UNIVERSITAT POLITÈCNICA DE CATALUNYA BARCELONATECH

ETSEIB

Tesis Doctoral

Estudio de la actividad antioxidante de diversas plantas aromáticas y/o comestibles

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Barcelona, septiembre 2016

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DEDICATORIA

Este trabajo quiero dedicarlo con mucho cariño y amor, a mi hija, la gordita que me llena de motivación cada día, a mi esposo que siempre ha estado apoyándome, dándome su amor y fortaleza, a mis padres porque gracias a ellos soy lo que soy y a mi hermana que sirvió de inspiración para embarcarme en este gran proyecto, sin ellos esto no hubiera sido posible.

El optimismo es la fe que conduce al logro. Nada puede hacerse sin esperanza y confianza.

Helen Keller

AGRADECIMIENTOS

En estas líneas quiero expresar la gratitud que siento a todas las personas que han estado presentes estos años. Esta tesis ha requerido de mucho esfuerzo y dedicación y no hubiese sido posible sin la cooperación de todas y cada una de las personas que nombraré, muchas de las cuales han sido un fuerte soporte.

Quiero agradecer de forma muy especial a mi directora de tesis, la profesora María Pilar Almajano, por haberme abierto las puertas a su grupo de investigación hace 4 años y darme la oportunidad de realizar esta tesis doctoral. Agradezco su confianza y apoyo en todo momento, sus consejos y su dedicación a mi formación como investigadora, la manera en la que ha guíado mis ideas y el haberme facilitado siempre los medios para llevarlas a cabo.

Quiero agradecer a toda mi familia. Con todo mi amor gracias a Jhonnatan, mi esposo, por su continua compresión aún en los momentos más difíciles y ser mi apoyo incondicional, porque a su lado la tristeza se transforma en alegria. A mi bebe Victoria porque incluso estando en la barriguita me permitió seguir adelante con mis experimentos y es mi gran ilusión. A mis padres, por su aliento, respaldo y por sus consejos, por haberme apoyado en todas las metas que me he propuesto. A Carol, mi hermana, por ser esencial siempre y confiar en mi capacidad para llegar hasta aquí. A mis suegritos, por su ánimo y su apoyo siempre.

También muestro mi agradecimiento a todo el personal del Departamento de Ingeniería Química del ETSEIB, Secretaria, Dirección, Mantenimiento, Biblioteca y a la Escuela de Doctorado, ya que cada uno siempre ha colaborado de forma muy positiva sin ponerme ningún impedimento y a la UPC por haberme concedido la beca FPI-UPC y haber permitido la financiación de este proyecto.

De manera muy especial quiero agradecer a la Universidad de Santiago de Compostela, al Insituto de Investigación de Análisis Alimentarios (IIAA), a todos sus estudiantes doctorales por su colaboración y al profesor Isaac Rodríguez, por haber hecho posible la realización de mi estancia doctoral y a quién le agradezco mucho su disposición, su voluntad y su sabiduría en mis primeros pasos por la técnica de espectroscopía de masas.

Al personal del Parc Cientific y del CCit quiero agradecer su estrecha colaboración, así como su completa disposición a resolver cualquier duda o problema que se haya podido presentar.

Gracias a mis compañeros doctorandos del grupo de investigación: Francisco, Monika, Aini y Sara. Gracias a todos chicos por haber formado parte de este gran equipo y siempre haber sido buenos compañeros.

Y a todos aquellos que no he nombrado pero que de una u otra manera han formado parte de este gran sueño.

RESUMEN

Las especies vegetales analizadas en este trabajo, plantas aromáticas (*Rosmarinus officinalis*, *Thymus vulgaris* y *Lavandula officinalis*) y otros tipos de plantas (*Caesalpinia decapetala*, *spinosa, Morinda citrifolia* (*Noni*)) tienen importantes propiedades beneficiosas para la salud y son útiles para la industria alimentaria y cosmética, ya que son ricas en compuestos polifenólicos. Estos compuestos poseen propiedades antioxidantes, capaces de inhibir procesos de oxidación en alimentos y de envejecimiento celular. Además pueden presentar propiedades bactericidas.

La oxidación de grasas y aceites hace que experimenten transformaciones que reducen su calidad nutritiva y se produzcan compuestos volátiles que generan olores y sabores indeseables. Esta oxidación puede ser minimizada por los extractos de las plantas, ya que actúan neutralizando los radicales libres que se forman en la primera etapa de la oxidación y ello permite que puedan sustituir, al menos parcialmente, a los antioxidantes sintéticos, potencialmente tóxicos. Este trabajo se centra en una profunda investigación de cada una de las plantas y su implementación en modelos de alimentos.

De forma inicial, se han analizado los polifenoles totales por Folin-Ciocalteau, la actividad antiradicalaria mediante los métodos TEAC (*Trolox Equivalent Antioxidant Capacity*), DPPH (*2,2-diphenyl-1-picrylhydrazyl*), ORAC (*Oxygen Radical Aborbance Capacity*) y FRAP (*Ferric Reducing Antioxidant Powe*r) y se han identificado los compuestos polifenólicos por HPLC (DAD y MS/MS) de cada uno de los extractos de las plantas, obtenidos a partir con una extracción sólido-líquida con etanol 50% como solvente, bajo condiciones de refrigeración a 4ºC durante 24h.

Seguidamente se han evaluado en sistemas alimentarios reales, como emulsiones de “aceite-en-agua” y productos cárnicos (hamburguesas y salchichas) para analizar la ralentización de la oxidación, seguida a través del valor de peróxido, el ensayo TBARS (*Thiobarbituric Acid Reactive Substances*) y el hexanal. En los sistemas cárnicos se han determinado otros factores, como pH, color, cantidad de metamioglobina y la variación de la actividad antioxidante en el propio sistema alimentario.

También se ha trabajado con envases activos. Se han fabricado films comestibles a base de una mezcla de gelatina y extracto etanólico de las plantas del género *Caesalpinia* y *Morinda*, con la finalidad de poder usarlo como película en contacto con el alimento, al ser comestible. Se caracteriza y evalúa tanto en la interacción con el alimento (hamburguesa), analizando el retraso en la oxidación, como por sus propiedades mecánicas y físico-químicas. Otro film que se ha fabricado es a base de ácido poliláctico (PLA) con adición de las plantas aromáticas romero y tomillo. Se caracterizó a través de sus propiedades físico-químicas y se analizó el

valor protector frente a la oxidación en el mismo sistema descrito de emulsiones “aceite-en-agua”.

De los extractos evaluados el de *Tara* ha presentado la mayor actividad antiradicalaria, con un valor frente al ensayo ORAC de 2 mmolesTE/g peso seco y el Noni la menor actividad con 0,017 mmoles TE/g peso seco. Así mismo, la Tara tuvo un gran efecto antioxidante en emulsiones “aceite-en-agua”. A una concentración de 0,5 % m/v la *Tara* redujo la formación de whidroperóxidos en un 96,17 %, aumentando la estabilidad de las emulsiones frente a la oxidación lipídica. Por su parte, la *C. decapetala* logró una reducción de la oxidación lipídica en hamburguesas de un 69,87% después de 11 días de almacenamiento a 4 ºC y al evaluar los films en este mismo tipo de producto, el film con extracto de *Tara* al 0,2 % produjo la mejor protección con una reducción de un 77,17% comparado al control.

Como conclusión, el extracto obtenido de todas las plantas, en especial la Tara, tiene una actividad antioxidante capaz de proteger a los alimentos analizados y los films fabricados con estos extractos son una alternativa a la adición directa en el alimento.

ABSTRACT

Plants analyzed in this study, aromatic plants (*Rosmarinus officinalis*, *Thymus vulgaris* and *Lavandula officinalis*) and other plants (*Caesalpinia decapetala*, *Caesalpinia spinosa* (*Tara*) and *Morinda citrifolia* (*Noni*)) have important beneficial properties for health and for the food industry and cosmetics, since they are rich in polyphenol compounds. These compounds possess antioxidant properties capable of inhibiting oxidation processes in food and cellular aging. They also may have antibacterial properties

Oxidation of fats and oils makes that they experiment transformations which reduce its nutritional quality and occur volatile compounds that generate undesirable odors and flavors. This oxidation can be minimized by extracts of plants, as they act to neutralize free radicals formed in the first stage of oxidation and may thus be at least partially replace the synthetic antioxidants, potentially toxic. For this reason this paper focuses on thorough research into the properties of each of the plants and their implementation in *Model Food Systems*.

Initially, the content of total polyphenols was analysed by a spectrophotometric method based on the Folin-Ciocalteu assay, and the antiradical activity was studied by the methods TEAC (Trolox Equivalent Antioxidant Capacity), DPPH (2,2-diphenyl-1-picrylhydrazyl), ORAC (Oxygen Radical Absorbance Capacity) and FRAP (Ferric Reducing Antioxidant Power) assays. Polyphenolic compounds in each of the plant extracts, obtained by a solid-liquid extraction with 50% ethanol as solvent, under refrigeration at 4ºC for 24h were identified by HPLC (DAD and MS/MS).

Plant extracts have also been evaluated in real food systems, such as "oil-in-water" emulsions and meat products (burgers and sausages), with the main objective being to observe the protective effect exerted against lipid oxidation over time. Primary oxidation was monitored by determining the peroxide value and secondary oxidation by the TBARS (Thiobarbituric Acid Reactive Substances) test and also hexanal determination. In meat systems other factors have been analyzed including pH, colour, quantity of metmyoglobin and the variation of antioxidant activity in the alimentary system itself.

The effects of extracts have also been studied in active packaging. In this regard, an edible film based on a mixture of gelatin and ethanol extract of plants of the genus *Caesalpinia* and *Morinda* has been investigated, with the aim of using as a film in contact with food and with the characteristic that it is also edible. This film is characterized and evaluated both in its interaction with food (meat patties) by inhibiting lipid oxidation developing in this food, and by effects on mechanical and physico-chemical properties. Similarly, a film based on polylactic acid (PLA) with the addition of rosemary and thyme was manufactured. The film was characterized by its physical and chemical properties and the protective effect against oxidation in the system described as "oil-in-water" emulsion was analyzed.

Of the extracts tested, Tara has presented the greatest antiradical activity, with a value against the ORAC assay of 2 mmolesTE / g dry weight and Noni lower activity with 0.017 mmol TE/g dry weight. Likewise, the Tara had a great antioxidant effect in emulsions "oil-in-water". Tara at a concentration of 0.5% m/v reduced hydroperoxide formation in a 96.17%, increasing the stability of emulsions against lipid oxidation. Meanwhile, C. decapetala achieved a reduction of lipid oxidation on burgers from a 69.87% after 11 days of storage at 4 ° C and when assessing the films in this type of product, the film with Tara extract at 0.2% produced the best protection with a reduction of 77.17% compared to control.

In conclusion, the extract obtained from all plants, especially Tara, has an antioxidant activity capable of protecting the food samples and films made from these extracts are an alternative to the direct addition in the food.

Palabras clave: polifenoles, actividad antirradicalaria, inhibición lipídica, envase activo, plantas aromáticas, *Caesalpinia decapetala*, *Tara*, *Noni*, emulsiones aceite-en-agua, productos cárnicos.

TABLA DE CONTENIDOS

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LISTA DE ABREVIATURAS

ABTS	2,2'-Azino-Bis 3-Etilbenzotiazolina-6-Ácido Sulfónico
AAPH	2,2,-Azobis(2-Metilpropinamida) Diclorhidrato
AUC	Area Under Curve
AOC	Antioxidant Capacity Assays
BHT	Butyl Hydroxy Toluene
BHA	Butyl Hydroxy Anisol
CA	Caffeic acid
C	Catechin
CD	*Caesalpinia decapetala*
CG	Catechin Gallate
CS	*Caesalpinia spinosa*
CE	Collision Energy
CTR	Control
DMPO	5,5-Dimethyl-1-Pyrroline *n*-Oxide
DG	Dodecyl Gallate
DPPH	2,2-Difenil-1-Picrilhidracil
DW	Dry Weight
EC	Epicatechin
ECG	Epicatechin Gallate
EGCG	Epigallocatechin Gallate
FRAP	Ferric Reducing Antioxidant Power
FTIR	Fourier Transform Infrared Spectroscopy
GAE	Gallic Acid Equivalent
GA	Gallic Acid
LL	Lavender Leaf
LR	Lavender Root
LF	Lavender Flower
MDA	Malodialdehyde

O/W	Oil-in-Water
MetMB	Metmyoglobin
NE	*Noni* Extract
ORAC	Oxygen Radical Antioxidant Activity
OG	Octyl Gallate
PBS	Phosphate Bufered
PG	Propyl Gallate
TP	Total Polyphenols
SEM	Scanning Electron Microscopy
RL	Rosemary Leaf
ROS	Reactive Oxygen Species
RR	Rosemary Root
RF	Rosemary Flower
RD	Rosemary Dry
RLE	Rosemary Lyophilized Extract
TE	Trolox Equivalent
TEAC	Trolox Equivalent Antioxidant Capacity
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TBHQ	Tert Butyl Hydroxiquinone
TLE	Thyme Lyophilized Extract
TGA	Thervogravimetric Analysis
TL	Thyme Leaf
TR	Thyme Root
TF	Thyme Flower
TD	Thyme Dry
TPC	Total Phenolic Content
T_{ini}	Initial Degradation Temperature
T_{max}	Maximum Degradation Temperature

Tg	Glass Transition Temperature
PV	Peroxide Value
WT	Whyte Tea
WVP	Water Vapour Permeability

Introducción

1. INTRODUCCIÓN

En este capítulo se explica la teoría relevante en el desarrollo de la Tesis, que se divide en las siguientes secciones: aplicación de plantas en los alimentos, antioxidantes, consideraciones generales de las especies vegetales trabajadas, comportamiento de *Model Food System* y envasado alimentario.

1.1. Aplicación de plantas en los alimentos

Las plantas y las especias, especialmente las especies aromáticas y sus extractos, han tenido un creciente interés tanto, en la industria alimentaria como en la investigación científica, debido a sus propiedades antioxidantes y antimicrobianas, que les permite competir con otros antioxidantes naturales y con los sintéticos utilizados actualmente [1]. Estas propiedades se deben a gran cantidad de sustancias, como por ejemplo, flavonoides, terpenoides, carotenoides, vitaminas, … [2]. Las hierbas y especias usadas, por lo general para condimentar platos, se han caracterizado por ser una excelente fuente de compuestos fenólicos [3–5], aportando grandes beneficios frente a la rancidez oxidativa e igualmente aportando un valor añadido al producto, al indicar en el etiquetado que sus compuestos antioxidantes son naturales, libre de aditivos sintéticos, característica muy deseable en los consumidores y que cada vez más esta marcando tendencia en el mercado.

Actualmente un pequeño número de plantas se usan como antioxidantes en la industria alimentaria. En vista de este gran auge, los estudios que se realizan en la búsqueda de antioxidantes seguros aumentan, para mantener la calidad del producto. La eficacia antioxidante de especies como el romero, tomillo, orégano y clavo [6–8] se han experimentado en diversos alimentos e incluso se han usado en el desarrollo de fitomedicamentos calificados como fitofármacos; por todo ello también representan un rol importante en la industria farmaceútica [9,10].

Los extractos de plantas ricos en compuestos fenólicos se consideran buenos candidatos para su uso como antioxidantes en productos cárnicos. Las propiedades antioxidantes de dichos compuestos se han probado con éxito tanto en sistemas modelo como en productos reales [11]. En su composición, principalmente se destaca el romero, debido a la presencia de carnosol, rosmanol, isorosmanol y rosmaridifenol, compuestos con elevado poder antioxidante. Esta planta es utilizada con éxito en carnes procesadas como las hamburguesas de vacuno, en las cuales el romero mostró una gran capacidad antioxidante además de cierto efecto antimicrobiano [12].

Asimismo, se ha demostrado que compuestos terpénicos con carácter fenólico aislados de especias y/o hierbas aromáticas poseen gran capacidad antioxidante. Por ejemplo, compuestos extraídos a partir de aceites esenciales de orégano, borraja y salvia han sido estudiadas por su

potencial antioxidante. Estos compuestos suelen mostrar actividad antimicrobiana que los hace útiles para mejorar la seguridad alimentaria del producto final.

En estudios precedentes se han demostrado que utilizar productos naturales como extractos de plantas, frutas, verduras y especias comestibles actúan sobre los procesos degradativos de lípidos [13–15], y son una alternativa a los productos sintéticos, que en algunos casos podrían ser perjudiciales para la salud y el medio ambiente.

1.2. Antioxidantes

Los radicales libres, especies químicas que poseen uno o más electrones desapareados, reaccionan con las moléculas adyacentes mediante reacciones de óxido-reducción para estabilizarse y formar especies menos reactivas. Las especies reactivas del oxígeno (*Reactive Oxygen Species, ROS*) incluyen radicales libres y ciertas especies no radicales que son oxidantes y/o se convierten fácilmente en radicales libres, como por ejemplo: HClO, HBrO, O_3, $ONOO^-$, O_2, o H_2O_2 [16].

Los antioxidantes son captadores de radicales libres y por ello retrasan o inhiben la etapa de iniciación del proceso de oxidación, lo que disminuye la consecuente formación de productos de descomposición volátiles (por ejemplo, aldehídos y cetonas) [17–19]. El potencial antioxidante de los compuestos fenólicos dependerá del número y disposición de los grupos hidroxilo en las moléculas de interés [20].

1.2.1. Compuestos fenólicos

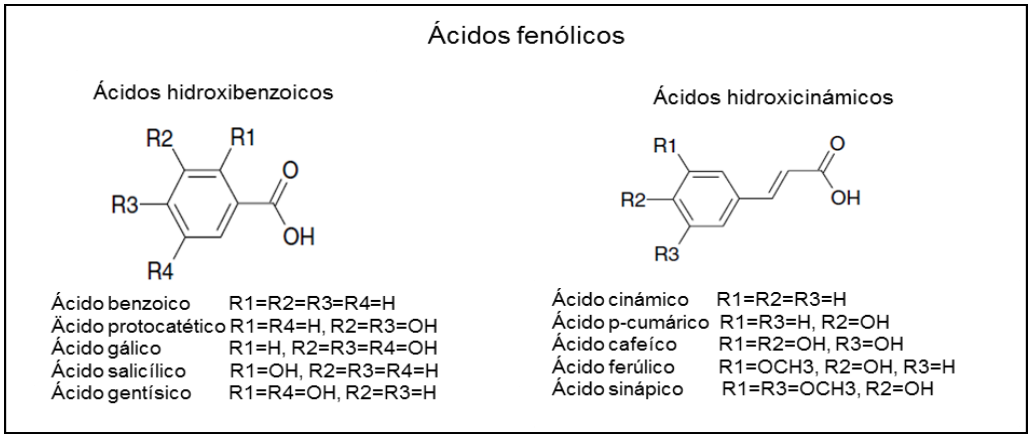
Los compuestos fenólicos constituyen una de las principales clases de metabolitos secundarios de las plantas, donde desempeñan diversas funciones fisiológicas. Entre otras, intervienen en el crecimiento y reproducción de las plantas y en procesos defensivos frente a patógenos [21,22].

Los compuestos fenólicos presentan un anillo benceno hidroxilado como elemento común en sus estructuras moleculares, las cuales pueden incluir grupos funcionales como ésteres, metil ésteres, glicósidos, etc. [23–25]. Las distintas familias de compuestos fenólicos se caracterizan principalmente por el número de átomos de carbono de su esqueleto básico molecular. De acuerdo a su estructura [26], los compuestos fenólicos se clasifican en:

- Ácidos cinámicos
- Ácidos benzoicos
- Flavonoides
- Proantocianidinas o taninos condensados
- Estilbenos
- Cumarinas
- Lignanos
- Ligninas

En la Fig. 1 se muestran las estructuras de los principales compuestos fenólicos sobre los cuales nos centraremos.

(a)



(b)

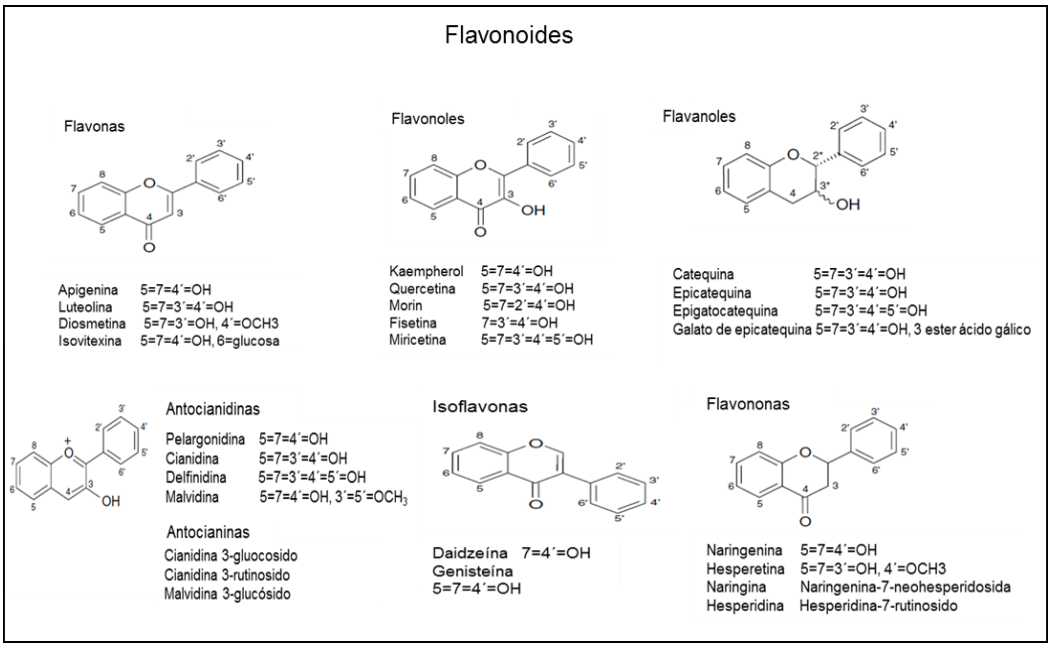


Fig. 1. Estructuras químicas de: (a) ácidos fenólicos y (b) flavonoides [27]

Los flavonoides son compuestos que constan de 15 átomos de carbono dispuestos en la configuración C6-C3-C6, compartiendo un esqueleto común (Fig. 2), compuesto por dos radicales fenilos (anillo A y B) ligados a través de un anillo C de pirano (heterocíclico). Los átomos de carbono en los anillos C y A se numeran del 2 al 8, y los del anillo B desde el 2' al 6' [28].

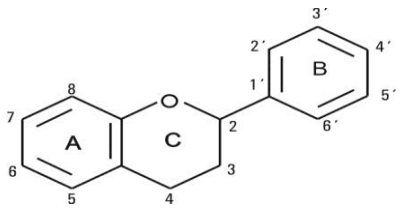


Fig. 2. Estructura básica de los compuestos fenólicos y sistema de numeración [29].

Los compuestos fenólicos están presentes en todo el reino vegetal y sus cantidades y tipos varían en función de diversos parámetros. Por ejemplo, de la especie vegetal y variedad, de la parte de la planta considerada (frutos, semillas, hojas, raíces, etc.), del grado de madurez, de las condiciones de cultivo, del procesado, del almacenamiento, etc [30,31].

La actividad antioxidante de los compuestos fenólicos se atribuye a su facilidad para ceder átomos de hidrógeno de un grupo hidroxilo aromático a un radical libre y a la posibilidad de deslocalización de cargas en el sistema de dobles enlaces del anillo aromático. Los compuestos fenólicos poseen además una estructura química ideal para captar iones metálicos (principalmente divalentes, como el hierro (II) y cobre(II)) y, por tanto, para inhibir la formación de radicales libres a través de reacciones tipo Fenton [32,33].

1.2.2.Efecto de los antioxidantes sobre la salud

La ingesta diaria promedio de polifenoles se ve afectada por los hábitos alimenticios; en la dieta mediterránea es de, aproximadamente, 1 g/día por persona; las principales fuentes son las frutas y, en menor medida, verduras y legumbres [34,35]. Estos compuestos ejercen efectos protectores frente algunas enfermedades graves como el cáncer y las enfermedades cardiovasculares. El estrés oxidativo impuesto por las ROS (tabla 1) desempeña, de hecho, un papel crucial en la fisiopatología asociada a neoplasia, aterosclerosis y enfermedades neurodegenerativas. En este sentido, los efectos de los compuestos químicos obtenidos a partir de frutas y verduras son muy activos y eficientes contra el cáncer (de colon, mama, colonorrectal…).

Los compuestos fenólicos así mismo presentan una amplia gama de propiedades fisiológicas, tales como anti-alergénico, anti-aterogénico, antiinflamatorio, antimicrobiano, antioxidante, anti-trombótico, cardioprotector y efectos vasodilatadores [36,37]. Los antioxidantes también pueden ejercer efecto oxidante dependiendo de la concentración y por tanto, ser perjudiciales para la salud [38].

Tabla 1. Nomenclatura de las principales ROS [16].

Radicales		No radicales	
Hidroxilo	$^{\bullet}OH$	Peróxidos orgánicos	ROOH
Alcoxilo	$RO^{\bullet}$	Oxígeno singlete	O_2
Hidroperoxilo	$HOO^{\bullet}$	Peróxido de hidrógeno	H_2O_2
Superóxido	$O_2^{\bullet}$-	Ácido nitroso	HClO
Peróxilp	$ROO^{\bullet}$	Catión nitrilo	HNO_2
Óxido nítrico	$NO^{\bullet}$	Peroxinitrito	NO_2^+
Dióxido de nitrógeno	$NO_2^{\bullet}$	Ácido Peroxinitroso	$ONOO^-$
		Alquil peroxinitritos	ONOOH
		Ozono	O_3
		Ácido hipobromoso	HBrO

1.2.3. Clasificación de los antioxidantes

Los antioxidantes de acuerdo a su origen se clasifican en dos grandes grupos: antioxidantes sintéticos y antioxidantes naturales (Fig. 3).

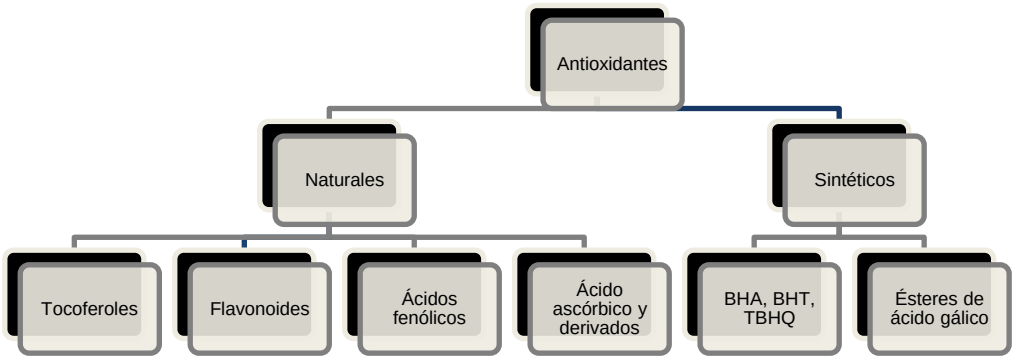


Fig. 3. Esquema de clasificación de los antioxidantes [27]

1.2.3.1. Antioxidantes sintéticos

De todos los antioxidantes sintéticos existentes en el mercado, sólo una parte han podido ser empleados en el campo alimentario dada la necesidad de comprobar la ausencia de toxicidad y actividad carcinogénica de sus formas oxidadas y de sus productos de reacción con los constituyentes del alimento. Los permitidos actualmente son el Butil Hidroxi Anisol (*Butyl Hydroxy Anisol*, BHA), Butil hidroxi tolueno (*Butyl Hydroxy Toluene,* BHT), galato de propilo (*Propyl Gallate,* PG), Terbutil Hidroquinona (*Tert Butylhydroquinone,* TBHQ), galato de octilo (*Octyl Gallate*, OG) y galato de dodecilo (*Dodecyl Gallate,* DG) [39]. Si bien estos antioxidantes sintéticos son altamente efectivos y económicos, su potencial efecto tóxico sobre la salud del consumidor, así como la necesidad de que “en ocasiones” el producto permanezca envasado durante un largo periodo de tiempo, ha llevado a la búsqueda de nuevas sustancias naturales sin efecto tóxico que reemplacen a los antioxidantes comerciales [7,40].

1.2.3.2. Antioxidantes naturales

Los antioxidantes naturales son principalmente, como se ha comentado, compuestos fenólicos. En general, los antioxidantes fenólicos se pueden dividir en varios grupos diferentes en función de su estructura básica. [41,42]. De entre los antioxidantes naturales, el ácido ascórbico (vitamina C) y sus derivados (ascorbato sódico, ascorbato de calcido, palmitato de ascorbilo), los tocoferoles, los ácidos fenólicos y los flavonoides son los antioxidantes naturales más comúnmente utilizados en aplicaciones alimentarias [43–46]. Otros compuestos presentes de forma natural también con actividad antioxidante son carotenoides y compuestos nitrogenados como los alcaloides, aminoácidos y aminas, así como ciertas proteínas [47].

1.2.4. Fuentes de antioxidantes

Los antioxidantes naturales se encuentran presentes en numerosas fuentes del reino vegetal, siendo frutas, vegetales, otros… las más importantes [48–51]. Los materiales ligno-celulósicos "provenientes de residuos agroalimentarios y forestales" pueden ser también considerados como fuentes naturales de este tipo de antioxidantes, a pesar de tratarse de matrices previamente procesadas [52].

Diversas matrices de fuentes de antioxidantes naturales se investigaron por nuestro grupo de investigación. Entre ellas cabe destacar los residuos de productos vegetales, como la semilla de aguacate y residuos de hojas de borraja, los cuales resultaron contener una considerable concentración de polifenoles y retrasar la oxidación de las grasas [53]. También residuos de frutas como la piña y el limón presentaron gran poder antioxidante con capacidad de proteger el ADN y las células del estrés oxidativo inducido. Los subproductos de la industria alimentaria pueden pasar de ser un simple residuo, a darle un valor añadido y convertirlo en un recurso aprovechable para evitar la oxidación de las grasas. Otras fuentes que hemos considerado como objeto de estudio, se han centrado en el aprovechamiento de plantas con grandes propiedades antioxidantes, como plantas de la familia: Gentianaceae, Convolvulaceae y Fabaceae, entre otras.

Entre todas las matrices de fuentes de antioxidantes naturales, los tés constituyen una de las más importantes, no sólo en función del número de antioxidantes presentes en los mismos (principalmente compuestos polifenólicos de la familia de las catequinas) sino también por la capacidad antioxidante de éstos [54].A continuación se describirán las diferentes especies vegetales utilizadas en particular para esta Tesis.

1.3. Consideraciones generales de las plantas estudiadas:

1.3.1. Romero (Rosmarinus officinalis)

Rosmarinus officinalis es una especie del género *Rosmarinus* de la familia Lamiaceae, comúnmente conocida como romero. La planta es originaria de los países que están bañados

por el Mediterráneo, pero crece actualmente en muchos países de clima cálido. Sus hojas son lineares parecidas a las de la lavanda, de haz oscuro y sus flores de azul claro, tubulares, agrupadas en ramilletes axilares (Fig. 4). Aunque tanto las hojas como las flores son muy aromáticas, parece que es en el cáliz donde se concentra la mayor parte de principios volátiles [55].

Fig. 4. Arbusto del romero (*Rosmarinus officinalis*) [56].

Esta planta se ha estudiado debido a la potente actividad antioxidante asociada a sus componentes [57–59]. Los compuestos presentes en los extractos del romero se pueden clasificar en tres grupos: diterpenos, flavonoides y ácidos fenólicos. La estructura del diterpeno está relacionada con el ácido carnósico (Fig. 5a), los flavonoides con las flavonas (apigenina y luteolina) y el ácido fenólico con el RA (Fig. 5b) [60,61]. Los ácidos rosmarínico y carnósico tienen una potente actividad antioxidante. Entre los diterpentos cabe destacar el carnosol, el rosmanol y el epi-rosmanol que influyen positivamente en la actividad antioxidante total [57].

(a) (b)

Fig. 5. Estructuras químicas del ácido carnósico (a) y el ácido rosmarínico (b) [62].

Los ácidos rosmanírico y carnósico son los compuestos más abundantes presentes en el romero [63]. Wellwood et al. [63] encontraron que el etanol usado como disolvente obtuvo una gran concentración de polifenoles (2,19 mg de RA/g peso seco y 29,77 mg de ácido carnósico/g peso seco). En otros estudios Yi et al. [64] y Emanuel et al. [65] demostraron que el etanol tuvo eficacia para la extracción de los compuestos polifenólicos mayoritarios presentes en esta planta, en especial empleando una concentración de 70-80 % [64,65].

Su aceite esencial es muy rico en componentes antioxidantes. Se han podido identificar alrededor de 90 componentes. Los principales han sido: 1,8-cineol, α-pineno, alcanfor, canfeno, β-pineno, linalool y limoneno [66–68]. Tutooolomondo et al. [69] también identificaron el acetato de bornilo y el tepinoleno, indicando que la composición química se vinculó a las características genéticas de los diferentes biotipos del romero**.** Por su parte, el extracto de romero obtenido a partir de una extracción con acetato de etilo, se demostró que era rico en CA, RA, éster metílico de RA, luteolina, hispidulin y apigenina [60,70]. En el extracto metanólico "además de los compuestos mencionados anteriormente" también se han identificado: ácido ferúlico, ácido cumárico, carnosol, ácido carnósico, hesperidina y genkwanin, los que se consideran como potencialmente muy útiles en los alimentos, cosméticos y productos farmacéuticos [71].

Ibarra et al. (2010) [72] obtuvieron una capacidad antirradicalaria del RA presente en el extracto de romero 1,5 veces superior en el ensayo ORAC y 4 veces mayor en el ensayo por FRAP que la obtenida en ácido carnósico. Sin embargo, para el método DPPH el extracto acuoso de romero mostró actividad antirradicalaria, comparable a antioxidantes como el alfa-tocoferol [73], mientras que el extracto metanólico contiene 30% de ácido carnósico, 16% de carnosol y 5% de RA presentó valores muy similares al BHA y BHT [74]. En cuanto a su aceite esencial, existe una variación en la actividad antirradicalaria dependiendo de tres estados de recolección de esta planta (etapa de floración, post-floración y etapa vegetativa), siendo más efectiva la etapa de floración [75].

Otros procesos de extracción mejoraron los resultados de actividad antirradicalaria. Por ejemplo, la extracción asistida por microondas y por ultrasonidos optimizaron la cantidad extraída de compuestos antioxidantes del romero [76,77]. Mediante extracción acelerada con metanol la actividad antiradicalaria fue mayor que la obtenida por extracción sólido/líquida [78].

Desde el punto de vista culinario y de preservación de los alimentos, el romero se consideró como la especia o hierba más común con fuertes propiedades bactericidas [55]. Posee aceites esenciales que contienen compuestos químicos tales como el carvacrol, cinamaldehído, eugenol y alcanfor, que son los componentes responsables de ejercer esta actividad. Microorganismos como la *Aeromonas sobria,* las cepas de Candida [79] y las bacterias *Lactobacillus curvatus*, *Lactobacillus sakei*, *Staphylococcus carnosus*, *Staphylococcus xylosus*, *Enterobacter gergoviae* y *Enterobacter amnigenus* son sensibles al aceite esencial de *R. officinalis* [80].

Además, cabe destacar que el extracto de romero presentó actividad bactericida frente a las bacterias transmitidas por alimentos, tales como, *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes* y *Staphylococcus aureus* [81]. Este efecto antimicrobiano se correlacionó con una concentración de 0,1% de extracto [82]. Por otra parte, también se demostró un efecto positivo en la protección de los alimentos frente a la contaminación del microorganismo *Vibrio alginolyticus* [83].

Además de los efectos comentados sobre los alimentos, diversos autores coinciden en destacar su efecto también si está presente en el material del envase [74,80,81,84–88]. Gok et al. [84] obtuvieron una disminución de la contaminación microbiana comparada al control (6,39 unidades formadoras de colonias (CFU)/g de carne) al tratar salchichas con extracto de romero y tocoferol a 500 ppm (6,03 CFU/g carne). Asimismo, muestras de filetes de pescado tratadas con romero permitieron un menor crecimiento bacteriano durante 13 días de almacenamiento, alcanzando 8,8 CFU/g pescado en las muestras tratadas con romero y 9,3 CFU/g pescado en la muestra control [85].Con referencia a los productos cárnicos, el extracto de romero es muy útil para retardar la oxidación lipídica En la carne de cerdo se encontró que la adición de extracto de romero logró la inhibición de la oxidación lipídica durante su almacenamiento en refrigeración [88–90] y la degradación de los pigmentos hemo causado por la cocción, después de 8 días de almacenamiento en refrigeración, donde Fernández-López et al. [91] obtuvieron valores de aproximadamente 75% de metamioglobina en el control versus un 45% de metamioglobina en la muestra con romero. Hernández-Hernández et al. [92] comentaron que el ácido rosmarínico es el principal compuesto que evita el deterioro del color . Así mismo, Lara et al. [93] no encontraron diferencias en las características sensoriales de este producto. Una mezcla de romero, ácido ascórbico, remolacha y lactato de sodio consiguió mantener el color inicial de la carne, así como inhibir la oxidación lipídica y el crecimiento microbiano en salchichas de cerdo. Por ello, Martínez et al. [94]consideraron que es una combinación efectiva para este tipo de productos.

En la carne de pollo también se han evidenciado buenos resultados. Salchichas de pollo tratadas con extracto de romero a 500, 1000 y 1500 ppm obtuvieron valores inferiores de TBARS comparados con las muestras control, después de 14 días de almacenamiento a 4 ºC [95]. Además tiene un efecto positivo en la apariencia y el sabor de la carne de pollo durante su almacenamiento [96].

En otros productos cárnicos como la carne de cordero nuevamente demostró su eficacia, permitiendo la estabilidad oxidativa de la carne cubierta con un film activo con extracto de romero, expuesta a un almacenamiento bajo iluminación durante 13 días a 1 ºC [88].

El pescado y los mariscos también fueron objeto de estudio. Muestras de besugo tratadas con extracto de romero en concentraciones de 2; 2,5 y 3% por peso seco mostraron menor índice de peróxidos y menores valores de TBARS (2,08; 1,57 y 1,16 mg MDA/kg pescado, respectivamente), después de 5 días de almacenamiento en refrigeración [97,98]. En condiciones de congelación a -18 ºC el romero también presentó efecto protector, alcanzando 120 días de almacenamiento [99]. También tuvo buenos resultados al usarse en filetes de tilapia como pretratamiento antes del proceso de salado [100]. Tironi et al. [101] publicaron que concentraciones de 500 mg/kg de extracto de romero alargaron la vida útil del salmón a 6 meses, y de 200 mg/kg a 3 meses, almacenados en congelación a -11ºC, evitando la oxidación de los lípidos y la pérdida del color rojo en el músculo. Una solución de extracto de romero con

quercetina, eritorbato de sodio y brotes de flores de acacia japonesa (*Sophora japonica*) utilizada por Dragoev et al. (2008) también inhibió satisfactoriamente la oxidación lipídica en muestras de caballa congelada [102]. En mariscos, como por ejemplo en los camarones se demostró su potente actividad antioxidante. Se marinaron con extracto de romero y se almacenaron a 1 ºC [86]. En esas condiciones, incluso después de 75 días, su análisis sensorial por panelistas expertos demostraron una menor oxidación en las muestras marinadas, frente a las muestras con ácido ascórbico, [103].

La adición de aceite esencial de romero reveló en la carne de res que la adición en concentración de 200 mg/kg carne, redujo significativamente los valores de TBARS durante su almacenamiento por 3 meses en congelación a -18 ºC, y las propiedades sensoriales se mantuvieron prácticamente exactas a las iniciales [104]. La combinación de vitamina C con extracto de romero aumentó la vida útil de filetes de carne fresca por 10 días, retrasando los procesos de oxidación [105]. Por sí solo, el extracto de romero mostró gran protección a la oxidación lipídica de la carne de res después de 12 días de almacenamiento en refrigeración, muy por encima que el extracto de limón y naranja [106].

En el campo de los aceites vegetales se comprobó también su estabilidad oxidativa. Yang et al. [107] obtuvieron una disminución del índice de peróxidos al incorporar extracto de romero (400 mg/kg aceite) en un aceite vegetal, retrasando igualmente la degradación de los ácidos grasos poliinsaturados en el aceite después de 24 días de almacenamiento en estufa a temperatura de 62ºC ± 1 ºC. Por su parte, Tohma et al. [108] obtuvieron buenos resultados al usar el romero como antioxidante para aumentar la vida útil del aceite de avellana usado para freír, no ocasionaron cambios en los resultados sensoriales de las patatas fritas y mejoraron la textura y el sabor de la fritura [109]. El extracto de romero mostró una actividad antioxidante significativa en comparación con el ácido cítrico, en la protección oxidativa del aceite de sésamo [110]. Igualmente, no interfirió en las características sensoriales del aceite de soja, con bastante aceptación por los consumidores [111]. Mediante el análisis “Oxytest" se reveló que el romero es una gran fuente de antioxidantes para proteger emulsiones de agua-en-aceite, empleando aceite de canola [112].

1.3.2.Tomillo (Thymus vulgaris.)

El *Thymus vulgaris L*. conocido comúnmente como tomillo, es una planta aromática del género *Thymus* y de la familia Lamiaceae, procedente de la región mediterránea, aunque actualmente está distribuida por todo el mundo [55].

Tiene unas hojas estrechas de un color verde agrisado oscuro, generalmente son aromáticas y las flores son tubulares de color malva, agrupadas en el extremo en ramas (Fig. 6)[55].

Fig. 6. Arbusto del tomillo (*Thymus vulgaris*) [113]

El tomillo y sus extractos se han usado a nivel digestivo para trastornos gastrointestinales. También es conocido su uso en diversas enfermedades como tos, bronquitis, laringitis y amigdalitis [5]. Además actúa como un agente antiparasitario, antihemítico, antiséptico, antiespasmódico y cicatrizante. [114]. El aceite esencial del tomillo posee dos componentes principales: el timol y el carvacrol (Fig.5) que son los compuestos aromáticos principales del tomillo. Según Perestrelo et al. [115] *T. vulgaris* tuvo un 67% de timol, mientras Bertoli et al. [116] detectaron entre 50-55% en las diferentes variedades de *T. vulgaris*. Los de menor concentración son los sesquiterpenos que se encuentran en menor cantidad en todas las partes de la planta del tomillo (hoja, flores y tallo). El número de sesquiterpenos detectado es de 12 en las hojas, 16 en las flores y 9 en los tallos, y el principal componente es el trans-cariofileno [117]. La planta contiene además ácidos fenólicos: ácido cafeico, clorogénico, cinámico, quínico y ferúlico; flavonoides: apigenina, derivados de la luteolina y quercetina [118,119]; saponinas triterpénicas (n-triacontano) y los ácidos ursólico y oleanólico [120].

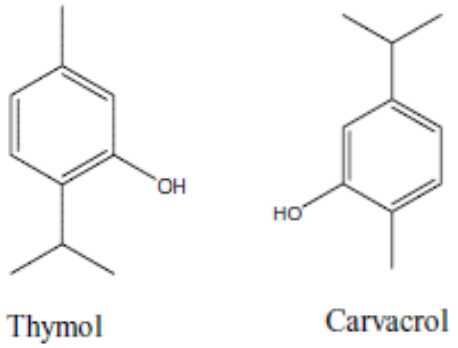


Fig. 5. Estructuras químicas del timol y del carvacrol [48].

Esta planta ha demostrado que posee actividad antiradicalaria [121]. La presencia de grupos aromáticos y del número de grupos ⁻OH parece coincidir con el potencial antioxidante de los compuestos presentes en la planta [48,122–128]. Esta actividad se determinó a través de

diversos ensayos como el DPPH y FRAP. Hossain et al. [129] demostraron que la actividad antioxidante del extracto de tomillo medida por el ensayo FRAP fue más efectiva usando una extracción presurizada, comparado a una extracción sólida/líquida. Ellos determinaron que el óptimo de temperatura fue de 129 ºC y un óptimo de metanol de 33 %. Chizzola et al. [130] indicaron que los extractos etanólicos de tomillo al 60 %, sometidos a un baño de agua a 40 ºC y ultrasonidos permitieron obtener buenos resultados sobre las pruebas DPPH (53,5-88,4 mgTrolox/g DW) y FRAP (35,7-93,8 mgTrolox/g DW). El aceite esencial de esta planta también tuvo buenos resultados en los métodos antiradicalarios. Grigore et al. [131] obtuvieron muestras con una actividad antiradicalaria elevada frente al método DPPH, con una concentración del aceite esencial de 3 mg/mL se exhibió una inhibición por encima del 50% del radical DPPH. La literatura indica que los compuestos responsables de esta actividad son los polifenoles, como el ácido rosmanírico, carvacrol, ácido cafeico, ácido ferúlico y timol entre otros [5].

El tomillo es ampliamente utilizado como especia para condimentar diversos plantas y como conservante de productos alimenticios [130]. Es una de las plantas aromáticas principales usadas en la alimentación, ya que su fragancia es persistente y sus propiedades bactericidas lo hacen indicado para patés, salchichas, carnes en conserva y encurtidos, además de poder ser utilizado para aromatizar aceites, vinagres y otros alimentos [55].

Generalmente, el aceite de tomillo y sus extractos tienen una fuerte actividad bactericida contra agentes patógenos portadores de enfermedades transmitidas por los alimentos (ETA) y organismos causantes de deterioro [132], como la *Escherichia coli* y *Enterococcus faecalis* [133]. Mattos de Oliveira et al. [134] indicaron que podría ser una posible alternativa frente a la *Listeria monocytogenes* en la industria cárnica. Los compuestos timol, *p*-cimeno y mezclas de ambos, presentaron efecto antibacteriano contra las cepas de Bacillus [135]. También muestra efecto frente a la bacteria *E. amylovora* [136]. Esto es debido a su alto porcentaje de compuestos fenolicos como timol, *p*-cimeno, carvacrol y *γ*-terpineno [122,128,135,137–141].

El aceite esencial del tomillo presentó una mejor acción como agente antifúngico que bactericida. Presentó mayor acción sobre hongos, como por ejemplo el *F. oxysporum y F. sp. Albedinis* [142]. Igualmente, sus extractos fueron eficaces contra los principales insectos portadores de los hongos causantes de formación de aflatoxinas en alimentos. Estas micotoxinas son potentes carcinógenos, teratógenos y mutagénicos, producidas por microorganismos del género Aspergillus [143]. En general, el aceite esencial ejerce actividad inhibitoria sobre bacterias Gram-positivas [144] y Gram-negativas [145].

Es notable el efecto antioxidante que posee esta planta sobre los alimentos. Sus oleoresinas protegieron el aceite de soja de la oxidación lipídica, medida por el ensayo Rancimat a una temperatura de 100 ºC. Así por ejemplo, una concentración de 3 g/kg de oleoresinas es suficiente para ejercer esta protección [146]. Incluso presentaron mejor efecto protector que el TBHQ [147]. Babovic et al. [148] aislaron fracciones de esta planta y comprobaron que a una

concentración de 200 mg/kg de aceite lograron mantener la estabilidad del aceite de girasol, después de 12 h a a 98 ºC. También probaron su extracto como pretratamiento en productos como patatas. Con una concentración de 1 g de extracto/Litro de aceite se logra reducir la acrilamida y se mantienen las propiedades sensoriales [149]. En productos cárnicos también resultó efectivo, inhibiendo la oxidación lipídica de carne de res, en una original mezcla de vino tinto, miel de tilo *Allium sativum* y *Armoracia rusticana* con tomillo[150].

1.3.3.Lavanda (Lavandula officinalis)

Lavandula officinalis es una especie del género *Lavandula* de la familia Lamiaceae, comúnmente conocida con el nombre de lavanda (Fig. 7). Es una planta arbustiva originaria del sur de Europa, en especial de las zonas costeras del Mediterráneo. La lavanda es un arbusto de hasta 60 cm de altura, de hojas grises, estrechas y aterciopeladas, las flores de un color azul agrisado. Las glándulas aromáticas se hallan localizadas en diminutos pelos estrellados que cubren en su totalidad hojas, flores y tallos, para liberar un poco de aceite (que enseguida se volatiliza). Entre las variedades del genus se encuentran principalmente: *L. officinalis* también conocida como *L. angustifolia* y *L. vera*, *L. x intermedia*, *L. latifolia*, *L. stoechas* y *L. spica* [55]. Desde el punto de vista medicinal la lavanda se ha usado como calmante, anti-séptico, anti-inflamatorio, antihistamínico, anti-diabético y digestivo.[151].

Entre los compuestos que presentan sus extractos, cabe destacar derivados terpénicos (ácido ursólico), cumarinas (herniarina), ácido cumárico, ésteres de la umbeliferona, cedreno, luteolina, ácido labiático, taninos, ácidos fenólicos (CA y clorogénico) y diversos principios amargos. Su aceite esencial contiene o-cimeno, diperteno, canfeno, cariofileno, linalol, geraniol, borenol, ésteres de linalol, acetato, butirato y valerianato de linalilo, geraniol y cineol [120]. Estos compuestos son los responsables de su actividad farmacológica [152–159] y pueden ser el punto de partida de posibles ingredientes naturales en cosméticos y fármacos para tratar enfermedades de la piel [160].

Otros compuestos detectados en su aceite esencal, de acuerdo a Yusufoglu et al. [161] fueron: alcanfor, β-felandreno, terpinoleno, α-tujen, n-hexanal, n-heptanal, metil-amil-cetona, etilamicetona, perialdehído, peril alcohol, borneol, α-terpineol, α-pineno, limoneno, varias lactonas y sesquiterpenos. Los principales componentes fueron el 1,8-cineol y el borneol [159,162]. Otros compuestos identificados en el extractode esta planta por otros autores fueron ácidos fenólicos: ácido ferúlico, RA, ácido *p*-cumárico y CA y flavonoides como quercentina, apigenina y kaempherol [163].

El aceite esencial mostró actividad inhibitoria muy elevada (más del 80%) sobre las enzimas acetilcolinesterasa y butirilcolinesterasa [164]. Así los aceites 1,8 cineol, α-pineno, eugenol, α-terpineol y terpinn-4-ol mostraron un IC_{50} para la enzima colinesterada de 0,015, 0,022, 0,48, 1,3 y 3,2 mg/mL, respectivamente [165].

Fig. 7. Arbusto de la lavanda (*Lavandula officinalis*) [166].

Los resultados de numerosos estudios demostraron que el aceite esencial de la lavanda tiene actividad bactericida. Su actividad se extiende a bacterias que producen enfermedades transmitidas por alimentos: *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans*, *Aspergillus Níger* [167]) y *Escherichia coli* [168]. Así mismo, su actividad antifúngica está también documentada, ya que inhibe el crecimiento de hongos tales como *Botrytis cinerea*, *Penicillium expansum*, *Rhizopus stolonifer* [169–173] y *Cryptococcus neoformans* [174]. Una alta capacidad inhibidora de los microorganismos productores del acné también se observó [175]. Además, sus aceites son fitotóxicos en cultivos de manzanas, usando una emulsión con 10% de aceite esencial de tomillo y 90% de agua destilada [173].

La lavanda puede ser un complemento valioso en la ingesta diaria por sus compuestos bioactivos [176]. Viuda-Martos et al. [177] obtuvieron un contenido de polifenoles totales de 913 mg GAE/L de aceite esencial. La capacidad antiradicalaria de sus extractos también se estudió, en este caso el total de polifenoles del extracto metanólico de la lavándula angustifolia fue de 5,2±0,02 mg GAE/g extracto y el % de inhibición medido por el ensayo DPPH fue de 35.4±1.7 mmolesTrolox®/g extracto [178]. Debido a estas propiedades antioxidantes la lavanda incorporada a distintos *food model systems* ha influído en la estabilidad de la oxidación lipídica. Rodriguez et al. [179] indican que el aceite esencial de lavanda evitó la oxidación del aceite de soja, mejorando los parámetros de calidad. Yang et al. [180] obtuvieron que el aceite de lavanda inhibió la peroxidación del ácido linoleico después de 10 días de almacenamiento. Por su parte, Kovatcheva-Apostolova et al. [158] demostraron que se reducía la oxidación lipídica de carne de pollo cocida tratada con extracto de *Lavandula vera* y almacenada en refrigeración.

1.3.4. Caesalpinia decapetala

La *Caesalpinia decapetala* es una planta del género *Caesalpinia* de la familia fabaceae. Está ampliamente distribuida en las regiones tropicales y subtropicales. Existen unas 17 especies de

amplia distribución en China y de ellas alrededor de 14 (por ejemplo, *Caesalpinia decapetala* se usa en China para el tratamiento del reumatismo y de enfermedades inflamatorias) [181].

Caesalpinia decapetala se cultiva y es natural de regiones tropicales, sabanas tropicales y bosques de China, Japón, Malasia y la India y en las tierras bajas de selva tropical en Nueva Gales del Sur, Australia. Es decir, suele encontrarse, generalmente, en localidades pantanosas y barrancos a 1800 metros, pudiéndose definir su rango de crecimiento desde el este de Asia-Himalaya hasta China (Fig.8) [182].

Las hojas de la *Caesalpinia decapetala* presentan una gran versatilidad de propiedades medicinales; es por ello que su rango de usos es muy amplio. Se utilizan en el tratamiento de quemaduras, exceso de bilis y en trastornos de estómago. También se conoce su aplicación como laxante, loción tonificante, antipirético y carminativo contribuye a la expulsión de gases [183]. Su extracto etanólico presenta efecto analgésico, antiinflamatorio y antipirético [183]. Igualmente, tiene grandes beneficios sobre enfermedades como trastornos neurodegenerativos, inflamación, infecciones virales y úlcera gástrica [184]. Esta planta presenta compuestos con actividad antitumoral contra la línea celular MGC-803 (cáncer gástrico), tales como, emodina, baicalín y apigenina, así como, compuestos con efectiva actividad antiradicalaria frente al radical DPPH como rutina, quercetina, baicalín y EC, incluso muy comparable con el ácido ascórbico [181].

De acuerdo a los estudios realizados hasta el momento los componentes químicos del genero *Caesalpinia* son más de 280. Entre ellos se puede encontrar diterpenos, triterpenos, flavonoides, fenoles aromáticos y fenilpropanoides [181]. Los diterpenos son los componentes predominantes en el género Caesalpinia. Los diterpenos son terpenos compuestos por 4 unidades de isopreno con fórmula molecular $C_{20}H_{32}$. Se encuentran en las plantas superiores, hongos, insectos y organismos marinos. Los diterpenos son la base de importantes compuestos biológicos tales como el retinol, retinal y el fitol. Algunos de los más importantes son los cassanos, presentes en la especie *Caesalpinia* [185]. Se conoce que las hojas de *Caesalpinia decapetala* contienen, el diterpeno caesaldecano.

También posee otros componentes que se han logrado aislar e identificar como: espatulenol, 4, 5-epoxi-8(14)-cariofileno, escualeno, lupeol, trans-resveratrol, quercetina, astragalin y estigmasterol [186], además de andrografólido, bergenina, rutin, emodina, betulina, baicalín, salicina y apigenina [181]. Li et al. [187] también aislaron 7 compuestos: acetato de lupeol, lupeol, ácido oleanólico, ácido pentadecanoico 2,3-dihidroxipropil éster, 1-(26-hidroxi hexadecanoico)-glicerol, estigmasterol y beta-sitosterol. En el extracto etanólico de sus tallos se han econtrado los componentes: 6'-hidroxi-3, 4- (1''-hidroxi-epoxi-propano)-2',3'-(1''β-hidroxi-2'''-carbonil-ciclobutano)-1,1'-difenil, octacosilo3,5-di-hidroxi-cinamato, daucosterol, β-sitosterol, 2',4,4'-tri-hidroxi-chalcona, bonducelina y 7,3',5'-trihidroxi flavona [44].

La *Caesalpinia decapetala* presentó actividad antiradicalaria importante, la cual fue dependiente de la concentración del extracto [188]. Unas óptimas condiciones de extracción se encontraron con etanol:agua 70:30 v/v a una temperatura de 65-70 ºC durante 48 h, en especial para extraer el GA [185].

Fig. 8. Árbol de la *Caesalpinia decapetala* [189]

1.3.5. Tara (*Caesalpinia spinosa*)

Caesalpinia spinosa comúnmente conocida como *Tara* (Fig. 9) es un árbol de origen peruano perteneciente al género *Caesalpinia* de la familia Fabaceae, que crece en las zonas secas. Sus flores son de color amarillo rojizo dispuestas en racimos, sus frutos son vainas aplanadas de color naranja de 8 a 10 cm de largo que contienen de 4 a 7 granos de semilla redondeadas de 0,6 a 0,7 cm de diámetro cada una. Crece en forma silvestre en la región andina y costa peruana [190].

La *Tara*, como fuente natural integral (tallo, hojas y frutos), fue utilizada desde la época prehispánica en la medicina folklórica o popular como astringente, cicatrizante, antidisentérico, y también y muy importante, como mordiente en teñidos de cuero. Estas propiedades se atribuyen a su gran potencial antioxidante.

Los taninos se dividen en taninos condensados y taninos hidrolizables. Los taninos se hidrolizan por acción de ácidos o enzimas originando un azúcar, un polialcohol y un ácido fenol carboxílico. La hidrólisis completa de los taninos de la *Tara* da cantidades importantes de GA (o derivados) y en menor concentración ácido elágico (o derivados), compuestos cuya capacidad antioxidante es mayor que la de los taninos. Varios estudios han determinado la capacidad antioxidante de los hidrolizados de los taninos de la *Tara* y han evaluado su eficacia en la conservación de productos oleosos [191]. Skowyra et al. [191] obtuvieron una concentración de 4,64 mg GAE/g peso seco a partir de una extracción con etanol al 75 % v/v y una actividad antiradicalaria de 10,17 y 4,29 mmoles TE/g peso seco frente a los ensayos ABTS y ORAC, respectivamente.

La transformación de los taninos de la *Tara* en compuestos fenólicos antioxidantes de bajo peso molecular puede ser una alternativa para la obtención de extractos de alto valor agregado cuya demanda actual es creciente por su utilidad como suplemento alimenticio, su aplicación farmacéutica y como sustituto de antioxidantes sintéticos empleados en la industria de alimentos. Según Srivastava et al. [192] el TA es un típico tanino hidrolizable que consiste en una mezcla de GA y ésteres de glucosa, y tiene efectos benéficos sobre la salud humana, incluida antimutagénesis, propiedades antioxidantes y acción anticancerígena. El aceite esencial de esta planta también es rico en monoterpenos, compuestos que podrían estar relacionados con su actividad antimicrobiana y antioxidante [193].

Las propiedades bactericidas y antioxidantes la hacen idónea para su uso en productos alimentarios. Aguilar et al. [194] obtuvieron que los galotaninos del extracto de la *Tara* y de su hidrólisis ácida durante 4 h, lograron buena inhibición de la *Escherichia coli*, por lo que demuestra su gran potencial sobre bacterias patógenas. Sus propiedades antioxidantes también se estudiaron en *Food Model System*. Su uso en estado seco como antioxidante natural mejoró la calidad y extendió el tiempo de vida útil de productos cárnicos como la carne de cerdo, durante su almacenamiento en refrigeración [195]. En un sistema de emulsiones aceite-en-agua también demostró su beneficioso efecto protector. La adición de 48 µg/mL del liofilizado del extracto etanólico de esta planta retrasó la oxidación lipídica de las emulsiones durante su almacenamiento a 38 ºC durante 18 días [191].

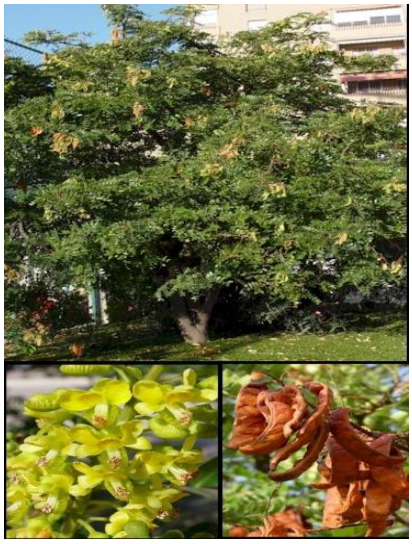


Fig. 9. Árbol de la *Tara* (*Caesalpinia spinosa*) [196]

1.3.6.Noni (Morinda citrifolia)

Morinda citrifolia conocida como *Noni* (español, portugués); *Noni*, Indian mulberry y cheese fruit (inglés); ba ji tian (chino), o morinde (francés), es un arbusto perteneciente al género *Morind*a de la familia Rubiaceae, de hoja perenne que en la madurez alcanza hasta 10 m de altura (Fig. 10). Nativo de Sureste de Asia y Polinesia tropical (Hawai, Nueva Zelanda e Islas de Pascua),

actualmente tiene una extensa distribución en las zonas tropicales del mundo y tolera una amplia gama de suelos y condiciones ambientales. Se compone de entre 6500 y 13000 especies de géneros [197]. La planta es identificada por sus grandes hojas, tallo recto y frutas de color amarillo-granada, son glabras, oblongas, de 20-45 cm de largo y 7-25 cm de ancho [198]. La familia es conocida por producir alcaloides, antraquinonas e iridoides, pero también se encuentran en sus extractos taninos, triterpenos y, con menor frecuencia, saponinas [199].

Entre las actividades biológicas descritas para *M. citrifolia*, se puede mencionar: actividad bactericida, antiviral, antihelmíntica, analgésica, hipotensora, antioxidante, hipoglucémica, antidepresiva y anti-inflamatoria [200], destacando entre todas ellas la actividad antitumoral [201]. Tiene grandes propiedades nutricionales [202,203]. Varios estudios revelaron que puede llegar a tener unos 200 compuestos fitoquímicos y entre los más abundantes se encuentran los ácidos fenólicos. Sin embargo, la composición química difiere ampliamente en función de la parte de la planta analizada [200]. Se han identificado los compuestos luteína, zeaxantina, beta-caroteno, criptoxantina beta, ECG, ácido siríngico, EGCG, ácido vaníllico, naringina y ácido cinámico [204]. Otro compuesto identificado fue la hiosciamina, que demostró elevada actividad antioxidante [205]. Los perfiles de ácidos grasos revelan que es una fuente rica de ácido linolénico, ácido esteárico, ácido oléico y ácido láurico, especialmente en sus semillas [206]. Pandy et al. [207] estudiaron que la escopoletina y la rutina, compuestos bioactivos del *Noni*, pueden ser los responsables de impedir los efectos de la dopamina y bloquear la respuesta de los receptores alfa-adrenérgicos a la noradrenalina. Algunos estudios *in vitro* e *in vivo* en animales sugierieron una posible sustancia no identificada en el jugo de la fruta de *Noni* sin pasteurizar qe puede tener un importante grado de actividad contra el cáncer [208].

