$ 0.02 <sup>bA</sup>	1.40 $\pm$ 0.01 <sup>aA</sup>
PWS (120 h)	0.40 $\pm$ 0.01 <sup>aB</sup>	0.69 $\pm$ 0.32 <sup>aB</sup>
PWSE (120 h)	4.34 $\pm$ 1.78 <sup>aA</sup>	1.41 $\pm$ 0.41 <sup>aA</sup>

Average  $\pm$  SD; different lowercase letters a, b and c represent significant differences by Tukey's test between the same sample at different hours. Different capital letters (A and B) represent significant differences between different samples (PSW and PSWE) at the same hours ( $P < 0.05$ ). \* GA: Gallic acid, EG: Ellagic acid. UV/MAX GA: 270.8, UV/MAX EG: 253.1. tR (min): GA: 2.65, EG:12.017. \*WF: without fermentation.

## Conclusion

The fungal SSF is a complex metabolic process that may be used as a strategy to produce valuable compounds from agro-industry wastes. This study demonstrated that the SSF of pecan walnut shell with *A. oryzae* represent a good alternative for the production and recovery of phenolic compounds especially when the material was previously treated by extrusion processes. This pretreatment could be a valuable alternative to reduce the SSF time and therefore, the energy costs. Moreover, the use of *A. oryzae*, has been used to generate different enzymes as proteases, cellulases, etc. In summary, the results obtained in this study will be a great alternative and opportunity to the biotechnological industry to use an agro-industry waste as antioxidants.

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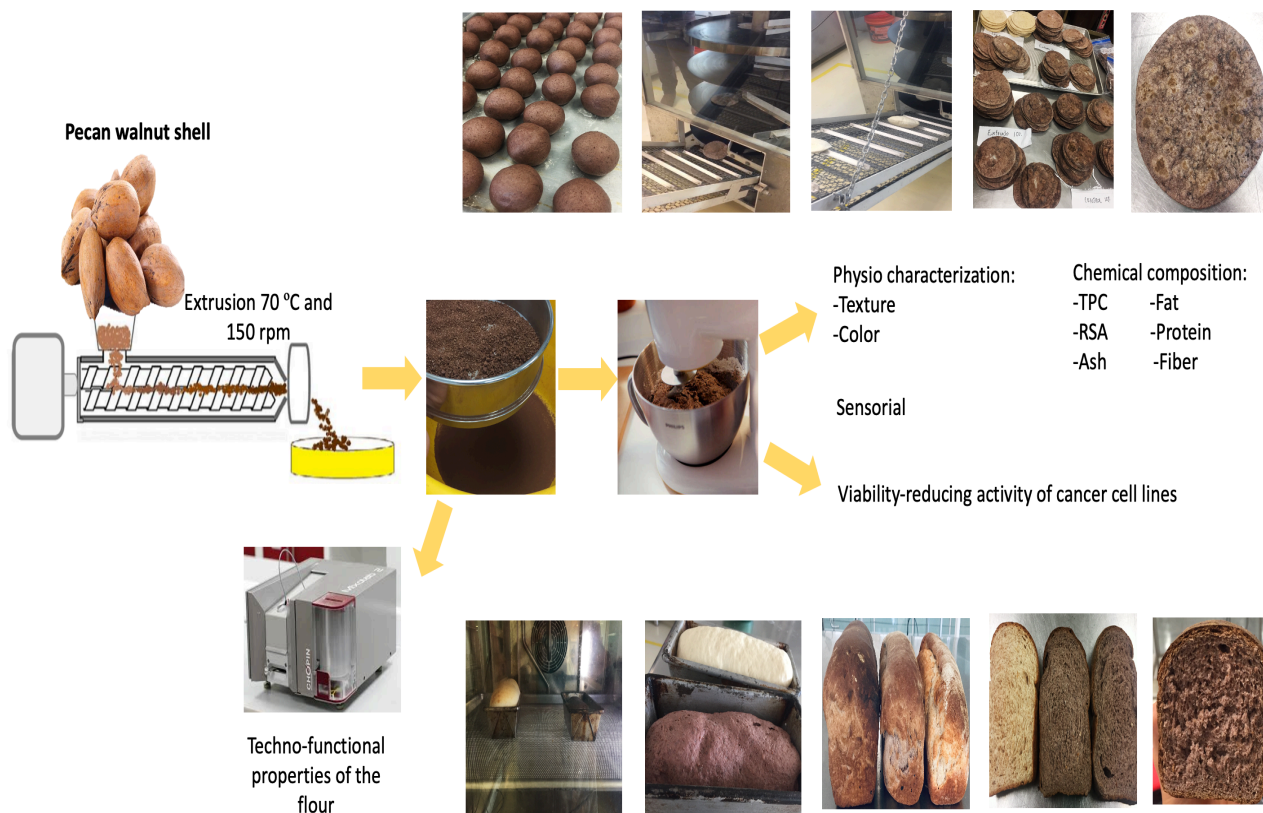
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### 6.3. Extruded pecan walnut shell (*Carya illinoensis*) flour in a traditional bread and tortilla recipe: Effects on functionality, phytochemical content and antiproliferative activities in human cancer cells.

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# Extruded pecan walnut shell (*Carya illinoensis*) flour in a traditional bread and tortilla recipe: Effects on functionality, phytochemical content and antiproliferative activities in human cancer cells

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Declarations of interest: none

## Abstract

This study evaluated the addition of different proportions (5, 10 and 15%) of flour proceeding from pecan walnut shell, both extruded (WSEF) and no-extruded (WSF), into a bread and tortilla formulation. The goal was to improve the total fiber content and the antioxidant activity. The properties of the dough were studied with Mixolab. After the physio characterization including texture, flour combinations were chosen: bread with 5% WSF or 5% WSEF and tortilla with 10% WSF or 10% WSEF. The chemical characterization, the viability of hepatocarcinoma cells and sensory evaluation were part of this study. Bread with 5% WSEF and tortilla with 10% WSEF had the highest total phenolic content (4.80 and 3.75 mg GAE/g), DPPH (402.26 and 180.42 TEAC in  $\mu\text{mols}/100\text{g}$ ), ABTS+ (518.34 and 305.73 TEAC in  $\mu\text{mols}/100\text{g}$ ), and total fiber content (11.19 and 16.04 in g/100g), respectively. The same treatments with a 10% (extract) reduced the viability of hepatic cancer cells by 53.46% (bread) and 44.97% (tortilla). The acceptability from panelists was higher than the control group.

**Keywords:** by-product, bread, tortilla, extrusion, antioxidants, fiber, hepatic cancer cell line

## 1.Introduction

The relationship between health and food products is gaining more relevance in the development of nutraceutical and functional foods (Buitimea-Cantúa, Daniel Mata-Ramírez, Sergio O. Serna-Saldívar, Javier Villela-Castrejón, Mary C. Villaseñor-Durán, 2018). Traditional bakery products are based on refined flours, sugars and fats. As a consequence, they are poor in dietary fibers (Miranda et al., 2014) and have a high glycemic index response (Matos Segura & Rosell, 2011). The addition of new ingredients with high phenolics and fiber contents in bakery's products may improve their potential biological activity (Quirós-Sauceda et al., 2014). However, for a successful design of these products, bigger attention needs to be paid to sensorial properties and consumer's habits; attributes as color, texture, flavor, etc., gain more relevance than the nutritional attributes. On

the other hand, bread is considered the staple food in most diets around the world, while the wheat tortillas are the second highest selling product in the packaged bread category in North America (Serna-Saldivar et al., 2004).

The pecan walnut shell is an important by-product of the food industry, characterized by high amounts of polyphenols (Villasante et al., 2019) with consequent benefits on health (Kureck et al., 2018). Previous studies determined that gallic acid, chlorogenic acid, p-hydroxybenzoic acid, epigallocatechin, epicatechin-gallate and tannins are the main compounds of the shell (Do Prado et al., 2014; Hilbig, Alves, et al., 2018; Hilbig, Policarpi, et al., 2018). Additionally, Do Prado et al. (2014) detected antimicrobial activity against *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio parahemolyticus* and *Bacillus cereus*. In previous work, in which the optimization of the extrusion temperature and screw speed on the pecan walnut shell was carried out, Villasante et al. (2019) demonstrated that a temperature of 70°C and a centrifugation at 150 rpm almost doubled the concentration of polyphenols and significantly increased radical scavenging activity compared to the non-extruded shell. Moreover, the mentioned study showed an increase of soluble fiber in the extrusion shell (Villasante et al., 2019).

The purpose of this study was to produce functional breads and tortillas with enhanced concentrations of fiber and phenolic compounds, using extruded and no-extruded pecan walnut shell. Effects of the inclusion of different concentrations of this by-product into the bread and tortilla on physical properties as texture were under study. Based on the texture results, the best treatments were chosen for both bread and tortilla, for which chemical composition, overall quality and acceptance of the product were determined. Finally, other evaluations were performed: retention of the total phenolic compounds and antiradical capacity in enriched bread and tortilla after the making process, and finally the effect on the reduction of viability of hepatic cancer cells (HepG2) line in vitro.

## **2. Materials and methods**

### **2.1. Raw materials**

The pecan walnut shells (*Carya illinoensis*) (WS) were collected in San Pedro, Coahuila, Mexico. The commercial refined all-purpose wheat (*Triticum aestivum* L) flour was purchased from Harina Selecta by Molinera de Mexico SA in CV.

### **2.2. Milling and particle size distribution**

The WS were ground using a Wiley mill (Arthur Thomas, Philadelphia, PA, USA) equipped with a 2 mm screen. Afterward, the particle size of the WS was determined by sifting 100 g through a set of sieves US No. 80 Rotap (Duratap Model DT 168, Advantech Mfg., New Berlin, WI, USA) that operated during 5 min.

### **2.3. Extrusion processing**

WS (after the section 2.2.) were processed in a twin-screw co-rotating extruder (BCTM-30 Bühler, Uzwil, Switzerland), using the same method and extrusion conditions (70 °C and 150 rpm) described by Villasante et al. (2019).

## 2.4. Technofunctional properties of the flour

A Mixolab instrument (Chopin, France) was used to determine the dough rheological properties according to the approved method AACCC 54-60.01, following the standard protocol Chopin+. This instrument measured the dough rheological properties and the amylograph during the mixing procedure of controlled heating and cooling (Rosell et al., 2013). The Mixolab profile started at 30°C with a speed mixing of 80 rpm. Dough mixing was carried out at 30°C for 8 min; eventually, the temperature was increased up to 90 °C, at a rate of 4°C/min. Bowl temperature was held at 90°C for 7 min, cooled to 50 °C at the rate of 4°C/min, and finally held at 50°C for 5 min. Parameters like water absorption, dough development time (min), dough stability (C1) range from 1.16 to 1.09 Nm, protein weakening (C2), starch gelatinization (C3), hot gel stability (C4) and retrogradation in the cooling phase (C5) were obtained from the Mixolab curves. The analysis was conducted twice per each of the samples.

## 2.5. Bread production

The formula of the control bread (CNB) used refined wheat flour (WF). The flour was replaced with walnut shell flour (WSF) or walnut shell extruded flour (WSEF) at 5%, 10% and 15% levels. The formulation used for bread was: 6% cane sugar (HEB de Monterrey, N.L, Mexico), 3.5% vegetable shortening (Inca, Unilever de Mexico S.A de C.V.; Tultitlán, Edo. de Mexico, Mexico), 2% dry milk (Nestle de Mexico, Mexico, D.F., Mexico), 2% salt (La Fina, Sales del Istmo, Coatzacoalcos, Veracruz, Mexico), 1.5% dry yeast “*Saccharomyces cereviceae*” (Nevada Roja, Panadis S.A de C.V. Monterrey, N.L., Mexico), 0.5% lecithin (Proveedores de Ingeniería Alimentaria S.A. de C.V. Monterrey, N.L., Mexico). Breads were prepared according to the method described by Chávez-Santoscoy, Lazo-Vélez, Serna-Sáldivar, & Gutiérrez-Urbe ( 2016). The amount of water added and the optimal mixing times were calculated using Chopin Mixolab according previous data (Tripette et Renaud, Paris, France) (table 1). After baking, the breads were left to cool down during 30 min at room temperature. Afterwards, the breads were cut into slices 15 mm thick and the slices were introduced into sealed polyethylene bags. The treatments were: CNB (100% WF), 5%WSB (with 5%WSF), 5%WSEB (with 5%WSEF), 10%WSB (with 10%WSEF), 10%WSEB (with 10%WSEF), 15%WSB (with 15%WSEF) and 15%WSEB (with 15%WSEF).

## 2.6. Tortillas production

The same proportion of WS and WSE used for the breads production was applied to the tortillas. Tortillas were produced according (Montemayor-Mora, Hernández-Reyes, et al., 2018), based on the following formulation: 13% vegetable shortening, 2% dry milk, 2% double acting baking (Rexal, Productos Mexicanos, Monterrey, NL, Mexico), 0.3% sodium stearoyl-2-lactylate (TECSA, Monterrey, NL, Mexico), and 0.2% carboxymethyl cellulose (PIASA, Monterrey, Mexico). The amount of water (W) and the treatments were: CNT (100% WF and 55.95% W), 5%WST (with 5%WSF and 56.53% W), 5%WSET (with 5%WSEF and 56.90%W), 10%WST (with 10%WSEF and 57% W), 10%WSET (with 10%WSEF and 57.21%W), 15%WST (with 15%WSEF and 57.47% W) and 15%WSET (with 15%WSEF and 57.95%W).The same mixing times of the bread production (table 1) were used for the tortillas. The baked tortillas were cooled on a porous surface at room temperature during approximately 30 min. Then, they were introduced into sealed polyethylene bags and stored at room temperature for further analysis.



## 2.7. Textural profile analysis (TPA) for the bread and texture parameters for the tortilla

Three slices of breads and tortillas of each treatment were randomly selected at days 0, 1 and 4 of storage. Texture analysis was conducted using a TA.XT2i Texture Analyzer (Stable Micro Systems, Godalming, England). The parameters evaluated for bread were: hardness, springiness, cohesiveness, chewiness, and resilience. The method followed indications described by (Buitimea-Cantúa, Daniel Mata-Ramírez, Sergio O. Serna-Saldívar, Javier Villela-Castrejón, Mary C. Villaseñor-Durán, 2018) but modifications were applied to bread thickness (15 mm).

The rupture force, the extensibility and the subjective rollability test was evaluated according to Montemayor-Mora, Hernandez-Reyes, Heredia-Olea, & Serna-Saldívar, Esther Pérez-Carrillo, Ana Antonieta Chew-Guevara, (2018). Values were registered from the average of five replicates.

## 2.8. Physical properties of bread and tortilla

Dimensions as diameter, thickness and weight of ten tortillas were determined by Montemayor-Mora, Hernandez-Reyes, Heredia-Olea, & Serna-Saldívar, Esther Pérez-Carrillo, Ana Antonieta Chew-Guevara, (2018). As for bread, the volume was determined by rapeseed displacement (National Manufacturing Co., Lincoln, NE, USA) according to method 10-05.01 of the AACC (1). The  $\Delta$ heights were determined by measuring the difference between the height of the bread before and after baking. The weights were determined 30 min after baking.

For color determination, ten tortillas and five slices of bread from each treatment were randomly selected and measured using the Konica Minolta colorimeter (model CM-600d) and calibrated with a standard series ( $X = 94.9$ ,  $y = 0.32$ , and  $x = 0.31$ ). The brightness was determined by  $L^*$ , red-green by  $a^*$ , and yellow-blue by  $b^*$ . The chrome ( $C^*$ ) and delta versus control ( $\Delta E$ ) were calculated according to eq. 1 and 2.

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (1)$$

$$E^* = \sqrt{a^{*2} + L^{*2} + b^{*2}} \quad (2)$$

## 2.9. Chemical composition of breads and tortillas

The moisture, ash, crude protein (a conversion factor of 5.81 was selected to calculate the protein content) and fat contents were determined by official AACC 2000 methods: 44-15, 08-01, 46-30 and 30-20, respectively. In order to analyze the digestibility of protein, samples were evaluated as reported by Hsu, Vavak, Satterlee, & Miller (1977) and Villasante et al. (2019). Soluble and insoluble dietary fibers were assayed using official AACC 2000 approved methods: 32-45.01 and 32-50.01, respectively.

All the characterization was determined on lyophilized, defatted and ground tortillas and bread samples.

## 2.10. Preparation of bread and tortilla extract for total phenolic content (TPC) and radical scavenging activity (RSA)

Lyophilized samples (1g) were mixed with 10 mL of 80% (v/v) aqueous methanol. The samples were stirred for one hour at 900 rpm and centrifuged (Thermofisher scientific SL 16R, Waltham, MA)

at 2500×g for 15 min at room temperature. The supernatant was removed, transferred into plastic vials, and kept in the freezer (-80°C) until analysis. Each sample was extracted by triplicate.

### 2.11. Determination of Total Phenolic Compound (TPC)

The total phenolic content was determined as reported by Mosca, Hidalgo, Villasante, & Almajano (2018) with some modifications, using the spectrophotometer Fluostar Omega (Paris, France) at 25° C and 750 nm; results were expressed in mg gallic acid equivalents/g of sample (mg GAE/g) ± standard deviation based on the calibration curve  $y = 0.891x - 0.0365$  with  $R^2 = 0.9929$ .

### 2.12. Determination of Radical scavenging activity (RSA)

Two methods were employed to measure the RSA. The DPPH (1,1-Diphenyl-2-Picrylhydrazyl) of controls and the treatments were measured according to the method described by Villasante, Girbal, Meton, & Almajano (2018). The methanolic extracts reacted with 200 µL of DPPH in methanol. The absorbance was measured at 517 nm after a period of 0 min (A0) and 75 min (A1) using a UV-Vis microplate reader spectrophotometer Fluostar Omega (Paris, France). The inhibition percentage of radical scavenging activity of each treatment was calculated by equation 4.

$$\%inhibition\ of\ sample = \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \quad (4)$$

Where A0 = initial absorbance of DPPH solution, A1 = absorbance with sample after 75 min.

The ABTS was determined spectrophotometrically, following the Segovia, Hidalgo, Villasante, Ramis, & Almajano (2018). The methanolic extracts were added in 200 µL, and then the absorbance was measured at 734 nm after 10 min. Results were expressed as µmol Trolox equivalents (TE)/g sample ± standard deviation by referring to a Trolox standard calibration curve  $y = 0.0025x + 0.0148$  with  $R^2 = 0.9963$ . Each sample was analyzed by triplicate.

### 2.13. Cell viability assays

#### 2.13.1. Preparation of bread and tortilla extracts for cell viability assays

The same procedure was used for the extraction of TPC and RSA. The methanol of the final supernatant was evaporated with nitrogen. Bread and tortilla extracts were weighed and resuspended in 2 mL of water. All samples were filtered (0.22µm; Tecknochroma).

#### 2.13.2. Cell culture and viability.

Human tumor derived from HepG2 (liver hepatocarcinoma); the cell line was cultured at 37°C in 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's (DMEM) medium supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) of fetal bovine serum. HepG2 cells were seeded in 12-well plates at a density of 2x10<sup>4</sup> cells/well. After 24 hours, two different concentrations (2 and 10%) of the following extracts were added to the cell culture: CNT, 10%WST, 10%WSET, CNB, 5%WSB and 5%WSEB. Water and cells were non-treated (CTR) were used as control. The cells with the extracts and water were incubated during 48 hours; eventually, the cell viability was determined by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described Gallego et al. (2017). 0.63 mM of MTT and 18.4 mM of sodium succinate were added and

left during 3 h at 37° C. After removal of the medium, formazan was resuspended with dimethyl sulfoxide (DMSO) and supplemented with 0.57% CH<sub>3</sub>COOH and 10% sodium dodecyl sulphate. Spectrophotometric determinations were performed at 570 nm in a Cobas Mira S analyzer (Hoffman-La Roche, Basel, Switzerland). The results were expressed as a percentage of cell survival relative to non-treated control cells.

#### 2.14. Preference sensory analysis

Hedonic sensory test was conducted on a taste panel consisting of 70 untrained panelists, aged from 18 to 60, staff and students from Tecnológico de Monterrey, campus Monterrey. All participants declared that they do not suffer from dried fruits allergy. Participants rated, on a five-point hedonic scale, one-day-old tortilla (CNWT, 10%WST and 10%WSET) and bread (CNWB, 5%WSB and 5%WSEB) samples, evaluating color, texture, flavor, odor, and overall quality. The samples were presented simultaneously on polystyrene plates, labeled with random codes and presented to the consumers in a randomized order (Serna-Saldivar, 2012). For the sensory analysis, the controls were produced using whole wheat flour. The aim was to compare the products containing walnut shell with a fiber-rich counterpart, produced with a flour available in the market, instead of using a refined flour. This method prevented from obtaining biased results depending on the fiber-rich or no-fiber preference of the consumers.

#### 2.15. Statistical analysis

The average value and standard deviation were calculated from the data obtained from the samples for each treatment. Where significant differences were analyzed by one-way ANOVA, average values were compared using Turkey's test at level of significance of 95%. All statistics were performed using Minitab-18.

### **3. Results and discussion**

#### 3.1. Rheology of the flour

All the samples were stable during 8 min-dough mixing. The table shows that the control (CNF) had a lower water absorption (WA) value (58.5%) and higher dough development time compared with samples with walnut shell. The time of the dough development is highly related to the gluten matrix strength and to the water absorption capacity (Rosell, Collar, & Haros, 2007) (Rosell et al., 2007). The increase of WA could be due to the presence of hydroxyl groups in the structure of fiber in the walnut shell (Kuchtová, Kohajdová, Karovičová, & Lauková, 2018). A similar behavior was also shown by other authors after the preparation of wheat dough enriched with by-products as grape skin, mango peel powder, apple fiber, lemon and orange (Ajila et al., 2008; Kohajdová Zlatica, Karovicova Jolana, Jurasová Michaela, 2011; Kuchtová et al., 2018). Increased water absorption in dough development has positive effects on the baking industry, thanks to the higher yield of the tortilla and bread (Sun et al., 2019).

The C2 parameter of samples showed values between 0.51 and 0.86 Nm. With the addition of walnut shell flour (WSF) and walnut shell extruded flour (WSEF), the increase of C2 was significant ( $p < 0.05$ ) in comparison with the control. Kuchtová et al. (2018) studied the effects of flour replacement with cellulose fiber on rheological properties of wheat dough. The results showed a

similar behavior with the addition of shell walnut powder. Rosell, Santos, & Collar (2010) mentioned that the increase of C2 is the result of some obstacles in the protein unfolding. One possible reason is the high fiber content in the walnut shell.

C3 measures the maximum torque during the heating stage. According to Dubat, (2010); and Heo, Lee, et al. (2013), this value could be related to the starch gelatinization and the amylose activity of the flour. As shown in table 1, the addition of WSF and WSEF increased significantly the C3 parameter compared to the control. The reason was probably the quick rupture of starch granules, thus leading to a higher pasting consistency and to lower pasting temperature, as occurred with the wheat flour enriched with cellulose (Lauková et al., 2017).

The control had the lowest value of C4. Moreover, the difference between the C4 and C5 (starch degradation) values decreased. The lowest values (C5-C4) obtained for shell walnut indicate a higher resistance to degradation (Hahnadev et al., 2013).

The value C5 represents the retrogradation of the starch; this effect can produce a fast staling in the tortilla and the bread. The table 1 shows the results of the treatments. In almost all the cases, the C5 value was not significative in comparison to the control. However, the treatments with WSEF had the lowest values, probably because the shell extruded flour had more soluble fiber (Villasante et al., 2019). In fact, a high soluble fiber content may keep the dough system hydrated and hence, delay retrogradation (Ma et al., 2019).

**Table 1.** Dough mixing behavior of pecan walnut shell flour (extruded and no-extruded) and wheat flour using Mixolab

Parameter	CNF	5%WSF	5%WSEF	10%WSF	10%WSEF	15%WSF	15%WSEF
Moisture %	13.00±0.02 <sup>f</sup>	13.40±0.01 <sup>c</sup>	13.30±0.04 <sup>d</sup>	13.20±0.02 <sup>c</sup>	13.70±0.01 <sup>b</sup>	13.00±0.01 <sup>g</sup>	13.80±0.02 <sup>a</sup>
Mixolab profile							
Water absorption (14%)	58.5±0.80 <sup>d</sup>	59.5±0.00 <sup>c</sup>	59.5±0.01 <sup>c</sup>	60.00±0.02 <sup>bc</sup>	60.0±0.00 <sup>bc</sup>	60.5±0.02 <sup>ab</sup>	61.0±0.03 <sup>a</sup>
Dough development time(min)	2.96±0.39 <sup>a</sup>	1.51±0.04 <sup>b</sup>	1.50±0.01 <sup>b</sup>	1.48±0.15 <sup>b</sup>	1.45±1.04 <sup>b</sup>	1.39±0.02 <sup>b</sup>	1.37±0.02 <sup>b</sup>
C2(protein weakening. Nm)	0.51±0.02 <sup>c</sup>	0.59±0.02 <sup>d</sup>	0.63±0.01 <sup>d</sup>	0.76±0.02 <sup>c</sup>	0.84±0.01 <sup>ab</sup>	0.78±0.01 <sup>bc</sup>	0.86±0.14 <sup>a</sup>
C3(starch gelatinization. Nm)	2.02±0.01 <sup>d</sup>	2.16±0.03 <sup>c</sup>	2.14±0.00 <sup>c</sup>	2.22±0.01 <sup>b</sup>	2.19±0.00 <sup>bc</sup>	2.28±0.00 <sup>a</sup>	2.24±0.00 <sup>ab</sup>
C4(hot gel stability. Nm)	1.92±0.44 <sup>b</sup>	2.03±0.02 <sup>a</sup>	2.04±0.00 <sup>a</sup>	2.07±0.00 <sup>a</sup>	2.05±0.02 <sup>a</sup>	2.02±0.05 <sup>a</sup>	1.99±0.02 <sup>ab</sup>
C5(starch retrogradation in the cooling phase.Nm)	3.91±0.02 <sup>ab</sup>	3.84±0.02 <sup>abc</sup>	3.77±0.00 <sup>abc</sup>	3.94±0.03 <sup>a</sup>	3.72±0.07 <sup>bc</sup>	3.74±0.11 <sup>abc</sup>	3.65±0.01 <sup>c</sup>
C4-C3 (Cooking stability range)	-0.10±0.02 <sup>a</sup>	-0.13±0.02 <sup>a</sup>	-0.11±0.02 <sup>a</sup>	-0.15±0.02 <sup>ab</sup>	-0.14±0.02 <sup>a</sup>	-0.26±0.02 <sup>c</sup>	-0.25±0.02 <sup>bc</sup>

Data is mean ± standard deviation ( $n = 3$ ). Values with similar superscript in a column do not differ significantly according to ANOVA (Tukey's test) at  $p < 0.05$ . CNF: 100% wheat flour, 5%WSF: 95% wheat flour with 5% walnut shell flour, 5%WSEF: 95% wheat flour with 5% extruded walnut shell flour, 10%WSF: 90% wheat flour with 10% walnut shell flour, 10%WSEF: 90% wheat flour with 10% extruded walnut shell flour, 15%WSF: 85% wheat flour with 15% walnut shell flour, 15%WSEF: 85% wheat flour with 15% extruded walnut shell flour.

