UNIVERSITAT POLITÈCNICA DE CATALUNYA
BARCELONATECH
Departament d'Enginyeria Química

Tesis Doctoral

# Aprovechamiento de residuos agro-industriales: Preparación de extractos, caracterización y uso en alimentos

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Departamento de Ingeniería Química

Universitat Politècnica de Catalunya

Barcelona, Septiembre 2015

UNIVERSITAT POLITÈCNICA DE CATALUNYA
BARCELONATECH
Escola de Doctorat

**Curs acadèmic:**

# Acta de qualificació de tesi doctoral

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Unitat estructural responsable del programa Departament d'Enginyeria Química

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Reunit el Tribunal designat a l'efecte, el doctorand / la doctoranda exposa el tema de la seva tesi doctoral titulada

**Aprovechamiento de residuos agro-industriales: Preparación de extractos, caracterización y uso en alimentos.**

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**AGRADECIMIENTOS**

Esta Tesis Doctoral no habría sido posible gracias a todas esas personas que me han ayudado todo este tiempo:

En especial a mi directora, Profesora María Pilar Almajano. Quiero agradecerle encarecidamente toda la orientación y el apoyo académico que me ha prestado. Su tiempo y su paciencia han sido inestimables. También quiero agradecerle su optimismo, su entusiasmo y la confianza que depositó en mí desde el primer momento, el darme todo el soporte necesario para cumplir con esta asombrosa tarea, la libertad de aprender dentro del laboratorio todo lo que ahora sé e inculcarme el amor a la investigación. Para usted profesora, mis respetos, lealtad y admiración.

A mi esposa e hijos, inspiradores de los logros, tranquilizadores de oficio, gracias por ese apoyo incondicional, por agregarle ese toque acogedor a este trayecto, compartir mis alegrías y animarme cuando pensaba que no podía llegar. Además, desde el fondo de mi corazón dar gracias a la inquitud de mi esposa por querer siempre estar mejor intelectual y sentimentalmente.

A mis padres y hermanos, que con su esfuerzo y visión de familia, me han acompañado en este camino de éxito.

A mis compañeros en el laboratorio: Aini, Sara, Gaby y Monika. Gracias por darme la oportunidad de conocerlos y contribuir a mi desarrollo.

Gracias a todos los estudiantes que han compartido conmigo este trabajo: Marie, Carlos, Juan José, Xisca, Laia, Ana y Julia.

Quiero dar las gracias al personal del CCiT UB en el departamento de técnicas separativas, por su disponibilidad y profesionalismo.

Y a todos aquellos que directa o indirectamente participaron en este logro. Muchas gracias.

“Un sutil pensamiento erróneo puede dar lugar a una indagación fructífera que revela verdades de gran valor” Isaac Asimov

## INDICE

# GLOSARIO

| | |
|---|---|
| AAPH | 2,2'-azobis (2-amidinopropano) dihidroclórico. |
| ABTS | 2,2'-azino-bis (ácido 3-etilbenzotiazolina-6-sulfónico). |
| ANOVA | Análisis de la varianza. |
| BE | Extracción por lotes. |
| BHA | Hidroxibutilanisol. |
| BHT | Butilhidroxitolueno. |
| BSA | Albúmina de suero bovino. |
| CCD | Diseño central compuesto. |
| CE | Extracción continua. |
| CPE | Extracción punto de nube. |
| DMPO | 5,5-dimetil-pirrolina N-óxido |
| DPPH | Difenilpicrilhidrazilo. |
| DSC | Calorimetria diferencial de barrido |
| DW | Peso seco. |
| EC | Epicatequina. |
| ECG | Epicatequingalato. |
| EDTA | Ácido etilendiaminotetraacético. |
| EFSA | Autoridad Europea para la Seguridad de los Alimentos. |
| EPR | Resonancia paramagnética electrónica. |
| EtOH | Etanol. |
| EVOH | Etilen-Vinil-Alcohol. |
| FA | Ácido ferúlico. |

| | |
|---|---|
| FRAP | Poder de reducción antioxidante del ión férrico. |
| FW | Peso fresco. |
| GAE | Equivalente de ácido gálico. |
| GC | Cromatografía de gases. |
| HDL | Lipoproteina de alta desidad. |
| HPLC | Cromatografía líquida de alta resolución. |
| IP | Punto de inducción. |
| LDL | Lipoproteina de baja densidad. |
| MAP | Empaque de atmósfera modificada. |
| MDA | Malondialdehído |
| MeOH | Metanol. |
| O/W | Aceite en agua. |
| OIT | Tiempo de oxidación inducida. |
| ORAC | Capacidad de absorción de radicales de oxígeno. |
| p-AV | Valor de p-anisidina. |
| PEF | Campo de pulso electromagnético. |
| PG | Propil galato. |
| PGR | Pirogalol rojo. |
| PUFA | Ácido graso poliinsaturado. |
| RMSE | Error cuadrático medio. |
| RSM | Metodoligía de superficie de respuesta. |
| SAE | Extracto de semilla de aguacate. |
| SSE | Suma de los cuadrados. |
| TBARS | Sustancias reactivas al ácido tiobarbitúrico. |

| | |
|---|---|
| TBHQ | Tert-butil-hidroquinona |
| TE | Equivalente de trolox. |
| TPC | Contenido total de polifenoles. |
| UABE | Extracción por lotes asistida con ultrasonidos. |
| UACE | Extracción continua asistida con ultrasonidos. |
| VP | Valor de peróxidos. |
| W/O | Agua en aceite. |
| Zp | Índice de desintegración de célula. |

# 1 INTRODUCCION

## 1.1 La industria de alimentos y sus sub-productos

El consumo de vegetales y frutas ha ido en aumento, en gran parte debido a todas las propiedades terapéuticas que han sido asociadas a ellos [1]. Algunas de estas frutas y verduras son consumidas de forma procesada, bien sea en conserva o en algún otro producto listo para comer. En este proceso se producen sub-productos, epicarpios (cortezas) y/o semillas, partes no comestibles, entre otros, los cuales suelen ser desechos difíciles de manejar ya que poseen una gran carga orgánica y llevan un costo adicional para su tratamiento [2,3]. Estos sub-productos pueden ser utilizados como materia prima para la obtención de otros productos, debido a la procedencia que tienen. Azúcares, espesantes (pectinas y almidones), aceites esenciales, sustancias bioactivas son algunos de los compuesto que se pueden encontrar en estos desperdicios de la agroindustria [4–6].