Las propiedades antiradicalarias del *Noni* han sido estudiadas en su fruto demostrando que el extracto metanólico y metanol/agua tuvieron los mejores resultados [209] y presentaron buena actividad medida por el ensayo ORAC (9,56 ± 0,9 µmoles de TE/g fruta) y DPPH (3,29,5 ± 0,09 µmoles de TE/g fruta) [210].

Aunque hay menos información científicamente fiable disponible en las propiedades del *Noni* que de las propiedades de otros productos botánicos utilizados en la presente Tesis Doctoral, el jugo de *Noni* puede ser importante en el creciente mercado de las bebidas funcionales. La planta tropical *M. citrifolia* está clasificada en la U.E. como nuevo alimento. La Autoridad Europea de Seguridad Alimentaria (*European Food Saety Athority*, *EFSA*) determinó que sus hojas, tostadas y secadas para hacer infusiones, son seguras, así como los zumos que se elaboran con este fruto [211].

Actualmente no se disponen de muchos estudios con esta planta en los alimentos, la mayoría están basados en investigaciones con su fruta. Tapp et al. [212] indicaron el gran potencial de esta fruta para mejorar la estabilidad del color y la vida útil de carne de res. En otro estudio se determinó que el tiempo de cosecha óptimo para el uso de estos frutos, fue de 126 días

después del desarrollo de la inflorescencia [213]. No obstante, la eficacia antioxidante de sus hojas no ha sido aún estudiada.

Fig. 10. Árbol del *Noni* (*Morinda citrifolia*) [214]

1.4. Comportamiento en un sistema de alimentos

1.4.1. Comportamiento en el seno de una emulsión

Las emulsiones se describen como *Model Food System*. Este sistema es una representación en el laboratorio en el que se controlan todos los parámetros y componentes, de lo que puede ser un alimento real. La emulsión se llama así por ser el medio de dispersión un líquido (agua) y su fase dispersa (aceite). Esta dispersión requiere de la adición de un agente emulsionante [215]. Se diseña una emulsión de aceite en agua (10 % de aceite) con Tween-20 como agente emulsionante. A dicha emulsión se le añaden distintas proporciones del extracto sujeto a estudio, que se compara con el control (sin extracto). Se sigue la evolución de su oxidación a lo largo del tiempo con condiciones de almacenamiento de agitación, temperatura de 37 ºC y ausencia de luz.

Las emulsiones permiten seguir la oxidación de los lípidos. En la primera fase de la oxidación lipídica, los radicales libres lipídicos reaccionan con el oxígeno y se forman hidroperóxidos [216]. Los hidroperóxidos son componentes inodoros y que afectan poco a las características organolépticas de las emulsiones [217]. Estos productos normalmente se vuelven a oxidar formando cetonas, aldehídos, alcoholes y ácidos que afectan negativamente al sabor, aroma, valor nutricional y calidad sensorial global del producto, bajando su pH. Por otra parte, se sabe que los polifenoles y otros antioxidantes naturales de los aceites mejoran significativamente la estabilidad, porque tienen la capacidad de dar un hidrógeno al radical libre y así evitar que éste se oxide [216].

Se ha observado que la descarboxilación o esterificación de Trolox® (un análogo hidrosoluble de la vitamina E): ácido 6-hidroxi-2,5,7,8-tetrametil-2-cromanocarboxílico), con su consecuente disminución de polaridad, aumenta su actividad en un sistema de emulsión [218,219].

1.4.2. Comportamiento en un sistema modelo cárnico cocido y sin cocción

La exposición al oxígeno y a la luz es uno de los principales factores que originan la aparición de fenómenos oxidativos en la carne y/o productos cárnicos. El oxígeno (O_2) constituye el punto de partida para el daño celular, consecuencia de un desequilibrio entre la producción de especies reactivas y los mecanismos de defensa antioxidante [220]. Tanto lípidos como proteínas son susceptibles de sufrir dicha consecuencia.

La peroxidación lipídica se inicia cuando un radical ataca a un carbono de la cadena alifática de un ácido graso. A este primer proceso le siguen una serie de reacciones de propagación y terminación para finalmente dar lugar a productos más estables como el Malondialdehido (MDA) (Fig. 11a) [221]. Los cambios asociados a la oxidación lipídica constituyen la principal causa de deterioro de la carne y/o productos cárnicos, ya que provocan la aparición de olores y sabores desagradables y la alteración del color, y en general, una reducción de la calidad organoléptica del producto. Así mismo dan lugar a una disminución del valor nutritivo de la carne y la generación de compuestos potencialmente nocivos para la salud relacionados con el riesgo de padecer diversas patologías [222].

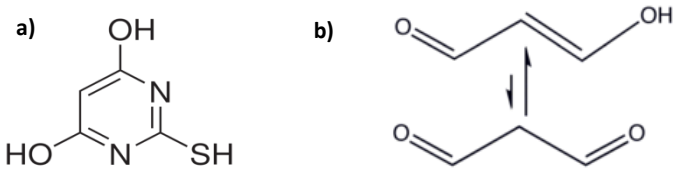


Fig. 11 a) Ácido Tiobarbitúrico (TBA) / b) Malondialdehído (MDA) [75,223].

Un método eficiente para determinar el grado de rancidez oxidativa de un producto cárnico durante el almacenamiento es la prueba: Sustancias Reactivas al Ácido Tiobarbitúrico (*Thiobarbituric Acid Reactive Substances, TBARS*) (Fig. 11b). En este ensayo se determina el producto principal de la oxidación de los fosfolípidos: el MDA por espectrofotometría a 531 nm (absorbancia del cromógeno rosa).

1.5. Envasado alimentario

1.5.1. Polímeros en el envasado de alimentos

Además de añadir los antioxidantes directamente en los alimentos, existe una tendencia creciente a diseñar films comestibles que mantengan la calidad y la frescura de los productos alimenticios a lo largo del tiempo [224,225]. En esta línea, los films de biopolímeros, son una alternativa para proteger los alimentos. Cabe destacar que el hecho de ser biodegradables

aumenta su valor desde el punto de vista ecológico, ya que son respetuosos con el medio ambiente.

A los alimentos se les debe proteger de los distintos factores externos (físicos, químicos y microbiológicos). Evidentemente se han de utilizar diferentes barreras, pero una de las fundamentales son los diferentes tipos de envases, entre ellos los films.

Los films se pueden producir de manera natural a partir de una gran variedad de productos, como polisacáridos, proteínas, lípidos y resinas. Además existe la posibilidad de adicionar otros componentes como plastificantes o también como agentes tensioactivos. Existe la posibilidad de que su origen sea sintético, como algunos provenientes del ácido poliláctico [226], lo cual no impide que puedan descomponerse en corto tiempo por la acción de microorganismos y/o enzimas. Krochta et al. [227] clasificaron os componentes de los films alimenticios en tres categorías, en función de su composición: con lípidos, con hidrocoloides y mezclas de ambos. Los hidrocoloides incluyen proteínas, derivados de celulosa, alginatos, pectinas, almidones y otros polisacáridos. Los lípidos incluyen ceras, acilgliceroles y ácidos grasos. Las mezclas son las que contienen componentes lipídicos e hidrocoloides.

Los films a base de proteínas se adhieren fácilmente a superficies hidrofílicas. Las fuentes más comunes son de origen animal, como colágeno, gelatina, suero de leche, albúmina, caseína, aunque también pueden ser derivados de plantas como el gluten de maíz, gluten de trigo o proteína de la soja [228,229]. Estos flms resultan buenas barreras frente al O_2 y CO_2 y proporcionan estabilidad mecánica; además sirven como vehículo para incorporar los antioxidantes [230]. Los polímeros de origen sintético biodegradables son resultado de la fermentación de polímeros primarios, utilizados como sustrato por diversos microorganismos; por ejemplo, se pueden obtener productos como el Ácido Poliláctico (*Polylactic Acid, PLA*), los polihidroxialcanoatos, ...

Por otro lado, un factor importante es la incorporación de un plastificante en el film, ya que actúa de forma positiva sobre las propiedades mecánicas y la permeabilidad y también influye en la estructura, la movilidad de la cadena y los coeficientes de difusión de gases o agua en el film. Normalmente la permeabilidad al agua suele ser una de las aplicaciones principales por la que se mejoran y analizan los films. En función del carácter polar de las proteínas se obtendrán unas u otras propiedades barreras de los films. Los grupos hidrofílicos libres favorecen las operaciones de absorción y adsorción y la transferencia de vapor de agua a través del film. Sin embargo, no suelen permitir la transferencia de los gases [231]. Los plastificantes más usados son: polioles (sorbitol, glicerol), lípidos y derivados (ácidos grasos, fosfolípidos y surfactantes) y monosacáridos, disacáridos y oligosacáridos (glucosa, jarabe de glucosa y miel entre otros) [232].

Los envases activos, de acuerdo a Lee et al. (2008) [233] pueden clasificarse en: (1) sistemas de absorción (si se usan films o bolsas para eliminar los gases presentes en los envases, tales

como, humedad, etileno, dióxido de carbono); (2) sistemas de emisión, (si se añaden compuestos con propiedades específicas, como bactericidas, antioxidantes, enzimas e incluso sabores o compuestos nutraceúticos que permiten mejorar la calidad del producto), y (3) otros sistemas (donde se incluyen indicadores de temperatura, sistemas de auto calentamiento o enfriamiento, entre otros).

El producto envasado se considera como un sistema ternario (entorno-envase-alimento), en el que ocurren interacciones tanto beneficiosas como perjudiciales. Estas interacciones corresponden a 3 fenómenos: permeación, sorción y migración (Fig. 12).

La permeación se refiere a la transferencia de materia y energía a través del material que constituye el envase. Puede ser de gases, humedad o aromas y se realiza en ambas direcciones, lo que puede provocar reacciones de oxidación, pardeamiento enzimático o degradación de vitaminas [234].

Las operaciones de absorción y adsorción se basan en la transferencia de sustancias desde el entorno o el alimento al envase, donde son retenidas. Engloba dos fenómenos, la adsorción (tiene lugar en la superficie) y la absorción (se da en el seno del envase).

Por último, la migración consiste en la transferencia de materia desde el envase al alimento. Estos compuestos pueden incidir en la calidad y seguridad del producto, como es el caso de los antioxidantes y productos bactericidas [235].

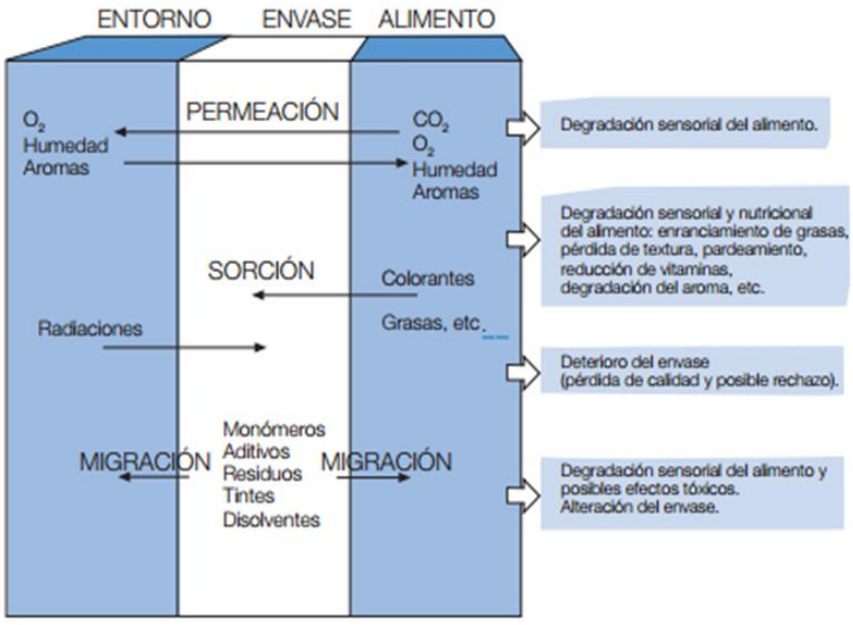


Fig. 12 Interacciones entorno-envase-alimento [236].

1.5.2.Aditivos en envases: Antioxidantes

La incorporación de los productos activos extraídos de plantas, frutas y vegetales en film es un tema actual de investigación en materiales. Los films pueden servir además de portadores de compuestos activos, tales como bacterianos y antioxidantes así como y potenciadores de sabor y conservadores de la textura [237,238].

Un aditivo debe cumplir ciertas características para ser añadido a un envase polimérico. Cabría destacar [239]:

- Ser eficientes en su acción.
- Estables en las condiciones de procesado.
- Estables en las condiciones de uso.
- No experimentar exudado o sangrado.
- No ser tóxicos ni dar lugar a olor o sabor.
- Ser económicos.
- No afectar negativamente a las propiedades del polímero.

Los films de biopolímeros son excelentes vehículos para la incorporación de una amplia variedad de aditivos. Se han empleado numerosas sustancias para mejorar las características de los polímeros [240,241]. Por ejemplo, la Tabla 2 recoge los tipos más comunes de aditivos empleados y las funciones básicas que presentan [242].

Tabla 2. Funciones de distintos aditivos empleados en las formulaciones de polímeros como films [242]

TIPO DE ADITIVO	FUNCIÓN
Antioxidantes	Inhibir las reacciones de oxidación
Estabilizantes a la luz	Evitar el deterioro del polímero por la exposición a la luz UV
Plastificantes	Aumentar la flexibilidad y resistencia al impacto
Agentes antiestáticos	Proteger al polímero frente a las descargas electrostáticas
Captadores de ácido o antiácidos	Neutralizar residuos ácidos catalíticos
Desactivadores metálicos	Desactivar residuos metálicos presentes en la formulación de las poliolefinas
Agentes nucleantes	Mejorar la claridad, transparencia y propiedades mecánicas
Retardadores de llama	Alterar el proceso de combustión originado por un importante flujo término
Colorantes	Proporcionar color al polímero
Agentes "*antiblocking*"	Evitar la adhesión entre films como consecuencia del frío o de la electricidad estática
Lubricantes	Disminuir la viscosidad y evitar el sellado del polímero a las superficies metálicas
Agentes "slip"	Impedir el sellado entre films mediante lubricación y reducir la carga electrostática
Agentes espumantes o de soplado	Disminuir la densidad del polímero por formación de gas en el procesado del polímero

El uso de antioxidantes tiene un especial interés en los materiales poliméricos destinados a envases de alimentos. En la actualidad se han desarrollado muchos estudios en la búsqueda de materiales de envasado con adición de antioxidantes que mantengan o extiendan la calidad de los productos alimenticios [243–246]. En su mayoría, son incorporados antioxidantes sintéticos como el BHA y BHT, pero la creciente preocupación acerca de los riesgos potenciales para la salud causados por estos aditivos ha llevado a un renovado interés en el uso de antioxidantes de origen natural, como ya se ha comentado [247].

De esta manera, algunos autores incorporaron extractos naturales en los films y evaluaron cómo éstos migran y/o retardan el proceso oxidativo de los alimentos durante el almacenamiento. La incorporación de plantas, como por ejemplo el extracto de romero, puede incluso ser más efectivo que el uso directo de los aditivos sobre la superficie, por ejemplo en el caso de la carne [248]. Otros extractos provenientes del orégano, té verde, cúrcuma, uva y propoleo demostraron que aumentan la estabilidad de los diferentes productos a base de carne y extienden su vida útil [249–251]. Ya se realizaron experimentos en pescado y pollo, por ejemplo la adición del aceite esencial de limón en una matriz de carragenina permitió reducir el crecimiento microbiano e inhibir la oxidación lipídica de filetes de trucha. El uso del aceite esencial de orégano y clavo en film permitió extender el tiempo de vida útil de pechugas de pollo [252].

Objetivos

2. OBJETIVOS

El objetivo de la presente Tesis es determinar la capacidad antioxidante y antirradicalaria de los extractos de distintas plantas aromáticas y/o comestibles: *Rosmarinus officinalis* (romero), *Thymus vulgaris* (tomillo), *Lavandula officinalis* (lavanda), *Caesalpinia decapetala*, *Caesalpinia spinosa* (*Tara*) y *Morinda critrifolia* (*Noni*). Además, comparar la actividad antioxidante en *Model Food System*, con el fin de demostrar la eficacia de estas plantas como posibles sustitutas de los antioxidantes sintéticos en la industria alimentaria. Este objetivo general se desglosa en los siguientes objetivos específicos:

- Cuantificar el contenido de polifenoles totales de los extractos de las plantas, determinar la capacidad antiradicalaria de cada uno de los extractos de las plantas por diferentes métodos y analizar la equivalencia entre ellos. Así como, identificar los compuestos antioxidantes presentes en los extractos mediante cromatografía líquida de alta resolución (HPLC-DAD y LC-MS).
- Evaluar y cuantificar la oxidación en el seno de una emulsión aceite-en-agua, como sistema modelo, con incorporación directa de los extractos vegetales en la emulsión, mediante la oxidación primaria y la oxidación secundaria.
- Evaluar el retardo en la oxidación de las grasas en un sistema cárnico (hamburguesas y salchichas), por incorporación directa de las plantas.
- Desarrollar, caracterizar y evaluar las propiedades de un envase activo comestible (films de gelatina) con incorporación de los extractos: *C. decapetala*, *C. spinosa* y *M. citrifolia*, así como analizar su capacidad antioxidante en un sistema cárnico (hamburguesas).
- Caracterizar y evaluar la capacidad antioxidante de un film biodegradable, a base de ácido poliláctico, con incorporación de *R. officinalis* y *T. vulgaris*, en el seno de una emulsión aceite-en-agua.

Materiales y Métodos

3. MATERIALES Y MÉTODOS

En el presente capítulo se profundiza la metodología empleada en los estudios experimentales, considerando aspectos como: el material vegetal utilizado, reactivos químicos, modo de extracción de las distintas especies vegetales, métodos para evaluar la actividad antirradicalaria, métodos para identificar compuestos polifenólicos, métodos de medición de la actividad antioxidante en *Model Food Systems*, procedimiento de fabricación y caracterización de films y análisis estadísticos.

3.1. Material vegetal

Las plantas aromáticas utilizadas fueron: romero (*R. officinalis*), tomillo (*T. vulgaris*) y lavanda (*L. officinalis*) y las comestibles no aromáticas: *C. decapetala*, *Tara* (*C. spinosa*) y *Noni* (*M. citrifolia*). Las aromáticas fueron recolectadas en la comarca del Barcelonés, Catalunya (España). Las plantas del género *Caesalpinia* fueron recibidas en estado seco del Instituto de Fitoterapia Americano (Perú) y la planta *Noni* fue recolectada en Venezuela y enviada en estado seco. Todas fueron recolectadas en los meses de primavera. Las plantas se diferenciaron en función de su origen botánico. En las plantas aromáticas se trabajó con las hojas, raíces y flores, exceptuando el romero que sólo se empleó las hojas y raíces. En el caso de las plantas del género *Caesalpinia* y la del *Noni* se trabajó únicamente con las hojas. Posteriormente se trituraron, se procedió a realizar una extracción sólido-líquida y el extracto se liofilizó y se almacenó en oscuridad en un desecador a temperatura ambiente hasta el momento de su uso. La nomenclatura usada en cada planta (siglas en inglés) se especifica en la Tabla 3:

Tabla 3. Resumen de las referencias de las plantas estudiadas

Referencia de la muestra	Tipo de muestra
RL	Hoja de romero
RR	Raíz de romero
RD	Hojas de romero seco
RLE	Extracto liofilizado de hojas de romero
TL	Hoja de tomillo
TR	Raíz de tomillo
TD	Hojas de tomillo seco
TLE	Extracto liofilizado de hojas de tomillo
TF	Flor de tomillo
LL	Hoja de lavanda
LR	Raíz de lavanda
LF	Flor de lavanda
CD	Hoja de *C. decapetala*
CS	Hoja de *C. spinosa*
NE	Extracto de la hoja de *Noni*

3.2. Reactivos químicos

Los reactivos usados en los métodos fueron: Folin-Ciocalteu, metanol, carbonato de sodio anhidro, alumina (óxido de aluminio), fluoresceína ($C_{20}H_{10}Na_2O_5$), tampón fosfato salino, ácido clorhídrico 37 %, Tween-20, ácido tricloroacético, ácido acético glacial, acetonitrilo calidad HPLC, ácido fórmico calidad HPLC, metanol calidad HPLC, todos ellos fueron suministrados por Panreac.

Los reactivos ácido tiobarbitúrico, GA, ABTS (2,2'-Azino-Bis 3-Etilbenzotiazolina-6-Ácido Sulfónico), Trolox® (6-Hidroxi-2,5,7,8-Tetrametilcromo-2-Ácido Carboxílico), DPPH (2,2-Difenil-1-Picrilhidracil), Butil Hidroxi Tolueno (BHT), AAPH (2,2,-Azobis(2-Metilpropinamida), dihidrocloruro, etanol parcialmente desnaturalizado, tiocianato de amonio, acetato de sodio 3-hidrato ($CH_3COONa{\cdot}3H_2O$), cloruro de hierro (III) hexahidratado ($FeCl_3{\cdot}6H2O$), TPTZ (2,4,6-Tripyridyl-s-Triazine) y glicerol se obtuvieron de Sigma-Aldrich.

El aceite de oliva de uso comercial y la gelatina tipo A se compraron en supermercado. El ácido poliláctico fue obtenido de Hycail Finland Oy (HM1011) y el cloroformo (grado HPLC) de Fischer Scientific.

3.3. Extracción de las muestras

La extracción general de las plantas se hizo en la relación de 1,5 g por 25 mL de disolvente. El disolvente que se empleó fue etanol 50% ($EtOH:H_2O$). La mezcla se sometió a agitación continua durante 24 horas a temperatura de 4 °C. Una vez finalizado el periodo las muestras se centrifugaron. A partir de la centrifugación, se tomó una parte del sobrenadante para su posterior uso en la determinación de la capacidad antiradicalaria.

El sobrenadante restante se separó, se rotaevaporó y se congeló a -80 °C durante 24 horas, seguidamente se liofilizó. El liofilizado obtenido fue pesado y mantenido protegido de la luz en un desecador hasta su posterior uso.

3.4. Determinación de polifenoles totales (Folin-Ciocalteu):

El método Folin-Ciocalteu usado actualmente fue propuesto por Singleton y Rossi [253] en el año 1965. Este ensayo es una modificación de un método desarrollado en 1927 por Folin y Ciocalteu empleado para la determinación de tirosina, el cual se basaba en la oxidación de los fenoles por un reactivo de molibdeno y tungstano sódico), que reaccionan con cualquier tipo de fenol, formando complejos fosfomolíbdico-fosfotúngstico. La transferencia de electrones a pH básico reduce los complejos fosfomolíbdico-fosfotúngstico en óxidos cromógenos de color azul intenso, de tungsteno (W_8O_{23}) y molibdeno (Mo_8O_{23}), siendo proporcional este color al número de grupos hidroxilo de la molécula [254]. La coloración azul se cuantifica por espectrofotometría en base a una recta de calibrado de GA [255].

Este método es criticado porque diversas sustancias de naturaleza no fenólica pueden interferir en las determinaciones y pueden dar lugar a concentraciones aparentemente elevadas de polifenoles debido a la presencia de otros compuestos. Por ello debe hacerse una eliminación previa de estas sustancias. Entre ellas destacan las proteínas, el ácido ascórbico ($C_6H_8O_6$), el ácido úrico ($C_5H_4N_4O_3$), algunos aminoácidos y nucleótidos, azúcares, aminas aromáticas y algunas sales inorgánicas. Sin embargo, como principal ventaja, cabe destacar que es un método simple, preciso y sensible [255]. Por ello, a pesar de estos inconvenientes, el ensayo de los fenoles totales se emplea con frecuencia en el estudio de las propiedades antioxidantes de alimentos vegetales y es un parámetro que generalmente muestra una estrecha correlación con diferentes métodos de medida de la actividad antioxidante [256,257].

Para realizar los ensayos, se tomaron muestras de los extractos de las plantas (7 % volumen) en la dilución adecuada y se agregó el reactivo Folin-Ciocalteu (30,8 % volumen) y una solución de carbonato de sodio al 20 % (30,8 % volumen) y agua Milli-Q (30,8 %). Las muestras se realizaron por triplicado, se dejaron reaccionar durante 1 hora en oscuridad y se midió la absorbancia en un espectrofotómetro FLUOstar OMEGA, Perkin–Elmer, Paris, France a λ=765 nm. Las unidades se expresaron como miligramo equivalente de ácido gálico (GAE) por gramo de extracto liofilizado.

3.5. Determinación de flavonoides totales

Los flavonoides son metabolitos secundarios que se encuentran en todas las partes de las plantas y cumplen una función protectora de ella y sus frutos. Por ejemplo, los flavonoides se encargan de proteger a los vegetales de la incidencia de rayos ultravioletas y visibles, protege a la planta también de los insectos, hongos, virus y bacterias. Además ejercen un efecto antioxidante, controlan la acción de hormonas vegetales y agentes alelopáticos, son inhibidores de enzimas e incluso son atrayentes de insectos polinizadores [258].

Para conocer el contenido de compuestos flavonoides totales se utilizó el método fotocolorimétrico descrito por Zhishen et al. [259], con una ligera modificación. De acuerdo a este método la muestra del extracto (10,6 % volumen), se mezcló en una dilución adecuada con 14,9 % de una solución al 5 % de $NaNO_2$. Al cabo de 5 minutos se añadieron 31,9 % de una solución de $AlCl_3$ al 10 %, 10,6 % de NaOH 1 M y 63,8 % de agua destilada. La absorbancia se midió a λ= 510 nm. Los resultados se expresaron en mg de Catequina por g de peso seco.

3.6. Determinación de la capacidad reductora fente al Fe(II) FRAP (*Ferric-Reducing Antioxidant Power*)

El análisis del FRAP (F*erric-Reducing Antioxidant Power*) fue introducido por Benzie y Strain [260] para medir la actividad antioxidante total. Se basa en la capacidad de los polifenoles para reducir Fe^{3+} a Fe^{2+} en los complejos formados con TPTZ (Fig. 13).

Este método se modificó recientemente para su uso en microplacas de 96-pocillos [261], dando una mejor reproducibilidad de muestras. El análisis se basa en el poder reductor de un antioxidante que reduce el ion Fe^{3+} al ion Fe^{2+}. Una absorción alta a una longitud de onda de 593 nm indica un poder de reducción alto del fitoquímico, es decir una actividad antioxidante alta [262].

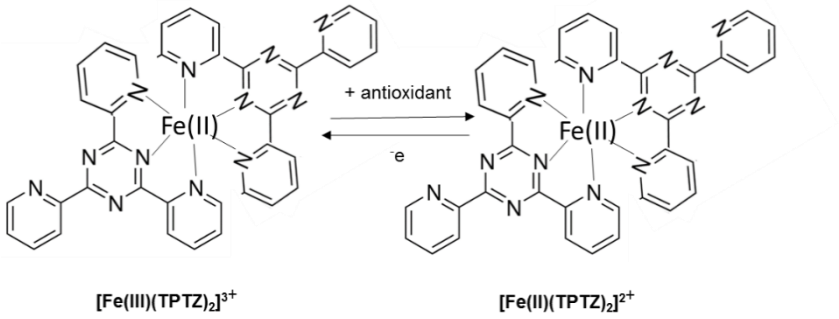


Fig. 13. Reacción FRAP [263].

Se realizó la determinación en microplaca, mezclando el reactivo FRAP incubado a 37°C con las muestras (en una dilución adecuada para que quede dentro de rango). El reactivo FRAP se preparó a partir de buffer acetato 300 mM, y los reactivos TPTZ 10 mM y cloruro de hierro (III) 20 mM. Éstos se mezclaron en la relación 10:1:1. Se realizó lectura de la absorbancia en el espectrofotómetro FLUOstar OMEGA, Perkin–Elmer, Paris, France, midiendo a λ= 593 nm.

3.7. Ensayo TEAC

El ensayo TEAC (*Trolox Equivalent Antioxidant Activity*) o ensayo del ABTS [2,2-Azinobis-(3-Ethylbenzothiazoline-6-Sulphonic Acid)] se basa en la captación por los antioxidantes del radical catiónico verde-azulado $ABTS^{\cdot+}$ (Fig. 14), cuyo espectro muestra un máximo de absorción a las longitudes de onda 415, 645, 734 y 815 nm. De todas ellas, las longitudes de onda empleadas más frecuentemente son 415 y 734 nm [255]. El método fue realizado de acuerdo a Almajano et al. [264] Como patrón se empleó el ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico (Trólox®), un análogo sintético hidrosoluble de la vitamina E.

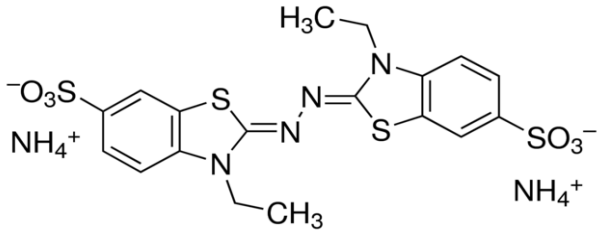


Fig. 14. Radical ABTS [263].

Se pueden utilizar dos estrategias para el desarrollo del método: inhibición y decoloración. En la primera los antioxidantes se añaden previamente a la generación del radical $ABTS^{+\cdot}$ y lo que se determina es la inhibición de la formación del radical, que se traduce en un retraso en la aparición de la coloración verde-azulada

En la segunda estrategia, usada en esta Tesis, los antioxidantes se añaden una vez el $ABTS^{+\cdot}$ se ha formado y se determina entonces la disminución de la absorbancia debida a la reducción del radical, es decir la decoloración de éste [265].

Para proceder al ensayo se preparó el radical catiónico ABTS (7 mM) y persulfato de potasio (24,24 mM). Se mezclaron. La absorbancia del radical ABTS se ajustó, diluyéndolo con PBS 10 mM (pH=7,4) incubado a 30 °C. Esta absorbancia se mantuvo entre 0,72 ± 0,01.

El ensayo se realizó en una microplaca, mezclando la disolución que contiene el radical (90% volumen) con la muestra del extracto (10% volumen) en las diluciones adecuadas para que la absorbancia quede dentro del rango de lectura del aparato. Se midió la absorbancia en el espectrofotómetro FLUOstar OMEGA, Perkin–Elmer, Paris, France a λ=734 nm durante 15 minutos. Se tomó como resultado final el minuto 5, a partir del cual ya no se observó disminución de la lectura.

A partir de una recta de calibrado con Trolox®, se representó el porcentaje de inhibición (%) *versus* la concentración de Trolox® (moles de Trolox® por unidad de volumen) y se obtuvieron los TE de la muestra analizada.

Con la absorbancia, se determinó el porcentaje de inhibición de los blancos y de cada muestra según la siguiente expresión:

$$\% \text{ Inhibición blanco} = (t_0 - t_5/t_0) \times 100 \quad \text{(Ec. 1)}$$

$$\% \text{ Inhibición muestra} = (t_0 - t_5)/t_0 \times 100 - \%\text{Inhibición} \quad \text{(Ec. 2)}$$

Donde:

t_0= representa el valor de la absorbancia al tiempo inicial.

t_5= representa el valor de la absorbancia a los 5 minutos de lectura.

3.8. Ensayo ORAC.

El ensayo ORAC (*Oxygen Radical Antioxidant Activity*) se aplica para evaluar la capacidad antioxidante en muestras biológicas y de alimentos. Se basa en la inhibición del radical peroxilo oxidativo inducido por descomposición térmica de compuestos azo como el AAPH [2,2'-Azobis-(2-Aminopropano)-Dihidrocloruro] (Fig. 15). El mecanismo de reacción se basa en la transferencia de un átomo de hidrógeno del antioxidante al radical libre. Para ello se usa un radical iniciador (AAPH) para generar el radical peroxilo (ROO·). En presencia de un

antioxidante, el radical capta, preferiblemente, un átomo de hidrógeno del antioxidante (AH), originando un compuesto hidroperóxido (ROOH) y un radical antioxidante estable (A·). Como consecuencia, la disminución de la fluorescencia por acción del radical peroxilo es disminuida o inhibida [255]. [255].

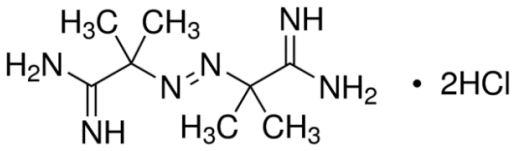


Fig. 15. Radical AAPH

La protección del antioxidante se mide a partir del área de fluorescencia bajo la curva (A*rea Under Curve, AUC*) (Fig. 16) de la muestra en comparación con la AUC del blanco (en la cual el antioxidante no está presente). La AUC del calibrado se genera usando diferentes patrones (con diferentes concentraciones estándar) de Trolox® [266].

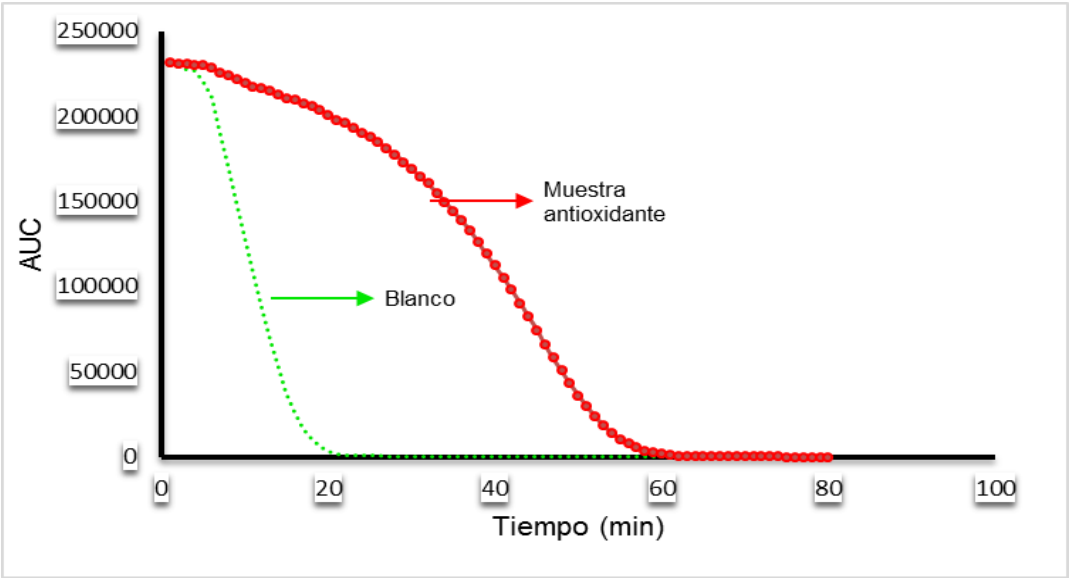


Fig. 16. Actividad antioxidante ORAC de una muestra con antioxidante medida como AUC

El ensayo se realizó de acuerdo a Skowyra et al. [191]. Las muestras del extracto de las plantas se diluyeron para mantenerse dentro del rango del equipo. Se colocaron en una microplaca (20 % volumen) y se agregó Fluoresceína (60 % volumen). Se realizó una lectura inicial en el equipo de análisis (FLUOstar OMEGA, Perkin–Elmer, Paris, France), preparado a una temperatura de incubación de 37 ºC. Una vez realizada esta, se agregó el AAPH (0,3 M) (20 % volumen) y se continuó con las mediciones durante 2 horas. Se calculó el AUC, mediante la siguiente ecuación:

$$AUC = ((0,5 + \sum_{i}^{Nc} = 1fi)/ fi) \times tc \quad \text{(Ec. 3)}$$

Donde:

AUC= área bajo la curva de la muestra del pocillo.

fi = unidades de fluorescencia (*fi*, corresponde al valor de la primera lectura).

Nc= número de ciclos.

tc: tiempo de cada ciclo, en este caso tc = 2 (2 minutos).

Seguidamente se calculó la disminución de la fluorescencia a través de la ecuación 4. Los resultados son expresados como micromoles TE/gramos de extracto liofilizado:

$$\text{Disminución}_{\text{fluorescencia}} = AUC - AUC_{Bl} \quad \text{(Ec. 4)}$$

AUC= área bajo la curva de la muestra del pocillo.

AUC_{Bl}=área bajo la curva del blanco.

3.9. Ensayo DPPH

El ensayo DPPH, permite evaluar la actividad de compuestos específicos o extractos usando el radical libre estable 2,2-difenil-1-picrilhidracilo (DPPH$^{\bullet}$) (Fig. 17) en una solución metanólica.

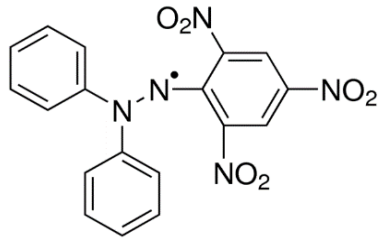


Fig. 17. Radical DPPH [267].

La reducción del DPPH$^{\bullet}$ se monitorea por la disminución en la absorbancia a una longitud de onda característica. En su forma de radical libre, el DPPH$^{\bullet}$ absorbe a 515 nm y cuando sufre reducción por un antioxidante, esta absorción desaparece. En consecuencia, la desaparición del DPPH$^{\bullet}$ proporciona un índice para estimar la capacidad del compuesto para atrapar radicales. El modelo que explica la actividad de un compuesto como antirradical se ejemplifica con la siguiente ecuación [268].

$$DPPH^{\bullet} + (AH)n \longrightarrow DPPH\text{-}H + (A^{\bullet})n$$

Donde AH es un antioxidante que actúa como antirradical donando átomos de hidrógeno (tal es el caso de los fenoles), lo que da como resultado radicales con estructuras moleculares relativamente estables , ya que se produce la deslocalización, que detendrán la reacción en

cadena. El nuevo radical formado ($A^{\bullet}$) puede interactuar con otro radical para formar moléculas estables (DPPH-A, A-A). La reacción entre el $DPPH^{\bullet}$ y un compuesto, depende de la conformación estructural del mismo, por lo que las comparaciones cuantitativas no siempre son apropiadas [268].El ensayo DPPH se realizó según Skowyra et al. [269] para determinar la capacidad que tienen los extractos para inhibir el radical DPPH. Se preparó una solución de DPPH al 5,07 mM en metanol puro. Se efectuaron las diluciones adecuadas para las muestras en estudio. Seguidamente en una microplaca, se agregó la disolución de DPPH, y las muestras (en la concentración de 10 % v/v de muestra y 90 % v/v del radical). Se midió la absorbancia en el espectrofotómetro FLUOstar OMEGA, Perkin–Elmer, Paris, France a λ=517 nm, durante 75 min.

3.10. Ensayo de resonancia paramagnética electrónica (*Electron Paramagnectic Resonance*, EPR):

La extracción de las plantas para efectuar este ensayo se realizó en MeOH en la proporción 1:10 (m/v). Este procedimiento se aplicó únicamente para *C. decapetala*. El sobrenadante de esta extracción se usó en el ensayo.

Para generar la reacción [270] se efectuó la mezcla de reactivos en el siguiente orden: 100 µL de DMPO (35 mM); 50 µL de H_2O_2 (10 mM); 50 µL del extracto de la planta a diferentes concentraciones o 50µL de ácido ferúlico como control positivo (0-20g/L) o 50µL de MeOH puro como control negativo y 50µL de $FeSO_4$ (2mM).

Las soluciones finales (125µL) se pasaron a través de un estrecho tubo de cuarzo (diámetro interior = 2 mm) y se introdujeron en la cavidad del espectrómetro de EPR. El espectro se registró 10 min después de la adición de la solución de $FeSO_4$, ya que en ese momento la señal del aducto radical es mayor.

Los espectros se registraron con un espectrómetro Bruker EMX 10/12-Plus en las siguientes condiciones: frecuencia de microondas de 9,8762 GHz; potencia de microondas de 30,27 mW; campo central de 3522,7 G; anchura de barrido de 100 G; ganancia del receptor de 5,02 × 104; frecuencia de modulación de 100 kHz; amplitud de modulación de 1,86 g; constante de tiempo de 40.96 ms y tiempo de modificación de 203,0 ms.

3.11. Identificación de compuestos polifenólicos mediante LC-MS

La identificación de los compuestos polifenólicos presentes en las plantas se realizó usando un equipo LC-ESI–QTOF-MS Agilent serie 1200 (Wilmington, DE, USA). El equipo está conformado por un sistema automático de inyección de muestras, dos bombas isocráticas de alta presión, un desgasificador y un horno cromatográfico. El espectrómetro de masas QTOF es un Agilent modelo 6520, con una fuente de ionización por electroespray dual.

Los componentes se separaron en una columna C_{18} Zorbax Eclipse XDB (100 mm × 2.1 mm, 3.5 m) de Agilent y conectada a una precolumna C_{18} (4 mm × 2 mm) de Phenomenex (Torrance, CA, USA). Las fases móviles usadas fueron: agua ultrapura (fase A) y acetonitrilo (fase B), ambos acidificadas con 0,1 % con ácido fórmico. El gradiente fue: 0-2 min, 3 % B; 25-27 min, 100 % B; 28-38 min 3 % B. La fase móvil tuvo un flujo de 0,2 mL min^{-1}. El volumen de inyección fue 10 µL y la temperatura de la columna fue de 30 ºC.

El analizador de masas TOF se operó a 2GHz. La exactitud en las masas fue dada por infusión continua, a través de un segundo nebulizador, de una solución de referencia de masas (Solución de calibración A de Agilent) en la fuente ESI. La identificación de los compuestos activos en los extractos de las muestras se realizaron por la selección de los picos más intensos en los cromatogramas obtenidos a partir de cada planta, seguido por la generación de su fórmula empírica y la adquisición de los iones producto para cada ion precursor obtenidos en los espectros, sometidos a diferentes colisiones de energía. Para la identifiación de los compuestos se utilizó la combinación de: (1) los valores de masa exactos para los iones precursores ($[MH]^-$), (2) la fragmentación de los patrones observados mediante el espectro (MS/MS), y (3) la existencia de espectros en la base de datos

3.12. Preparación de emulsiones

3.12.1. Eliminación de los antioxidantes del aceite.

Con una antelación de 24 horas al procedimiento de filtración del aceite, se colocó alúmina en una estufa a 200 ºC para garantizar la ausencia total de agua y la activación. Se dejó enfriar en un desecador hasta que alcanzó la temperatura ambiente.

Para la eliminación de los antioxidantes naturales del aceite, se hizo pasar a través de la alúmina 2 veces. Se almacenó a -80 °C hasta su uso.

3.12.2. Preparación de la emulsión.

Las emulsiones trabajadas fueron del tipo “aceite-en-agua” según Yoshida et al. [271]. Para ello, se disolvió Tween-20 (1 %) en agua Milli Q y se añadió aceite (10 %). Las muestras fueron añadidas en forma liofilizada re-disueltas en EtOH 50 % v/v. En todos los casos se usó un control negativo preparado sin la muestra y un control positivo (Trolox®, BHA o BHT) disuelto en etanol. Para formar la emulsión, el aceite se añadió gota en gota y en forma continua, manteniéndolo en frío (en hielo) y mediante sonicación durante 10 minutos.

3.12.3. Determinación del Valor de Peróxidos

El Valor Peróxido (*Peroxide Value,* PV) se determinó por el método del ferrocianuro [272]. Los reactivos utilizados fueron etanol (96%), cloruro de hierro (II) tetrahidratado, tiocianato de

amonio y ácido clorhídrico al 37%. Un primer reactivo se preparó con ácido clorhídrico y cloruro de hierro (II) y un segundo reactivo con tiocianato de amonio y agua MilliQ.

El ensayo consistió en pesar una gota de emulsión mantenida en un rango entre 0,007-0,01 g y diluirla en etanol. De esta disolución se tomó en una cubeta la cantidad necesaria, de acuerdo al grado de oxidación de la muestra y se adicionó etanol (96 %). Posteriormente se añadieron los reactivos preparados, en la proporción de 1,875 %v/v cada uno. La medición de la absorbancia se realizó en un espectrofotómetro marca Zuzi modelo UV4200/51 a λ=500 nm. El cálculo del valor de peróxidos fue:

$$VP = (((Abs \times dil)/m) + b)/a \quad \text{(Ec.5)}$$

Donde:

a= representa la pendiente de la recta de calibrado.

Abs= es la absorbancia obtenida.

b= es la ordenada en el origen de la recta de calibrado.

dil= es la dilución de cada emulsión.

m= peso de la muestra de emulsión tomada (g)

La recta de calibrado se determinó a partir de una serie de muestras de aceite oxidado, de acuerdo al método oficial AOCS Cd 8-53 [272].

3.12.4. Determinación de la oxidación secundaria mediante TBARS en emulsiones

El método se realizó como describe Grau et al. [273]. Se preparó el reactivo TBARS (15 % ácido tricloroacético, 0,375 % ácido tiobarbitúrico y ácido clorhídrico 2,1 %). Se tomó una cantidad de cada emulsión y se añadió BHA. Posteriormente se añadió el reactivo TBARS en la relación 1:5. Inmediatamente las muestras fueron introducidas en baño de ultrasonidos (equipo marca Prolabo) durante 10 minutos e inmersas en un baño de agua precalentado a 95 °C por 10 minutos más. Las muestras fueron centrifugadas y a partir del sobrenadante se midió la absorbancia a λ= 531 nm en un espectrofotómetro marca Zuzi modelo UV4200/51. Los resultados se expresaron como mg Malonaldidehído (MDA)/kg de emulsión.

3.12.5. Determinación de volálites en emulsiones por la técnica del espacio de cabeza

Se midió la concentración de hexanal presente en las emulsiones utilizando un cromatógrafo de gases TRACE GC, equipado con un espectrómetro de masas DSQII (Thermo Fisher Scientific) con un inyector de espacio de cabeza TRIPLUS automático. Se introdujo una concentración de 0,8 mL en viales de vidrio de 10 mL y se cerraron con tapas de aluminio con

septos de PTFE / silicona. Posteriormente, se agitaron y se calentaron a 60 °C durante 30min antes de la medición. Seguidamente 1ml de la fase de vapor se introdujo y se mantuvo a 65 °C. Las concentraciones de hexanal se determinaron usando una recta de calibrado preparada a partir de hexanal.

3.13. Preparación de hamburguesas de ternera

La carne picada se obtuvo de una carnicería local, la cual se pasó 3 veces por el molino. La carne se mezcló con sal (1,5 %). Posteriormente se separaron varias cantidades de carne y a cada una de ellas se le añadió la formulación correspondiente, pudiendo ser la planta en estado seco a diferentes concentraciones o controles positivos (BHT, extracto de té blanco). Los ingredientes fueron mezclados manualmente con la carne y se formaron hamburguesas pequeñas. Las hamburguesas se colocaron en bandejas, cubiertas con plástico transparente y mantenidas a 4 ºC hasta su evaluación. En el caso de los films, las hamburguesas fueron cubiertas con una película del film tanto por la parte superior de la hamburguesa como por la parte de abajo. En uno de los estudios las hamburguesas se almacenaron en atmósfera rica en oxígeno que potenció el color rojo de la carne.

3.13.1. Ensayos realizados en hamburguesas:

3.13.1.1. Ensayo de la capacidad antioxidante

Este ensayo se evaluó por el método FRAP. Para ello, de acuerdo como describe Linden et al. [274] se realizaron dos tipos de extracción para extraer los antioxidantes: lipofílica (con acetona/etanol/agua destilada; 5:4:1 v/v) e hidrofílica con agua destilada. Cada extracción se denominó como $FRAP_{water}$ y $FRAP_{lipid}$.

En el caso de la carne, para proceder a la extracción, en primer lugar se homogeneizó con un politrón (marca Ultraturrax). La carne (5 g) se mezcló con 5 mL de cada disolvente y se homogeneizó con el politrón por 30 segundos. Las dos extracciones se realizaron siguiendo esta metodología. La mezcla obtenida se centrifugó por 30 minutos a 4 ºC. El sobrenadante se filtró y se usó para el ensayo FRAP. Las medidas se hicieron por triplicado.

3.13.1.2. Ensayo de metamioglobina

Las muestras de carne (5 g) se homogenizaron con 25 mL de tampón fosfato 0,04 M (pH 6,8) durante 10 segundos usando un politrón. La mezcla homogeneizada se almacenó por 1 h a 4 ºC y se centrifugó a 4500 g por 20 minutos a 4 ºC usando una centrifuga refrigerada. La absorbancia del sobrenadante se filtró y se leyó en un espectrofotómetro marca Zuzi modelo UV4200/51 a 572, 565, 545 y 525 nm. El porcentaje de metamioglobina fue determinado usando la fórmula de Krzywicki [275]:

MetMB (%)= [2,514(A572/A525)+0,777(A565/A525)+0,8(A545/A525)+1,098]x100 (Ec.6)

Donde:

A_{572}= lectura a longitud de onda de 572

A_{525}= lectura a longitud de onda de 525

A_{565}= lectura a longitud de onda de 565

A_{545}= lectura a longitud de onda de 545

3.13.1.3. Medición del color

La colorimetría tiene por objeto detectar y cuantificar la sensación del color con la ayuda de unos parámetros. Para ello, se pueden determinar 3 parámetros: luminosidad, tono y pureza, que caracterizan a un color. Los colores que pueden presentar una muestra se cuantifican empleando distintos métodos. Uno de los más extendidos es el sistema cromático CieLAB, donde las coordenadas cromáticas que se determinan son L* (indica luminosidad de la muestra), a* (cuantifica la dimensión rojo-verde) y b* (cuantifica la dimensión amarillo-azul) [276].

Las medidas del color se realizaron en 4 puntos de la superficie de las hamburguesas de carne. El color se midió a lo largo de todo el estudio de la carne. Previamente se hizo un blanco usando una placa blanca y negra. Las muestras se realizaron por triplicado y con el instrumento se hicieron 3 medidas en 4 puntos diferentes. En cada punto medido el equipo realizó 9 mediciones (“disparos”). La media y desviación estándar se calculó para cada muestra.

3.14. Fabricación de film de gelatina

3.14.1. Método de producción del film

Para la elaboración de los films con las plantas del género *Caesalpinia* y *Noni*, se realizó una extracción etanólica en la proporción de 6 g de planta seca en 90 mL de EtOH:H_2O 50 % v/v en el caso de CD, *Noni* y 1,2 g de planta seca en el caso de la CS. Además, se realizó un film como control positivo a base de té blanco (WT), en la proporción de 6 g de planta seca en 90 mL de EtOH:H_2O 50 % v/v. . En todos los casos la extracción sólido-líquido se mantuvo a 4 ºC durante 24 h. Posteriormente, la mezcla se centrifugó a 2500 rpm y el sobrenadante se recogió para añadirlo en el film.

Los films se elaboraron de acuerdo a Bodini et al. [250] con gelatina de tipo A (2 g/100 g de solución filmogénica), la cual se hidrató a 25 ºC por 10 min y se solubilizó a 55 ºC por 15 min

en un baño termostático. Después se añadió el glicerol (20 g/100 g de gelatina). La solución se agitó y entonces se añadió el extracto. Nuevamente la solución se mezcló. La solución se dejó secar en superficies de dimensiones 355x240x17 mm, sobre polietileno (60 g solución filmogénica por bandeja) y se secaron en una estufa a 30 ºC durante 24 h. Se realizaron diferentes formulaciones:

- 3 formulaciones para las plantas CD y CS: CD1 (0,3 % m/v de extracto de planta en film), CD2 (0,7 % m/v de extracto de planta en film) and CD3 (1 % m/v de extracto de planta en film) and CS1 (0,07 % m/v de extracto de planta en film), CS2 (0,1 %) y CS3 (0,2 % m/v de extracto de planta en film).
- 2 controles positivos con BHA (0,001 %) and WT (1 %).
- 1 control negativo (sin extracto ni adición de ningún antioxidante)
- 1 formulación para *Noni*: NE (1 %)

3.14.2. Caracterización del film de gelatina

3.14.2.1. Determinación de la concentración de polifenoles totales

Para comprobar la difusividad de los compuestos polifenólicos del film se usaron simulantes alimentario: etanol 50 % v/v y agua y se determinó la concentración de polifenoles totales en el film de acuerdo a Bodini *et al.* [250]. Las muestras de films se pesaron y se diluyeron en una solución de etanol al 50 % , se tomaron muestras diariamente y se analizaron mediante los ensayos Folin-Ciocalteu, ORAC y TEAC.

3.14.2.2. Ensayo FTIR

Esta técnica se basa en el análisis de las frecuencias específicas de las vibraciones que presentan los enlaces químicos de las moléculas tras su interacción con la radiación en la longitud de onda del infrarrojo. Dichas frecuencias corresponden a niveles de energías y permiten identificar de modo inequívoco los grupos funcionales más importantes de las moléculas orgánicas [277,278].

El ensayo se realizó como describe Gallego et al. [279]. Para ello, se empleó un equipo FTIR (Golden Gate, Specac Ltd, Orpington, Reino Unido), con un accesorio de reflexión total atenuada (*attenuated total reeflection, ATR),* a una temperatura controlada. Los films se colocaron directamente en el cristal y se realizaron 8 exploraciones. Se obtuvieron espectros en la región del infrarrojo entre 4000 cm^{-1} y 400 cm^{-1} .

3.14.2.3. Propiedades mecánicas

Dentro de los diversos tipos de ensayos mecánicos que se pueden llevar a cabo en los polímeros, el de tracción permite calcular el estiramiento de una muestra colocada en un sistema de mordazas a una velocidad constante hasta su rotura (resistencia a la tracción del

material) [280]. El ensayo de tracción permite también conocer el grado de elongación del material antes de su rotura y el módulo de elasticidad o módulo de Young, que relaciona la proporción existente entre la tensión y la deformación del polímero [281]. Generalmente un incremento en la cantidad de plastificante da como resultado películas con menor fuerza de tensión y una mayor elongación [282–284].

Las propiedades mecánicas del film se evaluaron usando un equipo Zwick BZ2.5/TN1S (Zwick GmbH & Co. KG, ULM, Germany) y se midieron a una velocidad de deformación de 10 mm/min. Las muestras se cortaron manteniendo un espesor de 15,2 mm con una longitud de 10 mm y un ancho de 5 mm. La media se hizo sobre un mínimo de 10 mediciones de cada muestra. Se obtuvieron los resultados de resistencia a la tracción (T), alargamiento (E) y el módulo elástico (módulo de Young).

3.14.2.4. Permeabilidad al vapor de agua

El ensayo se realizó según Abdollahi et al. [285]. Los films se cortaron en forma circular y se fijaron en la parte superior de un vaso de precipitados. Éste se colocó en un desecador junto a un vaso de precipitados con agua destilada. El desecador se introdujo en una incubadora provista de control de temperatura (25 ºC). Las muestras se pesaron a diferentes intervalos de tiempo hasta completar 101 h, determinando la ganancia de masa de los films.

3.14.2.5. Ensayo de transmisión de luz (transparencia del film)

Este ensayo se realizó siguiendo el método propuesto por Fang *et al.* (2002) [286]. Los films se cortaron en rectángulos pequeños y se introdujeron en cubetas. Las cubetas se situaron en un espectrofotómetro Zuzi UV4200/51, AUXILAB, S.L., Navarra, Spain y se midieron a una longitud de onda de 600 nm por triplicado. La opacidad se calculó mediante la siguiente ecuación:

$$\text{Opacidad} = Abs_{600}/x \qquad \text{(Ec.7)}$$

Donde Abs_{600} es el valor de la abosorbancia (*Abs*) a 600 nm y *x* es el espesor del film (mm) calculado con un medidor manual de grosor marca Sauter.

3.14.2.6. Microscopia electrónica de barrido (*Scanning Electron Microscopy*, SEM):

Esta técnica consiste en hacer incidir un barrido de haz de electrones sobre la muestra, previamente recubierta de oro o carbón (le otorga propiedades conductoras). Al alcanzar el haz la superficie de la muestra se generan electrones secundarios (*Secundary Electron Image*, ESI). El microscopio dotado internamente de un detector, permite recoger la energía y transformarla en imagen [287].

La morfología de la película se evaluó por microscopía electrónica de barrido [250] en un equipo Nano Nova 230, FEI, Hillsboro, Oregón, EE.UU. Las películas se cortaron en trozos de 5 mm x 5 mm. Las muestras se recubrieron de oro y se observaron usando un voltaje de aceleración de 20 kV.

3.14.2.7. Propiedades de color en el film

Los parámetro de color CIE lab se midieron mediante un equipo colorimétrico Konica Minolta CM-3500d (Konica Minolta Sensing, INC.,, Milton Keynes, UK). Las medidas se tomaron en fiferentes puntos del film y los valores resultaron como media de 9 mediciones.

3.15. Fabricación del film con base de PLA

3.15.1. Elaboración del film de PLA

Además de los films de gelatina, se fabricó un film a base de PLA. En este film se incorporaron las plantas aromáticas romero y tomillo. Ambas plantas se añadieron en estado seco y su extracto etanólico liofilizado. Para ello, se prepararon un total de 9 films diferentes. La nomenclatura usada en las formulaciones de los films se muestra en la Tabla 4. Los films fabricados fueron:

- 1 film solo de PLA (como control).
- 4 films con las plantas aromáticas (2 con el extracto de la planta liofilizado y 2 con la planta seca).
- 4 con patrones antioxidantes (BHT, RA, CA y EGCG).

Tabla 4. Nomenclaturas usadas en los film de PLA

Referencia del film	Contenido
TD	Tomillo Seco
TLE	Extracto de Tomillo Liofilizado
RD	Romero Seco
RLE	Extracto de Romero Liofilizado
BHT	Butil Hidroxi Tolueno
RA	Ácido Rosmarínico
CA	Ácido Cafeico
EGCG	Galato de Epigalocatequina

Las plantas secas y los extractos liofilizados se añadieron en la concentración de 3 % (w/w) al PLA. La muestra de cada planta se trituró con la ayuda de un mortero. La muestra de tomillo seco se tamizó (tamaño orificio 140 micras). Los patrones (BHT, RA, CA, EGCG) se diluyeron y se añadieron al PLA en la concentración de 1,5 % (w/w).

El PLA se disolvió junto a la planta y/o muestra patrón en cloroformo, calentándose a 40 °C con agitación continua. La solución obtenida se vertió en placas de Petri (18,5 cm de diámetro) y se

dejó secar durante una semana. A continuación, los films se colocaron en un horno de vacío y se dejaron secar durante una semana más.

Considerando el diámetro de las cápsulas de Petri, se utilizó la relación de 2,9 g de PLA y 40 ml de cloroformo para la fabricación del film.

Los films elaborados con los patrones (RA, CA y EGCG) sólo se utilizaron para comparación en los ensayos TGA y los ensayos de migración.

3.15.2. Ensayo de migración

Los ensayos de migración se realizaron con los simulantes alimentarios: agua (grado HPLC) y etanol 95 % v/v. Los films se cortaron en tiras de 50 mg en el caso de los films con planta seca y 25 mg en los films con el extracto liofilizado y la muestra patrón. Las tiras de films se situaron en unos tubos de ensayo bordeando el interior del tubo. Se vertieron 5 mL en los tubos de los simulantes alimentarios y se taparon con tapones de rosca. Las muestras se colocaron en un horno a 37 ºC durante 10 días.

3.15.3. Pérdida y ganancia de masa del simulante alimentario

El peso inicial de los films se midió antes de la migración. La pérdida de masa se refirió al peso inicial. El peso húmedo de las películas se midió nuevamente después de 10 días de la migración, mientras que el peso seco de las muestras se midió después de 7 días de haberse sometido a vacío (0 Pa a 25 °C) [288].

3.15.4. Desorción/ionización láser asistida- tiempo de vuelo- espectrometria de masas (laser desortion ionization-time of flight-mass spectrometry)

La prueba de desorción/ionización láser asistida – tiempo de vuelo – espectrometría de masas (*Laser Desortion IonizationTtime of Flight- Mass Spectrometry*, LDI-ToF-MS) se realizó de acuerdo a Wu and Hakkarainen [289]. Para ello, se usó un espectrómetro de masas Bruker Ultra Flex con una fuente de iones SCOUT-MTP (Bruker Daltonics, Bremen, Alemania), equipado con una fuente láser de nitrógeno 337nm. La expulsión de iones se estableció en 200ns y se realizaron 1000 disparos en cada análisis.

3.15.4.1. <u>LDI-ToF-MS en las plantas:</u>

El procedimiento se llevó a cabo para obtener la distribución de los compuestos de bajo peso molecular presentes en las plantas en estado seco y en el extracto liofilizado. Cerca de 1 mg de material vegetal se disolvió en 1 ml de EtOH 75 % v/v y luego 1 µL se colocó en una placa MALDI de acero inoxidable.

3.15.4.2. LDI-ToF-MS en los films:

Se realizó este ensayo en los films sometidos a los simulantes alimentarios. Se analizaron dos muestras de cada material después de 18 horas, 68 horas y 240 horas. Mediante una jeringa Hamilton se inyectaron 5 µL del simulante alimentario. Los 5 µL de los simulantes alimentarios se detectaron en una placa MALDI de acero inoxidable. El blanco que se utilizó fue etanol y agua.

3.15.4.3. Ensayo termogravimétrico (*Thervogravimetric Analysis*, *TGA*)

Es una técnica que mide la masa de una muestra mientras se calienta, enfría o se mantiene a la misma temperatura en una atmósfera conocida, habitualmente aire u oxígeno y en algún caso, una atmósfera inerte de N_2. Se usa principalmente en el análisis cuantitativo de productos. Una curva típica de TGA nos muestra la pérdida de masa al aumentar la temperatura y se relaciona con la pérdida de componentes volátiles, descomposición del polímero, combustión y posibles residuos finales [290].