### 3.2.Physical properties of bread and tortilla

The specific volume represents an index of the expansion of bread. After cooling to room temperature, bread volume was determined by the seed displacement technique. As shown in the Table 2, the highest specific volume of the bread was obtained by the 5% WSEB sample. The specific

volume of breads with 10% and 15% of WSF and WSEF were significantly lower ( $p < 0.05$ ) than the control and the breads with 5% WSF and WSEF. Similar results were obtained for bakery products enriched with different percentages of citrus dietary fiber, extruded orange pulp and apple pomace (Kohajdová Zlatica, Karovicova Jolana, Jurasová Michaela, 2011; Larrea et al., 2005; Sudha et al., 2007). The fig 1 (supplementary material) showed the similarity in height and specific volume of the treatments 5%WSB and 5%WSEB versus the control group. A similar behavior was observed with breads delta height. A 15% WSF content reduced by 67% ( $p < 0.05$ ) the height compared to the control. The addition of flour with a high proportion of fiber (Villasante et al., 2019), like the walnut shell, causes a dilution of starch and gluten, thus decreasing the loaf volume (Filipovic et al., 2007; Lafarga et al., 2019). Huang & Ma (2019) explained that a high fiber content could result in a limited expansion during fermentation. Similar effects have also been observed for bread made with additions of other products with high content of fiber, such as calamondin pomace, by-products from the flaxseed industry, and fenugreek gum (Roberts et al., 2012). Color lightness ( $L^*$ ) and yellowness ( $b^*$ ) of the crumb decreased proportionally to the increase of the WSF and WSEF. However, the redness in the crumb ( $a^*$ ) increased significantly ( $p < 0.05$ ), almost 98% more than the control. The  $\Delta E^*$  was significantly higher with the addition of WSEF, compared to the treatment with WSF. These results are due to the pecan high heat used could produce reactions, such as Maillard, caramelization, or hydrolysis (Kureck et al., 2018; Villasante et al., 2019). The pecan walnut shell has a high tannins content (Kureck et al., 2018). In a study of oenological tannins on color stability of a Romanian red wine, Bichescu Cezar (2019) demonstrated that the use of tannins, in different doses, accentuates the violet tones.

A similar behavior was observed in the crust. The color imparted come from the ingredients used in bread formulation, in this case pecan walnut shell. Also, the brown color formation could be caused by the crust caramelization and Maillard reactions. Martins et al. (2017) studied the fortification of wheat bread with agroindustry by-product and found that, by the addition of 7% elderberry extract, the values of  $L^*$ ,  $a^*$  and  $b^*$  of the crust decrease compared to the control (Martins et al., 2017).

Regarding the tortillas, the addition of walnut shell powder did not show any significant effects ( $p < 0.05$ ) on diameter, thickness and weight (table 2) in comparison to the control. Similar results were shown by Zhu et al. when adding extruded wheat flour in the tortillas production (Zhu et al., 2017). The high heat, used in the processing of tortillas, might inactivate the enzymes that degrade the gluten; also, a lack of mechanical regression might be connected to the addition of fiber. Substantially higher rollability scores were measured over time (4 days) in tortillas made from 10% WSF and WSEF, but they were not statistically significant ( $p < 0.05$ ). The  $L^*$  and  $b^*$  of the tortilla generally decreased with WSF and WSEF. Opacity (low values of  $L^*$ ) is an important quality trait for tortilla; according to Alviola & Awika (2010), more opaque tortillas are preferred by the consumer.

**Table 2.** Physical properties of tortilla and bread added with pecan walnut shell extruded and no-extrude

Bread	CNB	5%WSB	5%WSEB	10%WSB	10%WSEB	15%WSB	15%WSEB
ΔHeight (mm)	2.59 ± 0.01 <sup>a</sup>	2.68 ± 0.24 <sup>a</sup>	2.42 ± 0.09 <sup>a</sup>	1.44 ± 0.06 <sup>b</sup>	1.05 ± 0.14 <sup>bc</sup>	1.33 ± 0.19 <sup>bc</sup>	0.85 ± 0.07 <sup>c</sup>
Specific volume (cm <sup>3</sup> )	798.13 ± 11.49 <sup>a</sup>	760.63 ± 25.6 <sup>ab</sup>	800.0 ± 14.1 <sup>a</sup>	668.10 ± 18.6 <sup>bcd</sup>	677.50 ± 24.7 <sup>bc</sup>	566.88 ± 2.65 <sup>d</sup>	623.10 ± 53.9 <sup>cd</sup>
Weight (g)	142.14 ± 2.26 <sup>a</sup>	142.43 ± 3.39 <sup>a</sup>	140.52 ± 1.85 <sup>a</sup>	144.48 ± 2.80 <sup>a</sup>	142.20 ± 2.09 <sup>a</sup>	144.95 ± 0.07 <sup>a</sup>	144.45 ± 0.64 <sup>a</sup>
Crumb color							
L*	76.59 ± 0.59 <sup>a</sup>	59.30 ± 1.51 <sup>b</sup>	55.95 ± 0.98 <sup>c</sup>	45.21 ± 0.40 <sup>d</sup>	42.78 ± 0.30 <sup>e</sup>	38.38 ± 0.48 <sup>f</sup>	36.79 ± 0.24 <sup>g</sup>
a*	0.14 ± 0.17 <sup>g</sup>	6.52 ± 0.30 <sup>f</sup>	7.53 ± 0.26 <sup>e</sup>	8.33 ± 0.12 <sup>d</sup>	9.67 ± 0.20 <sup>c</sup>	8.86 ± 0.36 <sup>b</sup>	11.13 ± 0.12 <sup>a</sup>
b*	15.80 ± 1.05 <sup>a</sup>	11.83 ± 0.18 <sup>c</sup>	12.99 ± 0.48 <sup>c</sup>	11.71 ± 1.12 <sup>f</sup>	13.09 ± 0.45 <sup>b</sup>	11.37 ± 0.20 <sup>g</sup>	12.61 ± 0.41 <sup>d</sup>
C*	15.80 ± 1.50 <sup>c</sup>	13.52 ± 0.30 <sup>g</sup>	15.02 ± 0.52 <sup>d</sup>	14.37 ± 1.60 <sup>f</sup>	16.28 ± 0.45 <sup>b</sup>	14.41 ± 0.82 <sup>c</sup>	16.82 ± 0.12 <sup>a</sup>
ΔE*		18.85 ± 1.62 <sup>f</sup>	22.10 ± 0.60 <sup>e</sup>	32.69 ± 0.40 <sup>d</sup>	35.23 ± 0.23 <sup>c</sup>	39.44 ± 0.70 <sup>b</sup>	41.41 ± 0.21 <sup>a</sup>
Crust color							
L*	42.18 ± 0.15 <sup>a</sup>	37.28 ± 0.14 <sup>b</sup>	33.51 ± 0.13 <sup>g</sup>	36.80 ± 0.20 <sup>c</sup>	35.47 ± 1.14 <sup>c</sup>	36.73 ± 0.19 <sup>d</sup>	34.12 ± 0.13 <sup>f</sup>
a*	11.31 ± 1.00 <sup>a</sup>	7.76 ± 0.31 <sup>d</sup>	7.99 ± 0.39 <sup>c</sup>	5.54 ± 0.30 <sup>g</sup>	8.93 ± 0.51 <sup>b</sup>	5.81 ± 1.39 <sup>f</sup>	6.82 ± 0.19 <sup>e</sup>
b*	16.86 ± 0.91 <sup>a</sup>	16.34 ± 0.51 <sup>b</sup>	10.72 ± 1.32 <sup>e</sup>	10.14 ± 0.9 <sup>f</sup>	12.93 ± 0.52 <sup>c</sup>	9.77 ± 0.9 <sup>g</sup>	11.71 ± 0.9 <sup>d</sup>
C*	20.30 ± 0.58 <sup>a</sup>	18.09 ± 1.01 <sup>b</sup>	13.38 ± 0.31 <sup>e</sup>	11.55 ± 0.58 <sup>f</sup>	15.71 ± 0.01 <sup>c</sup>	11.37 ± 2.31 <sup>g</sup>	13.56 ± 0.31 <sup>d</sup>
ΔE*		6.07 ± 0.58 <sup>f</sup>	11.13 ± 0.50 <sup>a</sup>	10.37 ± 0.40 <sup>d</sup>	8.14 ± 0.50 <sup>c</sup>	10.51 ± 0.51 <sup>c</sup>	10.57 ± 0.51 <sup>b</sup>
Tortilla	CNT	5%WST	5%WSET	10%WST	10%WSET	15%WST	15%WSET
Diameter (cm)	13.46 ± 0.50 <sup>ab</sup>	12.78 ± 0.24 <sup>ab</sup>	12.16 ± 0.01 <sup>b</sup>	12.96 ± 0.55 <sup>ab</sup>	13.54 ± 0.12 <sup>a</sup>	13.20 ± 0.44 <sup>ab</sup>	13.37 ± 0.04 <sup>ab</sup>
Thickness (cm)	2.86 ± 0.32 <sup>a</sup>	2.98 ± 0.29 <sup>a</sup>	2.93 ± 0.10 <sup>a</sup>	3.21 ± 0.34 <sup>a</sup>	3.19 ± 0.05 <sup>a</sup>	3.21 ± 0.01 <sup>a</sup>	3.19 ± 0.28 <sup>a</sup>
Weight (g)	31.38 ± 0.0 <sup>a</sup>	31.58 ± 0.01 <sup>a</sup>	31.73 ± 0.27 <sup>a</sup>	31.39 ± 0.00 <sup>a</sup>	31.28 ± 0.04 <sup>a</sup>	31.27 ± 0.34 <sup>a</sup>	31.13 ± 0.10 <sup>a</sup>
Rollability (day 4)	3.90 ± 0.14 <sup>a</sup>	3.50 ± 0.71 <sup>a</sup>	3.30 ± 0.42 <sup>a</sup>	4.20 ± 0.28 <sup>a</sup>	4.10 ± 0.14 <sup>a</sup>	3.60 ± 0.57 <sup>a</sup>	3.40 ± 0.28 <sup>a</sup>
Color							
L*	81.75 ± 1.66 <sup>a</sup>	54.92 ± 1.29 <sup>b</sup>	54.46 ± 4.29 <sup>bc</sup>	50.54 ± 0.57 <sup>bc</sup>	49.46 ± 1.04 <sup>bc</sup>	47.74 ± 0.17 <sup>bc</sup>	47.04 ± 0.55 <sup>c</sup>
a*	0.688 ± 0.21 <sup>c</sup>	6.91 ± 0.41 <sup>b</sup>	7.46 ± 0.62 <sup>ab</sup>	8.056 ± 0.30 <sup>ab</sup>	8.580 ± 0.24 <sup>a</sup>	8.20 ± 0.40 <sup>ab</sup>	8.66 ± 0.01 <sup>a</sup>
b*	17.66 ± 0.91 <sup>a</sup>	11.54 ± 0.95 <sup>b</sup>	11.05 ± 0.18 <sup>b</sup>	11.98 ± 1.17 <sup>b</sup>	12.12 ± 0.46 <sup>b</sup>	10.91 ± 0.15 <sup>b</sup>	10.84 ± 0.46 <sup>b</sup>
C*	17.67 ± 0.91 <sup>a</sup>	13.45 ± 1.03 <sup>b</sup>	13.33 ± 0.20 <sup>b</sup>	14.44 ± 1.14 <sup>b</sup>	14.85 ± 0.24 <sup>ab</sup>	13.65 ± 0.36 <sup>b</sup>	13.87 ± 0.36 <sup>b</sup>
ΔE*		28.21 ± 0.32 <sup>b</sup>	28.89 ± 2.83 <sup>b</sup>	32.57 ± 1.05 <sup>ab</sup>	33.70 ± 0.64 <sup>ab</sup>	35.49 ± 1.57 <sup>a</sup>	36.27 ± 2.08 <sup>a</sup>

Data is mean ± standard deviation ( $n = 10$ ). Values with similar superscript in a column do not differ significantly according to ANOVA (Tukey's test) at  $p < 0.05$ . CNB or CNT: Bread or Tortilla with 100% wheat flour, 5%WSB or WST: Bread or Tortilla with 95% wheat flour with 5% walnut shell flour, 5%WSEB or WSET: Bread or Tortilla with 95% wheat flour with 5% extruded walnut shell flour, 10%WSB or WST: Bread or Tortilla with 90% wheat flour with 10% walnut shell flour, 10%WSEB or WSET: Bread or Tortilla with 90% wheat flour with 10% extruded walnut shell flour, 15%WSB or WSET: Bread or Tortilla with 85% wheat flour with 15% walnut shell flour, 15%WSEB or WSET: Bread or Tortilla with 85% wheat flour with 15% extruded walnut shell flour.

### 3.3. Textural profile analysis (TPA) of bread

Results obtained from the TPA analysis of slices of bread of different treatments are shown in the table 3. Hardness in bakery is an important mechanical parameter to be taken into account; it is defined as the force needed to deform deep into the product (Wirkijowska et al., 2020). The lowest hardness values at day 0 was observed in treatments CNB, 5%WSB, 5%WSEB and 10%WSB, the highest hardness values reported in treatments 10%WSEB, 15%WSB and 15%WSEB. The hardness of 5%WSB and 5%WSEB, determined at the day 1 after baking, did not cause any significant changes ( $p < 0.05$ ) in comparison to the control. After 4 days of storage, hardness was increasing, however there is not difference between CNB, 5%WSB and 5%WSEB. These results could be having relation with the values obtained of specific volume (table 2), according De la Hera et al., bread with lower specific volume results more compact cells and denser crumb, thus increase the bread hardness.

In terms of springiness, the addition of WSF and WSEF were not significant ( $p < 0.05$ ) (the average was around 0.98-0.96 N). The cohesiveness, is the resistance or cohesion of internal bonds of the bread structure (Monthe et al., 2019). The bread cohesiveness at day 0 decreased with the addition of 10% and 15% of WSF and 15% WSEF. After 4 days, the CNB was significantly ( $p < 0.05$ ) more cohesiveness in comparison with the other samples with the addition of WSF and WSEF. The cohesiveness decreasing in parallel to the increase of the water content in formulation (table 1) (De La Hera et al., 2014).

In the majority of the cases, the addition of WSF and WSEF did not alter the chewiness of the bread at day 0 and 4. Only in the case of two samples: 5%WSB and 10%WSB, the values of chewiness decrease significantly ( $p < 0.05$ ) compared with the control at the day 4. This result could be positive because according Wang et al. a lower chewiness is associated with better tasting property (Wang et al., 2020).

Therefore, the addition of 5% of WSF and WSEF, didn't affect significantly the hardness. The values obtained were similar than the control at the different days. The springiness was not significantly difference between the samples and the control. Also, in the case of the cohesiveness the addition of 5% of WSF and WSEF were the more similar to the control. However, the values of cohesiveness presented by the control were significantly difference with comparison with the others samples. Finally, with the addition of 5%WSF the chewiness was better. In summary, the samples with 5% of WSF and WSEF had similar values than the control or in some cases better.

#### 3.4. Texture parameters of tortilla

Large breaking force and longer distance indicate a stronger and more stretchable tortilla (Liu et al., 2017). The subjective rollability test is used to evaluate the textural changes and the extent of cracking or breaking during storage (Bejosano et al., 2016). As shown in table 3, fresh tortillas (day 0) had the highest breaking force and extensibility values. Furthermore, there was no significant ( $p > 0.05$ ) difference in subjective rollability (day 0) among tortillas made with different flour blends. One day after baking, in the treatments with 10% and 15% WSF and 15% WSFE, the breaking force decreased significantly ( $p < 0.05$ ) compared to the control. After the same period, the extensibility decreased by adding the shell flour except with the addition of 10% WSEF. However, apart from the subjective rollability, the 10% SWET showed the highest values of breaking force and extensibility along the 4 days period. These results were agreed with previous data from Mixolab values, as C5 (measures starch reorganization in the cooling phase). By replacing white flour with WSEF, the content of fiber increased and the values of C5 decreased. The presence of fiber decreases the starch available for crystallization and gelatinization; this effects potentially beneficial, since it slows down the staling of tortillas during storage (Liu et al., 2017; Santos et al., 2008).

After analyzing the texture of the tortillas, the samples with the addition of 10% of WSF presented the best results at the day 4. Also, in some case similar than the control.

Based on the results obtained on the texture and the physical properties, the following studies were performed only on: CNB, 5%WSB, 5%WSEB, CNT, 10%WST and 10%WSET.

**Table 3.** Texture profile analysis (TPA) of bread and tortilla made with different blends of wheat flour and walnut flour or extruded walnut flour along storage time.

Bread traits	Hardness (N)			Cohesiveness			Chewiness (N)		
	Day 0	Day 1	Day 4	Day 0	Day 1	Day 4	Day 0	Day 1	Day 4
CNB	8.56±0.67 <sup>cdB</sup>	10.64±0.94 <sup>dAB</sup>	21.03±3.16 <sup>bcdA</sup>	0.64±0.08 <sup>BA</sup>	0.51±0.36 <sup>abA</sup>	0.48±0.00 <sup>BA</sup>	5.47±0.85 <sup>abA</sup>	5.32 ± 0.63 <sup>abA</sup>	9.73 ± 1.40 <sup>BA</sup>
5%WSB	9.07±1.03 <sup>cdB</sup>	13.01±1.34 <sup>bdAB</sup>	20.20±2.10 <sup>cdA</sup>	0.54±0.05 <sup>abA</sup>	0.38±0.06 <sup>CA</sup>	0.36±0.04 <sup>bcA</sup>	4.74±0.30 <sup>BA</sup>	4.87±0.57 <sup>BA</sup>	7.22±0.93 <sup>bcA</sup>
5%WSEB	8.11±1.10 <sup>dB</sup>	12.19±0.99 <sup>cdAB</sup>	17.46±1.08 <sup>dA</sup>	0.64±0.08 <sup>BA</sup>	0.47±0.04 <sup>abA</sup>	0.38±0.02 <sup>bcA</sup>	5.13±0.56 <sup>BA</sup>	5.56±0.35 <sup>abA</sup>	9.77±1.42 <sup>BA</sup>
10%WSB	10.74±0.96 <sup>bcB</sup>	12.99±1.15 <sup>b-dAB</sup>	27.33±4.02 <sup>abA</sup>	0.48±0.06 <sup>BA</sup>	0.39±0.04 <sup>bcA</sup>	0.34±0.02 <sup>bcA</sup>	4.98±0.69 <sup>BA</sup>	4.95±0.84 <sup>abA</sup>	5.32±0.46 <sup>CA</sup>
10%WSEB	11.87±1.5 <sup>abA</sup>	14.41±2.05 <sup>a-cA</sup>	27.29±3.15 <sup>abA</sup>	0.51±0.03 <sup>abA</sup>	0.43±0.05 <sup>a-cA</sup>	0.30±0.03 <sup>bcA</sup>	5.85±0.47 <sup>abA</sup>	5.91±0.49 <sup>abA</sup>	8.04±1.04 <sup>abA</sup>
15%WSB	14.62±1.41 <sup>abA</sup>	17.03±0.84 <sup>BA</sup>	26.91±4.31 <sup>abA</sup>	0.48±0.04 <sup>BA</sup>	0.37±0.02 <sup>CA</sup>	0.35±0.02 <sup>bcA</sup>	6.88±0.85 <sup>BA</sup>	6.20±0.50 <sup>BA</sup>	8.97±1.30 <sup>abA</sup>
15%WSEB	12.82±1.52 <sup>abA</sup>	15.22±2.5 <sup>abA</sup>	28.61±4.05 <sup>BA</sup>	0.47±0.05 <sup>BA</sup>	0.38±0.02 <sup>CA</sup>	0.30±0.02 <sup>CA</sup>	5.77±0.43 <sup>abA</sup>	5.50±0.97 <sup>abA</sup>	7.88±0.93 <sup>abA</sup>
Tortilla traits	Breaking force (g)			Extensibility (mm)			Subjective rollability		
	Day 0	Day 1	Day 4	Day 0	Day 1	Day 4	Day 0	Day 1	Day 4
CNT	1045.60±100.20 <sup>BA</sup>	791.40±73.20 <sup>abA</sup>	570.0±69.60 <sup>bcdA</sup>	26.02±1.35 <sup>BA</sup>	16.50±1.80 <sup>BB</sup>	10.15±1.01 <sup>cB</sup>	5.00 ± 0.00 <sup>BA</sup>	4.90±0.14 <sup>BA</sup>	3.90±0.14 <sup>BA</sup>
5%WST	1132.9±119.20 <sup>abA</sup>	894.10±95.60 <sup>BA</sup>	664.9±85.40 <sup>abA</sup>	24.1±2.00 <sup>b-cA</sup>	12.76±1.32 <sup>BB</sup>	12.24±1.43 <sup>abB</sup>	5.00±0.00 <sup>BA</sup>	4.60±0.07 <sup>BA</sup>	3.50±0.42 <sup>BA</sup>
5%WSET	1040.10±125.90 <sup>BA</sup>	814.90±87.40 <sup>BA</sup>	570.8±73.80 <sup>bcdA</sup>	20.10±1.63 <sup>dA</sup>	13.08±1.2 <sup>abB</sup>	11.82±1.2 <sup>abB</sup>	5.00±0.00 <sup>BA</sup>	4.85±0.00 <sup>BA</sup>	3.30±0.42 <sup>BB</sup>
10%WST	1004.00±119.00 <sup>BA</sup>	580.30±99.80 <sup>CA</sup>	423.50±43.30 <sup>BA</sup>	21.4±1.80 <sup>cdA</sup>	12.95±1.25 <sup>BB</sup>	10.2±0.39 <sup>bcB</sup>	5.00±0.00 <sup>BA</sup>	4.40±0.03 <sup>abB</sup>	4.10±0.28 <sup>BB</sup>
10%WSET	1239.00±127.70 <sup>BA</sup>	829.60±132.40 <sup>BA</sup>	733.80±83.00 <sup>BA</sup>	24.4±2.77 <sup>abA</sup>	14.7±1.82 <sup>abA</sup>	12.39±1.05 <sup>BA</sup>	5.00±0.00 <sup>BA</sup>	4.70±0.00 <sup>BA</sup>	4.20±0.14 <sup>BB</sup>
15%WST	989.80±167.50 <sup>BA</sup>	607.00±84.40 <sup>CA</sup>	472.0±62.60 <sup>cdA</sup>	20.92±1.67 <sup>dA</sup>	13.15±0.96 <sup>BB</sup>	9.25±0.70 <sup>BB</sup>	5.00±0.00 <sup>BA</sup>	4.25±0.63 <sup>BA</sup>	3.60±0.56 <sup>BA</sup>
15%WSET	1022.00±79.70 <sup>BA</sup>	651.10±118.10 <sup>bcdA</sup>	456.60±42.80 <sup>dA</sup>	22.69±2.0 <sup>b-dA</sup>	13.87±1.2 <sup>abB</sup>	9.75±1.10 <sup>cB</sup>	5.00±0.00 <sup>BA</sup>	4.10±0.07 <sup>BA</sup>	3.40±0.28 <sup>BA</sup>

Letters on the top right corner of each value indicate significantly different values between samples (minuscules) and the same sample between days (capital letters), according to ANOVA (Tukey's test) at  $P < 0.05$ . Data is mean  $\pm$  standard deviation ( $n = 3$ ). Values with similar superscript in a column do not differ significantly according to ANOVA (Tukey's test) at  $p < 0.05$ . CNF:100% wheat flour, 5%WSF:95% wheat flour with 5% walnut shell flour, 5%WSEF: 95% wheat flour with 5% extruded walnut shell flour, 10%WSF: 90% wheat flour with 10% walnut shell flour, 10%WSEF:90% wheat flour with 10% extruded walnut shell flour, 15%WSF:85% wheat flour with 15% walnut shell flour, 15%WSEF:85% wheat flour with 15% extruded walnut shell flour.

### 3.5. Chemical composition of breads (5%WSB and 5%WSEB) and tortillas (10%WST and 10%WSET)

The WSF and WSEF 5% composite breads showed no significant differences in protein, fat, and ash (table 4). Similar trend in the case of the protein and ash content found Purić et al., (2020) with the partial replacement of wheat flour by 5% of ground defatted apple seed cakes on wheat bread.

However, the supplemented breads with 5% of WSF and WSEF contain significantly ( $p < 0.05$ ) lower starch (%) than the control. A similar behavior was observed with the percentage of starch in tortilla treatments with 10% substitution.

Nevertheless, the protein and the fat content in tortillas decreased significantly ( $p < 0.05$ ) with the addition of WS and WSE. These results could be due to the low protein and fat content of the pecan walnut shell (Villasante et al., 2019).

The total fiber in both cases (bread and tortilla) increases significantly ( $p < 0.05$ ) with the addition of WSF and WSEF. In the case of the bread enriched with 5%WSF and 5%WSEF the soluble fiber increase 30 and 55% respectively with respect the control. The same samples increased the insoluble fiber content compared to the control by 65 and 70% respectively. According to the EC Regulation (2006) No. 1924, a claim that a food is a "source of fiber" or "high in fiber", may only be made where the product contains at least 3 or 6 g of total fiber per 100 g of bread (Union, 2006). The bread enriched with WSF and WSEF complies with this requirement. In tortilla with 10% of WSF or WSEF the soluble fiber increased 30 and 55% respectively with respect the control. In the case of



the insoluble fiber tortillas with WSF or WSEF showed significant differences, but between WSF and WSEF was no significant.

In their research, Alba et al. (2020) suggest that with the incorporation of dietary fiber into bread dough leads to change the bread characterizes as loaf volume, crumb, hardness and sensory properties. On the other hand, Lin et al. (2016) propose that the soluble fiber originated from fruits are habitually bound to phenolic compounds. They found that the pectin derived from blueberries bound anthocyanins that may have enhanced bioavailability for colonic microflora fermentation.

### 3.5.1. TPC and RSA

The total phenols, showed in table 4, demonstrated significant variations between the controls of the breads and tortillas, and the products containing walnut shell powder. Nevertheless, the samples with WSEF had the highest polyphenols contents in comparison to the controls, they presented almost 34% more in tortilla and 20% more in bread. However, between samples with the addition of WSF and WSFE didn't present significantly differences in the TPC. This could be for the high content of polyphenols in the pecan walnut shell. According De la Rosa et al. the shell is compounding by more of 60 mg GAE/g of sample. On the other hand, with the addition of WSFE in the bread and tortilla, the radical scavenging activity (DPPH) increment significantly ( $p < 0.05$ ) in comparison with the control and the products with WSF. Villasante et al. (2019) demonstrated that the extrusion process in walnut shell increment the radical scavenging activity. Brennan, Derbyshire, & Tiwari (2011) explain that the increase in extruded products is frequently due to their release from the cell wall matrix and to the temperature, screw speed and moisture used in the process. Anton, Ross, Lukow, Fulcher, & Arntfield (2008) showed the same results when adding different concentrations of red, black, pinto, or navy bean flours to wheat tortillas, the TPC and RSA increase. The reason could be for the content of phenolic compounds as flavonol glycosides, anthocyanins, and condensed tannins (Anton et al., 2008). In the case of bread, the values of percent of inhibition (DPPH) and ABTS+ of the treatments: 5%WSB and 5%WSEB were significantly higher than the control (table 4). In a study that worked with the addition of 5% raw mango peel powder to whole wheat bread, the DPPH (% inhibition) triplicated, from 21.51% to 68.54% inhibition, compared to the control (without mango).

On the other hand, the high content of fiber in the shell could be increment the antiradical capacity and the bioavailability of phenolic compounds.