Por ejemplo, en la industria del procesado de la piña al elaborar conservas y zumos, no se aprovecha aproximadamente el 60% del total. En estos desperdicios se pueden encontrar mayoritariamente enzimas (bromelina), azúcares (fructosa) y polifenoles, entre ellos el ácido ferúlico [7,8]. En el procesado del aguacate, la semilla, que representa entre el 13-18% se considera un sub-producto. Sin embargo, posee gran cantidad de polifenoles (ácido clorogénico, epicatequina, epicatequingalato) que pueden tener diversas utilidades en la industria alimentaria [9–11]. En la industria de los cítricos, limón y naranja, la piel constituye uno de sus principales sub-productos, de la que se pueden obtener pectinas y aceite esenciales que son muy preciados por otras empresas alimentarias [12]. En el procesado de la borraja se descartan las hojas que constituyen el 60% de la planta, y precisamente en ellas se ha determinado que se pueden encontrar gran variedad de polifenoles [13]. Por último, como otro ejemplo de los muchos que se encuentran sobre los residuos obtenidos en la industria agroalimentaria, en el procesado de la alcachofa se desechada aproximadamente el 30 % de la planta. Estos residuos están siendo utilizados como ingredientes de alimentos funcionales debido a la alta fibra que contiene [14].

Los polifenoles son uno de los componentes más activos de los componentes que se pueden encontrar en los desperdicios de la industria agroalimentaria, Son metabolitos secundarios de las plantas e influyen y en la calidad sensorial y nutricional de vegetales y frutas [15], son los responsables de muchas de las cualidades antioxidantes y efectos beneficiosos [16], como antiespasmódicos, antihipertensivos y también son reguladores del colesterol,

retardadores de células cancerígenas [17–19]. Por otra parte, el extracto elaborado a partir de vegetales y frutas es utilizado para evitar la oxidación lipídica en sistemas de emulsiones y otros sistemas alimentarios [11,20–22] y para proteger los alimentos del crecimiento de microorganismos [23]. El extracto de romero es uno de los que ha sido estudiado por la EFSA como aditivo alimentario, detectando que a los niveles de concentración estudiados (desde 180 a 400 mg de extracto/Kg de peso de persona/equivalente día), éste no representa un problema de seguridad alimentaria [24].

## 1.2 Los polifenoles

### *1.2.1 Definición y clasificación*

Los polifenoles son moléculas que poseen un anillo aromático con uno o más grupos hidroxilos unidos a él. Sus fórmulas van de un anillo fenólico hasta estructuras más complejas de alto peso molecular [15], como se puede observar en el esqueleto general representado en la figura 1 (caso concreto de los flavonoides). Son metabolitos secundarios sintetizados por las plantas durante su crecimiento normal o cuando son sometidas a *stress* por infecciones, heridas, radiación ultravioleta, entre otras [16]. Además, pueden tener funciones de protección, de pigmentación de plantas y de atracción de agentes polinizadores [15]. Poseen una gran cantidad de aplicaciones y se les atribuyen muchas propiedades terapéuticas.

La distribución de los polifenoles en las células no es uniforme; en función de la solubilidad forman parte de la pared celular o se encuentran dentro de la célula [16]. Según la disposición de los grupos hidroxilos o la complejidad de la molécula formada, los polifenoles poseen diferente clasificación. Entre ellos se pueden encontrar: Flavonoides, ácidos fenólicos, taninos, estilbenos y lignanos [15,25].

### *1.2.2 Los flavonoides*

Los flavonoides son los polifenoles más frecuentemente encontrados en los alimentos, si bien en bajas concentraciones. Los más representativos son la quercetina y el kaempferol [25] compuestos de bajo peso molecular con 15 átomos de carbono según una configuración C6-C3-C6 (figura 1). La estructura consiste en dos anillos aromáticos unidos por un puente de 3 átomos de carbono, usualmente en forma de un anillo heterocíclico [15].

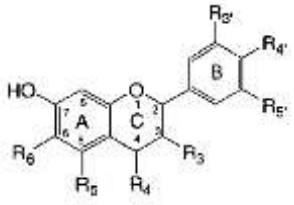
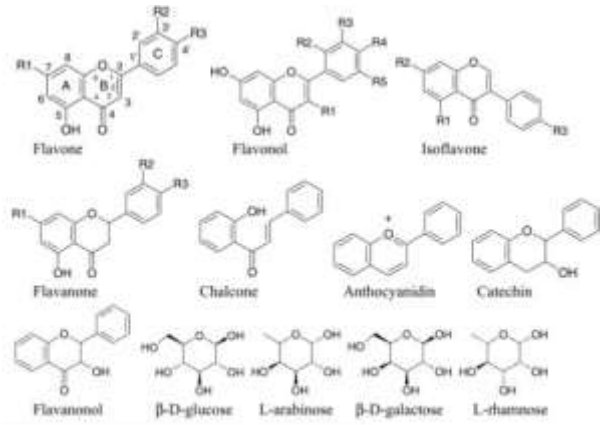



**Figura 1.** Estructura general de los flavonoides.

Dentro de los flavonoides podemos encontrar sub-divisiones que dependen de cómo se encuentran distribuidos los distintos grupos en la molécula central (figura 1). Entre ellos se encuentran: Flavanonas, isoflavonas y antocianinas. En la figura 2, se pueden observar las diferentes formas y distribución que tienen los flavonoides.




| | Flavanone | $R_1$ | $R_2$ | $R_3$ | MW |
|---|---|---|---|---|---|
| 1 | Hesperetin | OH | OH | $OCH_3$ | 302 |
| 2 | Hesperidin | 7-O-Rha-glu | OH | $OCH_3$ | 610 |
| 3 | Naringin | 7-O-Rha-glu | H | OH | 580 |
| 4 | Naringenin | OH | H | OH | 272 |
| 5 | Eriocitrin | 7-O-Rha-glu | OH | OH | 596 |
| 6 | Eriodyctol | OH | OH | OH | 288 |
| 7 | Isosakuranetin | OH | H | $OCH_3$ | 286 |

| | Flavone | $R_1$ | $R_2$ | $R_3$ | MW |
|---|---|---|---|---|---|
| 8 | Chrysoeriol | OH | $OCH_3$ | OH | 300 |
| 9 | Chrysin | OH | H | H | 254 |
| 10 | Apigenin | OH | H | OH | 270 |
| 11 | Luteolin | OH | OH | OH | 286 |
| 12 | Acacetin | OH | H | $OCH_3$ | 284 |
| 13 | Genkwanin | $OCH_3$ | H | OH | 284 |