El ensayo por TGA (Wu and Hakkarainen [289]) se realizó para evaluar la estabilidad termo-oxidativa de los films y el efecto que poseen las plantas estudiadas sobre los films de PLA. El equipo que se usó es un Mettler-Toledo TGA / SDTA 851e. Las muestras se cortaron en forma circular y se colocaron en vasos de aluminio sin tapa. Las muestras se calentaron desde 50 °C a 600 °C a una velocidad de calentamiento de 10 °C/min. El flujo del gas de oxígeno durante el análisis fue de 50 mL/min.

3.15.5. Ensayo de la calorimetría diferencial de barrido (Differential Scanning Calorimetry, DSC):

Es una técnica en la que la diferencia de calor entre una muestra y una referencia se miden en función de la temperatura. La muestra y la referencia se mantienen aproximadamente a la misma temperatura y se mide el flujo de calor. El equipo DSC se utiliza para caracterizar polímeros, productos farmacéuticos, biológicos y químicos; se pueden medir las propiedades físicas de los materiales como entalpía, energía de fusión, calor específico, cristalinidad, pureza o estabilidad a la oxidación.

Los films se analizaron mediante un equipo DSC (Mettler-Toledo DSC 820). Para ello, se pesaron 2-5 mg del film y se colocaron en recipientes de aluminio y se cerraron para asegurar una atmósfera de nitrógeno (para evitar la degradación debido a la oxidación) (Wu and Hakkarainen [289]).

Las muestras se calentaron desde -30 °C a 240 °C con una velocidad de calentamiento de 10 °C/min y se mantuvieron a 240 °C durante 2 minutos. Después se enfriaron a -30 °C a una velocidad de 10 °C/min. Las muestras se mantuvieron a -30 °C durante 2minutos antes de

recalentarlos desde -30 °C a 240 °C con la misma velocidad (10 °C/min). Se fijó un flujo de gas de nitrógeno de 50 mL/min.

La temperatura de transición vítrea (Tg) se tomó como el punto medio de la transición durante la segunda etapa del calentamiento. La temperatura de fusión (Tm) se obtuvo de la primera etapa de calentamiento. Todas las muestras se evaluaron por triplicado. Así mismo, el grado de cristalinidad se calculó usando la siguiente ecuación (con $\Delta H^o f = 93\ J/g$):

$$WC = (\Delta Hf/\Delta H^o f) \times 100 \qquad \text{(Ec.8)}$$

3.16. Análisis estadísticos

Los datos de los análisis se expresaron como media ± desviación estándar. Los análisis estadísticos se realizaron con el software Minitab. Las diferencias significativas se determinaron por el análisis de varianza ANOVA (de una sola vía) mediante comparacion con el test Duncan ($p < 0.05$).

Resultados y Discusión Experimental

4. RESULTADOS Y DISCUSIÓN EXPERIMENTAL

A continuación se presentan los resultados experimentales realizados con las plantas aromáticas y/o comestibes empleadas. Los estudios están clasificados en función de la familia de planta con la que se ha trabajado. En este caso tenemos las plantas de la familia Lamiaceae (*R. officinalis*, *T. vulgaris* y *L. officinalis*), Fabaceae (*C. decapetala* y *C. spinosa*) y Rubiaceae (*M. citrifolia*).

Los resultados se muestran en forma de artículo, en las revistas que son:

- Antioxidant properties of three aromatic herbs (Rosemary, Thyme and Lavender) in Oil-in-Water Emulsion. Publicado en Journal American Chemists´ Society.

- Antioxidant Activity of Polylactic Acid (PLA) Film Prepared with Rosemary and Thyme in Oil-in-Water Emulsions. Artículo enviado a European Food Research and Technology.

- Oxidative stability of O/W emulsions with Caesalpinia decapetala antioxidant capacity by EPR. Publicación aceptada en Pharmaceutical Biology en septiembre.

- Analytical characterization of polyphenols from *Tara* and Caesalpinia decapetala as tabilizers of O/W emulsions. Artículo impress en Journal of Food Science.

- Caesalpinia decapetala extracts as inhibidors of lípid oxidation in beef patties. Publicado en Molecules.

- Gelatine-based antioxidant packaging containing Caesalpinia decapetala and Tara as a coating for grodund beef patties. Publicado en Antioxidants.

- Active packaging incorporationg *Noni* extract to maintain the oxidative stability of meat Burger. Artículo enviado a Journal of Food Science.

Estudios con Plantas de la Familia Lamiaceae

(*R. officinalis*, *T. vulgaris* y *L. officinalis)*

- Antioxidant properties of three aromatic herbs (Rosemary, Thyme and) in Oil-in-Water Emulsion.
- Antioxidant Activity of Polylactic Acid (PLA) Film Prepared with Rosemary and Thyme in Oil-in-Water Emulsions.

4.1. Antioxidant properties of three aromatic herbs (rosemary, thyme and lavender) in oil- in- water emulsion

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Publicado en Journal American Chemists´ Society (2013): 90:1559-1568; doi: 10.1007/s11746-013-2303-3

4.1.1. Introduction

The susceptibility of lipids to oxidation is one of the main causes of deterioration in the quality of food emulsions. This deterioration includes undesirable changes in flavors, textures, shelf life, appearance, and nutritional profiles [291]. In addition, it may cause degradation of nutritional quality, and even affect food safety. Antioxidants are a group of substances that, in low concentrations, inhibit or retard oxidative processes through a mechanism that usually involves oxidation of the antioxidant [292].

Polar compounds have been reported to impact negatively on the oxidative stability of the oils. Free fatty acids accelerate the oxidation rate of oils and oil in water emulsions (O/W) through the ability of the carboxylic acid group to accelerate the decomposition of hydroperoxides and form prooxidative complexes with metals [293].

Synthetic antioxidants, such as Butyl Hydroxy Anisole (BHA), Butyl Hydroxy Toluene (BHT) and Propyl Gallate (PG) are used in many foods to prevent rancidity. However, their effects on health have been questioned. Natural antioxidants are presumed to be safe because they occur in nature and in many cases are derived from plant sources. Natural antioxidants have many advantages: they are accepted by consumers, are considered safe and have less regulatory requirements. The antioxidant properties can be due to many substances including some vitamins, flavonoids, terpenoids, carotenoids and phytoestrogens [2].

The use of herbs and spices to inhibit the development of oxidative reactions in food systems has recently become popular. Rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*) and lavender (*Lavandula angustifolia*) have been the center of focus as sources of natural antioxidants, and it has been reported that the antioxidant properties of spices were attributed to their phenolic contents. The compounds present in these extracts can be classified into three groups: diterpenes, flavonoids and phenolic acids. Compounds such as rosmarinic and carnosic acid are major components [63].

Aromatic herb extracts act as antioxidants to retard oxidation of fats, and their activity can be assessed in oil in water emulsions (O/W) as a model system. An emulsion is a dispersion of droplets of one liquid in a second immiscible liquid. It does not form spontaneously and it requires the addition of an emulsifying agent to be stable. The use of emulsions to assess the activity of natural antioxidants is a model for foods such as mayonnaise, where the antioxidant

capacity of samples containing additives can be compared with a control emulsion under controlled storage conditions. Lipid oxidation can be monitored in emulsions. In the first phase of lipid oxidation, lipid free radicals react with oxygen to form hydroperoxides [294].

These products usually are further oxidized to form ketones, aldehydes, alcohols and acids that negatively affect the taste, aroma, nutritional value and overall sensory quality of the product, and also lower the pH. Moreover, it is well known that polyphenols and other natural antioxidants significantly enhance the stability of edible oils, because they have the capacity to donate one hydrogen atom to a free radical and reduce propagation of the radical chain reaction [294].

Secondary oxidation of fats can be followed by testing for TBARS (Thiobarbituric Acid Reactive Substances), which has been widely used to determine the degree of oxidative rancidity of a product during storage [75]. Determination of the level of lipid peroxidation in a system can be quantified by measuring the main product formed by the oxidation of polyunsaturated fatty acids, namely Malondialdehyde (MDA). This can be detected and quantified when it reacts with thiobarbituric acid. Measurement of thiobarbituric acid reactive substances is a good method to determine the ability of different antioxidants to inhibit lipid peroxidation [75].

The main objective of this study was to determine and compare the antiradical capacity of extracts from three aromatic plants: rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris)* and lavender (*Lavandula angustifolia*), with a study of the individual parts (leaf, flower and stem). The antioxidant activity in emulsions was compared in order to demonstrate the ability of extracts from these plants to be used as possible substitutes for synthetic antioxidants in the food industry.

4.1.2.Results and discussion

4.1.2.1. Analysis of Total Polyphenols.

The concentration of total polyphenols in the extracts was determined, and the results are shown in Table 5. The extract from the leaves of thyme had the highest polyphenol content with 334 ± 18.4mg Gallic Acid Equivalent (GAE)/g lyophilized extract, with the extract from thyme flowers and lavender leaves next in polyphenol content with no significant difference between the samples (p= 0.751). Lavender flowers had a lower polyphenol content (52 ± 2.1 mg GAE / g lyophilized extract) than the leaves. The phenolic content of *R. officinalis* was in the range 198 ± 14.5 and 219 ± 6.1 mgGAE/g lyophilized extract. Several authors have reported the polyphenol content of this plant. Dorman et al. [295] reported a similar content in rosemary leaves (185 mg GAE / g extract), when determined following extraction with water.

However, studies involving methanol extraction of rosemary leaves have reported lower values than those obtained in the present study, in the range of 2.6 to 59. 6 mgGAE/g Dry Weight (DW) (relative to the whole plant, not lyophilized), with 80% methanol (MeOH:H_2O) used as extracting

solvent [296]. Extraction with 50 % ethanol (EtOH: H_2O) allows higher recovery of the phenolic compounds [177]. Moreno et al. [59] also found a lower polyphenol content of 120 mgGAE/mL in a methanol extract.

Table 5. Content of total polyphenols, and antioxidant activity assessed by the ORAC, TEAC, FRAP and DPPH assay for the studied extracts (means ± s.d.).[a]

	FOLIN-CIOCALCETAU (mg GAE/g lyophilized extract)	**TEAC** (mM Trolox/g lyophilized extract)	**ORAC** (mM Trolox/g lyophilized extract)	**FRAP** (mM Trolox/g lyophilized extract)	**DPPH** (mM Trolox/g lyophilized extract)
RL	219 ^{e} ± 6.1	0.8 ^{b} ± 0.04	2.9 ^{d} ± 0.10	2.0 ^{e} ± 0.02	1.1 ^{d} ± 0.06
RR	198 ^{d} ± 14.5	1.2 ^{e} ± 0.08	3.2 ^{e} ± 0.12	1.7 ^{d} ± 0.02	1.0 ^{d} ± 0.08
TL	334 ^{g} ± 18.4	1.1 ^{d} ± 0.04	4.6 ^{f} ± 0.17	3.1 ^{g} ± 0.03	1.6 ^{g} ± 0.09
TR	132 ^{b} ± 4.4	0.8 ^{b} ± 0.07	2.0 ^{b} ± 0.06	1.1 ^{b} ± 0.02	0.6 ^{b} ± 0.04
TF	288 ^{f} ± 10.1	1.4 ^{f} ± 0.10	4.4 ^{f} ± 0.17	2.3 ^{f} ± 0.02	1.2 ^{e} ± 0.06
LL	295 ^{f} ± 10.1	1.3 ^{e} ± 0.09	4.6 ^{f} ± 0.22	2.3 ^{f} ± 0.02	1.5 ^{f} ± 0.06
LR	162 ^{c} ± 5.2	0.9 ^{c} ± 0.06	2.6 ^{c} ± 0.07	1.5 ^{c} ± 0.02	0.9 ^{c} ± 0.07
LF	52 ^{a} ± 2.1	0.3 ^{a} ± 0.01	0.8 ^{a} ± 0.03	0.4 ^{a} ± 0.01	0.2 ^{a} ± 0.01

[a] Values with the same superscript letters are not significantly different ($p < 0.05$) (mean for triplícate samples). The abbreviations of the plant extracts are: rosemary leaf (RL), rosemary root (RR), thyme leaf (TL), thyme root (TR), thyme flower (TF), lavender leaf (LL), lavender root (LR), lavender flower (LF).

Thyme is a rich source of polyphenols, especially in its leaves. Gramza-Michalowska et al. [297] reported a value of 229.63 mg GAE/g lyophilized powder after ethanol extraction. This value is lower than those found in the present study, 334 ± 18.4 mg GAE/g lyophilized powder for leaves and 288 ± 10.1 mg GAE/g lyophilized powder for flowers. These results are also higher than those reported for other species such as leaves of *T. argaeus*, which were reported to contain 83.31 ± 0.59 mg GAE/g methanol extract [148].

The ethanolic extract of *L. officinalis* demonstrated the high phenolic content in this plant, particularly in the leaves (295 ± 10.1 mg GAE/g lyophilized extract). The polyphenol content of extracts of this species extracted with other solvents (like 70% acetone) were lower (74 mg/g extract) [298]. Also, Miliuauskas et al. [178] published a value of 5.4 ± 0.2 mg GAE/g extract after extraction with methanolic solvents.

Solvent can have an important effect on polyphenol content and antioxidant activity. Several procedures for extraction have been described proposing different times of extractions, solvents and weight/volume ratios, but organic extracts give higher yields of Rosmarinic Acid (RA) from herbs than extraction with water [299]. Extracts of rosemary in organic solvents have lower DPPH radical scavenging activity than aqueous extracts [300]. Miliauskas et al. [178] found that yields of extract from *L. angustifolia* with different solvents were in the order methanol>acetone>ethyl acetate, but the radical scavenging activity was relatively weak compared with other plant extracts, reflecting the low flavonoid content of the extracts.

Considering the co-presence of polar and nonpolar phenols, it was decided to extract with 50 % aqueous ethanol. Compositions of each extract of RA and Caffeic Acid (CA) are presented in table 6.

Table 6. Amount of RA and CA quantified by HPLC

Extracts	Content of RA ppm (mg/L extract)	Content of CA ppm (mg/L extract)
RL	15.14 ± 19	41.42 ±51
RR	156.61 ± 65	112.40 ± 51
TL	392.21 ± 1	179.65 ± 8
TR	80.16 ± 12	67.00 ± 4
TF	104.20 ± 3	82.27 ± 12
LL	81.04 ± 1	113.61 ± 1
LR	52.14 ± 3	55.99 ± 6
LF	40.97 ± 1	29.08 ± 1

[a]The abbreviations of the plant extracts are: rosemary leaf (RL), rosemary root (RR), thyme leaf (TL), thyme root (TR), thyme flower (TF), lavender leaf (LL), lavender root (LR), lavender flower (LF).

The analysis showed that RA and CA were important phenolic compounds in the herbs. The highest content of RA was found in TL (396.2 mg.kg-^{1}), TF (102.2 mg.kg-^{1}), followed by RR (85.2 mg.kg-^{1}) and LL (81.6mg.kg-^{1}). RA possesses a broad spectrum of biological activities and is known as an antiviral, antibacterial, antioxidant, antinflammatory and immunostimulating agent [301,302]. Among the *Lamiaceae* species investigated by Janicsák et al. [303], thyme was an abundant source of RA, followed by lavender, which is in agreement with our findings.

Like RA, CA is also a common constituent of the plants of the *Lamiaceae* family. CA was identified in extracts of TL (71.6 mg.kg-^{1}), TR (169.2 mg.kg-^{1}) and in LR (114.1 mg.kg-^{1}). These results are in agreement with those of Janicsak et al. [303] who also reported a lower content of CA than RA in the *Lamiaceae* plants.

CA, luteolin, RA and hispidulin were present in a thyme extract [5], whereas CA, kaempferol and myricetin were present in the extract of lavender flowers [304]. Phenolic diterpenes (carnosic acid, carnosol, 12-O-methylcarnosic acid), caffeoyl derivatives (RA) and flavones (isoscutellarein 7-O-glucoside and genkwanin) were identified in rosemary leaves, but the flavones and 12-O-methylcarnosic acid were absent from the stems [305].

4.1.2.2. Antioxidant Activity

The antioxidant capacity of extracts from leaves, stems and flowers of rosemary, thyme and lavender was investigated by the TEAC, ORAC. DPPH and FRAP assays (Table 10).

The antioxidant capacity of the extracts was in the order LL, TL, TF> RR>RL> LR>TR>LF by the ORAC assay; TF>RR,LL>TL>LR>TR,RL>LF in the TEAC assay and TL>TF, LL>RL>RR>LR>TR>LF in the FRAP assay and TL>LL>TF> RL, RR>LR>TR>LF in the DPPH assay. This compares with the total phenol content in the order: TL> TF, LL>RL>RR>LR>TR>LF. For all 4 antioxidant assays, the TF, LL and TL extracts had the highest antioxidant capacity, with minor differences in the order of activity depending on the

assay.This is consistent with the highest polyphenol content being present in these extracts.The LF and TR extracts had the lowest antioxidant capacity, and again this is consistent with their low polyphenol content.The RR, RL, and LR extracts were intermediate in polyphenol content and also in antioxidant capacity with minor differences in the order depending on the assay used. The main differences were in the ratio of antioxidant activity for RL and RR which had values of 0.91, 0.67, 1.18, 1.10 for the ORAC, TEAC, FRAP and DPPH assays, when the Folin-Ciocalteu assay indicated the ratio of polyphenols was 1.08. Thus the FRAP and DPPH values were consistent with the polyphenol content, whereas the ORAC and TEAC assays were more responsive to the polyphenols in RR. Phenolic diterpenes (carnosic acid, carnosol, 12-O-methylcarnosic acid), caffeoyl derivatives (id) and flavones (isoscutellarein 7-O-glucoside and genkwanin) were identified in rosemary leaves, but the flavones and 12-O-methylcarnosic acid were absent from the stems [305]. The presence of *o*-dihydroxy substituents in the aromatic ring of carnosic acid, carnosol and RA would suggest that these were more active antioxidant components than the flavones and 12-O-methylcarnosic acid, and this would be consistent with the increased activity of the stem extract in the ORAC and TEAC assays which depend on radical-scavenging activity. The FRAP assay is a measure of reducing capacity and the DPPH assay is sensitive to steric hindrance, with different antioxidants reacting at different rates, and can give misleading results.

The polyphenol content assessed by the Folin-Ciocalteu assay correlated with antioxidant capacity assessed by the FRAP, TEAC and ORAC assays (Fig. 18). The correlation coefficients were $\mathbf{r^2}$= 0.980 (FRAP), $\mathbf{r^2}$= 0.821 (TEAC), and $\mathbf{r^2}$= 0.983 (ORAC).

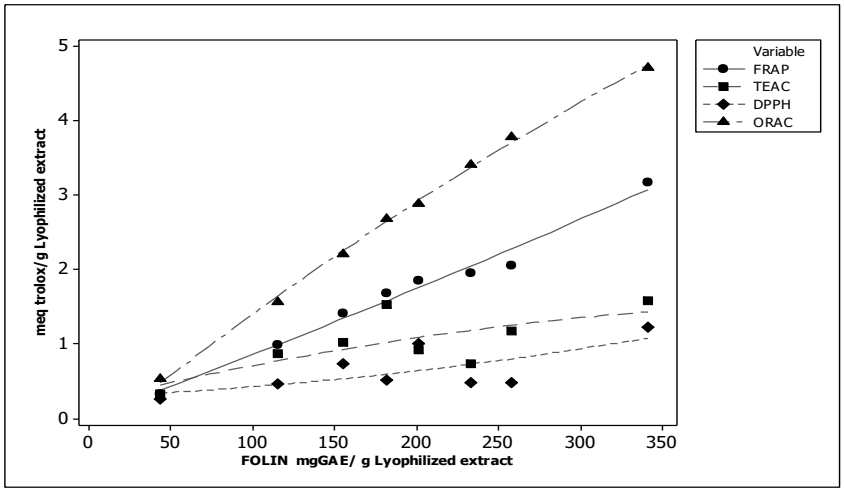

Fig. 18. Plot of the association between the polyphenol content determined by the Folin-Ciocalteu analysis and antioxidant activity assessed by the FRAP, DPPH, TEAC and ORAC assays.

DPPH is a stable nitrogen-centered radical, and many antioxidants do not react rapidly with DPPH because of steric hindrance due to bulky substituents in the chemical structure around the radical, which makes small antioxidant molecules generally show greater activity [306]. The

polyphenol content assessed by the Folin–Ciocalteu assay correlated with antioxidant capacity assessed by the FRAP, TEAC and ORAC assays (Fig. 2). The correlation coefficients were r^2= 0.980 (FRAP), r^2= 0.821 (TEAC), and r^2= 0.983 (ORAC).

In the case of the DPPH method the correlation was not so good (r=0.711). This may be partly due to reaction of the DPPH radical with components other than the polyphenols present in the solution, but steric hindrance probably also contributed.

There is extensive literature on the antioxidant capacity of herb and spice extracts. The values obtained by the TEAC assay for rosemary (leaves and stems) were 0.8 ± 0.04 and 1.2 ± 0.08mmol Trolox Equivalent (TE)/ g lyophilized extract respectively. These values are much higher than the values of 0.274 and 0.324 mmol TE/ g extract, reported by Tawaha et al. [296] for rosemary after extraction with 80 % methanol or hot water.

Tsai et al. [304] reported a TEAC value value of 0.274 mmol/g for an extract from *L. angustifolia* flowers, which is similar to the value of 0.3 mmol TE/ g lyophilized extract found in the current study. There are not many literature reports using the ORAC assay in the plants studied. Zheng and Wang [5] reported a value of 19.49µmol/g fresh weight of thyme. This value is lower that our study where the range is between 30-100 µmol TE / g fresh weight. Brewer [26] reported that the ORAC values for thyme (*T. vulgaris*), both fresh and dry, were 0.27mmol TE/ g fresh weight and 0.15 mmolTE/ g dry weight, respectively.This value for dry thyme (*T. vulgaris*) is much lower than those found in the current study, which has values between 0.3 for the stems and 0.6 mmolTE/ g DW for the leavesThe extracts with the highest percentage inhibition by the DPPH assay were the leaves of rosemary and thyme, with values of 81.7 % and 73.5 % respectively. These extracts contained components that reacted relatively slowly with the DPPH radical. The percentage inhibition during the 75 minutes of the experiment did not reach a limit and components continued to react with the radical after this time. The remainder of the extracts reached stability after about 70 minutes, presenting a faster kinetics.

Babovic et al. [307] reported that the DPPH radical scavenging activity of thyme leaves was greater than that of rosemary leaves, which agrees with this study. Lavender showed a percentage inhibition (%) between 23 to 56 % in the DPPH assay. This compares with the percentage inhibition of 23 % for an ethanol extract [48], which is comparable to the values obtained in this study.

The strong antioxidant power of *T. vulgaris* and *R. officinalis* measured by the FRAP assay was reported in the literature [308,309]. The antioxidant power for extracts of rosemary (*R. officinalis*) nd thyme (*T. vulgaris*), extracted with 80 % v/v methanol was 0.01 mmol TE/g dry extract for both. The recovery of polyphenolic compounds and the antioxidant capacity for extracts of the plants in our study in which 50 % aqueous ethanol was used for extraction were higher.

4.1.2.3. Antioxidant Effects in Stored Emulsions

In the study the antioxidant activity of rosemary, lavender and thyme extracts was analyzed in oil-in-water emulsions, as a Model Food System (O/W). Each part of the plants was studied separately (leaves, flowers and stems). The oxidation was followed by assessment of the primary oxidation products (Peroxide Value, PV) and the secondary oxidation products (TBARS value). In addition the change in pH was monitored, since pH tends to fall during oxidation.

4.1.2.3.1. Evolution of Peroxide Value

Fig. 19 shows the evolution of PV *versus* time. The control (without extract added) was oxidized first, and the second sample oxidized was the positive control (Trolox 250 ppm). The sample containing Trolox, and the samples containing extracts were not significantly oxidised during the first 7-9 days. After this first period, the sample containing Trolox was oxidised more rapidly than the samples containing extracts. The time required for the emulsions to reach a peroxide value of 10 meq hydroperoxides / kg of emulsion was determined as a measure of stability. This value was taken since the limit for products of edible fats (animal, plant and anhydrous), margarine, and fat preparations, to guarantee quality is <10 meq hydroperoxides/kg. The first sample to reach 10 meq hydroperoxides / kg of emulsion was the control sample, and this occurred rapidly (2 days). The next samples to reach this level of deterioration were the positive control (Trolox) and LR. Other samples followed the order of stability: LF ≈ LL ≈ TR < TF < RR ≈ TL ≈ RL.

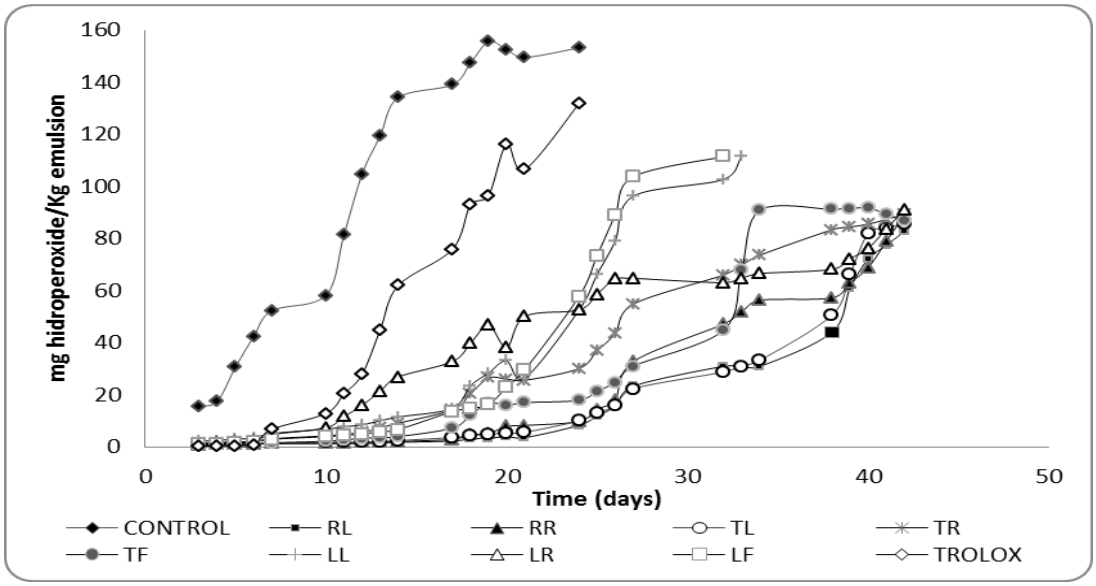

Fig.19. Changes in peroxide value of emulsions during storage for 42 days.

It is interesting to compare this order of stability with the results of the antioxidant assays where TF, LL and TL were most active followed by RR, RL, LR and then LF and TR were least active. There are clear differences in the activity in the homogeneous antioxidant assays and in the emulsion. This can be explained by the phenomenon known as the polar paradox, where antioxidants with less polar characteristics are more effective in an emulsion than in

homogeneous solution [310]. The reduced activity of TF and LL in the emulsion is consistent with the presence of more polar antioxidants in these extracts. Blazekovic et al., [311] reported that RA was a major polyphenol in an ethanolic extract of *L*. *angustifolia* leaves. It has previously been reported that RA is more active than carnosol and carnosic acid in oil but is a less effective antioxidant in an emulsion because of its relatively high water solubility [312]. The antioxidant activity of rosemary extracts is associated with the presence of various phenolic diterpenes, such as carnosic acid, carnosol, rosmanol, and rosmaric diphenol rosmariquinone. Del Bano et al. [305] and Frankel et al. [312] reported that the carnosol and carnosic acid content was higher than the RA conent in rosemary leaves. Carnosic acid has been described as a lipophilic antioxidant that scavenges hydroxyl radicals and peroxyl radicals, preventing lipid peroxidation [313]. Hence, the carnosol, and carnosic acid in RR and RL help the extracts to retain their antioxidant properties in the emulsion whereas the TF, which relies more on its RA content for its antioxidant effectiveness in the homogeneous antioxidant assays, becomes less effective in the emulsion. Ferulic and GA were also reported to be major antioxidants in extracts from the flowers of the related species *T.capitata* and these phenolic acids are known to be polar, and hence if these were also present in thyme (T.*vulgaris*), they would be less effective in an emulsion [314].

4.1.2.3.2. Evolution of pH over time.

The pH of the samples was measured as a parameter to investigate its correlation with PV, since some hydroperoxide decomposition products are acidic (Fig. 20). The pH change was inversely proportional to the PV. Many antioxidants have the disadvantage of being less effective as antioxidants when the pH is low. A significant effect of pH on the antioxidant activity of carnosic acid and carnosol (main components of rosemary) was reported with a high antioxidant activity at pH 4 to 5 [312]. Lipid oxidation in emulsions is slower at higher pH and the speed is accelerated as pH decreases [312].

Following the order of primary oxidation, the pH experienced a decline in accordance with oxidation. All samples started with a near neutral pH. The negative control (without extract) and positive control (Trolox) were the first to change. The control sample had a marked decrease in pH from day 6 and the Trolox from day 14. The lavender extract was oxidized more rapidly with a fall in pH detectable from 20 days and the pH decreased in the order LR> LL> LF. . Parts of thyme (leaves and stems) had a similar behavior to the other extracts until time=20 days, when the pH decreased for TR, and day 27 when it decreased for TF. The stability of samples containing RR, RL and TL was similar. There was a reduction between 33 to 34 days, but at this time the pH of the RL was higher than that of the others.

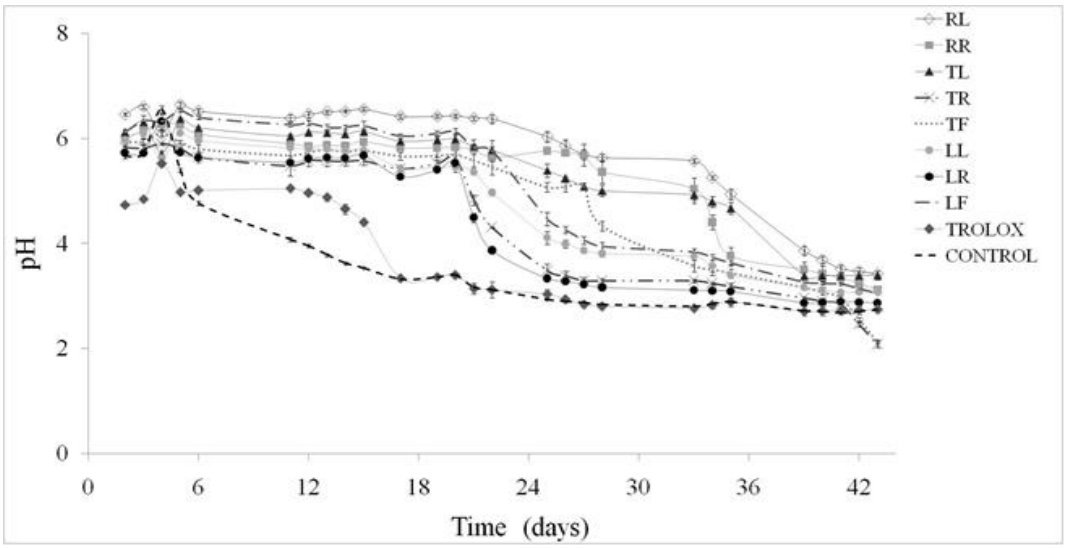

Fig. 20. pH evolution during 42 days of study.

4.1.2.3.3. Evolution of TBARS.

Hydroperoxides decompose to form secondary oxidation products, which are responsible for the flavor, the rancid odor and undesirable taste of oxidised fats [315]. Secondary oxidation products were monitored by measurement of the TBARS (Fig. 21). According to analysis after 5 weeks, TBARs values of emulsions containing added herb extracts were lower than that of the control (4.35 mg MDA/kg) and the Trolox (4.23 mg MDA/kg). The TBARS value increased from 3 weeks. RL (1.79 mgMDA/kg) and TL (2.50 mg MDA/kg) were the most effective antioxidants, followed by TF (2.50 mg MDA/kg) and RR (2.59 mg MDA/kg). The highest TBARS value for the herbs, were for LR, TR, LL and LF. It can be concluded that RL and TL had the best antioxidant effect based on findings from the PV and TBARS measurements.

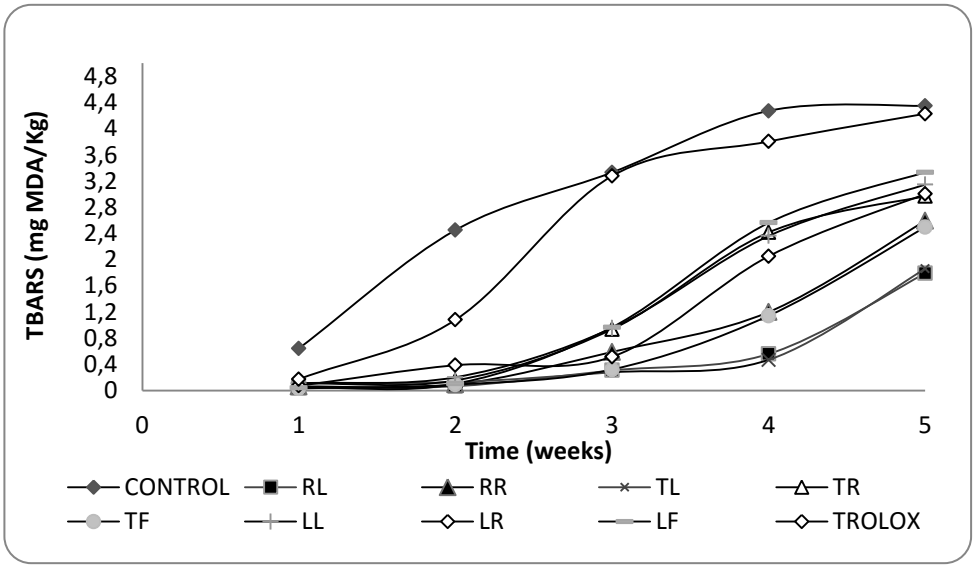

Fig. 21. Changes in TBARS values during the study.

4.1.3.Conclusions.

This study proved the antioxidant activity of extracts from the aromatic plants. The results obtained showed that there are significant differences between different parts of the plants studied. The highest content of total polyphenols and antioxidant capacity assessed by homogeneous antioxidant assays was in the extracts from thyme flowers, lavender leaves and thyme leaves, followed by the stems and leaves of rosemary. The sample with the lowest concentration of polyphenols and lowest antiradical capacity was the lavender flower extract.

Rosemary leaves and thyme leaves were most effective at protecting the emulsion against oxidation followed by the rosemary stems and thyme flowers. No significant differences between the leaves of thyme and rosemary were detected.

This research has demonstrated the antioxidant properties of the plant tissues of these species, and they may represent an alternative to synthetic antioxidants in preservation of food, as well as in the pharmaceutical industry and cosmetics.

4.2. Antioxidant activity of polylactic acid (PLA) film prepared with rosemary and thyme in oil-in-water emulsions

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Artículo enviado a European Food Research and Technology en 2016

4.2.1. Introduction

Active packaging is a highly relevant innovative proposal applied that could increase the consumer satisfaction. It can be defined as a system in which the product, the package and the environment interact in a positive way to extend the shelf life of the product or to achieve some characteristics that cannot be obtained otherwise [316].

Previous studies by Boonnattakorn et al. [317] with ethylene vinyl acetate matrix with incorporation of Mangiferin (potent natural antioxidant) show that matrices with varying defree of vinyl acetate could potentially be used to control magniferin release rate for packaging with antioxidant properties. It is important to emphasize that natural products with radical scavenging activity used in the preparation of the film continues to maintain its antioxidants activity [318]. Some authors have found that films can have chelating activity of Fe (II) in oil in water emulsions and open a wide field of work and study to design films that protect such emulsions [319].

Polylactic Acid (PLA) is one of the few commercially available bio-based and biodegradable thermoplastic packaging materials. The use of PLA as a food-packaging material is gaining interest due to environmental reasons and higher demands on sustainability of materials and products. PLA can offer a sustainable alternative for food packaging across a wide range of commodity applications in response to consumers' demands and market trends in the use of renewable resources [319].

It has been shown that PLA protects the products sufficiently against quality changes, which has allowed its application in other fields, such as, including biomedical applications, such as, drug delivery systems and tissue engineering due to its biocompatibility, degradability and significant characteristics.

Current innovations in food packaging research include the development of active packaging systems based on materials, which can involve a variety of additives such as antioxidants, anti-microbials, vitamins, flavours and colourants with the aim of improving the appearance, mainly, and, also to extend the foodstuff shelf-life. The increasing demand for natural additives has resulted in studies based on natural active compounds, such as plant extracts or essential oils, which are categorized as "Generally Recognised as Safe" (GRAS) by the US Food and Drug Administration as well as the current European Legislation for materials intended to be in contact with food (EU N10/ 2011 Regulation) [320].

The phenolic compounds in herbs act as antioxidants due to their redox properties, allowing them to act as reducing agents, hydrogen donors, free radical quenchers and metal chelators. Crude extracts of fruits, herbs, cereals and other plant materials, rich in phenolics, are of great interest for the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. In this sense, aromatic herbs such as rosemary (*Rosmarinus officinalis*) and thyme (*Thymus vulgaris*) have been widely studied because of their potent antioxidant activity [107].

However, few works have reported the combination of natural antioxidants with biopolymer for use in active food packaging applications. The use of these materials could be a promising alternative to extend the shelf-life of foodstuff inside the bioplastic packaging. Therefore, the aim of this study is to evaluate the influence of PLA films that contain either rosemary or thyme extracts in the oxidative stability of Oil-in-Water (O/W) emulsions stored with these films protection, as well as the characterization of the prepared films for their basic properties and migration behavior.

4.2.2.Results and discussion

4.2.2.1. Determination of Peroxide Value (PV).

The most influential deteriorative reaction that could shorten the shelf life of oil in general is lipid degradation. In this study, the effectiveness of the fabricated films to facilitate the oxidative stability of soybean oil was investigated by the determination of Peroxide Value (PV). PV is one of the most commonly used techniques for determining the degree of lipid degradation and it is determined by measuring the formation of hydroperoxides, the primary product of lipid oxidation.

Oxidative stability of the emulsions O/W in contact with PLA films with rosemary and thyme, dry and lyophilized, was monitored during 35 days of storage (Fig. 22). The behavior of the samples is clearly differentiated into two groups. A first group where samples CTR, pure PLA, Thyme Dry (TD) and Rosemary Dry (RD) films show an increase in the oxidation from the first days, and a second group formed by Rosemary Lyophilized extract (RLE) and Thyme Lyophilized extract (TLE) film stabilized against oxidation especially during the initial period of 18 days. At this period, the TLE film experience a rise considerable compared to RLE film.

The control sample and pure PLA reached a maximum in hydroperoxide production after 13 days (50.54 and 48.34 meq hydroperoxides/kg emulsion, respectively), which indicates that the samples began to form secondary oxidation products. Samples with RD and TD films also experienced relatively rapid oxidation of the emulsion (low induction time) showing similar behavior to pure PLA. Sample with RLE PLA had best protective effect against formation of primary oxidation products relatively closely followed by TLE PLA. The emulsions with RLE PLA maintained the stability of the emulsion until the 22 day (6.20 meq hydroperoxides/kg emulsion).

As determined by the Codex Alimentarius, the maximum acceptable level of PV for refined vegetable oils is 10 miliequivalent O_2/kg oil [321].

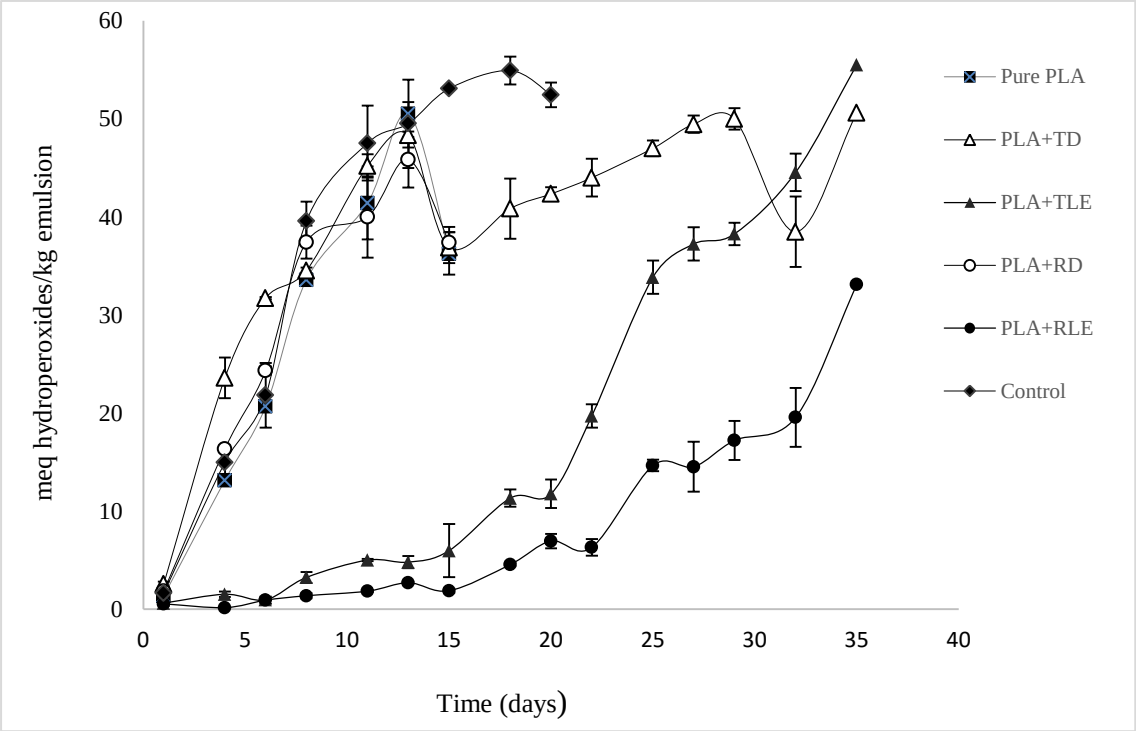

Fig. 22. Changes in peroxide value of emulsions during storage at 33±1°C for 30 days. Bars represent standard deviation (*n*=3).

Compared to other studies with natural plants, Samsudin et al. [322] studied PLA incorporated with 2 wt. % marigold flower extract gave compliance with the Codex Alimentarius having a shelf life of 5 days, which is well below our study of PLA with RLE, which gave a shelf life of 22 days. Likewise in other study with marigold flower Colín-Chavez et al. [323] investigated soybean oil packaged in pouches made of polyethylene-bases films coaining marigold flower extract, showing a protection of soybean oil for 4 days (exceeds 10 miliequivalent O_2/kg oil), less effective than rosemary.

RLE has great antioxidant power, in previous studies with these plants in emulsions Gallego et al. [324] tested the power of oxidation inhibition of these plants incorporated in emulsions O/W, showing that *R. officinalis* leaves were the best of 3 plants studied (rosemary, thyme and lavender) at protecting the emulsion from oxidation. The protective effect of film with plant extracts on lipid oxidation in oil-in-water system is a consequence of the presence of active phenolic compounds in the extracts. Rosemary extract is associated with the presence of various phenolic diterpenes, such as carnosic acid, carnosol, rosmanol, and rosmaric diphenol rosmariquinone. It is thought that carnosic acid is a lipophilic antioxidant that scavenges hydroxyl radicals and peroxyl radicals, preventing lipid peroxidation [313].

The lyophilized ethanolic extract of rosemary contain approximately between 10 to 20% of antioxidant compounds. Even compounds such as diterpenes of rosemary have been compared

with common antioxidants such as α-tocopherol, demonstrating a markedly greater antioxidant activity [325].

4.2.2.2. Evolution of pH of emulsions over time

The pH of the samples was measured as a parameter to investigate its correlation with PV, since some hydroperoxide decomposition products are acidic, showed in Fig. 23. The pH decreases as the PV increased. Many antioxidants have the disadvantage of being less effective as antioxidants when the pH is low. A significant effect of pH on the antioxidant activity of carnosic acid and carnosol (main components of rosemary) was reported with a high antioxidant activity at pH 4–5. Lipid oxidation in emulsions is slower at higher pH and the speed is accelerated as pH decreases [326].

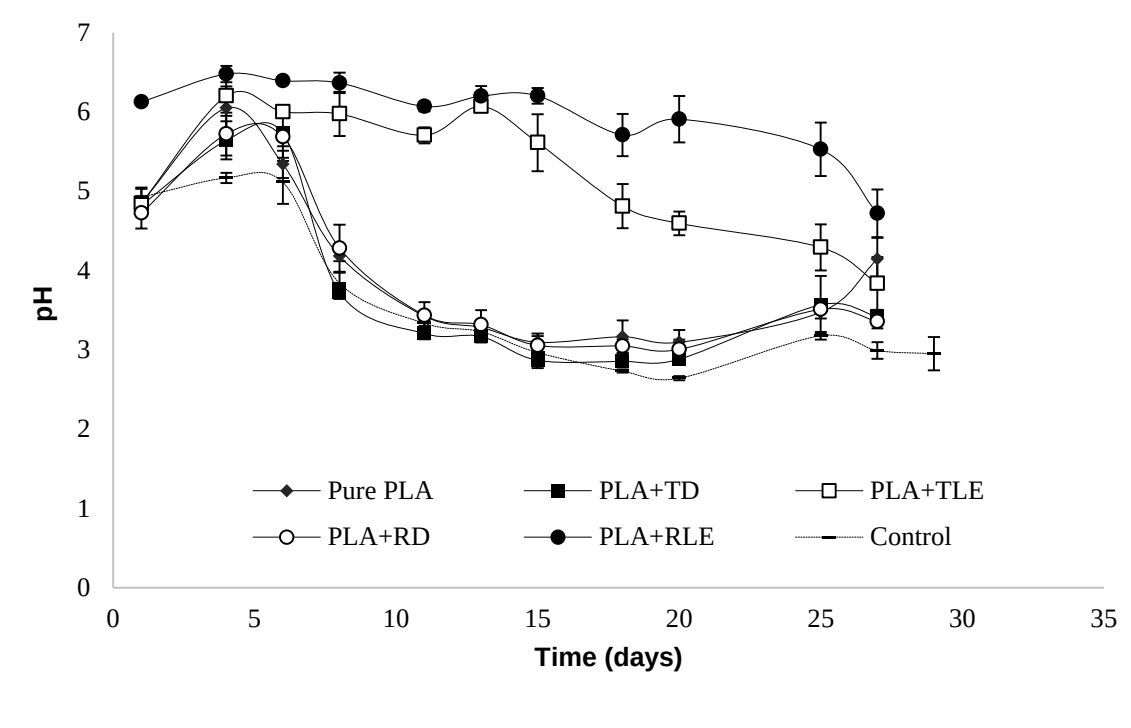

Fig.23. pH evolution during 30 days of study. Bars represent standard deviation (*n*=3).

Following the order of primary oxidation, the pH experienced a decline in accordance with oxidation. The negative control (without extract) and with pure PLA film had a marked decrease in pH from day 8. The emulsions with PLA films containing RD and TD behaved similar to the control. The emulsions with PLA films containing RLE and TLE exhibited more stable pH with a fall detectable first from 18 days and 27 days, respectively. The pH values were most stable for the emulsion in contact with films containing RLE.

4.2.2.3. Determination of secondary oxidation by TBARS.

Peroxides can form alcohols, aldehydes and acids, or dehydration reactions may occur, that form ketones, while peroxyl radicals can lead to the formation of dimers, trimers, epoxides,

ethers and these compounds are indicator compounds for oxidised fat. This secondary oxidation has a major impact from the sensory point of view and these compounds are responsible for the flavour, the rancid odor and undesirable taste of oxidised fats [315].

The TBARS method was conducted from the first week after preparation of the emulsion, where the hydroperoxides could have reacted to form Malondialdehyde (MDA), and end products of lipid peroxidation. The evaluation was conducted weekly for four weeks (Fig. 24). The emulsion with the control and pure PLA showed the highest production of secondary products (9.59 meq malondialdehyde/kg emulsion and 11.75 meq MDA/kg emulsion, respectively), this behaviour could be ascribed to a faster hydroperoxides decomposition with the formation of aldehydes and other products, leading to unpleasant off-flavours. The emulsions with PLA films containing RD and TD exhibited similar TBARS values as PLA and control. However, films containing TLE and RLE had significantly lower values, 2.54 meq malondialdehyde/kg emulsion and 4.07 meq malondialdehyde/kg emulsion, respectively, after four weeks. By taking into consideration the concentration of secondary oxidation products over time the following ranking could be determined: TD > RD > RLE> TLE. PLA with TLE, thus, showed the best effect in O/W emulsions.

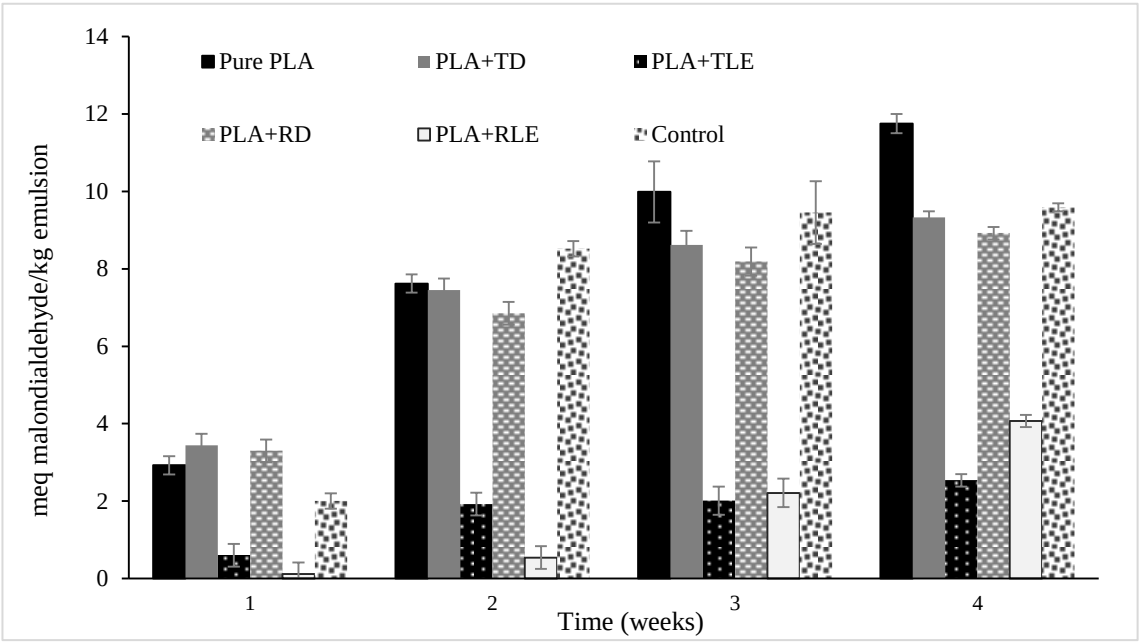

Fig. 24. Changes in TBARS values during the study. Bars represent standard deviation (*n*=3).

4.2.2.4. Migration testing-mass loss

Migration studies are commonly conducted with food simulants, providing uniform contact of the packaging with the food. Migration of antioxidants is of interest because these compounds and their degradation products can migrate from plastics into food during processing and storage [327] and become indirect food additives. In the case of active packaging this could be a desired phenomenon. Table 7 includes mass loss of the films in contact with two food

simulants, water and 95 % ethanol. The pure PLA films contained small amounts of cyclic oligomers [288]. These cyclic oligomers are soluble in ethanol, but not in water. This migration of cyclic oligomers into ethanol is the probable reason for the generally higher mass loss observed in ethanol as compared to mass loss in water and is in accordance with study of Bor et al. [328], where a higher mass loss was observed during storage at room temperature in ethanol as compared to storage in water for 7 days. The different PLA films in contact with ethanol show variation behaviour. The mass loss of PLA with TD is the same that the pure PLA, while the mass loss of PLA with RD, TLE and RLE is somewhat higher, indicating some migration of plant components. In general, the PLA films show lower water absorption due to the hydrophilic nature of the plant material, presenting larger holes (SEM image). Here, the water fills these void spaces and results in a wet mass, much larger than that of PLA film subjected to ethanol (have smaller holes).

Table 7. Food simulant uptake and mass loss of the films from ethanol migration

Samples	Food simulant uptake (%) in water	Food simulant uptake (%)in EtOH 95%	Mass loss (%) in EtOH 95%	Mass loss (%) in water
PLA	3.80 ± 0.25	6.30 ± 0.57	9.5 ± 0.30	7.0 ± 0.46
TD	17.20 ± 3.72	8.20 ± 0.65	9.5 ± 0.37	8.1 ± 0.15
TLE	25.10 ± 2.00	11.20 ± 0.25	11.0 ± 0.15	9.2 ± 0.08
RD	11.70 ± 1.03	8.60 ± 0.60	10.3 ± 0.23	8.3 ± 0.62
RLE	27.70 ± 0.10	11.50 ± 0.41	10.7 ± 0.21	8.3 ± 0.17
BHT	3.70 ± 0.23	8.80 ± 0.16	11.6 ± 0.32	8.4 ± 0.20
CA	3.70 ± 0.36	10.00 ± 0.29	13.1 ± 0.10	8.6 ± 0.35
EGCG	6.60 ± 0.15	10.20 ± 0.57	11.8 ± 0.13	9.5 ± 0.35
RA	5.30 ± 0.80	11.30 ± 0.60	12.0 ± 0.20	8.8 ± 0.18

[a] The abbreviations for the samples used are: film only with Polylactic Acid (PLA), film with Dry Thyme (TD), film with Thyme Extract Lyophilized (TLE), film with Rosemary Dry (RD), film with Rosemary Extract Lyophilized (RLE), film with Butyl Hydroxy Toluene (BHT), film with Caffeic Acid (CA), film with Epigallocatechin Gallate (EGCG) and film with Rosmarinic Acid (RA).

From PLA films with plants added subjected at water food simulant. PLA films with TLE had the biger mass loss in water, which correlates well with the best antioxidant effect of these films in contact with O/W emulsions. The mass loss of pure PLA is explained by hydrolysis of PLA or the cyclic oligomers and migration of the resulting linear oligomers [116]. The films with lyophilized extracts exhibited a higher mass loss compared to the film with dry plants after water migration which indicates that the lyophilized extracts contain more water soluble compounds. The results suggest that the release kinetics depend on the affinity between the active agents and the food simulants, similar to mentioned by Muriel-Galet et al. [329].

Of the films with the standards, the BHT film had relatively low mass loss after migration in water which is explained by the insolubility of BHT in water. The solubility of BHT is however high in ethanol. Similar behavior was also reported by other studies with PLA-based functional

film regarding the diffusivity of antioxidants like a tocopherol, resveratrol, Catechin (C), Epicatechin (EC), and BHT into ethanol-based simulants [330–332].

The BHT in PLLA is according to Iñiguez-Franco et al. [330] expected to have a higher diffusion rate in 95% ethanol compared to C and EC in Poly-L-Lactic Acid (PLLA), which could be explained by the number of hydroxyl groups interacting with the polymer: C and EC have five compared to only one in BHT. C and EC did not migrate to water in detectable amounts in the above mentioned study. The ethanol goes in between the PLLA chains and can therefore reduce the interaction of C/EC and PLLA resulting in migration of the AOs. Ethanol as food simulant for PLLA is therefore questioned concerning whether if it is correctly simulating the food packaging interaction [330].

4.2.2.5. LDI-ToF-MS analysis of low molecular weight compound profiles

LDI-ToF-MS analysis (Fig. 25) was performed to show the general profile of low molecular weight compounds in the original dry and lyophilized plants. Comparison of the spectra of the low molecular weight compounds in the dry plants and in the lyophilized extracts shows that the lyophilized extracts contain larger fraction of molecules with molecular weight < 300 g/mol as compared to the dry plants. This could be expected based on the extraction process for preparation of lyophilized samples and it is likely connected to the higher antioxidant effect observed for the films with lyophilized plants. In addition, more interestingly the obtained spectra clearly illustrate that TLE contains the largest fraction of compounds with lowest molecular weight followed by RLE and then very similar products profiles are observed for the whole plants TD and RD, which closely correlates with the antioxidant effect of the corresponding films.

4.2.2.6. Thermo-oxidative stability of the PLA films

The thermo-oxidative stability of the different PLA films was studied by Thermogravimetric Analysis (TGA) (Fig. 26 and 27). Noticeable differences were observed for the initial degradation temperature (T_{ini}) and maximum degradation temperature (T_{max}) values depending on the additive. These results show that the addition of plants could also significantly improve the thermo-oxidative stability of the PLA films.

Onset of degradation temperature for the pure PLA film was about 313.4 °C. The addition of herbs, inclusive RD, TD, RLE and TLE as well as the model natural antioxidant Rosmarinic Acid (RA), increased the onset of degradation in all cases. However, the highest improvement was observed after addition of TLE and RA. These films exhibited the highest onset temperatures 334 °C and 332 ºC, respectively, i.e. around 20 °C improves compared to pure PLA films. Interestingly the traditional antioxidant, BHT did not have any stabilizing effect on the films. At the beginning of the TGA analysis, there was also a mass loss of several percentage This is attributed to the evaporation of water because of the hydroscopic nature of PLA and other low

molecular weight compounds like cyclic lactide oligomers, acetaldehyde and carbon monoxide [333].

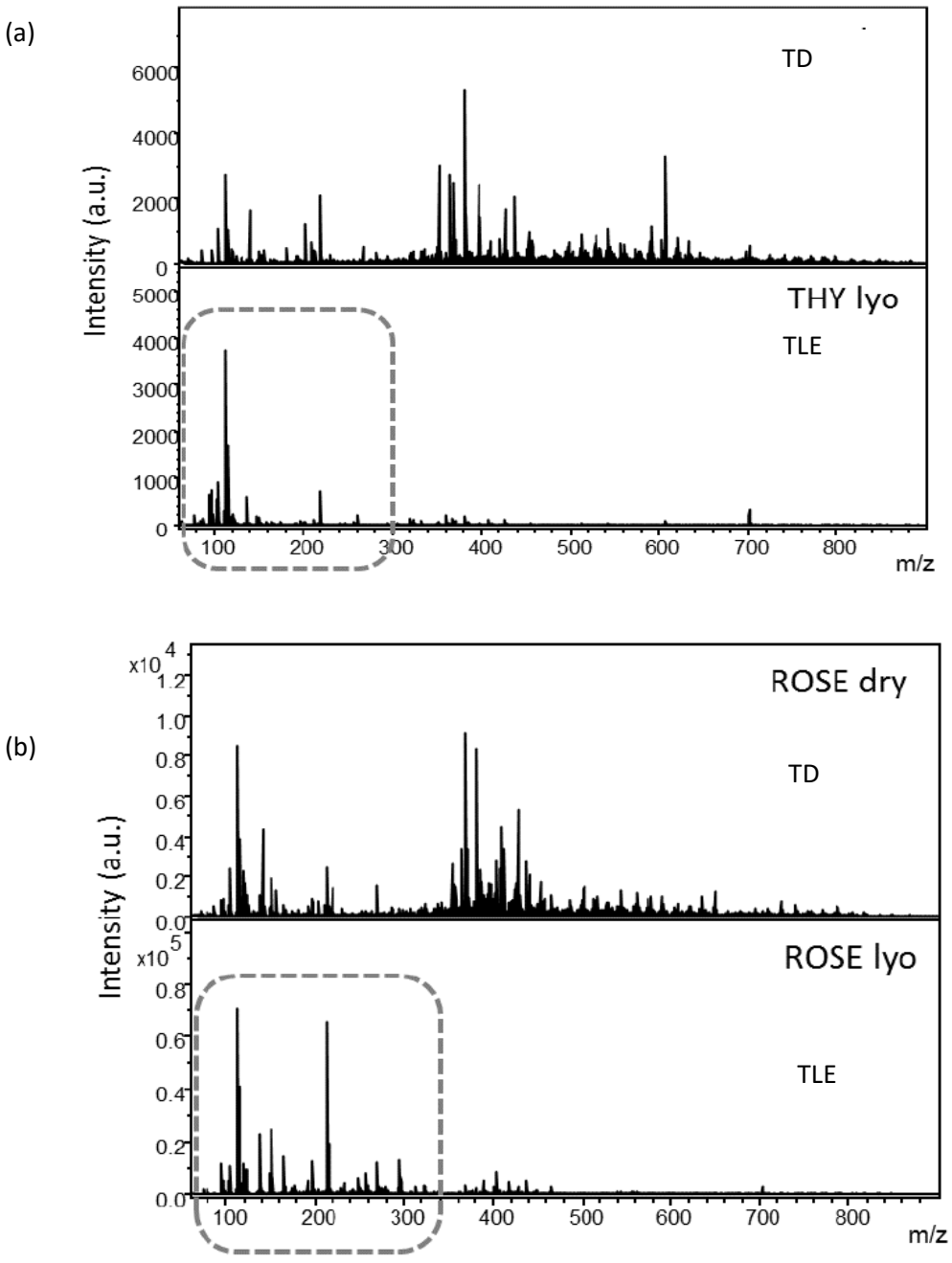

Fig. 25. Plant material before migration, comparison between TD and TLE (a) and between RD and RLE (b).

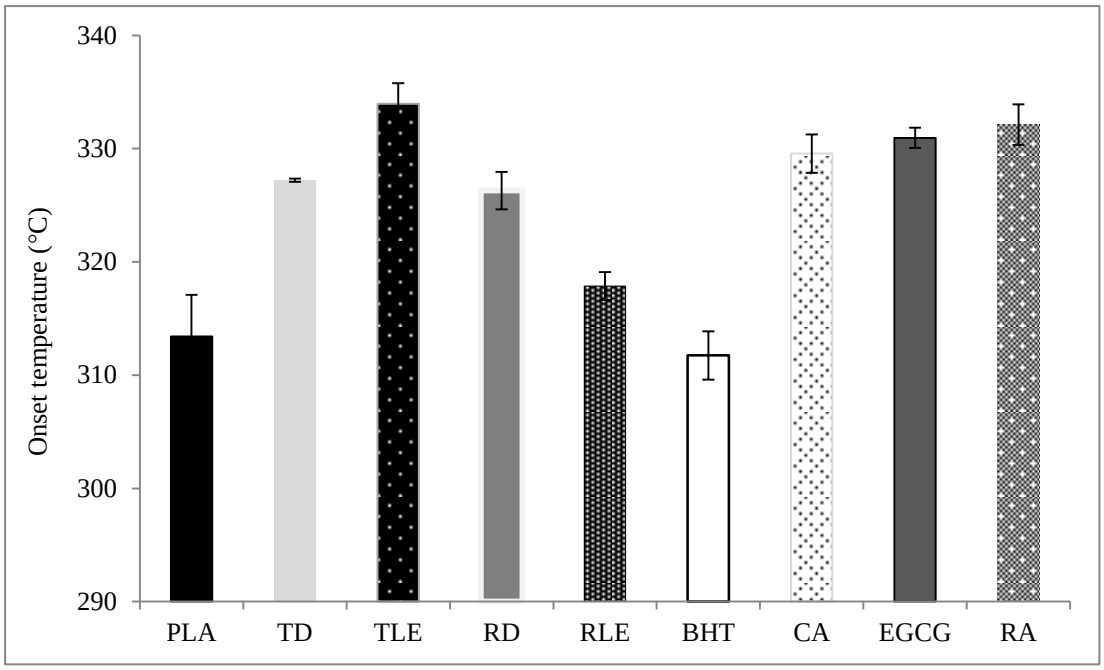

Fig. 26. Onset of degradation temperature for thermo-oxidation. Bars represent standard deviation (*n*=3).