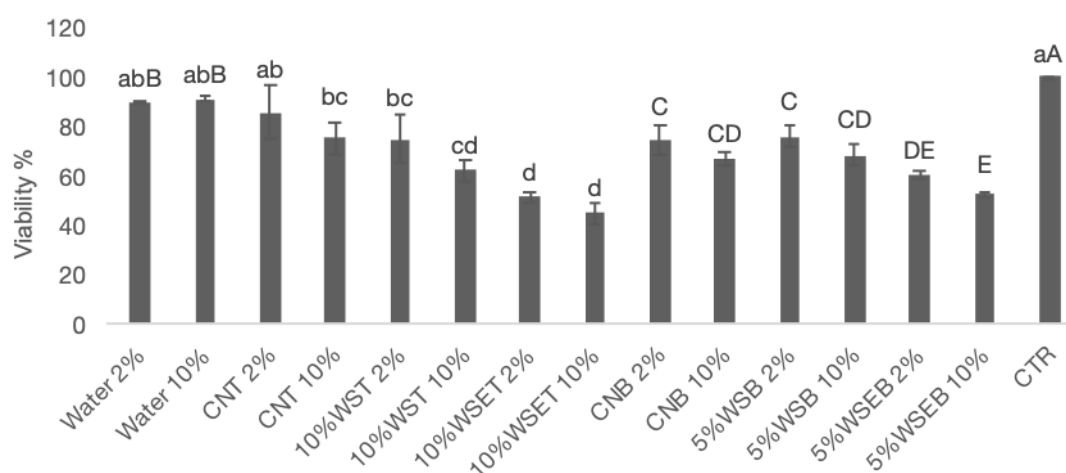
**Table 4.** Chemical composition, Total Phenolic Compounds (TPC) and Radical Scavenging Activity (RSA) of tortilla and bread added with WSF and WSEF

Parameter	CNB	5%WSB	5%WSEB	CNT	10%WST	10%WSET
Moisture (%)	32.22 ± 0.02 <sup>a</sup>	30.40 ± 0.01 <sup>b</sup>	29.92 ± 0.04 <sup>c</sup>	13.81 ± 0.02 <sup>a</sup>	12.28 ± 0.33 <sup>b</sup>	12.29 ± 0.01 <sup>b</sup>
Protein (%)	14.49 ± 0.34 <sup>a</sup>	13.23 ± 1.50 <sup>a</sup>	12.69 ± 0.35 <sup>a</sup>	12.65 ± 0.03 <sup>a</sup>	12.44 ± 0.04 <sup>b</sup>	12.16 ± 0.05 <sup>c</sup>
Fat (%)	4.19 ± 0.21 <sup>a</sup>	4.36 ± 0.39 <sup>a</sup>	4.26 ± 0.86 <sup>a</sup>	13.15 ± 0.02 <sup>a</sup>	12.64 ± 0.00 <sup>c</sup>	13.04 ± 0.02 <sup>b</sup>
Ash (%)	0.99 ± 0.01 <sup>a</sup>	1.04 ± 0.03 <sup>a</sup>	1.06 ± 0.01 <sup>a</sup>	1.98 ± 0.02 <sup>a</sup>	2.05 ± 0.12 <sup>a</sup>	1.91 ± 0.08 <sup>a</sup>
Starch (%)	64.30 ± 0.02 <sup>a</sup>	59.71 ± 0.02 <sup>b</sup>	57.99 ± 0.01 <sup>b</sup>	59.06 ± 0.31 <sup>a</sup>	49.60 ± 0.29 <sup>b</sup>	49.29 ± 0.27 <sup>b</sup>
Total fiber (%)	3.70 ± 0.10 <sup>c</sup>	9.32 ± 0.13 <sup>b</sup>	11.19 ± 0.18 <sup>a</sup>	5.64 ± 0.24 <sup>c</sup>	13.74 ± 0.61 <sup>b</sup>	16.04 ± 0.01 <sup>a</sup>
Soluble fiber (%)	0.90 ± 0.01 <sup>c</sup>	1.29 ± 0.01 <sup>b</sup>	2.00 ± 0.10 <sup>a</sup>	1.82 ± 0.02 <sup>c</sup>	2.62 ± 0.13 <sup>b</sup>	4.01 ± 0.00 <sup>a</sup>
Insoluble fiber (%)	2.80 ± 0.01 <sup>c</sup>	8.03 ± 0.14 <sup>b</sup>	9.20 ± 0.28 <sup>a</sup>	3.81 ± 0.22 <sup>b</sup>	11.12 ± 0.48 <sup>a</sup>	12.03 ± 0.01 <sup>a</sup>
TPC (mg GAE/g)	3.83 ± 0.01 <sup>b</sup>	4.53 ± 0.06 <sup>a</sup>	4.80 ± 0.19 <sup>a</sup>	2.46 ± 0.24 <sup>b</sup>	3.68 ± 0.13 <sup>a</sup>	3.75 ± 0.10 <sup>a</sup>
DPPH (%)	25.22 ± 0.17 <sup>c</sup>	35.81 ± 0.37 <sup>b</sup>	50.07 ± 0.84 <sup>a</sup>	15.33 ± 0.01 <sup>c</sup>	20.53 ± 0.20 <sup>b</sup>	30.11 ± 0.09 <sup>a</sup>
DPPH (TEAC in μmols/100 g)	135.97 ± 0.01 <sup>c</sup>	225.95 ± 0.37 <sup>b</sup>	402.26 ± 0.89 <sup>a</sup>	18.46 ± 0.01 <sup>c</sup>	74.06 ± 0.20 <sup>b</sup>	180.42 ± 0.09 <sup>a</sup>
ABTS <sup>+</sup> (TEAC in μmols/100 g)	477.49 ± 0.11 <sup>b</sup>	501.17 ± 0.12 <sup>a</sup>	518.34 ± 0.39 <sup>a</sup>	266.22 ± 0.06 <sup>b</sup>	288.48 ± 0.06 <sup>a</sup>	305.73 ± 0.16 <sup>a</sup>

Data is mean  $\pm$  standard deviation ( $n = 10$ ). Values with similar superscript (in the same product) in a column do not differ significantly according to ANOVA (Tukey's test) at  $p < 0.05$ . CNB or CNT: Bread or Tortilla with 100% wheat flour, 5%WSB: Bread with 95% wheat flour with 5% walnut shell flour, 5%WSEB: Bread with 95% wheat flour with 5% extruded walnut shell flour, 10% WST: Tortilla with 90% wheat flour with 10% walnut shell flour, 10%WSET: Tortilla with 90% wheat flour with 10% extruded walnut shell flour. DP: Digestible protein; GI: Glycemic index; DPPH:2,2-diphenyl-1-picrylhydrazyl; ABTS<sup>+</sup>: 2, 2'-Azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid); GAE: Gallic acid equivalent; TE: Trolox equivalent.

### 3.6. Viability-reducing activity of bread and tortilla extracts against human cancer cell lines.

The cytotoxicity of WSF and WSEF in bread and tortilla were evaluated in the cancer cell line (HepG2, hepatocellular carcinoma) according to the MTT assay (fig. 1). The samples showed some toxicity against human tumor cell lines. However, at the tested concentrations for tortilla, the sample 10%WSET at 10% inhibited more than 50% of the growth of the tumor cells (fig 1). The number of studies related to the anticancer activity of corn tortilla is limited; wheat tortilla has never been studied. Herrera-Sotero et al., (2017) worked with blue corn tortilla extracts and found antiproliferative effects against HepG2 at 1000  $\mu\text{g}/\text{mL}$ . For the bread, 5%WSEB extract significantly inhibited almost the 50% cell proliferation at 10% in comparison with the CTR. Glei et al. characterized in vitro the chemoprotective potential of an aqueous extract of bread, enriched with a green coffee extract in HepG2 cancer cell line; they found that, after 24 hours of incubation, the cell number decreased by approximately 50% (Palafox et al., 2019). In all the cases, the viability (%) was lower in extruded pecan walnut shell treatments, for both tortilla and bread. The results shown in the fig 1. were consistent with the findings in the table 4, demonstrating that the extrusion process in walnut shell increased the TPC and the radical scavenging activity (Villasante et al., 2019). Moreover, little information is available regarding the cytotoxicity of nuts shell in cancer cell lines. Sorice et al. (2016), Jung, Lee, Na, & Yu (2015) and Cacciola et al. (2019) had positive results in this regard; they worked with chestnut shell extracts in different cancer cell lines, including HepG2.



**Fig 1.** Growth inhibitory potency of wheat bread and tortilla enriched with different percent of pecan walnut shell extruded and non-extruded in comparison to wheat bread and tortilla in cancer hepatic cell line (HepG2).

Cell viability was assayed 48 h at two different concentrations (2 and 10%) of each extract. Control cells were non-treated (CTR), other control treated with 2 and 10% of water. Bread and tortilla

prepared with 5% or 10% (w/w) replacement of wheat flour with extruded or no-extruded pecan walnut shell. Each bar represents the mean  $\pm$  SD of three replicates. Different letters indicate significant differences between treatments ( $p < 0.05$ ).

### 3.7. Preference sensory analysis

The results of hedonic tests on the control with whole wheat flour and shell walnut breads and tortillas are shown in Table 5. Overall, no significant ( $p < 0.05$ ) difference was observed in the texture and acceptability parameters for any bread formulation. Chávez-Santoscoy et al. (2016) showed similar results with the production of bread with 0.5% black bean extract. The color attribute in all the samples was remarkably different ( $p < 0.05$ ) in comparison to the control; this was probably caused by the brown color of the shell. Rashida et al. obtained similar results in the production of breads with the addition of black gram flour (Ali et al., 2018). The flavor of bread was one of the most important parameters influencing its acceptance by consumers. This attribute depends on different variables, such as the ingredients and the process of bread production (Callejo, 2011; Quílez et al., 2006). Regarding bread, the samples with WSF and WSEF presented significantly ( $p < 0.05$ ) better results than the control.

On the other side, the addition of WSF and WSEF to the tortillas did not show a significant difference ( $p < 0.05$ ) in comparison to the control. However, the tortillas with 10%WSE received better evaluation than the tortilla with 10%WS. This result could be based on the TPA analysis at day 0 (table 3). Regarding the color, similar results were obtained for the bread: the panelists preferred the control tortillas. On the other hand, the results obtained for the flavor parameter and the acceptability of the CNT and 10% WSET were significantly ( $p < 0.05$ ) better than the treatment with 10%WST. Previously, it was demonstrated that the addition of grape pomace flour or red wheat flour contributed to the bitter and astringent flavor; this might be caused by the phenolic compounds of these flours (Avbelj et al., 2017; Grain et al., 2020).

**Table 5.** Score of sensory analysis of breads and tortillas with the addition of WS and WSE

Bread traits	CNB	5%WSB	5%WSEB
Texture	3.89 $\pm$ 0.92 <sup>a</sup>	3.84 $\pm$ 1.14 <sup>a</sup>	4.02 $\pm$ 1.00 <sup>a</sup>
Color	4.44 $\pm$ 0.80 <sup>a</sup>	3.68 $\pm$ 0.98 <sup>b</sup>	3.59 $\pm$ 1.10 <sup>b</sup>
Flavor	3.62 $\pm$ 1.02 <sup>b</sup>	3.76 $\pm$ 1.07 <sup>ab</sup>	4.06 $\pm$ 1.0 <sup>a</sup>
Acceptability	3.90 $\pm$ 0.86 <sup>a</sup>	3.87 $\pm$ 0.98 <sup>a</sup>	3.97 $\pm$ 0.93 <sup>a</sup>
Tortilla traits	CNT	10%WST	10%WSET
Texture	4.56 $\pm$ 0.70 <sup>a</sup>	4.25 $\pm$ 0.90 <sup>a</sup>	4.37 $\pm$ 0.89 <sup>a</sup>
Color	4.56 $\pm$ 0.67 <sup>a</sup>	3.71 $\pm$ 1.16 <sup>b</sup>	3.83 $\pm$ 1.06 <sup>b</sup>
Flavor	4.19 $\pm$ 0.91 <sup>a</sup>	3.88 $\pm$ 1.07 <sup>b</sup>	4.04 $\pm$ 0.91 <sup>ab</sup>
Acceptability	4.04 $\pm$ 0.94 <sup>a</sup>	3.46 $\pm$ 1.96 <sup>b</sup>	4.12 $\pm$ 0.86 <sup>a</sup>

Letters on the top right corner of each value indicate significantly different values between samples, according to ANOVA (Tukey's test) at  $p < 0.05$ . *Note.* Panelists scored using a 5-point hedonic scale: 1, nothing; 2, a little bit; 3, regular; 4, moderate; 5: a lot.

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**Supplementary material**

Fig 1.



Breads a) Control, b) 5%WSB and c) 5%WSEB

## **Chapter 7. Pecan kernel and shell packaging and encapsulation process**

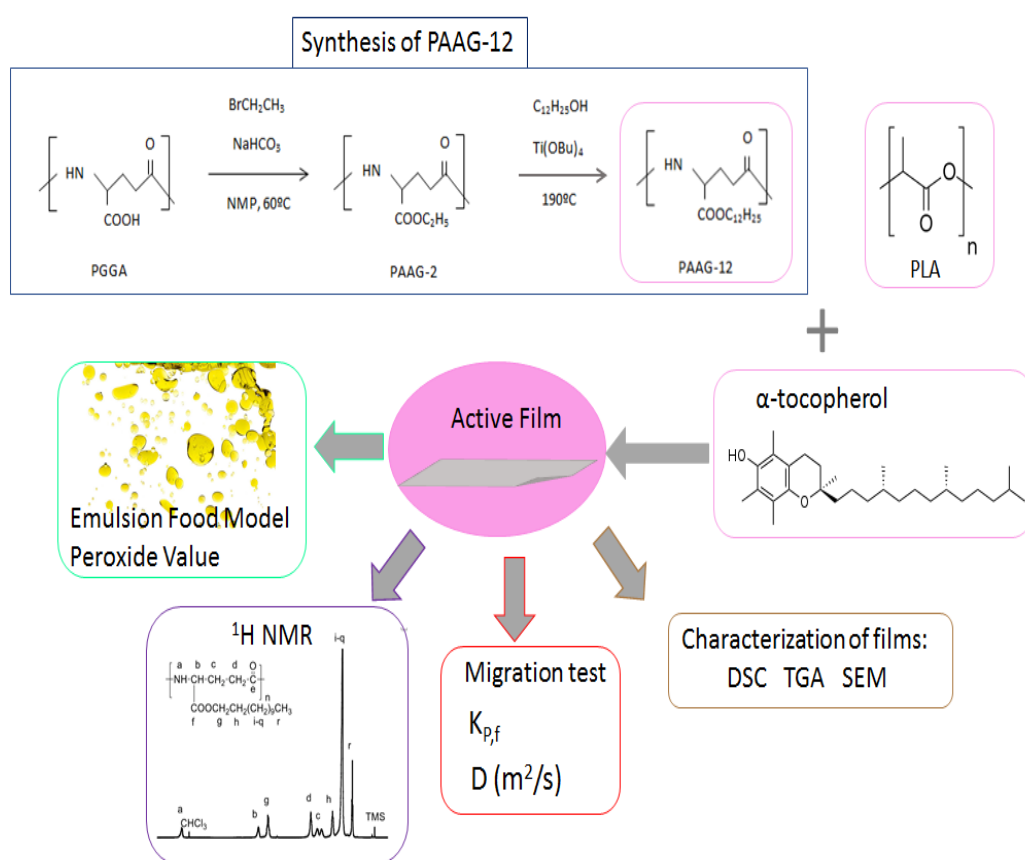
## 7.1. Poly ( $\alpha$ -Dodecyl $\gamma$ -Glutamate) (PAAG-12) and Polylactic Acid Films Charged with $\alpha$ -Tocopherol and Their Antioxidant Capacity in Food Model

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Article

# Poly ( $\alpha$ -Dodecyl $\gamma$ -Glutamate) (PAAG-12) and Polylactic Acid Films Charged with $\alpha$ -Tocopherol and Their Antioxidant Capacity in Food Models

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**Abstract:** Poly ( $\alpha$ -dodecyl  $\gamma$ -glutamate) (PAAG-12) was successfully synthesized from poly ( $\gamma$ -glutamic acid) (PGGA) according to Nuclear Magnetic Resonance (NMR) analyses. PAAG-12 films were prepared and enriched with 5%  $\alpha$ -tocopherol, with the aim of using them as novel antioxidant active packaging for food applications. Thermogravimetric Analysis (TGA) characterization determined that  $\alpha$ -tocopherol improved thermal stability of films, which is beneficial for industrial processing. Polylactic Acid (PLA) films prepared with the same amount of  $\alpha$ -tocopherol were used as a standard and both types of films were applied to two different food models to assess their protective action against oxidation. Water, 50% ethanol (EtOH) and 95% EtOH were used as food simulants and HPLC analyses were performed to determine diffusion and partition coefficients in PLA and the novel polymer, the latter exhibiting slower release rates. Primary oxidation was measured with peroxide value, which revealed that PAAG-12 films with  $\alpha$ -tocopherol protected oil-in-water (O/W) emulsions up to 29 days, complying with the Codex Alimentarius.

**Keywords:** active film; food packaging; characterization; NMR analyses; controlled release; antioxidant; emulsion; food simulant; polymer

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## 1. Introduction

Oxidation taking place in both raw and processed foods is a current concern to the food industry and market and, therefore, to researchers in this field. When the product is degraded, several oxidation products are created and the quality of food is decreased, due to texture and/or color change, taste deterioration and reduction in nutritional quality. Traditionally, synthetic antioxidants were directly added to food systems to protect them from oxidation. However, years of intense research have focused on effective natural additives, and synthetic antioxidants are currently being replaced by natural alternatives for food conservation [1,2].

There is a wide variety of antioxidants that can effectively protect food systems from oxidation.  $\alpha$ -Tocopherol is a lipophilic natural antioxidant used as an additive in many foods and cosmetics. It is the most active form of vitamin E, which acts as an antioxidant reacting with free radicals solubilized in membrane lipids. Its active center is located at the hydroxyl group in position six of the aromatic ring [3].

Ongoing research focuses on new methods to maintain properties of products by using natural antioxidants delivered gradually, which has been demonstrated to improve the efficiency of the antioxidant [4]. This progressive transmission of the additive is achieved by its loading to a matrix or carrier, which is usually a polymer film to simplify the applicability to food packaging, leading to the term active packaging [5–7].

This concept is gaining a lot of popularity specifically when the matrix is a biopolymer, due to several advantages such as the non-toxicity and environmental considerations. Among the wide range of available biopolymers, Polylactic Acid (PLA) is a renewable, biodegradable polyester easily obtained from carbohydrate sources such as corn starch, sugar cane and biomass byproducts [8]. It is used for multiple applications and exhibits good mechanical properties even when natural antioxidants are added [9–11].

The inclusion of  $\alpha$ -tocopherol in PLA films and the subsequent application in food models is a widely studied topic [12–15], but there are other polymers with promising characteristics that could be interesting carriers for this antioxidant, and that are currently being studied [16].

Comb like polymers are a special type of branched polymers that have been attracting a lot of attention recently due to their ability to order in a periodical layered structure [17]. Poly ( $\alpha$ -dodecyl  $\gamma$ -glutamate) (PAAG-12) is obtained from poly ( $\gamma$ -glutamic acid) (PGGA) by a two-step esterification. The latter is synthesized by bacteria of the genus *Bacillus*, which can be produced as a secretion product, either kept retained in the microorganism capsule or liberated in the medium [18].

In this study, PAAG-12 and PLA films were prepared and charged with 5% (w/w)  $\alpha$ -tocopherol. This amount was the optimum considering that a higher concentration made the films too brittle for manipulation and lacked applicability. The main goal of this study was to analyze the antioxidant activity against oil-in-water emulsions, both of the new synthesized film (PAAG-12) and of the PLA with  $\alpha$ -tocopherol, and to analyze the delivery of  $\alpha$ -tocopherol in different situations. Results showed that PAAG-12 has a slower release behavior than PLA and has proved to be a suitable film matrix for  $\alpha$ -tocopherol release into oily foods.

To our knowledge, this is the first study where PAAG-12 is applied as an active film for the conservation of food models.

## 2. Materials and Methods

### 4.1. Chemicals

Semicrystalline polylactic acid (PLA),  $M_w \approx 140000$  g/mol; Poly( $\gamma$ -glutamic acid) (PGGA) were supplied by Meiji Co., (Tokyo, Japan). The following reactants were purchased from Sigma-Aldrich Company Ltd.: 1-dodecanol ( $\text{CH}_3(\text{CH}_2)_{11}\text{OH}$ ), >98%;  $\alpha$ -tocopherol ( $\text{C}_{29}\text{H}_{50}\text{O}_2$ ); ethyl bromide ( $\text{C}_2\text{H}_5\text{Br}$ ), 98%; titanium tetrabutoxide ( $\text{Ti}(\text{OBu})_4$ ), 97%; iron (II) chloride tetrahydrate ( $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ ), 99%; alumina (aluminum oxide) ( $\text{Al}_2\text{O}_3$ ), 98%; hydrochloric acid (HCl), 37%; deuterated chloroform

(CDCl<sub>3</sub>), 99% D atoms; trichloroacetic acid (TCA) (Cl<sub>3</sub>CCOOH), 99%; trifluoroacetic acid (TFA) (CF<sub>3</sub>COOH), 99%; trimethylchlorosilane ((CH<sub>3</sub>)<sub>3</sub>SiCl), >98%; sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), >99%.

Sodium bicarbonate (NaHCO<sub>3</sub>), 99%; Polyoxyethylene (20) sorbitan monolaurate (Tween-20) (C<sub>58</sub>H<sub>114</sub>O<sub>26</sub>); solvent N-methylpyrrolidone (NMP) (C<sub>5</sub>H<sub>11</sub>N), 97%; and chloroform (CHCl<sub>3</sub>), >99%, were bought at Merck (Darmstadt, Germany). Ammonium thiocyanate (NH<sub>4</sub>SCN), 98%; toluene (C<sub>6</sub>H<sub>5</sub>CH<sub>3</sub>), >98%, were purchased from Panreac Química S.L.U (Barcelona, Spain). Ethanol (CH<sub>3</sub>CH<sub>2</sub>OH), 96%, was purchased from Solvech. Sunflower oil was purchased from a local supermarket

#### **4.2. Preparation of Films**

First, 5 g of Poly (γ-glutamic acid) (PGGA) was suspended in 0.2 L of N-methylpyrrolidone and stirred at 80 °C for 2 h. Then the mixture was cooled to 60 °C and 13.15 g (156 mmol) of NaHCO<sub>3</sub> and 15 mL of ethyl bromide were added (the latter in a continuous flow of 3 mL/h over 5 h). The mixture was stirred at 60 °C for 22 h. The ethylation reaction was followed by <sup>1</sup>H Nuclear Magnetic Resonance (NMR). After removing the NaBr precipitate by filtration, the reaction solution was poured into 1.5 L of cool HCl (pH = 1.5) to precipitate PAAG-2, which was then separated by filtration. The resulting powder was repeatedly washed with cool water and finally dried under vacuum at 50 °C.

PAAG-2 and 1-dodecanol were mixed at 1:35 molar ratio with Ti (OBu)<sub>4</sub> in 10% molar concentration of the polymer and left to stir under nitrogen at 190 °C for 8 h. The reaction progress was monitored by <sup>1</sup>H-NMR. Once the reaction was finished, the final solution was poured into ethanol at 40 °C. The mixture was further stirred for 2 h and was left to cool at room temperature. The precipitated polymer (PAAG-12) was separated from the supernatant solution by filtration. This polymer was then dissolved in chloroform to purify it and this step was repeated twice. Purification was accomplished by re-precipitation with methanol and finally dried under vacuum to obtain a white powder. Once the polymer was obtained, chloroform was used as solvent to prepare the film, with a ratio of 12 g of polymer for every 100 mL. Then 5% (w/w) α-tocopherol was prepared by diluting 500 mg of α-tocopherol in 5 mL of chloroform. It was added to the mixture and then spread onto Petri dishes (5 cm Ø, 0.3 g each) and left to dry at room temperature for 24 h. A film without α-tocopherol was also prepared. <sup>1</sup>H and <sup>13</sup>C NMR spectra were measured on a Nuclear Magnetic Resonance spectrometer (Bruker, model AMX-300, Germany); operating at 300 MHz for <sup>1</sup>H and 75 MHz for <sup>13</sup>C. All spectra were obtained in CDCl<sub>3</sub>/TFA, with chemical shifts expressed in ppm and coupling constant (J) in Hertz (Hz). To prepare the PLA film, semi crystalline PLA (5.125 g) was dissolved in chloroform (100 mL) and stirred until complete dissolution of the polymer. 5% α-tocopherol was added and the homogenized mixture was spread onto Petri dishes (11 cm Ø, 1.025 g each) and left to dry at room temperature. A negative control was prepared (without α-tocopherol).

#### **4.3. Characterization of PAAG-12 Films**

PAAG-12 films were characterized by Thermogravimetric Analysis using a thermogravimetric analyzer (METTLER-TOLEDO, model DSC/TGA 1 StarSystem), and by Differential Scanning Calorimetry (DSC) using a differential scanning calorimeter (PEKIN ELMER, model PYRIS 1 DSC). The antioxidant they contain, α-tocopherol, was also characterized in order to provide a comparison between films with and without the natural additive. SEM micrographics were also performed to observe the superficial morphology of both PAAG-12 and PLA films. Film thickness was determined.

To perform Thermogravimetric Analysis (TGA), samples of 10–15 mg of  $\alpha$ -tocopherol, PAAG-12 film with and without  $\alpha$ -tocopherol were introduced in aluminum cups. The samples were heated from 25 to 600 °C with a heating rate of 10 °C/min. The gas flow during the TGA was 20 ml/min, and it was performed in both air (21% O<sub>2</sub>) and nitrogen atmospheres to observe the differences. DSC analysis thermograms were obtained from 4–6 mg samples at heating and cooling rates of 10 °C·min<sup>-1</sup> under a nitrogen flow of 20 mL·min<sup>-1</sup>. Indium and zinc were used as standards for temperature and enthalpy calibration. The samples were heated from -60 to 150 °C at a speed of 10 °C/min. The temperature was maintained for 1 min before decreasing it again to the starting point and keeping it there for 1 min. The initial temperature rise was repeated once more, and after 1 min it was decreased to -75 °C at 150 °C/min. This temperature was maintained for 5 min and then raised again at 20 °C/min until 150 °C. Finally, the calorimeter was left to cool at room temperature. For  $\alpha$ -tocopherol, the DSC procedure involved a temperature increase from -60 to 150 °C at 10 °C/min and subsequent cooling at room temperature.

#### **4.4. Preparation of Food Simulants**

The first food model consisted of three solvents: 2 food simulants (water and 50% aqueous ethanol) and 95% aqueous ethanol. Each of them represented a different polarity, and therefore varying capability of extracting lipophilic substances from the carrier, such as  $\alpha$ -tocopherol from PAAG-12 and PLA films. The 2 food simulants were selected according to European Commission regulation (EU) No 10/2011 on plastic materials and articles intended to come into contact with food. The 95% aqueous ethanol was used to simulate extreme conditions such as very fatty foods, high temperatures, etc., because it is a concentration that can also be used.

To perform experiments using these solvents, loaded films were cut into rectangular pieces with a total area of 4 cm<sup>2</sup>. Food simulant was then added to vials and substances left to migrate naturally at a constant temperature (30 °C) and mild shaking speed. The liquid volume/area ratio was 6.25 mL/cm<sup>2</sup> complying with ASTM D4754-98, which establishes a ratio between 155 and 0.31 mL/cm<sup>2</sup>. Daily samples were taken over 13 days from the vials to analyze by HPLC the amount of  $\alpha$ -tocopherol present in the food simulant and were kept frozen (-80 °C). This time span was selected to enable the observation of the slow release with time of the antioxidant from the matrix.