| | Flavonol | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | MW |
|---|---|---|---|---|---|---|---|
| 14 | Rutin | 3-O-Rha-glu | H | OH | OH | H | 610 |
| 15 | Kaempferol | OH | H | H | OH | H | 286 |
| 16 | Quercetin | OH | H | OH | OH | H | 302 |
| 17 | Morin | OH | OH | H | OH | H | 302 |
| 18 | Isorhamnetin | OH | H | $OCH_3$ | OH | H | 316 |
| 19 | Myricetin | OH | H | OH | OH | OH | 318 |
| 20 | Fisetin | OH | H | H | OH | OH | 286 |

| | Isoflavone | $R_1$ | $R_2$ | $R_3$ | MW |
|---|---|---|---|---|---|
| 21 | Biochanin A | OH | OH | $OCH_3$ | 284 |
| 22 | Sissotrin | OH | 7-O-Glu | $OCH_3$ | 446 |
| 23 | Genistein | OH | OH | OH | 270 |
| 24 | Genistin | OH | 7-O-Glu | OH | 432 |
| 25 | Formononetin | H | OH | $OCH_3$ | 268 |
| 26 | Ononin | H | 7-O-Glu | $OCH_3$ | 430 |
| 27 | Daidzein | H | OH | OH | 254 |
| 28 | Daidzin | H | 7-O-Glu | OH | 416 |

Glu=Glucoside, Rha-glu=Rhamnoglucosyl

**Figura 2.** Diferentes formas de los flavonoides.

Esta disposición de los grupos químicos le confiere a la molécula diferentes funciones. Por ejemplo, las isoflavonas son moléculas parecidas a los estrógenos (estradiol) que han demostrado su eficacia en contrarrestar los efectos de la menopausia [26]. Las catequinas, se encuentran en los extractos del té y son, en gran parte, responsables de la actividad antioxidante que tiene esta planta [27]. Las antiocianinas son responsables de aportar color a algunos vegetales. Su uso como colorante natural se ha incrementado últimamente, ya que, además de ser colorante, tiene propiedades beneficiosas para la salud [28].

### *1.2.3 Los ácidos fenólicos*

Estos compuestos, que representan un tercio de los polifenoles consumidosen la dietadiaria, se encuentran presente en plantas en sus formas libres o unidos a otros compuestos. Se pueden clasificar en dos subgrupos: ácidos hidroxibenzoicos y ácidos hidroxicinámicos [15]. En la figura 3 puede observarse la estructura general y las combinaciones para formar distintos ácidos fenólicos.

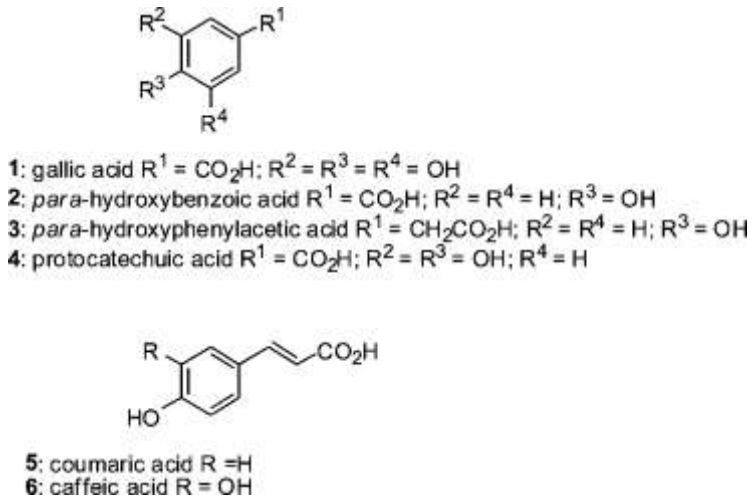



**Figura 3.** Fórmula general de los ácidos fenólicos [29].

Los ácidos hidroxibenzoicos se encuentran mayoritariamente en la plantas de forraje y se caracterizan por tener una estructura central de C6-C1. Dentro de éstos ácidos fenólicos podemos encontrar: gálico, p-hidroxibenzoico, p-hidroxifenilacetico y los ácidos protocatéquicos. En las plantas de forraje estos compuestos se pueden encontrar aislados, pero la forma más habitual es glicosilados y ampliamente distribuidos [29]. El ácido salicílico, uno de los

primeros fármacos conocidos por su poder antiinflamatorio, está relacionado directamente con los ácidos hidroxibenzoicos [30].

Los ácidos hidroxicinámicos se encuentran mayoritariamente en plantas y frutas, por ejemplo el cumárico, cafeico, ferúlico, sinápico. En el forraje se encuentra el ácido clorogénico. Tiene un esqueleto central de C6-C3. Está asociado directamente al efecto protector frente al cáncer de colon [31]. En la figura 3 se muestran dos ejemplo de estos ácidos fenólicos.

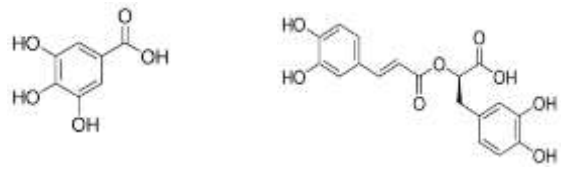
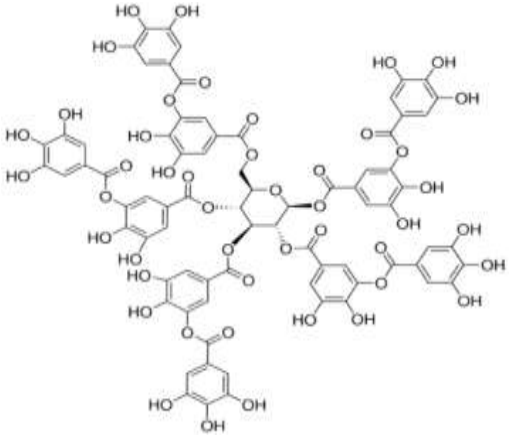


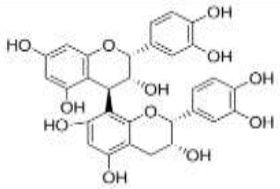

**Figura 4.** Ejemplo de Ácidos Fenólicos.

### *1.2.4 Los taninos*

Los taninos son compuestos de peso molecular relativamente alto. Se pueden dividir en dos subgrupos: Los condensables y los hidrolizables. Los taninos condensables se llaman proantiocianidinas, son dímeros, oligómeros o polímeros de la catequina, un flavonoide, que se encuentra en las semillas y piel de las uvas, entre otras muchas plantas. En combinación con las proteínas de la saliva son los responsables del sabor astringente de algunas frutas y sus bebidas [32]. Los taninos derivados de la familia del ácido gálico son los llamados hidrolizables, son polímeros de la glucosa con el ácido elágico, gálico y/o hexahidroxidifénico [15]. Se encuentran en cantidades significativas en frutos como la mora, fresa, frambuesa y almendra [33].