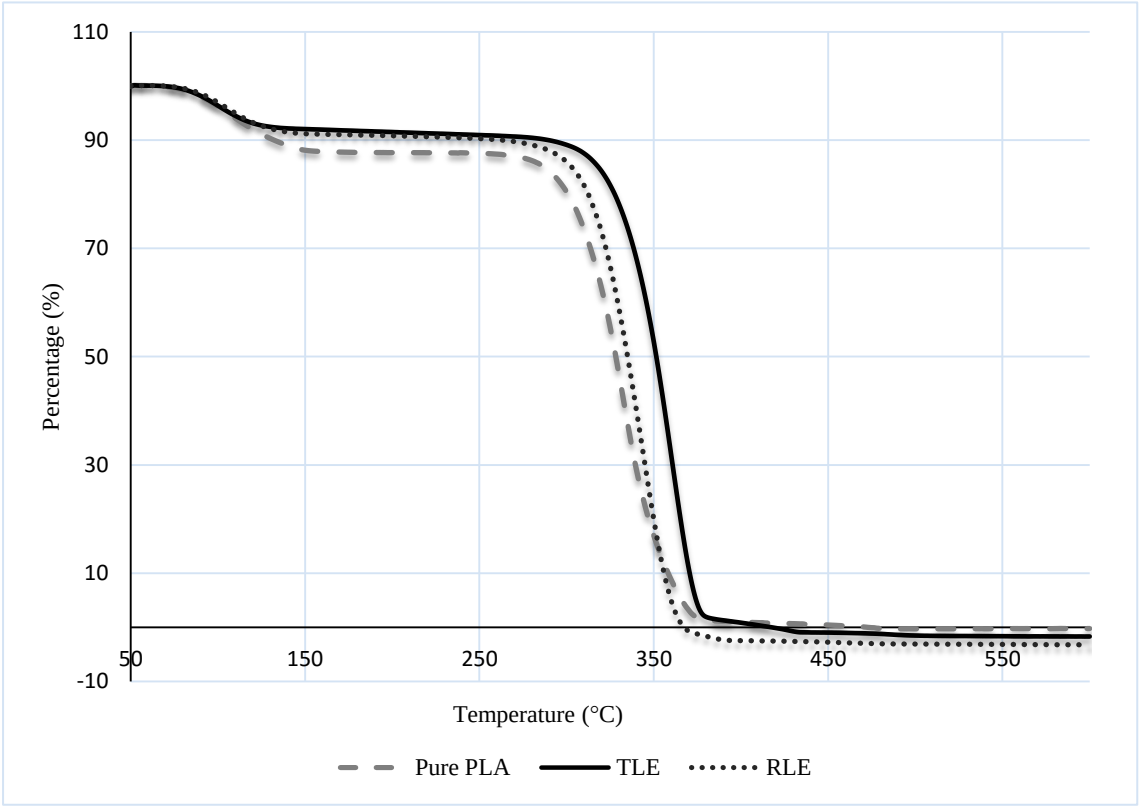

Fig. 27. TGA curves for the plants TLE and RLE.

Because of the sensitive nature of PLA, additives to prevent degradation during processing at high temperatures are of great interest, especially natural ones, like plants and plant extracts. Both, high temperatures and hydrolysis, are factors that threaten ester bonds. The main mechanism of thermal degradation is by random scissions of the main-chain [321].

The polyphenols in the plants could protect the PLA from radicals that would otherwise degrade it. Although the degradation is mainly caused by nonradical processes, protecting effects on polymers have been observed in previous studies by adding free radical inhibitors, which slowed down the degradation rate [334]. Thereby, studies confirm that the addition of antioxidants compounds in the development of active packaging not significantly modify the thermal stability [335].

4.2.2.7. Thermal properties and crystallinity of the PLA films

The glass transition temperatures (Tg), as determined by DSC for pure PLA and PLA films containing rosemary and thyme are depicted in Fig. 28. Tg for the different films were quite similar. Pure PLA exhibited a Tg around 59.3 ºC. The Tg was reduced with the addition of TD and RD and additionally reduced after addition of lyophilized plants TLE and RLE and thyme.

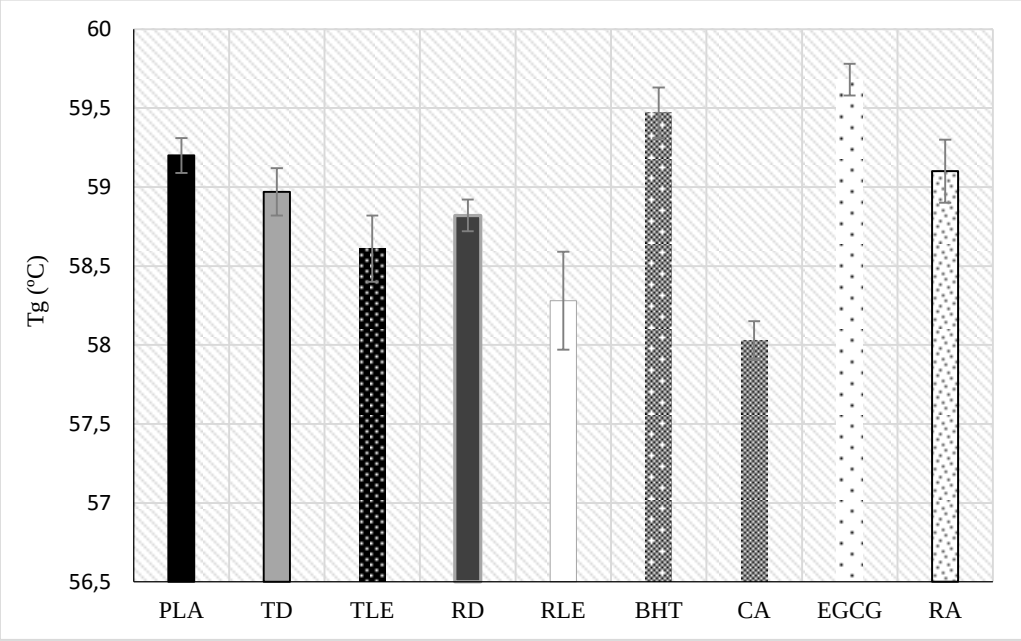

Fig. 28. Glass transition temperature. Bars represent standard deviation (*n*=3).

The results show that the crystallinity of the films was also influenced by the plants or antioxidant added. It decreased ($p < 0.05$) with the addition of TD, TLE, RD and BHT but increased ($p < 0.05$) with the addition of RLE. The maximum degree of crystallinity (13 %) was found for the films containing RLE and the films with TD have the lowest crystallinity (4.7 %).

Gonçalves et al. [336] obtained a similar result where the degree of crystallinity of PLA film decreased after the addition of BHT. In another study with different antioxidants Sawalha et al. [337] reported that the addition of eugenol increased the PLA film crystallinity, while limonene decreased it, due to the different interactions with PLA. They state that the high crystallinity obtained with eugenol can be explained by the film formation process: the eugenol remained inside the film after evaporation of the solvent, and remains there for some time (while slowly diffusing outwards). The high mobility in the film, due to the high swelling, gives ample time for

crystallization to take place. Thus, the final film will have higher crystallinity than those films in which the chain mobility was reduced more quickly.

Reduction of crystallinity is a negative point with respect to the mechanical and barrier properties and positive point for faster degradation of PLA in nature or in biomedical applications. In many applications, increasing the crystallization amount of PLA is desired because in its amorphous form, the range of application of PLA is severely limited. Thus for these cases the crystalline form is required to increase the high temperature stability of PLA [290].

4.2.3.Conclusion

A higher degree of lipid oxidation was noted in emulsions in contact with pure PLA compared to the emulsions in contact with RLE and TLE. These plants were successful in decreasing the lipid oxidation of the emulsions. In the migration assay the mass loss in ethanol was more extensive than the mass loss in water. However, the migration of plant materials and/or antioxidants was faster in contact with water as predicted from the difference in mass loss for pure PLA and PLA plant films. LDI-MS of the dried and lyophilized plants showed that TLE had the highest content of low molecular weight compounds followed by RLE, which correlated well with the determined antioxidant effects. In addition to decreasing the lipid oxidation the plants could improve the thermo-oxidative stability of PLA. Even here TLE was the most effective antioxidant and the addition of TLE could increase the onset of degradation temperature from 313 °C for pure PLA to 334 °C, i.e. by 20 °C. Plant material can therefore be used as an additive for thermo-oxidative stabilization of the PLA. Based on this these plants TLE and RLE, could be proposed as ecofriendly and biocompatible additives for development of sustainable active PLA packaging.

Estudios con Plantas de la Familia Fabaceae

(*C. decapetala* y *C. spinosa*)

- Oxidative stability of O/W emulsions with Caesalpinia decapetala antioxidant capacity by EPR.
- Analytical characterization of polyphenols from *Tara* and Caesalpinia decapetala as tabilizers of O/W emulsions.
- Caesalpinia decapetala extracts as inhibidors of lípid oxidation in beef patties.
- Gelatine-based antioxidant packaging containing Caesalpinia decapetala and Tara as a coating for grodund beef patties.

4.3. Oxidative stabiliy of O/W emulsions with *Caesalpinia decapetala*- antioxidant capacity by EPR

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Publicación aceptada en Pharmaceutical Biology en septiembre de 2016

4.3.1. Introduction

Oil-in-Water (O/W) emulsions form the basis for many food products, such as milk, beverages, sauces and dressings. Lipid oxidation in O/W emulsions is believed to be more likely to occur due to the higher interfacial area of emulsions, where it has been suggested the oxidative reaction is initiated [338].

The incorporation of antioxidants, which are molecules capable of preventing and/or delaying oxidative lipid damage when used in proper conditions, represents a key approach to overcome the quality deterioration of lipid-based foods products, which occurs mainly due to the attack of Reactive Oxygen Species (ROS) [339].

Depending on various factors, such as the physico-chemical characteristics of the medium where they are located and their interactions with other compounds, antioxidants can act as retarders, when they counteract lipid oxidation by protecting target lipids from oxidation initiators; or by hindering the propagation phase, which is a property of the so-called ‘‘chain-breaking’’ antioxidants. Amongst the molecules considered as antioxidants, phenolic compounds are particularly important because of their high redox potentials, and also, because they are the most abundant antioxidants found in the diet [339]. Therefore, various phenolic antioxidants are usually incorporated into O/W emulsions to improve their oxidative stability. Synthetic antioxidants such as Butyl Hydroxy Anisole (BHA), Butyl Hydroxy Toluene (BHT), and Tert Butyl Hydroquinone (TBHQ) are very effective in retarding lipid oxidation [340].

Recently, there has been growing interest in the use of natural polyphenols to retard lipid oxidation due to their possible health-promoting properties and remarkable antioxidant activity [299,48]. Natural additives extracted from nuts, fruits, and herbs, and spices have been studied [341,109].

Caesalpinia decapetala (Fabaceae) (CD) has been used since ancient times in traditional Oriental medicine. It possesses antipyretic, anti-inflammatory, laxative, tonic, carminative, anticancer, antioxidant, and several others activities [181]. Previous chemical investigations on CD had revealed that the main chemical components were terpenoids and flavonoids. Many chemical compounds that occur in this plant have antitumor activities. This plant is a rich source of tannins. The leaves of CD contain cassane diterpenoid, caesaldecan, spathulenol, 4,5-

epoxy-8(14)-caryophyllene, squalene, lupeol, resveratrol, quercetin, astragalin and stigmasterol [186].

The functional properties of CD have been known and studied in various fields. However, there are no studies about the effects of addition of an extract of CD into a food system. This plant may be useful in the development of new antioxidant strategies for retaining the quality of O/W emulsions. Therefore, the aim of this study was to evaluate the antioxidant capacity and to test the oxidative stability of O/W emulsions containing ethanolic extracts of CD.

4.3.2.Results and Discussion

4.3.2.1. Total Phenolic and Total Flavonoid Content

Phenolic compounds with various structures and molecular weights are formed as secondary metabolites in plants and contribute to the innate flavour of food. Flavonoids are one of the most diverse and widespread groups of natural compounds. The flavones, isoflavones, flavonols, anthocyanins, and catechins are likely to be the most widespread natural phenolics [10].

The total phenolic content of CD expressed as mg of gallic acid per g of dry plant was 31.58 ± 1.4 mg/g (Table 8). Pawar & Surana (2010) reported that the concentrations of phenolic compounds in the wood and pericarp of CD were significant, with extraction with methanol giving a yield of 13.28 ± 0.006 mg of gallic acid per g of dry weight (DW) in the wood and 12.68 ± 0.005 mg of gallic acid per g of dry plant in the pericarp. In another study with the species *C. digyna,* Srinivasan et al. [10] reported that extraction with methanol and water gave a yield of 44.70 ± 14.2 mg/g DW. The results show that the extract is rich in phenolic compounds, and these medicinal plants exhibited quite high antioxidant capacities when compared with some fruits, seeds and other medicinal plants reported in the literature [10]. The extract of CD had a polyphenol content (165.54 ± 7.4 mg gallic acid/g DW) comparable with those of the stem of thyme (132 ± 4.4 mg gallic acid/g DW) and stem (162 ± 5.2 mg gallic acid/g DW) and flower (52 ± 2.1 mg gallic acid/g DW) of lavender, reported by Gallego et al. (2013).

The total flavonoid content of CD as mg of catechin per g of dry plant was 1.96 ± 0.008 mg/g. Pawar & Surana [11] reported values that varied from 3.93 ± 0.005 mg quercetin/g DW (wood of CD) to 5.26 ± 0.005 mg quercetin/g DW (pericarp of CD). It is evident from our analysis that the plant is rich in flavonoids.

Table 8. Content of total polyphenols, and antioxidant activity assessed by the ORAC, TEAC, FRAP and DPPH assay for the studied extract.

Total polyphenols (mg GAE/g DW)		**31.58**	**(1.40)**
FLAVONOIDS	**(mg C/g DW)**	1.96	(0.01)
TEAC	**(µmol Trolox/g DW)**	360	(10)
ORAC	**(µmol Trolox/g DW)**	700	(70)
FRAP	**(µmol Trolox/g DW)**	200	(10)
DPPH	**(µmol Trolox/g DW)**	300	(20)

Mean values of three replicates (*n*=3); standard deviations are included in brackets.The antioxidant activity may also be influenced by some particularly active individual phenolic compounds other than flavonoids. Many studies have revealed that phenolic contents in plants are related to their antioxidant activity, and the antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers [12].

Figure 29 shows the ESI(−)–MS/MS spectra for the compounds. The analysis of the components in the CD extract showed that samples contained catechin (a), quercetin (b), *p*-coumaric (e), 4-hydroxybenzoic acid (d) and gallic acid (c). Gallic acid was present at the highest concentration (Table 2). Wei et al. [8] reported the presence of quercetin and rutin in CD extracts, although no rutin was detected in this study.

Previous chemical analysis of this plant had revealed that the main components were terpenoids and flavonoids. Recently, the antitumor activity of the chemical constituents of CD was tested to validate the medicinal use of CD [8].

Numerous studies of the properties of species of the genus *Caesalpinia* (Fabaceae) have confirmed their effectiveness as a natural source of bioactive compounds with therapeutic applications. The genus *Caesalpinia* contains several classes of chemical compounds including flavonoids, diterpenes and steroids. Gallic acid is an active constituent in this genus [13].

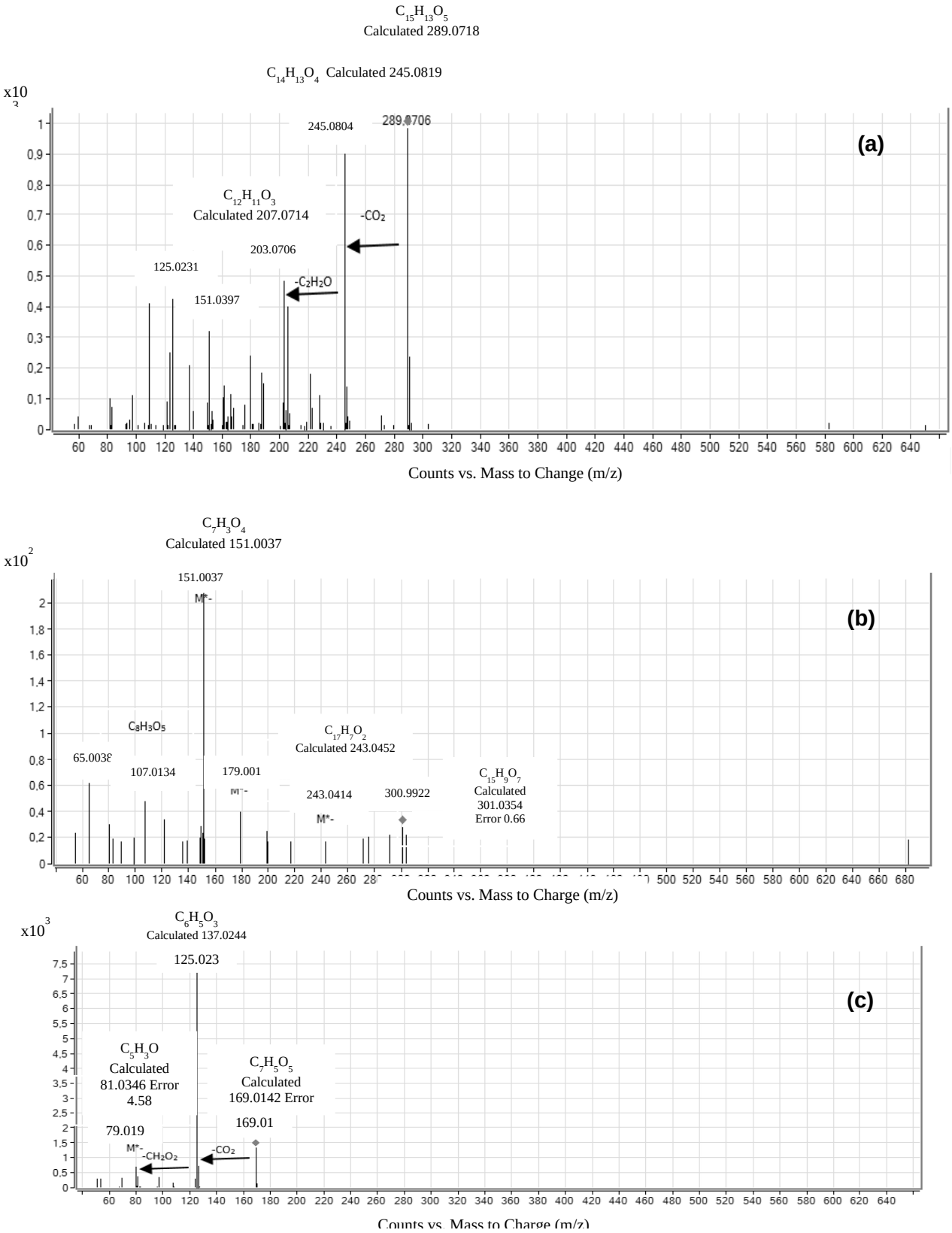

Fig. 29. ESI (-)-MS/MS spectra corresponding to C (a), quercetin (b), GA (c), 4-hydroxybenzoic acid (d), and p-coumaric acid (e).

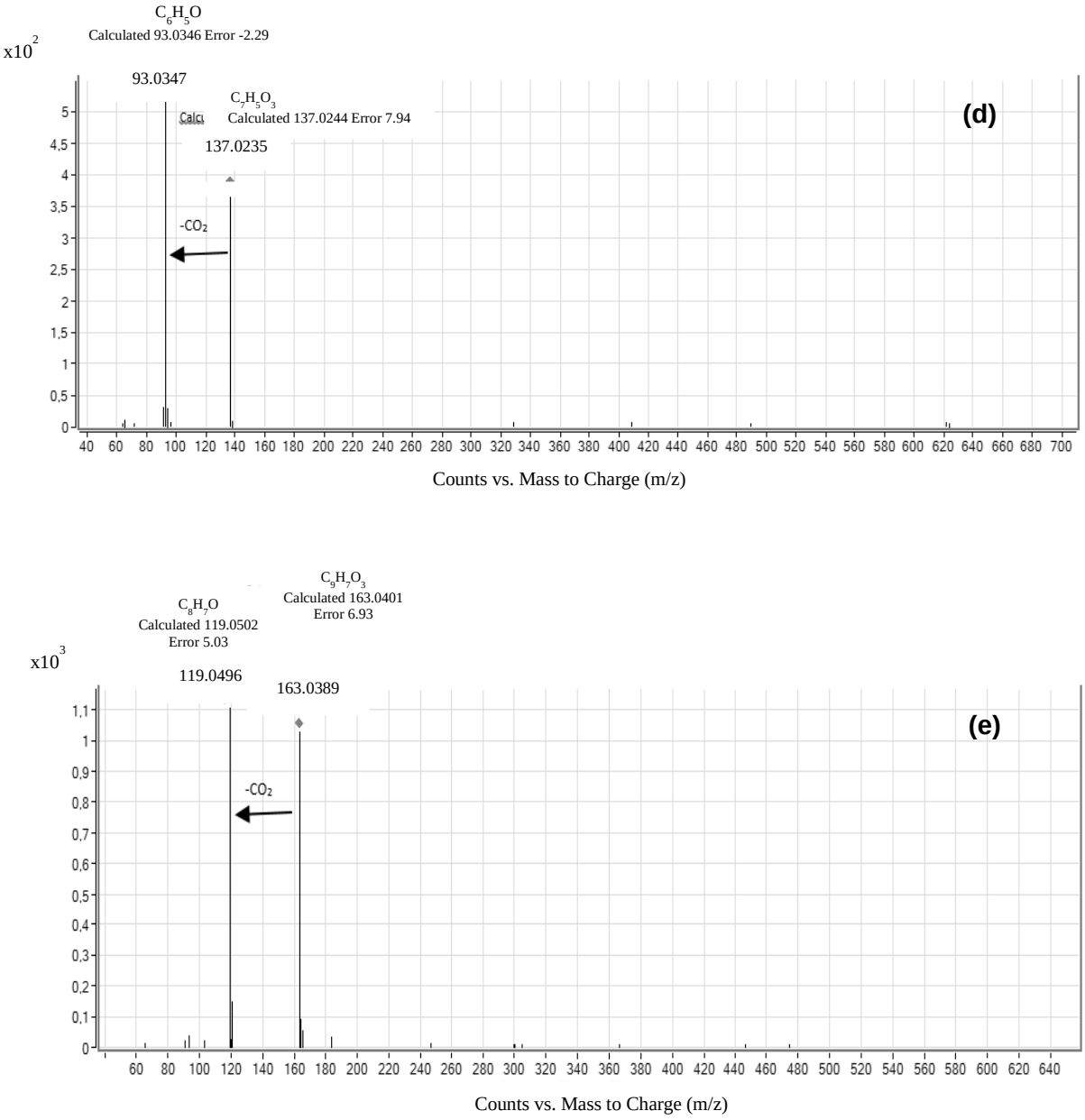

Fig. 29. ESI (-)-MS/MS spectra corresponding to catechin (a), quercetin (b), gallic acid (c), 4-hydroxybenzoic acid (d), and *p*-coumaric acid (e).

4.3.2.2. Antioxidant Capacity Determination

The antioxidant activity of a compound can be measured by the ability of the compound to intercept free radicals by radical scavenging. Antioxidants may act by one or more of several possible mechanisms including sequestration of free radicals; hydrogen donation; metal ion chelation; electron transfer; or even acting as a substrate for radicals such as superoxide or hydroxyl.

The antioxidant capacity of CD extract was investigated by the TEAC, ORAC, DPPH and FRAP assays (Table 9). The order of antioxidant activity compared to Trolox fell in the order ORAC >

TEAC > DPPH > FRAP value. The ORAC assay measures the loss of fluorescence of a probe (fluorescein) in the presence or absence of an antioxidant. In the presence of antioxidant, the FL decay is inhibited and a typical ORAC assay kinetic curve is produced. Data reduction from the ORAC assay is achieved by (1) calculating the *Area Under Curve* (AUC) and net AUC (AUCsample - AUCblank), (2) obtaining a standard curve by plotting the concentration of Trolox and the AUC (linear or quadratic fit between 0.78 and 12.6 µM Trolox), and (3) calculating the Trolox equivalents of a sample using the standard curve. A major strength of this assay is that unlike many other assays which rely on the use of non-biological stable radicals, the ORAC assay uses peroxyl radicals, a biological species formed by the decomposition of 2,2-Azobis-2-Methyl-Propanimidamide Dihydrochloride (AAPH), which would then react with a fluorescent probe, forming a non- fluorescent product [14].

Table 9. Polyphenols identified by LC-MS in CD extract.

Name	Empirical formula	Monoisotopic molecular weight (Da)	Retention time (min)	Concentration (ng mL^{-1})	
Catechin	$C_{15}H_{14}O_6$	290.0790	11.6	669.40	(7)
Quercetin	$C_{15}H_{10}O_7$	302.0427	16.8	64.80	(5)
Gallic acid	$C_7H_6O_5$	170.0215	3.1	77824.60	(40)
4-hydroxybenzoic acid	$C_7H_6O_3$	138.0317	10.8	155.80	(10)
p-coumaric acid	$C_9H_8O_3$	164.0473	13.5	155.70	(9)

Mean values of three replicates (*n*=3); standard deviations are included in brackets.

The antioxidant activity of the extract by this method was 700 ± 70 µmol Trolox/g dry plant. The ORAC value is a measure of the radical scavenging capacity of the plant. The ORAC value of CD was higher than those found in commonly consumed herbs with high antioxidant capacity including basil, marjoram, oregano, ginger, thyme and black tea (0.048 mmol/g DW; 0.27 mmol/g DW, 0.14 mmol/g dry plant, 0.39 mmol/g DW, 0.27 and 0.013 mmol/g DW respectively) (Brewer 2011). The TEAC, FRAP and DPPH assays involve an electron transfer reaction. The TEAC assay is based on monitoring the decay of the radical-cation $ABTS^+$ produced by the oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) caused by the addition of a phenolic-containing sample. $ABTS^+$ has a strong absorption in the range of 600–750 nm and can be easily determined spectrophotometrically. In the absence of phenolics, $ABTS^+$ is rather stable, but it reacts energetically with a H-atom donor, such as phenolics, being converted into a non-colored form of ABTS (Fig. 2a) [15]. In this assay the antioxidant activity of CD was 360 ± 10 µmol Trolox/g DW. Our results are lower than the value reported by Gan et al. [16], who found that the antioxidant capacity of the genus *C. sappan L*. (Fabaceae) was 417.48 ± 10.57 µmol Trolox/g DW, although the results are of similar magnitude, and natural variability occurs due to growing conditions etc. According to the literature, the genus Caesalpinia

provides strong antioxidant activity, which is confirmed. Also the antioxidant capacity was tested using the “stable” free radical DPPH. This otherwise-stable free radical is reduced from violet to yellow in the presence of antioxidants, and the change can be monitored spectrophotometrically [17]. The extract gave a relatively low percentage inhibition, with values of 39.8%. The percentage inhibition during the 75 minutes of the experiment did not reach stability and components continued to react with the radical after this time. Hence the low DPPH value is likely to be partly due to steric inhibition of the reaction between the bulky antioxidants and the DPPH radical, which has two bulky aromatic groups attached to the nitrogen radical.

The amount of plant extract needed to decrease the initial $DPPH^{\bullet}$ concentration by 50% (IC_{50}) is a parameter widely used to measure the antioxidant activity. The lower the IC_{50} value, the higher is the antioxidant power. The amount of plant extract needed to decrease the initial $DPPH^{\bullet}$ concentration by 50% (IC_{50}) was 1 mg/ mL. This result was similar to the value reported by Muñoz-Ortiz et al. (2011) who found an IC_{50} of 1.30 mg/mL for leaves of *Caesalpinia pluviosa* (Leguminoseae), which were collected in Ballivian Province and were deposited in the National Herbarium of Bolivia in La Paz (number of vouchers VM6). However, the IC_{50} value for *Caesalpinia bonducella* (Fabaceae) was much smaller at IC_{50}=74.73 µg/mL [18], as for the bark of *C. pyramidalis* (Fabaceae) IC_{50} = 16.98 ± 1.34 µg/mL, which was incorporated in the Herbarium UFP Geraldo Mariz, Department of Botany, Federal University of Penambuco with the number 60.195 [19], indicating more active antioxidants in the bark of this plant. The FRAP value is a measure of the capacity of the antioxidant to reduce ferric (III) to ferrous (II) ions (Fig. 2b). The FRAP assay also takes advantage of electron-transfer reactions. In this assay a ferric salt, Fe(III)(TPTZ)2Cl_3 (TPTZ) 2,4,6-tripyridyls-triazine), is used as an oxidant. The redox potential of the Fe (III) salt (~0.70 V) is comparable to that of $ABTS^{\bullet-}$ (0.68 V). Therefore, essentially, there is not much difference between the principles of the TEAC assay and the FRAP assay except the TEAC assay is carried out at neutral pH and the FRAP assay under acidic (pH 3.6) conditions [20].

The antioxidant capacity measured by this assay was lower than that measured by the other assays (200 ± 10.0 µmol Trolox equivalent/ g DW). This value is lower than the values reported for *Caesalpinia sappan* of 313.50 ± 44.66 µmol and 0.324 mmol Trolox equivalent/g DW (Gan et al. 2010). However, the FRAP value reported by Gan et al. [16] was in the whole plant, whereas our result is specific to the leaves of the medicinal plant CD.

The radical-scavenging activity of phenolic acids depends partly on the number of electron donor hydroxyl and methoxy substituents which increase the stability of the phenoxy radicals. Gallic acid with three hydroxyl groups and a carboxyl group is very active in reducing free radicals [21].

According to the literature, the medicinal plant species Caesalpinia has many biological activities. For example, *C. sappan* showed antibacterial activity, and had the potential to be developed into an antibiotic. Plants of this species are often used for the prevention and

treatment of cardiovascular and cerebrovascular diseases because they improve blood circulation or stop bleeding [22]. Because of their high antioxidant capacities, it is possible that these plants will be beneficial for cardiovascular and cerebrovascular diseases caused by oxidative stress, and they might be developed into a functional food or drug in the future. However, further evidence relating to their bioavailability and bioactivity is required.

4.3.2.3. Electron Paramagnetic Resonance (EPR) Study

The EPR radical scavenging method has been developed to evaluate the concentration of free methoxy radicals ($CH_3O^{\bullet}$) generated in the Fenton reaction with the CD extract. The OH radical is the most ROS found in both plant and animal cells with a very short half-life [23].

Figure 30a shows the decreasing EPR signal with the increase of CD extract concentration. The free radical scavenging activity of CD extracts was investigated against methoxy ($CH_3O^{\bullet}$) radical by a competitive method in the presence of 5,5-Dimethyl-1-Pyrroline n-Oxide DMPO as spin trap, using EPR spectroscopy. $CH_3O^{\bullet}$ was generated according to the Fenton procedure but due to its relatively short half-life it was identified by EPR by its ability to form a stable nitroxide adduct with DMPO, DMPO-OCH_3 (hyperfine splitting constants, a_N = 13.9 G and a_H = 8.3 G). This stable DMPO-OCH_3 compound can be detected by the double integration value of the EPR signal.

The CD extract at different concentrations competes with the spin trap in the scavenging of methoxy radicals. Thus, the effect reduces the amount of radical adducts and, accordingly, reduces the intensity of the EPR signal. The graph indicates that the area of the signal of the spectrum decreased as the amount of CD increased. The EPR assay had great correlation (Fig. 30b) with TEAC assay (r^2=0.84) and ORAC assay (r^2=0.99)This study confirmed that the scavenging activity of the CD extracts containing polyphenol constituents could be measured by the decrease of the intensity of the spectral bands of the adduct DMPO-OCH_3 in the EPR spectrum as the amount of antioxidant was increased.

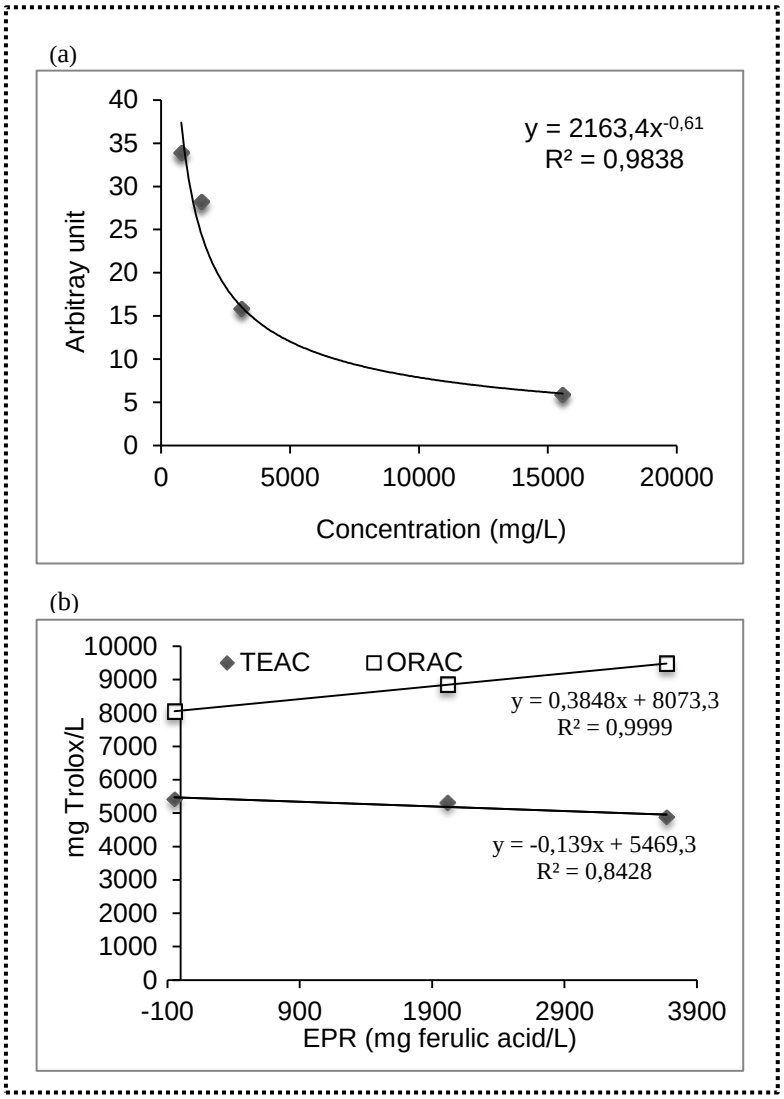

Fig. 30. (a) Antioxidant activity determined by EPR assay to CD extract at different concentrations and (b) correlation between ORAC and TEAC assays with EPR assay

4.3.2.4. Effect of CD Extract on Oxidative Stability of Emulsions

Edible oils containing a high percentage of polyunsaturated fatty acids are very sensitive to auto- and photo-oxidation. Since autoxidation proceeds via a free radical chain reaction, antioxidants that transfer electrons and/or hydrogen atoms may retard the process. Primary oxidation was measured by the Peroxide Value (PV), and secondary oxidation was assessed by the Thiobarbituric Acid (TBA) reaction and the hexanal content in our study.

The yield of the extract of CD was 19%. The antioxidant effect of the extract of CD in oil-in-water emulsions was studied at three concentrations in the range 0.002-0.2%. The PV of emulsion samples increased throughout storage at 33ºC ± 1ºC (Fig. 31).

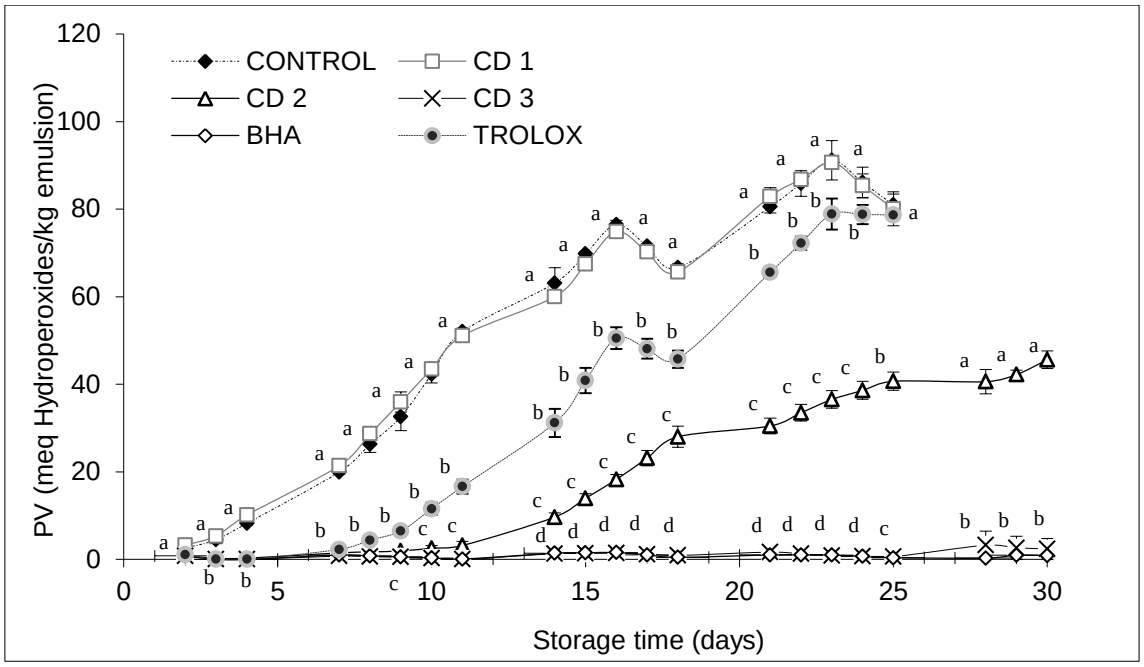

Fig. 31. Changes in peroxide value of emulsions during storage at 33°C for 31 days. Bars represent standard deviation (*n*=3). Different letters in the same day (a, b,c,d) indicate significant differences between samples.

The PV of emulsion samples containing CD at concentrations of 0.02% (CD2) or 0.2% (CD3) was lower than that of the control or sample containing Trolox (0.02%), but at 0.002% (CD1) concentration the emulsion containing CD deteriorated at a similar rate to the control (Table 10). The emulsion stability, assessed by hydroperoxide formation, increased with increasing concentration of the natural extract. Emulsions containing extract concentrations of 0.02% and 0.2% showed a higher stability than those prepared with the positive control Trolox (0.02%) and the extract at a concentration of 0.2% exhibited a similar antioxidant effect to that of BHA at 0.004%.

The PV in all the emulsions increased during storage (31 days). At day 1, the PV of the control and the sample containing the lowest concentration of CD extract were already significantly higher than the values for the other samples and this shows that even in the preparation step itself these two samples had oxidized. As the storage progressed, the PV showed a gradual increase in all the emulsions and the PV of the control reached 80.98 meq/kg emulsion at the end of the storage period. Emulsions containing plant extracts had PV values of 80.07 meq/kg emulsion (CD1), 48.98 meq/kg emulsion (CD2) and 2.14 meq/kg emulsion) (CD3). The PV of the emulsions containing the positive controls were 0.81 meq/kg emulsion (BHA) and 78.66 meq/kg emulsion (Trolox) at the end of the storage period.

Table 10. VP (a) and TBARS value (b) of emulsions containing CD extract at different storage times.

(a)

	Storage time (days)			
Samples	4	10	15	25
CONTROL	8.24^{a} (0.48)	42.30^{a} (1.97)	69.79^{a} (0.30)	80.98^{a} (2.49)
CD 1	10.12^{a} (0.30)	43.51^{a} (1.25)	67.44^{a} (0.35)	80.07^{a} (3.90)
CD 2	0.26^{b} (0.11)	2.55^{b} (0.60)	13.96^{b} (1.04)	40.68^{b} (2.09)
CD 3	0.01^{b} (0.07)	0.27^{c} (0.05)	1.37^{c} (0.17)	0.61^{c} (0.10)
BHA	0.01^{b} (0.20)	0.33^{c} (0.09)	1.49^{c} (0.09)	0.37^{c} (0.08)
TROLOX	0.01^{b} (0.33)	11.56^{d} (1.37)	40.85^{d} (2.87)	78.66^{d} (0.80)

(b)

	Storage time (weeks)			
Samples	**1**	**2**	**3**	**4**
CONTROL	0.27^{a} (0.15)	1.06^{a} (0.13)	1.57^{a} (0.15)	2.30^{a} (0.02)
CD 1	0.37^{b} (0.05)	1.01^{a} (0.09)	1.54^{a} (0.05)	2.42^{b} (0.02)
CD 2	0.05^{c} (0.10)	0.45^{b} (0.05)	0.63^{b} (0.10)	1.19^{c} (0.02)
CD 3	0.06^{c} (0.01)	0.23^{c} (0.00)	0.05^{c} (0.01)	0.05^{d} (0.03)
BHA	0.03^{c} (0.01)	0.27^{c} (0.00)	0.05^{c} (0.01)	0.05^{d} (0.02)
TROLOX	0.08^{c} (0.01)	0.98^{a} (0.08)	1.03^{d} (0.01)	1.60^{e} (0.02)

Mean values of three replicates (*n*=3); standard deviations are included in brackets. For each descriptor and for each time, mean values followed by different letters (a–e) denote significant differences (*P*<0.05).

PV=Peroxide Value/ TBARS= Thiobabituric Acid Reactive Substances.The protective effect of plant extracts on lipid oxidation in oil-in-water system is a consequence of the presence of active phenolic compounds in the extract. Several research groups have determined the antioxidant and protective effects of herbs on fats and oils. Plants of the Lamiaceae family contain active antioxidants with rosemary being a very good source, although oregano, sage and thyme also have good antioxidant capacity [24]. Rosemary extracts are relatively effective in oils, but much less effective in emulsions due to the polar nature of the antioxidants. Oregano extract was also more active in oil than emulsion, although sage extract was relatively effective in both media [25].

Malonaldehyde and other TBA reactive substances are produced as a result of oxidation of polyunsaturated fatty acids. According to analysis after 31 days, the TBA values of emulsion samples containing added plant extracts at > 0.02% were lower than that of the control, with the order of activity of additives similar to that determined by the PV (Fig.32). No significant differences were found between the TBARS values for emulsions containing BHA and

CD3 antioxidants ($p < 0.05$). The order of TBARS values was CD1 > Control > Trolox > CD2 > CD3 > BHA at the end of the storage period.

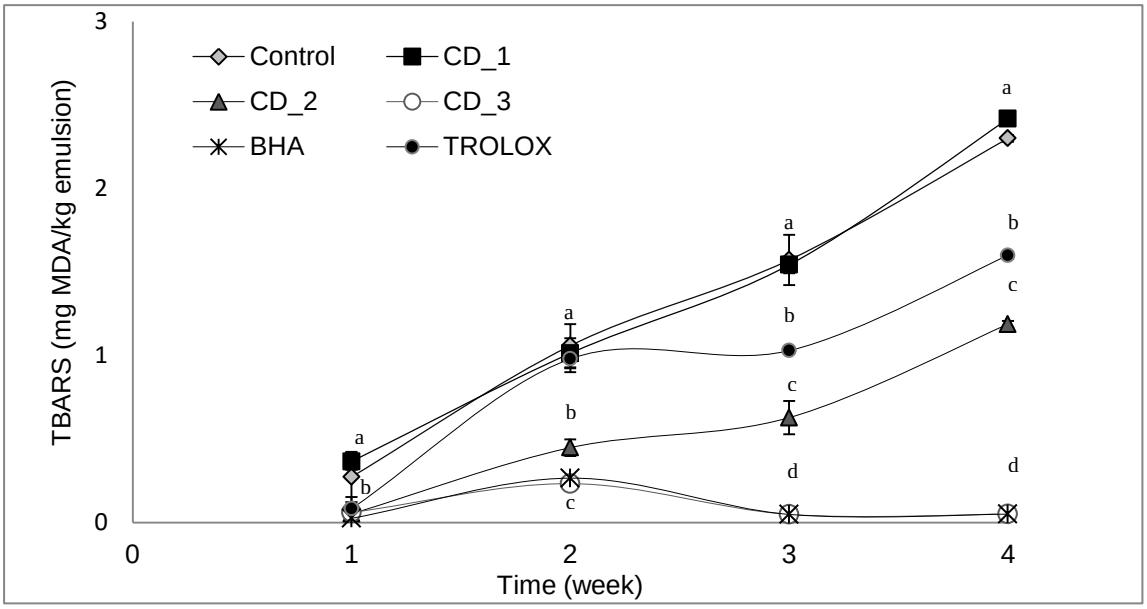

Fig. 32. Changes in TBARS values of emulsions during the study. Bars represent standard deviation (*n*=3). Different letters in the same day (a,b,c,d) indicate significant differences between samples

4.3.2.5. Headspace Volatile Analysis

The concentration of volatile secondary oxidation products increases during oxidation, and hexanal is generally the main volatile produced from sunflower oil (Laguerre et al. 2007). Initially in the first week the concentration of hexanal was low in all O/W emulsion samples and it increased gradually during the storage period (Fig. 33).

Emulsions containing additives, especially those with added lyophilized CD extract showed, in general, lower concentrations of volatile compounds (702 µM for CD2 or 69.3 µM for CD3) than the negative control (823.4 µM) during the experiment. The sample containing the lowest concentration (CD1 = 0.002% of extract) had no significant antioxidant effect. Furthermore, samples containing Trolox and CD2 concentration maintained a similar behaviour until the fourth week. The behavior of emulsions containing CD3 and BHA was similar, as was evident in week 4 when hexanal values for CD3 (69.40 µM) increased, but the samples containing additives were more effective than the Trolox (681.325 µM) control. Both TBARS values and hexanal concentrations are measures of the secondary products of oxidation, although TBARS values include both volatile and non-volatile aldehydes. A linear correlation between TBARS values and the concentration of hexanal was found (r^2 = 0.96%).

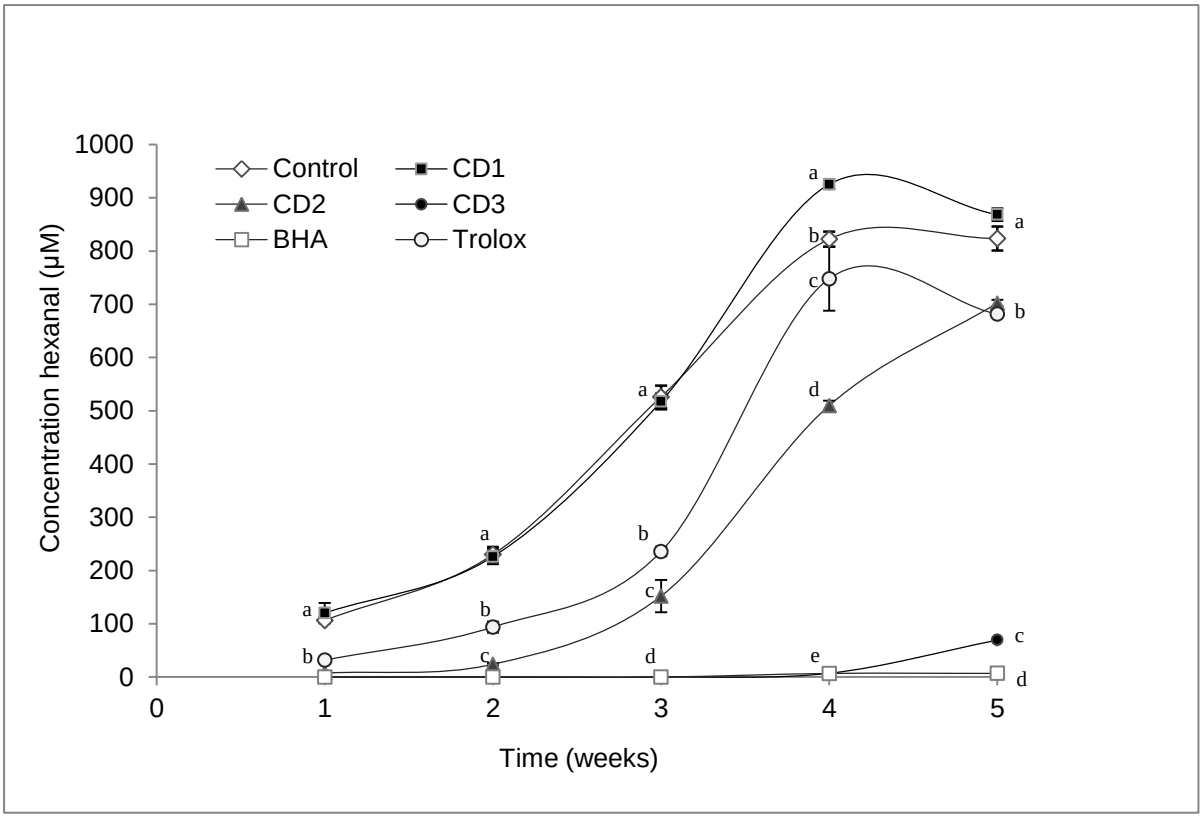

Fig. 33. Change in headspace hexanal concentration for oil-in-water emulsions during the study. Bars represent standard deviation (*n*=3). Different letters in the same day (a,b,c,d,e) indicate significant differences between samples.

The results showed that emulsions containing extracts from CD have good oxidative stability during storage, which can be attributed to the antioxidant activity of the phenolic compounds in the herb including phenolic acids, and the flavonoids catechin and epicatechin. A good proportion of gallic acid is present at the interface in oil in water emulsions stabilized by Tween 20, so the antioxidant is located at the correct location to react with free radicals generated in the aqueous phase [26]. The results show that CD phenolics were effective in inhibiting formation of lipid hydroperoxides and hexanal in the oil-in-water emulsions. This is important because in order for an antioxidant to be effective, it must be able to inhibit the formation of volatile secondary lipid oxidation products that are perceived as off-flavors.

4.3.3.Conclusions

The results of this study show that ethanolic extracts of CD exerted a significant effect on the stability of oil-in-water emulsions, especially at 0.2% concentration, and were more effective than Trolox (0.02%) and comparable in activity to BHA (0.004%). The extract had strong antioxidant activity and was rich in polyphenols. The presence of gallic acid, catechin, epicatechin and vanillic acid contributed significantly to the antioxidant activity of the extract.

Consumer demand for healthy food products provides an opportunity to develop antioxidants as new functional foods. The finding of high antioxidant activity of CD extracts in emulsions provides a possible alternative to synthetic preservatives for the food industry and could be recommended as an alternative antioxidant for the conservation of fats.

4.4. Analytical characterization of polyphenols from *Tara* and *Caesalpinia decapetala* as stabilizers of O/W emulsions

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Artículo impress en Journal of Food Science en agosto de 2016

4.4.1. Introduction

Oxidation products from lipids are responsible for deterioration in the sensorial properties and the nutritional value of foods. The grade of lipids oxidation is greatly affected by many factors, such as (1) the content of unsaturated fat, (2) matrix types, such as Oil-in-Water (O/W) emulsions or bulk oils, and (3) the presence of pro-oxidative metal ions [357,358]. In order to prevent oxidative reactions, a number of aromatic and medicinal plants contain active compounds exhibiting antioxidant properties. Most of these compounds are phenolic species present in different parts of the plants [188].

Caesalpinia is a pantropical genus that presents around 140 species[359]. The genus is anatomically characterized by the presence of different types of secretory structures throughout the plant [360]. Such structures, in combination with other anatomical characters, have been used for taxa delimitation within *Caesalpinia* [359,361].

C. decapetala (CD) has great properties in traditional medicines, e.g. leaves are used in treatment of burns, biliousness, and stomach disorders. It is also employed as laxative, tonic, carminative, and antipyretic [188]. The antioxidant, antitumor and anti-fertility activities of CD have been also reported in the literature [181,184,185]. The leaves of CD contain several other active constituents including cassane diterpenoid, squalene, caesaldecan, spathulenol, lupeol, resveratrol, quercetin, stigmasterol and astragalin [186]. Although the existing scientific data are still not enough to support the traditional medicinal uses of CD, the content of phenolic species make this plant valuable for the nutraceutical industry.

Another plant from the family *Caesalpinia* with antioxidant properties is *C.spinosa* (CS), also known as *Tara*. CS infusions have been traditionally and extensively used by the Peruvian folk medicine to treat inflamed tonsils, fever, cold and stomachaches [362]. *Tara* pods, which represent up to 62% of the plant weight, are a good source to produce tannic, gallotannic and gallic acid [363]. Pods of CS contain a high percentage of tannins (between 40% and 60%) which are of the hydrolysable type, rendering gallic acid (GA) as the main constituent under acidic hydrolysis conditions [364]. Tannins in general are phenolic compounds with astringent, antiviral, antibacterial, antiparasitic, and antioxidant properties [365].

Naturally occurring polyphenols, from vegetable sources, are of great interest for the pharmaceutical and food industries in order to be used as feeding supplements and/or food preservatives. On the other hand, the properties of these natural extracts are strongly affected

by their chemical composition, which, in many cases, remains unknown. Mass Spectrometry (MS) constitutes a powerful tool for the identification of unknown compounds in complex plants extracts [366,367].

The aims of this study are (1) to characterize and (2) to evaluate the suitability of extracts from CD and CS leaves to prevent the oxidation of O/W emulsions. The Folin-Ciocalteu method and the Peroxide Value (PV) were employed to evaluate the effect of different extraction conditions in the total polyphenolic content of extracts and in the stability of O/W emulsions. LC-QTOF-MS was used as analytical technique for identification of phenolic species in extracts and to follow their time-course during aging of O/W emulsions.

4.4.2.Results and Discussion

4.4.2.1. Total phenolic content

Fig. 34 shows the total polyphenols contents, expressed as mg of Gallic Acid Equivalent (GAE) per g of dried plant tissues. Obtained data revealed (1) the much higher concentration of polyphenols in CS leaves versus CD ones, and (2) the enhanced efficiency of the ethanol: water (50:50) solution for extraction purposes. Ethyl acetate performed also quite well for the extraction of CS leaves; however, it turned to be the worst solvent for CD. Such behavior points out to the possibility that polyphenols existing in both species display different structures, that is, they belong to different chemical families. Obviously, the ethanol: water (50:50) solution was selected as extraction mixture for further experiments.

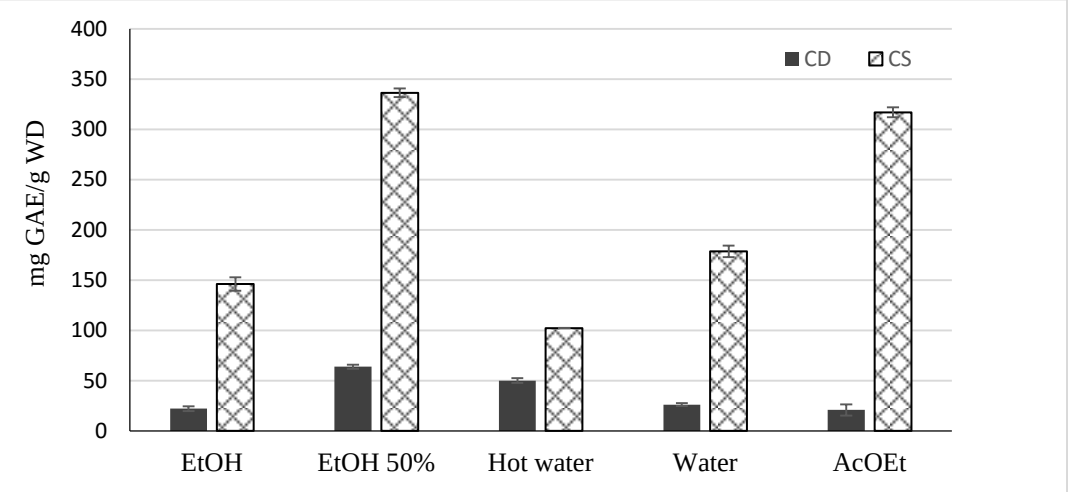

Figure 34.Total polyphenolic contents in CS and CD leaves extracts as function of extraction conditions (at a temperature of 4 ºC, except for the extraction in hot water at 100 ºC). Bars represent standard deviation (*n*=3).

Under optimal conditions, a value of 336.4 ± 4.5 mg GAE/g dry sample was measured in CS leaves. Similar results were obtained by Skowyraet al. [191], who reported 464 mg GAE/g dry CS pods using ethanol (75 % in water) as extraction solvent. Also, Veloz-García et al. (2004)

reported a polyphenol extract yield of 48±3% referred to dry weight in cascalote (*Caesalpinia cacalaco*) pods, a plant of the same genus, using a ternary extraction mixture of water–methanol–acetone (8:1:1). The total phenolic content of CD (EtOH 50%) was 63.8 ± 2.1 mg GAE/g DW, Fig. 35. Pawar et al. [188] reported a much lower concentration in the wood and pericarp, giving a yield of 13.28 ± 0.006 mg GAE/g DW and 12.68 ± 0.005 mg GAE/g DW, respectively.

4.4.2.2. Identification of phenolic compounds by LC-QTOF-MS

sSome relevant data of phenolic species identified in the extracts from CS and CD leaves obtained as discussed above. Tabla 11 showed compounds are organized in order to facilitate the discussion of their identities. Reported data correspond to theoretical values (masses) obtained for precursor ($[M-H]^-$) ions, experimental *m/z* ratios for product ions, their empirical formulae and retention times. The score column indicates how well the experimental MS spectrum fits the calculated one for the empirical formula assigned to each species. This score, in a scale from 0 to 100, accounts for mass accuracy, isotopic profile and spacing between the cluster of ions corresponding to the $[M-H]^-$ species. The column corresponding to Collision Energy (CE) contains the value suggested to acquire the product ion scan spectrum of each compound, maintaining the signal of the precursor ion, at the same time that several product ions are generated.

Table 11. Database of phenolic compounds identified in the extracts from CS and CD leaves.

Compound number/group	Retention time (min)	[a][M-H]⁻ ion	Empirical formula	Normalized Score (0-100)	[c]MS/MS fragments	CE (V)	Proposed identity	CS	CD
1	3.4	169.0142	$C_7H_6O_5$	96.2	125.0239, 79.0190	15	Gallic acid	X	X
2	2.2, 3.4, 4.8	343.0665	$C_{14}H_{16}O_{10}$	99.7	191.0555, 169.0140	15	Monogallolyl quinic acid	X	-
3	10.4, 10.9-12.0	495.0780	$C_{21}H_{20}O_{14}$	98.5	343.0665, 191.0553, 169.0131	15	Digallolyl quinic acid	X	-
4	12.0, 12.2-13.1	647.0890	$C_{28}H_{24}O_{18}$	98.8	495.0770, 343.0665, 169.0130	15	Trigallolyl quinic acid	X	-
5	12.8-14	799.0999	$C_{35}H_{28}O_{22}$	98.3	647.0872, 495.0774, 343.0656	15	Tetragallolyl quinic acid	X	-
6	11.3	183.0299	$C_8H_8O_5$	98.7	168.0054, 124.0160	15	Methyl gallate	X	-
7	11.4, 11.9	321.0252	$C_{14}H_{10}O_9$	99.5	169.0134, 125.0238	5	Digallic acid	X	-
8	13.3	473.0362	$C_{21}H_{14}O_{13}$	99.8	321.0247, 169.0133	5	Trigallic acid	X	-
9	13.8	300.999	$C_{14}H_6O_8$	97.5	283.9949, 145.0280	35	Ellagic acid	X	-
10	2.1-3.2	331.0671	$C_{13}H_{16}O_{10}$	99.4	271.0449, 211.0237, 169.0131	15	Glucogallic acid	X	-
11	9.7, 10.3, 10.6	483.0780	$C_{20}H_{20}O_{14}$	98.8	331.0663, 211.0238, 169.0133	15	Gallic acid galloylglucoside	X	-
12	13.9	441.0827	$C_{20}H_{18}O_{10}$	98.0	289.0705, 169.0149, 125.0207	10	Epicatechin gallate	X	-
13	16.5	301.0354	$C_{15}H_{10}O_7$	70.0	178.9973, 151.0023, 121.0293	25	Quercetin	X	X
14	10.9	137.0244	$C_7H_6O_3$	87.1	93.0345	5	*p*-hidroxybenzoic acid	-	X
15	13.5	163.0389	$C_9H_8O_3$	90.0	119.0500	5	*p*-Cumaric acid	-	X
16	11.7	289.0718	$C_{15}H_{14}O_6$	99.4	245.0807, 203.0705, 179.033	25	Catechin	-	X
17	14.8	445.0776	$C_{21}H_{18}O_{11}$	80.0	269.0447, 175.0235, 113.0241	15	Apigenin glucuronide	-	X
18	17.5	269.0455	$C_{15}H_{10}O_5$	84.0	225.0556, 151.0029, 117.0340	25	Apigenin	-	X
19	13.6	431.0984	$C_{21}H_{20}O_{10}$	81.0	341.0662, 311.0546, 283.0597	25	Vitexin	-	X

Calculated m/z value for the compound [b] Normalized score values for the most intense peak of each group for 0.05% aqueous solutions of CS and CD freeze-dried extracts. [c] Experimental m/z values

In the case of CS extracts, up to 13 major phenolic species have been noticed in the LC-ESI (-)-MS chromatograms. Eleven species correspond to derivatives of GA (compound number 1) and their identification is based on spectral MS/MS information, previous literature data and high resolution MS/MS databases (e.g. Metlin database). Compounds **2-5** are esters generated from reaction between one hydroxyl group of quinic acid and the carboxylic moiety in the molecule of GA. In all cases, several peaks with the same MS/MS spectrum were noticed. Quite often, these peaks are not baseline resolved resulting in broad chromatographic bands with several valleys and apexes. In these situations, retention times provided in Table 15 correspond to the start and the end of the whole chromatographic band. Transitions appearing in the MS/MS spectra of these species (compounds **25**) reflect consecutive replacements of one unit of Gallic Acid (GA) ($C_7H_5O_4$) by hydrogen. Thus, calculated mass differences between these product ions correspond to 152.0110 Da. This fragmentation route ends with the product ions corresponding to quinic and GA (calculated m/z 191.0561 and 169.0142 Da, respectively). Fig. 31A shows a possible structure for trigalloyl quinic acid, together with its MS/MS spectra. Mass errors corresponding to differences between calculated and experimental masses for product ions remained below 1.5 mDa. The existence of complex, conjugated forms between gallic and quinic acids in CS extracts agrees with previous reports using low resolution tandem MS detection [369]. LC separation of such mixtures becomes a complex task and requires chromatographic analysis times above 120 min. Thus, in this study, the sum of peak areas corresponding to galloly quinic acids with the same empirical formula was used as response variable to follow their time-course during emulsions storage.