Samples were injected directly into an HPLC (Waters Alliance 2695 HPLC) with Diode-Array Detector Waters 996, equipped with an autosampler. The column was Ultrabase 100, 3 microns, ODS2 100 × 4.6 mm (Akady). The mobile phase was 100% methanol, therefore isocratic procedure was employed, at a flow rate of 0.8 mL/min. The test lasted 12 min and the injection volume was 20  $\mu$ L. Signals at a wavelength of 292 nm were stored and collected by Software Empower (Waters).

##### **2.4.1. Estimation of Partition Coefficients and Diffusivities**

Results of the release study were quantified by calculating the partition coefficient ( $K_{p,f}$ ) and diffusivity ( $D$ ) for each type of film.

From the three general cases of mass transfer theory [19], the one selected was the one in which the film is in contact with an infinite volume of food/simulant and the external mass transfer coefficient is negligible. This assumption is done considering  $K_{p,f} < 1$ , when the migrant, in this research  $\alpha$ -tocopherol, has higher affinity towards the food system (this antioxidant is lipophilic therefore has an affinity for targeted oily foods).

The estimation of diffusivities can be performed using Fickian diffusion models, in which simplifications can be made [20] and the equation becomes Equation (1):

$$\frac{M_{f,t}}{M_{f,\infty}} = 1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp\left[-\frac{(2n+1)^2}{4L^2} D\pi^2 t\right] \quad (1)$$

where  $M_{f,t}$  is the mass of the migrant in the food (simulant) at a particular time  $t$ ,  $M_{f,\infty}$  is the mass of the migrant in the food (simulant) at equilibrium,  $L$  is the film thickness,  $D$  is the migrant diffusivity and  $t$  is time (International System of Units, SI).

$$\frac{M_{f,t}}{M_P} = \frac{4}{L} \left(\frac{Dt}{\pi}\right)^{0.5} \quad (2)$$

where  $M_P$  is the initial amount of migrant in the film.

The partition coefficient  $K_{P,f}$  is defined as the ratio of the migrant concentration in the film ( $C_{p,\infty}$ ) to the migrant concentration in the food simulant ( $C_{f,\infty}$ ), both at equilibrium:

$$K_{P,f} = \frac{C_{p,\infty}}{C_{f,\infty}} \quad (3)$$

(migration results are presented using  $D$  ( $m^2/s$ ) and  $K_{P,f}$  values).

#### **4.5. Preparation of Oil-In-Water (O/W) Emulsions and Determination of Primary Oxidation**

Oil-in-water emulsions were prepared using sunflower oil according to previous research [22] and served as the second food model. Samples were analyzed by peroxide value.

Primary oxidation was assessed by peroxide value using the ferric thiocyanate method with calibration using the official method of the Association of Official Analytical Chemists (AOAC)', according to methodology described in previous research [23]. Analyses were performed using a UV/Vis absorbance spectrometer (FLUOstar Omega), equipped with a temperature-controlled incubation chamber and a fluorescence multiplate reader (BMG Labtech, Ortenberg, Germany). Results are expressed as milliequivalents (meq) of hydroperoxide per kg of emulsion.



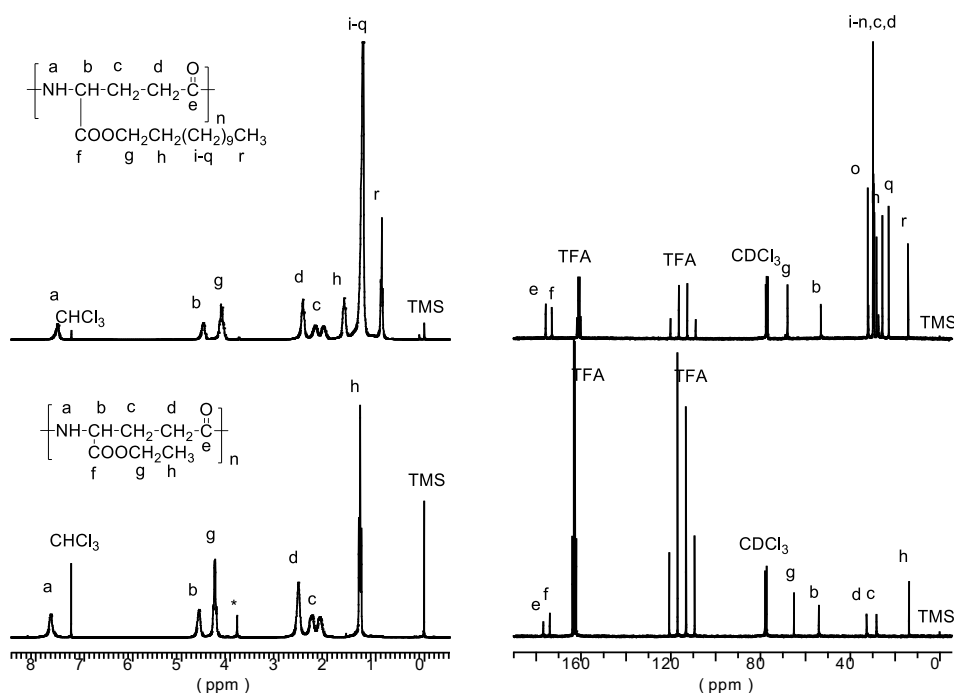
#### 4.6. Statistical Analyses

Peroxide value was determined in triplicate. Data were analyzed using one-way analysis of variance (ANOVA) and Tukey's comparison test was used to determine significantly different values ( $p < 0.01$ ) using Minitab 18 software.

### 3. Results and Discussion

#### 3.1. PAAG-12 Synthesis and Film Preparation

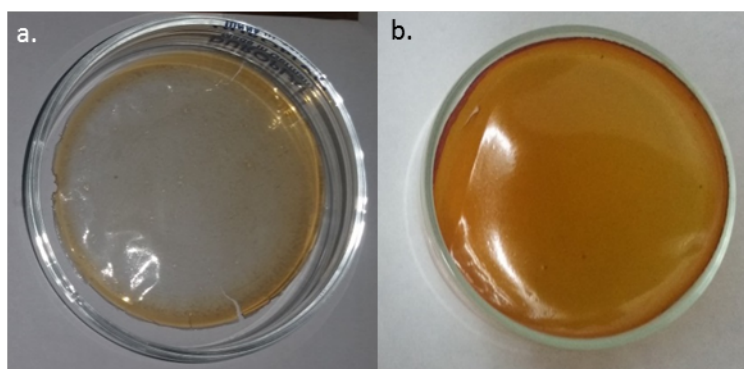
Esterification of PGGGA with ethyl bromide was checked by  $^1\text{H}$  NMR. Signals corresponding to methylene of ethyl group were compared with the  $\alpha$ -CH group of PGGGA to determine the degree of esterification. Both the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of PAAG-2 with peak assignments obtained at the end of the reaction are shown in Figure 1 (bottom). This polymer was then modified by transesterification with 1-dodecanol, where the ethyl group was totally replaced after 8 hours of reaction at  $190\text{ }^\circ\text{C}$ . The degree of transesterification was checked by comparing the signals of the  $\alpha$ -CH group and the signals of the side methylenes and methyl of the dodecyl side chain. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of purified PAAG-12 with peak assignments are shown in Figure 1 (top).



**Figure 1.**  $^1\text{H}$  (left) and  $^{13}\text{C}$  (right) Nuclear Magnetic Resonance (NMR) spectra of Poly( $\alpha$ -dodecyl  $\gamma$ -glutamate) (PAAG-12) (top) and PAAG-2 (bottom) with peak assignments.  $\text{CDCl}_3$ : deuterated chloroform; TFA: trifluoroacetic acid; TMS: tetramethylsilane.

Figure 2 depicts the prepared films. The main difference between them is the color. PAAG-12 films are transparent (Figure 2a), and when adding  $\alpha$ -tocopherol, they have an amber tint (Figure 2b.). They can be also told apart by touch, as control films are more brittle than the loaded ones,

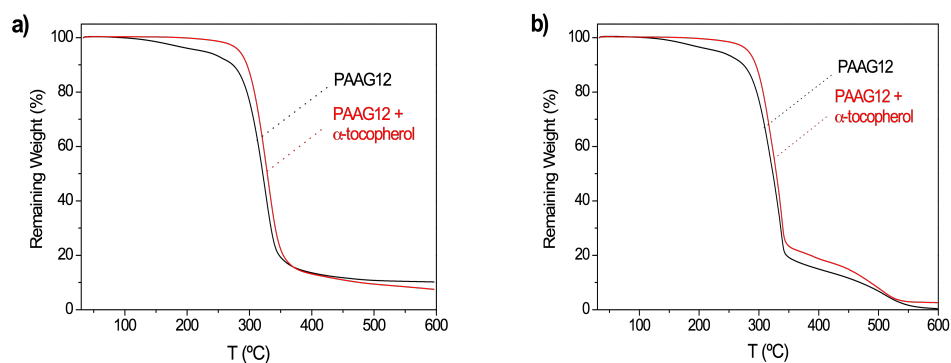
which present a gummy, moldable texture. The thickness of the PAAG-12 film with  $\alpha$ -tocopherol was 0.142 mm.



**Figure 2.** PAAG-12 control film (a) and film with 5%  $\alpha$ -tocopherol (b).

#### 4.7.3.2. PAAG-12 Film Characterization Using TGA and DSC

Thermogravimetric analyses were performed in a nitrogen atmosphere for both PAAG-12 films and were compared, as depicted in Figure 3. Both nitrogen and air atmospheres were used to test the effect of different oxidant and inert atmospheres in the thermal degradation.



**Figure 3.** Thermogravimetric Analysis (TGA) of the PAAG-12 control film and the film charged with 5%  $\alpha$ -tocopherol in (a) nitrogen and (b) air atmospheres.

**Table 1.** Values of TGA of the PAAG-12 control film, the film charged with 5%  $\alpha$ -tocopherol and  $\alpha$ -tocopherol film in nitrogen and air atmospheres.

Sample	Atmosphere	TGA a		
		$\sigma$ Td (°C)	maxTd (°C)	Rw (%)
PAAG-12	N2	226.4	325.6	10.2
	Air	229.1	337.4	0.4
PAAG-12 + 5% $\alpha$ -tocopherol	N2	284.1	328.9	7.5
	Air	279.4	338.7	2.6
$\alpha$ -tocopherol	N2	275.8	365.7	0
	Air	291.1	341.1	0.2

<sup>a</sup> Thermal decomposition temperature measured at 5% of weight loss ( $^{\circ}T_d$  ( $^{\circ}C$ )) and at maximum weight loss rate ( $^{\max}T_d$  ( $^{\circ}C$ )).  $R_w$ : weight (%) remaining after heating at 600  $^{\circ}C$ .

PAAG-12 shows a thermal decomposition that starts at about 200  $^{\circ}C$ , with a maximum decomposition rate at 226.4 and 229.1  $^{\circ}C$  for both nitrogen and air atmospheres. It is observed that there is not much effect of the atmosphere on the thermal stability of this polymer. When these films were charged with  $\alpha$ -tocopherol the thermal stability was substantially increased with an escalation of the onset temperature of about 58 and 50  $^{\circ}C$ , respectively, and slight rises in the maximum weight loss rates temperature. The enhanced thermal stability of this antioxidant on other polymers has been reported and correlated to its ability to efficiently deactivate all damaging free radicals, primarily alkylperoxyl and alkyl radicals generated in the thermal degradation processes [24]. These results are very promising because they show that the incorporation of a natural antioxidant such as  $\alpha$ -tocopherol in this polymer improves its low thermal stability, thus allowing a better processability at higher temperatures.

In order to see if the thermal behavior of  $\alpha$ -tocopherol or PGGGA-12 was affected by the mutual blending, DSC thermograms were recorded. Unfortunately, no transitions were observed for both PGGGA-12 and  $\alpha$ -tocopherol or their blends, which prevents conclusions from being drawn from these DSC thermogram analyses.

### **3.3. Food Models**

Two food models were used to test the antioxidant capacity of PAAG-12 films charged with 5%  $\alpha$ -tocopherol. PLA films charged with the same amount of antioxidant were also used as a comparison.

#### **3.3.1. Food Simulant**

The first food models were three types of solvents: water, 50% aqueous EtOH (as food simulants) and 95% aqueous EtOH (it was used to simulate extreme conditions such as very fatty foods, high temperatures, etc., and it is a concentration that can also be used). HPLC was used to determine  $\alpha$ -tocopherol concentration.

Solvents in contact with PAAG-12 and PLA films were analyzed by HPLC and results demonstrated there was a progressive liberation of  $\alpha$ -tocopherol in both ethanolic solvents, as summarized in Table 2. However, the migration was much higher for 95% EtOH than 50% EtOH. This was corroborated by  $^1H$  NMR analyses conducted on PAAG-12 films after application to food simulants (exhausted films); the remaining antioxidant in the polymer matrix is lower for 95% EtOH medium than 50% EtOH. This tendency was also observed in previous studies with synthetic phenolic antioxidants in PLA films [25]. Due to the lipophilic nature of  $\alpha$ -tocopherol, no migration was observed when water was used as food simulant. Other substances added to PLA films such as cinnamaldehyde exhibited high rates of liberation at 20 days of contact [26] when using ethanol 50% as food simulant.

**Table 2.** Diffusion ( $D$ ) and partition coefficients ( $K_{p,f}$ ) for  $\alpha$ -tocopherol migration from PAAG-12 and PLA films into food simulants.

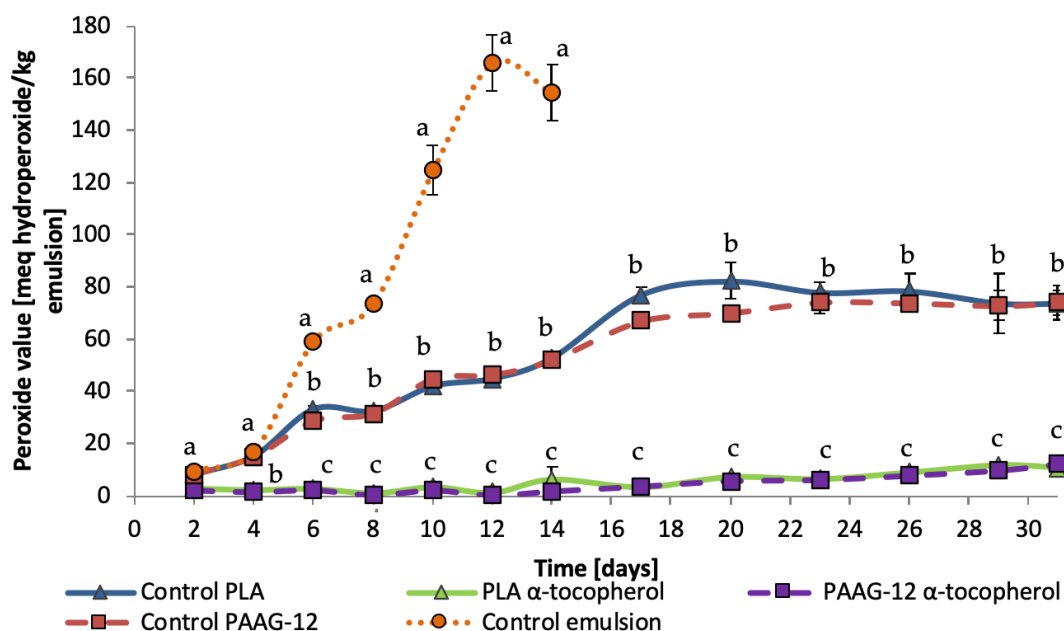
Polymer	Simulant	$K_{p,f}$	$D$ ( $m^2/s$ )
PAAG-12	EtOH 50%	1.05	$1.12 \times 10^{-11}$
	EtOH 95%	0.37	$5.58 \times 10^{-10}$
PLA	EtOH 50%	0.94	$1.11 \times 10^{-10}$
	EtOH 95%	0.36	$1.12 \times 10^{-10}$

It was observed that PLA active films allowed higher migration of  $\alpha$ -tocopherol to solvents, and this was especially marked with the 50% EtOH simulant. Several authors have found higher diffusion rates from films in higher amount of ethanol simulants, apparently due to the increased swelling of the polymer, thus creating void spaces favoring the migration of compounds [13,27].

When comparing both films, PAAG-12 exhibits lower release rates than PLA with the same simulant conditions. This indicates this novel biopolymer could be an alternative polymer matrix that can provide progressive release of antioxidants, for applications where slow liberations are intended

### 3.3.2. Primary Oxidation of O/W Emulsions Determined with Peroxide Value Assay

Thirty-one days tests were performed on emulsions protected with the films. Results were plotted and expressed as milliequivalents of hydroperoxide/kg emulsion with time, as can be seen in Figure 4



**Figure 4.** Primary oxidation value by peroxide assay of oil-in-water (O/W) emulsions protected by control emulsion, control PAAG-12, control PLA, PLA  $\alpha$ -tocopherol and PAAG-12  $\alpha$ -tocopherol films. Samples with different letters denote significant differences ( $p < 0.01$ ).

In Figure 4, three different behaviors are observed; on the one hand, the emulsion without any film (control emulsion) exhibits the highest oxidation rates. Oxidation measurements were stopped after 14 days as the peak of primary oxidation was reached. Further measurements would display parallel reactions of decomposition of chemical substances produced by oxidation, diminishing meq hydroperoxide, which would alter interpretation.

On the other hand, there are the O/W emulsions protected with films that do not contain  $\alpha$ -tocopherol. They undergo less oxidation than the control emulsion, implying that the polymer itself has an antioxidant effect. This represents a 72% reduction in oxidation at day 12 and 55.7% at the end of the experiment, day 31.

Finally, there are the films containing  $\alpha$ -tocopherol. It should be noted that after 31 days they have only reached a peroxide value (PV) of 10 meq hydroperoxide/kg emulsion, which indicates that these active films are very powerful antioxidants, protecting the emulsion up to 92.6% compared to the control.

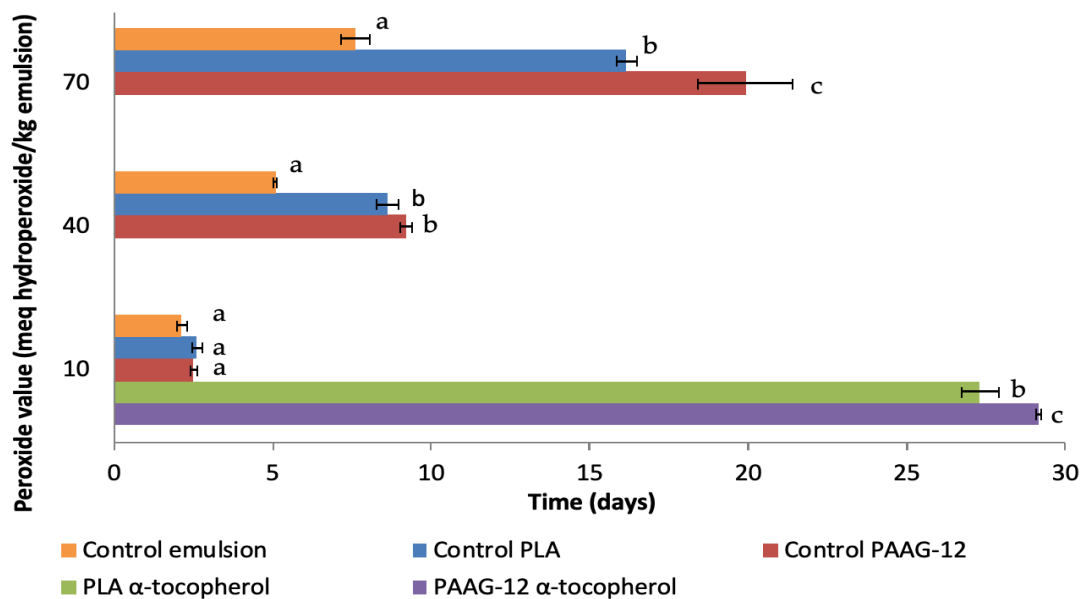
In order to provide a comparison of the films studied, the time to reach PV = 10, 40 and 70 meq hydroperoxide/kg emulsion has been calculated and is displayed in Figure 5. The different peroxide values were selected taking into consideration the compliance with the Codex Alimentarius, in which the maximum acceptable level of PV for refined vegetable oils is 10 milliequivalent/kg [28].

It can be observed that the addition of 5%  $\alpha$ -tocopherol to both films provides a strong protection, increasing the duration of the good condition of the emulsion (10 meq/kg) up to 29 days for PAAG-12 films and 27 days for PLA films. This is the reason for the lack of data for these two samples at 40 and 70 meq/kg in Figure 5, as those high oxidation values were not achieved.

The protection levels offered by the film without the antioxidant are only visible at higher peroxide values, as for the 10 meq/kg there were no significant differences between control emulsion and control films.

Previous studies conducted in our research team exhibited antioxidant protection with PLA active films in O/W emulsions up to 20 days [22] when using thyme and rosemary lyophilized extracts.

Other authors conducted similar peroxide value experiments using marigold flower extract instead of  $\alpha$ -tocopherol and prepared PLA pouches containing soybean oil [29] but surpassed the 10 meq/kg value before day 5.



**Figure 5.** Time for samples to reach different peroxide values (graph). Samples with different letters denote significant differences ( $p < 0.01$ ).

#### 4. Conclusions

PAAG-12 was successfully synthesized from PGGA as demonstrated by NMR spectra. The increase of the onset temperature of PAAG-12 film when adding the natural antioxidant  $\alpha$ -tocopherol validates the improvement of the thermal stability of the branched polymer, which entails a better processability for industrial applications for food packaging.

When assessing the first food model, higher migration of  $\alpha$ -tocopherol from films to food simulants was observed when those simulants had higher ethanol concentration. This was demonstrated by HPLC analyses of food simulants and NMR of exhausted films. It was also revealed that PLA allowed a higher migration of antioxidant into the food simulant medium than PAAG-12 at short contact times, which demonstrates that this novel polymer is a promising matrix for active packaging applications. The peroxide value assay of O/W emulsions proved the extraordinary protection provided by active films, capable of increasing the lifespan in compliance with the Codex Alimentarius (10 meq/kg) up to 29 days.

**Author Contributions:** Conceptualization, S.M. and M.A.; Data curation, J.V., E.C. and G.I.H.; Formal analysis, J.V. and A.M.; Funding acquisition, M.A.; Investigation, J.V., E.C. G.I.H.; Project administration, M.A.; Resources, M.A.; Supervision, A.M., S.M. and M.A.; Writing—original draft, G.I.H.; Writing—review and editing, J.V., G.I.H., A.M. and M.A.

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**Conflicts of Interest:** All authors have approved the final manuscript.

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## 7.2. Characterization and application of gelatin films with pecan nut and nut shell extract (*Carya illinoensis*)

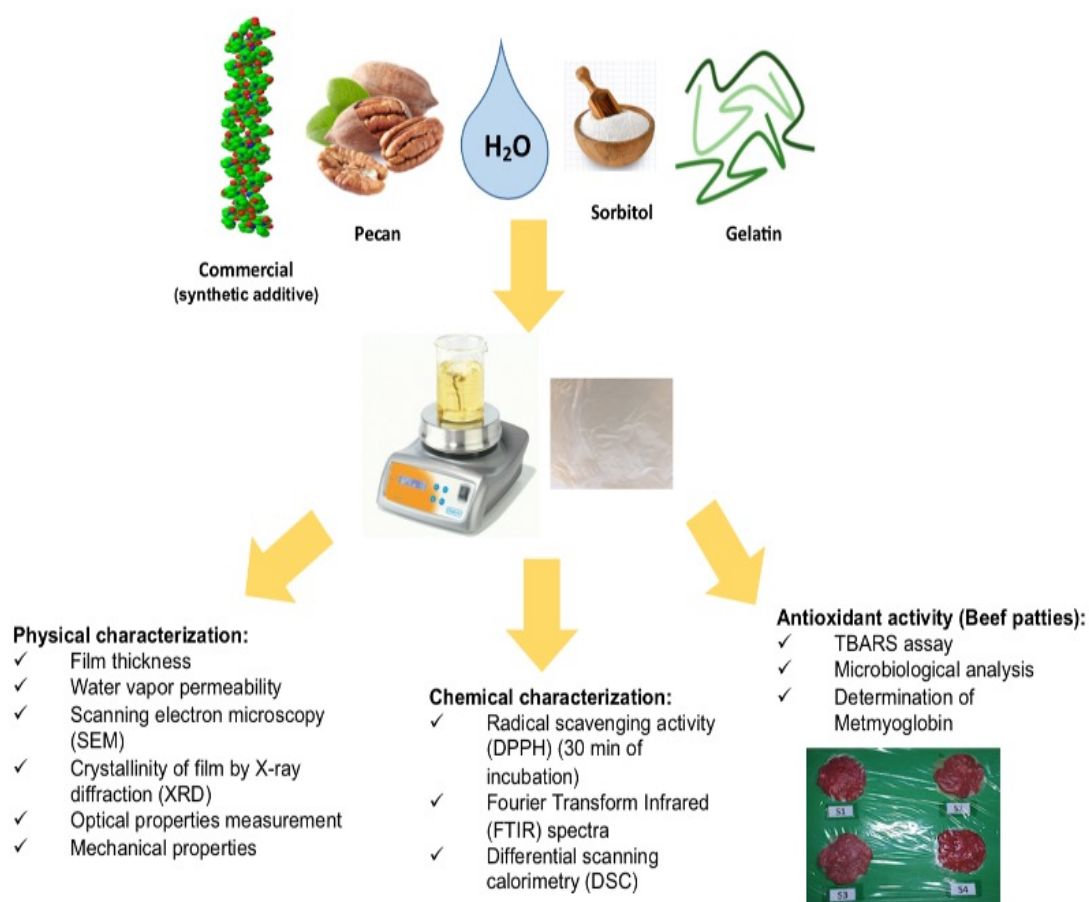
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Article

# Characterization and Application of Gelatin Films with Pecan Walnut and Shell Extract (*Carya illinoensis*)

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**Abstract:** Phenolic compounds that come from natural products are a good option for minimizing lipid oxidation. It should be noted that these can not only be introduced directly into the food, but also incorporated into edible biofilms. In contact with food, biofilms extend its useful life by avoiding contact with other surfaces and preventing deterioration due to air, one of the main objectives. In particular, gelatin is a biopolymer that has a great potential due to its abundance, low cost and good film-forming capacity. The aim of this study has been to design and analyse gelatin films that incorporate bioactive compounds that come from the pecan walnut and a by-product, the pecan walnut shell. The results showed that mechanical and water vapor barrier properties of the developed films varied depending on the concentration of the pecan walnut, shell and synthetic antioxidant. With increasing pecan walnut concentration (15%) the permeability to water vapor ( $0.414 \text{ g}\cdot\text{mm}/\text{m}^2\cdot\text{day}\cdot\text{Pascal}$ ,  $\text{g}\cdot\text{mm}/\text{m}^2\cdot\text{day}\cdot\text{Pa}$ ) was significantly lower than the control ( $5.0368 \text{ g}\cdot\text{mm}/\text{m}^2\cdot\text{day}\cdot\text{Pa}$ ). Furthermore, in the new films the elongation at the break and Young's modulus decreased by six times with respect to the control. Films with pure gelatin cannot act as an antioxidant shield to prevent food oxidation, but adding pecan walnut (15% concentration) presents 30% inhibition of the DPPH stable radical. Furthermore, in the DSC, the addition of pecan walnut (15 and 9% concentrations), showed the formation of big crystals; which could improve the thermal stability of gelatin films. The use of new gelatin films has shown good protection against the oxidation of beef patties, increasing the useful lifetime up to nine days, compared to the control (3–4 days), which provides opportunities for the commercialization of meat products containing lower concentrations of synthetic products.