Tanino Hidrolizable
(Ácido Tánico)



Tanino Condensable
(1,2,4-proantocianidina)

**Figura 5.** Dos tipos de Taninos: encontrados en el vino (ácido tánico) y los arándanos rojos (1,2,4-proantocianidina).

En general los taninos tienen diversos efectos en sistemas biológicos, tales como, atrapar y encapsular metales, pueden actuar como agentes precipitantes y también como antioxidantes [15].

### *1.2.5 Estilbenos y lignanos*

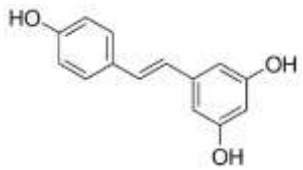


**Figura 6.** Estructura del Resveratrol

Los estilbenos se encuentran en muy pocas cantidades en la dieta humana El más destacado de todos es el resveratrol [32] que puede existir en sus formas isoméricas, cis y trans, siendo esta última la que posee mayor actividad biológica. Lo producen las plantas en situaciones de *stress* y ante infecciones de microorganismos. Se encuentran en frutos tales como las uvas, las bayas y el cacahuete. Su efecto biológico no ha sido determinado con claridad ya que los estudios realizados no muestran datos concluyentes. Este polifenol se ha asociado a la prevención de enfermedades cardiovasculares y ciertos beneficios en procesos

inflamatorios; sin embargo, los datos obtenidos son contradictorios[34,35]. Por otra parte su ingesta es tan pequeña que se puede considerar insuficiente para tener efecto [32,34,35].

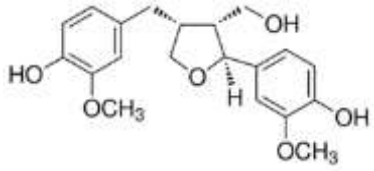



**Figura 7.** Lariciresinol un lignano que se encuentra en la semilla del sésamo [36]

Los lignanos están formados por dos unidades de fenilpropano. La principal fuente de estos polifenoles son las semillas de linaza en forma de secoisolariciresinol diglucósido [18], que se hidroliza en el colon para su absorción. También puede encontrarse en varios cereales, granos y frutas, pero en bajas concentraciones [32]. El consumo de esta semilla ha estado relacionado con la mejora del perfil lipídico en sangre, reducción del LDL, HDL y triglicéridos [37].

## 1.3 Propiedades de los polifenoles.

Los polifenoles poseen diversas propiedades debido a su constitución y su estructura molecular [32]. Entre las más importantes cabe destacar el efecto antioxidante y la relación que éste tiene en los beneficios proporcionados a la salud, en la prevención de enfermedades cardiovasculares, de enfermedades antiinflamatorias, entre otras; también tiene efecto antimicrobiano.

### *1.3.1 Antioxidantes*

Las especies reactivas de oxígeno y los radicales libres son los principales agentes de la oxidación y se pueden considerar dañinos para los sistemas biológicos [38], si bien son necesarios ya que el metabolismo es oxidativo. En muchos experimentos científicos se ha comprobado *in vitro* que los polifenoles encontrados en plantas son buenos antioxidantes y pueden ayudar al sistema antioxidante endógeno [39].

En los alimentos la oxidación lipídica es una reacción indeseada que disminuye la calidad al producto, ya que, entre otras cosas, se alteran sus cualidades

organolépticas [40]. La reacción de la oxidación lipídica se produce a través de un mecanismo de radicales libres que incluye tres procesos: Iniciación, propagación y terminación [41].

En la iniciación, los lípidos insaturados (LI) y los hidroperóxidos (LOOH) producen radicales libres ($L^{\bullet}$) por la extracción del hidrógeno en presencia de varios agentes iniciadores, tales como el calor, la luz, las radiaciones y los metales de transición:

$$LH \xrightarrow{Iniciador} L^{\bullet} + H^{\bullet} \quad (1)$$

La formación de radicales libres a partir de la descomposición de los hidroperóxidos es enérgicamente más favorable que la reacción directa.

$$LOOH \xrightarrow{calor} LO^{\bullet} + OH^{\bullet} \quad (2)$$

O por la acción de metales de valencia variable, donde se producen radicales de alcoxilo ($LO^{\bullet}$) y radicales de peroxilo ($LOO^{\bullet}$).

$$LOOH + M^{+} \longrightarrow LO^{\bullet} + OH^{-} + M^{++} \quad (3)$$

$$LOOH + M^{++} \longrightarrow LOO^{\bullet} + H^{+} + M^{+} \quad (4)$$

Por otra parte, éstos también pueden producir radicales libres por la acción de la luz:

$$LOOH \xrightarrow{hv} LO^{\bullet} + OH^{\bullet} \quad (5)$$

Por lo anteriormente visto, los hidroperóxidos son los iniciadores fundamentales en la oxidación lipídica [41].

En la segunda fase, la de propagación, el radical libre reacciona con el oxígeno de la atmósfera:

$$L^{\bullet} + O_2 \leftrightharpoons LOO^{\bullet} \quad (6)$$

Este radical de peróxilo puede reaccionar con un LH para formar nuevamente un hidroperóxido y un radical.

$$LOOH^{\bullet} + LH \longrightarrow LOOH + L^{\bullet} \quad (7)$$

Debido a que esta reacción es lenta, los radicales de peróxilo atacan selectivamente a los enlaces de hidrógenos más fácilmente rompibles de los ácidos grasos mono y poli-insaturados [41].