Compound number **6** is the methylated form of GA and species **7-8** corresponded to esterification of two and three molecules of GA. Several peaks have been noticed for any of these species (compounds **7-8**) depending on whether the hydroxyl moiety in meta- or para-position to the carboxylic group is involved in the esterification reaction. Again, the molecular ion for the $[M-H]^-$ form of GA at m/z 169.0142 Da is present in the MS/MS spectra of these two compounds, spectrum not shown. Compound number **9** arises also from esterification between two molecules of GAd to generate a tetracyclic structure. The compound presents a very rich product ion scan spectra which allows is unambiguous identification as ellagic acid using the Metlin accurate MS/MS database.

Compound **10** corresponds to a molecule of GA bonded, through a phenolic group, to glucose. It displays a high polarity which is in agreement with a short retention time. Species number **11** corresponds to esterification of the above structure (compound **10**) with one additional molecule of GA. Compound number **12** is again an ester between Epicatechin (EC) and GA. Quercetin is a flavonoid structurespecies identified in the extracts from CS leaves. Detailed interpretation of the product ion scan spectrum assigned to this peak is shown in Fig. 36B. Again, mass errors for the precursor and the two most intense product ions stayed below 1.5 mDa. Also, the experimental MS/MS spectrum in Fig. 35B agrees with that existing in the MassBank data base.

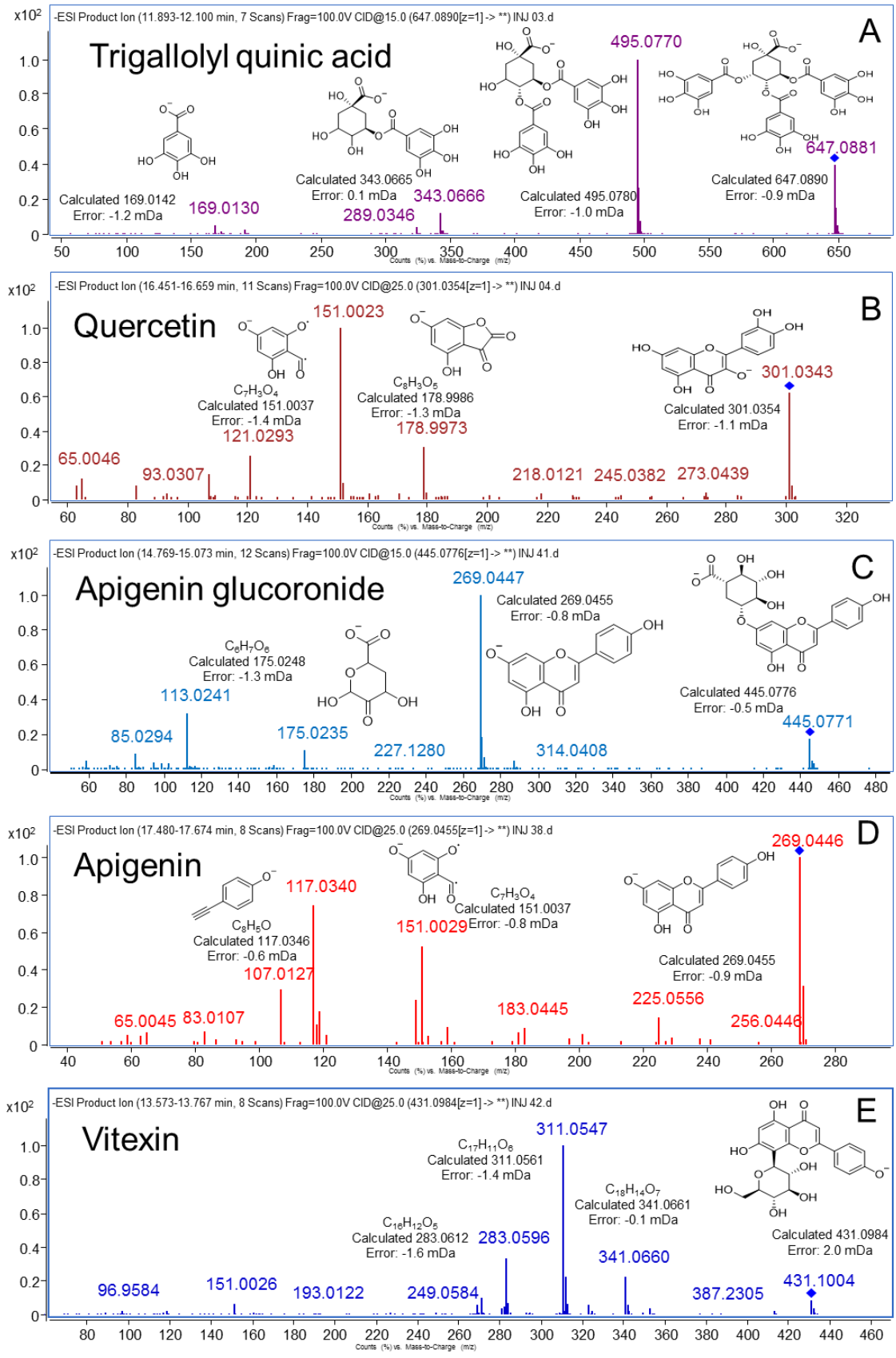

Fig. 35. Product ion scan spectra for CS and CD

In summary, phenolic species rendering the most intense LC-MS peaks in the aqueous solutions of CS correspond to free GA itself, its oligomers and conjugated forms with quinic acid, glucose and flavonoids. Previous studies have also reported the existence of a number of gallic and ellagic acids derivatives in other plants of the *Caesalpinia* family, such as *C. ferrea* [370].

As regards CD extracts, eight compounds could be identified, Table 16. Two of them (GA, and quercetin) were also found in CS extracts reaching higher chromatographic responses in this latter sample. The *p*-hydroxybenzoic acid and *p*-cumaric acids rendered weak peaks under LC-MS conditions employed in this study; however, they identities could be confirmed by injection of pure standards. Also, their product ion spectra displayed a neutral loss of CO_2 as the most intense transition, figure not shown. On the other hand, CD did not contain noticeable levels for any of the conjugated derivatives of GA reported for CS extracts. Other compound was found in CD extracts identified as Apigenin-7-glucuronide. Detailed interpretation of fragments in the product ion spectrum of this compound is shown in Fig. 36C. Despite the very low mass errors, the proposed structure is just tentative since it is not possible to establish which phenolic moiety is bonded to the glucuronide group. The empirical formula assigned to compound 18 ($C_{15}H_{10}O_5$) is compatible with three polyphenols: emodin, baicalein and apigenin. Interpretation of its product ion spectrum and database comparison (MassBank database) permitted to assign this species to the flavone apigenin. The product ion at m/z 151.0029 Da observed in the experimental spectrum of this compound, Fig. 36D, is present in the MS/MS spectra of 5,7-dihydroxyflavones, such as crysin and apigenin but is not observed for the 5,6,7-trihydroxyflavone baicalein. Finally, compound 19 was identified as vitexin, a glucoside of apigenin, Fig. 36E. Again, the experimental spectrum matched that existing in Metlin database for Vitexin.

4.4.2.3. Oxidative stability of oil-in-water emulsions

The formation of primary oxidation products, hydroperoxides, measured in the different emulsions, containing a 0.5 % (w/v) of freeze-dried plants extracts, during storage at 33 ºC is shown in Fig. 36A. The PV of emulsion samples containing the plant extracts was lower that the control. The control PV reached 176.16 meq hydroperoxides/kg emulsion at the end of the storage period. Emulsions containing CD and CS extracts showed a similar behavior until 18 days. Thereafter, the CS extract was much more efficient to reduce the formation of hydroperoxides. After 20 days of incubation, PV levels of 18.21 and 6.73 meq hydroperoxides/kg emulsion were measured for CD and CS, respectively. The maximum acceptable level of PV for refined vegetable oils, established by the Codex Alimentarious, is 10 miliequivalent O_2/kg oil. Emulsions with CD reached this limit after 18 days. In the case of CS extracts containing emulsions, such value was not exceeded at the end of the experiment.

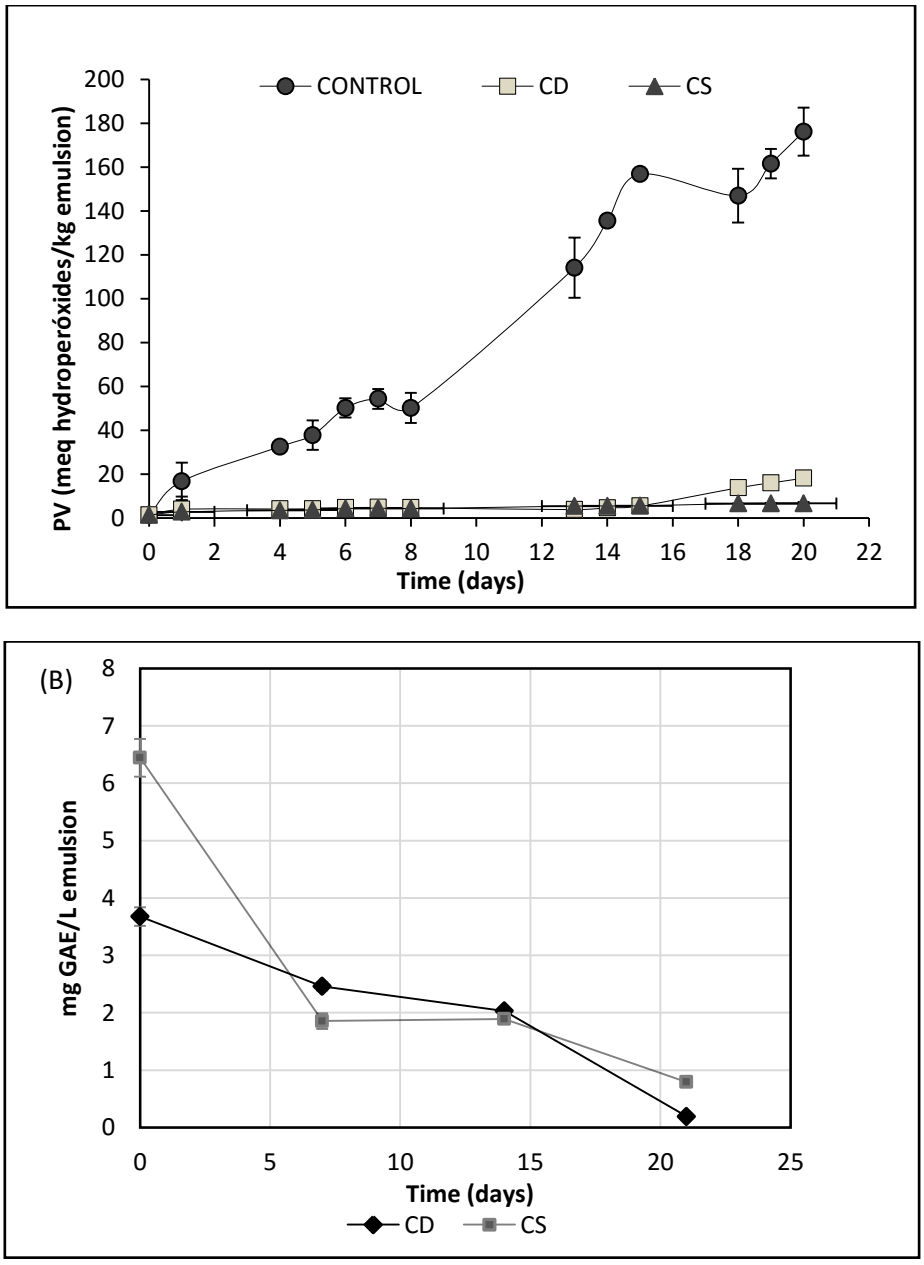

Fig. 36. (A) Peroxide value of emulsions with CD and CS and (B) Time-course of the total polyphenol content during storage of oil-in-water emulsions containing CD or CS extracts (0.5%) as stabilizers. Bars represent standard deviation (*n*=2)

In comparison with previously published data, CS and CD extracts proved to be more effective slowing down the formation of hydroperoxide radicals in oil-in-water emulsions. For instance, Roedig-Penman et al. [371] measure a PV of 30 meq /Kg emulsion after 40 days of storage, at 30 ºC, of oil-in-water emulsions containing a 0.03 % (w:v) of tea extracts. In other studies with species of *Caesalpinia*, Maisuthisakul et al. [372] reported the effect of the *Cratoxylum formosum* dry extract in oil-in-water emulsion (0.01% w:w). The PV value reached 50 meq/kg emulsion after 4.55 days maintained at 60ºC. Also, in previous studies with the CS, we found that one with a lower concentration (48mg/L emulsion) forming hidroperoxides remains below 20 meq / Kg emulsion in 8 days at 38ºC.

4.4.2.4. Variation of polyphenols compounds in emulsions

According to Shahidi and Zhong [247] antioxidants act as free radical scavengers at the water-oil interface where they create a protective barrier and thus 'block' the entrance of molecular species capable of accelerating lipid oxidation into the organic phase. In order to further understand the effect of plants extracts in the stability of the emulsions, the time-course of (1) the total polyphenolic content, determined following the Folin-Ciocalteu method, and (2) that of selected compounds in acetonitrile extracts obtained from emulsions, determined by LC-QTOF-MS, was investigated.

The initial total polyphenol measured (Fig. 37B) showed higher values at week 1 of emulsion preparation containing samples (3.68±0.2 mg GAE/L emulsion to CD and 6.44±0.3 mg GAE/L emulsion to CS). Storage significantly decreased the total polyphenols of both emulsions with very little differences after 2 weeks, despite the different measured PV depending on the employed plant extract, see Fig. 36A.

Evolution of selected compounds (those showing the larger variations) in emulsions containing CS extracts are showed in Fif. 38. Responses for GA (A) and the mono-substituted ester with quinic acid (B) increased steady with storage time. Likely, such behavior results from hydrolysis of the poly-substituted esters (particularly, tri- trigalloly quinic acids, Fig. 37C). This kind of reactions might compete with lipids oxidation, contributing to stabilize the oil-in-water emulsions. Decay in the responses of the oligomeric species trigallic acid (Fig. 37D) and the conjugated of GA with glucose (Fig. 37F) also contributes to increase the response obtained for the free form of GA. Hydrolysis reactions also explain the dramatic reduction in the normalized signal for the ester of EC and GA (Fig. 37E). Finally, the response measured for quercetin increased after 7 days, which suggest a probable degradation of a derivative species, containing the flavonoid structure of quercetin. Such precursor could not be identified in this study; however, the Metlin database contains several hundreds of conjugated species between quercetin and different saccharides. It is worth noting that responses measured for most compounds in Fig. 37 remained stable in aqueous infusions containing the same amount of CS extract stored under same conditions as emulsions, figure not shown. Thus, time-course profiles depicted in Fig. 38 are related to lipids oxidation reactions. The exception to the above behavior corresponded to Epicatechin Gallate (ECG), whose response also decreased with storage time for aqueous solutions; anyhow, the decrease rate of this species in this matrix was lower than that observed for O/W emulsions.

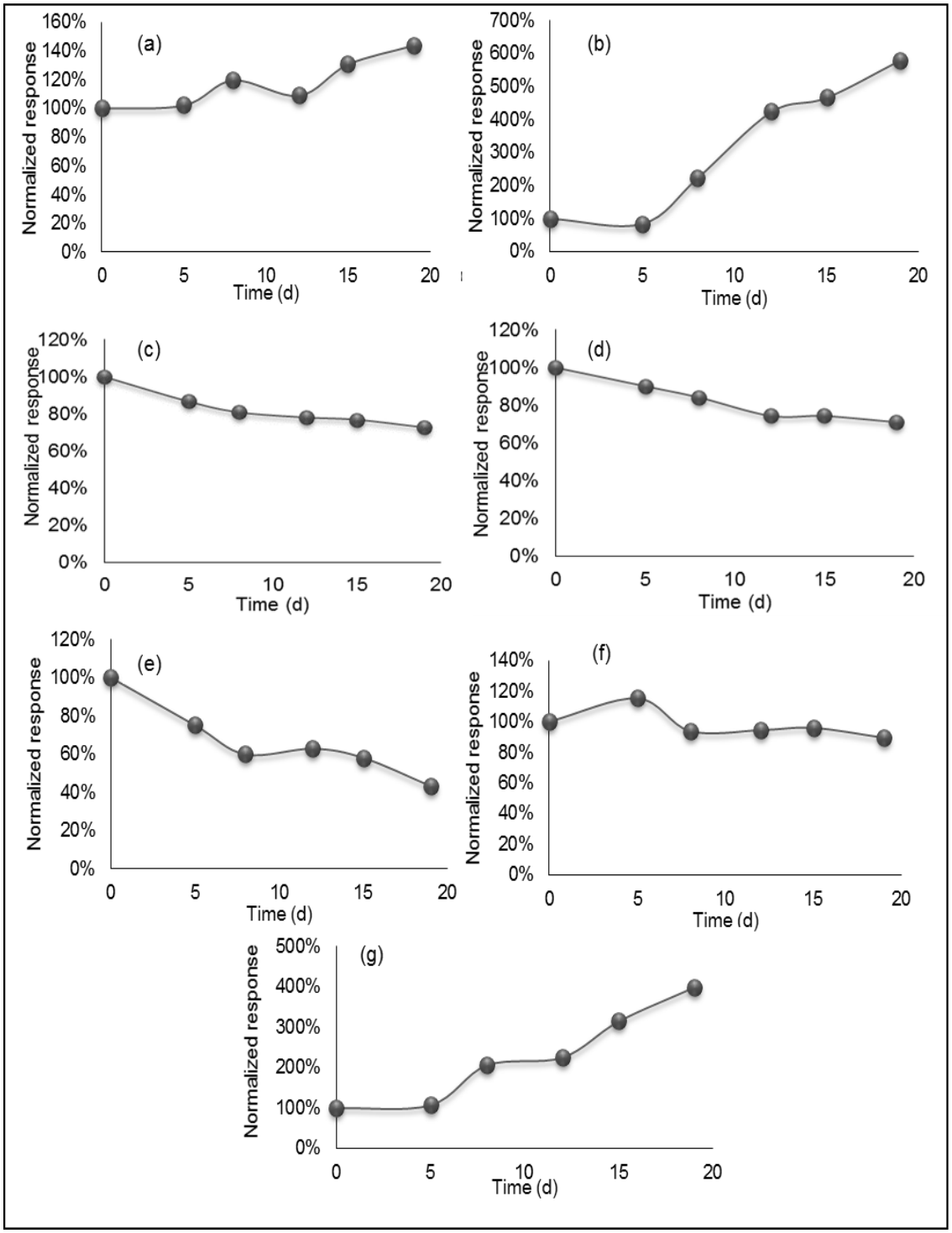

Figure 37. Time-course of selected compounds in oil-in-water emulsions containing a 0.5% of freeze-dried CS extract: (A) GA, (B) Monogalloyl quinic acid, (C) Trigalloyl quinic acid, (D), Trigallic acid, (E) ECG, (F) 3-glucogallic acid, (G) Quercetin. Average values for duplicate experiments.

The evolution of several phenolic species in emulsions stabilized with CD extracts is displayed in Fig. 39. In this case, the lowest variations (below 20%) corresponded to apigenin glucuronide, vitexin and C (Fig. 39A, 39B and 39F). Conversely to the behavior depicted in Fig. 38, the

response of GA (Fig. 38C) decreased in CD containing emulsions. Likely, such reduction is due to the absence of oligomers and esters of GA in this plant. Quercetin (Fig. 38D) increases dramatically with time following the same trend as in CS stabilized emulsions (Fig. 38G) supporting the above reported idea of its generation from hydrolysis of an unknown precursor. This latter comment is also valid to explain the trend of *p*-cumaric acid (Fig. 38E). Also, the analysis revealed the presence of rutin, with precursor ion at m/z 609,1461 Da., but this peak had a very low signal.

The highest concentration of polyphenols was observed at the beginning of emulsion, as indicated in Fig. 37 and Fig. 38. In both varieties a bigger variation of polyphenols compounds were observed throughout 20 days storage period. This is attributed to the fact that reactions between phenolic substances of plants are mainly polymerization reactions rather than degradation or oxidation reactions. We show the changes made by the compounds between 5 and 20 days.

The polyphenols compounds were identified by comparison of their retention times. In the emulsions with CS the compounds suffer a greater degradation are gallotanins and polyphenol ECG. However, the end of the study was obtained a considerable increased of quercetin, quininic acid and mono galloyl. Emulsions with CD showed a similar pattern of GA compounds and quercetin dihydrate. Likewise, a decrease in Catechin polyphenols apigenin-7-glucuronide, vitexin and GA were observed. *p*-cumaric acid and quercetin compounds showed a great increase over time.

Thus, some polyphenols compounds slowed during emulsions oxidation, possibly due to higher scavenging levels of free radicals due to the added antioxidants. The moieties of polyphenol structures easily form hydrogen bonds with radicals produced by oil oxidation, resulting in degradation of polyphenols. The content of catechins decreased over time in the emulsions. It is well known that catechins can undergo degradation, oxidation, epimerization and polymerization and many factors could contribute to these chemical changes [373]. In our work the ECG had a reduction over the time, this polyphenol compound could have suffered an epimerization, Catechin Gallate (CG) is a product result of the epimerization of ECG. Sharma and Zhou [374] observed that ECG decreased while their epimers CG increased in green tea during thebiscuit making process. This can be due to the combined effects of pH and temperature.

In the emulsions occurs a significant variation of GA, there are an increased the contents of mono-galloyl and galloylquininic acid as a consequence of its release through thermal degradation of GA. The majority of tannins compounds can be derivatives of this molecule. The galloyl quinic acid of Tara can be divided in to different groups: mono-, di-, and tri-galloyl [369].

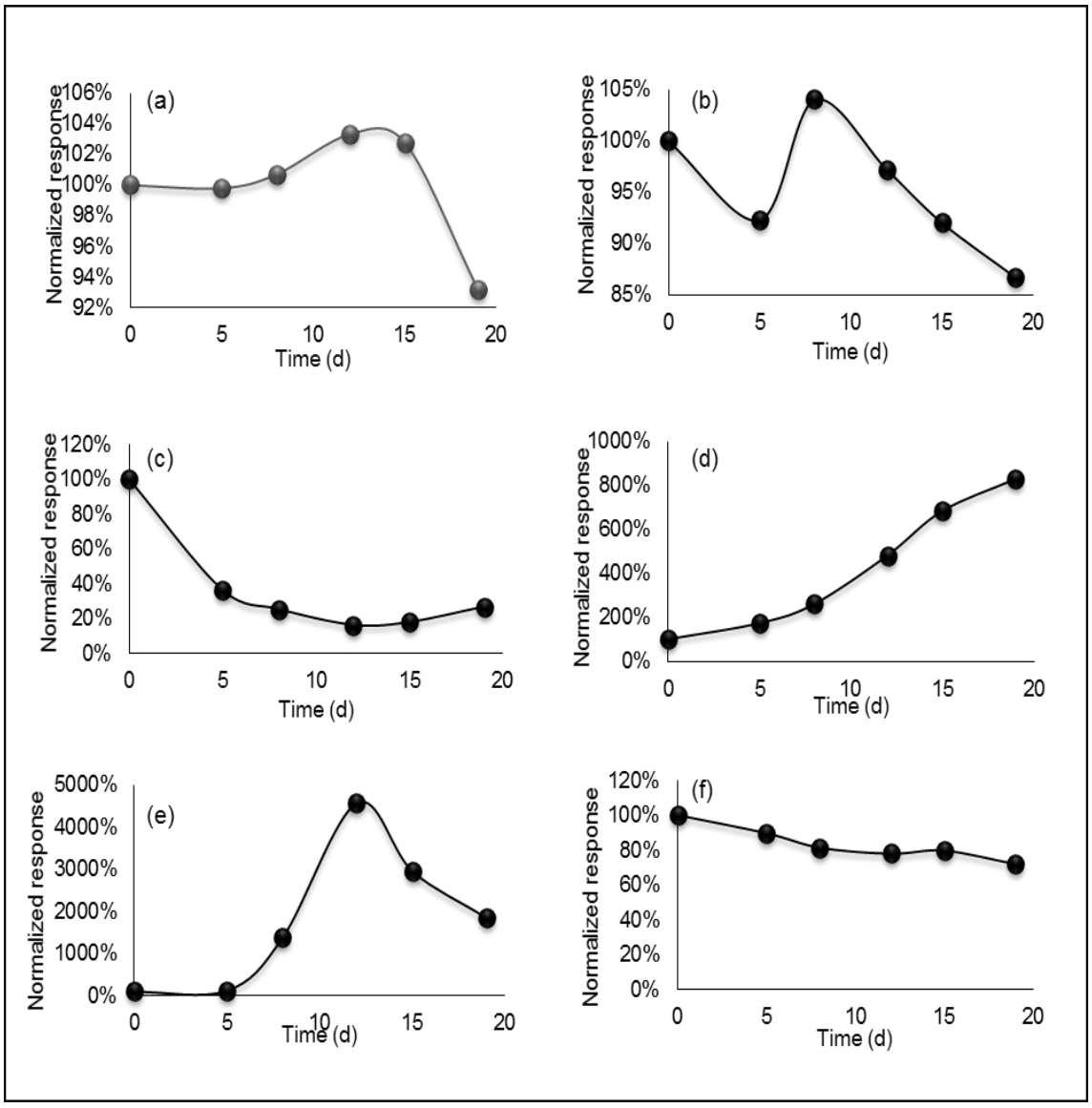

Figure 38. Time course of selected compounds in oil-in-water emulsions containing a 0.5% of freeze-dried CD extract: (A) Apigenin glucuronide, (B) Vitexin, (C) GA, (D), Quercetin, (E) *p*-cumarico and (F) C.

Some compounds over time may result transformation to other compounds to be influenced by factors such as temperature, hydrolysis, etc. Quercetin over time suffer a decrease, one possible reason relates to the rutin present in the samples, which could have suffered decomposition and its transformation into quercetin. The 85% of rutin is transformed to quercetin [375].

In the case of *p*-coumaric acid Pryce [376] considers that *p*-cumaric acid is a precursor metabolite of other polyphenolic compounds more complex. Thereby *p*-hydroxybenzoic acid is produced through three ways: directly from chorismic acid from 4-oxo-cyclohexanecarboxylic and through the *p*-cumaric acid [377]. This explains the decrease of the *p*-cumaric acid in the emulsions at end of the study.

Another compound which showed a decrease in time was the apigenin glucuronide. Although this behavior the variation available is very mild, it is remaining stable throughout the study.

4.4.3.Conclusions

The total polyphenol content and the stabilities of emulsions with the vegetal extracts were investigated. Emulsions containing 0.5 % of CD and CS leaf extracts showed much lower PV indexes than non-stabilized, control emulsions. After 20 days of storage at 33 ºC, the CS extract exerted a higher radical scavenger activity than the CD one. Time-course changes in polyphenolic species identified in CS extracts suggested the hydrolysis of conjugated and oligomeric forms to free GA. The responses of some flavonoids,e.g. quercetin, also increased during storage of oil-in-water emulsions; nevertheless, such pattern could not be correlated with parallel reduction in the responses of a precursor molecule. QTOF-MS provides valuable information for identification of major compounds in the LC chromatograms corresponding to CD and CS extracts; however, a comprehensive characterization of these chromatograms requires the use of more systematic approaches aiming to (1) identify molecular features in the chromatograms, even if they display the same retention time, (2) generate their product ion scan spectra and (3) compare the experimental product ion spectra with those existing in high resolution database.

The total polyphenols in the emulsion with CS indicates the highest antioxidant activity for the gallic acids. This correlated with the polyphenolic compounds present in the plant extract.

In the study, the efficacy of these extracts was found to protect against lipid oxidation of O/W due to the stability and/or increase certain polyphenolic compounds at study end. The results reveal that the herbs selected are rich sources of phenolic compounds with a high radical scavenging activity. This outcome favors the application of natural extracts as natural additives in different fields.

4.5. *Caesalpinia decapetala* Extracts as Inhibitors of Lipid Oxidation in Beef Patties

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Publicado en Molecules (2015): 20: 13913-13926; doi:10.3390/molecules200813913

4.5.1. Introduction

Lipid oxidation, one of the major causes of quality deterioration, is also important because it can negatively affect sensory attributes such as color, texture, odor, and flavor as well as the nutritional quality of the product. Meat mincing, cooking and other processing prior to refrigerated storage disrupt muscle cell membranes facilitating the interaction of unsaturated lipids with pro-oxidant substances such as non-heme iron, accelerating lipid oxidation leading to rapid quality deterioration and development of rancidity. Initially lipid oxidation in meat products results in a cardboard flavor and progresses with the development of painty, rancid and oxidized flavors [378].

Antioxidants are substances that at low concentrations retard the oxidation of easily oxidizable biomolecules, such as lipids and proteins in meat products, thus improving the shelf life of products by protecting them from deterioration caused by oxidation [379].

Synthetic antioxidants such as Butyl Hydroxy Anisole (BHA), Butyl Hydroxy Toluene (BHT), Tert Butyl Hydroquinone (TBHQ), and Propyl Gallate (PG) have been used as antioxidants in meat and poultry products, but synthetic antioxidants have fallen under scrutiny due to potential toxicological effects [379].

Natural extracts have been developed in response the recent demand for natural products and consumers' willingness to pay significant premiums for natural foods. Many plants have been recognized as possessing antioxidant activity, including barks of cinnamon (*Cinnamomum iners*), buds of clove (*Syzygium aromaticum*), rhizomes of ginger (*Zingiber officinale* Rosc.), leaves of green tea (*Camellia sinensis*) and leaves of thyme (*Thymus vulgaris*) [380].

C. decapetala (CD) is a climbing shrub that belongs to the genus *Caesalpinia* of the *Fabaceae* family. *C. decapetala*is widely distributed around the world, but mainly distributed in the southern regions of the Yangtze River in China. The plant is locally known as "Yan wang ci" in Guizhou Province, China. The roots are used in folk medicine to treat bronchitis, prevent colds, and as an antimalarial agent. Previous chemical investigations on *C. decapetala*revealed that the main chemical components were terpenoids and flavonoids [44]. Recently, the chemical constituents have been systematically investigated and the antitumor activities of the compounds have been tested to validate the medicinal use of this plant. *C. decapetala* has been shown to contain antioxidants. The leaves contain cassane diterpenoid, caesaldecan,

spathulenol, 4,5-epoxy-8(14)-caryophyllene, squalene, lupeol, resveratrol, quercetin, astragalin and stigmasterol [186].

Our objective in this study was therefore to evaluate the effectiveness of *C. decapetala* extract in preventing or reducing lipid oxidation as well as color changes in ground beef patties during storage at a chilled temperature (4 °C).

4.5.2.Results and Discussion

4.5.2.1. Antioxidant Capacity Assays (AOC)

AOC determined by the ferric reducing antioxidant power (FRAP) assay at 24 h and after 11 days are presented in Figure 40. In order to obtain an accurate value for the total antioxidant activity (Table 12), both the hydrophylic and lipophilic antioxidant activity analyses were done on the same samples.

The hydrophylic and lipophilic antioxidant activity values were higher in the sample containing *C. decapetala* leaf extract (0.5 %). The hydrophylic antioxidant activity (0.20 ± 0.003 mol Trolox Equivalent (TE)/mL sample) had a higher value than the lipophilic FRAP value with no significant difference ($p < 0.05$) from the sample of BHT (0.21 ± 0.01 mol TE/mL sample). The sample with the lowest antioxidant activity as expected was the control.

The FRAP value is a measure of the capacity of the antioxidant to reduce Ferric (III) ions to Ferrous (II) ions [260]. In our study the final hydrophylic and lipophilic values of antioxidant activity of the sample containing *C. decapetala* (0.5 %) were higher than those reported by Topuz et al. [381]. They studied the effect of addition of sauces containing olive oil and pomegranate juice into marinated anchovy to retain the initial quality, by preventing undesired chemical and oxidative alterations during storage at 4 °C. The total antioxidant activity value of CD2 (0.39 ± 0.03) was similar to those (0.31 ± 0.05) reported by Bubonja-Sonje et al. [382].

The capacity activity of the hydrophylic and lipophilic extracts can be attributed to different polyphenol compounds. The hydrophylic extract contains antioxidants such as phenolic derivatives of Benzoic Acid and cinnamic acid or flavonoids [383]. In the lipophilic extract the major contributors to the antioxidant activity are hydrophobic compounds such as carotenoids, tocopherols, polymeric proanthocyanidins and high molecular weight tannins [384].

The extracts showed a higher ability to reduce Fe^{3+}. The assay showed higher AOC values in the assay carried out with the lipophilic extract compared to the values for the hydrophylic extract.

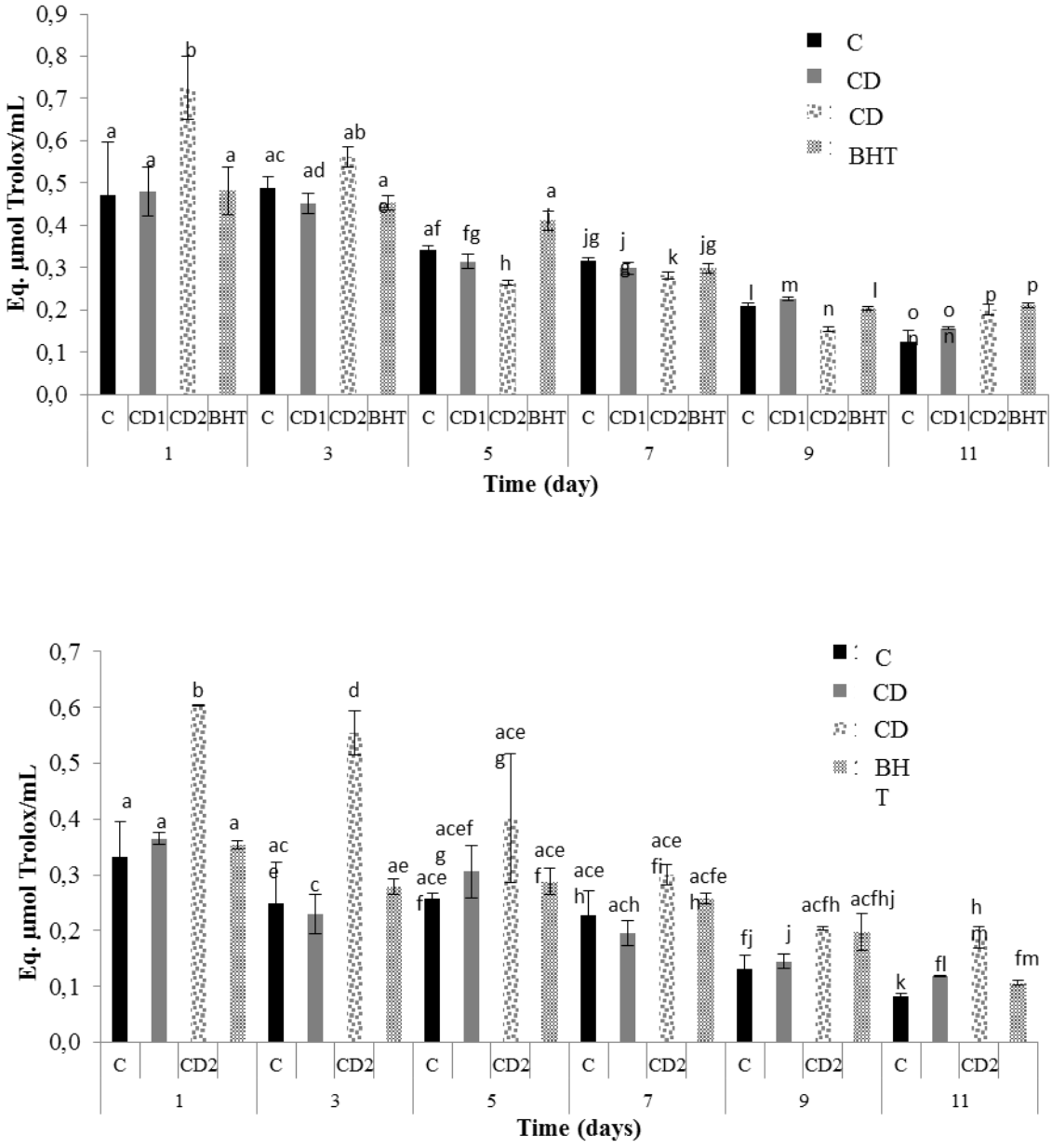

Fig. 40. (a) AOC measured by FRAP water; (b) and FRAP lipid; assay for each treatment: Control (C), CD1 (0.1%), CD2 (0.5%) and BHT after 11 days of storage. The values represent mean ± standard error; treatment means that do not share a common letter are different (p < 0.05).

Table 12. The antioxidant activities of *C. decapetala* (0.1% and 0.5%) and BHT in beef patties after 11 days of storage.

Sample	**Antioxidant Activities (µmol Trolox/mL Sample)**		
	Hydrophylic	Lipophilic	Total
Control	0.13 (0.03) [a]	0.08 (0.01) [a]	0.21 (0.19) [a]
CD1	0.16 (0.003) [b]	0.12 (0.002) [b]	0.28 (0.07) [b]
CD2	0.20 (0.01) [c]	0.19 (0.02) [c]	0.39 (0.03) [c]
BHT	0.21 (0.01) [c]	0.11(0.004) [b]	0.32 (0.01) [d]

Results of sample concentrations (µmol Trolox/mL sample) are expressed as mean (SD). Different letters (a–d) in the same column denote significant differences among samples ($p < 0.05$).

4.5.2.2. Effects on Metmyoglobin Formation

The effect of *C. decapetala* and BHT on Metmyoglobin (MetMB) percentage in beef patties is presented in Figure 41. The relative MetMb percentage increased with time for the 11 days of refrigerated storage. The samples treated with leaf extract and BHT had a lower ($p < 0.05$) concentration of MetMb compared to the control, thus demonstrating some ability to inhibit formation of MetMb. After 10 days, the control sample exhibited higher MetMb concentration (73.48 ± 0.20). No significant difference was found between the control and sample CD 1 0.1%. Antioxidant effect was best in samples containing leaf herb extract (66.57 % ± 0.3 % for *C. decapetala* at 0.5 %). The sample with BHT had a very similar behavior to the CD2 extract, with no significant difference between these samples at the end of the study.

Although many factors can influence the color stability of meat and meat products, the susceptibility of myoglobin to autoxidation is a predominant factor. The discoloration of meat from red to brown during storage results from the oxidation of OxyMb to MetMb [385].

The radical species produced during muscle phospholipid oxidation may act to promote OxyMb autoxidation. Conversely, superoxide anion released from oxidized OxyMb can dismutate to hydrogen peroxide and hydroxyl radical, which are potent lipid pro-oxidants [385]. The free radical scavenging effects of phenolic compounds occurring in *C. decapetala* leaf extract are the most likely reason for the retardation of MetMb formation.

In a previous study, Sánchez et al. [12] reported that beef patties treated with rosemary did not exceed 40 % of metmyoglobin after day 8 of storage. Significant correlations (95%) were

observed between metmyoglobin formation and values from the thiobarbituric acid reactive substance (TBARS) assay. This confirms that both parameters reflect the oxidation rate for the samples during the study period, showing the control as the most oxidized sample. The addition of 0.5% *C. decapetala* was effective in inhibiting myoglobin oxidation and maintained the redness of the beef patties due to its ability to maintain oxymyoglobin stability, and to reduce the formation of metmyoglobin.

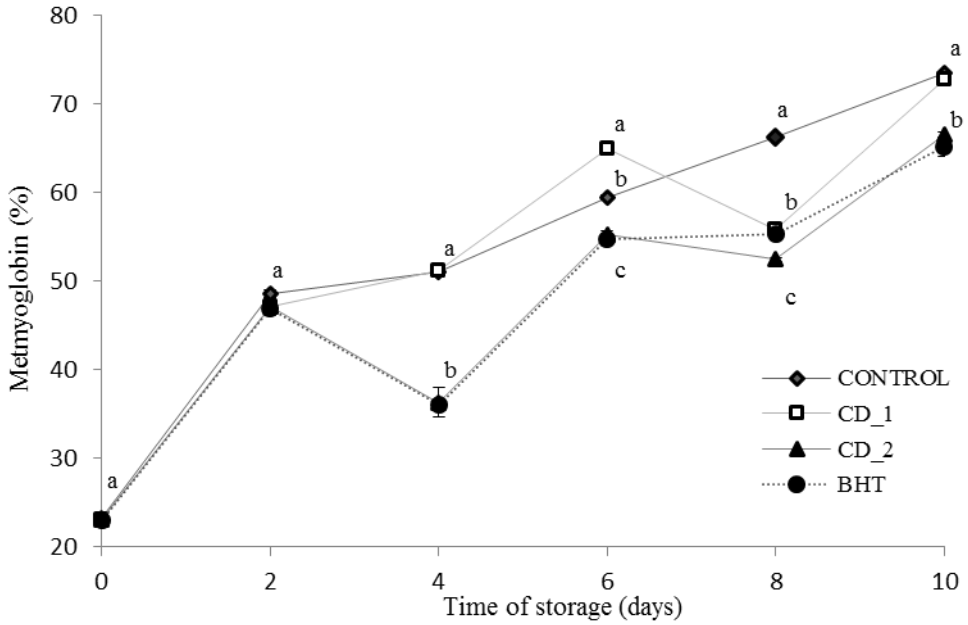

Fig. 41. Effects of *C. decapetala* extract added at 0.1% and 0.5% (*w*/*w*) and BHT on metmyoglobin changes in beef patties during 11 days of refrigerated storage at 4 °C. Results are given as mean ± standard error. Different letters in the same day (a–d) indicate significant differences between samples.

4.5.2.3. Volatile Compounds

The hexanal content increased together with the TBARS values, thereby suggesting lipid oxidation development (Fig. 42). The hexanal content of meat stored at 4 °C increased rapidly over the first four days of storage. The trend observed for hexanal values was as follows ($p < 0.05$): control > CD1 > CD2 = BHT. The antioxidant herb extract added to beef patties reduced the amounts of volatile compounds formed. After eight days, the control and CD1 sample showed the highest hexanal concentration throughout the storage period. CD2 (0.5 %) extract and BHT samples, which also had the lowest TBARS values, formed the least volatiles with 7.16 ± 0.1 and 6.89 ± 0.1ppm hexanal, respectively.

Flavor and aroma compounds found in meat include a broad array of compounds, including hydrocarbons, aldehydes, ketones, alcohols, furans, thiophenes, pyrroles, pyrazines, oxazoles,

thiazoles, and sulfurous compounds. Also, flavor and aroma are attributes most easily detected and assessed by consumers as either acceptable or not [386].

Aldehydes are the most prominent volatiles produced during lipid oxidation and have been used to successfully follow lipid oxidation in meat or meat products where they are reported to contribute tothe overall off-flavor of oxidized meat. Hexanal is reported to be the most sensitive indicator for lipid oxidation [387]. Hexanal and heptanal are degradation products from the oxidation of long chain polyunsaturated fatty acids *n* − 6, mainly linoleic acid [388]. Long chain polyunsaturated fatty acids are known to be less stable towards oxidation than monounsaturated fatty acids, and the high hexanal values observed in this study can be attributed to degradation of linoleic acid.

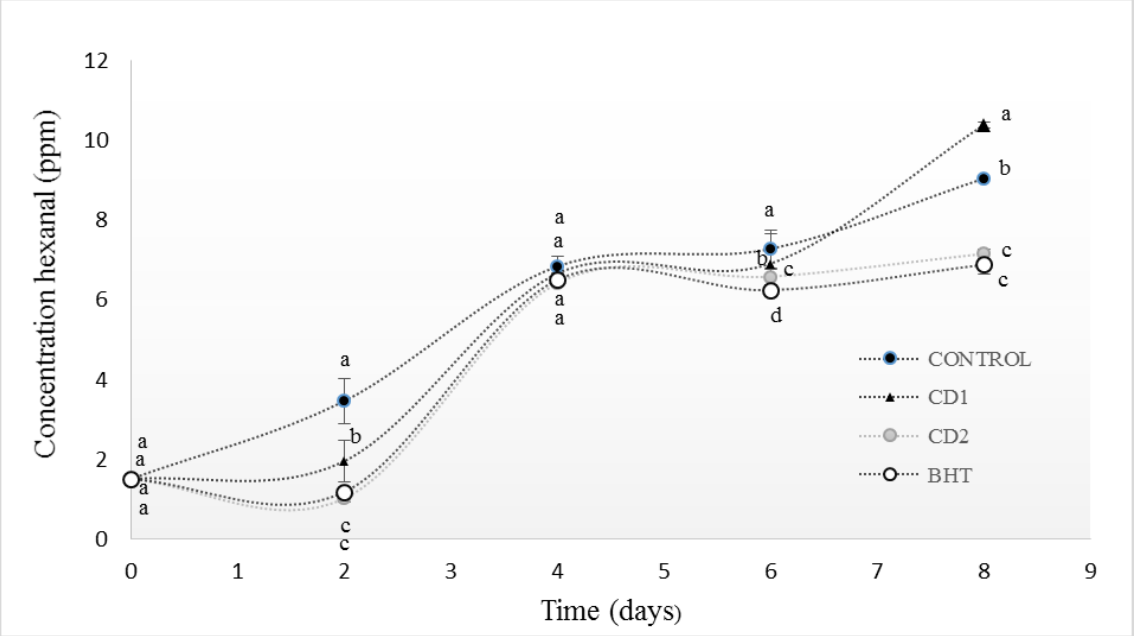

Fig. 42. Hexanal in beef patties with *C. decapetala* extract added at 0.1% and 0.5% (*w*/*w*) concentration and BHT packaged in a modified atmosphere and stored at 5 °C. Results are given as mean ± standard error. Different letters in the same day (a–d) indicate significant differences between samples.

Similar observations have been also made by Juntachote et al.[389] in cooked ground pork sausages with various added antioxidants. Sampaio et al.[390] indicated that natural antioxidants including honey, oregano and sage exhibited greater antioxidant efficacy than that shown by BHT, when assessed by hexanal formation.

4.5.2.4. Effect on Lipid Oxidation and the Color of Beef Patties

4.5.2.4.1. Thiobarbituric Acid Reactive Substance (TBARS) Value

The antioxidant effects of *C. decapetala* leaf extracts and the synthetic antioxidant BHT in ground beef patties (0.1 % and 0.5 % *w*/*w*) are shown in Figure 43. The extracts showed

effective antioxidant activity against lipid oxidation, although the TBARS content of the patties treated with edible plant extract (0.5%) was lower than that of the patties treated with BHT. As expected, the TBARS values of the control sample increased most by 5.6 mg malondialdehyde/kg sample after 11 days, whereas the TBARS values of patties containing 0.1% and 0.5% *C. decapetala* extract increased by 2.9 and 1.7 mg malondialdehyde/kg sample, respectively, after 11 days—significantly less than the control ($p < 0.05$).

The ethanolic extract of *C. decapetala* was moderately antioxidant at both 0.1 % and 0.5 % in beef patties, with significantly lower ($p < 0.05$) TBARS values than the control, and the concentration 0.5% was more effective as an antioxidant than BHT treatment. We concluded that a 0.5 % *C. decapetala* leaf extract is more capable than BHT of maintaining lipid stability and efficiently delaying lipid oxidation in refrigerated beef patties.

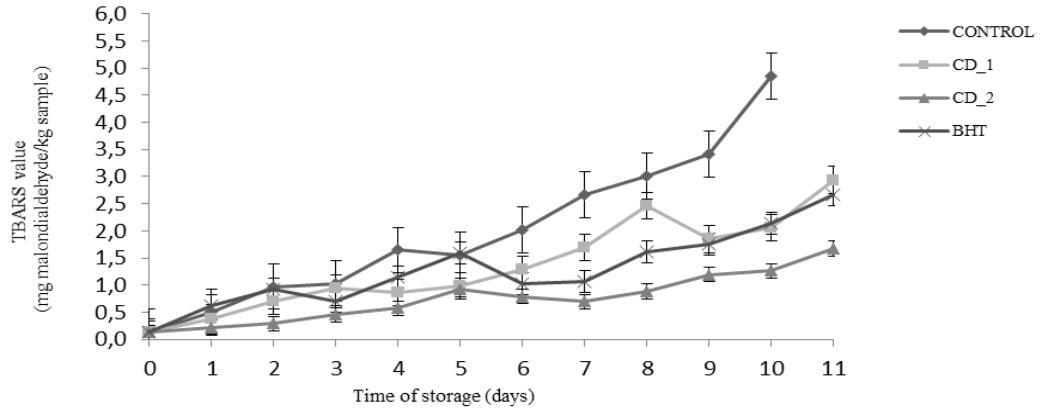

Fig. 43. Effects of two concentrations of *Caesalpinia decapetala* added at 0.1% and 0.5% (*w*/*w*) and BHT added at 0.01% (*w*/*w*) on TBARS value (mg MDA/kg sample) of raw beef patties during 11 days of refrigerated storage at 4 °C. Results are given as mean ± standard error. Different letters in the same day (a–d) indicate significant differences between samples.

Our results are consistent with various other studies, all of which reported that natural antioxidants from culinary herbs and edible plants were effective at controlling lipid oxidation and extending the shelf life of meat products. Fasseas et al.[391] reported that both oregano essential oil (3 %) and sage essential oil (3 %) significantly reduced oxidation. Mitsumoto et al.[392] reported that adding tea catechins (200 or 400 mg/kg) to minced meat inhibited lipid oxidation in both raw and cooked beef. Similar results to ours were reported by McCarthy et al.[393], where an addition of rosemary extracts (0.2 %) to beef patties stored in refrigeration had antioxidant activities similar to BHA/BHT (0.01%/0.1 %). Formanek et al.[340] noted that rosemary extracts worked synergistically with vitamin E to inhibit the formation of malondialdehyde (TBARS). Han and Rhee [394] showed that 0.25% (*w*/*w*) extracts of rosemary, sappanwood, and red or white peony almost completely inhibited lipid oxidation in raw beef patties.

In general, the effectiveness of these natural antioxidants is proportional to the number of –OH groups present on the aromatic rings. If their solubility is compatible with a particular meat system, the fact that they are natural and have antioxidant activity that is as good as or better than the synthetics makes them particularly attractive for meat products.

4.5.2.4.2. Changes in pH of Raw Beef Patties

Figure 44 shows the effects of *C. decapetala* added at two concentrations (0.1 % and 0.5 %) w/w and BHT (0.1 %) on the pH values in raw beef patties during cold storage for 11 days. The control sample had the highest pH value (5.50), and the pH values of the other treatments decreased with storage time. Samples treated with *C. decapetala* (0.5 %) had the lower pH value after storage (5.39). The changes in pH value during storage might be due to acidity produced by bacterial action on the muscle glucose and accumulation of the microbial metabolites due to bacterial spoilage in pork meat patties [394].

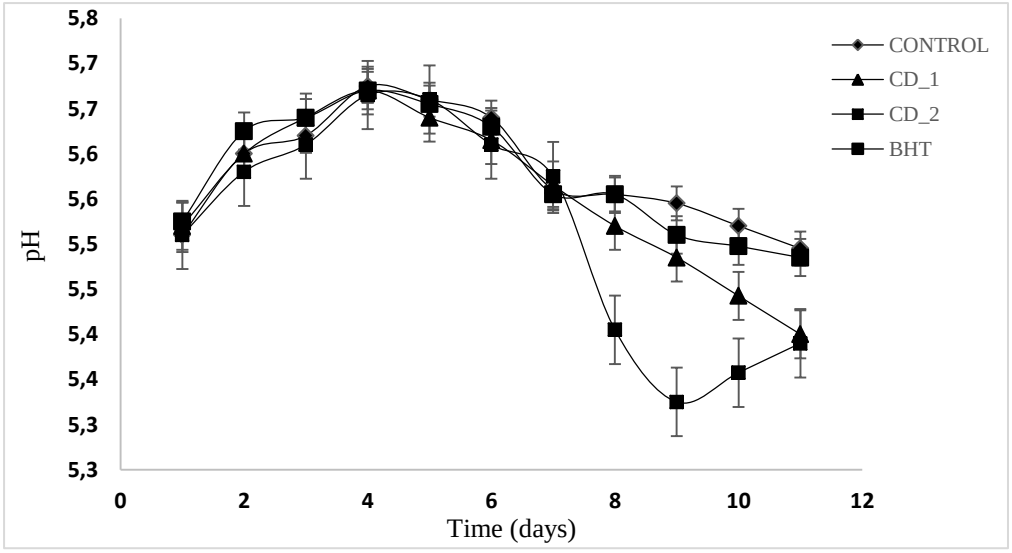

Fig. 44. Effects of *Caesalpinia decapetala* added at 0.1% and 0.5% (*w*/*w*) and BHT added at 0.01% (*w*/*w*) on the changes in pH values of raw beef patties. Data is presented as mean ± standard deviation. Different letters at the same time (a–d) indicate significant differences between samples.

4.5.2.4.3. Color Changes

The *Commission Internationale de l'Éclairage* (in French, (CIE) color values in raw ground beef samples with/without spice extracts are shown in Table 13. The color of meat and meat products after slaughter and manufacturing is altered by increased metmyoglobin.

Table 13. Color changes of beef patties containing extracts at 4 °C.

Trait	Days	Control		CD1		CD2		BHT	
Lightness (*L**)	0	37.53	(3.19) ^{a}						
	1	37.84	(3.24) ^{a}	38.86	(1.75) ^{a}	37.42	(2.47) ^{a}	42.42	(5.14) ^{b}
	2	36.42	(2.22) ^{a}	39.73	(2.18) ^{b}	39.35	(1.77) ^{b}	38.71	(2.90) ^{b}
	3	39.37	(5.18) ^{a}	38.74	(3.55) ^{a}	40.17	(3.08) ^{a}	40.93	(3.41) ^{a}
	4	38.47	(5.18) ^{a}	40.92	(2.60) ^{b,a}	34.53	(2.35) ^{c}	38.52	(2.99) ^{b}
	5	42.43	(2.66) ^{a}	41.34	(3.05) ^{a}	36.78	(2.06) ^{b}	37.29	(4.37) ^{b}
	6	44.33	(3.36) ^{a}	41.74	(3.22) ^{b}	40.05	(1.74) ^{b}	44.23	(2.59) ^{a}
	7	44.38	(4.94) ^{a,d}	46.36	(2.85) ^{b,d}	40.47	(1.76) ^{c}	45.94	(1.88) ^{d}
	8	42.85	(2.50) ^{a}	44.83	(3.30) ^{b,a}	40.59	(2.56) ^{c}	35.58	(4.99) ^{d}
	9	45.71	(3.32) ^{a}	48.14	(3.92) ^{b,a}	42.17	(2.65) ^{c}	44.63	(2.12) ^{a}
	10	44.31	(3.35) ^{a}	48.68	(2.14) ^{b,a}	46.76	(3.76) ^{b,a}	43.07	(3.03) ^{a,c}
	11	43.80	(3.66) ^{a}	50.90	(1.88) ^{b}	43.01	(4.15) ^{a}	44.46	(7.98) ^{a,b}
Redness (*a**)	0	3.33	(0.58) ^{a}						
	1	5.08	(1.73) ^{a}	5.92	(1.88) ^{b}	4.33	(1.82) ^{c}	6.42	(2.08) ^{d}
	2	6.20	(0.63) ^{a}	4.63	(1.18) ^{b}	2.35	(1.05) ^{c}	6.30	(1.34) ^{a}
	3	4.41	(1.67) ^{a}	6.52	(1.63) ^{b}	4.56	(1.03) ^{a}	4.20	(1.33) ^{a}
	4	5.04	(2.14) ^{a}	4.48	(1.73) ^{b,a}	2.57	(0.88) ^{c}	3.81	(1.23) ^{d}
	5	4.15	(0.43) ^{a}	2.43	(0.96) ^{b}	1.86	(0.59) ^{c,b}	0.87	(0.59) ^{d}
	6	2.68	(0.87) ^{a}	1.75	(0.81) ^{b}	0.83	(0.59) ^{c}	2.11	(0.29) ^{d}
	7	1.24	(0.28) ^{a}	0.44	(0.24) ^{b}	0.82	(0.45) ^{b,a}	0.72	(0.36) ^{b,a}
	8	1.48	(0.35) ^{a}	0.53	(0.20) ^{b}	0.38	(0.29) ^{c,b}	0.66	(0.24) ^{d,b}
	9	1.27	(0.38) ^{a}	0.95	(0.49) ^{a,b}	0.80	(0.40) ^{a,b}	0.63	(0.27) ^{b}
	10	0.58	(0.44) ^{a}	0.21	(0.19) ^{b}	−0.58	(0.50) ^{b}	0.50	(0.40) ^{a,b}
	11	−0.47	(0.31) ^{a}	−0.78	(0.34) ^{b}	−0.70	(0.27) ^{a,b}	0.41	(0.23) ^{a,b}
Yellowness (*b**)	0	3.33	(0.82) ^{a}						
	1	6.57	(1.73) ^{a}	6.89	(2.45) ^{a}	6.44	(2.17) ^{a}	8.20	(1.85) ^{b}
	2	8.21	(2.08) ^{a}	6.46	(2.56) ^{b}	8.39	(1.07) ^{a}	7.00	(2.01) ^{c}
	3	8.15	(2.86) ^{a}	10.50	(1.75) ^{b}	8.71	(1.47) ^{a}	5.89	(2.05) ^{c}
	4	6.62	(2.36) ^{a,b}	7.38	(2.28) ^{a,b}	8.48	(2.43) ^{a}	5.56	(2.68) ^{b}
	5	8.34	(0.97) ^{a}	7.38	(2.50) ^{b}	7.60	(1.61) ^{b,a}	3.32	(2.25) ^{c}
	6	9.60	(2.01) ^{a}	10.25	(1.99) ^{b,a}	6.17	(1.04) ^{c}	9.11	(1.83) ^{a,b}
	7	7.02	(3.36) ^{a}	6.75	(2.09) ^{b}	9.49	(2.27) ^{c}	9.00	(1.61) ^{c}
	8	10.46	(0.98) ^{a}	9.89	(1.55) ^{a}	9.55	(1.30) ^{a}	2.90	(2.00) ^{b}
	9	7.76	(2.59) ^{a}	8.74	(2.99) ^{b}	8.56	(1.42) ^{b}	7.39	(1.97) ^{a}
	10	7.69	(1.16) ^{a}	8.90	(1.87) ^{b,a}	11.59	(3.01) ^{c}	9.70	(1.50) ^{d,b}
	11	4.52	(1.35) ^{a}	12.30	(2.42) ^{b}	10.57	(3.04) ^{c,b}	6.23	(4.06) ^{d,a}

Results of color changes are expressed as mean (SD). Means with different letters (a–d) in the same day are significantly different at $p < 0.05$.

The formation of metmyoglobin is associated with the oxidation of oxymyoglobin (light pink color) during storage. *L** values showed a small difference for all samples throughout the storage period. Different authors refer to these slight changes in the values of *L** in meat through the storage time [395,396]. The *a** value (redness) is the most important color parameter in evaluating meat oxidation, as a decrease in redness makes the meat product unacceptable to consumers. In all samples, the redness (*a** value) decreased as storage time progressed. At the end of the study period (Day 11), the intensity of each color parameter was lower than the value measured at Day 0 as a result of the oxidation process, leading in this way to a change in color. It is clear that the protective effects of the test extracts against the color loss (*a** value decrease) in stored beef patties were not as pronounced as their effects against lipid oxidation. At the end of storage, the *a** values of the CD1 and CD2 samples (−1.02 ± 0.33) were significantly lower ($p < 0.05$) than those of others samples. BHT displayed the highest value of *a** at the end of the experiment. Therefore, the natural plant extracts affected meat

color, specifically redness, and are therefore potentially useful in prolonging the shelf life of the meat product. Several authors have reported an *a** value decrease in different meat and meat products stored under a modified atmosphere [396,397]. The samples had an initial yellowness (*b**) value of 3.33 ± 0.82. Significant differences ($p < 0.05$) were observed in *b** values in all samples throughout storage.

4.5.3. Conclusions

Our experiments with raw beef patties indicated that the *C. decapetala* extract may be promising as a source of natural antioxidants for meat products. The analysis of TBARS, fatty acid degradation, antioxidant activity and concentration of volatile compounds provides a complete assessment of the consequences of lipid oxidation in burger patties. The addition of this extract to the beef patties at 0.5 % was the most effective antioxidant. This concentration inhibited formation of TBARS and volatile compounds more effectively than the synthetic antioxidant BHT over the course of 11 days. Using this herb extract as an ingredient in burger patties may be an efficient strategy to enhance the nutritional value and safety of these meat products.