**Keywords:** gelatin based films; pecan walnut; shell; antioxidants; physicochemical; meat

## 1. Introduction

The food industry has been gaining interest in biodegradable food packaging, not only because they protect the product, but because they can also incorporate bioactive compounds that help lengthen the food's shelf-life. Studies confirm that protein based biodegradable films present excellent

mechanical properties and a good barrier towards oxygen, microorganisms and humidity [1]. Furthermore, they are natural and safe [2].

The partial acid/base hydrolysis of collagen leads to gelatin production, which comes from bones and skin from different sources such as fish or stock (bovine, porcine and avian) [3]. This protein is of great interest, due to its low cost and easy production. The main disadvantage of gelatin based films is their hydrophilicity and, as a result, high water permeability [4]. Adding hydrophobic compounds would allow improvement of this trait. Several studies have shown that adding natural compounds to gelatin based films allows a controlled release of the active components (tocopherols, phenolic compounds and essential oils) and also helps prevent lipid oxidation and microbial contamination [5]. Examples of this are the addition of some compounds such as a curcumin derivative [6], rosmarinic acid, cinnamon bark essential oil [7], green tea, grape seed, ginger, ginkgo leaf extract [8] and acerola powder [9]. The possibilities and studies are very vast. Some have shown very positive results, because there has been an increase in total polyphenols, radical scavenging activity and antimicrobial activity in the foods that it has acted upon. Furthermore, there are other investigations that show that the interaction between the gelatin protein and polyphenols increases their stability and bioactivity [10].

The pecan walnut (*Carya illinoensis*) belongs to the Juglandaceae family. It comes from the south of the United States and the north of Mexico. It is a high nutritional value nut, due to its polyunsaturated fats, such as linoleic acid [11]. It is also a protein, vitamin and mineral source and an excellent source of tocopherols, sterols, carotenoids and aliphatic alcohols [12]. A recent study has proved that using pecan walnut as an antioxidant in sardines prevented their deterioration [13]. Other studies have shown that including the use of pecan walnut in diets can reduce the incidence of cardiovascular diseases, like type 2 diabetes, Alzheimers, Parkinson and cancer [14–16].

Other parts of the pecan walnut, such as the leaves, the fruit, the green shell and the dry shell contain a high content of photochemical compounds (phenols, condensed tannins, flavonoids and procyanidins) [17–20]. The shell of the fruit represents 40–50% of the pecan walnut's weight [21]. Nowadays, the shell is used for infusions and it is also considered an organic residue from the food industry, which can cause serious problems to the environment [22–24].

The aim of this study is the development of gelatin based film for food packaging with the addition of different concentrations of pecan walnut, shell and commercial preservatives, characterizing them and studying how they affect the oxidation of beef burgers when they are brought into contact at a cooling temperature. The addition of this residue aims to improve not only the physical and mechanical properties of films but also to increase the antioxidant capacity.

## **2. Materials and Methods**

### **2.1. Materials**

All the chemical reagents including gallic acid, 2,2-diphenyl-1-picrylhydrazyl, Trolox, methanol, ethanol, triptone soya agar and Ringer solution were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Sorbitol was purchased from Calbiochem; Neutral Gelatin from Hacendado (Barcelona, Spain); pure water (Millipore-Q system, Barcelona, Spain). Synthetic antioxidant "Commercial" (dextrin, dextrose, 5.7% SO<sub>2</sub>, potassium metabisulfite, sodium ascorbate and sodium citrate) is commonly used in the meat industry (Barcelona, Spain).

## **2.2. Sample and Extracts Preparation**

Pecan walnut (PW) and shell (PWS) were collected in the north of Mexico, they were frozen with liquid nitrogen and shredded with a mortar. Extracts were prepared in order to assay radical scavenging activity and total phenolic content, and a concentrated extract (extract with the ethanol evaporated) to incorporate in the gelatin films. PW and PWS were weighed (3 g) and extracted with 20 mL of 50:50 (v/v) ethanol:water. The extracts were stirred for 90 min at room temperature. Both extracts were centrifuged and the supernatants were stored at  $-80\text{ }^{\circ}\text{C}$ . To prepare the concentrated extracts, ethanol was completely evaporated with Rotavapor R-200 with vacuum control V-800.

## **2.3. Film Preparation with PW and PWS Extracts**

To prepare the film forming solution, 1.2 g of neutral gelatin and 0.24 g of sorbitol as plasticizer were dissolved in 30 mL of 50:50 ethanol:water solution. Then, the solution was stirred for 10 min at  $40\text{ }^{\circ}\text{C}$  until an even mix was formed. Afterwards, different volumes of PW extract were added: 30, 20 and 10 mL (final percentages in the film forming solution 50%, 30% and 10%) that represent 15%, 9% and 3% of initial dry PW, respectively. For the PSW films, only 10% of the extract was used (3% of dry PSW) because higher concentrations were operationally impaired. The solutions were made up to 60 mL with the ethanolic solution and stirred for 15 min. Then, the solutions were applied to plates (23 cm  $\times$  15 cm) and covered with parafilm paper. They were dried at room temperature for 5 days.

## **2.4. Chemical Analysis**

### **2.4.1. Total Polyphenol Content (TPC) of PW and PWS Extract**

The Folin–Ciocalteu colorimetric methodology with several modifications was followed in order to determine the TPC by absorbance in a spectrophotometer [25]. A 1:10 diluted sample with milli-Q water was stirred and the absorbance was measured at 765 nm with Fluostar Omega (BMG Labtech, Ortenberg, Germany). The concentration was expressed as mg of gallic acid equivalents (GAE)/g dry weight (DW) for the total polyphenolic content (TPC). All samples were analysed in triplicate. The standard curve was constructed by plotting the absorbance against the gallic acid concentration, over the range from 0.11 to 1.72 mM.

### **2.4.2. Radical Scavenging Activity of PW and PWS**

For the radical scavenging activity, the DPPH (2,2-diphenyl-1-picrylhydrazyl) method was used [26]. Results are expressed in  $\mu\text{mol}$  Trolox Equivalents (TE)/g DW. Measurements were done in triplicate for each sample.

### **2.4.3. Radical Scavenging Activity of the Films Measured by DPPH**

DPPH radical scavenging activity was determined according to the method of Jongjareonrak et al. with some modifications [27]; 0.1 g of the sample was dissolved in 5 mL of methanol for 30 min. Then an aliquot of 20  $\mu\text{l}$  of the methanolic extract was mixed with 200  $\mu\text{l}$  of 0.06 mM DPPH methanol. It was stirred and left to rest at  $25\text{ }^{\circ}\text{C}$  in darkness for 75 min. The absorbance of the mix was measured at 517 nm using a Fluostar Omega (BMG Labtech, Ortenberg, Germany).

The inhibition of the stable DPPH radical was calculated by using the following equation:

$$Inhibition(\%) = \frac{A_0 - A_s}{A_0} \cdot 100 \quad (1)$$

where  $A_0$  is the absorbance of the initial DPPH solution at 517 nm, and  $A_s$  is the absorbance of the DPPH solution exposed to the sample at time 75 min.

#### 2.4.4. Fourier Transform Infrared (FTIR) Spectra

The method followed was reported by Staroszczyk et al., with some modifications [28]. FT-IR analysis was performed with the Spectrum Two™ FT-IR Spectrometer (PerkinElmer, Waltham, USA). The spectra were determined in an infrared region between 4000  $\text{cm}^{-1}$  and 400  $\text{cm}^{-1}$  (transmittance). Each sample was measured eight times.

#### 2.4.5. Differential Scanning Calorimetry (DSC)

The thermal properties of the films were determined by DSC following the methodology reported by Ramis [29], using a Thermal Analysis System, Mettler–Toledo DSC30 (Schwerzenbach, Switzerland). The films ( $10.00 \pm 0.25$  mg) were put into an aluminum DSC open crucible. All samples were cured in a nitrogen atmosphere with an air flow of 50 mL/min. The studies were performed in a temperature range of 30–250°C, with a heating rate of 10 °C/min. The fusion enthalpies were calculated with the integration of the calorimetric signal using a straight base line, with the STARE software (Mettler Toledo company, Barcelona, Spain).

### 2.5. Physical Analysis

#### 2.5.1. Film Thickness

The film thickness was measured by a handheld micrometer Mitotuyo, No. 7327 (Kawasaki, Japan) with 0.001 mm accuracy. The film was measured at five random positions. The average of these values was accepted as the film thickness.

#### 2.5.2. Water Vapor Permeability (WVP)

The permeability values to water vapor were measured as they were by Shiku et al., with some modifications [30]. Permeation cups with silica gel were used; then, the films were cut and put into the discs. The supports (the cups) were introduced into an oven that had a relative humidity of 99% at  $25.0 \pm 0.1$  °C for 24 h. They were weighed before and after introducing them into the drier and the entire procedure was repeated twice. To calculate the permeability to water vapor, the following formula was used:

$$WVP\left(\frac{g \cdot mm}{m^2 \cdot days \cdot Pa}\right) = \frac{(W_f - W_o) \cdot x}{A \cdot t \cdot (P_2 - P_1)} \quad (2)$$

$W_f$  is the final weight (g),  $W_o$  is the initial weight (g),  $x$  is the film thickness (mm),  $A$  is the area of exposed film ( $m^2$ ),  $t$  is the time of incubation (days) and  $(P_2-P_1)$  is the vapor pressure differential across the film (Pascal: Pa).

### 2.5.3. Scanning Electron Microscopy (SEM)

The morphology of the surface and the transversal section of the samples were observed using the Scanning Electron Microscope (SEM) (Nano Nova 230, FEI, Hillsboro, OR, USA EE.UU). The lyophilized films were cut into two fragments of 2 cm diameter to place them in the aluminum supports adapted to the machine. A gold coating was applied to increase their conductivity through the sample. The applied accelerating voltage was set on 15.0 kV, with an increase of 500 and 2000  $\times$  [31]. The experiment was carried out in triplicate.

### 2.5.4. Crystallinity of Film by X-Ray Diffraction (XRD)

XRD analysis was used to study the structural properties of the films. The instrument used was a dust diffractometer analytical X'Pert PRO MPD (Frenchay, Bristol United Kingdom) with 240 mm of radius. For the transmission method, a configuration of convergent beam was used with a focalized mirror and a transmission geometry with flat samples. The  $K\alpha$  radiation of Cu was used ( $\lambda = 1.5418 \text{ \AA}$ ) with a measuring time of 300 s per step and two repetitions were performed. The same configuration was used for the reflexing method, but with a Cu radiation of  $K\alpha$  ( $\lambda = 1.5406 \text{ \AA}$ ). Again, the theme assuring time was 100 s per step. In both methods, the same operating power of 45 kV–40 mA was used.

### 2.5.5. Optical Properties Measurement

The color measurements in films were performed using Datacolor International Colorimeter (Lucerne, Switzerland). They were scanned from 400 nm to 700 nm in order to obtain the reflectance, with a standard light (D65 10 Deg). The luminosity was determined by  $L^*$ , red–green by  $a^*$  and yellow–blue by  $b^*$ . The determinations were carried out in triplicate. The  $L^*$ ,  $a^*$  and  $b^*$  parameters were used to calculate total color difference ( $\Delta E$ ) according to the following equation [32].

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \quad (3)$$

where  $\Delta L^*$ ,  $\Delta a^*$  and  $\Delta b^*$  are the differentials between a sample color parameter and the color parameter of a standard is used as the film background (without any extract)

### 2.5.6. Mechanical properties

Ultimate Tensile Strength (UTS), Elongation at Break (EB) and Young's Modulus (YM) of each film sample were measured with a Zwick/Roell (Ulm, city, Germany) model ProLine with software testXpert II, according to the legislation ISO 527-1:2012 [33]. Rectangular shapes with dimensions of 57  $\times$  13 mm in width were used. YM (MPa), UTS (MPa) and EB (%) were calculated using the following equations:

$$YM = \frac{\Delta\sigma}{\Delta\varepsilon} \quad (4)$$

$$UTS = \frac{F}{A_0} \quad (5)$$

$$EB = \frac{\Delta l}{l_0} \quad (6)$$

where  $\sigma$  is the uniaxial force per unit surface and  $\varepsilon$  the proportional deformation,  $F$  (N) is the applied force,  $A_0$  is the area of the sample ( $m^2$ ),  $\Delta l$  is the elongation variation between clamps (mm) and  $l_0$  is the initial elongation between clamps (mm).

## 2.6. Evaluation of Beef Patties

### 2.6.1. Preparation of Beef Patties

Beef meat for the patties was purchased from a local food market in Barcelona, Spain. Beef patties were formulated with 1000 g of different sets of ground beef, each one taken from the round part of different cuts, and was minced three times through 4 mm industrial plates. The meat was mixed aseptically for 3 min with 15 g of salt (1.5% m/m final concentration). Subsequently, the mix was flattened and cut by a round cutter into small hamburger patties, each one around 8 g of weight and for microbiology experiments 10 g. The hamburger patties were placed in plastic trays, one control (sample prepared with no wrapping; meat sample only) and the other beef patties were covered on both sides with the different film treatments: control (only gelatin), 15% PW, 9% PW, 3% PW, 3% PWS and 1% commercial. Then the samples were stored in the dark at  $4 \pm 1$  °C.

### 2.6.2. TBARS Assay

The TBARS value was determined according to the methodology of Villasante et al. [34] with several modifications; 0.5 g of sample were homogenized for 1 min with 0.5 mL EDTA 0.3% solution and 2.5 mL TBARS reagent and an Ultra-Turrax blender was used (Ika-Werke, GmbH & Co, Staufen, Germany). While the determination of all samples was taking place, all samples were maintained in an ice bath. Afterwards they were filtered through Whatman filters no. 1 and the reaction got started with the insertion of the tubes in a water bath at  $95 \pm 1$  °C for 10 min. After cooling, a UV/VIS microplate reader spectrophotometer Fluostar (Paris, France) was used to read the absorbance of the samples at 531 nm. Values of TBARS were shown as mg of malonaldehyde per kg of sample. The experiment was performed in triplicate.

### 2.6.3. Microbiological Analysis

Ten grams of each sample was dissolved with 90 mL of ringer solution and placed in a stomacher bag (Stomacher® Lab system Seward, London, United Kingdom country). Then, they were homogenized for 5 min using a Seward stomacher 400 (Seward Medical UAC House, London, United Kingdom). Several dissolutions were prepared and 100  $\mu$ L of each dissolution was spread on a triptone soya agar coated plate. Afterwards, the coated plates were incubated at  $35 \pm 1$  °C for 48 h.

Once the incubation finished, the colony forming units (CFU) were determined and reported as log of colony forming units per gram (log CFU/g).

2.6.4. Determination of Metmyoglobin (MetMb)

The determination of metmyoglobin was carried out according to the procedure described by Ouerfelli et al. [35] with some modifications. One gram of meat patty was homogenized with 5 mL of 0.04 M phosphate buffer (pH 6.8) for 30 s using an Ultra-Turrax (IKA, Staufen, Germany). The mixture was refrigerated at 4 °C for 1 h and centrifuged at 12,000× g for 10 min at 4 °C. The absorbance of the upper phase was read at 572, 565, 545 and 525 nm using a UV/VIS microplate reader spectrophotometer Fluostar (Paris, France).

The percentage of metmyoglobin was determined using the following formula:

$$MetMb(\%) = [2.514 \times \frac{A_{572}}{A_{525}} + 0.777 \times +0.8 \times +1.098](7) \quad (6)$$

where the absorbances:  $A_{572}$ ,  $A_{525}$ ,  $A_{565}$  and  $A_{545}$  were at a wavelength of 572, 525, 565 and 545 nm, respectively.

### 2.6.5. Statistical Analysis

The samples were analysed in triplicate. The mean and the standard deviation were obtained from each measurement. The significant differences were found by one-way ANOVA. Means were contrasted using Tukey's test  $p < 0.05$ . All statistics were carried out using Minitab-18 (Minitab Ltd., Coventry, United Kingdom) for Windows.

## 3. Results and Discussion

### 3.1. Total Polyphenol Content (TPC) and Radical Scavenging Activity (RSA) of PW and PWS

The total polyphenol content (TPC) and the radical scavenging activity (RSA) of PW and PWS, determined in ethanolic aqueous extracts (50:50), have been found to be tightly correlated. Table 1 shows the results of the TPC and RSA from the PW and PWS extracts. Both the TPC and the RSA are three and five times greater for the shell than for pecan walnut. These are not novel values, since similar results have been reported by different authors in the bibliography, where the pecan walnut and shell extracts have been found to have seven times more polyphenol content in the shell (92.5 mg GAE/g sample) than in the fruit (11.9 mg GAE/g sample) [20], all of them originating from different parts of Mexico. The researcher Flores-Córdova et al. obtained similar results working with two different varieties of the pecan walnut, Western and Wichita [36]. All the studies conclude that the values obtained for the shell are several times greater than the ones obtained for the fruit. Nevertheless, the exact values and obtained quantities differ between publications due to the different varieties of pecan walnut studied, the geographic area, the year of harvest, soil, agricultural practices and environmental influences, among others.



**Table 1.** Total Polyphenol Content (TPC) and radical scavenging values from the PW (pecan walnut) and PWS (pecan walnut shell).

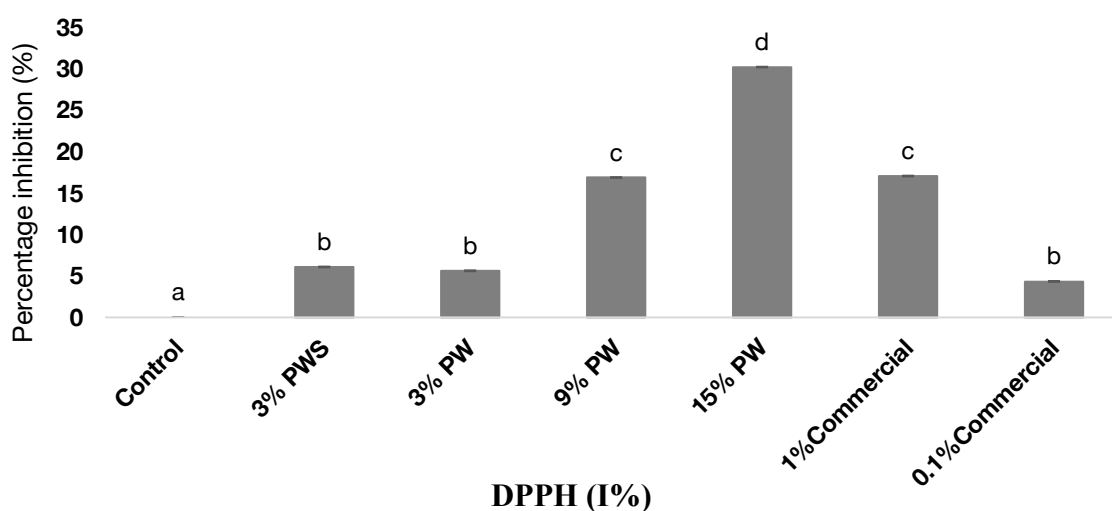
Treatments	TPC [mg GAE/g DW]	DPPH [ $\mu\text{mol TE/g DW}$ ]
PW	20.55 $\pm$ 0.03 <sup>a</sup>	37.63 $\pm$ 1.08 <sup>a</sup>
PWS	72.96 $\pm$ 0.02 <sup>b</sup>	205.12 $\pm$ 3.00 <sup>b</sup>

\*Mean  $\pm$  SD ( $n = 3$ ). Different letter means within each column with different superscripts are significantly different ( $p < 0.05$ ).

### 3.2. RSA of the Films by DPPH (2,2-diphenyl-1-picrylhydrazyl)

Nowadays there is an increased interest in food packaging that offers antioxidant properties to avoid or delay lipid oxidation. Methodologies such as the DPPH method are used to calculate the delay in the production of the free radicals' formation and determine the radical scavenging activity [37].

Figure 1 shows the radical scavenging activity measured by the DPPH methodology in films with different shell and pecan walnut concentrations and also compared to the commercial antioxidant in films. The control, gelatin film without any added extract, has very little radical scavenging properties due to particular amino acids such as glycine and proline [38].



**Figure 1.** Radical scavenging activity of the films: DPPH (1%). Different letter means are significantly different ( $p < 0.05$ ). PW: Pecan walnut; PWS: Pecan walnut shell.

Data indicated that DPPH' radical-scavenging activity of films increases in a dose-dependent way, incrementing the final content from 3% to 9%, leading to a significant improvement in the radical scavenging activity, which was boosted from about 5 to 30%. Similar results were obtained for the 3% shell concentration, 3% walnut concentration and 0.1% of the commercial additive which is the

concentration usually used by the meat industry. The 9% PW concentration offers a percentage of inhibition similar to the 1% commercial sample. As for the 15% PW concentration, it is more than thirty times greater than the control. The PW extract contains a significant amount of phenolic compounds such as catechin and epicatechin, among others and phenolic acids such as gallic and ellagic acid which have shown antioxidant characteristics [15].

### 3.3. Fourier Transform Infrared (FTIR) Spectra

Fourier Transform Infrared spectra were used to determine the chemical characteristics of molecules that were present in the films. Table 2 shows the FTIR spectra of the control, PW, PWS and commercial films. A strong broad band at 3289.11 and 3281.09  $\text{cm}^{-1}$  represents the OH and N-H groups [43] and was observed in all samples, even though the peak got more intense after the addition of the PW and the commercial preservative. Two more peaks were observed between 3007.26 and 3007.59 and also 2934.24 and 2923.27  $\text{cm}^{-1}$ , which are due to the presence of aliphatic and unsaturated hydrocarbons related to terpenoid compounds [44]. The 3007.26–3007.59  $\text{cm}^{-1}$  peak is not found in the control nor in the shell extract, which indicates that it is a compound that can only be found in the pecan walnut, like vitamin E. The second peak 2934.24–2923.27  $\text{cm}^{-1}$  is also not found in the shell extract, which could be due to the amorphous cellulose found in the shell [45].

The peak between 1630.86 and 1645.51  $\text{cm}^{-1}$  is attributed to a C=O stretch [6], which could represent amide I or an unsaturated ester and carboxylic acid [46]. In the PWS extract, the peak is 36.26% stronger with respect to the control film. The same tendency is observed in the peak between 1548.37 and 1541.41  $\text{cm}^{-1}$ , which represents the amide I

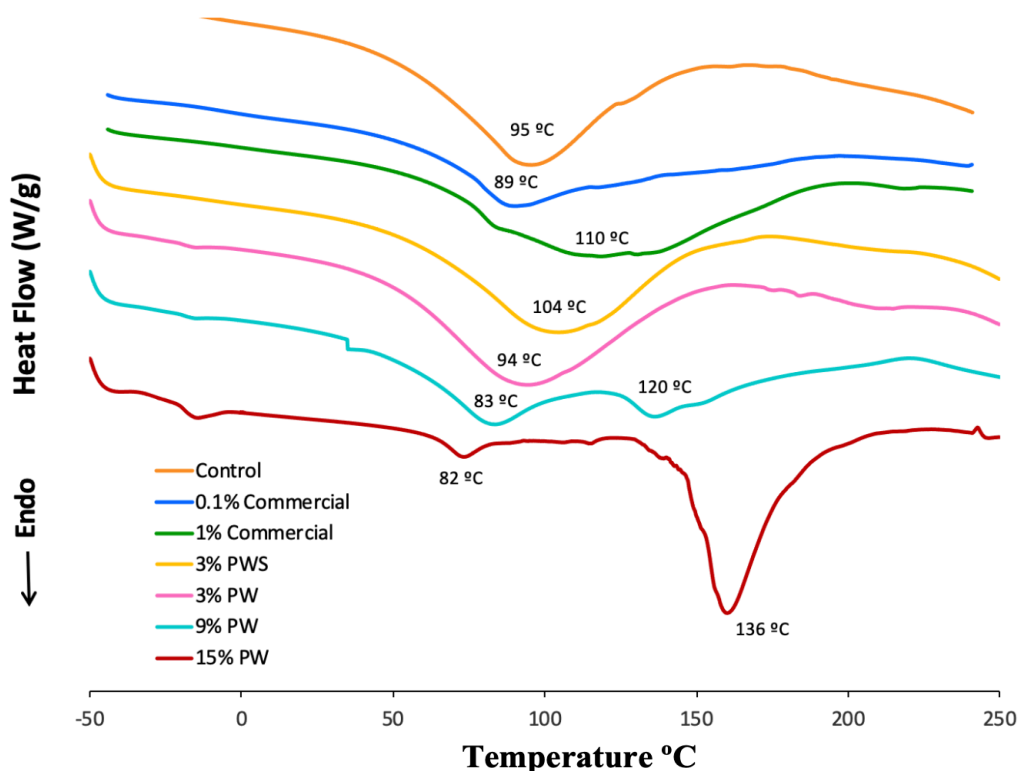
**Table 2.** FT-IR spectroscopy of gelatin film with PW (pecan walnut) and PWS (pecan walnut shell)

PEAK ( $\text{CM}^{-1}$ )	N.º	FUNCTIONAL GROUP	BOND	CONTROL FILM	3% PW FILM	9% PW FILM	15% PW FILM	3% PWS FILM	1% COMMERCIAL FILM	0.1% COMMERCIAL FILM
3289.11–3281.09	1	Amines/Amides/-OH	N-H/O-H	81.09 %	93.48 %	93.59 %	95.67 %	--	88.07 %	93.73 %
3007.26–3007.59	2	Aromatic	C-H	--	--	93.90 %	93.2 %	--	--	--
2934.24–2923.27	3	Alkyl	-C-H	91.09 %	91.84 %	68.08 %	71.25 %	--	92.82 %	95.14 %
1743.74–1743.29	4	Acid carboxylic	C=O	--	95.32%	66.68 %	72.24 %	--	--	--
1630.86–1645.51	5	Carbonyl	C=O	62.12 %	86 %	89.37 %	85.49 %	98.37 %	80.55 %	71.14 %
1548.37–1541.41	6	Amines blending	N-H	67.68 %	87.51 %	90.89 %	88.05 %	98.15 %	83.08 %	83.66 %

### 3.4. Differential Scanning Calorimetry (DSC)

The thermodynamic characteristics are useful to determine the quality of films when they are kept under different conditions such as temperature, gas pressure or atmospheres (reducing or oxidant). The effect of pecan walnut and shell extracts and the addition of commercial preservatives on the thermal stability of the gelatin films was examined. The samples and control were maintained at equal temperature throughout the analysis. Figure 2 shows the heat flow versus the temperature and several peaks can be observed. The first peaks (89.29–117.51 °C) in all the samples correspond to the natural crystallization of the gelatin. In addition, the transition coil-helix of the gelatin film

facilitates disruption of the molecular ordered structure [47]. The values of the fusion transition temperature of the control (94.89 °C) were similar to those obtained by Wang (92.12 °C) [48], who worked with gelatin films incorporating high amylose corn starch. The films with 15 and 9% pecan walnut concentrations had two different peaks, 91.64–133.43 °C and 83.14–135.91 °C respectively. That indicates that they have two points of fusion, which could be due to bigger crystal formation. The samples with 3% PW and PWS concentration do not show significant differences compared to the control, so the fruit quantity and the residue incorporated in the film is not enough to induce the formation of more crystals. The films with commercial preservatives showed similar results.



**Figure 2.** Differential scanning calorimetry (DSC).

The addition of PW and PWS could improve the thermal stability of gelatin films. The effect on thermal properties depends on the internal structure. Maybe, films containing PW and PWS have higher physical stability which requires more energy to break the structure, resulting in a higher thermal stability [49].