Por último, en la fase de terminación, los radicales formados reaccionan entre ellos mismos formando productos tales como cetonas, alcoholes, aldehídos y otros compuestos hidrocarbonatos que aportan nuevas propiedades sensoriales características de los aceites oxidados [42]

$$LOOH^{\bullet} + LOOH^{\bullet} \longrightarrow >C{=}O + O_2 + >CH\text{-}OH \quad (8)$$

Los polifenoles poseen diferentes formas, mecanismos, de contrarrestar la oxidación, entre los que se identifican tres: inactivación de los radicales libre por transferencia del átomo de hidrógeno, por transferencia del electrón y/o por acción del secuestro de los metales de transición [43]. En el primer mecanismo los antioxidantes *AH* reaccionan con el radical libre $R^{\bullet}$ según la reacción:

$$R^{\bullet} + AH \longrightarrow RH + A^{\bullet} \quad (9)$$

donde, el radical $A^{\bullet}$, es menos reactivo que el radical $R^{\bullet}$. El desprendimiento del *H* va a depender de la energía a la cual se encuentra unido al *O* del antioxidante y eso en gran medida es una muestra de la capacidad antioxidante del compuesto fenólico [44]. En el otro mecanismo, el *AH* dona un electrón al radical libre, quedando éste con carga negativa:

$$R^{\bullet} + AH \longrightarrow R^{-} + AH^{\bullet +} \quad (10)$$

Así el radical libre, es una especie estable mientras el antioxidante es menos reactivo. Estas dos especies, $A^{\bullet}$ y $AH^{\bullet +}$, pueden reaccionar con otros radicales o entre ellas mismas y formar compuestos no reactivos [41].

$$A^{\bullet} + LOO^{\bullet} \longrightarrow LOOA \quad (11)$$

$$A^{\bullet} + A^{\bullet} \longrightarrow A\text{-}A \quad (12)$$

El tercer mecanismo tiene que ver con la capacidad de algunos polifenoles de atrapar iones metálicos, ya que atraen a los metales con carga positiva a zonas con densidad de carga negativa del polifenol, los “secuestran” e imposibilitan al metal para reaccionar y formar radicales.

Se han descrito diversos estudios en los que los flavonoides presentan capacidad en secuestrar iones metálicos por lo que podrían ser usados en lugar de los quelantes sintéticos [43]. Es conocido que los metales de transición pueden acelerar el proceso de oxidación de los aceites al reducir la energía de activación en el primer paso de la oxidación. Además pueden reaccionar directamente con los aceites para formar radicales alquilo. También pueden producir especies oxígeno-reactivas, hidróxidos y peróxidos y éstos, a su vez, acelerar la oxidación lipídica [38,43,45]:

$$Fe^{3+} + RH \longrightarrow R^- + H^+ + Fe^{2+} \quad (13)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^\bullet + OH^- \quad (14)$$

$$Fe^{2+} + ROOH \longrightarrow RO^\bullet + OH^- + Fe^{3+} \quad (15)$$

$$Fe^{3+} + ROOH \longrightarrow ROO^\bullet + H^+ + Fe^{2+} \quad (16)$$

El catión $Fe^{2+}$ es mucho más activo que el catión $Fe^{3+}$ en la descomposición de los hidroperóxidos lipídicos y para catalizar la autooxidación. El catión $Fe^{3+}$, además, causa la descomposición de compuestos fenólicos tales como ácido cafeico en aceite de oliva y disminuye la estabilidad oxidativa del aceite [45].

### *1.3.2 Antioxidantes en aceite*

En el caso del aceite, la oxidación ocurre por el contacto del mismo con el aire o con pequeñas burbujas suspendidas en su interior. Por ello los polifenoles menos hidrofóbicos tienen un buen efecto protector ante a la oxidación del mismo, debido a que son compuestos polares y éstos se ubican en la interfase, la parte de contacto entre el aire y el aceite lo cual evita o disminuye el contacto entre el aceite y el oxígeno, figura 8 (a). Sin embargo, los polifenoles hidrofóbicos se encuentran disueltos en el aceite y no ofrecen esta protección [41,46] incluso a pesar de que el aire sea menos polar que el aceite.

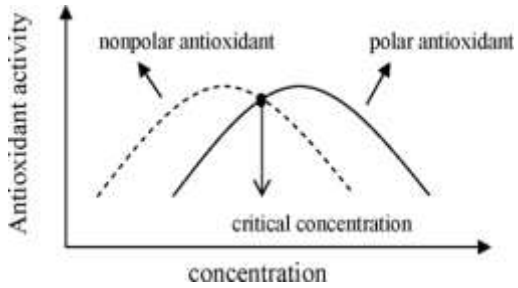



**Figura 8.** Actividad antioxidante de polifenoles según su concentración en aceites [47].

Polifenoles tales como el ácido cafeico, son muy efectivos en la prevención de la oxidación de los alimentos elaborados con aceite cuando se compara con el antioxidante sintético no polar BHT. Es más, los ésteres de polifenoles altamente antioxidantes como el hidroxitirosol no son tan eficaces frente a la oxidación como el propio polifenol hidroxitirosol lo que pone en evidencia y explica la teoría de la llamada "paradoja polar", debido a que el éster es menos polar [46]. Sin embargo, la teoría de la "paradoja polar" tiene sus limitaciones, ya que existen estudios que han demostrado que el poder antioxidante en aceites depende del tipo de polifenol y de su concentración en el aceite. Por ejemplo, el ácido ascórbico es más antioxidante a bajas concentraciones, pero el ascorbil palmitato es más antioxidante a altas concentraciones en el aceite de maíz [47]. En la figura 8 se puede observar mejor el comportamiento antes descrito. Además, se observa una concentración crítica donde la naturaleza del antioxidante, polar o no polar, ofrece protección dependiente de la concentración.