4.6. Gelatine-Based Antioxidant Packaging Containing Caesalpinia decapetala and Tara as a Coating for Ground Beef Patties

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Publicado en Antioxidants el 31 de marzo de 2016, 5, 10; doi: 10.3390/antiox5020010

4.6.1. Introduction

Research into the development of edible films and coatings to extend the shelf-life of food products has attracted increasing interest in recent years due to several factors, such as consumer demand for high quality food [398], government demand for reducing packaging waste and marketing demand for new products. Additionally, the food industry is constantly seeking new and improved packaging systems and materials in a further attempt to retard deteriorative changes of food quality and, consequently, extend food shelf-life [399].

These films can help maintain and improve the quality of fresh, frozen and processed meat foods by reducing moisture loss, lipid oxidation and colour deterioration and acting as carriers for antimicrobial and antioxidant food additives [400].

Oxidation processes represent some of the most significant mechanisms that cause food spoilage. Lipid oxidation is one of the major limiting processes responsible for the reduction in food shelf-life, since it leads to off-flavour, off-odour and has been linked to oxidation reactions that cause product discoloration and loss of vitamins. Synthetic antioxidants have been used to prevent lipid oxidation, but the increasing demand for natural products has renewed the interest in natural polymers as raw materials for edible coatings or films due to their potential to extend the shelf-life of food and reduce the complexity and cost of packaging systems [401].

Traditional packaging technologies used for fresh meat and processed meat products have consisted chiefly of vacuum packaging, Modified Atmosphere Packaging (MAP) and air-permeable packaging. In recent decades, technological advancements in materials, methodology and machinery have enhanced the efficiency and function of the packaging of meat products [298]. Gelatine is a protein-based polymer, widely used in the manufacture of edible films. As a consequence, the properties of films made from mammalian (chiefly porcine and bovine) gelatines have been widely studied [402]. Antioxidant active material is one type of “active packaging”, an innovative technology for food preservation based principally on mass transfer interactions between systems “food/packaging” [403]. The effect of active coating treatments with natural antioxidants on the uptake of lipid oxidation of food has been investigated [404]. Several studies have evaluated how antioxidants Butyl Hydroxy Toluene (BHT), Butyl Hydroxy Anisole (BHA), alpha-tocopherol and natural extracts) incorporated in packaging film migrate out of the film and retard lipid oxidation in the stored foodstuff [405]. Different plant or herb extracts have been incorporated in gelatine films to enhance the

antioxidant and/or antimicrobial properties, such as green tea extract [406,407], longan seeds and leaves [404], oregano or rosemary aqueous extracts [408] and murta ecotypes leaf extracts [409].

In order to meet consumer demands for more natural, disposable, potentially biodegradable and recyclable food packaging materials, research has focused on the incorporation of two plants rich in polyphenolic compounds, namely *Caesalpinia decapetala* (CD) and *Caesalpinia spinosa* (CS) (*Tara*), into the coating film. The genus *Caesalpinia* has long been used in Chinese traditional medicine. Plants of this genus have proven to be a rich source of compounds, such as diterpenoids, triterpenes, flavonoids, *etc.* However, information on their application to films is limited; thus, the aim of this work was to investigate the effectiveness of gelatine film to maintain the physicochemical properties of beef patties, maintaining the oxidative stability of these fresh food products.

Therefore, the objectives of this work were to develop a new type of active gelatine film enriched with Caesalpinia extract with added concentrations of ethanolic extract of CD and CS and to determine the lipid inhibition on ground beef patties during chilled storage, in addition to analysing the optical, mechanical, barrier and antioxidant properties of these films.

4.6.2.Results and Discussion

4.6.2.1. Total Phenolic Content and Antioxidant Activity

Folin-Ciocalteu phenol reagent was used to obtain an estimate of the phenolic groups present in the gelatine film containing plant extracts. The control films (without extract) contained no phenolics (data not shown). The total polyphenol content of gelatine film with herb extracts is shown in Figure 39a. As expected, total phenol content increased significantly by the incorporation of more extract ($p < 0.05$). The phenol content as Gallic Acid Equivalent(GAE) per gram of films ranged from 178 to 515 mg GAE/g film for CS and 61 to 191 mg GAE/g film for CD. The highest value (515 ± 27 mg GAE/g film) was for the film formulated with CS3 (0.2% extract), and the lowest value (61 ± 12 mg GAE/g film) was for the film with CD1 (0.3 % extract).

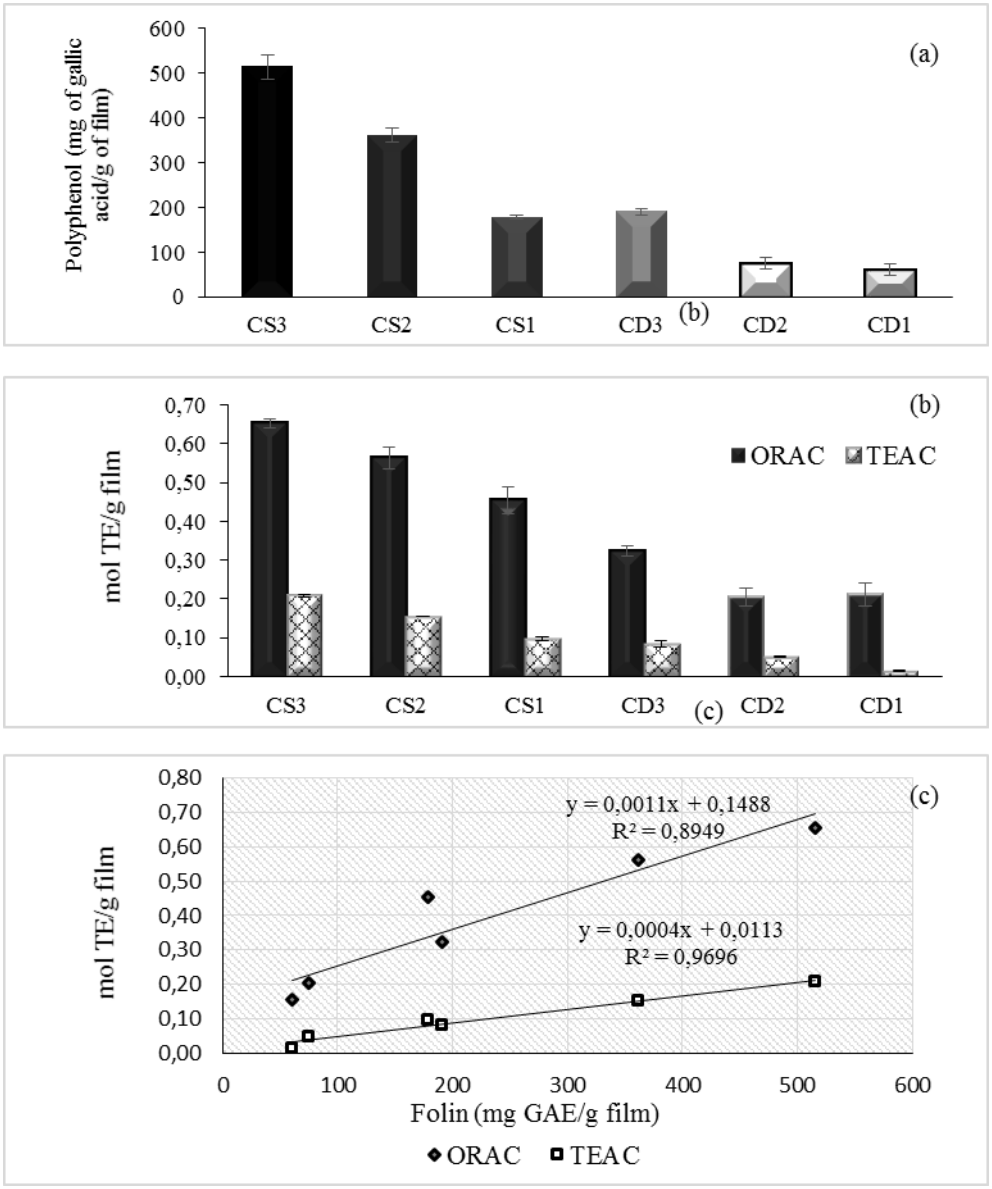

Fig. 39. (a) Polyphenol concentration of films with different concentrations of plants extracts: *Caesalpinia spinosa* 1 (CS1) (0.07%), CS2 (0.1%), CS1 (0.2%), *Caesalpinia decapetala* 1 (CD1) (0.3%), CD2 (0.7%), CD1 (1%). (b) Antioxidant activity by oxygen radical absorbance capacity (ORAC) and Trolox equivalence antioxidant capacity (TEAC) assays of films with different concentrations of plant extracts. (c) Correlation for polyphenol content from the Folin assay with antioxidant activity assessed by the TEAC and ORAC assays.

The antioxidant activity was measured by the ORAC and TEAC assay (Fig. 39b). The ORAC method measure the loss of fluorescence of a probe (fluorescein) in the presence or absence of an antioxidant. The ORAC value was 0.65 ± 0.01 mol Trolox Equivalent (TE)/g film for CS3 at a concentration of 0.2 % and 0.32 mol TE/g for CD3 at a concentration of 1 %. Moreover, when using the TEAC assay, based on the ability of an antioxidant to reduce the $ABTS^{\bullet+}$ radical [410], the antioxidant activity was higher at 0.21 ± 0.007 mol TE/g film for CS3 and lower at 0.02 ± 0.001 mol TE/g film for CD3.

The polyphenol content assessed by the Folin-Ciocalteu assay correlated with antioxidant capacity assessed by the TEAC and ORAC assays (Fig. 45c). The TEAC assay was the antioxidant assay that best correlated with the total phenolic content in the extracts (r^2 = 0.9696). The correlation coefficient for the ORAC assay was r^2=0.8949.

Packaging with antioxidant properties is a promising technique to extend the shelf-life and maintain the quality of food. The Folin assay is a useful tool to know the total polyphenols, and the ORAC and TEAC assays are important techniques for measuring antioxidant activity.

The Folin values were higher than those for other films containing natural extracts with antioxidant power, as the film incorporated with aqueous chitosan green tea extract (20% *w*/*v*), where the total polyphenol content was about 33 mg GAE/g film [411]. Similarly, according to the study by Araujo et al.[318] with ethanolic extract propolis, the polyphenols were proportional to the extract incorporated in films. The antioxidant capacity of the films should be strongly related to the portion of film that can be dissolved in water and, consequently, to the release of active compounds.

The antioxidant capacity assessed by the ORAC and TEAC assays was higher in the CS3 (0.2%) extract films. The antioxidant activity is in direct relation to the concentration of polyphenols in the herb extract. Comparing to other plants, the ORAC value for the film containing CS was higher than the values found for commonly-consumed herbs with high antioxidant capacity, including basil, marjoram, oregano, ginger, thyme and black tea (0.048 mmol/g DW, 0.27 mmol/g DW, 0.14 mmol/g DW, 0.39 mmol/g DW, 0.27 and 0.013 mmol/g DW, respectively). The results obtained for these assays are consistent with the CS composition, which contains a high proportion of phenolic compounds. The phenolic compounds are free radical acceptors that delay or inhibit the initiation step of autoxidation or interrupt the autoxidation propagation step [412]. The high antioxidant capacity is due to the rich content of polyphenols contained in these plants, especially CS. The CS tree was traditionally considered the second-richest tannin feed stock after *Schinopsis balansae* [413]. Many studies over recent years have demonstrated that the antioxidant activity of plants is caused mainly by phenolic compounds [414].

The results indicated that incorporation of CS and CD into gelatine film enhanced the antioxidant activity of the film. Several studies show that the incorporation the ethanolic extracts of plants improve the antioxidant power of film. Norajit et al.[415] observed that the incorporation of a ginseng ethanolic extract improved the antioxidant activity of film compared to the control film. Gómez-Estaca et al.[408] showed that the incorporation of borage ethanolic extract to gelatine films gave rise to a high reducing ability with FRAP.

4.6.2.2. Characterization of Bioactive Films

4.6.2.2.1. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of films are shown in Fig. 40. Samples showed the amide A band located at about 3300 cm^{-1}, the amide I band located between 1700 and 1600 cm^{-1}, the amide II band located between 1600 and 1500 cm^{-1} and the amide III band between 1200 and 1400 cm^{-1}.

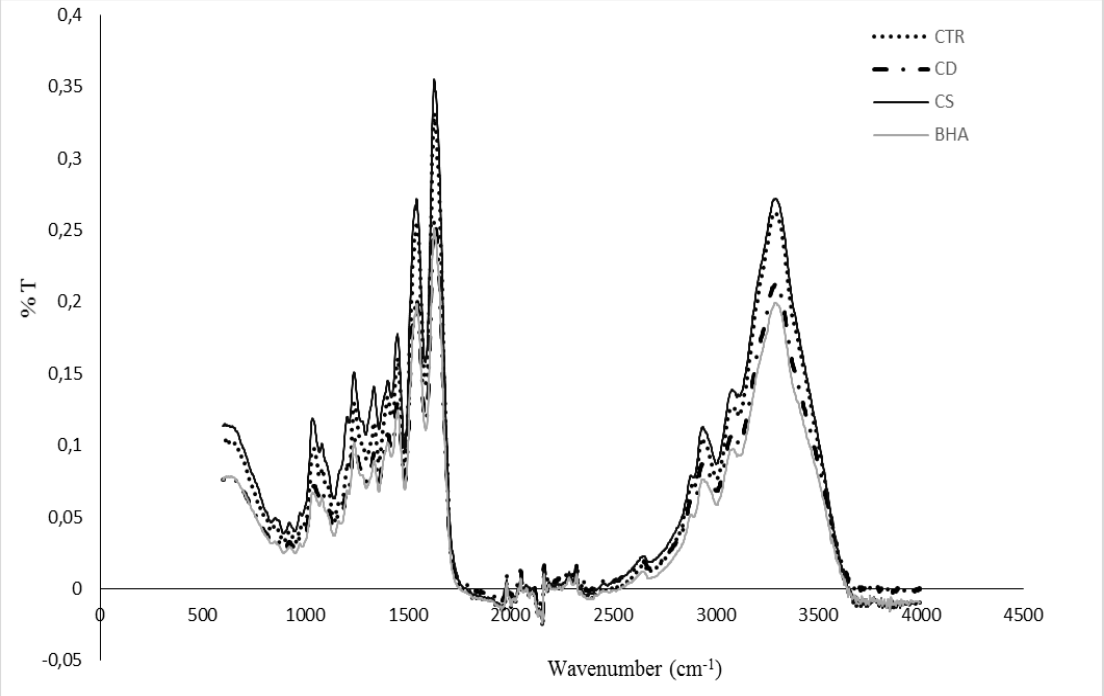

Fig. 40. Infrared spectra of gelatine-based films treated with different samples: CTR (control), BHA, CS3 and CD3.

FTIR spectra showed that there was no interaction between the functional groups of plants and gelatine. The absorption bands in the spectra were situated in the amide band region. Amide A is attributed to the stretching of the N-H group. The amide I band is related to the stretching CO, and the amide II band is related to the stretching of C-N and the angular distortion of the N-H bond. The samples containing CD and CS showed similar spectra to the control gelatine film, suggesting that there is no interaction between the functional groups of the extracts and gelatine. Similar results are in agreement with [250,416].

4.6.2.2.2. Mechanical Properties

The effects of CD and CS addition on the Tensile Strength (TS) and Elongation at Break(EAB) of the film are presented in Table 14. The addition of CD1 (0.3 %), CS1 (0.07 %) and CS2 (0.1 %) significantly increased tensile strength. CD2, CD3 and CS3 samples had values below that of the control sample. It was observed that values of EAB were inversely proportional to the concentration of the plant extracts added in the film, showing a decrease by increasing the

concentration of the plants, and in the case of TS, it was directly proportional to the concentration of plant extracts.

Table 14. The effects of CD and *Tara* at different concentrations on the tensile strength (TS) and elongation at break (EAB) of gelatine films.

Sample	TS (Mpa)	EAB (%)
CTR	69.8 ± 9.5 $^{a, c}$	95.6 ± 13.5 ^{a}
CD 1	96.4 ± 6.2 ^{b}	108.0 ± 15.9 ^{a}
CD 2	68.5 ± 18.2 ^{a}	231.1 ± 82.6 ^{b}
CD 3	57.0 ± 5.8 ^{c}	404.4 ± 51.8 ^{c}
CS 1	123.8 ± 1.5 ^{d}	178.9 ± 53.3 ^{b}
CS 2	86.6 ± 10.1 ^{a}	58.6 ± 5.8 ^{d}
CS 3	55.7 ± 10.6 ^{c}	357.4 ± 40.1 ^{c}
BHA	109.5 ± 12.5 ^{e}	62.9 ± 15.2 ^{d}

Different lowercase letters (a–e) in the same column indicate significant differences ($p < 0.05$) between samples.

Different antioxidant agents can influence the mechanical properties of the films. Our results for mechanical properties are consistent with various other studies. Bodini et al. [250] in their study with gelatine films using propolis extract suggested that the ethanol propolis extract acted as a plasticizing agent, increasing the mobility of the polymer matrix, which, in turn, promoted a reduction in tensile strength and increased film elongation. Nuñez et al. [417] reported that the addition of lignin produced an evident plasticizing effect, as deduced from significant decreases in TS in the composite films, alongside a marked increase in EAB. Hoque et al. [418] studied the mechanical properties of films prepared from gelatine and partially hydrolysed gelatine containing different herb extracts. Films made from gelatine containing cinnamon, clove and anise showed higher TS, but lower EAB. Lim et al. [419] reported that the TS of agar-based films containing nano-clays decreased with an increased concentration of grape seed extract in the film matrix up to 1.2 %. Similar results have been also reported for agar-based films containing green tea extracts [406].

In general, the incorporation of polyphenol-rich aqueous extracts reduces the mechanical properties of the films [406]. However, there is a relationship between the concentration of the plant extract and the film's mechanical properties.

4.6.2.2.3. Water Vapour Permeability (WVP)

WVP is one of the most important parameters for biodegradable films. This parameter was studied to evaluate the combined effect of CD and CS on the barrier properties of the gelatine film. The WVP of the gelatine-based film containing CD3 (1 %), CS3 (0.2 %) and BHA is shown in Table 15. As can be seen from this table, the control film showed the highest WVP, followed by the film containing added CS. The lowest WVP was found in the gelatine film treated with CD and BHA. There were no significant differences between the CD film and the BHA-treated film ($p > 0.05$).

Table 15. Water Vapour Permeability (WVP) of the different gelatine films.

Sample	WVP
Control	1.94 [a] ± 0.1
CD 3	1.11 [b] ± 0.2
CS 3	1.32 [c] ± 0.2
BHA	1.12 [b] ± 0.1

Different lowercase letters in the same column indicate significant differences ($p < 0.05$) between samples.

The results obtained showed that the addition of CS extract improved the barrier properties of gelatine films. Rattaya et al. [420] said that the chemical nature of the macromolecule, the structural/morphological characteristics of the polymeric matrix, the chemical nature of the additives, as well as the degree of cross-linking all affect the barrier characteristics of the film. Wu et al. [407] reported that the incorporation of green tea extract into gelatine film caused the resulting film to have lower WVP. They hypothesized that polyphenolic compounds could fit into the gelatine matrix and establish cross-links with the reactive groups of the gelatine through hydrogen bonds or through hydrophobic interactions. Bodini et al. [250] reported that the incorporation of ethanol-propolis extract led to a significant reduction in WVP in relation to the control gelatine film. The WVP should be as low as possible for food packaging in order to avoid or at least to reduce moisture transfer between the food and the atmosphere, or between two components within a heterogeneous food product [421].

4.6.2.2.4. Light Absorption

Table 16 shows the absorption of samples at 600 nm and the opacity. The lowest transmittance was for the film containing BHA. The film with CD3 had the lowest percent transmittance (%T) for UV radiation (64.86 %), and the opacity value was higher (14.46 %), compared to the control, so it is clear that inclusion of the extract at this concentration into gelatine improved the light barrier properties.

Table 16. Light transmission (%T) and opacity of gelatine films with CD and CS extracts at different concentrations of the different gelatine films.

Samples	%T	Opacity
Control	87.00	4.46 [a] ± 0.02
CD 1	86.30	4.92 [d] ± 0.02
CD 2	85.51	5.23 [c] ± 0.03
CD 3	64.86	14.46 [b] ± 0.01
CS 1	88.10	4.23 [g] ± 0.02
CS 2	87.30	4.54 [f] ± 0.03
CS 3	85.90	5.08 [e] ± 0.02
BHA	79.98	7.46 [h] ± 0.01

Different lowercase letters in the same column indicate significant differences ($p < 0.05$) between samples.

Transparent packaging allows the oxidation and degradation of nutritional compounds, because light acts as a catalyst for these processes. Therefore, opaque packaging and packaging containing specific compounds that absorb light in the UV-VIS spectrum have been developed to prevent these reactions. Plant extracts are commonly used to provide colour and opacity to polymers. A food packaging film is required to protect food from the effects of light, especially UV radiation [414].

In general, light transmission at 600 nm for all films was in the range of 64.86%–87%. With the addition of herb extracts, variations in light transmission of the resulting films were observed. The incorporation of a higher concentration of the extracts in the film produces an increase in opacity of the film. The observed differences in the transparency of the films can be attributed to differences in concentration, colour, polyphenols present in extracts and the extracts' interaction with the gelatine film [27].

4.6.2.2.5. Scanning Electron Microscopy

Figure 47 shows the Scanning Electron Microscopy (SEM) of gelatine films treated with CD, CS and BHA. It was observed that the surface of the control film was very homogeneous without bubbles. The incorporation of the synthetic antioxidant BHA allowed the formation of crystals in the film matrix. The addition of more plant extracts to the film generated a more heterogeneous surface. In the case of film containing CD1 and CD2 extracts, the samples showed an appearance very similar to the control, and the same was observed in the film treated with CS1 *Tara* extract.

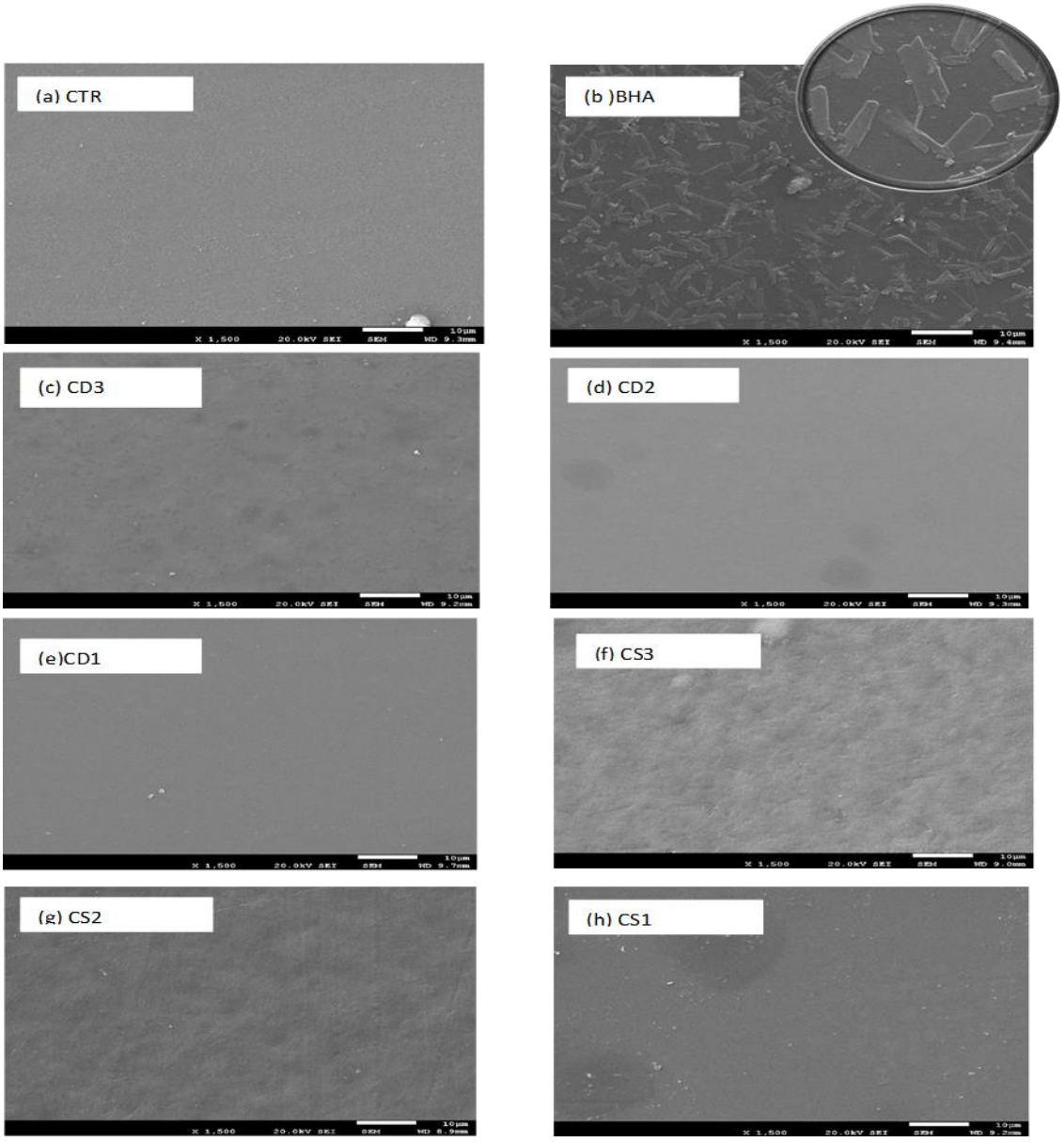

Fig. 41 Scanning electron microscopy of gelatine films with different concentrations of plant extracts (1500×).

Higher concentrations of the extracts used in the films (CD3, CS2 and CS3) formed a more heterogeneous structure with pore formation in the matrix. The formation of a heterogeneous surface can be related to a reduction of the dissolution of extract in gelatine when the concentration is higher. Similar results have been reported by other authors. Bodini et al.[250] showed that increased ethanol-propolis extract concentrations produced an increase in the porosity of the matrix, which was probably associated with distribution of extract in the polymer matrix. Hoque et al.[418] showed that a smooth surface was also obtained in the film prepared from gelatine treated with star anise. Furthermore, Li et al.[414] reported that the micrographs of films displayed a heterogeneous surface and porous appearance after the addition of natural antioxidants (grape seed extract, gingko leaf extract, green tea extract) at a concentration of 1.0

mg/mL. In general, the smooth surface became rougher when the concentration of herb extracts was increased.

4.6.2.2.6. Colour Properties

In practical applications, colour quality might influence the appearance of edible films, which in turn affects the acceptance of foods by consumers. The incorporation of plant extracts improves the film's ability to block UV and visible light. The *Commission Internationale de l'Éclairage* (in French, (CIE) colour values in the films with or without herb extracts are shown in Table 17. L* values did not differ significantly between samples. For example, the L* values for control, CD film and CS film were 74.12 ± 1.21, 72.35 ± 1.04 and 75.16 ± 0.81, respectively. With the addition of phenolic compounds, the L* values of composite films were practically unchanged. The control had an initial yellowness (b*) value of 4.51 ± 1.47, and the addition of the extracts caused a significant increase ($p < 0.05$) in this value.

Table 17. Colour parameters of gelatine films: control, CD3 (1 %), CS3 (0.2 %) and BHA.

Samples	L*	a*	b*
Control	74.12 ^{a} ± 1.21	0.86 ^{a} ± 0.30	4.51 ^{a} ± 1.47
CD 3	72.35 ^{b} ± 1.04	−1.39 ^{b} ± 0.63	8.37 ^{b} ± 1.45
CS 3	75.16 ^{a} ± 0.81	2.33 ^{c} ± 1.33	8.15 ^{b} ± 1.52
BHA	73.93 ^{a} ± 1.53	−1.69 ^{d} ± 0.41	7.77 ^{b} ± 0.80

Mean values are showed for color parameters (lightness, L*; redness, a*; yellowness, b*)

Different lowercase letters ($^{a–d}$) in the same column indicate significant differences ($p < 0.05$) between samples.

As a general trend, the addition of phenolic compounds caused an increase of blueness for composite films as indicated by increased b* values and caused a decrease of redness (a* values), except for the CS film.

Previous authors have reported that the addition of phenolic compounds caused a reduction of the brightness of edible films [238]. Ahmad et al.[422] showed that the incorporation of lemongrass oil into fish skin gelatine films increased its total colour difference. However, no marked effect on the colour parameters of films was obtained when ginger oil was added [423]. The type of plant used influenced the colour of the gelatine film, depending on the type and concentration incorporated.

4.6.2.3. Evaluation of Antioxidant Activity in Food

4.6.2.3.1. TBARS Assay

Fat content is one of the most important quality indicators of minced beef products. The minced beef has approximately 17,3 % of fat [424]. A direct method. Thiobarbituric Acid Reactive Substances (TBARS) was used to evaluate the effectiveness of the antioxidants in preventing

oxidative degradation of lipids with the production of compounds, such as conjugated hydroperoxides and aldehydes. The Thiobarbituric Acid (TBA) values were calculated as mg Malondialdehyde (MDA)/kg meat. TBARS indices were significantly ($p < 0.001$) affected by storage time and the active packaging system. The TBARS values of all samples increased continuously up to 12 days of storage (Fig. 48a). TBARS values of control, CD3 (1%), CS3 (0.2%), WT (1%) and BHA were 0.59 ± 0.04, 0.33 ± 0.01, 0.30 ± 0.00 and 0.26 ± 0.01 mg MDA/kg at Day 3 and increased to 1.27 ± 0.10, 0.53 ± 0.01, 0.27 ± 0.02, 0.27 ± 0.04 and 0.98 ± 0.01 mg MDA/kg at the end of storage (Day 12). The highest rate of increase was observed in the patties treated with the control gelatine film (without plant extract), while the lowest TBARS value was found in the patties stored with gelatine film treated with CS, followed by samples treated with WT.

It was noted that the films containing natural extracts did not reach a value of 1.5 mg MDA/kg by the end of the long storage period (Day 12). It has been reported that an index of 1.5 is closely related to perceptible and unacceptable off-odour of meat [94].

In our study, samples stored with WT film and CS film behaved similarly with no significant difference ($p > 0.05$). However, when compared to the sample stored under CD film, there was a noticeable difference, since this was less effective. In this study, the samples reduced lipid oxidation relative to the control in the following order: CS>WT>CD>BHA. These results are consistent with polyphenol concentrations obtained with the Folin method, which gave values for CD (27.71 ± 1.03 mg GAE/g DW) and CS (368.90 ± 0.6 mg GAE/g DW) and with antioxidant activity assessed by the ORAC method that showed the same order for the plant extracts.

The mechanism by which the antioxidant-treated packaging lowers the number of molecules reactive to TBA is currently under investigation. Inactivation of free radicals by either migration of antioxidant molecules from the active film to the meat or scavenging of those oxidant molecules from the meat into the active film may be considered as hypotheses for its mechanism of action. Our results are in agreement with various other studies in films, all of which reported that natural antioxidants from culinary herbs and edible plants were effective at controlling lipid oxidation and extending the shelf life of meat products. However, Lorenzo et al.[425] studied the effect of antioxidant active systems on lipid stability of foal steaks during storage. Active films with oregano essential oil (2 %) resulted in a decreased lipid oxidation of foal steaks, and these were more efficient than those treated with green tea (1 %). Similarly, Camo et al.[88] reported that fresh lamb steaks were treated with three different preparations of natural antioxidants containing rosemary and oregano, which resulted in the enhanced oxidative stability of lamb steaks. Furthermore, similar results have been obtained when other compounds were incorporated, such as essential oils [422] and grapefruit extracts [426]. In all cases, the oxidation rates decreased, maintaining an acceptable quality in meat, poultry or fish products.

These results indicated that lipid oxidation in beef patties could be minimized by the use of gelatine film treated with natural extracts. We found that a concentration of 0.2 % of CS extract in the film showed strong antioxidant activity against lipid oxidation in ground beef patties.

4.6.2.3.2. Metmyoglobin

The rates of formation of surface metmyoglobin in fresh beef patties are shown in Figure 42b. Metmyoglobin percentage for the control increased rapidly in the first seven days of storage, reaching values above 39.5%, but in samples stored under films treated with antioxidants, the increase in TBARS values was slow and steady.

The discoloration that occurs during the display of red meat cuts is generally associated with the accumulation of metmyoglobin in the meat surface. During refrigerated storage, MetMb accumulation and meat discoloration largely depend on the presence of reducing systems in meat and on lipid oxidation [427].

Djenane et al.[105] reported that a consumer panel rejected samples of fresh beef with a percentage of MetMb greater than 40%. Using this limit, the presence of natural antioxidant extracts led to a significant ($p < 0.05$) inhibition of metmyoglobin formation. The film treated with CS reduced the % of metmyoglobin (37.40 %) to less than 40 %, and this value was very similar to that obtained in treatment with WT (37.19 %), showing the positive effect of the natural extracts in the active films on the colour of refrigerated fresh patties.

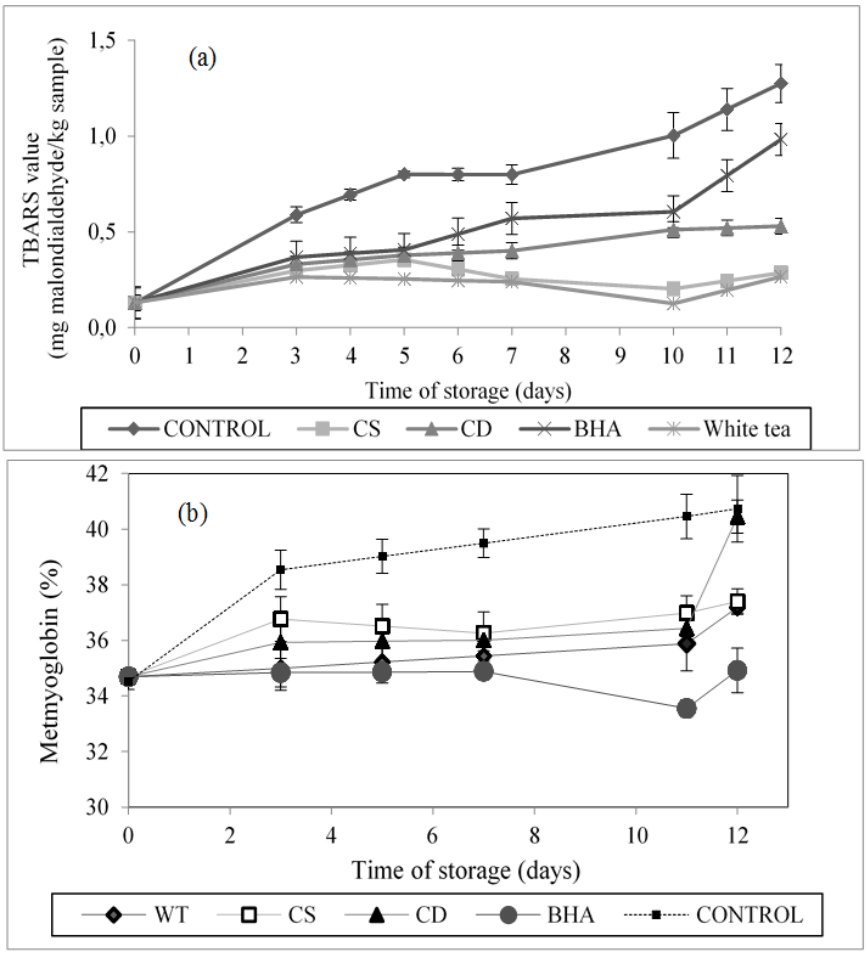

Fig. 42. (a) Effects of films with CD and CS added at 1% and 0.2%, respectively, on the TBARS value (mg MDA/kg of sample) of raw beef patties during 12 days of refrigerated storage at 4 °C and (b) effects of CD and CS extract added on metmyoglobin changes in beef patties during 12 days of refrigerated storage at 4 °C. Results are given as the mean ± standard error.

4.6.3.Conclusions

From the results presented, it is possible to conclude that CD and CS incorporation caused an improvement of the gelatine film properties, reducing tensile strength, and the CS extract reduced the permeability to water vapour of films relative to the control. Films exhibited high antioxidant activity, especially for films prepared with the addition of 0.2 % of CS and 1 % of CD. The analysis of TBARS provided a complete assessment of the consequences of lipid oxidation in beef patties. The CS film was the most effective antioxidant for the beef patties, inhibiting the formation of TBARS more effectively than the synthetic antioxidant BHA over the course of 12 days. Results indicated that biodegradable gelatine films containing CD and CS have good potential for utilization in food packaging. Advances in active packaging materials based on renewable sources, such as gelatine containing natural extracts, will open new lines of research for the development of improved eco-friendly materials.

Estudios con Plantas de la Familia Rubiaceae

(*M. citrifolia*)

- Active packaging incorporationg *Noni* extract to maintain the oxidative stability of meat Burger.

4.7. Active packaging incorporating *Noni* extract to maintain the oxidative stability of meat burger

M.Gabriela Gallego, Francisco Segovia, María Pilar Almajano

Artículo enviado a Journal of Food Science en septiembre de 2016

4.7.1. Introduction

Morinda citrifolia L. (*Noni*) grows in tropical and sub-tropical regions. This plant has positive effects against cancer, diabetes and obesity [77,428]. Approximately 200 phytochemical compounds were identified; the most abundant compounds are organic acids and alkalis. However, the chemical composition largely differs depending on the part of the plant [200].

It is very well known that meat is the muscle tissue of slaughter animals composed of water, proteins, lipids, minerals and a small proportion of carbohydrates. Meat and meat products are susceptible to quality deterioration due to their rich nutritional composition [429].

From the foregoing, it is necessary to protect the meat. Specifically, the most important packaging technologies used to increase the shelf life of meat are vacuum packaging, modified atmosphere packaging, intelligent packaging, active packaging, bioactive packaging, etc. Active packaging is a novel technique used to preserve various foods including meat by the release of the active agents which have been incorporated into the package during its preparation [243,403]. The release of active agents can be controlled over an extended period of time to maintain or extend the quality and shelf-life of products, without the need for direct addition of any substances to the foodstuff [430]. Increasing demand for synthetic packaging materials has put tremendous pressure on the environment because of their poor biodegradability and non-renewability [296,431]. Studies considering alternative systems for food protection that use biopolymers have increased significantly in the last years because these materials are entirely biodegradable and often edible and have few adverse environmental effects [432].

Bioactive packaging involves the use of edible packaging material with the incorporation of edible active substances. A number of studies have reported the use of purposefully adding antioxidants for extending the shelf life of meat products. Nisa et al. [246] reported that the addition of green tea extract resulted in decreases in Thiobarbituric Acid Reactive Substances (TBARS) values. Garrido and others [433] applied red grape pomace to pork burgers. Hayes et al. [434] applied lutein, sesamol, ellagic acid and olive leaf extract in fresh and cooked pork sausages. The oxidative stability of meat can be extended by using antioxidants and proper packaging [435].

Therefore, the objectives of this work were to develop an active packaging film of gelatin with *Noni* extracts. Characterization of films was carried out by the determination of physical, barrier,

morphological and mechanical properties. Films were also applied to fresh beef to study their effect on lipid oxidation.

4.7.2.Results and Discussion

4.7.2.1. Total Phenolic Content (TPC) and Antioxidant Activity

Table 18 showed the Total phenolic content of *Noni*. The value in *Noni* leaves extract was 4.76 mg Gallic Acid Equivalent (GAE)/g Dry Weight (DW). Different polyphenolic compounds have been found in this plant and its fruit. The main ones are, scopoletin that is found in *Noni* fruit juice, with anti-inflammatory and antioxidant activities [202,436,437] and the rutin, a flavonoid with a high antioxidant potential [213].

Table 18. Content of Total Polyphenols (TPC), and antioxidant activity assessed by the DPPH, ORAC and TEAC assay for the *Noni* extract.

Total polyphenols (mg GAE/g DW)	**4.73**	**(0.3)**
TEAC (mmol Trolox/g DW)	0.09	(0.002)
ORAC (mmol Trolox/g DW)	0.017	(0.01)
DPPH (µmol Trolox/g DW)	0.017	(0.0004)

Mean values of three replicates (*n*=3); standard deviations are included in brackets.

The TPC obtained in the leaf extract of *Noni* was comparable with different studies of this plant. Chan-Blanco et al. [438] and Correia et al. [439] obtained values in *Noni* pulp between 0.51-2.16 mg GAE/g. Likewise, Krishnaiah and others [440](2015) obtained a content of phenolic in the pulp dehydrated of 4.31 mg EAG/g. Costa and others [441] who determinated the antioxidant activity in pulp, bark and seeds of *Noni*, found a content of phenolic compounds for ethanolic extracts of 0.20 mg GAE/g. These values were lower than the obtained in the present study. Nevertheless, Žugić et al. [209] determined the best solvent to obtain greater antioxidant capacity of the *Noni* fruit cultivated in Paraná and they reported a considerable TPC value of 9.67 mgGAE/g.

The discrepancies in the levels of values of phenolic compounds, according to Soares et al. [442], They can be attributed to several factors, including the degree of ripeness of the fruit, crop conditions, the storage form, geographical area. This is where, according to the findings by Assanga et al. [443], the phenolic content of the *Noni* fruit varied with the stages of maturation and climate seasons, with the highest levels were found in mature fruits collected in spring, summer and autumn.

HPLC chromatogram (Fig. 43) of the *Noni* showed he existence of the following polyphenolic compounds: vanillin (4), gallic acid (1), vanillic acid (2), ferulic acid (5) and rutin (3), see Table 19. The concentration of rutin was the highest (0.31 mg/g DW).

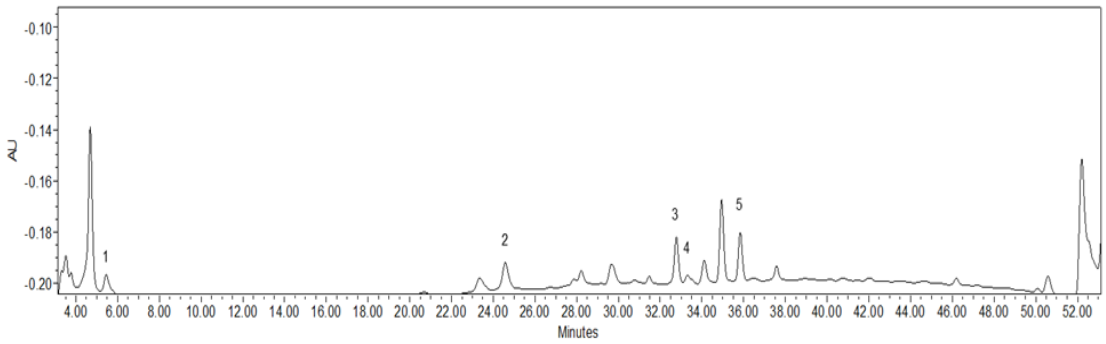

Fig. 43. HPLC chromatogram of ethanol extract of *Noni* leaf. For peak annotations, refer to Table 2.

Table 19. Quantification by HPLC of antioxidant compounds in *Noni* leaf extract.

Peak	Retention Time	Compound	Area	mg/g DW
1	5,45	Gallic acid	190570	0,08
2	24,57	Vanillic acid	226600	0,14
3	32,78	Rutin	266400	0,31
4	33,31	Vanillin	67920	0,03
5	35,84	Ferulic acid	267219	0,14

Previous studies reported the presence of rutin, vanillin and vanillic acid in this plant [210,444]. The concentrations of phenolic compounds were–higher than those described in the literature. Dussossoy et al. [210] studied the antioxidative and anti-inflammatory actives and characterized the *Noni* juice from Costa Rica, reporting that *Noni* juice contained 0.04 mg/g fresh weight of rutin, 0.35 mg/g fresh weight of vanillin and 0.0026 mg/g fresh weight of vanillic acid. Pandy et al. [207] who investigated the intervention of *Noni* and its bioactive compoundson the dopaminergic and noradrenergic systems, found in methanolic extract of *Noni* fruit 1.66 µg/mg DW of rutin. This compound is a important bioactive constituent in the pharmacological field (Pachauri and others 2012). Rutin is a flavonoid that plants synthesize and use it as a mechanism to protect against ultraviolet radiation and diseases [446], with great antioxidant power [375]. In the *Noni* has been described the presence of quercetin and kaempferol rutinoside [210]. The phenolic groups are reported in playing a vital role in antioxidant capacity.

Table 18 showed the antiradical activity measured by the methods: DPPH, measuring DPPH radical reduction by electron transfer of the antioxidant sample ABTS; It measures the ability of the antioxidant to reduce the ORAC assay ABTS and measures the ability of the antioxidant to neutralize the peroxyl radical. The DPPH and the ORAC assays gave rather similar results for the extracts (0.02 mmol TR/gDW). The results from TEAC assay differed from the two other assays with a value of 0.09 mmol TR/ g DW. The results are higher than the reported by Dussossoy and others (2011) to *Noni* juice 0.01 mmol TR/g for ORAC and 0.003 mmol TR/g for DPPH.If we compare our results with some fruit juices with high antioxidant properties, we can

say that the total polyphenols obtained are higher than those obtained for example, in orange juice, apple juice and tomato juice. [447] and lower than other plant used in culinary, like ginger [448]. However, the values obtained for TEAC assay were higher than plants from the *Lamiacea*e, *Rutaceae*, *Asteraceae* families and other plant species with medicinal properties [296].

4.7.2.2. Characterization of bioactive films

1.1.1.1.1. Fourier transform infrared spectroscopy (FTIR)

Spectra measured by FTIR is showed in Figure 44. The control film and with*Noni* added showed typical bands of the gelatin. Among them, the most common were the amide A to 3300cm-1, amide I located between 1700 to 1600 cm-1, amide II between 1600-1500 cm-1 and amide III between 1200-1400 cm-1 [250].

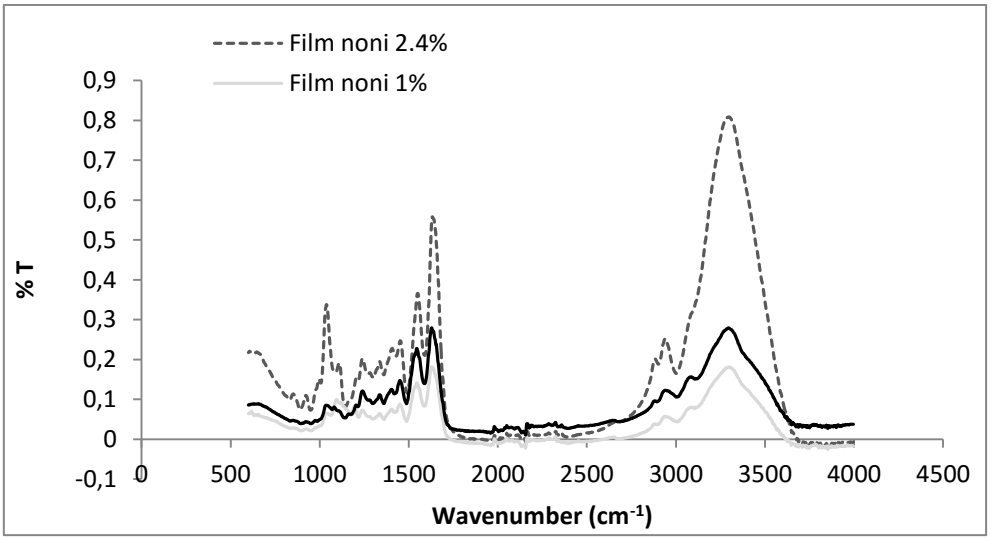

Fig. 44. Infrared spectra of gelatin-based films treated with different samples: CTR (control), NE1 and NE2.

The characteristics bands show that the amide A is due to stretching of NH, amide I is due to the stretching of C=O group together with a contribution of vibration deformation of the links CN and NH, amide II is due to stretching CN and NH deformation and amide III is due to the vibration of NH bond of amino groups It was observed that samples of film with NE1 control and show very similar spectra, this indicates that there is no interaction between the functional groups of the extracts and gelatin used. The film with NE2 sample compared to the control showed a slight change in the wavenumber. Similar results agree with Gallego and others [279].

1.1.1.1.2. Mechanical properties

Mechanical propertires of films are showed inTable 20. The thickness of the films was 15.2µm. The extract of *Noni* has no effect on film thickness [449].

The Elongation at Break (EAB) and Tensile Strength (TS) of the samples was determined. NE concentration used in the films affected the TS and EAB with increasing the concentration of NE it was observed a slight decrease in the TS value and increased EAB value. The up most TS value was found in the film incorporated with NE2 (2.4 %) and the lowest value was found in the control film.

Table 20. The effects of *Noni* extract at different concentrations on Tensile Strength (TS) and Elongation at Break (EAB) of gelatin films.

Sample	TS (Mpa)	EAB (%)	WVP (10-8g.mm/h.cm2.Pa)
CTR	85,51 ± 4,8	119.68 ± 37.4	1.81 a ± 0.1
NE1	83,31 ± 27,33	672,67 ± 34,58	1.31 b ± 0.2
NE2	80,58 ± 20,09	846,99 ± 312,1	1.96 c ± 0.2

Different lowercase letters (a-e) in the same column indicate significant differences ($p < 0.05$) between samples.

Lim and others [419] indicated low values of TS to increase the concentration of seed extract agar incorporated into film grape, which could be due to loss of interactions between molecules agar. Similarly, Vichasilp et al. [449] obtained lower TS when the quantity of longan seed extract incorporated into film increased. Different studies have reported that the natural additive incorporated into films acts as plasticizer [279]. Bodini et al. [250] indicated that the addition of propolis extract reduced the value of TS and incrmentó the value of EAB, because acted as a plasticizer. Núñez-Flores et al. [450] found that increasing the concentration of lignin in the film a plasticizing effect occurs, reducing the TS and increased EAB

1.1.1.1.3. Water Vapor Permeability (WVP)

WVP of films are showed in Table 23. The concentration added of NE in films influenced values of WVP. of NE (p<0.05). The highest WVP (1.96 10^{-8}g.mm/h.cm^2.Pa) was obtained by incorporating 1 % of NE and the lower WVP (1.31 10^{-8}g.mm/h.cm^2.Pa) in the film incorporated with 2.4 % of NE.

The inclusion of natural extract in the gelatin film can decrease the WVP. Giménez et al. [406] observed that adding green tea extract in the gelatin films agar WVP values decreased. Is said to various factors such as, the chemical nature, morphology and characteristics of the structure of the polymer matrix and the nature of the additives incorporated can affect the barrier properties [420]. Vichasilp et al. [449] observed a lower WVP values when the concentration of seed extract of longan was higher (500 ppm). Wu et al. [407] informed that the values of WVP decreased with the addition of green tea extract. Polyphenols can fit in a gelatin matrix and establish crosslinks by hydrogen bonds or hydrophobic interactions with reactive groups of the gelatin matrix

4.7.2.2.1. Scanning Electron Microscopy (SEM)

The results of SEM image shown in Figure 45. Control film has homogeneous and compact surface without observing pores in the structure less homogeneous when NE was incorporated especially when the concentration of the plant is greater. However, the surface was fairly compact and uniform, even in the film with the greatest concentration of NE. In the film surface with EN2, large spheres were observed, possibly due to the concentration of the extract.

Wu et al. [407] said that the structure can be more compact, when increased the interaction between protein and polyphenols is give. Nagarajan et al. [451] mentioned that the presence of voids in the polymer matrix is related to the less interaction between the protein molecules. Jridi et al. [452] reportedthat the structure of the film depends on the distribution of molecules within the network of the film. In this sense it relates to the molecular weight of the gelatin chain and with the interactions in the polymer matrix.

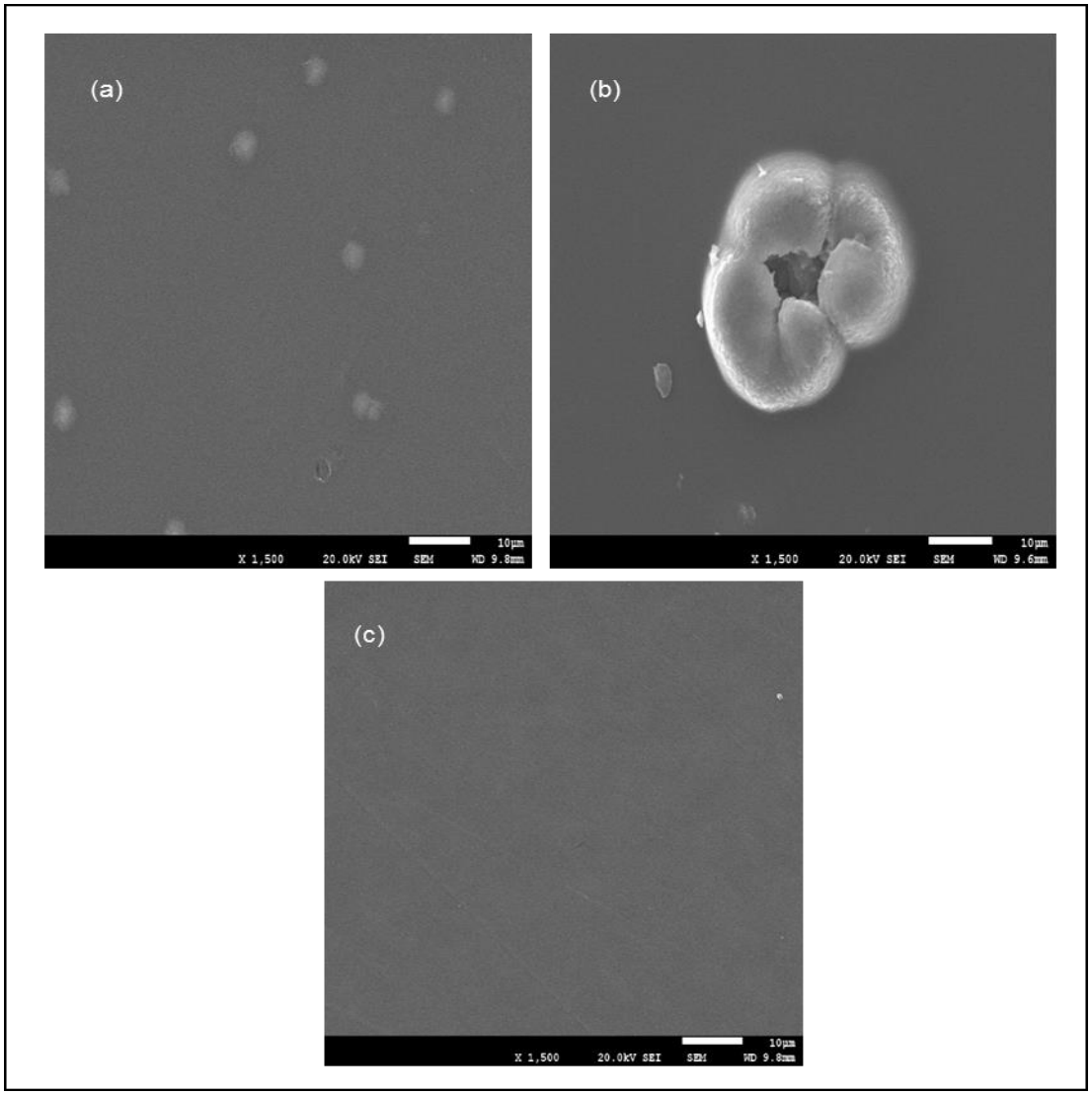

Fig. 45. Scanning electron microscopy of gelatin films with different concentrations of *Noni* extracts (1500x)

1.1.1.1.4. Color properties and Light absorption

Table 21 showed the effects of the concentration of *Noni* on the color parameters: L, a*, and b*of coating. Concentration of NE added in the film influenced in the color. The addition of NE had a considerable effect. Was observed that adding NE 2.4%, the values of lightness (L) in films were lower. The control sample has the highest values of L. The redness (a*) values increased with the concentration of plant extract. The yellowness (b*) of the film was higher when was incorporated NE extract. Similarly results are reported by Vichasilp et al. [449] who studied films with addition of longan seed extract. Gallego et al. [279] reported that the addition of extract of *C. decapetala* caused an increment in b* values and a declinein a* values compared to the control sample. Similar results were obtained in the film with NE1.The transparency in film with NE was in the range of 91.15 % to 98.61 %. The lower transparency was observed in the control film, increased with the addition of plant extract. The opacity parameter is important in films particularly if they are used in food products [453]. The incorporation of natural extracts in the films can improve the barrier against light, which has been the subject of various studies [407].

Table 21. Color parameters and transparency of gelatin films: Control, NE (1%) and NE (2.4%).

	L	a*	b*	% Transparency
Control	73.20 ± 0.5 a	0.9 ± 0.7 a	5.1 ± 0.6 a	98.61 ± 0.2 a
NE1 (1%)	72.89 ± 1.4 ab	-1.8 ± 0.3 b	9.45 ± 1.5 b	96.23 ± 0.1 b
NE2 (2.4%)	71.50 ± 0.5b	-1.5 ± 0.5 b	10.13 ± 1.1 b	91.15 ± 0.1 c

Different lowercase letters in the same column indicate significant differences ($p < 0.05$) between samples.

4.7.2.3. Evaluation of antioxidant activity in food by TBARS assay

The lipid oxidation is measured by the TBARS assay mainly. At the end of the experiment (day 9) the control sample presented higher values of TBARS compared to the samples packaged with the film with NE. The results are shown in Figure 46. In this sense, the TBARS values of the burgersamples packaged with film with added NE at 1 % and 2.4 % increased from 0.15mg Malondialdehyde (MDA)/kg meat to 0.90 and 0.86mgMDA/kg meat, respectively. Furthermore, there were no significant differences between the film with NE 2.4% and the positive control (commercial synthetic antioxidant). The samples coated with the film turned out to be better than the samples mixed with dry plant directly,–which at the end of the study reached 1.10mgMDA/kg meat.

Different authors confirm the inhibiting lipid in meat products using plant extracts in films. Kim et al. [454] reported that the samples packaged with incorporated green tea extract film showed lower TBARS values. It is well known that polyphenolic compounds present in tea have a high antiradical activity [455]. Nisa et al. [246] also reported that the green tea extract incorporated

into a film based on potato starch, produces a protective effect against lipid oxidation. Ojagh et al. [456] described that the trout fillets with chitosan-cinnamon film had significantly lower TBARS value. the mechanism by which MDA is blocked is related to the antiradical capacity possess polyphenolic compounds. The hydroxyl group present in the aromatic ring of these compounds is able to give electrons and neutralize free radicals [457]. Polyphenolic compounds are found primarily in plants. Among these we can mention: catechins, ellagic acid and resveratrol which have two aromatic rings and favonoles have three rings. One feature that makes them effective is the number of -OH groups on the aromatic ring [458].

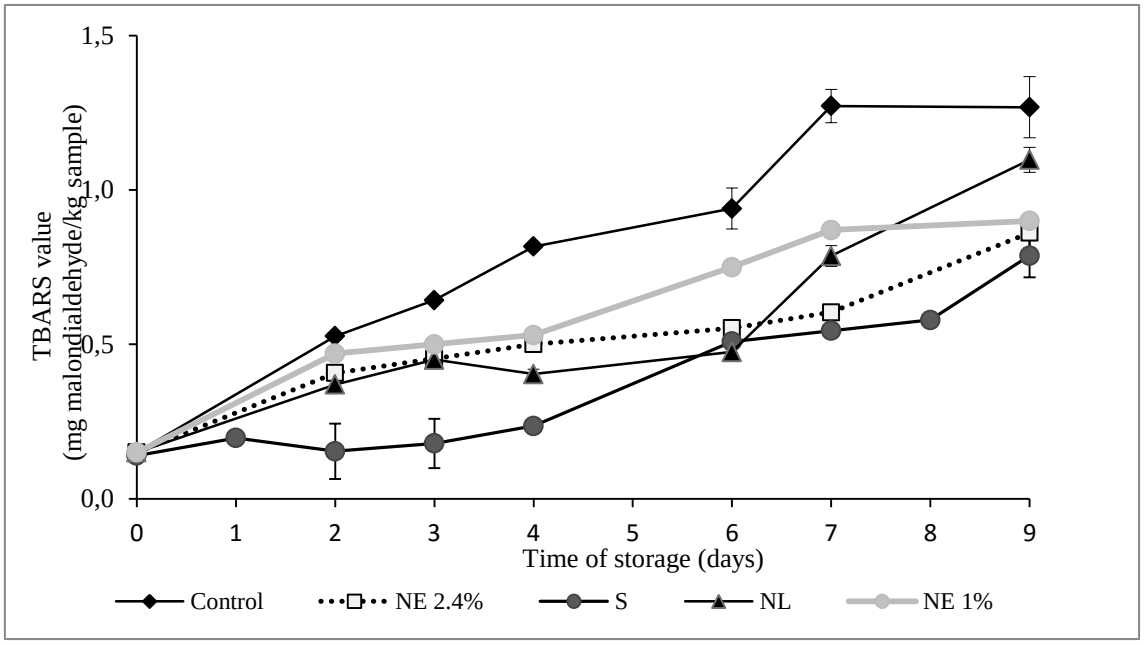

Fig. 46**.** Effects of films with NE at 1% and 2.4% on TBARS value (mg MDA equivalent/kg sample) of raw beef patties during refrigerated storage at 4°C.

The natural antioxidants are derived from plants and include alpha tocopherol (vitamin E; which each have one aromatic ring), catechins, ellagic acid and resveretrol (which each have two aromatic rings), and flavonols (which have three aromatic rings) have three aromatic rings). In general, the effectiveness of these natural antioxidants is proportional to the number of –OH groups present on the aromatic rings [458].

4.7.3. Conclusion

Gelatin film incorporated with extract of *Noni* exhibited good antioxidant properties on the fat oxidation in a concentration of 2.4 %. Films with incorporation of *Noni* extract mecáncias improved film properties, the water vapor permeability and opacity. Adding *Noni* extract 2.4 % in films, it optimizes overall film properties and provides antioxidant activity in meat products. Active packaging materials based on natural antioxidants opens the door for materials research that will increase the quality of foodstuffs naturally and are environmentally friendly.