### 3.5. Physical Analysis

#### 3.5.1. Film Thickness

The film thickness could be correlated with the water vapor permeability and mechanical properties of the film [50]. Table 3 shows the thickness of the gelatin films with the addition of the different extract concentrations. The films obtained showed a thickness range from 0.032 to 0.150 mm. Significant differences ( $p < 0.05$ ) were found between the control and the samples with 15% and 9% PW and 3% PWS concentrations, whereas the ones with added artificial preservatives do not show any significant differences. Given that all the samples were prepared with different concentrations of extracts that contain different solid components (dry mass), this resulted in thicker

films after evaporation of the solvent. Similar trends were reported for gelatin film containing different apple-peel nanoparticles [49].

**Table 3.** Thickness of the samples.

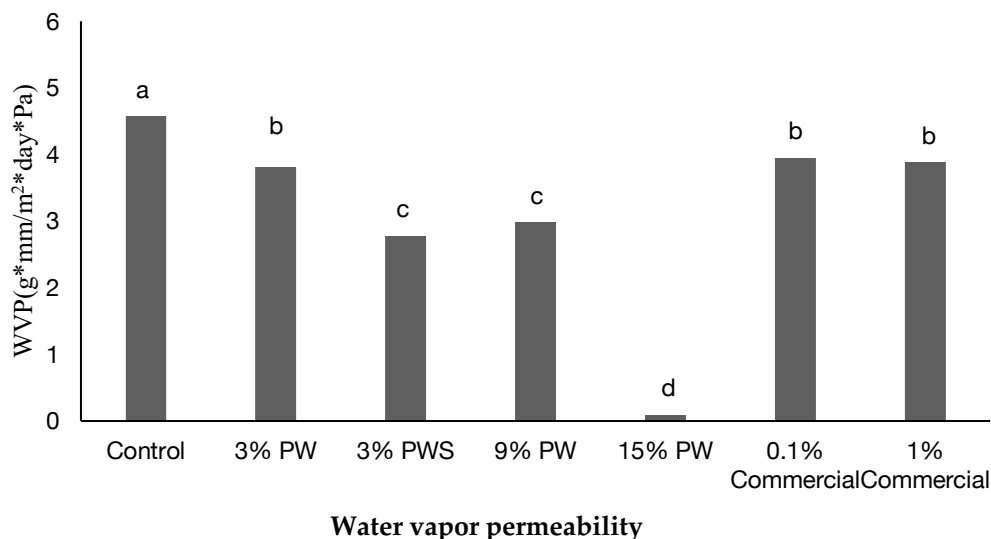
Sample	Thickness (mm)
Control	0.032 ± 0.01 <sup>d</sup>
15% PW	0.150 ± 0.02 <sup>a</sup>
9% PW	0.079 ± 0.00 <sup>b</sup>
3% PW	0.047 ± 0.00 <sup>d</sup>
3% PWS	0.076 ± 0.00 <sup>bc</sup>
1% Commercial	0.051 ± 0.00 <sup>cd</sup>
0.1% Commercial	0.047 ± 0.01 <sup>d</sup>

\*Mean ± SD (n = 3). Different superscripts letter in the column means that are significantly different (p < 0.05).

PW: Pecan walnut; PWS: Pecan walnut shell.

### 3.5.2. Water Vapor Permeability (WVP)

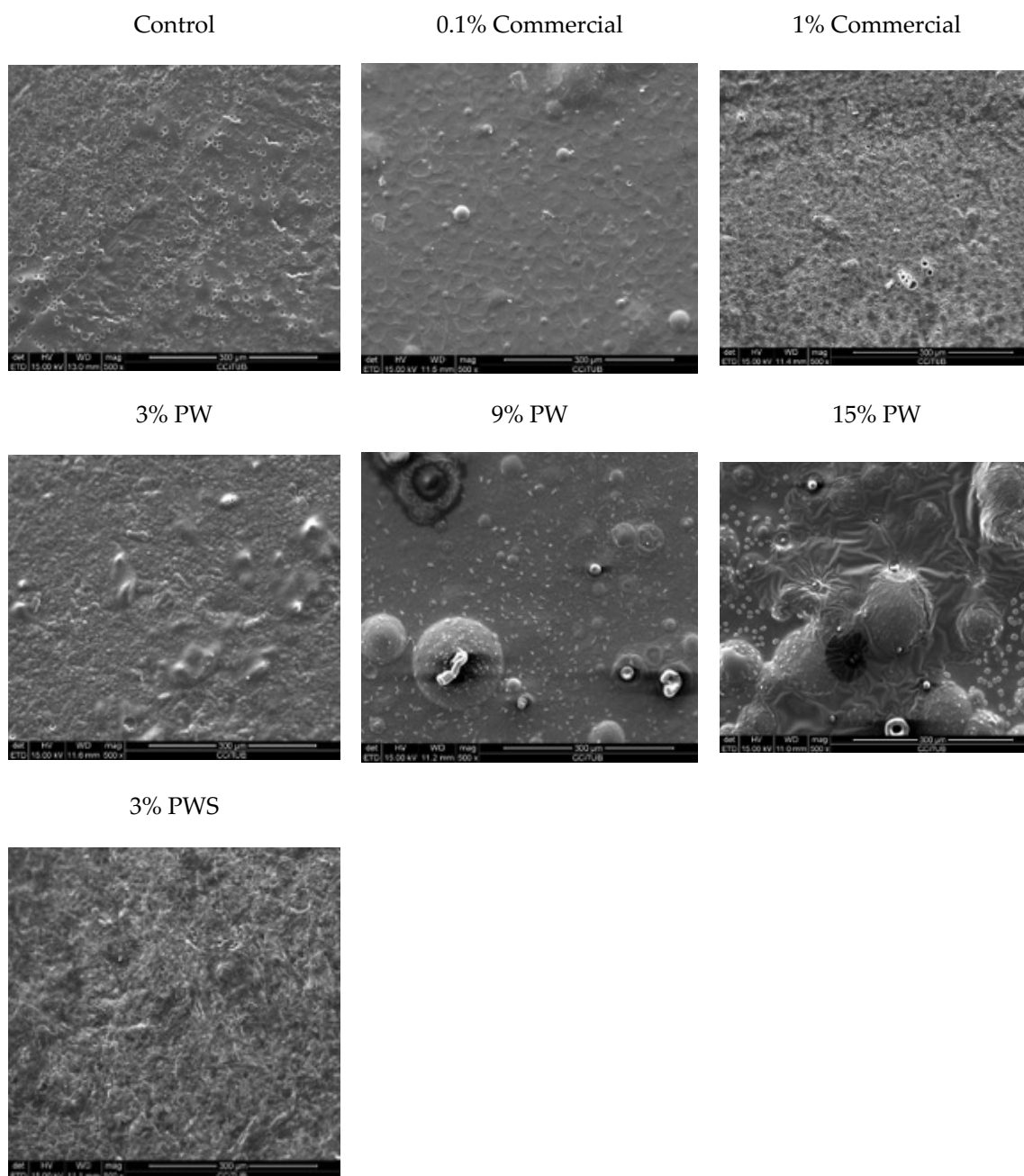
Films with gelatin are hydrophilic, so they tend to retain water in their structure, which causes a swelling, showing an insufficient performance. When plasticizing agents such as sorbitol and fatty acids are incorporated, they make the films more permeable to water vapor [51]. Figure 3 shows the water vapor permeability is inversely proportional to the extract concentration. This could be due to the fact that the pecan walnut is rich in triacylglycerols containing monounsaturated fatty acids and tocopherols such as vitamin E [52]. The lipids are hydrophobic compounds that exert a greater barrier to water vapor. In this case, the PWS films present, as expected, a higher permeability to water vapor because it is mainly fiber [21]. The commercial films (both concentrations) showed the same behavior as the 3% PW; this could be due to the dextrose and dextrin content in the commercial additive [53].



**Figure 3.** Water vapor permeability (WVP). Different letter means are significantly different ( $p < 0.05$ ). PW: Pecan walnut; PWS: Pecan walnut shell.

### 3.5.3. Scanning Electron Microscopy (SEM)

Mechanical properties and water vapor permeability could be affected due to the films' microstructure, like the morphology and homogeneity of the matrix [54]. Figure 4 shows SEM images of the films' surface. SEM images show that the outer layer of the control film was flat, uniform, ordered and with a homogeneous structure without bubbles or pores. It can be observed that, with increasing PW concentration, the matrix was more heterogeneous. This is due to a phase separation, because the gelatin and the PW have a hydrophilic and a hydrophobic nature, respectively. Acosta et al. [55] performed some studies in which they added esters of fatty acids to gelatin and yucca starch films and a phase separation also occurred [55]. Other authors reported a similar phenomenon [56]. It is clear from the SEM analysis, that the films that contained extracts had an altered structure and presented some discontinuities in the matrix (Figure 4). Nano-particles were formed when the ethanolic extract was blended with water due to the fast evaporation of non-water-soluble extract components. Gonçalves et al. studied the hydrophobic properties of some antioxidants, which could lead to the microprecipitation of this hydrophobic unit [57]. Morphological changes in the films such as a rougher surface were observed due to changes in solubility. Similar results were found by Pastor et al. and Chang-Bravo et al. who studied biopolymer films containing ethanolic extracts of propolis [58,59]. In addition, Riaz et al. also obtained similar results, which show that the samples with the lowest concentration of apple peel ethanolic extract have a good dispersion in the film, whereas in the other samples, agglomerates of nanoparticles were described. In fact, a low extract concentration resulted in a more homogeneous particle size distribution [49]. Teodoro et al. studied the fabrication of cassava starch films containing acetylated starch nanoparticles as reinforcement [60].



**Figure 4.** SEM images of the cross-sections or surface of gelatin films with the addition of different concentrations of pecan walnut, shell and commercial preservative. PW: Pecan walnut; PWS: Pecan walnut shell.

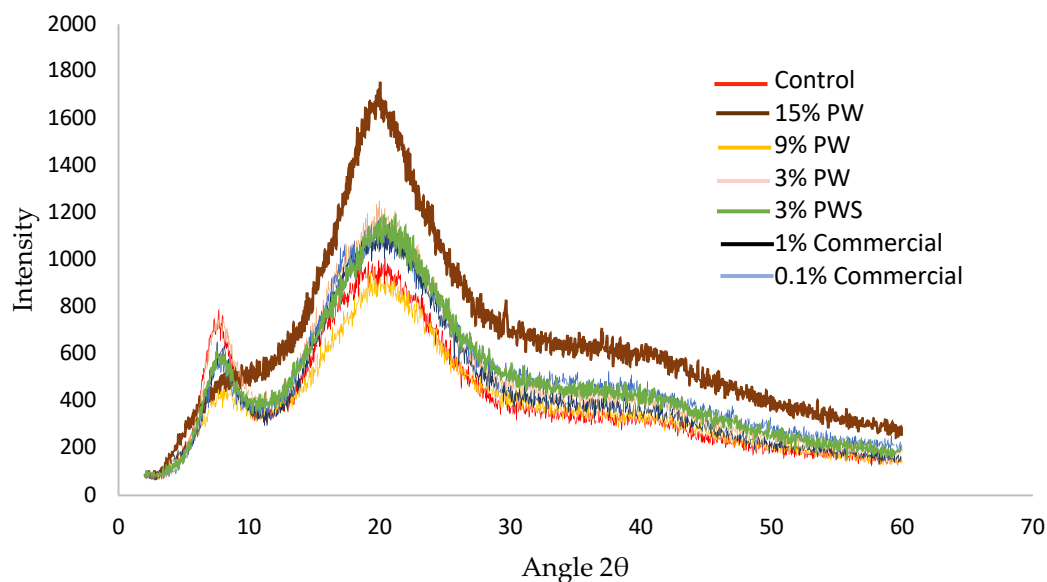
In this work, the shell presents a more homogenous but roughened disposition. This is concordant with results obtained by Iahnke et al. with the incorporation of beetroot in gelatin films [61] and Harini et al. who studied a film based on walnut shell fiber and cashews [45].

The film with a 0.1% commercial preservative shows a more homogeneous dissolution, whereas the 1% commercial film shows porosity and roughness.

#### 3.5.4. Crystallinity of Film by X-ray Diffraction (XRD)

Depending on the internal structure formed by intermolecular bonding, solids can be classified as amorphous, polycrystalline or crystalline. The structure was studied by X-ray diffraction. Figure 5

shows an acute and dominant peak between 7.5 and 8.2° ( $2\theta$ ) in all samples, which decreases its intensity on increasing the PW or PWS concentration. The other peak is between 18 and 25° ( $2\theta$ ) also in all the samples. These two peaks are found in  $d = 11.5$  and 4.5 Angstrom (Bragg's Law), respectively [62], which indicated the mean difference between the reticular planes (it is not crystalline, so it cannot be specifically named distance between planes).



**Figure 5.** X-ray of gelatin films with the addition of different concentrations of pecan walnut, shell and commercial preservative. PW: Pecan walnut; PWS: Pecan walnut shell.

These peaks indicate the reconstitution of the collagen-like triple-helix structure of partially crystalline gelatin. The intensity of this peak was assigned to the content of triple helix [63,64].

Similar results were shown by Pępczyńska et al. with salmon gelatin film—a high intensity peak is observed at the diffractive angle  $2\theta \sim 9^\circ$ —that they concluded, similar to Rivero et al., could be due to the formation of triple helices (in the process of salmon gelatin film formation). In the same study Pępczyńska et al. found a broader diffractive peak in a  $2\theta$  diffraction angle range of 11–30° [65,66].

On the other hand, similar results were found for gelatin films with different concentrations of epigallocatechin gallate fabricated by thermo-compression molding. Nilsuwan et al. found that with a high concentration of this phenolic compound there was a higher peak intensity. They suggested that this result could be due to a higher number of interactions between gelatin and the epigallocatechin gallate via hydrogen bonding [67].

### 3.5.5. Optical Properties Measurement

Color is one of the most important sensory quality attributes for consumers. Usually, protein and polysaccharide-based films are colorless, depending on the concentration and the type of amino acids present and the treatment applied. Uranga et al. worked with anthocyanins. In their study, anthocyanins were applied to gelatin films, with the control (only gelatin) having similar values to the ones shown in Table 4 ( $L^* = 94.86$ ,  $a^* = -0.11$  and  $b^* = 4.98$ ) [44].

The addition of PW and PWS in gelatin films caused an increase in the yellowness ( $b^*$ ), in the redness ( $a^*$ ) and the total color difference value ( $\Delta E^*$ ) compared to the control. There are no

significant differences between the two commercial concentrations, but when the PW and PWS concentration increase, the luminosity (\*L) was reduced compared to the control. This is because the PW and PWS is opaque, due to its polyphenolic compounds; the coffee color comes from compounds that the shell and pecan walnut contain, such as tannins [68,69].

**Table 4.** Optical properties measurement.

Film	CIE L*	CIE a*	CIE b*	CIE ΔE
Control	93.75 ± 0.23 <sup>a</sup>	-0.43 ± 0.34 <sup>de</sup>	3.55 ± 0.43 <sup>e</sup>	0.00 ± 0.00 <sup>e</sup>
3% PW	91.72 ± 0.92 <sup>ab</sup>	0.01 ± 0.16 <sup>de</sup>	7.48 ± 1.82 <sup>cde</sup>	4.78 ± 2.04 <sup>cde</sup>
9% PW	89.20 ± 0.87 <sup>bcd</sup>	0.33 ± 0.16 <sup>de</sup>	12.77 ± 1.67 <sup>b</sup>	10.32 ± 1.89 <sup>b</sup>
15% PW	84.03 ± 0.60 <sup>e</sup>	2.49 ± 0.35 <sup>ab</sup>	18.51 ± 0.66 <sup>a</sup>	18.08 ± 0.93 <sup>a</sup>
3% PWS	86.71 ± 0.44 <sup>de</sup>	3.19 ± 0.25 <sup>a</sup>	12.21 ± 0.41 <sup>bc</sup>	11.73 ± 0.65 <sup>b</sup>
1% Commercial	93.61 ± 0.21 <sup>a</sup>	-0.61 ± 0.23 <sup>e</sup>	4.39 ± 0.45 <sup>de</sup>	0.87 ± 0.32 <sup>de</sup>
0.1% Commercial	93.62 ± 0.07 <sup>a</sup>	-0.54 ± 0.02 <sup>e</sup>	4.66 ± 0.12 <sup>e</sup>	1.13 ± 0.13 <sup>e</sup>

CIE L\* represents the vertical coordinate of a three-dimensional arrangement of colors and has a value range from 0 (black) to 100 (White). CIE a\* is the horizontal coordinate and a values range from -80 (Green) to +80 (red). CIE b\* is the horizontal coordinate and the values go from -80 (blue) until +80 (yellow). The total difference in color is represented by CIE ΔE\*. Diverse letters in the same column indicate that there is a significant difference between them ( $p < 0.05$ ). PW: Pecan walnut; PWS: Pecan walnut shell.

### 3.5.6. Mechanical Properties of the Films

The food industry is developing new food packaging that is capable of maintaining the food properties and their integrity during the life of the product [70-72]. For this reason, the mechanical properties of the packaging such as rigidity, tension and percentage of deformation have a relevant role. The results of mechanical parameters (Young's modulus, tensile strength and elongation at break) of the analyzed films are presented in Table 5. It can be observed that the control film, 3% PW and PWS and the two different commercial concentrations do not present significant differences regarding the Young's modulus, UTS and EB. Additionally, they showed higher YM and UTS parameters, which means that they present a greater resistance to elastic deformation, so they are more rigid and show a higher deformation tension. Due to this, their percentage of deformation is smaller, whereas for the films with a higher PW concentration, the breaking tension and the Young's modulus decreased compared to the control, up to six and eight times, respectively. The elastic deformation increased significantly ( $p < 0.05$ ). Results with the same tendency were reported in different studies: Ge et al., who added rosmarin extract to gelatin films [73], Phebe and Ong worked with starch-chitosan films incorporated with oregano essential oil [72], Putsakum et al. studied the properties of gelatin films developed from neem extract [74] and Bonilla et al. studied the effect of eugenol oil and ginger addition in gelatin films [75]. In all these studies, it can be concluded that, when polyphenolic compounds and fatty acids are added, they provoke an interaction with the gelatin matrix



(protein), forming hydrogen bridges and covalent bonds with amine and hydroxyl groups and inducing the reduction of protein–protein interactions [8]. Similar effects have been found in the addition of oxidized linoleic acid compared with the control, presenting a lower elastic modulus and tensile strength (8–42% and 21–57 %) but higher elongation at break (366–956%) [76]. This study claims that incorporation of fatty acids such as linoleic acid to films could reduce the intermolecular interactions among gelatin molecules, resulting in increasing chain mobility and free volume in the film matrix. In this study, tannins are the main polyphenolic compounds in the pecan walnut. Cano et al. added tannins from oak bark to gelatin based films; they found that the resistance to break and elastic modulus significantly decreased. They mentioned that this fact could be due to the precipitation of the gelatin–tannin complexes, which is caused by the presence of strong electrostatic connections between the different charged groups (gelatin: Positive; tannins: negative) [41]. In contrast, the incorporation of 0.1 and 1% commercial preservatives did not show significant differences with respect to the control ( $p < 0.05$ ).

Therefore, the incorporation of PW and PWS concentration could affect the mechanical properties of gelatin films differently. It depends on the method used in the extract addition and the concentration incorporated.

**Table 5.** Effect of the addition of different concentrations of PW and PWS, and commercial preservative on Ultimate Tensile Strength (UTS), Elongation at Break (EB), Young's Modulus (YM).

Sample	YM (MPa)	UTS (MPa)	EB (%)
Control	797.92 ± 250.90 <sup>ab</sup>	39.93 ± 13.56 <sup>ab</sup>	6.39 ± 0.12 <sup>d</sup>
3% PW	615.47 ± 172.79 <sup>abc</sup>	37.51 ± 2.70 <sup>ab</sup>	8.68 ± 2.57 <sup>cd</sup>
9% PW	358.96 ± 16.24 <sup>bcd</sup>	16.74 ± 2.90 <sup>bc</sup>	25.70 ± 1.99 <sup>b</sup>
15% PW	96.70 ± 17.65 <sup>d</sup>	5.95 ± 1.05 <sup>c</sup>	31.19 ± 4.28 <sup>bc</sup>
3% PWS	513.99 ± 34.01 <sup>abcd</sup>	21.84 ± 1.83 <sup>abc</sup>	6.39 ± 0.03 <sup>d</sup>
1% Commercial	883.24 ± 16.84 <sup>a</sup>	43.79 ± 3.68 <sup>a</sup>	7.17 ± 1.39 <sup>d</sup>
0.1% Commercial	964.25 ± 232.44 <sup>a</sup>	48.06 ± 19.76 <sup>a</sup>	8.29 ± 3.76 <sup>cd</sup>

\*Mean ± SD (n = 3). Values that share the same superscript letter in each column are not significantly different ( $p < 0.05$ ). PW: Pecan walnut; PWS: Pecan walnut shell.

### 3.5.7. Film Protection in Beef Patties

Edible films and coatings reduce lipid oxidation, which preserves the quality of meat products during their storage time [77]. In order to have a higher concentration of antioxidants in a patty's surface, antioxidant extracts can be added to the formulation of films. Protein films have a tightly packed, organized hydrogen-bonded network structure and therefore are good oxygen barriers. Gelatin films are a type of protein based polymer, which has been broadly used to form edible films.

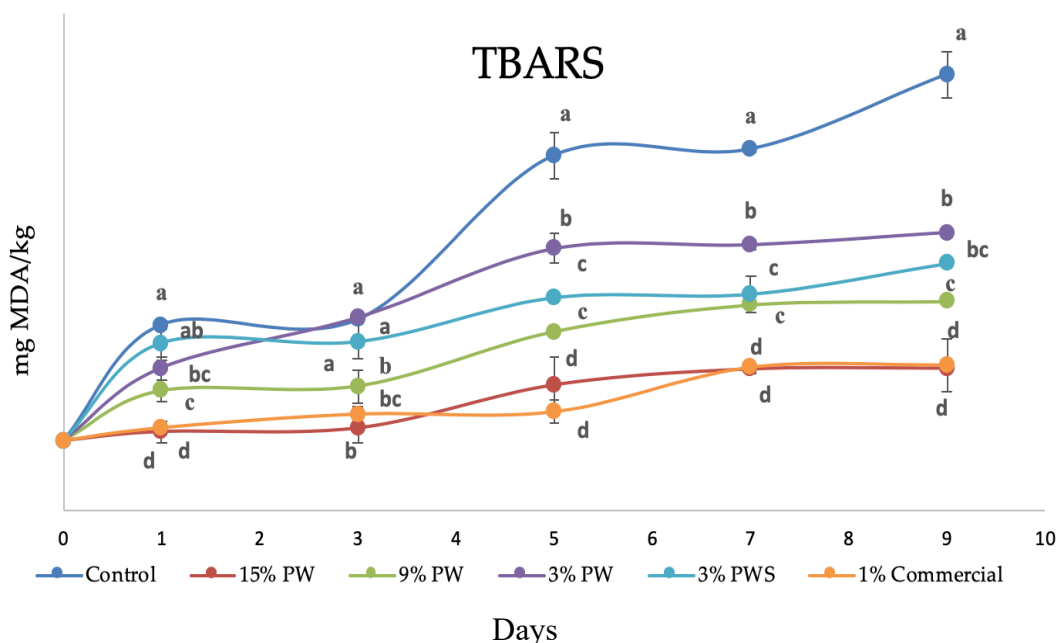
Films made from proteins are excellent oxygen barriers because of their tightly packed, ordered hydrogen-bonded network structure. A particular case is gelatin films, a protein based polymer widely used as a starting material for edible film formation.

In the previous sections, it has been shown that films (both the ones that contain the pecan walnut shell and the ones that contain the pecan walnut itself) have radical scavenging activity and good physical properties. Therefore, they are potentially useful to protect food against oxidation once in contact with the food itself. To show its antioxidant activity in a real food, an experiment has been designed in which different films are set above and under small beef patties (8 g, 3 cm of diameter). Six small patties are set in any tray; five of them are protected with one of the different films designed and the control sample does not have a film. Each full tray is covered with transparent film suitable for food.

Periodically, one tray is removed to do the analysis on secondary oxidation through the TBAR compounds. TBARs were measured as an indicator of the secondary phase of lipid oxidation as mg malondialdehyde per kg of sample (mg MDA/kg beef). The deterioration of the secondary lipids would alter the flavor and contribute a rancid odor and unfavorable taste to the food [78].

The metmyoglobin as the compound responsible for the loss of red color and the apparition of a brown coloration has also been followed and analyzed.

The results of the evolution in time of the compounds reactive to thiobarbituric acid (expressed in mg of malondialdehyde (MDA)/kg of meat) against time (nine days in total) are summed up in Figure 6.



**Figure 6.** Thiobarbituric acid reactive substance (TBARS) values of raw beef patties during storage at  $4 \pm 1$  °C.

Results show the mean of triplicate determinations ( $n = 3$ ) and are shown as a mean value  $\pm$  SD. Different letters in a day illustrate significant differences between the samples at  $p < 0.05$ , whereas different capital letters show significant differences between storage days at  $p < 0.05$  for the same sample. PW: Pecan walnut; PWS: Pecan walnut shell.

It is interesting to point out the static situation that appears after seven days; four different types of samples can be distinguished. The oxidized sample (which reaches higher values than 1,2 mg MDA/kg of meat) is, as should be expected, the control, that is, the mini-hamburger that does not have any gelatin film protection (it does have protection with the commercial film, common to all patties).

These TBAR values make the sample unacceptable for its consumption, due to the MDA quantities between 1.0–2.0 mg /kg, which make the product have off-flavors that make it unacceptable [37,79].

The next sample in terms of deterioration (significantly different even with  $\alpha = 0.01$ ) is the one with the lower pecan walnut quantity (3% PW). It has an oxidation value of approximately 0.75 mg MDA/ kg of meat. According to the report by Brychcy [80], rancidity and off-flavor were noticed when the TBAR value was in the range of a 0.5–1.0 mg MDA/kg sample. For this reason, it could be said that the pecan walnut extract concentration within the film is not enough to avoid an oxidation process that causes the meat to be not acceptable after a week.

The films 3% PWS and 9% PW do not show different behaviors statistically speaking. Both reach values near the limit of sensory acceptability after seven days (values near 0.6 mg MDA/kg of sample). These values are in the same ranges (also considered up to seven days) as the ones obtained by Huiyun Zhang et al. [81], who prepared a natural active chitosan coating loaded with nano-encapsulated *Paulownia tomentosa* essential oils and investigated the effects of the coating treatments in pork chops.

Lastly, there is the group formed by the film with the commercial antioxidant and the film that contains the greater walnut concentration (1% commercial, 15% PW, respectively). In this case the oxidation up to seven days is close to 0.4 mg MDA/kg of meat. This fact shows that the oxidative protection that the film containing 15% PW transfers is very similar to that provided by the commercial antioxidant (1%). Both prevent oxidation, making the beef patty free of off-flavors and rancidity.

Other films, also gelatin ones, where phenolic antioxidants have been added, but tested in a different matrix, such as cod-liver oil, show similar results after 10 days, even though the control is less oxidized (values near 1 mg MDA/kg sample). However, the protection is maintained longer, up to 20 days, at which time the control, without a film applied, has five times higher oxidation [82].