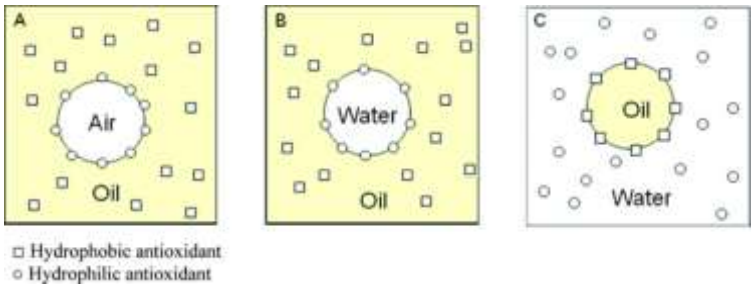



**Figura 9.** Comportamiento de la actividad antioxidante de polifenoles en aceites y emulsiones [46].

### *1.3.3 Antioxidantes en emulsiones*

Una parte de los alimentos que consumimos diariamente son emulsiones: mayonesa, leche, cremas, helados, entre otras. En las emulsiones se encuentran suspendidas pequeñas gotas de aceite (aceite en agua, O/W) o pequeñas gotas de agua (agua en aceite, W/O) y se necesita un emulsionante, compuesto que hace posible la estabilidad de la emulsión, ya que actúa de conexión entre las dos fases, inmiscibles entre sí, la acuosa y la oleosa [46]. Las reacciones de oxidación de los aceites en el seno de alimentos que contienen (o que son) emulsiones les confieren cualidades no deseadas, debido a la formación de nuevos compuestos (off-flavours). De ahí la necesidad de agregar antioxidantes para que actúen de protectores frente a la oxidación [48].

Los antioxidante pueden ser sintéticos, tales como el BHA, el BHT o el ácido gálico, o pueden ser extractos naturales de plantas, tales como extractos de té o de romero [21,49]. De acuerdo con la teoría de la paradoja polar, los compuestos no polares son más efectivos cuando se usan en emulsiones O/W. Esto es debido a que se colocan en la interfase formada por el agua y el aceite, mientras que los compuestos polares se encuentran en el agua. Al colocarse en la interfase actúan como una barrera que impide o retarda la formación de radicales libres que a su vez formarían los compuestos indeseados [46], figura 9 (b) y (c). Sin embargo, la efectividad del antioxidante también puede depender de la naturaleza del agente usado para la emulsión, de su polaridad, lo que puede fomentar la mejor organización de las moléculas del polifenol [41]. Por otra parte, muchos de los emulsionantes usados en la industria alimentaria poseen actividad antioxidante en sí mismos, ya que tienen la capacidad de ordenar el sistema en el que se encuentran, retardando la actividad de los radicales libres [50]. Además forma una capa protectora, que no permite la entrada al aceite de metales que catalizan la reacción de oxidación. Sin embargo, también pueden promover la oxidación lipídica por ofrecer mayor área de contacto entre el aceite y los agentes oxidantes [41]. Esto demuestra que las emulsiones son sistemas complejos que dependen de muchos factores.

## 1.4 Métodos para determinar polifenoles y la actividad antioxidante

A continuación se dan a conocer los métodos más comunes utilizados para determinar la capacidad antioxidante de extractos de plantas y sub-productos de la industria alimentaria

### *1.4.1 Métodos espectrofotométricos de determinación de polifenoles, flavonoides y antocianinas*

Se han desarrollado un gran número de métodos espectrofotométricos para determinar el contenido total de polifenoles en extractos y plantas. Estos métodos se basan en diferentes principios para poder detectar los polifenoles [15,28]. El método más ampliamente aplicado en la comunidad científica para determinar cantidad de polifenoles totales es el método desarrollado por Otto Folin y Vintila Ciocalteu en 1927 para medir la tirosina, y luego adaptado por Vernon Singleton y Joseph Rossi en 1965 para poder medir los polifenoles totales. Esta metodología se basa en la reducción química por una mezcla de óxidos de tungsteno y molibdeno [51]. El reactivo Folin-Ciocalteu (F-C) se prepara disolviendo 100g de tungstato de sodio y 25g de molibdato de sodio en 700 ml de agua destilada. La solución se acidifica con 50ml de HCl concentrado y 50 ml de ácido fosfórico al 85%. La solución acidificada se hierve durante 10h, se enfría y se añaden 150g de sulfato de litio tetrahidratado. El resultado es una solución de un amarillo intenso que es el reactivo F-C. La naturaleza de la reacción de este reactivo no ha sido realmente definida. Se asume que la reacción con el sustrato envuelve una reacción de reducción reversible de uno o dos electrones. Durante el ensayo F-C, la reacción entre los polifenoles totales y el reactivo ocurre a pH 10 (conseguido al agregar el carbonato de sodio), y bajo esas condiciones básicas, la disociación de un protón fenólico conduce a la formación de un ion de fenolato, que es capaz de reducir el reactivo F-C[52]. Esta reacción produce compuestos que dan un color azúl a la solución, cuya intensidad se mide por la absorbancia de la solución a la longitud de onda de 765 nm. La intensidad de la absorción está directamente relacionada con la cantidad de polifenoles que se encuentran en la solución [51]. Este ensayo puede tener problemas de interferencia con otras sustancias que se encuentren en la solución, tales como, el ácido ascórbico, ácido dehidroascorbico y azúcares reductores (glucosa y fructosa). Tanto el ácido ascórbico como el ácido dehidroascorbico son un problema a la hora de determinar la cantidad de polifenoles en frutas como la naranja, el kiwi y la fresa. Estas sustancias reaccionan rápidamente con el reactivo F-C proporcionando la coloración azul sin ser causada por los polifenoles presentes. Por tanto, la presencia de azúcares reductores es un gran problema, especialmente si la cantidad de polifenoles es baja en el producto que se requiere analizar. Por ello se ha de hacer una separación previa de los azúcares pasando el extracto por una columna de separación, por ejemplo Oasis de Waters (Milford, USA) para evitar que la coloración azul se deba a estos azúcares [52,53].

La cantidad de flavonoides totales puede ser cuantificada también por un método colorimétrico donde el compuesto fenólico forma un complejo con el aluminio [15,16]. El extracto se hace reaccionar con $AlCl_3$ a pH 3,1. El contenido total de flavonoides se determina midiendo la absorbancia de la solución a 407 nm. Una modificación del ensayo de $AlCl_3$ propuesto por Zhishen et al. incluye la reacción del extracto fenólico con nitrato de sodio seguido por la formación de complejo flavonoide-aluminio, entonces la absorbancia de la solución se mide a una longitud de onda de 510 nm [54,55]. El patrón de calibración comúnmente utilizado es la quercetina [4,56,57]. La desventaja principal de este método es que solamente estima la cantidad total de flavonoides y no sus compuestos derivados que pueden estar en la muestra [15]. En la figura 10 se puede observar la formación del complejo coloreado.