Discusión Global de los Resultados

5. DISCUSION GLOBAL DE LOS RESULTADOS

En este capítulo se discuten en forma general los resultados obtenidos en los distintos estudios con las especies vegetales: romero, tomillo, lavanda, *C.decapetala*, *Tara* y *Noni*, considerando un análisis de los polifenoles totales, actividad antiradicalaria, actividad antioxidante en emulsiones y productos cárnicos y envasado activo con extractos de plantas. Esta sección se divide en los siguientes segmentos:

5.1. Análisis de Polifenoles Totales e identificación de los diferentes compuestos polifenólicos

Antes de efectuar la extracción polifenólica de las diversas plantas a estudiar (romero, tomillo, lavanda, *C. decapetala*, *Tara* y *Noni*), se realizó una extracción con diferentes concentraciones del disolvente etanol:agua, para determinar la concentración más efectiva. El método escogido para estimar la concentración adecuada fue el ensayo Folin-Ciocalteu. Ello permitió determinar el rango de concentraciones que representan una mejor recuperación de polifenoles totales. De los posibles (entre 35-60 % de etanol no había diferencias significativas) se escogió EtOH:H_2O 50% v/v por ser la más efectiva en trabajos previos del grupo de investigación y estudios previos consultados en la bibliografía [459].

Las plantas estudiadas presentaron diferencias significativas en los polifenoles totales (expresados, como Equivalente de Ácido Gálico (GAE), empleando el mencionado extracto etanólico al 50 % v/v). En las plantas aromáticas romero, tomillo y lavanda (Tabla 5, página 66), los extractos fueron significativamente diferentes ($p< 0,05$) incluso entre los distintos órganos de una misma especie. El extracto de las hojas del tomillo presentó el mayor contenido en polifenoles totales (334 ± 18,4 mg de GAE/g peso liofilizado). La lavanda es la planta que presentó menor contenido de polifenoles totales, en especial sus flores (52 ± 2,1 mg de GAE/g peso liofilizado). Los polifenoles totales de los diferentes órganos del romero se mantuvieron entre 198 ± 14,5 y 219 ± 6,1 mg GAE/ g peso liofilizado.

Teniendo en cuenta estos resultados, se puede decir que *T. vulgaris* es una fuente rica de polifenoles, especialmente sus hojas, mediante extracción etanólica. Estudios como los de Gramza-Michalowska et al. [297] presentaron resultados similares, obteniendo valores de 334 ± 18,4 mg GAE/ g extracto liofilizado en las hojas y 288 ± 10,1 mg GAE/ g peso liofilizado en flores. Además, los valores fueron superiores a los obtenidos para otras especies, como el *T. argaeus* con un contenido de 83,31 ± 0,59 mg GAE/ g peso liofilizado [141].

El contenido polifenólico de las hojas del *R. Officinalis* fue superior a sus flores y raíces, con un valor de 219 ± 6,1 mg GAE/ g extracto liofilizado. Estudios previos, como los de Hinneburg et

al. [380] describieron un contenido similar en hojas de romero (185 mg GAE/g peso liofilizado), mediante extracción realizada únicamente con agua.

Por su parte, la lavanda mostró valores inferiores al tomillo y romero. Considerando los resultados obtenidos en el extracto etanólico liofilizado, los valores fueron superiores a estudios publicados por otros autores [163]. Los polifenoles determinados en extractos de esta especie con otros solventes (como acetona al 70 %) fueron muy inferiores (74 mg/ g extracto) [298]. Así mismo, autores como Miliuauskas et al. [178] publicaron, en el extracto metanólico de esta planta, un contenido mínimo de 5,4 ± 0,2 mg GAE/ g peso liofilizado.

El elevado contenido de polifenoles en los extractos de las plantas aromáticas se confirmó por HPLC-UV. Los extractos presentaron elevadas cantidades de RA y CA; los valores de RA fueron de 396,2 mg/kg peso liofilizado (hojas del tomillo), 102,2 mg/kg peso liofilizado (flor del tomillo), 85,2 mg/kg peso liofilizado (raíz del romero) y 81,6 mg/kg peso liofilizado (hojas de lavanda). A su vez, los valores de CA estuvieron en rangos de 71,6 mg/kg (hojas de tomillo), 169.2 mg/kg (raíz del tomillo) y 114.1 mg/kg (raíz de lavanda). Estos compuestos fueron los constituyentes principales de las plantas de la família Lamiaceae. Los resultados están en concordancia con la bibliografía consultada. Por ejemplo, Janicsák et al. [303] encontraron que el contenido de RA y de CA en plantas de la familia Lamiaceae, variaba en rangos de 0,01 a 9,30 mg/g (RA) y de 0 a 0,62 mg/g DW (CA) [303].

En el caso de la *C. decapetala* y la *Tara* también se demostró que tienen un gran contenido de polifenoles totales, con valores en el rango de 31,58-63,83 mg GAE/g DW para *C. decapetala* y de 336,36 ± 4,5 mg GAE/g DW para la *Tara*. Estos resultados fueron superiores a los encontrados en la bilbliografía para el género *Caesalpinia*. Veloz-García [368] encontraron valores de 48±3 % referido al peso seco de *C. cacalaco*. El análisis de los componentes de la *C. decapetala* mostró que contiene C, quercentina, GA, ácido 4-hidroxibenzoico y ácido *p*-cumárico. Por su parte, en la *Tara* se encontraron cantidades destacables de GA y ácidos taninos [194].

La cantidad polifenólica obtenida en las hojas del *Noni* resultó ser bastante elevada al compararla con su fruto. En las hojas se obtuvo una concentración de 4,73 mgGAE/g peso seco muy superior a un 0,48 mg/g peso fresco encontrado en el zumo de *Noni* [210]. Estos resultados van en el mismo rango, teniendo en cuenta que el 90% de la planta es agua. Se identificaron muchos compuestos. Caben destacar rutina, GA, ácido vanillico, vanillina y ácido ferúlico, con concentración de rutina (0,31 mg/g DW) relativamente superior a las anteriores. La rutina es un glucósido de flavonoide con elevado potencial antioxidante [460]. También se encontraron otros compuestos como escopoletina, 6-metoxi-7-hidroxicumarina [461]; este último posee actividad anti-inflamatoria y antioxidante [437] y es capaz de capturar el rádical superóxido [436].

5.2. Evaluación de la capacidad antiradicalaria

Los antioxidantes pueden actuar por uno o varios mecanismos como por ejemplo secuestro de radicales libres, donación de hidrógeno, quelación de iones metálicos o transferencia de electrones [410]. La actividad antiradicalaria de los extractos etanólicos del romero, tomillo, lavanda (hojas, flores y raíces) y las hojas de *C. decapetala* y *Noni*, se analizó por los métodos TEAC, DPPH, FRAP y ORAC.

Para los cuatro ensayos realizados los extractos de las hojas y flores del tomillo y hojas de la lavanda tuvieron la más alta capacidad antiradicalaria, con pequeñas diferencias entre los distintos ensayos. En general, los valores se encontraron en un rango de 0,7-3,1; 0,4-2,3; 0,6-2,3 mmol TE de Trolox/g peso seco para las hojas del tomillo, las flores del tomillo y las hojas de la lavanda, respectivamente. Esto se corresponde con el elevado contenido polifenólico en las muestras. Entre estas plantas existe una excelente correlación entre los polifenoles totales y los métodos ORAC (r^2=0,983), TEAC (r^2=0,821) y FRAP (r^2=0,980). Las flores de la lavanda y las raíces de tomillo presentaron la menor capacidad antirradicalaria.

La *C. decapetala* presentó una actividad antiradicalaria inferior a las plantas aromáticas estudiadas. Los valores obtenidos por los diferentes métodos fueron: 0,7±0,03 (ORAC), 0,4±0,02 (TEAC), 0,3±0,02 (DPPH) y 0,2±0,01 (FRAP) expresado en mmol TE/g peso seco. Cabe resaltar que estos resultados fueron superiores a estudios realizados con plantas comúnmente consumidas y conocidas por su alta capacidad antioxidante como, por ejemplo, la albahaca, mejorana, orégano, jengibre, tomillo y el té negro [48].

Se estudió ampliamente la relación entre la estructura química y la actividad antioxidante de los flavonoides [462,463]. De manera general, los aspectos que determinan la potencia antioxidante son: (1) una estructura catecol en el anillo B, (2) un doble enlace entre las posiciones 2 y 3 conjugado con el grupo 4-oxo o un 3-OH en el anillo C, y (3) la presencia de grupos hidroxilo en las posiciones 3´, 4´ y 5´en el anillo B (ver Fig. 2, página 7). La presencia del grupo catecol está relacionada con la capacidad de donar un hidrógeno o electrón para estabilizar la especie radical. Es el caso de la quercetina (presente en la *C. decapetala*) y luteolina (presente en el tomillo).

Los ácidos fenólicos y sus derivados también son compuestos con alta capacidad antiradicalaria [299]. Los ácidos cinámicos (ver Fig. 1, página 6) muestran mayor actividad que los ácidos benzóicos, lo cual puede deberse a la presencia de dobles enlaces que favorecen esta actividad. Dentro del grupo de los ácidos cinámicos, los derivados del CA son más antiradicalarios, en general, que los derivados del ferúlico y estos a su vez más que los *p*-cumáricos, lo cual indica que la presencia de hidroxilos libres en posición meta en el anillo aromático es importante para que se produzca una elevada actividad antirradicalaria. Esto explica la elevada actividad antirradicalaria del tomillo frente a la *C. decapetala*, por su gran contenido de CA; la *C. decapetala* es, sin embargo, más rica en *p*-cumárico.

Las hojas del *Noni* presentaron igualmente actividad antirradicalaria frente a los diferentes radicales libres analizados. Según los resultados obtenidos, el ensayo ORAC mostró un valor de 0,017±0,001 mmol TE/g DW. Dussossoy et al. [210] obtuvieron en el zumo de la fruta un valor de 0,0095 mmol Trolox/g peso fresco y los análisis con el radical DPPH fueron de 0,0032 mmol TE/g peso fresco. Es importante detacar que los resultados obtenidos con el extracto de las hojas, fueron superiores a frutas como el tomate, la fruta de la pasión y el fruto de la planta *Physalis peruviana* [464]. Los ensayos de FRAP y TEAC obtuvieron valores de 0,06 mmol Trolox/g peso seco y 0,09 mmol TE/g peso seco, respectivamente. Es de mencionar que en las hojas de esta planta se identificaron compuestos flavonoides y ácidos fenólicos que poseen alto poder antioxidante, entre ellos la rutina, debido a la presencia del doble enlace entre las posiciones 2 y 3 conjugado con el grupo 4-oxo en el anillo C.

5.3. Protección de los extractos frente a la oxidación en *Model Food Systems*

La oxidación en sistemas lipídicos es de gran interés para la industria alimentaria. Un número elevado de alimentos está constituído por lipídos en proporción variable. Los ácidos grasos insaturados, si son afectados por factores adversos, producen los hidroperóxidos. Éste es el punto de inicio para la formación de numerosos compuestos de alteración en los productos grasos. Los hidroperóxidos pueden transformarse progresivamente en compuestos como aldehídos y cetonas, ocasionando cambios indeseables en las grasas [465]. Un sistema lipídico típico susceptible de experimentar esta alteración es el de las emulsiones de “aceite-en-agua”. Éstas forman parte de un sinfín de alimentos como leches, natas, mayonesas y salsas, entre otros; de ahí la importancia de su análisis. Se evaluaron diversos extractos etanólicos en este sistema alimentario, de romero, tomillo, lavanda, *C.decapetala* y *Tara*.

Todos los extractos estudiados, a las concentraciones analizadas, demostraron eficacia en la inhibición de la oxidación lipídica de las emulsiones a lo largo del tiempo de estudio, con resultados similares o incluso mejores que los que presentaba el control positivo (Trolox® y/o BHA) y con un porcentaje de inhibición de la oxidación del doble o triple al presentado por el control (emulsión idéntica sin agente antioxidante). Respecto a las plantas aromáticas, su actividad protectora frente a productos de oxidación primaria sigue el siguiente orden: lavanda<tomillo<romero. El romero y el tomillo (100 ppm) fueron los que mejor protegieron a la emulsión de la oxidación, siendo las hojas el órgano más eficaz, donde a partir de los 24 días empiezan a llegar a un PV=10 meq hidroperóxido/kg emulsión, mientras que el Trolox (250 ppm) lo alcanza a los 10 días

En cuanto al género *Caesalpinia*, las dos plantas analizadas ejercieron un efecto protector. *C. decapetala* se estudió a 0,002 %, 0,02 %, 0,2 % y 0,5 %; la concentración más efectiva fue la del 0,2%, donde pasados 31 días no se alcanzarón los 10 meq hidroperóxido/kg emulsión. Sin embargo, un 0,5% ejerció un efecto pro-oxidante alcanzando dicho valor en un periodo de

18 días. En cambio, la *Tara* a una concentración de 0,5 % ejerció un gran poder protector superando los 20 días sin haber alcanzado los 10 meq hidroperóxido/kg emulsión. De todas las estudiadas cabe destacar que la *Tara* es la que logra mejores resultados en la inhibición lipídica, con una concentración de 0,5 %.

Se analizó la variación de los compuestos polifenólicos en las emulsiones con las plantas del género *Caesalpinia* con LC-MS. Se observó el comportamiento de los principales polifenoles a través del tiempo, tanto en infusión como en la propia emulsión. Los antioxidantes actuaron como capturadores de radicales libres en la interfase agua-aceite por lo que crearon una barrera protectora, bloqueando la entrada de especies capaces de acelerar la oxidación de los lípidos [247].

En las emulsiones se produjo una disminución significativa del GA en el caso de la *C. decapetala* y un aumento en la *Tara*. También se observó un aumento del contenido del ácido mono-galoil quínico como consecuencia de su liberación debido a la degradación térmica del GA. Los taninos encontrados fueron precisamente derivados del GA. El ECG presentó un descenso a lo largo del tiempo, seguramente por experimentar epimerización; el compuesto resultante fue el CG. Wang et al. [466] obtuvieron resultados similares en el estudio de la cinética de degradación y epimerización del EGCG, estudiado en un sistema acuoso sometido a diferentes intervalos de temperatura. Por otra parte, el compuesto *p*-cumárico disminuyó en la etapa final (del tiempo que duró el estudio) lo que seguramente hizo generar ácido *p*-hidroxibenzoico. Además, se observó un notable aumento del compuesto quercetina en las muestras. La quercetina presenta las 3 características estructurales principales para desarrollar buena actividad antioxidante: presencia en el anillo B de la estructura catecol, presencia de un doble enlace en posición 2-3 y la presencia de grupos hidroxilo en las posiciónes 3 y 5 [467].

Los productos cárnicos son alimentos muy sensibles a la oxidación lipídica, una de las principales razones del deterioro de su calidad durante su almacenamiento, lo que ocasiona cambios indeseables tanto a nivel sensorial como nutricional. Inicialmente la oxidación desarrolla un sabor a cartón y progresa con el desarrollo de un color marrón y sabor a rancio y oxidado [468].

La actividad antioxidante de *C. decapetala* y *Noni* se evaluó en un sistema cárnico, formado por hamburguesas de carne de ternera. *C. decapetala* se evaluó en dos concentraciones (0,1 % y 0,5 %, peso seco) y el *Noni* a 0.5 % peso seco. Ambas poseen gran efecto protector en los productos cárnicos, en particular a la mayor concentración analizada, 0.5 %. Al cabo de 11 días de almacenamiento en refrigeración a 4 ºC alcanzarón valores de 1,7 y 0,86 mg malondialdehido/kg muestra, respectivamente. Estos valores fueron inferiores al antioxidante sintético BHT (0,01%), donde las muestras alcanzaron los 2,66 mg malondialdehido/kg muestra en el mismo periodo de tiempo. Sin embargo, fueron superiores a una mezcla comercial de antioxidantes sintéticos usados en la industria cárnica, que se añadió al 0,5 % (dextrina,dextrosa, 5,7 % SO_2, E301, E331),.

Los antioxidantes naturales provenientes de plantas son efectivos en el control de la oxidación lipídica y incremento del tiempo de vida útil de productos cárnicos [469,470]. La oxidación tiene influencia en el color que presenta la carne. Con el tiempo se observó una decoloración de color rojo a marrón, como resultado de la oxidación de OxyMb a MetMb [385]. *C.decapetala* al 0,5 % logró obtener un %MetMb (66,57 % ± 0,3 %) inferior al control (73,48 % ± 0,2 %) y muy comparable con el obtenido para BHT al 0,01 % (65,09 % ± 1 %). Estas plantas se pueden considerar posibles ingredientes para controlar la oxidación de hamburguesas de carne de ternera.

5.4. Envases activos con extractos de plantas

La protección de alimentos con envases activos que presentan propiedades antioxidantes es una tecnología prometedora para incrementar la durabilidad de los alimentos [471]. Se han desarrollado y evaluado materiales de envasado que proveen al alimento de una protección frente a la oxidación, a través de la transferencia de los antioxidantes naturales del material de envase al alimento. En el estudio se analizaron dos tipos de film con gran potencial para ser usado en la industria alimentaria: un film elaborado con ácido poliláctico y un film comestible a base de una solución de gelatina y glicerol (como plastificante).

En los films desarrollados con ácido poliláctico se incorporaron las hojas de romero y tomillo por su eficaz capacidad antioxidante. Tanto las plantas en estado seco como sus extractos etanólicos (50 % v/v) liofilizados se probaron en los films a una concentración del 3 % (m/m). Los films se caracterizaron y se estudiaron sobre emulsiones de “aceite-en-agua”. Se evaluó la oxidación primaria durante 35 días. Los films con el extracto liofilizado de tomillo y romero presentaron un comportamiento muy similar hasta los 18 días de almacenamiento. Los films con adición de las plantas liofilizadas mostraron resultados más eficaces respecto a los films con las plantas secas. El film de PLA con el extracto de romero liofilizado fue el que tuvo un mejor comportamiento ya que únicamente alcanzó un valor de 33,10 meq hidroperóxidos/kg emulsion al cabo de 35 días, frente al control (PLA puro) que obtuvo un valor de 65,59 meq hidroperóxidos/kg emulsion. El tiempo en alcanzar los 10 miliequivalentes O_2/kg aceite fue de 18 días, mientras que la muestra control lo alcanzó en 4 días. Evidentemente, el efecto protector del film (con los extractos liofilizados de las plantas) en la emulsión es una consecuencia de la presencia de compuestos polifenólicos en los dichos extractos. El de romero se asocia a la presencia de varios polifenoles como ácido carnósico, carnosol, rosmanol, etc. El ácido carnósico es un antioxidante lipofílico que captura los radicales peroxilo e hidroxilo previniendo la peroxidación lipídica [313]. En este sentido, el ensayo LDI-MS demostró la presencia de compuestos de bajo peso molecular en los extractos liofilizados. Ciertos compuestos como los flavonoides con gran actividad antioxidante son de bajo peso molecular [472]. Igualmente, las plantas mejoran la estabilidad termo-oxidativa del PLA [334]. El extracto liofilizado de tomillo aumentó la temperatura de inicio de degradación 20ºC más que el PLA puro (313 ºC).

Por otro lado, se desarrolló un envase activo comestible, donde se incorporaron los extractos de las plantas *C. decapetala*, *Tara* y *Noni*. Los films se fabricaron con una mezcla de gelatina y glicerol en la que se incorporó el extracto etanólico 50 % de estas muestras.

Los films de gelatina con el extracto de *C. decapetala* y *Tara* se fabricaron a distintas concentraciones: 0,3, 0,7 y 1 % (m/m) para *C. decapetala* y 0,07, 0,1 y 0,2 % (m/m) para *Tara*. Cuando los films se disolvieron en un simulante alimentario (etanol) se obtuvo un contenido polifenólico elevado[1] en el rango de 178-515 mgGAE/g film para la *Tara* y 61-191 mgGAE/g film para *C. decapetala*. Estos resultados mostraron mayor potencial que otras plantas de gran poder antioxidante. Por ejemplo, extractos acuosos de té verde (20 % m/v) obtuvieron un contenido polifenólico sobre 33 mg GAE/g film [411]. La actividad antioxidante de los films es directamente proporcional a la concentración de polifenoles en los extractos. Partícularmente el film con *Tara* presentó un gran potencial antioxidante. Esta planta se considera tradicionalmente el segundo material con mayor contenido en taninos después de *Schinospsis balansae* [196].

La incorporación de *C. decapetala* y la *Tara* en los films mejoró sus propiedades mecánicas, reduciendo la resistencia a la tracción. Además, el extracto de *Tara* redujo la permeabilidad al vapor de agua en relación al control. Los films con los extractos de plantas a concentraciones 1 % (*C.decapetala*) y 0,2 % (*Tara*) se evaluaron también en hamburguesas de carne de ternera. El film con *Tara* redujo el número de valor de sustancias reactivas al TBA, seguramente por la migración de las moléculas antioxidantes del film de envasado a la carne. Los valores de TBARS fueron notablemente inferiores al control (muestra de carne únicamente con sal y sin film específico). En el experimento que se trabajó con carne de ternera protegida con film que incorporaba CS (0,2 %), evaluado durante 12 días, se obtuvo unos valores de 0,29±0,02 mg MDA/kg, muy comparable con la carne con film de té verde (1 %) 0,27±0.04 mg MDA/kg (el antioxidante natural de los más potentes conocidos) y mucho más efectivo que la carne protegida con el antioxidante sintético BHA (0,001) (0,98±0,05 mg MDA/kg). La muestra control al cabo de 12 días presentó valores de 1,27±0,1 mg MDA/kg de carne. Los resultados obtenidos en las hamburguesas con los films con extractos de plantas no alcanzarón valores de 1,5 mg MDA/kg al final del tiempo de estudio (12 días). Existen estudios que indican que un índice de 1,5 mg MDA/kg está estrechamente relacionado con olores desagradables que son fácilmente perceptibles e inaceptables en la carne [473].

El film de gelatina-glicerol con extracto añadido de *Noni* se fabricó a una concentración de 1 % y 2,4 % m/m. Ambos films fueron caracterizados y evaluados en hamburguesas de ternera. La incorporación del extracto de *Noni* al film proporcionó una reducción de la oxidación lipídica en las muestras de carne. Una vez finalizado el estudio (9 días), las muestras con film al 1 % y

[1] Los films fabricados con el extracto de tara al 0,3% obtuvieron un contenido polifenólico de 178 mgGAE/g film y con *Caesalpinia decapetala* al 0,07 de 61 mgGAE/g film.

2,4 % no tuvieron diferencias significaticas, con valores de oxidación secundaria de 0.86±0.05 y 0,9±0,01 mg MDA/kg de carne, respectivamente. Estos films mostraron una reducción del grado de oxidación con respecto al control entre 29-32 %. También se evidenció la eficacia de esta planta al compararse con un control positivo (mezcla comercial de antioxidantes sintéticos: dextrina,dextrosa, 5,7 %SO_2, E301, E331), donde la muestra con film al 2,4 % no tuvo diferencia significativa respecto a ésta. La presencia de una capa protectora en la superficie del producto provoca la absorción de humedad y oxígeno, por lo que la cantidad que llega al producto es inferior, disminuyendo la velocidad de oxidación [474]. Además, la adición de extracto de *Noni* rico en polifenoles que migran del film al producto, protege la carne frente a la oxidación.

La incorporación del extracto de *Noni* aumentó la eficacia de la permeabilidad al vapor de agua, barrera de luz y las propiedades mecánicas de la película de gelatina. La concentración del extracto de *Noni* incorporado en el film mejoró la resistencia a la tracción (*tensile strength, TS*) y el alargamiento a la rotura (*elongation at break, EAB*). Los valores de TS tendieron a disminuir y los valores de EAB a aumentar cuando la concentración del extracto de *Noni fue* mayor, obteniendo valores de TS más altos en el film con *Noni* al 2,4 % y más bajos en el film control.

Conclusiones

6. CONCLUSIONES

En este capítulo se plantean las conclusiones pertinentes en base a los resultados obtenidos, y se divide en las siguientes secciones: actividad antiradicalaria de los extractos de las especies vegetales, composición química en las plantas estudiadas, actividad antioxidante en *Model Food System* y actividad antioxidante en un sistema de envasado activo.

6.1. Actividad antirradicalaria de los extractos de las plantas romero, tomillo, lavanda, *C.decapetala*, *Tara* y *Noni*l

- El contenido en polifenoles totales en las plantas estudiadas fue de 336,36 mg GAE/ g peso seco para la *Tara*, el mayor de todos los analizados, seguida de *C. Decapetala* (63,83 mg GAE/ g peso seco) y las hojas del tomillo (54,39 mg GAE/ g peso seco). Las que presentaron menor concentración de polifenoles fueron las hojas de *Noni* (4,46 mg GAE/ g peso seco) y las flores de lavanda (17 mg GAE/ g peso seco). Los extractos etanólicos estudiados demostraron actividad antiradicalaria frente a los radicales de los métodos estudiados (TEAC, DPPH y ORAC). De las plantas aromáticas, el extracto proveniente de las hojas del tomillo presentó el mayor poder antioxidante, en un rango que va de 0,7a 3,1 mmoles TE/g peso seco. La *Tara* presentó una capacidad antirradicalaria en el rango de 2,0-8,5 mmoles TE/g peso seco. El extracto del *Noni* presentó valores comparables a las plantas aromáticas (0,017-0,9 mmoles TE/ g peso seco). La actividad antiradicalaria medida por el ensayo ORAC, fue de 4,6 mmoles TE/g peso liofilizado para las hojas del tomillo, 0,7 mmoles TE/g peso seco y para las hojas de la *C. Decapetala* y 0,017 mmoles TE/g peso seco para el *Noni*.

6.2. Composición química en las plantas estudiadas

- En las plantas aromáticas estudiadas se identificaron ácido cafeico y ácido rosmarínico. El contenido mayor de ácido rosmarínico se encontró en hojas del tomillo (396,2 mg/kg), seguido de flores del tomillo (102,2 mg/kg), raíces del romero (85,2 mg/kg) y hojas de la lavanda (81,6 mg/kg). De igual manera, el contenido en ácido cafeico fue superior en hojas del tomillo (71,6 mg/kg), seguido de raíces del tomillo (169,2 mg/kg) y raíces de la lavanda (114,1 mg/kg).
- En la muestra de *Tara* se identificaron principalmente taninos hidrolizables y quercetina. Sin embargo, en *C. decapetala* se encontraron ácidos fenólicos (ácido *p*-cumárico y ácido *p*-hidroxi benzóico), además de catequina, apigenina, glucurónido de apigenina y el compuesto vitexina.

6.3. Actividad antioxidante en *Model Food System*

- Las hojas de romero (*R. officinalis*) y las hojas de tomillo (*T. vulgaris*), fueron las que protegieron con mayor eficacia la emulsión frente a la oxidación, no existiendo diferencias significativas entre ambas, y con una formación de 82 y 85 meq hidroperóxidos/kg de emulsión, respectivamente, al cabo de los 42 días de estudio.
- La *C. decapetala* y la *Tara* presentaron una gran capacidad de proteger las emulsiones frente a la oxidación. La *C. decapetala* al 0,2 % m/v permitió reducir en un 97,36 % la formación de hidroperóxidos, obteniendo 2,13 meq hidroperóxidos/kg de emulsión al transcurrir 31 días. La *Tara*, a una concentración de 0,5 % m/v, presentó una formación de 6,73 meq hidroperóxidos/ kg de emulsión, al cabo de 20 días de estudio, lo que significó que redujo la formación de hidroperoxidos en un 96,18 %.
- En un sistema cárnico, la *C. decapetala* y el *Noni* al 0,5 % protegieron de manera similar las hamburguesas. Al final de los 11 días de almacenamiento, en refrigeración a 4ºC, *C. decapetala* logró una reducción de 69,87 % y el *Noni* de un 69,99 %. La efectividad en la protección frente a la oxidación fue inferior a una mezcla de antioxidantes sintéticos (87,45 %) al 0,5 %, pero evitó los efectos indeseables en el producto y fue altamente efectiva para ser un producto de origen natural

6.4. Actividad antioxidante en un sistema de envase activo de alimentos

- Se han obtenido films de PLA con antioxidantes naturales provenientes de plantas aromáticas, las cuales han proporcionado una alta protección frente a la degradación oxidativa en emulsiones aceite-en-agua. La mejor protección fue dada por el extracto de romero liofilizado (este film logra una inhibición lipídica 30 veces superior al film control).
- Las propiedades térmicas de los film de PLA revelaron que la incorporación de extractos naturales de *C. decapetala* y *Tara* como agentes antioxidantes mejoraron la estabilidad térmica del polímero.
- Se desarrollaron satisfactoriamente films de mezcla de gelatina-glicerol utilizando el extracto de hojas de *C. decapetala*, *Tara* y *Noni* como agentes activos para el retardo de la oxidación lipídica en hamburguesas de carne de ternera. El film con extracto de *Tara* (0,2 %) desarrolló una elevada protección de las muestras de carne, logrando una reducción de un 77,17 % frente al control, muy comparable con el film de té verde al 1% m/m (control positivo) (78,71 %). Por su parte, el *Noni* (2,4 % m/m) logró reducir en un 34,49 % la oxidación lipídica, lo que indica una protección inferior a la obtenida por la *Tara*.
- El estudio de propiedades mecánicas de los films de gelatina-glicerol apuntó a que los extractos de *C. decapetala* y *Tara* mejoraron dichas propiedades, reduciendo la resistencia a la tracción. A su vez la *Tara* redujo la permeabilidad al vapor de agua.

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Anexos

8. ANEXOS

8.1. Participación en congresos

II Encuentro de la juventud investigadora (EDI-USC). 2014. Antioxidant properties of three aromatic herbs (rosemary, thyme and lavender) in oil-in-water emulsions). María Gabriela Gallego Iradi. Universidad de Santiago de Compostela, Santiago de Compostela.

Effect of solvent on the polyphenol content and antioxidant activity of Betula Alba and Convolvulus Arvensis. Mohd A. Nurul, Francisco Segovia, Gabriela Gallego, José Casanova, MaríaPilar Almajano. Departamento de Ingeniería Química, ETSEIB – Universitat Politècnica de Catalunya – Av. Diagonal 647, 08028 Barcelona

Evaluation of antioxidant activity of three plants in the Pyrenees: Betula alba, Convolvulus arvensis and Malva Slyvestris. Mohd A. Nurul, Francisco Segovia, Gabriela Gallego, José Casanova, MaríaPilar Almajano. Departamento de Ingeniería Química, ETSEIB – Universitat Politècnica de Catalunya – Av. Diagonal 647, 08028 Barcelona.

8.2. Publicaciones de segundo y tercer autor

Monika Skowyra, Maria Gabriela Gallego, Francisco Segovia and Maria Pilar Almajano. 2014. Antioxidant Properties of *Artemisia annua* Extracts in Model Food Emulsions. *Antioxidants* 2014, *3*, 116-128.

Nurul Aini Mohd Azman, Maria Gabriela Gallego, Luis Juliá, Lluis Fajari and MaríaPilar Almajano. 2015. The Effect of *Convolvulus arvensis* Dried Extract as a Potential Antioxidant in Food Models. *Antioxidants*, *4*, 170-184.

Francisco Segovia Gómez, Sara Peiró Sánchez, Maria Gabriela Gallego Iradi, Nurul Aini Mohd Azman and María Pilar Almajano. 2014. Avocado Seeds: Extraction Optimization and Possible Use as Antioxidant in Food. *Antioxidants*, *3*, 439-454.

Antioxidants **2014**, *3*, 116-128; doi:10.3390/antiox3010116

OPEN ACCESS

antioxidants

ISSN 2076-3921

www.mdpi.com/journal/antioxidants

Article

Antioxidant Properties of *Artemisia annua* Extracts in Model Food Emulsions

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Received: 16 December 2013; in revised form: 13 February 2014 / Accepted: 14 February 2014 / Published: 3 March 2014

Abstract: *Artemisia annua* is currently the only commercial source of the sesquiterpene lactone artemisinin. Although artemisinin is a major bioactive component present in this Chinese herb, leaf flavonoids have shown a variety of biological activities. The polyphenolic profile of extract from leaves of *A. annua* was assessed as a source of natural antioxidants. Total phenolic content and total flavonoid content were established and three assays were used to measure the antioxidant capacity of the plant extract. The measurement of scavenging capacity against the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation, the oxygen radical absorbance capacity (ORAC) and the ferric reducing antioxidant power (FRAP) were 314.99 μM Trolox equivalents (TE)/g DW, 736.26 μM TE/g DW and 212.18 μM TE/g DW, respectively. *A. annua* extracts also showed good antioxidant properties in 10% sunflower oil-in-water emulsions during prolonged storage (45 days) at 32 °C. Artemisia extract at 2 g/L was as effective as butylated hydroxyanisole (BHA) at 0.02 g/L in slowing down the formation of hydroperoxides as measured by peroxide value and thiobarbituric acid reactive substances. The results of this study indicate that extract of *A. annua* may be suitable for use in the food matrix as substitutes for synthetic antioxidants.

Keywords: *Artemisia annua*; antioxidants; oil-in-water emulsions; lipid oxidation

1. Introduction

Lipid oxidation is of great concern to the consumer because it causes physical and chemical deterioration of food quality, such as undesirable changes in taste, texture, appearance and development of rancidity, losses of important nutritional values and formation of potentially harmful components including free radicals and reactive aldehydes [1,2]. Especially, this process is favored in oil-in-water emulsions because of the large contact surface between the oxidizable lipid hydroperoxides in emulsion droplets and water-soluble prooxidants resulting in the propagation of oxidation reactions [3]. To avoid this problem, synthetic antioxidants are commonly used, such as butylated hydroxytoluene and butylated hydroxyanisole [4]. However, in recent years there has been an increasing interest in the use of naturally occurring substances for the preservation of food. Aromatic plants have been the subject of study, particularly by the chemical, pharmaceutical and food industries, because of their potential use in food for two principal reasons: (i) safety considerations regarding the potentially harmful effects of the chronic consumption of synthetic compounds in food and beverages; and (ii) "natural" additives are perceived as beneficial for both quality and safety aspects and also possible beneficial effects on human health [5].

Artemisia annua (*Asteraceae* family) commonly known as "annual wormwood" is a plant used for many centuries in Chinese folk medicine for the treatment of malaria and fever. Its health-promoting effects have been mainly attributed to its content of artemisinin, a sesquiterpene lactone used as the raw material for production of artemisinin-based combination therapy, used against drug-resistant *Plasmodium falciparum* in areas where malaria is endemic. *A. annua* is also a rich source of antioxidant flavonoids that are thought to play an important role in potentiating the effects of artemisnin drugs against cancer and parasitic diseases [6]. Moreover, *A. annua* leaves have a high content of essential oil (EO) containing cineole, α-pinene, camphene, camphor and artemisia ketone [7]. The essential oil of *A. annua* is referenced as having antifungal and antimicrobial activity [8]. *A. annua* also shows anti-inflammatory, antipyretic [9], antioxidant [10], anticancer [11,12] and cytotoxic [13] activities. Although not yet reported in the literature, *A. annua* extracts, being a rich source of various phenolic compounds could therefore be incorporated in model emulsions as a source of natural antioxidant to prolong quality and stability.

The aim of this paper is to report a study of the antioxidant properties of *Artemisia annua* extracts in model emulsions stored for long periods, which can be representative of real food systems and their expected shelf life. Lipid oxidation was determined by following the formation of peroxide values (PV) as the primary oxidation products and thiobarbituric acid reactive substances (TBARs) as the secondary products.

2. Experimental Section

2.1. Materials

Artemisia annua was grown in a greenhouse (Balaguer, Spain). Leaves of *A. annua* were collected, dried and ground to a homogenous powder in collaboration with the company Pinnies Hortícoles. Refined sunflower oil was purchased in a local market. All reagents and chemicals were of analytical grade supplied by Sigma–Aldrich Company Ltd. (Gillingham, UK) or Panreac (Barcelona, Spain).

2.2. Extraction

Air-dried and finely ground *Artemisia annua* was weighed (2 g) and extracted with 50 mL of ethanol-water mixture at 50:50 (v/v). The mixture was stirred continuously for 24 h at 4 °C. After that, all samples were centrifuged (Sigma 6K10, Osterode am Harz, Germany). Part of the supernatant was used to determine the antiradical capacity. The volume of the remaining supernatant was measured and the solution was evaporated, frozen at −80 °C for 24 h and lyophilized for 3 days. Samples were then weighed and kept protected from light in a desiccator until used to prepare an oil-in-water emulsion system.

2.3. Total Phenol and Flavonoid Content

Total polyphenol content (TPC) of extracts was determined by colorimetry following the Folin-Ciocalteu method [14]. The absorbance was measured at 725 nm using a UV–vis spectrophotometer (Fluostrar Omega, Perkin-Elmer, Paris, France) and the results were expressed in gallic acid equivalents, GAE, using a gallic acid standard curve (10–70 μM).

Total flavonoid content (TFC) of extracts was measured according to the method of Zhishen *et al.* [15]. The absorbance at 510 nm was measured using spectrophotometer UV-4201/20 (Zuzi, AuxiLab, S.L., Navarra, Spain). Values were determined from a calibration curve prepared with catechin (ranging from 6 to 60 mg/L) and expressed as mg of catechin equivalent per gram of dry weight of plant (CE/g DW).

2.4. Antioxidant Capacity Determination

Three different methods were used for the evaluation of the antioxidant activity of the extracts: 2,2'-azino-bis-(3-ethylbenzthiazoline)-6-sulphonic acid ($ABTS^{\bullet+}$) assay [16], Oxygen Radical Absorbance Capacity (ORAC) assay [17] and Ferric Reducing Antioxidant Power (FRAP) method [18]. Results were expressed as μM of Trolox equivalent (TE) per gram of dry weight of plant (DW).

2.5. Liquid Chromatography-Mass Spectrometry

LC-MS analyses of the *A. annua* extracts were carried out using LC-QTOF-MS instrument, acquired from Agilent (Wilmington, DE, USA). The LC was an Agilent 1200 Series, consisting of a vacuum degasser unit, an autosampler, two isocratic high pressure mixing pumps and a chromatographic oven. The QTOF mass spectrometer was an Agilent 6520 model, furnished with a Dual-Spray ESI source. The mobile phase was composed of 0.1% formic acid (v/v) in water (eluent A) and 0.5% formic acid (v/v) in acetonitrile (eluent B). Separations were performed on a reversed-phase Zorbax Eclipse XDB-C18 column (100 mm × 2.1 mm, 3.5 μm) acquired from Agilent and connected to a C18 (4 mm × 2 mm) guard cartridge supplied by Phenomenex (Torrance, CA, USA). The temperature of the column was maintained at 30 °C, the mobile phase flow was 0.2 mL/min, and the following gradient was used: 0–10 min, 3% B; 10–25 min, 100% B; 27–38 min, 3% B. The injection volume for samples was 10 μL. Nitrogen (99.999%), used as nebulizing (35 psi) and drying gas (330 °C, 10 °C/min) in the dual ESI source, was provided by a high purity generator (ErreDue srl, Livorno, Italy). Nitrogen (99.9995%), for collision-induced dissociation experiments (MS/MS measurements), was purchased

from Carburos Metálicos (A Coruña, Spain). The QTOF instrument was operated in the 2 GHz (Extended Dynamic Range, mass resolution from 4500, at *m/z* 100, to 11,000, at *m/z* 900) mode and compounds were ionized in positive ESI, applying capillary and fragmentor voltages of 3500 and 160 V, respectively. A reference calibration solution (Agilent calibration solution A) was continuously sprayed in the source of the QTOF system, through a second nebulizer. The Mass Hunter Workstation software was used to control all the acquisition parameters of the LC-ESI-QTOF-MS system and also to process the obtained data. Full scan MS spectra were acquired in the range from 100 to 1700 *m/z* units, during the whole chromatographic run, considering an acquisition rate of 1.4 spectra/s. The identification (caffeic acid, apigenin and rutin) was based on the accurate masses, isotopic abundances and spacing of signals in their ($[M + H]^+$) cluster of ions, obtained in the MS mode, as well as, on their MS/MS fragmentation patterns and the exact mass of products ions.

2.6. Oil-in-Water Emulsion System

2.6.1. Removal of Tocopherols from Sunflower oil

Tocopherols were removed from sunflower oil by column chromatography using activated alumina, as described by Yoshida *et al.* [19]. The oil was stored at −80 °C prior to emulsion preparation (up to 2 days).

2.6.2. Preparation of Emulsions and Storage Conditions

Oil-in-water emulsions were prepared with 1% of Tween 20 as emulsifier and 10% of sunflower oil (2.7.1). Emulsions were prepared by dropwise addition of oil to the water phase, with sonication using a UP200S ultrasonic (Hielscher Ultrasonics GmbH, Teltow, Germany) while cooling in an ice bath for 10 min. It was necessary to repeat sonication 7 times (7 × 10 min) to have enough volume of emulsion. Freeze-dried powder of the *A. annua* extract was redissolved in ethanol 50% (v/v) and added directly to the emulsion and homogenized, obtaining final concentrations of 0.20, 0.65 and 2 g/L (C1, C2 and C3, respectively). For the negative control no extract was added, and the positive controls were prepared with Trolox (0.02 g/L) and BHA (0.02 g/L) dissolved in ethanol.

All emulsions were stored in triplicate in 30 mL amber bottles in the dark, with constant elliptical movement and allowed to oxidize at 32 ± 1 °C for 45 days.

2.6.3. Measurement of Primary Oxidation by Peroxide Value (PV) and pH

Peroxide value (PV) was measured periodically (every 2 or 3 days during the time of storage) using aliquots of 0.007–0.01 g of each sample and determined by the ferric thiocyanate method [20], after calibrating the procedure with a series of oxidized oil samples analyzed by the AOCS Official Method Cd 8-53 [21].

The pH of the samples was measured (pH-meter GLP21, Criston Instruments, Barcelona, Spain) as a parameter to investigate its correlation with PV.

2.6.4. Measurement of Secondary Oxidation by TBARs Method

The thiobarbituric acid reactive substances (TBARs) assay was performed as described by Maqsood and Benjakul [22] with some modifications. One mililiter of oil-in-water emulsion sample was mixed with a TBARs solution containing 0.375% thiobarbituric acid and 15% trichloroacetic acid in 0.25 N HCl solution (5 mL). The samples were placed immediately in an ultrasonic bath (Prolabo brand equipment, Lutterworth, UK) for 5 min and then heated in a water bath (95 °C) for 10 min. The mixture was centrifuged (Sigma 3K30, Sigma Laborzentifugen GmbH, Osterode am Harz, Germany) at room temperature at 4000 rpm for 10 min. The absorbance of the supernatants was measured at 532 nm (Spectrophotomter UV-4201/20, Zuzi, Navarra, Spain). The TBARs values were expressed as mg of malondialdehyde (MDA) per kg of emulsion calculated using 1,1,3,3-tetraethoxypropane (Sigma-Aldrich, St. Louis, MO, USA) as the standard.

2.7. Statistical Analysis

TPC, TFC, $ABTS^{+}$, ORAC and FRAP measurements were performed in triplicate on triplicate samples. PV and TBARs measurements were performed once on triplicate samples.

Mean values for different parameters were calculated and compared by analysis of variance (one-way ANOVA) using commercial software (Minitab 16). Moreover, statistical differences between mean values were identified at the 95% of confidence level ($p < 0.05$). Person's correlation analysis was performed using the same statistical package.

3. Results and Discussion

3.1. Phenolic Content and in-Vitro *Antioxidant Activity of Extract*

The total polyphenols (TPC) and flavonoids (TFC) in extracts of *A. annua* leaves obtained with 50% ethanol are shown in Table 1. The *A. annua* extract contained 23.36 ± 0.92 mg gallic acid (GAE)/g dry weight (DW) and 2.68 ± 0.07 mg catechin/g DW (TPC and TFC, respectively).

Table 1. Polyphenol and flavonoid content and antioxidant activity of *A. annua* extracts.

Method	Amount detected *
Total polyphenol content (mg GAE/g DW)	23.36 ± 0.92
Total flavonoid content (mg CE/g DW)	2.68 ± 0.07
ABTS (μM TE/g DW)	314.99 ± 7.70
ORAC (μM TE/g DW)	736.26 ± 17.55
FRAP (μM TE/g DW)	212.18 ± 6.02

* Results are expressed as mean ± standard deviation ($n = 3$).

A recent paper on the analysis of extracts of *A. annua* [23] found a TPC values (384.1 ± 6.7 to 521.2 ± 5.4 mg GAE/100 g DW) for methanol and acetone extraction, respectively, much lower than what we report here for ethanolic extract. However, studies involving hexane and methanol extraction of *A. annua* leaves have reported higher values than those obtained in the present study, in the range of 90.12–134.50 mg GAE/g DW [24]. In addition the same authors found higher TFC value (6.14 mg

epicatechin/g DW) in the methanolic extract. Consequently, the extraction method and the solvent used play a key role in the extraction of polyphenols and flavonoids from plant material.

Antioxidant activity of the extracts from *A. annua* was assessed by three different methods: ABTS, ORAC and FRAP. The use of several methods provides more comprehensive information about the antioxidant properties of the original product because there are substantial differences in sample preparation, extraction of antioxidants (solvent, temperature, *etc.*), selection of end-points and expression of results [5]. For the ABTS assay the value obtained was 314.99 ± 7.70 μM TE/g DW, a value 2 times lower than that found in the ORAC assay which was 736.26 ± 17.55 μM TE/g DW. It is quite usual to obtain higher values in the ORAC test, due to differences in the sensitivity of these methods. Finally, for the FRAP assay the value found was 212.18 ± 6.02 μM TE/g DW. Gouveia and Castillo [23] found the ABTS value of 477.0–2197.3 μM TE/100 g DW in *A. annua* leaves using extraction with methanol and acetone, respectively, which is much lower than that we found in the current study. Also Zheng and Wang [25] found the ORAC value (15.69 ± 0.57 μM TE/g fresh weight) in the phosphate buffer extract much lower than what we report here for the alcoholic extract. Viuda-Martos [5] described the ferric reducing capacity and metal chelating ability of the *A. annua*, finding strong reducing power and effectivity in metal chelating (62.25%–98.03%) of essential oils from *A. annua*. They also reported determination of oxidative stability of fat (Rancimat assay), finding that 5–50 g/L *A. annua* essencial oils showed pro-oxidant activity.

A few recent reports indicated that *A. annua* was one of the four medicinal plants with the highest ORAC level, the ORAC value of *A. annua* leaves and inflorescences extracts was reported as 1125 and 1234 μM TE/g, respectively, which is half to two thirds of the ORAC of oregano extracts [6].

LC-MS analysis of the plant extract of *A. annua* showed the presence of several phenolic compounds quantified in the following increasing order: caffeic acid, rutin and apigenin (Table 2). The concentrations of caffeic acid (1.352 μg/g DW), rutin (0.765 μg/g DW) and apigenin (0.135 μg/g DW) in *A.annua* extract were lower than those reported in the literature. Carvalho *et al.* [26] reported that the *A. annua* leaves conteined 80 μg/g of DW of catechins, 2 μg/g of DW of flavonols, 75 μg/g of DW of hydroxycinnamic acids and 430 μg/g of DW of hydroxybenzoic acids. Carbonara *et al.* [27] found in water extracts of *A. annua* 3.11 ± 0.02–4.10 ± 0.06 mg/g DW of caffeic acid. Morover, Ivanescu *et al.* [28] reported that *A. annua* had 1.144 mg/100 g DW of apigenin.

Table 2. Liquid chromatography-mass spectrometry (LC-MS) parameters and amount of selected antioxidant compounds in *A. annua* extracts.

Compounds	Rt (min)	Linear regression equation	R^2	Linear range (ppm)	MS (m/z) [M − H]	Content μg/g DW
Rutin	5.33	$y = 333.54x + 2184.6$	0.998	0.1–1	609	0.764
Caffeic acid	5.41	$y = 588.03x + 198.38$	0.999	0.1–1.5	179	1.353
Apigenin	7.85	$y = 1028.4x + 37085$	0.991	0.1–0.5	269	0.135

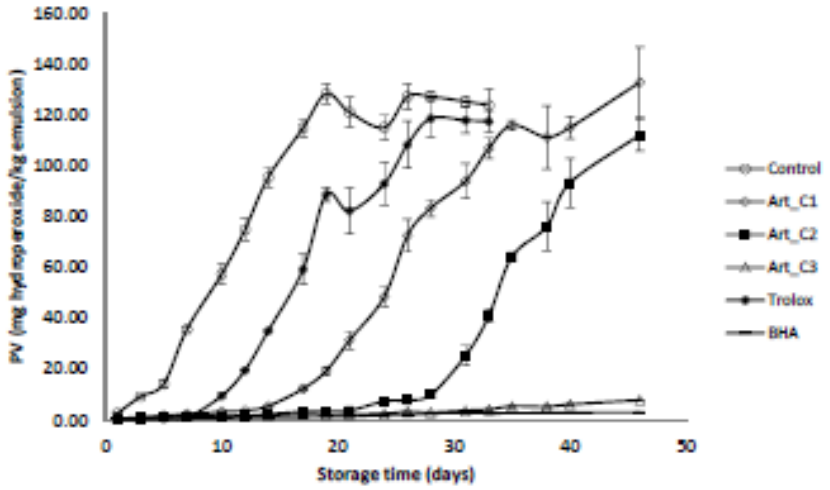
3.2. Antioxidant Activity of Extracts in Model Emulsion System

In this study, to accelerate the oxidative damage, emulsions were stored at 32 ± 1 °C. Oxidative stability was assessed by periodic analysis of primary and secondary oxidation products (measured by

the peroxide and the thiobarbituric acid reactive substances values, respectively). In addition the change in pH was monitored, since pH tends to fall during oxidation.

Peroxide values in the oil emulsions increased significantly faster in the sample without any antioxidant addition (Figure 1), reaching 10 meq hydroperoxides/kg of emulsion (this value is the allowed limit for products containing edible fats) after four days. The next samples to reach this level of deterioration were Trolox at 0.02 g/L (after 10 days), Art_C1 at 0.20 g/L (after 16 days) and Art_C2 at 0.65 g/L (after 28 days). Other samples: Art_C3 and BHA were stable until the end of the experiment (after 45 days, PV was <10 meq/kg). *A. annua* extracts added to oil-in-water emulsions were very effective in stablilzing the emulsion with 2 g/L *A. annua* extract being similar to BHA (0.02 g/L) in activity during 45 days of storage at 32 °C. Kiokias *et al.* [29] reported peroxide values between 45.60 and 51.15 meq/kg after two months in 10% sunflower oil-in-water emulsions with 2 g/L of different carotenoids including β-carotene, lycopene, paprika, lutein and bixin. Ramful *et al.* [30] found that *Eugenia pollicina* leaf extract at a concentration of 0.02% was also effective in slowing down hydroperoxide formation in soybean oil emulsion during 13 days of storage at 40 °C. Roedig-Penman *et al.* [31] reported that tea extracts added to sunflower oil-in-water emulsion were very effective in its stabilization, the tea extract (0.03%) being similar to BHT (0.02%) and taking 40 days of storage at 30 °C to reach a PV of 30 meq/kg.

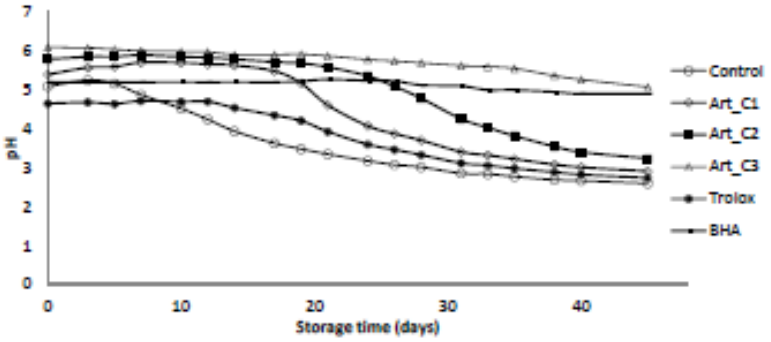
Figure 1. Evaluation of primary oxidation (peroxide value) in a model food system (O/W emulsion 10% of oil) with different concentrations of *A. annua* (C1: 0.20 g/L; C2: 0.65 g/L and C3: 2 g/L).



pH can affect oxidative reactions by influencing prooxidant (e.g., iron solubility increases with decreasing pH) and antioxidant (the pH can alter the charge of antioxidants, which can affect solubility and chelation capacity) activity. The pH of oxidation models should therefore be similar to the food of interest [32]. In addition, since it is known that many antioxidant molecules are less effective when the

pH is low [33], this parameter was also measured as a potential indicator of oil-in-water emulsions oxidation. From an initial average value of 5.5, the samples without any antioxidant addition and with Trolox tended to stabilize their pH at 2.60 and 2.74, respectively, after 45 days (Figure 2). In the Art_C1, Art_C2, Art_C3 and BHA samples the pH slowly decreased during storage, but in Art_C1 and Art_C2 it decreased rapidly after 25 and 33 days, reaching the value of 2.90 and 3.24, respectively. Observing this relationship confirmed that the pH fell as PV increased. Gallego *et al.* [18] and Sorensen *et al.* [34] reported that lipid oxidation increased when pH was decreased from 6 to 3 in a 10% oil-in-water emulsion.

Figure 2. Evaluation of pH in a model food system (O/W emulsion 10% of oil) with different concentrations of *A. annua* (C1: 0.20 g/L; C2: 0.65 g/L and C3: 2 g/L).



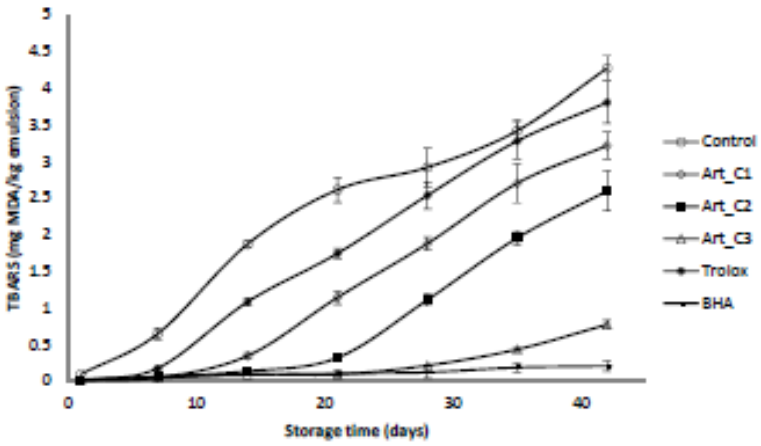
Secondary oxidation products in the emulsions were monitored by measurement of the TBARs (Figure 3). After 6 weeks, TBARs values in emulsions containing *A. annua* extracts and BHA were lower than that those in the control (4.27 mg MDA/kg) and the Trolox-containing sample (3.80 mg MDA/kg). BHA was the most effective antioxidant followed by *A. annua* extract Art_C3, Art_C2 and Art_C1. Garcia-Iñiguez *et al.* [35] reported that a lyophilized aqueous extract of *Melissa officinalis* (lemon balm) at 620.6 ppm was as efficient as BHA at 200 ppm in controlling the TBARs formation in oil-in-water emulsions made with a mixture of algae and lineseed oils upon storage during 15 days at 20 °C. Dimakou and Oreopoulou [36] found that polar (paprika, marigold, bixin) and hydrophobic (β-carotene, lycopene) carotenoids exerted antioxidant effect measured by TBARs test during thermally accelerated autooxidation (60 °C) of sunflower oil-in-water emulsions stabilized by Tween 20.

In the present study positive correlation between PV and TBARs (R^2 = 0.9200) levels in oil-in-water emulsions was found.

The activity of phenolic compounds as antioxidants in food systems (such as oil-in-water emulsions) depends not only on the structure (*i.e.*, number and position of hydroxyl groups bound to the aromatic ring) and chemical reactivity of the phenolics but also on other factors such as their physical location, interactions with other food components, and environmental conditions, for example pH [2,34,37]. Natural plant antioxidants can protect food components from oxidation under the stress

of heating and storage. The most effective antioxidants are those that interrupt the free radical chain reaction. Usually containing aromatic or phenolic rings, these antioxidants donate H˙ to the free radicals formed during oxidation becoming radicals themselves. These radical intermediates are stabilized by the resonance delocalization of the electron within the aromatic ring and formation of quinone structures. In addition, in many of the phenolics positions suitable for molecular oxygen attack are not available. Both synthetic (BHA and BHT) and natural plant antioxidants contain phenolic (flavonoid) functions. Plant extracts with antioxidant activity generally quench free radical oxygen with phenolic compounds as well [4]. However, the addition of polyphenols to lipid dispersions has been shown to result not only in antioxidant effects [38], but also in pro-oxidant activity [39].

Figure 3. Evaluation of secondary oxidation (TBARs) in a model food system (O/W emulsion 10% of oil) with different concentration of *A. annua* (C1: 0.20 g/L; C2: 0.65 g/L and C3: 2 g/L).



Phenolic compounds such as caffeic acid, rutin and apigenin have received increasing interest due to their potential antioxidant activity. Caffeic acid has a single aromatic ring with two –OH groups that are capable of donating H˙. In addition it is a polar compound with a strong ability for chelating metals [4]. Rutin is a compound that contains an *o*-diphenol group in their molecular structure (*o*-diphenol groups are able to chelate metal ions such as iron) [34].

The antioxidant capacity of natural extracts in food emulsions bas been ascribed to a number of influential factors, including the different polarities and antiradical activities of mixed phenolics. The presence of water in the emulsion results in the partition of antioxidants between polar and apolar phases, a fact influencing the antioxidant activity. According to the "polar paradox", hydrophilic antioxidants are more effective in nonpolar media, whereas lipophilic compounds are better antioxidants in polar media. However, several authors have reported that some compounds do not comply with the polar paradox and interpreted the behavior of phenolic compounds in emulsified systems using a different approach known as the "cutoff theory" [40,41]. Sorensen *et al.* [34] reported

that caffeic acid and rutin inhibited the development of PV during the entire storage period in Citrem-stabilized emulsions at pH 6. Furthermore, the most water-soluble compound, caffeic acid, showed different effects depending on pH and emulsifier type. Thus, it was a strong pro-oxidant at pH 3 (with or without iron), but at pH 6 its effect depended on the emulsifier type and on the presence of iron. In addition Medina *et al.* [37] reported that at pH 6, caffeic acid was able to reduce the amount of peroxides formed in emulsions containing Tween, but increased the formation of volatiles. Conde *et al.* [2] found that caffeic acid (5 mmol/kg emulsion) showed good antioxidant properties in 30% sunfloweroil-in-water emulsions at pH 5.4 during storage at 50 °C. The same author [40] reported that higher concentrations of rutin and apigenin in the rafined extracts produced from chestnut burs retarded the formation of hydroperoxides in oil-in-water emulsions.

The ability of a compound to inhibit lipid oxidation could be influenced by its interactions with other antioxidants [42]. Synergy between antioxidants has been reported in a range of different media, including oils, emulsions, liposomes, microemulsions, fish and meat muscles. In some reports, the effects of antioxidants used in a combination could only be described as additive, but the term synergy should be restricted to situations where the mixture of antioxidants has a greater impact than the sum of their separate effects. Synergy between antioxidants may vary both with the medium and the nature of the lipids. Caffeic acid was effective in protecting α-tocopherol in retarding lipid oxidation in the fish muscle [43,44]. α-Tocopherol showed a strong synergistic effect with queretin in the methyl oleate in water emulsion, but the effect was reduced in phospolipd liposomes and the combination of α-tocopherol and quercetin had a shorter induction time than quercetin alone, when the oxidative stability was assessed in oil by the Rancimat test [45].

4. Conclusions

This study showed that the extract of *Artemisia annua* provides protection against the oxidative deterioration of oil-in-water emulsion. In addition, food emulsions appear to be useful vectors in supplying the daily dosage of *A. annua* extract in consumers, which may positively affect their health. Moreover, considering consumer's preference for antioxidants from natural sources, these results could offer the basis for their more systematic use by food industry. Further research into the enrichment of food products with bioactive substances extracted from *A. annua* should be conducted because we still have no sufficient knowledge about their activity during food processing, or about their interactions with other food components.

Acknowledgments

The authors would like to thank Pàmies Hortícoles for kindly supplying us the plants.

Conflicts of Interest

The authors declare no conflict of interest.

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Antioxidants 2015, *4*, 170-184; doi:10.3390/antiox4010170

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antioxidants

ISSN 2076-3921

www.mdpi.com/journal/antioxidants

Article

The Effect of *Convolvulus arvensis* Dried Extract as a Potential Antioxidant in Food Models

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Academic Editors: Maria G. Miguel, João Rocha and Ehab A. Abourashed

Received: 31 October 2014 / Accepted: 25 February 2015 / Published: 10 March 2015

Abstract: In this study, the antioxidant activity of the *Convolvulus arvensis Linn* (CA) ethanol extract has been evaluated by different ways. The antioxidant activity of the extract assessed by 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation, the oxygen radical absorbance capacity (ORAC) and the ferric reducing antioxidant power (FRAP) was 1.62 mmol Trolox equivalents (TE)/g DW, 1.71 mmol TE/g DW and 2.11 mmol TE/g DW, respectively. CA ethanol extract exhibited scavenging activity against the methoxy radical initiated by the Fenton reaction and measured by Electron Paramagnetic Resonance (EPR). The antioxidant effects of lyophilised CA measured in beef patties containing 0.1% and 0.3% (w/w) CA stored in modified atmosphere packaging (MAP) (80% O_2 and 20% CO_2) was determined. A preliminary study of gelatine based film containing CA showed a strong antioxidant effect in preventing the degradation of lipid in muscle food. Thus, the present results indicate that CA extract can be used as a natural food antioxidant.

Keywords: *Convolvulus arvensis*; lipid oxidation; active packaging film; antioxidant activity

1. Introduction

Free radicals produced in the human body result from natural biochemical reactions and, together with external attacks due to stress, smoke and unbalanced diets, among other factors, could cause an imbalance between oxidants and antioxidants. For this reason, it is necessary to supplement the diet with antioxidant based food. This excess of radicals is associated with aging and many diseases such as heart problems, diabetes, neurodegenerative disorder and cancers. Previous studies indicate that the consumption of plant foods rich in antioxidants is beneficial for health and helps to prevent degenerative processes which contribute to many diseases [1–3]. Due to the increasing awareness of the benefits of consuming healthy food, many food companies are using antioxidants as an alternative approach, instead of using synthetic preservatives which at high doses may have toxic effects on the consumer.