Bermúdez-Oria et al. also worked with gelatin films and beef meat. The film used pectin–fish gelatin (in this case they came from fish and incorporated pectin) and the specific antioxidants added, hydroxytyrosol and 3,4-dihydroxyphenylglycol [83]. The protection against oxidation (measured through the TBAR formation) was less because the values obtained were between 1 and 2.5 mg MDA/kg meat (compared with the control, which had values near 4 mg MDA/kg meat).

As a conclusion regarding the oxidation, it can be said that films designed with the incorporation of 9 and 15 per cent of PW, like the one with 3% of shell, stabilized the beef patties so that after one week the degree of oxidation made them suitable for consumption.

Regarding the microbial contamination, it has been seen that after a week the log of the CFU/g meat is 4.12 for the control. All samples protected with gelatin films have similar values (expressed in the same units, log of CFU/g) between 3.5 (for the protected meat with the film that incorporates

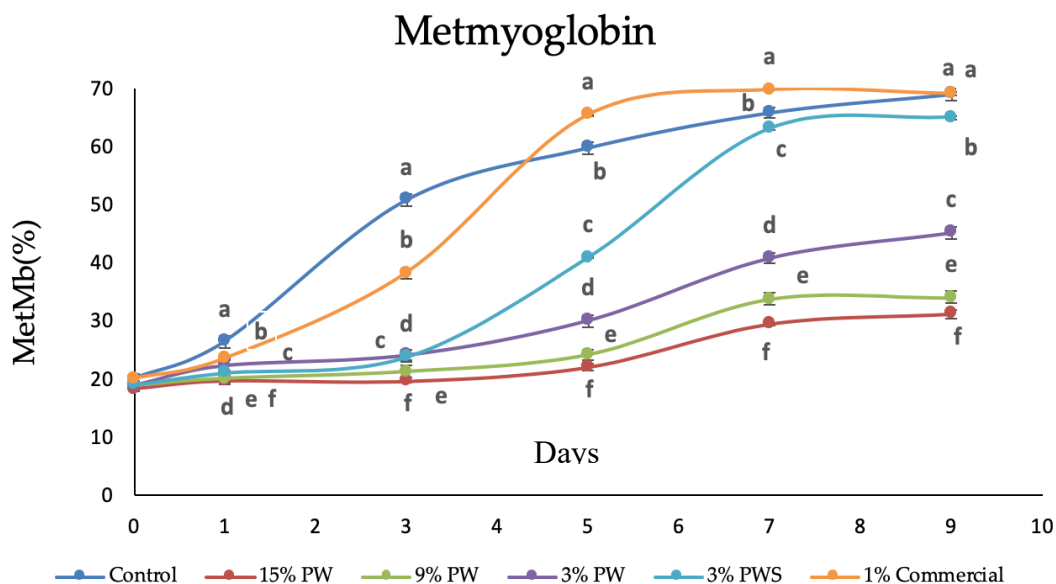
15% of PW) and 3.97 for the beef covered by the film that incorporates the shell. No significant differences were observed between them.

The processing of the patties was carried out working in sterile conditions, which favors the initial contamination being low (around 100 of CFU/g meat), and therefore the growth found is not very important.

There are reported cases where an antimicrobial agent is added to the gelatin film to extend the useful life of the beef meat from eight to 14 days (with respect to the microbial contamination and taking into consideration that it does not refer to ground meat, but to slices of meat) [84]. However, there are authors, like Bonilla Lagos et al. [85], who have studied samples with an initial contamination higher than 1000 CFU/g and after seven days some samples protected with film incorporating natural extracts reached levels between 5 and 7 (Log of CFU/g sample). However, it can be said that the films did not give significant protection against microbial contamination.

Metmyoglobin was analyzed. Myoglobin in fresh red meat can be found as deoxymyoglobin, oxymyoglobin and metmyoglobin. Deoxymyoglobin is responsible for the purple color in the meat. When exposed to oxygen it can be rapidly oxidized to bright red oxymyoglobin. Further oxygenation results in the formation of metmyoglobin, which is related to a loss of freshness in the meat and can be identified as a brown coloration.

Figure 7 shows the myoglobin percentage of the different samples. After a week, all the samples were significantly different ( $p < 0.05$ ) and two big groups could be distinguished. The first group is the control sample, the commercial 1% and the 3% PWS. They all present a metmyoglobin percentage higher than 60%, which correlates to the color shown by the different patties after a week of storage (a dark color, unpleasant at first sight).



**Figure 7.** Metmyoglobin values of raw beef patties during storage at  $4 \pm 1$  °C. Results represent the mean of three replicates ( $n = 3$ ) and are expressed as mean value  $\pm$  SD; different letters in the same day indicate significant difference between samples at  $p < 0.05$ , different capital letters indicate significant difference between storage days at  $p < 0.05$  for the same sample. PW: Pecan walnut; PWS: Pecan walnut shell.

The other group comprised all the samples that incorporate films with PW, with a difference depending on the percentage of PW. The hamburger protected with the film that has more PW (15%) exhibited a lower metmyoglobin value, less than 30%, whereas the other two were between 34% and 41% for the film sample with 9% and 3%, respectively.

This fact indicates that there is no clear correlation between the oxidation (measured through TBARs) and the color (measured by % of metmyoglobin). The samples covered with film containing PW showed lower concentrations of metmyoglobin, which could be due to some phenolic compounds that the PW contains. De la Rosa et al. identified, in pecan kernels, phenolic acids such ellagic acid, ellagic and gallic acid derivatives and monomeric flavan-3-ols. However, in the shells, they only identified ellagic and gallic acids [20]. These compounds in the kernel could slow down metmyoglobin formation.

Several authors, like Bentayeb et al. found that surface metmyoglobin formation was depressed by active packaging containing rosemary, although the increase of rosemary concentration in the film did not cause significant differences [86].

#### 4. Conclusions

The results showed that mechanical and water vapor barrier properties of the developed films varied depending on the concentration of the pecan walnut, shell and synthetic antioxidant. With higher pecan walnut concentration (15%), the permeability to water vapor was significantly lower than the control. Furthermore, with the addition of natural extracts and synthetic preservatives, the elongation at the break and Young's modulus were lower than for the control. FTIR analysis allowed characterization of the molecular interactions occurring after the inclusion of pecan walnut and pecan walnut shell. The microstructural observations by SEM clearly demonstrated that the structure of the film containing walnut was heterogeneous, whereas the shell extract and commercial additive showed a homogeneous structure. Films with pure gelatin cannot act as an antioxidant shield to prevent food oxidation, but adding pecan walnut (15% concentration) improved stability of the stable DPPH radical by 30%. Furthermore, in the DSC, the addition of pecan walnut (15 and 9% concentrations), allowed the formation of big crystals, which improved the thermal stability of gelatin films. The use of gelatin films with the incorporation of bioactive compounds coming from the pecan walnut and the walnut shell have shown good protection against the oxidation of beef patties, which opens up a big field for the commercialization of meat products with lower quantities of synthetic products. However, the antimicrobial protection with the addition of pecan walnut and walnut shell extracts was low.

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**Abbreviations:** PW, pecan walnut; PWS, pecan walnut shell; TPC, total polyphenols content; GAE, mg of gallic acid equivalents; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Trolox, (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid); DW, dry weight; UTS, Ultimate tensile strength; EB, elongation at break; YM, Young's modulus;  $\Delta E^*$ , total color differences value; XRD, X-ray diffraction; SEM, scanning electron microscopy; WVP, water vapor permeability; DSC, differential scanning calorimetry; DW, dry weight.

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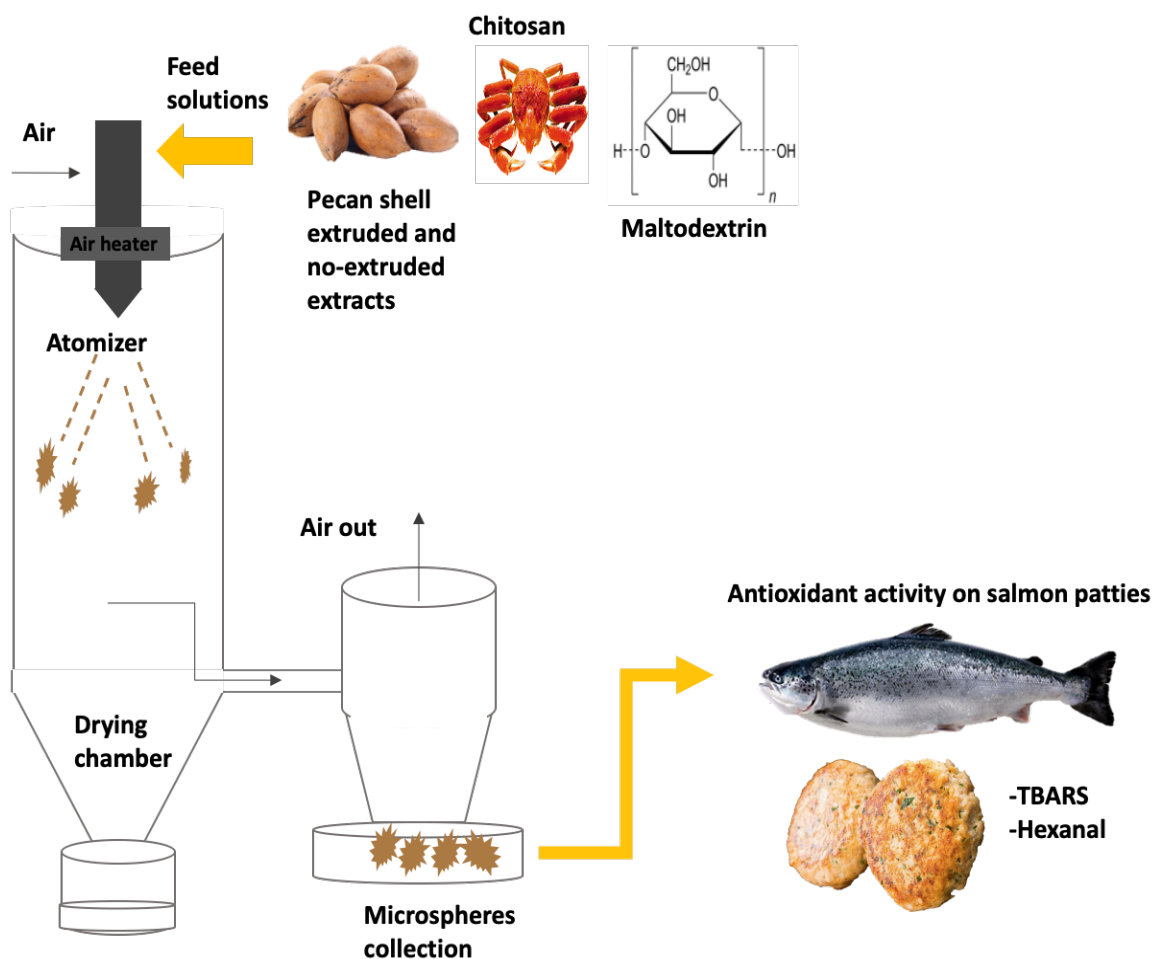
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### 7.3. Effects of Spray Drying Encapsulation of Extruded Pecan Walnut Shell (*Carya illinoensis*) on Salmon Patties (*Salmon salar*)

Juliana Villasante



# Effects of Spray Drying Encapsulation of Extruded Pecan Walnut Shell (*Carya illinoensis*) on Salmon Patties (*Salmon salar*)

## Introduction

The pecan shell (*Carya illinoensis*) is a natural source of polyphenols compounds with antioxidant properties. In our previous study, we have determined gallic, p-hydroxybenzoic and ellagic acid as the major phenolics in pecan walnut shell (extruded and no-extruded) [1]. A characteristic of these compounds is that they are subject to rapid inactivation or degradation, limiting their use as natural preservative [2]. They are unstable at high temperatures or in the presence of light or oxygen [3]. On the other hand, they are interfering with sensory characteristics when added directly or as extract to food products [4]. To solve these problems, several authors use encapsulation by spray-drying, for example: Sarabandi and Seid (2020) produced microencapsulation of peptide fractions derived from enzymolysis of flaxseed protein [5]; Radünz et al. (2020) evaluated the antioxidant and antimicrobial potential of encapsulated and unencapsulated thyme essential oil [4]; Papoutsisa et al. (2018) developed capsules of lemon by-product aqueous extracts by spray-drying, and also, they determined antioxidant capacity of the capsules [6].

This technique is an effective method that could be used to increase physicochemical stability and preserving the effectiveness of many biologically active molecules [7]. Some of the advantages of the spray-drying are low cost, rapid, reproducible, allowing easy scale-up, available equipment and efficiency [5].

Microcapsules stability are mostly dependent on the wall material [8]. Chitosan is a natural polycationic molecule with physicochemical properties as: positive charges via its amino groups, degradable and biocompatible, its permeability increases with decrease of pH, no sensory influence on food, antimicrobial and antifungal capacity. These characteristics make this biopolymer a potential coating microencapsulation for applications in food industry [7]. However, one encapsulating agent alone could not have all the required properties, a synergy of agents may be used [9,10]. A carbohydrate like maltodextrin can be selected as an encapsulating material.

In this study, the encapsulation of aqueous-ethanolic extracts of pecan walnut shell (extruded and no-extruded) were evaluated for the antioxidant activity in salmon patties, in order to explore food applications using chitosan and maltodextrin as encapsulating agent.

## Methods

### **Extract preparation for preparation of microspheres**

Pecan walnut shell (PSW) or extruded pecan walnut shell (PSWE) (extruded at 70 °C and 150 rpm) were weighed (3 g) and extracted with 20 mL of 50:50 (v/v) ethanol-water. The extracts were stirred for 90 min at room temperature. Both extracts were centrifuged, and the supernatants were

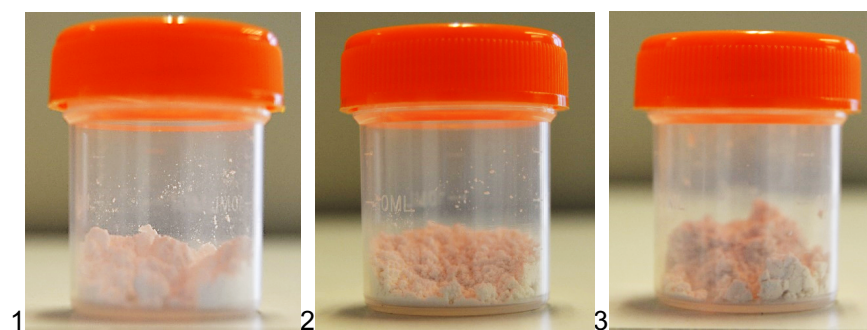
stored at  $-80^{\circ}\text{C}$  in darkness. To prepare the concentrated extracts, ethanol was completely evaporated with Rotavapor R-200 with vacuum control V-800.

### ***Microspheres preparation***

The microspheres were prepared using chitosan (0.5%) and acetic acid (0.5%) in water. Then the solution was heated at  $40^{\circ}\text{C}$ , during approximately eight hours at 700 rpm. Afterwards, the rest of the water (20%) and the maltodextrin (7.5%) were mixed with the solution of chitosan.

The total solution was separated into three parts, the first was the control (CTR). The extract of PSW and PSWE were added to the second and the third solution, respectively. The pH was measured before drying the samples, in order to ensure it was higher than 4.

Solutions (CNB, PSW and PSWE) (200 mL) were fed to the Büchi Mini Spray Dryer B-290 (Advanced 60 Hz unit, Switzerland) at a rate of 2.5 mL/min with an average processing time of 30 minutes each AT  $80^{\circ}\text{C}$ . After the process, the microspheres were collected with a toothbrush and vegetable paper, and they were kept at room temperature in desiccators during 1 day before characterization.

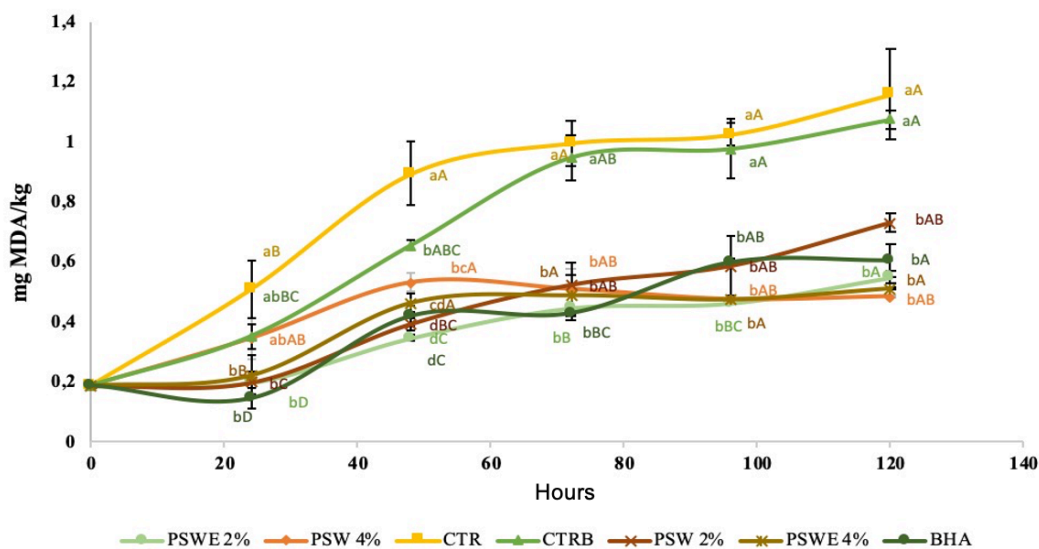


**Fig 1.** Microspheres from PSW extracts, chitosan and maltodextrin: CTRB (1), PSW (2) and PSWE (3).

### ***Statistical Analysis***

The mean value and standard deviation were calculated from the data obtained from the three samples for each treatment. Where significant differences were detected by one-way Anova, means were compared using Tukey's test  $p < 0.05$ . All statistics were performed using Minitab-16 for Windows (company, city, state abbrev if USA, country).

## Results and discussion



**Fig 2.** Evolution of TBARS value in salmon samples with different treatments kept on storage ( $4 \pm 1$  °C) in a period of 120 h.

<sup>a-d</sup> means by different letters indicate significant difference ( $p < 0.05$ ) on mg MDA/kg values between the samples at the same hours. <sup>A-D</sup> means by different letters indicate significant difference ( $p < 0.05$ ) on MDA/kg values in the same sample at different times. CTR: Salmon without microspheres. CTRB: Salmon with microspheres without antioxidant. 2% or 4% PSW: Salmon with microspheres with 2% or 4% of pecan walnut shell. 2% or 4% PSWE: Salmon with microspheres with 2% or 4% of extruded pecan walnut shell. BHA: Salmon with 0.1% of BHA (butylated hydroxyanisole).

The TBARS method is used to determine the level of compounds developing at the secondary stage of fat and oil oxidation in foods [1,11]. Malondialdehyde and related compounds are formed when the concentration of hydroperoxides is appreciable in lipids [12]. Results shown in the figure and table above indicate that after 24 h incubation in the fridge, samples containing pecan walnut shell extract (extruded and non-extruded) 4% (PWS) were significantly less oxidized ( $p < 0.05$ ) than the two controls (CTR and CTRB). After 72 h, all treatments, except the controls (CTR and CTRB), followed a similar trend, being significantly less oxidized ( $p < 0.05$ ); also, at the end of the experiment (120 h), almost all the samples with pecan walnut shell (extruded and non-extruded) were less oxidized than the commercial additive (BHA). These results could be due to the high content of total phenolics, condensed tannins, antioxidant and antimicrobial activity in the pecan walnut shell (in previous results presented in this Thesis). Similar encapsulation of pecan walnut shell was made by Kureck et al. but using Zein, a grain protein (*Zea mays* L.) instead of chitosan [2]. They found that epigallocatechin, ellagic acid, epicatechin and gallic acid were the most abundant compounds; thermal analysis proved that the microparticles are stable above 200 °C [2]. On the other hand, both industry and science are focusing on the development and addition of microcapsules based on natural compounds such as essential oils or extracts from aromatics plants, fruits, vegetables and by-products in perishable foods. Alves et al. used grape seed extract-carvacrol microcapsules and they

studied their effects on the shelf-life of refrigerated salmon [13]. Castro et al. minimized the lipid oxidation of salmon with whey protein film incorporated with 1% of green tea extract [14].

Fig 2. shows a lower quantity of MDA/kg formed in salmon with CTRB, if compared to the sample without capsules (CTR). These differences could be attributed to the antimicrobial activity and antioxidant mechanism of chitosan [11,15].

Treatment	Hexanal ( $\mu\text{g}$ hexanal / kg of salmon)
CTR	128.24 $\pm$ 0.14 <sup>a</sup>
CTRB	59.11 $\pm$ 0.45 <sup>b</sup>
2% PSW	15.27 $\pm$ 0.23 <sup>d</sup>
2% PSWE	17.21 $\pm$ 0.24 <sup>c</sup>
4% PSW	14.20 $\pm$ 0.12 <sup>f</sup>
4% PSWE	14.57 $\pm$ 0.18 <sup>e</sup>
BHA	12.98 $\pm$ 0.14 <sup>g</sup>

**Table 1.** Hexanal content in samples of salmon with different treatments, after 144 hours. Results are expressed in  $\mu\text{g}$  of hexanal/kg of salmon. <sup>a-g</sup> means by different letters indicate significant difference ( $p < 0.05$ ) on mg of hexanal/kg of salmon values between the samples.

Volatile compounds produced from lipid oxidation such as hexanal can cause rancid flavors and odors. Due to the limitations of TBARS, the measurement of hexanal can be a good indicator of lipid oxidation. Hexanal is the dominant aldehyde produced during oxidation [16]. Studies have proposed that hexanal indicates lipid oxidation of meat or fish more effectively than any other volatile component (octanal, nonanal and pentanal) [17]. After 144 hours of storage, the quantity of hexanal decreased significantly ( $p < 0.05$ ) in the salmon treated with capsules with chitosan and extruded and non-extruded pecan walnut shell. However, the lowest values of hexanal compound were observed in the samples formulated with BHA. Similar to the effect on TBARS, 4%PSW was found to be the one of the most effective antioxidants, in addition to the commercial antioxidant (BHA). A similar trend to that found by analyzing TBARS was observed, since the hexanal after 144 hours in the samples with CTRB was significantly lower than the control (CTR).

There are a few studies related to salmon oxidation, but not yet with microspheres. However, these results agreed with those reported by Jónsdóttir et al. (2008), they worked with smoking salmon and concluded that the composition of volatile compounds produced in cold smoked salmon depends on the composition of phenolic compounds (guaiacol and syringol) in the wood that is used in the smoking procedure [18,19]. Supawong et al. (2018) demonstrated the effectiveness of 2% of rice bran hydrolysate in fried fish cakes, when they analysed rancid volatile compounds as hexanal, the levels were significantly ( $p < 0.05$ ) lower than for the control [20].



The phenolic composition in chitosan microspheres with pecan walnut shell allows their use for retarding formation of free radicals and reactive oxygen species, with the objective to prevent lipid oxidation and control the production of rancid volatile compounds in fresh salmon.

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## Chapter 8. General discussion

The secondary metabolites found in by-products from plants are gaining huge consideration due to their wide range of biological activities. The common thread of this dissertation has been to study the potential use of pecan (*Carya illinoensis*) shell and kernel to slow food deterioration and provide functional properties as a food additive. In addition, possible synergistic effects with other plant materials, such as roselle flower (*Hibiscus sabdariffa*) and cayenne pepper (*Capsicum annuum*) were addressed.

The pecan has antioxidant properties due to its remarkably high content of phenolics, flavonoids, tannins and other anti-radical classes. Chemical properties of the pecan walnut were previously reported by Medina-Juárez et al., and De la Rosa et al., who performed an extraction of total polyphenol content (TPC) using one gram of sample in 10 ml of solvent [1,2]. Medina-Juárez employed 70 % of methanol-water while De la Rosa et al. used 80 % of acetone-water; the reported values of TPC were 19.95 and 12.50 mg GAE/ g DW, respectively, flavonoids were 16.36 and 6.40 mg CE/g DW, and condensed tannins were 9.10 and 26.7 mg CE/ g DW. The results obtained for the anti-radical capacity were: 276.65 and 83.40  $\mu\text{mol TE/g DW}$  for ABTS and 287.67 and 108.70  $\mu\text{mol TE/g DW}$  for DPPH.

In our study, TPC extractions were done with defatted pecan kernel weight to solvent ratios at 0.1 and 0.05 g/mL, and ethanol-water 50% v/v as solvent. The results were 20.55 and 22.95 mg GAE/g DW, respectively. We can conclude that the types of solvents, methods, temperatures and sample weight to solvent ratio used in the extractions, could lead to significantly different results than those reported by other authors. Moreover, different geographical regions may contribute to different phenol contents, and consequently the formation of different secondary metabolites. In this regard, it is well known that the genetic level and phytochemicals formation are highly influenced by environmental factors [3].

Values related to the radical scavenging activity were 37.63 mg TE/ g DW (DPPH), which are similar to those obtained in previous reports for other nuts, such as pistachios (27.55 mg TE/ g DW) and pine nuts (76.33 mg TE/ g DW) [4,5].

From the nutritional point of view, the pecan nut is highly appreciated because it is a material of high fat density, mainly containing monounsaturated and polyunsaturated fatty acids. There is evidence that the dietary intake of MUFAs and PUFAs lowers blood cholesterol and has beneficial effects on vascular reactivity, thrombosis and inflammation [6]. Pecan nuts are a complex food and a source of multiple nutrients such as proteins, vitamins (tocopherols) and minerals (magnesium, phosphorus, zinc, copper, and manganese) [7].

Given that the fiber content is much higher in the pecan shell, we hypothesized that the optimal extraction process could be markedly different than for the kernel. To this end, a statistical study was designed. We optimized pecan shell extraction using a response surface methodology (RSM) to determine the total phenolic content (TPC) and the radical scavenging activity. Factors studied included sample weight to solvent ratio (w/V; ranging from 0.023 to 0.14) and solvent effect of different concentrations of ethanol in water (v/v %; ranging from 29 to 71). The method chosen to estimate the best concentration of TPC was the Folin-Ciocalteu test. The results indicated that at higher ethanol concentration, higher phenolic content was extracted until reaching the optimal concentration (36 %).

Above this value, it started to decline slightly. On the other hand, regarding the weight to solvent ratio, the surface plot for TPC response indicated a strong decline when sample dry weight to solvent ratio increased. In the case of the radical scavenging activity, the assay used was oxygen radical absorbance capacity (ORAC).

RSM was previously used in other studies to optimize the extraction process with the aim to extract high quantities of polyphenols. This methodology was successfully applied using by-products such as grape pomace skin, hazelnut skin, peanut skin, chestnut shell, and mango peel [8–12]. Using a ratio of pecan shell weight and solvent of 0.0234 g/mL and 44 % v/v ethanol-water or 100 % water, we obtained higher concentrations of polyphenols in the ethanolic extract (41 mg GAE/g DW) compared to aqueous (100 %) extract (20 mg GAE/g DW). In other studies, we used a weight to solvent ratio of 0.1 or 0.15 g/mL and solvent with ethanol-water of 50 % v/v; the results obtained were 96.81 and 72.96 mg GAE/g DW, respectively. The difference in values could be attributed to other factors involved in the extraction process, such as temperature, time, stirring speed and origin of the ethanol [13,14].