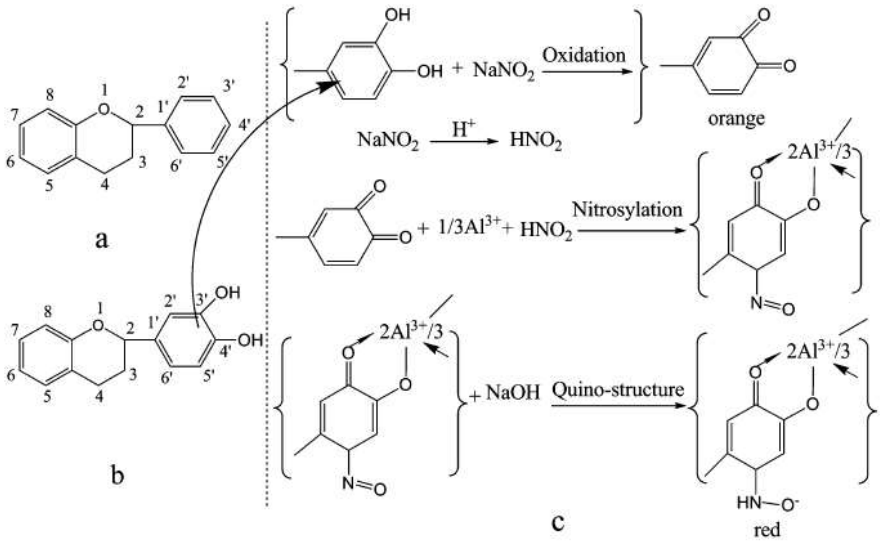



**Figura 10.** Reacciones de coloración de los flavonoides [55].

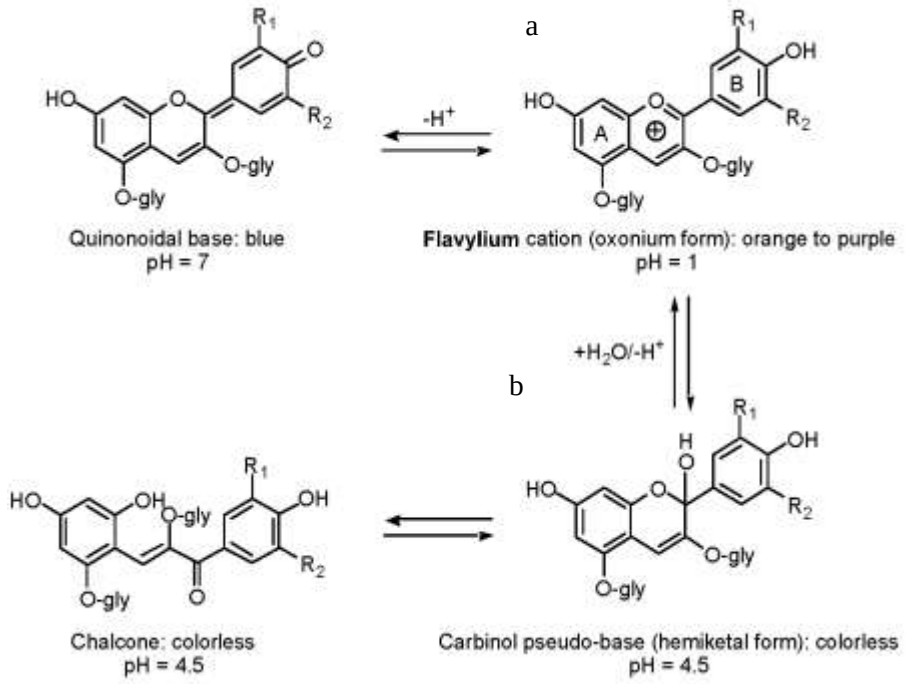



**Figura 11.** Comportamiento de las antocianinas con la variación de del pH [58].

Para determinar antocianinas, se utiliza la propiedad que tienen de cambiar de color según el pH de la muestra. [59]. Existe la forma oxonio, figura 11 (a), de color rojo en pH 1,0, y el hemicetal incoloro en pH 4,5, figura 11 (b). La diferencia en la absorbancia de los pigmentos a una longitud de onda 520 y en 700 nm es proporcional a la concentración de pigmento. Los resultados se expresan sobre una base cianidina-3-glucósido. Cuando las antocianinas son degradadas en la forma polimérica presentan resistencia a cambiar el color independientemente del pH y por esta razón, no se pueden analizar con este método, ya que absorben a pH 4.5, así como pH 1,0 [58]. Aplicando la siguiente fórmula se puede obtener la cantidad de antocianinas:

$$Antocianina\left({}^{mg}/_{l}\right) = \frac{A \times MW \times fd \times 10^{3}}{\varepsilon \times l} \qquad (17)$$

donde, A=($A_{520\ nm}$-$A_{700\ nm}$)pH 1.0- A=($A_{520\ nm}$-$A_{700\ nm}$)pH 4.5; MW=449,2 g/mol; fd= factor de dilución; l= largo de la cubeta; ε=26.900 coeficiente de extinción molar en l/mol×cm y $10^3$=factor de conversión de g a mg.

### *1.4.2 Métodos espectrofotométricos de determinación de captura de radicales libres*

Algunos de los métodos espectrofotométricos más usados para determinar la actividad antirradicalaria en extractos de plantas y frutas son los ensayos de: *2,2-Diphenyl-1-picrylhydrazyl* (DPPH), *2,2'-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid)* (ABTS) y el poder de reducción del ion $Fe^{3+}$ (FRAP).

En el DPPH, el color violeta de la solución del radical $DPPH^{\bullet}$ va disminuyendo proporcionalmente a la capacidad antioxidante que posea la muestra que se analiza [60]. La longitud de onda de lectura, que corresponde a un máximo de absorción, es 515 nm y se realizan lecturas hasta alcanzar el estado estacionario. Para obtener un porcentaje equivalente a la capacidad antirradicalaria de la muestra se puede aplicar la ecuación [61]:

$$\%DPPH^{\bullet}_{Rem} = 100x\frac{[DPPH^{\bullet}]_{Rem}}{[DPPH^{\bullet}]_{T=0}} \quad (17)$$

donde, $[DPPH^{\bullet}]_{Rem}$ es la concentración de $DPPH^{\bullet}$ remanente en estado estable después de la reacción y $[DPPH^{\bullet}]_{T=0}$ es la concentración del radical en el tiempo cero de la reacción.La gran ventaja de este método es su rapidez y sencillez. Las desventajas tienen relación con la sensibilidad del reactivo para reaccionar con las diferentes moléculas. Puede existir un cambio de color que no sea indicativo de la actividad antirradicalaria. Además los antioxidantes con moléculas pequeñas dan mejores resultados que los antioxidantes con moléculas grandes, debido al efecto estérico. Por otra parte, los agentes reductores pueden reducir al radical lo que hace que los resultados se muestren alterados. Por último, pueden ocurrir interferencias cuando se miden sustancias que absorben a la misma longitud de onda, como por ejemplo los carotenoides [61].