Natural antioxidants are compounds, generally from plants, that are used as food additives with the aim of inhibiting oxidation of the product [4]. Thus, the use of natural antioxidants as preservatives to maintain quality and nutritional traits is increasingly widespread, mainly in food that contains high levels of lipids, such as meat products. Therefore, the incorporation of natural antioxidants such as herbs could be an economical strategy to develop healthier meat products. Moreover, they can improve technological properties, as well as increase the eco-efficiency [5] in the food industry. Besides formulation of food with a natural antioxidant strategy, active packaging is also gaining interest for its potential to provide food quality and safety benefits. The combination of natural preservatives and biodegradable plastic into one food packaging formulation is a promising approach to extending product shelf life [6].

Plants rich in polyphenol constituents possess antioxidant activity by free radical scavenging. For instance, green tea can inhibit lipid peroxidation and chelate transition metals, consequently helping to prevent degenerative diseases. If incorporated into an edible film, it could help to maintain the quality of food products [7].

Convolvulus arvensis Linn (CA) is an annual (or sometimes perennial climber), commonly found as a weed throughout Europe and Asia. This plant is being used for many purposes. The root and the resin are cholagogue, diuretic, laxative and purgative [8]. The flower is laxative, used as a tea infusion and also in treatment of wounds and fever, whereas the leaf can be helpful during the menstrual period [9]. Meanwhile, Meng *et al.* showed that the ubiquitous CA extract could be considered as a promising anti-cancer agent, with over 50% inhibition of tumor growth activity at non-toxic doses [10]. CA also provided an immunostimulant effect when tested on rabbits and turned out to have cytotoxic effects on human cancerous cells [11,12]. In a preliminary study, Thrakal *et al.* reported the antioxidant activity of CA extract using the DPPH method, nitric oxide scavenging activity and the reducing power assay [13]. Furthermore, the CA extract showed abundant traces of phenolic compounds including *p*-hydrobenzoic acid, syringic acid, vanillin, benzoic acid and ferulic acid [14]. This high content of phenolic compounds may allow it to serve as an antioxidant source for the food industry. However, the antioxidant activity of the CA extract towards lipid oxidation has not been fully determined yet. Thus, our goals were (1) to evaluate the antioxidant activity of CA using *in vitro* assays including FRAP,

TEAC, ORAC and EPR scavenging activity and (2) to demonstrate the ability of CA extract to inhibit lipid deterioration in beef meat, by adding the dry extract directly in the patty composition or in the formulation with active packaging. One of the components in CA is an alkaloid, which is a compound that exhibits anti-cancer activity but may display toxic effects in the host at high doses. Therefore, the extraction of CA has been carried out according to the method described by Meng *et al.* [10] to reduce the presence of alkaloid in the extract before adding the lyophilized extract directly into the beef.

2. Experimental Section

2.1. Plant Material

Commercial dried CA was kindly supplied by Pàmies Horticoles (Balaguer, Spain), a registered herbal company. All reagents and solvents used were of analytical grade and obtained from Panreac (Barcelona, Spain) and Sigma Aldrich (Gillingham, England).

2.2. Extraction of CA Extract

Dried roots of CA were finely ground using a standard kitchen food processor. Ground CA was extracted in three different ways: (1) with 50:50 (v/v) ethanol:water; (2) with 75:25 (v/v) ethanol:water and (3) with 90:10 (v/v) ethanol:water, always in the ratio 1:30 (w/v). The extractions were performed at 4 °C ± 1 °C for 24 h, in the dark with constant stirring. The extract solutions of CA were recovered by filtration using Whatman Filter paper, 0.45 μm (Whatman, GE healthcare, Wauwatosa, WI, USA). Part of the supernatant was taken for subsequent use to determine the antiradical capacity. The volume of the remaining supernatant was measured and the excess of ethanol was removed under vacuum using a rotary evaporator (Buchi Re111, Switzerland) and kept frozen at −80 °C for 24 h. All extracts were dried in a freeze dryer (Unicryo MC2L −60 °C, Germany) under vacuum at −60 °C for three days to remove moisture. Finally, lyophilised CA was weighed to determine the soluble solids concentration (g/L) as described by Zhang *et al.* [15]

2.3. Determination of the Total Phenolic Content (TPC)

The Folin-Ciocalteu method was used to determine the total phenolic content (TPC) as reported by Santos *et al.* [16].

2.4. Determination of Free Radical Scavenging Activity Assays

2.4.1. *In-Vitro* Antioxidant Capacity Determination

Three different methods were used for the evaluation of the antioxidant activity of the extracts: 2,2'-azino-bis-(3-ethylbenzthiazoline)-6-sulphonic acid TEAC assay [17], Oxygen Radical Absorbance Capacity (ORAC) assay [18] and Ferric Reducing Antioxidant Power (FRAP) method [19]. Results were expressed as μM of Trolox equivalent (TE) per gram of dry weight of plant (DW).

2.4.2. Electron Paramagnetic Resonance (EPR) Spectroscopy Radical Scavenging Assay

EPR radical scavenging activity was measured following the method described by Azman *et al.* [20]. The extraction was executed in MeOH in 1:10 (w/v) ratio and the soluble concentration of CA was determined according to the procedure above. The spin-trapping reaction mixture consisted of 100 µL of DMPO (35 mM); 50 µL of H_2O_2 (10 mM); 50 µL CA extract at different concentrations or 50 µL of ferulic acid used as reference (0–20 g/L) or 50 µL of pure MeOH used as a control; and, finally, 50 µL of $FeSO_4$ (2 mM), added in this order. The final solutions (125 µL) were passed through a narrow (inside diameter = 2 mm) quartz tube and introduced into the cavity of the EPR spectrometer. The spectrum was recorded 10 min after the addition of the $FeSO_4$ solution, when the radical adduct signal is greatest.

X-band EPR spectra were recorded with a Bruker EMX-Plus 10/12 spectrometer under the following conditions: microwave frequency, 9.8762 GHz; microwave power, 30.27 mW; center field, 3522.7 G; sweep width, 100 G; receiver gain, 5.02×10^4; modulation frequency, 100 kHz; modulation amplitude, 1.86 G; time constant, 40.96 ms; conversion time, 203.0 ms.

2.5. Determination of Antioxidant Activity in Food Model

2.5.1. Preparation of Beef Patties

The meat consisted of flank of beef provided by "Embutidos La Masia", Barcelona. It was collected seven days after slaughter to allow it to mature and was kept at approximately −20 °C for further treatment. The extraction of CA was carried out according to the method used by Meng *et al.* to remove alkaloid compounds [10]. Fat and joint tissues were trimmed off lean meat (2000 g) and the meat was minced through 8 mm industrial plates. Then, the minced meat was divided into four batches and mixed with 1.5% of NaCl and either (i) control (no addition), (ii) 0.1% BHT, (iii) 0.1% lyophilised CA, (iv) 0.3% lyophilised CA. All batches were mixed vigorously for 2 min to attain an even distribution of additives throughout the meat. Each sample was moulded into smaller portions (about 20 g each), stuffed and packed with polystyrene B5-37 (Aespack) trays and placed in BB4L bags (Cryovac) of low gas permeability (8–12 $cm^3 \cdot m^{-2}$ per 24 h). The air in the packaged trays was flushed with 80:20 (v/v) O_2:CO_2 by EAP20 mixture (Carburos Metalicos, Barcelona). Samples were stored in the dark at 4 °C ± 2 °C for 10 days and the samples were analysed for oxidation by thiobarbituric acid reactive substances (TBARS) method, % metmyoglobin, colour, pH and microbial quality. Every measurement was carried out in triplicate each day for 10 days (except for microbiological analysis which was done every three days).

2.5.2. TBARS Assay

The TBARS method was used to measure the extent of lipid oxidation over the storage period as described by Grau *et al.* [21]. Samples (1 g) were weighed in a tube and mixed with 3 g/L aqueous EDTA. Then, the sample was immediately mixed with 5 mL of thiobarbituric acid reagent using an Ultra-Turrax (IKA, Germany); at 32,000 rpm speed, for 2 min. All procedures were carried out in the dark and all samples were kept in ice. The mixture was incubated at 97 ± 1 °C in hot water for 10 min and shaken for 1 min during the process to form a homogeneous mixture. The liquid sample was

recovered by filtration (Whatman Filter paper, 0.45 μm), and then it was cooled for 10 min. The absorbance value of each sample was measured at 531 nm using a spectrophotometer. The TBARS value was calculated from a malonaldehyde (MDA) standard curve prepared with 1,1,3,3-tetraethoxypropane and analysed by linear regression. All results were reported in mg malonaldehyde per kg of sample (mg MDA/kg sample).

2.5.3. Colour Measurement

Objective measurements of colour were performed using a CR 400 colorimeter (Minolta, Osaka, Japan). Each patty was cut and the colour of the slices was measured three times at each point. A portable colorimeter with the settings: pulsed xenon arc lamp, 0° viewing angle geometry and aperture size 8 mm, was used to measure meat colour in the CIELAB space (Lightness, L*; redness, a*; yellowness, b* (CIE, 1978). Before each series of measurements, the instrument was calibrated using a white ceramic tile.

2.5.4. Percentage of Metmyoglobin

The metmyoglobin method was based on that developed by Xu *et al.* [22]. Five grams of beef patties were homogenized with 25 mL of ice-cold 0.04 M phosphate buffer (pH 6.8) for 15 s using a homogenizer (Ultra-Turrax, IKA, Germany), which was set at speed setting 2 (18,000 rpm). The homogenised patty was allowed to stand at 4 °C for 1 h and centrifuged at 4500 g for 20 min at 4 °C using a high-speed freezing centrifuge (GI-20G, Anke, Shanghai, China). The absorbance of the filtered supernatant was read at 572, 565, 545, and 525 nm with a spectrometer (Fluostar Omega, BMG Labtech, Germany). The percentage of metmyoglobin was determined using the formula: MetMb (%) = [−2.514 (A572/A525) + 0.777 (A565/A525) + 0.8 (A545/A525) + 1.098] × 100

2.5.5. Development of Gelatin-Film with Antioxidant Coating

The fabrication of gelatin based film with antioxidant coating was adapted and characterized from Bodini *et al.* [23]. While the filmogenic solution was cooled after the solubilization of sorbitol, 0.75% (w/w) of CA extract / gelatin and 0.1% (w/w) BHT/gelatin were added.

2.6. Statistical Analysis

A one-way analysis of variance (ANOVA) was performed using Minitab 16 software program (Minitab Pty Ltd., Sydney, Australia) ($\alpha = 0.05$). The results were presented as mean values ($n \geq 3$).

3. Results and Discussion

3.1. Analysis of Total Polyphenols and Free Radical Activity Assays

On average, a higher weight of soluble solids was extracted from CA with 50% ethanol than with 75% and 90% of ethanol. The use of ethanol as extraction solvent is due to the fact that the solvent is recognized as a GRAS (Generally Recognized as Safe) component which can be safely used for applications in the food industry [24]. Ethanol also turned out to be effective in the extraction of flavonoids and their glycosides, catechols and tannins from raw plant materials. Generally, CA extracted

with 50% ethanol showed higher phenolic content and antioxidant activity values in ORAC, FRAP and TEAC. Our results showed that the total phenolic content correlated with the antioxidant activity determined by the assays. Nevertheless, the values obtained in the ORAC assay were higher than the ones in the FRAP and TEAC assays, which also showed the extract scavenging activity against peroxy radicals ($OOH^{\cdot}$) generated in the assay. Total phenolic content reported for the plant extract with ethyl acetate turned out to be higher than our present results with 244 mg GAE/g DW [24]. The presence of compounds with antioxidant potential in the ethanol extract (Table 1) was revealed in the measurement of total antioxidant capacity in this study. In previous studies, the antioxidant activity of CA has been analyzed using the DPPH method, nitric oxide scavenging activity and reducing power assay applied to both methanol and ethyl acetate solvent extracts [13,25]. To the best of our knowledge, this is the first report of the antioxidant activity of CA extracts assessed using the TEAC, ORAC and FRAP methods.

Table 1. Soluble solids concentration, total phenolic content (TPC) and antioxidant activity of *Convolvulus arvensis Linn* (CA) extract.

Activity *Convolvulus arvensis*	Extraction Solvent		
	50:50 EtOH:H_2O	75:25 EtOH:H_2O	90:10 EtOH:H_2O
Soluble concentration (g/L)	13.76 ± 0.05	13.61 ± 0.02	11.43 ± 0.05
Total phenolic content (g GAE/g DW)	13.0 ± 0.05	12.1 ± 0.03	9.9 ± 0.02
FRAP (mmol of TE/g DW)	1.62 ± 0.02	1.51 ± 0.06	0.98 ± 0.01
TEAC (mmol of TE/g DW)	1.71 ± 0.01	1.68 ± 0.01	1.41 ± 0.04
ORAC (mmol of TE/g DW)	2.11 ± 0.05	2.05 ± 0.05	1.71 ± 0.03

* Mean value $n = 3$. The standard deviation for each assay is less than 5%. Gallic Acid Equivalent (GAE), Trolox Equivalent (TE), Dry Weight (DW).

3.2. EPR Scavenging Radical Assay

The EPR radical scavenging method has been developed by Azman *et al.* to evaluate the concentration of free methoxy radicals ($CH_3O^{\cdot}$) generated in the Fenton reaction with the CA extract [20]. Figure 1 shows the decreasing signal of EPR with the increase of CA extract concentration. The free radical scavenging activity of CA extracts was investigated against methoxy ($CH_3O^{\cdot}$) radical by a competitive method in the presence of DMPO as spin trap, using EPR spectroscopy. $CH_3O^{\cdot}$ was generated according to the Fenton procedure with a relatively short half-life that was identified by EPR because of its ability to form a stable nitroxide adduct with DMPO, DMPO-OCH_3 (hyperfine splitting constants, $a_N = 13.9$ G and $a_H = 8.3$ G). This stable DMPO-OCH_3 compound can be detected by the double integration value of the signal from EPR. The presence of CA extract at different concentrations may compete with the spin trap in the scavenging of methoxy radicals. Thus, the effect reduces the amount of radical adducts and, accordingly, reduces the intensity of the EPR signal. The best fitting with intensity of EPR signal was shown as an exponential function (Figure 1) that, if concentration values are in g/L, corresponds to Equation (1):

$$y = 48.856\, e^{-0.001 x};\ R^2 = 0.953 \quad (1)$$

The graph indicates that the exponential value of the signal of the spectrum decreased as the amount of CA increased. This study confirmed that the scavenging activity of the *Convolvulus arvensis* extracts

containing polyphenol constituents could be measured by the decrease of the intensity of the spectral bands of the adduct DMPO-OCH_3 in the EPR spectrum with the amount of antioxidant.

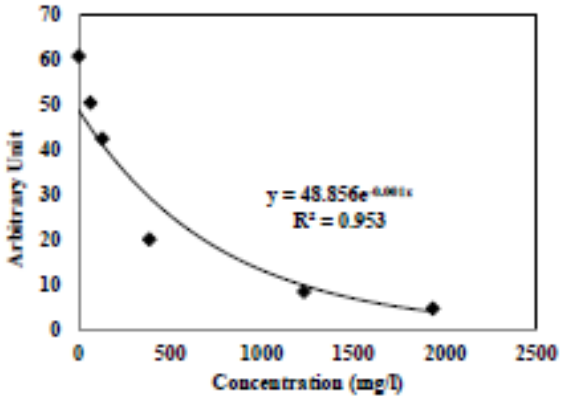

Figure 1. Antioxidant activity determined by the Electron paramagnetic resonance (EPR) spectrum of the radical adduct DMPO-OCH_3 generated from a solution of H_2O_2 (2 mM) and $FeSO_4$ (0.04 mM) with DMPO (14 mM) as spin trap in MeOH as solvent. The EPR signal decreases with the higher antioxidant activity.

3.3. Antioxidant Activity in Model Food

3.3.1. Colour and % Metmyoglobin

Meat colour is one of the most important traits that reflect the meat freshness and quality for consumers. The colour parameters representing lightness (L*), redness (a*), and yellowness (b*) are shown in Table 2. Generally, the value of colour (L*, a* and b*) decreased as the storage time increased. Initial mean lightness (CIE L*) was 38.68 ± 0.87, and control sample showed the lowest value of L* at the end of 10 days storage. There are marginally differences in L* with all samples throughout storage times. The slight change of L* value in meat storage was addressed by few authors [26,27]. The decrease of L* value indicates that a darkening developed, which may be due to the Maillard reaction or the effect of moisture content, which influences lightness values [28,29].

Table 2. Effect of CA extract and BHT on instrumental colour value (L*, a*, b*) of beef patties during 10 days of refrigerated storage at 4 °C. (Mean ± SE).

Assay	Sample	Days of Storages					
		0	2	4	6	8	10
L*	Control	38.68 ± 0.87 ^{a,1}	38.68 ± 1.50 ^{a,1}	37.89 ± 0.32 ^{b,3}	37.10 ± 1.23 ^{b,2}	36.23 ± 0.45 ^{c,2}	35.61 ± 2.22 ^{d,1}
	0.1% BHT	38.68 ± 0.87 ^{a,1}	39.06 ± 1.08 ^{b,2}	38.25 ± 0.97 ^{a,2}	38.43 ± 1.06 ^{a,1}	37.09 ± 1.19 ^{c,1}	36.18 ± 0.46 ^{c,2}
	0.1% CA	38.68 ± 0.87 ^{a,1}	38.60 ± 1.05 ^{a,1}	39.26 ± 1.46 ^{b,1}	38.63 ± 0.55 ^{a,1}	37.11 ± 1.02 ^{c,1}	37.06 ± 1.22 ^{c,3}
	0.3 % CA	38.68 ± 0.87 ^{a,1}	39.94 ± 0.71 ^{b,2}	39.79 ± 1.23 ^{b,1}	38.25 ± 1.40 ^{a,1}	38.91 ± 1.47 ^{a,3}	38.84 ± 1.13 ^{a,4}
a*	Control	7.49 ± 0.27 ^{a,1}	7.77 ± 0.29 ^{a,1}	6.54 ± 0.33 ^{b,1}	6.27 ± 0.16 ^{b,2}	4.71 ± 0.02 ^{c,1}	2.09 ± 0.01 ^{d,1}
	0.1% BHT	7.49 ± 0.27 ^{a,1}	8.18 ± 0.42 ^{b,2}	9.28 ± 0.28 ^{c,2}	7.05 ± 0.31 ^{a,1}	6.36 ± 0.37 ^{d,2}	2.87 ± 0.01 ^{e,1}
	0.1% CA	7.49 ± 0.27 ^{a,1}	7.61 ± 0.33 ^{a,1}	5.57 ± 0.26 ^{b,3}	6.25 ± 0.19 ^{c,2}	6.60 ± 0.33 ^{c,2}	3.31 ± 0.02 ^{d,2}
	0.3 % CA	7.49 ± 0.27 ^{a,1}	7.64 ± 0.21 ^{a,1}	7.20 ± 0.47 ^{a,4}	7.50 ± 0.20 ^{a,1}	7.61 ± 0.37 ^{a,3}	4.08 ± 0.01 ^{b,3}
b*	Control	7.42 ± 0.32 ^{a,1}	4.86 ± 0.01 ^{b,1}	7.68 ± 0.36 ^{a,1}	8.55 ± 0.19 ^{c,1}	9.95 ± 0.21 ^{d,1}	6.77 ± 0.02 ^{e,1}
	0.1% BHT	7.42 ± 0.32 ^{a,1}	6.68 ± 0.16 ^{b,2}	8.40 ± 0.27 ^{c,1}	8.39 ± 0.37 ^{c,1}	8.38 ± 0.24 ^{c,2}	6.10 ± 0.01 ^{d,1}
	0.1% CA	7.42 ± 0.32 ^{a,1}	8.00 ± 0.37 ^{b,3}	8.19 ± 0.33 ^{b,1}	5.17 ± 0.13 ^{c,2}	7.49 ± 0.07 ^{a,3}	4.35 ± 0.09 ^{d,2}
	0.3 % CA	7.42 ± 0.32 ^{a,1}	7.14 ± 0.49 ^{a,4}	7.59 ± 0.29 ^{a,2}	7.01 ± 0.21 ^{a,3}	7.99 ± 0.27 ^{a,3}	3.25 ± 0.01 ^{b,3}

Control: 1.5% salt (w/w); 0.1% BHT: 1.5% salt with 0.1% BHT (w/w); 0.1% CA: 1.5% salt with 0.1% CA (w/w) 0.3% CA: 1.5% salt with 0.3% CA (w/w). $^{a-d}$: Means within a row with different letters are significantly different ($p < 0.05$). $^{1-4}$: For each attribute, means within a column with different number are significantly different ($p < 0.05$). Mean value $n = 6$ and the standard deviation for each assay is less than 5%.

A reduction of the a* value was experienced by all samples in 10 days' storage ($p < 0.05$), indicating that a decrease in redness occurred in the meat. The 0.1% BHT displayed the highest value of a* during three days' storage and declined gradually afterwards ($p < 0.05$). This finding was expected due to the role of BHT as a synthetic antioxidant which is used to retain colour and delay lipid oxidation in the meat [30]. The redness of 0.3% CA was maintained around a value of 7 during the eight days before the colour faded rapidly in 10 days' storage ($p > 0.05$). At the end of storage, 0.3% CA showed the highest a* value followed by 0.1% CA ($p < 0.05$) and 0.1% BHT and control exhibited a low value with no significant difference between both samples ($p > 0.05$). Many features contributed to the red colour in the meat such as the influence of salt and oxygen composition that enhanced the red colour of beef patties [31,32]. The samples had an initial yellowness (b*) value of 7.42 ion that enhanced the red in both samples (eight days before $p > 0.05$). In general, no significant difference ($p > 0.05$) was observed in b* values in all samples throughout storage. The present findings seem to be consistent with other research which found that yellowness in meat patties is not influenced by storage time and packaging conditions [26,33].

The effect of CA extracts and BHT on relative MetMb percentage in beef patties are presented in Table 3. A significant correlation between MetMb (%) and the instrumental colour features was reported previously [22]. The MetMb percentage increased as the storage time increased throughout the 10 days' refrigeration, whereas the control showed the highest MetMb compared to all samples. The treated groups of CA extract and BHT had lower ($p < 0.05$) proportions of MetMb compared to the control at the end of storage. The acceleration of colour deterioration and lipid oxidation depended on many causes, including storage time, type of packaging and test system. Free radicals produced by lipid oxidation in meat are susceptible to initiating the reaction of oxidizing oxymyoglobin (red colour) to metmyoglobin (brown colour) which results in the discolouration of meat during storage. Previous research has indicated a relationship between lipid oxidation and myoglobin oxidation or discolouration in meat products [22,34]. A sufficient amount of antioxidant in the sample can delay the formation of metmyoglobin. The scavenging ability of samples treated with antioxidant can reduce the oxidation of metmyoglobin acting as scavengers of hydroxyl radicals produced from oxidation of oxymyoglobin. The 0.3% of CA extract displayed the lowest metmyoglobin percentage compared to all samples, and the change of % metmyoglobin was inversely proportional to the value of redness (a*).

3.3.2. TBARS Analysis in Beef Patties

In general, the levels of lipid oxidation in beef patties increased over time and the values followed the order: 0.3% CA < 0.1% BHT < 0.1% CA < Control (Figure 2). The presence of a controlled atmosphere with high oxygen packaging (MAP) resulted in higher TBARS values and increased the oxidation rate in muscle food [32,35]. No statistical difference was observed between 0.1% BHT and 0.1% CA on any of the storage days However, the TBARS values of both samples showed significant differences compared to those of the control samples ($p < 0.05$). From seven days onwards, the control reached the highest TBARS values of all samples, with values greater than 1.2 mg malonaldehyde/kg sample. The levels of lipid oxidation were the lowest in 0.3% CA in beef patties throughout storage and significantly lower than for all other samples. The oxidation rate of meat patties was more reduced for a higher concentration of CA extract, as shown by comparison of the rates for 0.1% and 0.3% addition.

The 0.1% BHT was added for comparison with the natural antioxidant bearing in mind the FDA guidelines for using BHT is ≤200 ppm in meat products. The effect of CA extract on lipid oxidation in meat has never been reported. The active properties of CA reported by Hegab and Ghareib [14] have been attributed to various phenolic acids such as ferulic acid, cinnamic acid and *p*-coumaric acid. The antioxidant activity of phenolic compounds is closely related to the hydroxyl group linked to the aromatic ring which is capable of donating hydrogen atoms with electrons and neutralizing free radicals. This mechanism blocks further degradation by oxidation to form MDA, which can be measured by the TBARS method [36]. This study confirmed the potential of CA extract to inhibit lipid degradation in beef patties.

3.3.3. TBARS Analysis in Meat under Active Packaging

The TBARS index (Figure 3) revealed that the coating of beef patties with edible films enriched with antioxidants lowered the oxidation rate during 17 days' storage. By comparison, the gelatin film without any added antioxidants did not display any protective effect. Lipid oxidation with respect to TBARS values of control, meat patties sample and those wrapped with CA and BHT incorporated film showed a significantly different TBARS value ($p < 0.05$) than the control sample. This result suggested that lipid oxidation in meat samples could be minimized by the use of a gelatin film containing CA probably due to the antioxidant activity of the CA extract. However, BHT and CA coated in gelatin film did not show any significant difference between the values for the different periods of storage.

Duthie *et al.* demonstrated the presence of phenolic acids measured using LC-MS in chicken patties mixed with vegetable powders including ferulic acid, *p*-hydrobenzoic acid, *p*-coumaric acid, caffeic acid and cinnamic acid [37]. In reviewing the literature, CA contained a great amount of phenolic compounds that may be responsible for its strong antioxidant activity in many assays. The constituents included *p*-hydroxybenzoic acid, syringic acid, vanillin, benzoic acid, ferulic acid found by Elnaawely and Tawata [25]. HPLC analysis done by Hegab and Ghareib showed traces of eight phenolic constituents including pyrogallic acid, protocatechuic acid, resorcinol, chologenic acid, caffeic acid, salicylic acid, *p*-coumaric acid and cinnamic acid [14]. These compounds lead to many pharmacological benefits to human health. Benzoic acid and its derivatives showed antimicrobial potential [38] while gallic acid and caffeic acid showed 50% inhibitory effects on cancer cell proliferation [39]. *p*-coumaric, ferulic acid and cinnamic acid and their derivatives bring many pharmacological benefits to humans including, anticancer and antioxidant effects [40,41]. Moreover, many constituents detected in the CA extract correlated significantly with antioxidant activity measured by ORAC and TEAC assays and have played an important role in the detoxification of endogenous compounds in humans [42].

Table 3. Effects of CA extract and BHT on metmyoglobin changes in beef patties during 10 days of refrigerated storage at 4 °C. (Mean ± SE).

Assay	Sample	Day of Storages					
		0	2	4	6	8	10
% Metmyoglobin	Control	23.38 ± 0.46 [1]	29.19 ± 0.71 [2]	37.56 ± 1.31 [2]	47.84 ± 1.21 [1]	53.9 ± 1.16 [1]	60.03 ± 2.82 [2]
	0.1% BHT	23.38 ± 0.46 [1]	25.69 ± 1.04 [4]	29.98 ± 0.81 [1]	37.5 ± 1.85 [2]	45.7 ± 1.53 [2]	57.6 ± 1.24 [1]
	0.1% CA	23.38 ± 0.46 [1]	27.96 ± 0.33 [1]	33.48 ± 0.79 [3]	44.81 ± 1.29 [3]	48.7 ± 1.67 [3]	57.1 ± 1.18 [1]
	0.3 % CA	23.38 ± 0.46 [1]	28.91 ± 0.81 [1,2]	30.71 ± 0.29 [1]	33.37 ± 0.94 [4]	40.1 ± 1.53 [4]	50.78 ± 1.56 [3]

Control: 1.5% salt (w/w); 0.1% BHT: 1.5% salt with 0.1% BHT (w/w); 0.1% CA: 1.5% salt with 0.1% CA (w/w) 0.3% CA: 1.5% salt with 0.3% CA (w/w). All samples values are significantly different throughout the storage time ($p < 0.05$) [1–4]: Means within a column with different numbers are significantly different ($p < 0.05$). Mean value $n = 6$ and the standard deviation for each assay is less than 5%.

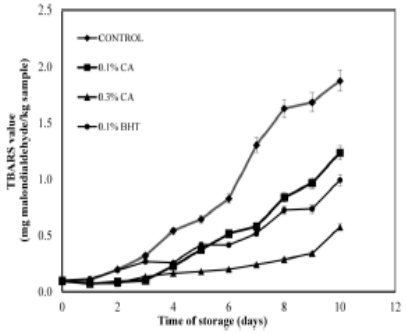

Figure 2. Changes in TBARS values (mg malondialdehyde/kg sample) of control and sample containing different concentrations (0.1% and 0.3% w/w) of CA extract in MAP atmosphere during 10 days storage at 4 ± 1 °C without light. Each sample was measured in triplicate and the average standard deviation for each sample was less than 5%.

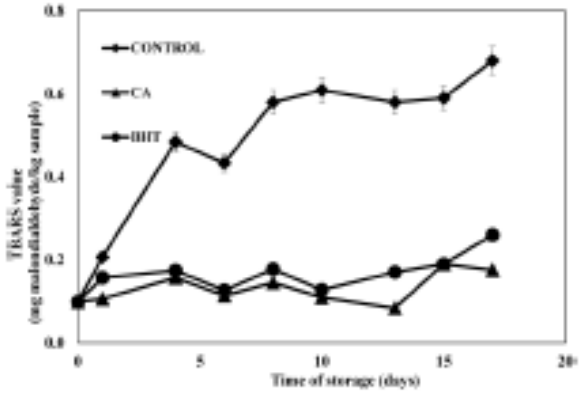

Figure 3. Changes in TBARS values (mg malondialdehyde/kg sample) of control and sample containing BHT and CA extract in MAP atmosphere during 17 days' storage at 4 ± 1 °C without light. Each sample was measured in triplicate and the average standard deviation for each sample was less than 5%.

4. Conclusions

The CA extract showed an excellent antioxidant activity in 50% aqueous ethanol measured by FRAP, TEAC and ORAC assays. This is also the first time that the radical scavenging activity has been evaluated in a CA extract against methoxy radical generated in the Fenton Reaction assessed by EPR.

The CA extract also showed a protective effect against lipid degradation in the muscle food model. Lyophilised CA (0.1% and 0.3% w/w) can be applied as an antioxidant in meat patties. It showed inhibition of lipid oxidation in MAP. 0.3% of CA retained meat redness and browning colour measured by the metmyoglobin assay which was much better than the control ($p < 0.05$) during 10 days' storage. A preliminary study of gelatin based film coated with CA showed there was a significant delay in the lipid degradation in beef ($p < 0.05$). Therefore, this study confirmed that CA could be used by the food industry as a source of antioxidants.

Acknowledgments

The authors acknowledge the special fund from The Technical University of Catalonia, University Malaysia Pahang, Malaysia Government and the Government of Andorra in the framework of the research grants of Working Community of the Pyrenees (ACTP016-AND/2012) for the financial support of this research. The authors acknowledge Monica Blanco, Marta Rosal and Michael H. Gordon for their help with the English.

Author Contributions:

Maria Pilar Almajano and Nurul Aini Mohd Azman conceived and designed the study. Maria Gabriela Gallego performed the LCMS analysis whereas Luis Julia and Lluis Fajari supervise the experimental work on the EPR study. Nurul Aini Mohd Azman wrote the paper with assistance of Maria Pilar Almajano who reviewed all the manuscript and the final version to be submitted.

Conflicts of Interest

The authors declare no conflict of interest.

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Antioxidants 2014, *3*, 439-454; doi:10.3390/antiox3020439

OPEN ACCESS

antioxidants

ISSN 2076-3921

www.mdpi.com/journal/antioxidants

Article

Avocado Seeds: Extraction Optimization and Possible Use as Antioxidant in Food

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Received: 25 February 2014; in revised form: 18 April 2014 / Accepted: 13 May 2014 / Published: 10 June 2014

Abstract: Consumption of avocado (*Persea americana* Mill) has increased worldwide in recent years. Part of this food (skin and seed) is lost during processing. However, a high proportion of bioactive substances, such as polyphenols, remain in this residue. The primary objective of this study was to model the extraction of polyphenols from the avocado pits. In addition, a further objective was to use the extract obtained to evaluate the protective power against oxidation in food systems, as for instance oil in water emulsions and meat products. Moreover, the possible synergy between the extracts and egg albumin in the emulsions is discussed. In Response Surface Method (RSM), the variables used are: temperature, time and ethanol concentration. The results are the total polyphenols content (TPC) and the antiradical power measured by Oxygen Radical Antioxidant Capacity (ORAC). In emulsions, the primary oxidation, by Peroxide Value and in fat meat the secondary oxidation, by TBARS (Thiobarbituric acid reactive substances), were analyzed. The RSM model has an R^2 of 94.69 for TPC and 96.7 for ORAC. In emulsions, the inhibition of the oxidation is about 30% for pure extracts and 60% for the combination of extracts with egg albumin. In the meat burger oxidation, the formation of TBARS is avoided by 90%.

Keywords: RSM; avocado pit; ORAC; extraction; emulsion; oxidation; meat

1. Introduction

Vegetables and fruits are essential foods in our diet and also have many compounds that are beneficial for health due to minor components. These minor components include phenolic substances [1]. These are secondary metabolites of plants. They have an aromatic ring with one or more hydroxyl groups. Their complexity may be high, as for example quercetin, which is one flavone with several aromatic rings. The properties depend on the arrangement and/or structure of the molecule [2].

In recent times, many plants have been studied in order to characterize them depending on the amount of polyphenols they have and on their potential use [3].

The polyphenols are associated with the potential prevention of diseases which are due to the presence of free radicals, such as cardiovascular insufficiency, hypertension, inflammatory conditions, asthma, diabetes and Alzheimer's [4], thanks to their antiradical power. For this reason, they are very useful in food products, since they prevent lipid peroxidation due to the attack of free radicals [5]. They also protect against oxidation, direct or indirect, caused by metal cations [6]. These cations stimulate the creation of reactive oxygen species (ROS), which are harmful to the health. In some cases, polyphenols have been used as preservatives, protecting against microorganisms [7].

The process of food, especially for IV and V gamma products, produces many byproducts and waste. This type of waste has a significant environmental impact due to the organic charge. It also has associated handling, transport and storage costs, among others. Therefore, more and more alternative uses for these residues are sought, as for instance animal feed and fertilizers, among others. In the present case, it is interesting to obtain, through an optimized extraction process, harmless substances with high antioxidant power. Thus, what was a waste becomes a "high value-added" product [8,9]. Previous examples already studied [10–12] are the orange juice industry, where a large amount of skin and seeds are produced with a high content of polyphenols and the industry of processed apple, pear and peach, with a significant amount of skin byproduct. There is evidence that the skin may even have a greater amount of polyphenols than the flesh [13]. Also, the waste from wine and beer production includes phenolic compounds [9]. Other studies have focused on the shells of nuts, rice and wheat in which large amounts of polyphenols are found [14].

In the avocado industry the pulp is used, while the skin and the seeds are discarded as waste. These residues are rich in polyphenols with antioxidant and antimicrobial power [15]. Among the polyphenols the (+)-catechin and (−)-epicatechin [16] and chlorogenic and protocatechuic acid, are included [14]. Previous studies on this residue have been applied to pork burgers and have been shown to be effective in preventing oxidation and microbial growth [15].

Given the above, it can be concluded that polyphenols obtained from these industrial wastes can be potent antioxidants and, in some cases, they are better than synthetic antioxidants such as BHA or BHT which in high doses can become toxic [17].

In order to optimize the extraction process, response surface methodology (RSM) has been used. Phenolic compounds extraction optimization from strawberries [18], apple pulp [9] and residues of

chestnuts [19], are examples of this. This method establishes a multivariable mathematic model to obtain the relationship between responses and independent variables [20,21] with the use of a minimal number of experiments.

This paper consists of two main objectives. First, a mathematical model was obtained to predict the best conditions of extraction of polyphenols from dried avocado seed. Second, an extract using these conditions was obtained and the effect of lyophilized powder in the delay oxidation in oil-in-water (O/W) emulsions and beef meat burgers analyzed.

2. Experimental Section

2.1. Materials

2,2′-Azo-bis(2-amidinopropane) dihydrochloride (AAPH), was used as peroxyl radical source. Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid), ethanol, fluorescein, AAPH, BHA, egg albumin, *p*-anisidine (4-amino-anisole; 4-methoxy-aniline), isooctane, potassium persulfate, acetic acid (glacial) and 2-thiobarbituric acid were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK). Folin-Ciocalteu reagent, sodium carbonate and 1,6-diaminohexane were supplied by Merck (Darmstadt, Germany). Trichloroacetic acid, hydrochloric acid and Tween® 20 (Panreac Quimica S.L.U, Barcelona, Spain) were acquired from Panreac Quimica S.L.U. (Barcelona, Spain). Refined sunflower oil, with no added antioxidants, was purchased from a local retail outlet. All compounds were of reagent grade.

2.2. Avocado Preparation

The avocado (*Persea americana*) was obtained in the local market; the seeds were separated from other edible parts. This waste was homogenized and frozen at −80 °C for lyophilization. Then the seeds were ground into a powder by using a Moulinex mill (A5052HF, Moulinex, Lyon, France). The particle size was standardized with a number 40 mesh sieve. Finally, the powder was stored in a dark bottle in a desiccator until use.

2.3. Extraction Procedure

Extraction was carried out in dark bottles: lyophilized sample powder (0.25 g) was blended with 15 mL of solvent of concentration specified by the experimental design (Table 1). This mixture was placed in a bath by stirring at the required temperature and time specified by the experimental design (Table 1). At the end, it was cooled in a refrigerator at 5 °C, centrifuged (Orto Alresa, Madrid, Spain) at 2500 rpm for 10 min, vacuum filtered and the lost solvent was replaced. The extract was stored at −20 °C until used for analysis.

2.4. Total Phenolic Content (TPC)

TPC was determined spectrophotometrically following the Folin-Ciocalteu colorimetric method [22]. A sample diluted 1:4 with milli-Q water was stirred in triplicate. The final concentration in the well (96 wells plate was used) was: 7.7% v/v sample, 4% v/v Folin-Ciocalteu's reagent, 4% saturated

sodium carbonate solution and 84.3% of milli-Q water, all mixed. The solution was allowed to react for 1 h in the dark and the absorbance was measured at 765 nm using a Fluostar Omega (BMG Labtech, Ortenberg, Germany). The total phenolic content was expressed as mg Gallic Acid Equivalents (GAE)/g dry weight.

Table 1. Experimental design and responses for extraction.

Temperature (°C)	Ethanol Concentration (%)	Time (min)	TPC (mg GAE/g dw)	ORAC (mg TE/g dw)
60.00	60.00	25.00	41.00 ± 0.97	104.16 ± 2.13
60.00	93.63	25.00	35.10 ± 0.24	116.12 ± 1.03
80.00	80.00	5.00	46.78 ± 0.59	153.17 ± 3.84
26.36	60.00	25.00	40.78 ± 0.17	70.54 ± 0.97
60.00	60.00	25.00	41.10 ± 0.57	106.10 ± 2.40
40.00	40.00	45.00	43.24 ± 0.76	104.01 ± 2.35
80.00	80.00	45.00	45.43 ± 0.49	144.94 ± 2.84
80.00	40.00	45.00	45.37 ± 1.39	130.08 ± 2.65
80.00	40.00	5.00	43.70 ± 0.66	150.03 ± 1.73
60.00	60.00	25.00	40.90 ± 0.47	104.28 ± 1.03
60.00	60.00	55.22	42.87 ± 0.70	158.77 ± 1.33
40.00	40.00	5.00	41.19 ± 0.55	99.17 ± 1.81
60.00	60.00	2.77	42.92 ± 1.13	155.44 ± 2.71
93.64	60.00	25.00	46.95 ± 0.09	126.23 ± 3.35
60.00	26.36	25.00	42.33 ± 0.10	129.78 ± 3.84
40.00	80.00	45.00	38.98 ± 0.45	100.72 ± 3.27
40.00	80.00	5.00	35.48 ± 0.55	91.01 ± 3.51

GAE: Gallic Acid Equivalents; TE: Trolox Equivalents; TPC: Total Phenolic Content; ORAC: Oxygen Radical Antioxidant Capacity.

2.5. ORAC Assay

Antioxidant activities of avocado seeds extracts were determined by the ORAC assay, as reported by Casettari *et al.* [23]. The assay was carried out using a Fluostar Omega equipped with a temperature-controlled incubation chamber. The incubator temperature was set to 37 °C. The extract samples were diluted 1:20 with milli-Q water. The assay was performed as follows: 20% of sample was mixed with Fluorescein 0.01 mM, and an initial reading was taken with excitation wavelength, 485 nm and emission wavelength, 520 nm. Then, AAPH (0.3 M) was added, measurements were continued for 2 h every 5 min. This method includes the time and decrease of fluorescence. The area under the curve (AUC) was calculated. A calibration curve was made each time with the standard Trolox (500, 400, 250, 200, 100, 50 mM). The blank was 0.01 M phosphate buffered saline (pH 7.4). ORAC values were expressed as mg Trolox Equivalents (TE)/g of dry weight.

2.6. Statistical Analysis

RSM was used to determine the optimal conditions of polyphenol extraction. A central composite design (CCD) was used to investigate the effects of three independent variables with two levels

(solvent concentration, extraction temperature, and extraction time) with the dependent variables (TPC, ORAC activity). CCD uses the method of least-squares regression to fit the data to a quadratic model.

The adequacy of the model was determined by evaluating the lack of fit, coefficient of determination (R^2) obtained from the analysis of variance (ANOVA) that was generated by the software. Statistical significance of the model and model variables were determined at the 5% probability level ($\alpha = 0.05$). The software uses the quadratic model equation shown above to build response surfaces. Three-dimensional response surface plots and contour plots were generated by keeping one response variable at its optimal level and plotting that against two factors (independent variables). Response surface plots were determined for each response variable. The coded values of the experimental factors and factor levels used in the response surface analysis are shown in Table 1. The graphics and the RSM analysis were made by software Matlab version R2013b (The MathWorks Inc., Natick, MA, USA, 2013). All responses were determined in triplicate and are expressed as average ± standard deviation. The answers have a percentage deviation less than 10%.

2.7. Water-Oil Emulsions

Oil-in-water emulsions (20.2 g) were prepared by dissolving Tween-20 (1%) in acetate buffer (0.1 M, pH 5.4), either with or without protein, namely egg albumin (0.2% w/w) and avocado seeds extracts (0.45% w/w, 0.225% w/w, 0.1125% w/w). The emulsion was prepared by the dropwise addition of oil (sunflower oil) to the water phase, cooling it in an ice bath with continuous sonication with a Vibracell sonicator (Sonics and Materials, Newtown, CT, USA) for 4 min. All emulsions were stored in triplicate in 60 mL glass beakers in the dark (inside an oven) at 30 °C in an incubator. Two aliquots of each emulsion (0.005–0.1 g, depending on the extent of oxidation) were removed periodically for determination of peroxide value (PV).

2.8. Peroxide Value (PV)

PV was determined by the ferric thiocyanate method [24] (after calibrating the procedure with a series of oxidized oil samples analyzed using the AOCS Official Method Cd 8-53). Data from the PV measurements were plotted against time.

2.9. Meat Preparation

Fresh beef meat was purchased from a local processor 96 h postmortem. All subcutaneous and inter-muscular fat and visible connective tissue were removed from the fresh beef muscle. Lean meat was ground through 0-4 mm plate using a meat grinder (PM-70, Mainca, Barcelona, Spain). The ground meat was divided into six portions for each experiment prior to the addition of the sodium chloride or different concentration of powder (freeze-dried extract of powder of avocado). The lyophilized avocado and the powder of direct avocado were mixed with the salt final concentration of 1.5% (w/w). Each portion of beef meat was mixed manually with each solid. Each mixed sample was divided into nine smaller portions (about 10 g each) and allocated onto trays. The meat was packed under MAP (20% CO_2 and 80% O_2) in polystyrene/EVOH/polyethylene trays, heat sealed with laminated barrier film and stored at 4 ± 1 °C for 8 days. Patties were evaluated for lipid oxidation.

2.10. Thiobarbituric Reactive Substances

Fat meat oxidation was determined by the concentration of thiobarbituric acid-reactive substances (TBARS) using the method described by Domenech Asensi (2013) [25] with some modifications. In the dark, 1 g of burger patty was dispensed in tubes and 1 mL of EDTA was added. The samples were homogenized for 5 min sin an Ultra-Turrax (Ika®-Werke, Staufen, Germany) with 5 mL of TBARS reactive (Trichloroacetic acid, 9.2%; Hydrochloric acid, 2%; Thiobarbituric acid, 0.22%, all w/w final). During homogenization, the tubes were placed in an ice bath to minimize the development of oxidative reactions. The sample tubes were heated at 90 °C in a boiling water bath for 20 min and then left to cool. Two milliliters of slurry was centrifuged (10,000 rpm for 10 min). The absorbance was measured at 531 nm in a Spectrophotometer Zuzi model 4201/20 (AUXILAB, SL, Navarra, Spain). The result is expressed in mg of MDA/kg sample.

3. Results and Discussion

3.1. Extraction Optimization

Experimental design was carried out to see the effects of temperature, solvent concentration (ethanol) and time in both TPC and radical scavenging (measured by ORAC). Several authors used ethanol/water as solvent to extract different raw material polyphenols, such as seeds, grape marc, fruits, among others [26–30]. Ethanol concentration with the highest polyphenols yield is in the range of 10%–60%. Ethanol, instead of methanol, is used when it is necessary to reduce the toxicity of extracts [18]. The time effect was measured between 5 and 45 min, because some research reported that it is enough to achieve the maximum amount of polyphenols [31,32]. Temperature bounds were taken between 40 and 80 °C, to achieve the maximum temperature that does not have a negative effect on the polyphenols stability [33]. All these parameters are collected in Table 1 which shows the experimental design for the variables temperature (T), ethanol concentration (% EtOH) and time (t), with responses of TPC and antiradical activity measured by ORAC.

Figure 1 shows the relationship between the variables T, % EtOH and t in polyphenol extraction. The process is favored by high temperatures and low concentrations of ethanol (in the studied range). This behavior can be attributed to the nature of the polyphenols present in the sample, mainly chlorogenic acid and protocatechnic acid [34] both highly soluble in water. The solvent plays an important role in mass transfer of the compounds; not all polyphenols show identical behavior in the extraction process, and the less polar polyphenols are favored by the highest concentration of ethanol [9].

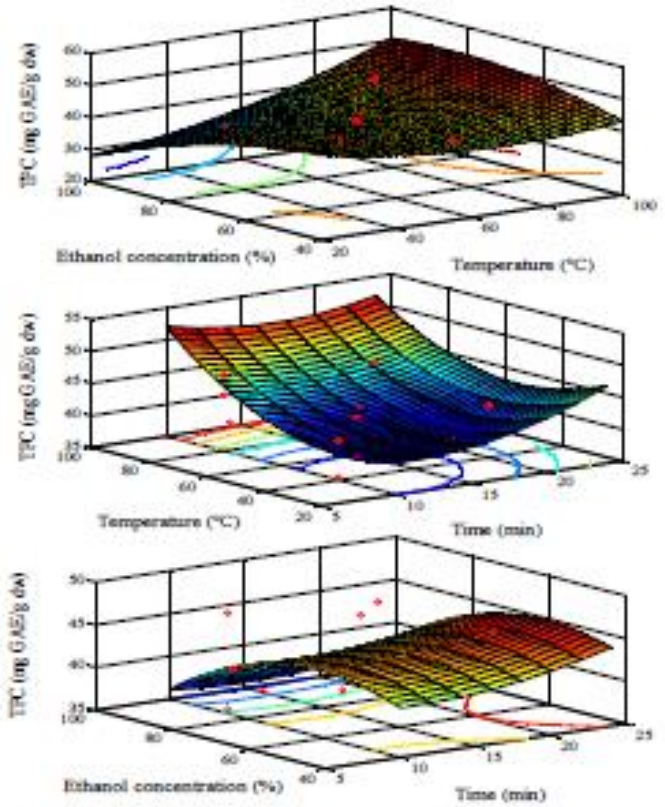
The effect of temperature on the extraction is associated with the solubility of the components present in the avocado pit. This variable, T, has a marked influence on the diffusivity of the substances [30]. Solubility increases with temperature. Time has no influence in the extraction process. This means that from the beginning, the extraction is governed by the solubility and diffusion, and both are almost complete after 5 min.

Figure 2 shows the effect of the parameters on the antioxidant power measured by ORAC. The ORAC increases with temperature. In the investigated range, the ORAC is increased about

44% (Table 1). Furthermore, as stated above, it is in accordance with the higher polyphenols solubility at high temperature. This means that these kinds of polyphenols are thermo-resistant [20].

The effect observed for the percentage of ethanol is similar to that described in the TPC. An increase in the ethanol concentration causes a decrease in antioxidant activity. It is not a new fact, because similar results were described in other studies and were justified by the polarity of the compounds of the extract [18].

Figure 1. Response surface model plot: the variable is the total phenolic content (TPC) of the extract. % EtOH with temperature; temperature with time; % EtOH with time.

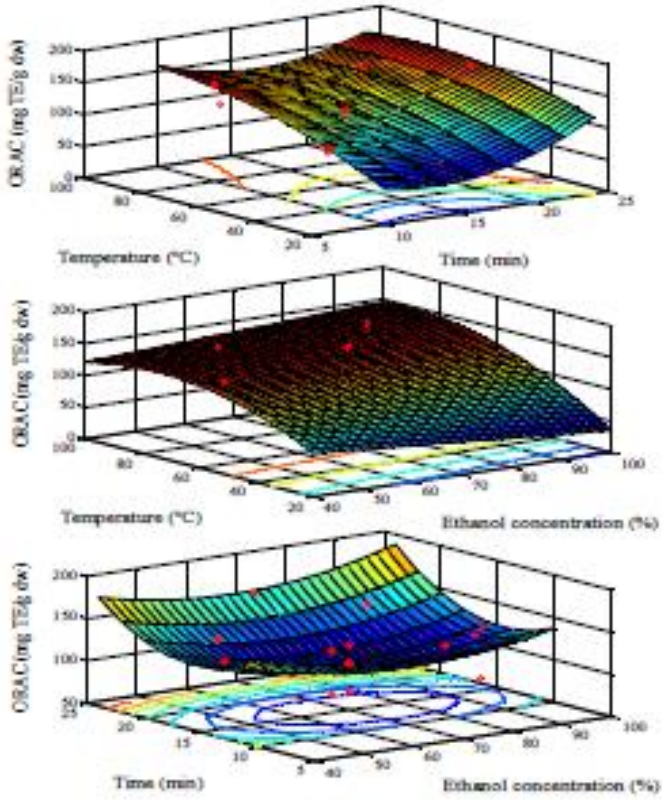


On TPC the variable t has no influence, while on ORAC small changes were observed, but all of them with similar final values. One possible explanation is that there are antioxidant compounds with slow solubilization and, therefore, the time promotes an increase in total extraction [35].

Table 2 shows the "*p* values" of the mathematical model for the coefficients, with the decoded variables. It starts with the complete model, taking the variables that have less influence, *i.e.*, with

$p > 0.05$. For TPC all those that are with % EtOH and t are involved. This means that the more important variable is T. However, on the ORAC, the variables that have more influence are % EtOH, *t*, and these quadratic terms. From the data, different iterations were made and less influential terms were eliminated; after which the values were recalculated. With these data the reduced model was obtained and provided a better fit. In ORAC the predicted R^2 becomes 77.88 which is within the range of a good set [36].

Figure 2. Response surface model plot: the variable is the Oxygen Radical Antioxidant Capacity (ORAC) of the extract. Temperature with time; % EtOH with temperature; % EtOH with time.



Therefore, with the exception of *T* × % EtOH for the TPC, all of the crossed terms disappear in the reduced model (which is used to adjust and to determine the optimal extraction conditions).

Additionally, the quadratic variables % EtOH × % EtOH and $t \times t$, as well as the linear variable t are eliminated for the TPC. The quadratic term $T \times T$ is eliminated from the model which determines the scavenging activity. This is summarized in Table 2.

Table 3 lists the completed model and the reduced model equations. The reduced model has a higher R^2 predicted which means that it is more reliable in estimating a response.

When the fitting was considered good enough, the experiment was performed in the laboratory to obtain the real value. Table 4 contains these values for the TPC and for the ORAC. The TPC is fitted with less than a 4% error (the predicted value is 43.6 mg GAE/g dw, compared to an experimental value of 45.01 mg GAE/g dw). This indicates that the initial hypothesis was correct, and demonstrates that T is the variable with the greatest influence on the maximum TPC extraction.

Table 2. *p*-Values for each of the constants in the equation of the mathematical model.

Term	*p*-Value	
	Response	
	TPC	ORAC
Complete Model		
Constant	0.001	0.006
Temperature (°C)	0.012	0.069
Ethanol (%)	0.291	0.022
Time (min)	0.804	0.001
Temperature (°C) × Temperature (°C)	0.014	0.135
Ethanol (%) × Ethanol (%)	0.622	0.046
Time (min) × Time (min)	0.068	0.000
Temperature (°C) × Ethanol (%)	0.003	0.186
Temperature (°C) × Time (min)	0.119	0.071
Ethanol (%) × Time (min)	0.610	0.435
Reduced Model		
Constant	0.000	0.000
Temperature (°C)	0.005	0.000
Ethanol (%)	0.001	0.031
Time (min)	-	0.000
Temperature (°C) × Temperature (°C)	0.029	-
Ethanol (%) × Ethanol (%)	-	0.033
Time (min) × Time (min)	-	0.000
Temperature (°C) × Ethanol (%)	0.004	-

TPC (mg GAE/g dw); ORAC (mg TE/g dw); GAE: Gallic Acid Equivalent; TE: trolox equivalent.

However, the values which maximize scavenging activity (ORAC) have a greater deviation. The value predicted by the reduced model was 200.66 mg TE/g dw, compared to an experimental value of 154.3 mg TE/g dw, which represents a deviation of 23.1%.

The best-fitting experimental conditions were then applied, *i.e.*, 23 min extraction with 56% EtOH and 63 °C. This extract was lyophilized and used in subsequent experiments.

Table 3. Mathematical equations from Response Surface Method (RSM) for each of the responses, with their respective value of R^2 and R^2-predicted.

Response	Equation	R^2 Value	
		R^2	R^2 Pred.
Complete Model			
TPC	$62.87 - 0.47\,T - 0.25\,[\%] - 0.14\,t + 0.003\,T^2 - 0.001\,[\%]^2 + 0.03\,t^2 + 0.006\,T \times [\%] - 0.007\,T \times t - 0.003\,[\%] \times t$	94.69	57.0
ORAC	$318.2 + 2.03\,T - 04.41\,[\%] - 019.5\,t - 00.009\,T^2 + 0.023\,[\%]^2 + 0.7\,t^2 + 0.012\,T \times [\%] - 00.053\,T \times t - 00.03\,[\%] \times t$	96.7	75.0
Reduced Model			
TPC	$69.7 - 00.53\,T - 00.39\,[\%] + 0.002\,T^2 - 00.006\,T \times [\%]$	85.7	66.76
ORAC	$345.7 + 1.01\,T - 03.92\,[\%] - 022.01\,t + 0.027\,[\%]^2 + 0.73\,t^2$	91.88	77.88

T: Temperature (°C); [%]: Ethanol concentration (%); *t*: Time (min); Pred.: response predicted by model. TPC in mg GAE/g dw and ORAC in mg TE/g dw.

Table 4. Optimal conditions for the extractions for TPC and ORAC, given by RSM.

Model	Conditions			Response		
	Temperature (°C)	Ethanol (%)	Time (min)	Predicted	Predicted RM	Experimental
TPC	63	56	23	51.75	43.6	45.01
ORAC	93.6	44.7	7	206.82	200.66	154.3

TPC in mg GAE/g dw; ORAC in mg TE/g dw.

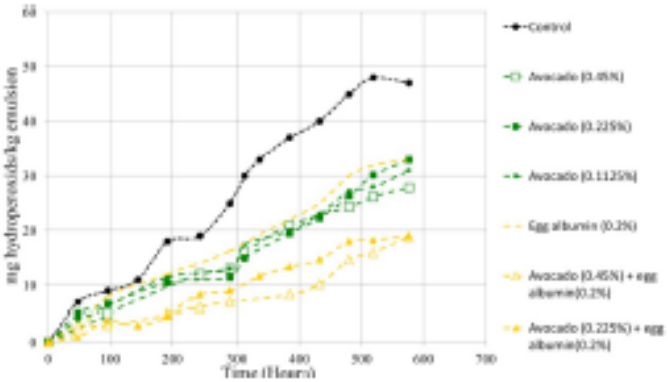
3.2. Extract Optimized Effect in Oil-in-Water Emulsions (O/W)

Figure 3 shows the evolution of peroxide value over time. In this case, the possible synergy between the extract (with different concentrations) and egg albumin was determined. Firstly, it should be noted that both albumin and various concentrations of the extract of avocado produce significant protection against oxidation. For example, within the 400 h of the experiment the amount of hydroperoxides produced is 90% higher in the control than in any of the samples (20 mg hydroperoxides/kg of emulsion hydroperoxides *vs.* 38 mg/kg for the emulsion control). Notably, there were no significant differences ($p < 0.05$) for the three tested avocado concentrations (0.1125%, 0.225% and 0.45% w/w), as well as egg albumin (0.2% w/w). This fact could be explained by the solubility of the hyophilized extract in water and the ability to coat the oil drop generated in the emulsion and prevent oxidation thereof. The necessary concentration that allows this protection is already achieved with 0.1125% and the results do not improve if increased. Similar behavior has been published elsewhere [37–39].

In fact, putting together two different compounds (avocado pit extract and egg protein) allows greater protection against oxidation and further differentiates the two concentrations of the tested extract. For example, the time required to reach 15 mg hydroperoxides/kg emulsion goes from 180 h of the control group up to 480 h for the sample containing 0.45% extract + 0.2 egg protein. This is an increase of 260% superior durability. In the intermediate areas three avocado extract concentrations

were tested, as well as the protein (an increase in the durability between 150% and 180%) and one that contains 0.225% of avocado and 0.2% protein, with an improvement of the durability of 220%. Almajano and Bonilo-Carbognin already published similar results of synergy [40,41]. As a summary, it can be said that increasing the concentration of the extract does not improve the durability. However, the incorporation of small amounts of protein allows significant differences to be found between the samples containing protein and those that do not contain it.

Figure 3. Peroxide values *vs.* time in the emulsions.



Avocado pits contain polyphenolic compounds (such as protocatechnic acid, chlorogenic acid, syringic acid and rutin), which are very strong antioxidants [34]. In 2010, Sasaki [42] studied the antioxidant power of chlorogenic acid in oil-water emulsions. The effects discovered are remarkable. The authors analyzed the presence of other compounds, which in that case were also polyphenolic compounds. Additionally, they demonstrated that the presence of several different compounds provided better results than the added individual effects.

As it was stated before, 1% of surfactant (Tween-20) was added to the emulsion prepared in the present work. This eases the dissolution of the polyphenolic compounds, thus increasing the antioxidant activity in the emulsion.

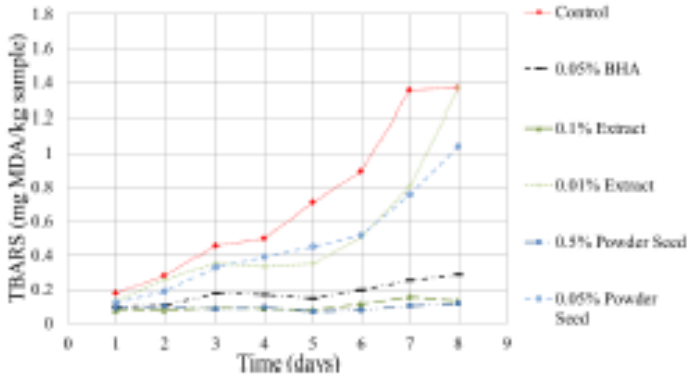
3.3. Effect of the Extract in Burger Meat

The TBARS method is widely used to determine the oxidation of fats and oils in foods [43–45]. In Figure 4, the evolution of TBARs *vs.* time for each of the studied beef burger meat patties is collected. Samples containing 0.1% lyophilized extract and 0.5% direct seed powder have no significant differences compared with the BHA (0.05%), but show a big difference compared with the control. The lower concentration (0.01% and 0.05% lyophilized extract powder direct seed) presented intermediate behavior, as expected. The duration of the experiment was 8 days and it was observed that the burger meat with 0.5% seed powder and 0.1% of lyophilized extract had no significant oxidation, or the protection is higher than 90%. These results are similar to those reported by Weiss *et al.* [46] for pork burgers. That study examined protecting fat oxidation also with excellent

results [46]. Additional results along the same lines have avocado oil added directly to the pork burgers. This shows a positive effect on the conservation of the burger [47].

It is not the first time avocado pits have been used in meat products. Rodriguez-Carpena *et al.* (2011) [15], prepared pork meat pies and inserted the grinded avocado pits to protect the meat against lipid oxidation. The authors indicated that one of the factors might be the formation of chelates with the copper and iron cations. These cations, in their free ionic state, could cause the creation of free radicals.

Figure 4. The TBARS (Thiobarbituric acid reactive substances) values for the meat emulsions.



4. Conclusions

RSM was used to identify the best conditions for the extraction of compounds with an antioxidant activity from an organic residue: the avocado pit. The reduced model obtained provides parameters that fit with those of the TPC (with a 3.13% error when compared to the experimental value).

The lyophilized extract was used as protection from the oxidation of oils (oil-in-water emulsions) and fat (beef burgers) with excellent results, especially in meat, in which the durability of the burger meat is significantly increased relative to oxidation.

These studies should encourage further exploration in this area of study in order to obtain a byproduct of the natural antioxidants that currently as waste are worthless.

Acknowledgments:

This research was conducted at the Department Chemical Engineering, University Polytechnic of Catalonia, Spain. The authors thank Universidad Experimental Politécnica "Antonio Jose de Sucre" and Carlos Barreiro, for their support in this research. The authors want to thank Mariona Vila for the support.

Author Contributions:

Francisco Segovia and Sara Peiró have done the experimental section and the design, data acquisition, analysis and data interpretation; Maria Gabriela Gallego and Nurul Aini Mohd, participate in revising it critically for important intellectual content. Finally, Maria Pilar Almajano, as director, had the initial idea, the constant support, the English revision and the final approval of the version to be submitted and any revised version.

Conflicts of Interest

The authors declare no conflict of interest.

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