The presence of polyphenols in pecan shell ethanolic extracts were confirmed with LC-MS-TOF. The samples contained several phenolics, including: gallic, ellagic, p-hydroxybenzoic, protocatechuic, pentose, methyl ellagic pentoside, epigallocatechin gallate, dimethyl ellagic rhamnoside, dimethyl ellagic acid, caffeic acid 3-glucoside, 3-p-Coumaroylquinic acid, lambertianin C isomer, coumarin derivate, quercetin, pedunculagin, vanillic acid, kaempferol derivate, castalin, carminic acid, myricetin, syringetin-3-o-glucoside, ferulic acid, and 1-O-galloyl castalagin. Some of them were also reported by others authors: Kureck et al., through a LC-DAD analysis, found catechin, epigallocatechin, epicatechin gallate, proanthocyanidins, chlorogenic acid, vaillic acid, caffeic acid, ellagic acid, 4-hydroxibenzoic acid and hydroxycynamic as the main phenolics in pecan shells [15]. Hilbig et al. found similar compounds in their study [16].

The TPC extraction from defatted pecan kernel was performed using weight to solvent ratios 0.1 and 0.05 g/mL, with a solvent ethanol-water 50% v/v. The results were 20.55 and 22.95 mg GAE/g DW, respectively. As for the pecan shell, the difference in the values could result from the extraction of TPC declining when sample dry weight to solvent ratio increased, or other factors. In any case, these values confirm the results obtained by De la Rosa et al.; 5- to 20-fold higher concentrations of phenolic compounds were found in pecan shells [17].

In the case of the roselle flower, acidified extraction increased the polyphenols extracted by almost 33%.

Lipid oxidation is one of the major causes of quality deterioration in natural and processed foods, because it affects many quality characteristics such as color, texture, and flavor (rancidity). Also, the oxidative instability of PUFAs often restricts their functional use as nutritionally beneficial lipids in food [18]. The food industry employs a variety of methods to increase the oxidative stability of their products. Addition of antioxidants (synthetic or natural) that chelate pro-oxidative metals and scavenge free radicals are the most usual methods used to delay lipid oxidation [19,20].

Most of the lipids in foods are dispersed as oil in water or water in oil. In this study, the shell was used in a food model as emulsion (oil in water). The results were very promising. A concentration of 6.26 g/L of shell extract avoided the onset of oxidation before 768 hours of incubation and offered

more protection than gallic acid at 0.78 g/L. Similar studies with by-products in emulsions, proved their potential as natural food preservatives [21–23].

One of the major causes of quality deterioration of meat products are microbial spoilage and lipid oxidation [24]. The rate and extent of oxidative deterioration can be reduced through various procedures like curing, vacuum packaging, modified atmosphere packaging and, most importantly, adding antioxidants [25]. The meat industry usually adds synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), ascorbate or sulphites, to fresh and processed meats, in order to prevent lipid oxidation. However, the potential risks to health associated with these synthetic compounds have created a growing demand for natural antioxidants [24,26–28]. On the other hand, the International Agency for Research on Cancer from the World Health Organization (WHO) reported the risk of carcinogenicity from consumption of red and processed meat. The possible mechanisms involved in the potential carcinogenicity of red and processed meat, could be due to the presence of compounds such as N-nitroso-compounds (NOCs), heme iron, polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic amines (HAAs) [29]. Several studies demonstrated that natural extracts with high antioxidant activity, reduced the potential carcinogenic substances in meat products. Examples of these products are: spices such as prickly ash peel, fennel, star anise, cumin, chili pepper, turmeric, ginger, and curry, clover, garlic, buckwheat, lemon juice, soy sauce, chitosan, vegetable oils, olive oil, sunflower oil, grape seed oil, and teas (green, white, yellow, oolong, dark, and black)[30–34].

In the present study, we worked with beef patties containing 4 % of pecan shell powder. The TBARS value (mg MDA/kg sample), after 7 days at  $4\pm 1$  °C, decreased by 81.5 % compared with the control.

Indeed, in recent years, there have been major improvements in the development of active packaging. The main function of packaging is to serve as a container for the food, but also it is fundamental to maintain the food quality and safety from production to final consumption.

Recent studies have focused on the incorporation of natural products containing bioactive compounds like phenols into packaging materials to produce active edible films. Moghadam et al. worked with films based on mung bean protein, enriched with pomegranate peel [35]. Rodríguez et al. developed an edible film based on papaya and integrated with *Moringa oleifera* and ascorbic acid [36].

In this thesis, we developed gelatine films with 3 % of pecan shell extract. The radical scavenging activity of these films was similar to the sample with 0.1 % of the commercial preservative. To show its antioxidant activity in real food, the films were set above and under small beef patties. We found that after 8 days at  $4\pm 1$  °C, the contents of mg MDA/kg sample decreased by 43 % compared to the control (with gelatine film only).

EPA and DHA are PUFAs that are present in relatively high concentrations in the flesh of marine fish. EPA and DHA were found to have positive effects on various cardiovascular diseases, such as blood clotting disorders and high blood pressure [37]. However, the high lipid content makes fish products very susceptible to lipid deterioration. Recently, a number of authors examined the use of natural antioxidants in different fish species such as sardine and salmon [38,39].

Delivery systems such as encapsulation could offer advantageous strategies to improve antimicrobial and antioxidant activities and enhance the shelf life of fish products. In this thesis, we encapsulated the pecan shell and the extruded pecan shell (extruded at 70 °C and 150 rpm). Eventually, the capsules were incorporated into fresh salmon patties. The concentration of MDA per kg of salmon patties with pecan shell (extruded and non-extruded) after 5 days at 4±1 °C, presented less oxidation than the commercial additive (BHA).

In beef patties, the possible synergistic effects of pecan shell and roselle flower (*Hibiscus sabdariffa*) and cayenne pepper (*Capsicum annuum*) may be used as natural antioxidant and antimicrobial food additive. Our findings demonstrated the efficiency of the synergy to prevent lipid oxidation, with low values of MDA and hexanal in beef patties stored at 4±1 °C for 13 days. Moreover, the mix was effective in reducing the microbial activity, the concentration of metmyoglobin and pH. In the same study, roselle flower exhibited high antimicrobial activity towards microorganisms that are commonly present in meat and fish products. In addition, roselle flower was used in synergy with pecan kernel in sardine patties. All analyses showed that samples treated with pecan nut and roselle flower had better quality parameters than the control. These results could be explained by the high content of tocopherols and phenolic compounds in the kernel combined with the antimicrobial capacity and the phenolic composition in the roselle flower.

Sensory evaluation provides useful information about the human perception when a product changes due to modification of an ingredient or its shelf life. According to Hui et al. in the meat industry sensory testing is often used for two important objectives [40]. Firstly, to help the marketing department know whether consumers will purchase a new or substantially modified product. Secondly, sensory evaluation must be performed in order to relate the consumer's acceptance of a product to the sensory descriptors that define that product [41]. In this study, we used a descriptive and ranking test. Some authors also used descriptive tests to evaluate natural antioxidants in meat [42,43].

Semi-trained panelists evaluated in different tastings the beef and sardine patties. In the first case, they evaluated different parameters of five beef patties with diverse mixes of pecan shell, roselle flower and cayenne pepper. The results showed that the control (without antioxidant) was the most acceptable patty. Possibly, the panelists did not have the habit of eating spicy and acidic products. For sardines with kernel and roselle flower, the panelists reported an acidic shade. Members of the panel from South American countries argued that it was a pleasant taste. Nguyen et al. developed cookies with the addition of roselle powder [44]. The sensory evaluation indicated that roselle was associated with a bitter taste, which affected the overall acceptance of the cookies.

To improve sustainability, the development of new products for the food industry needs to be addressed from the social, environmental, and economical point of view in addition to scientific and engineering perspectives [45,46]. The use of by-products affords considerable advantages, such as the reduction of waste production. Most of these wastes, including the pecan shell, derive from the agri-food industry and are rich in phenolic compounds and other valuable compounds such as fiber, protein, vitamins and minerals. The use of extrusion technology can enhance the bioavailability of certain compounds. Recently, food industry by-products have been used in extrusion processes. Some examples include apple pomace, carrot pomace, cherry pomace, pineapple peel and pomace, cassava bagasse, citrus peel, bagasse, and citrus seed [47–51]. In this thesis, we optimized the

extrusion process of the shell. In the case of TPC, we found that the temperature, screw speed, and the interaction between both showed no significant ( $p < 0.05$ ) effects in the process. Unlike TPC, the condensed tannin contents with the temperature and the interactions between temperature–screw speed were statistically significant, but not the screw speed ( $p < 0.05$ ). Finally, the extrusion process increased the radical scavenging activity in most of the treatments. The pecan shell extruded at 70 °C and 150 rpm was the optimal. With these conditions the total dietary fiber increased 3.7%. Additionally, the soluble dietary fiber in the extrudates was more than 3-fold higher than in the control (non-extruded shell). This type of fiber has an important role in human digestion because it increases the digesta viscosity and fermentation to produce short chain-fatty acids [52]. Similar results were obtained by Zhong et al. who found that the extrusion of lupin seed coat (a by-product) significantly increased the soluble fiber content from 29.03 g/kg dry basis to 90.28 g/kg dry basis [53].

Most of this fiber content in the pecan shell could be cellulose, hemicellulose and lignin. These types of fibers limit the application of these residues for certain processes, such as biomolecules and biofuels [54,55]. The solid-state fermentation with some microorganisms could be a good option to generate cellulolytic enzymes that degrade the inner regions of cellulose to disrupt the polymer chains of the pecan shell. Also, with this process filamentous fungi could biodegrade tannins and liberate phenolic compounds [56,57]. In this study, the fermentation with *A. oryzae* of extruded (113.67–130.30 mg GAE/g) and non-extruded (32.64–42.65 mg GAE/g) shell confirmed that the phenolic content increased significantly ( $p < 0.05$ ) from 0 to 120 hours. A similar tendency was presented by the radical scavenging activity with DPPH and ABTS methods. In the case of the condensed tannins, dependence on time was not clear. However, at the last hours of fermentation a decrease of the condensed tannins was observed. Catechin (mg of catechin/g of sample) in extruded pecan shell decreased from 275.19 (0 hour) to 218.11 (120 hours), while in non-extruded pecan shell the values decreased from 120.21 (0 hour) to 31.48 (120 hours). In all cases the values in extruded pecan shell were higher than in non-extruded pecan shell. This may contribute to greater fungal growth in extruded pecan shell.

Our findings support the notion that the extruded pecan shell can be used in clean-label products and can improve the nutraceutical value of bread and tortilla, two important products in the human diet, particularly in Mexico. With the addition of different percent's of extruded and non-extruded shell on the bread and tortilla formulation, we found that bread with 5% WSEF and tortilla with 10% WSEF had the highest total phenolic content (4.80 and 3.75 mg GAE/g), DPPH (402.26 and 180.42 TEAC in  $\mu\text{mols}/100\text{g}$ ), ABTS<sup>+</sup> (518.34 and 305.73 TEAC in  $\mu\text{mols}/100\text{g}$ ), and total fiber content (11.19 and 16.04 g/100g), respectively. The same treatments with 10% extract reduced the viability of hepatic cancer cells by 53.46% (bread) and 44.97% (tortilla). On the other hand, the results of hedonic tests showed no significant ( $p < 0.05$ ) difference in the texture and acceptability for any formulation of bread and tortilla. These results could be related to the increase of the dietary fiber content, which not only reduces and prevents several human diseases, but also improves the texture, increases the water-holding capacity and oil-binding capacity [58].

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## Chapter 9. Overall conclusion

1. The extraction conditions were optimized by response surface methodology; the variables were sample weight to solvent ratio (w/v) and ethanol concentration (%). Extraction with 0.0234 w/v gave the maximum value for total phenolic contents (TPC) and oxygen radical absorbance capacity (ORAC). The ethanol percentage was 36% for TPC and 51% for ORAC.

Different methods for the quantification of phenolic compounds and the scavenging capacity (TPC, ORAC, FRAP, DPPH and ABTS) of shell extracts (44% ethanolic and aqueous) were assayed. Total polyphenols in shell extracts were twice more than after aqueous extraction. The results for ORAC, FRAP, DPPH, and ABTS were 5-, 1.54-, 1.41- and 1.71-fold higher, respectively, in the ethanol extract than in the aqueous extract.

2. Sixteen shell constituents were identified in shell extracts by LC-MS-TOF. The main compounds detected were epigallocatechin, (+)- catechin, procyanidin, ellagic acid, phloridizinyll glucoside, and (-)- epigallocatechin gallate.

3. The effectiveness of shell and kernel extracts concerning protection against oxidation of oil in water emulsions was evaluated. The formation of hydroperoxides was significantly faster in samples without antioxidants. Control samples reached 10 meq/kg in less than 65 hours. Sample oxidation (reaching the hydroperoxide value of 10 meq of hydroperoxides/kg emulsion) followed this order: control (in the absence of walnut/shell or gallic acid extracts) > gallic acid at 0.21 g/L > walnut at 1.09 g/L > shell at 1.09 g/L > gallic acid at 0.39 g/L > gallic acid at 0.78 g/L > walnut at 4.35 g/L > walnut at 6.52 g/L > shell at 4.35 g/L > shell at 6.52 g/L. Emulsions containing higher concentrations of shell extract endured longer in a good condition and started the oxidation process at the end of the experiment (after 768 hours), and therefore were more protective than gallic acid at 0.78 g/L.

4. The extrusion cooking process was optimized with respect to the variable's temperature and screw speed. The extrusion process increased antioxidant activity in most of the treatments. The highest radical scavenging capacity (DPPH: lowest IC<sub>50</sub>) was obtained for samples processed at 70 °C and 150 rpm. These conditions increased the concentration of phenolic compounds, radical scavenging activity, and total dietary fiber including soluble fiber. In addition, the extrusion process changed the content of 9 detected phenolic compounds in the shell. Extrusion at 70 °C and 150 rpm significantly increased the presence of phenolic compounds compared to controls (non-extruded shell). Increased phenolic compounds included gallic acid, ellagic acid pentose, ellagic acid, and dimethyl ellagic acid. The major phenolic acid compound in the extruded shell was ellagic acid (1.74 µg/g dry sample). The soluble fiber in extrudates was more than 3-fold higher than in the control.

5. Solid-state fermentation with *A. oryzae* and as substrate pecan shell extruded and non-extruded increased the total polyphenols after 120 hours (shell extruded 75.35-130.30 mg GAE/g and non-extruded 34.99-42.65 mg GAE/g). Radical scavenging assessed by  $\mu\text{mol}$  equivalents of Trolox in the DPPH method increased in the extruded shell almost 3.3 times after 120 hours, and in the non-extruded shell 1.26 times after 120 hours of fermentation. Similar changes to those found with the DPPH method were observed with the ABTS method. However, the condensed tannins in most of the cases decreased.

6. The present study has demonstrated the potential of using a by-product (pecan walnut shell) as natural ingredient in the development of bakery products. Results showed that the incorporation of shell (extruded and no-extruded) significantly increased the antiradical capacity, phenolic and fiber content in wheat bread and tortilla. Also, the reduction of the viability of human cancer cell lines by extracts of the finished products can be assessed.

7. Total polyphenols, radical scavenging capacity (by DPPH) and antimicrobial activity using the diffusion method with roselle disk flowers and cayenne pepper extracts were quantified. The radical scavenging activity was significantly higher in roselle flower ( $73.06 \pm 0.01 \mu\text{mol TE/ g DW}$ ) than in cayenne pepper ( $98.29 \pm 0.04 \mu\text{mol TE/ g DW}$ ). The TPC for roselle flower was  $15.02 \pm 0.01 \text{ mg GAE /g DW}$ , while a lower value was observed for cayenne pepper,  $7.034 \pm 0 \text{ mg GAE /g DW}$ .

Antimicrobial activity revealed that roselle flower extracts inhibited the growth of *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Micrococcus luteus*, *Escherichia coli*, and *Salmonella enterica*. Cayenne pepper extracts showed less antimicrobial activity than the roselle flower. Cayenne pepper extracts simply showed a moderate effect against *L. monocytogenes* and *M. luteus*. On the other hand, pecan shell extracts showed antimicrobial capacity against five out of the six microorganisms studied: *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Micrococcus luteus*, and *Salmonella enterica*.

8. Shell powder, roselle flower, cayenne pepper and their mixtures can be successfully used to decrease lipid oxidation and improve the shelf life and color stability of model meat systems. On the last day of the experiment (day 12), three groups of samples with similar oxidation levels were observed. The more effective treatments were the commercial preservative 0.7%, roselle flower 2% and the mixture of shell 4% + cayenne pepper 0.35% + roselle flower 2%, with a percent inhibition of 79, 73 and 75, respectively, compared with the control. Indicators of secondary oxidation, such as hexanal formation, were not observed at the end of the experiment in the patties with commercial preservative 0.7% and roselle flower 2%. However, samples treated with shell, Cayenne pepper and roselle flower presented significant amounts of hexanal ( $p < 0.05$ ), although lower than in the control sample.

Results showed that the chroma ( $c^*$ ) values, calculated as  $(a^2 + b^2)^{1/2}$ , decreased as storage time progressed in controls and treated samples. The decrease of  $c^*$  followed a similar trend to that for  $a^*$  (red-green) and  $b^*$  (yellow-blue) values, which slowly decreased as a result of increasing amounts of shell, roselle flower and cayenne pepper. Particularly, the sample with the mix of shell

4%+ cayenne pepper 0.35% and roselle flower 2% was markedly effective in keeping the values of  $c^*$  of meat products stored at 4°C. In general, samples with roselle flower contained lower concentrations of metmyoglobin than other samples at the end of the experiment.

In regard of microbiological results, the samples with the mixes of shell, cayenne pepper and roselle flower presented less microbial growth than samples with only a mix of shell and cayenne pepper.

9. The addition of pecan kernel and roselle flower to sardine patties indicated that after 66 h of incubation at 4 °C, samples containing 10% of pecan kernel were significantly less oxidized (0.89 mgMDA/kg) than under any other condition. Samples with 5% of roselle flower (2.33 mgMDA/kg) and with 5% roselle flower + 5% pecan kernel (2.18 mgMDA/kg) were significantly less oxidized than the control (5.04 mgMDA/kg). Following 5 days of treatment, the control sample presented the highest hexanal levels, 20-fold greater than at day 0. The lower levels of hexanal were found in samples containing 10% of pecan kernel. The amount of biogenic amines (BA) in sardine flesh significantly varied depending on the treatment. After 5 days of incubation, samples containing 10% of kernel pecan had lower amounts of BA than the control and any other treatment. Samples containing 5% pecan kernel also presented better results in preventing the formation of most BA than 0.1% BHA. On the other hand, the fatty acid profile in the sardine's patties changed. Addition of pecan kernel to the fish flesh increased the amount of linoleic and oleic acid by 3- and 4-fold, respectively. Sensory analysis was also performed. The sample with 10% of pecan kernel had the higher taster's acceptability.

10. The preparation and characterization of gelatin films incorporating different pecan extracts were studied. Film thickness, morphology of the surface and the transverse section (by SEM), thermodynamic characteristics (by DSC), water vapor barrier, structure analysis (by XRD), as well as mechanical and optical properties of the developed films varied depending on the concentration of walnut, shell and synthetic antioxidant. The film with higher walnut concentration (15%) was 20-fold thicker than the control. The permeability to water vapor (0.414 gmm/m<sup>2</sup>·day·Pa) was significantly lower than the control (5.0368 gmm/m<sup>2</sup>·day·Pa). Furthermore, in newly formed films the elongation at break and Young's modulus decreased 6-fold. Additionally, the addition of pecan walnut (15% and 9%) led to the formation of big crystals by DSC, which could improve the thermal stability of gelatine films. The luminosity (\*L) of films containing pecan kernel and shell extracts was reduced compared to the control. The film with higher concentration (15%) of pecan kernel extract, presented 30% inhibition of the DPPH stable radical.

11. Oxidation of beef patties protected with different films was studied. Compared with controls, the 15% pecan kernel films displayed prolonged shelf life due to 30-fold increased reduction. Moreover, films with pecan kernel and shell extracts exhibited protection against microbial contamination, although at levels not significantly different than the gelatin film.

12. The antioxidant properties of PAAG-12 and PLA films with vitamin E as the main non polyphenolic pecan antioxidant were assessed in emulsions. The peroxide value of O/W emulsions after 31 days showed that PAAG-12 and PLA films with  $\alpha$ -tocopherol protected the emulsion up to 50% compared with films without  $\alpha$ -tocopherol. In addition, PAAG-12 showed thermal decomposition starting at about 200 °C, with a maximum decomposition rate at 226.4 and 229.1 °C for both nitrogen and air atmospheres. When the films were treated with  $\alpha$ -tocopherol, the thermal stability substantially increased with an increase in the onset temperature at about 58 (nitrogen) and 50 °C (air atmospheres).

13. The addition of chitosan and shell capsules to minced salmon showed that 24 h incubation at 10 °C significantly reduced oxidation in samples containing pecan walnut shell extract (extruded and non-extruded) compared with controls (samples without capsules and with empty capsules). At the end of the experiment (120 h), almost all the samples with pecan walnut shell (extruded and non-extruded) were less oxidized than those with commercial additives (0.1% BHA). Hexanal content after 144 hours of storage significantly decreased in salmon samples treated with capsules with chitosan and extruded and non-extruded pecan shell. Hexanal levels in the samples formulated with BHA were 90% decreased compared to the control (without capsules).



## Appendix: Conferences and publications

The following presentations on conferences have resulted from the research work presented in this thesis:

- Juliana Villasante, Ares Bobet Llobera and MaríaPilar Almajano

“Effects of pecan shell walnut (*Carya illinoensis*), roselle flower (*Hibiscus sabdariffa*), and cayenne pepper (*Capsicum annuum*) powder on lipid oxidation and antimicrobial activity of beef patties during refrigerated storage”

Congress: Euro Food Chem. Porto, Portugal. June 2019.

Organization: Food Chemistry Division of the European Chemical Society (FCD-EuChemS) and the Portuguese Chemical Society (SPQ)

\*Poster 3rd place
- Juliana Villasante, Tamara Alvarez, Xavier Ramis, MaríaPilarAlmajano

“Physicochemical and antioxidant properties of gelatin-based films containing pecan walnut and shell (*Carya illinoensis*)”

Congress: Food Technology. Atenas, Grecia. April 2019.

Organization: EuroSciCon Lt

\*Poster
- Juliana Villasante, MariaPilar Almajano, Esther Pérez, Erick Hereida

“Effects of extrusion pretreatment parameters on pecan walnut shell (*Carya illinoensis*)”

Congress: Agriculture and Food Chemistry. Roma, Italia. June 2018.

Organization: EuroSciCon Lt.

\*Poster 1st place
- Villasante Juliana, Almajano MP

“Antioxidant activity of pecan shell”.

7th European Symposium CONACYT, Strasburg, Francia. April 2018.

Organization: Consejo Nacional de Ciencia y Tecnología.

\*Oral Presentation

- Villasante Juliana, Kaur Lovepreet, Badía Laura, Rodero Lourdes, Almajano MP

“Antioxidant activity of walnuts (shell and seed) of different origins”

First International Conference on Bio-antioxidants (ICBA 2017), Young Scientists School on Bio-antioxidants (YSSBA 2017) Sofia, Bulgaria. June 2017

Organization: Institute of Organic Chemistry with Centre of Phytochemistry, BAS.

\*Oral Presentation
- Hidalgo Gador, Villasante Juliana, Almajano MP

“Antioxidant activity of red fruits”

First International Conference on Bio-antioxidants (ICBA 2017), Young Scientists School on Bio-antioxidants (YSSBA 2017) Sofia, Bulgaria. June 2017.

Organization: Institute of Organic Chemistry with Centre of Phytochemistry, BAS.

\*Oral Presentation
- Federica Mosca, Juliana Villasante, Xavier Ramis, Eva Codina, MaríaPilar Almajano

“Poly-lactic acid (PLA) films as active food packaging with antioxidant properties”

First International Conference on Bio-antioxidants (ICBA 2017), Young Scientists School on Bio-antioxidants (YSSBA 2017) Sofia, Bulgaria. June 2017.

Organization: Institute of Organic Chemistry with Centre of Phytochemistry, BAS.

\*Poster Presentation

**Publications that do not formed part of this thesis:**

- “Effect of Neem (*Azadirachta indica* L.) on Lipid Oxidation in Raw Chilled Beef Patties”

Ouerfelli, M. (M.O.); Villasante, J. (J.V.); Ben Kaâb, L.B. (L.B.B.K); Almajano, M. (M.A.)

*Antioxidants* 2019, 8, 305. <https://doi.org/10.3390/antiox8080305>

Author Contributions

Conceptualization, M.O. and M.A.; Formal analysis, M.O. and J.V.; Funding acquisition, M.A.; Investigation, M.O. and J.V.; Methodology, M.O. and J.V.; Project administration, M.A.; Supervision, M.A.; Validation, M.A.; Writing—original draft, M.O.; Writing—review & editing, M.A. Final advice L.B.

- “Avocado Seed: A Comparative Study of Antioxidant Content and Capacity in Protecting Oil Models from Oxidation”

Segovia, F.J. (F.J.S.); Hidalgo, G.I. (G.I.H.); Villasante, J. (J.V.); Ramis, X. (X.R.); Almajano, M.P. (M.P.A.) *Molecules* 2018, 23, 2421. <https://doi.org/10.3390/molecules23102421>

#### Author Contributions

Conceptualization, F.J.S. and M.P.A.; Validation, X.R. and M.P.A.; Formal Analysis, F.J.S., X.R.; Investigation, F.J.S., G.I.H. and J.V.; Resources, X.R. and M.P.A.; Writing-Original Draft Preparation, F.J.S.; Writing-Review & Editing, G.I.H., J.V.; Supervision, Project Administration and Funding Acquisition, M.P.A. All authors read and approved the final manuscript.

- “Continuous or Batch Solid-Liquid Extraction of Antioxidant Compounds from Seeds of *Sterculia apetala* Plant and Kinetic Release Study”

Mosca, F. (F.M.); Hidalgo, G.I. (G.I.H.); Villasante, J. (J.V.); Almajano, M.P. (M.P.A.) *Molecules* 2018, 23, 1759.

#### Author Contributions

M.P.A. conceived the idea, designed and led the experiments and coordinated the authors. F.M. performed the experiments, analyzed the data and wrote the manuscript. G.I.H. and J.V. collaborated in the performance of the experiments and review of the manuscript. All authors read and approved the final manuscript.

- “The Administration of Chitosan-Tripolyphosphate-DNA Nanoparticles to Express Exogenous SREBP1a Enhances Conversion of Dietary Carbohydrates into Lipids in the Liver of *Sparus aurat*”

Silva-Marrero, J.I.(J.I.S.-M.); Villasante, J. (J.V.); Rashidpour, A. (A.R.); Palma, M. (M.P.); Fàbregas, A. (A.F.); Almajano, M.P.(M.P.A.); Viegas, I. (I.V.); Jones, J.G. (J.G.J.); Miñarro, M.(M.M.); Ticó, J.R. (J.R.T.); Baanante, I.V.(I.V.B.); Metón, I (I.M.). *Biomolecules* **2019**, 9, 297.

#### Author Contributions

Conceptualization, I.M. and I.V.B.; methodology, J.I.S.-M., J.V., A.R., M.P., A.F., I.V., J.G.J., M.M. and J.R.T.; validation, M.P.A., I.V., M.M., J.R.T., I.V.B. and I.M.; formal analysis, J.I.S.-M., J.V., A.R., M.P. and A.F.; investigation, J.I.S.-M., I.V., M.P.A., I.V.B. and I.M.; writing—original draft preparation, J.I.S.-M. and I.M.; writing—review and editing, J.I.S.-M., J.V., A.R., M.P., A.F., M.P.A., I.V., J.G.J., M.M., J.R.T., I.V.B. and I.M.; project administration, I.M.; funding acquisition, I.M.