El método de DPPH, se ha usado con efectividad para medir actividad antioxidante en zumos, frutas y vegetales [12,56,62,63]; en extractos de plantas [64–66]; en alimentos [49,67,68]; y en subproductos de la industria alimentaria [69–71].

Otro método que actúa de manera similar al DPPH es el ABTS. En este método el ABTS es oxidado a radical catiónico, $ABTS^{\bullet+}$ por los radicales peróxidos (y otros oxidantes) lo que produce una solución intensamente coloreada. La presencia de antioxidantes en el medio produce una disminución de la intensidad de

ese color y permite medir la capacidad antioxidante de la muestra [72]. Las longitudes de onda utilizadas más frecuentemente son 415 y 734 nm. La absorbancia obtenida en el seno de la disolución como efecto de la reacción entre el radical y el antioxidante se compara con la que se obtiene en la reacción del estándar trolox, en las mismas condiciones. El resultado de este test se expresa en capacidad antioxidante en equivalentes de trolox [61,72]. Este método es popular entre los investigadores, pudiendo determinar la capacidad antioxidante en frutas [73], verduras [74], cereales [72], bebidas [75], residuos [76], extractos de plantas [77], entre otras.

Entre las ventajas que posee el método ABTS, cabe destacar la solubilidad del radical en soluciones acuosas y en solventes orgánicos, lo que hace que sea el método adecuado para determinar actividad antioxidante tanto en sistemas hidrofóbicos como los hidrofílicos; la rapidez en obtener los resultados, de aproximadamente 7 min [61]; y por último, el que esta reacción puede ser fácilmente adaptable a diferentes equipos y métodos de medición [61,78]. Como desventajas, el radical no representa una función fisiológica real en mamíferos. Además es térmicamente inestable pudiendo reducirse el radical. El punto final de la reacción puede variar, ya que las reacciones pueden ser más lentas en algunas muestras y quizás el valor descrito en un tiempo determinado es menor al valor real de la muestra [61]. Por ello es conveniente alargar la lectura unos minutos más, hasta comprobar estabilidad.

El método FRAP (*Ferric Reducing Antioxidant Power*) fue desarrollado para determinar la capacidad antioxidante del ácido ascórbico en plasma o suero. En este método el complejo $Fe^{3+}$- 2, 4, 6 tripiridil-s-triazina (TPTZ) se reduce y forma $Fe^{2+}$, bajo condiciones ácidas, lo que genera una coloración azul intensa que es medida en un espectrofotómetro a una longitud de onda de 593 nm[72]. Este método mide la capacidad que posee un antioxidante para transferir un electrón y así reducir un componente. Este tipo de método también se llama SET (*Single Electron Transfer*). El FRAP, en combinación con otros métodos para determinar la actividad antioxidante de un compuesto, nos puede indicar cómo ocurre la reacción o cual es el mecanismo de reacción que posee el antioxidante. La reacción que ocurre cuando es aplicado el método FRAP se muestra a continuación:

$$Antioxidantes + Fe^{3+} \leftrightharpoons Fe^{2+} + antioxidantes\ oxidado\ s \quad (18)$$

$$Fe^{2+} + Fe(CN)_6^{3-} \leftrightharpoons Fe[Fe(CN)_6]^- \quad (19)$$

O

$$Antioxidantes + Fe\ (CN)_6^{3-} \leftrightharpoons Fe(CN)_6^{4-} + antioxidantes\ oxidados \quad (20)$$

$$Fe\ (CN)_6^{4-} + Fe^{3+} \leftrightharpoons Fe\ [Fe(CN)_6]^- \quad (21)$$

Por otra parte, como los metales reducidos estimulan el efecto prooxidante de algunos polifenoles, es interesante utilizar ese método para determinar cuándo un polifenol o extractos con alto contenido de polifenoles puede llegar a tener este efecto prooxidante[61]. Entre las desventajas del FRAP cabe destacar que las reacciones de transferencia de electrón son largas y esto puede llevar a obtener medidas erróneas en la capacidad antioxidante de una muestra. Por otra parte, sólo se mide la actividad antioxidante en base al ion $Fe^{3+}$, medida que es incompleta, aunque si relevante en cuanto al mecanismo en la actividad fisiológica [61].

### *1.4.3 Método fluorimétrico*

El método se basa en detectar la disminución de la fluorescencia de la solución a lo largo del tiempo por acción de radicales o antioxidantes, ya que su reacción produce compuestos no fluorescentes [61,79]. En este método los radicales son elaborados por calentamiento de una azida. La descomposición de esta azida produce gas nitrógeno y dos radicales $R^{\bullet}$. El radical reacciona con el oxígeno presente formando radicales peróxidos $ROO^{\bullet}$, los cuales pueden reaccionar con la fluoresceína o con los antioxidantes. La siguiente secuencia muestra cómo se suceden las reacciones en el análisis [80].

$$R\text{-}N{=}N\text{-}R \xrightarrow{calor} N_2 + 2R^{\bullet} \xrightarrow{O_2} ROO^{\bullet} \quad (22)$$

$$ROO^{\bullet} + fluorescencia \rightarrow ROOH + no fluorescencia \quad (23)$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \quad (24)$$

$$ROO^{\bullet} + A^{\bullet} \rightarrow ROO\text{-}A \quad (25)$$

La competencia entre la reacción 23 y las reacciones 24 y 25 son las bases de este análisis, ya que al reaccionar el antioxidante con el radical, la fluorescencia se mantiene en el tiempo [80]. Los datos obtenidos de esta reacción son recogidos en una gráfica que expresa intensidad de fluorescencia *versus* tiempo y el área bajo la curva resultante describe la capacidad antioxidante que posee la muestra que se

está analizando [81]. A continuación se muestra una figura representativa del análisis. En ella se puede observar una muestra de trolox a una concentración determinada, un extracto de planta y el blanco. Para efectos del cálculo de la capacidad antioxidante el área del blanco debe ser restada al área total de la muestra.

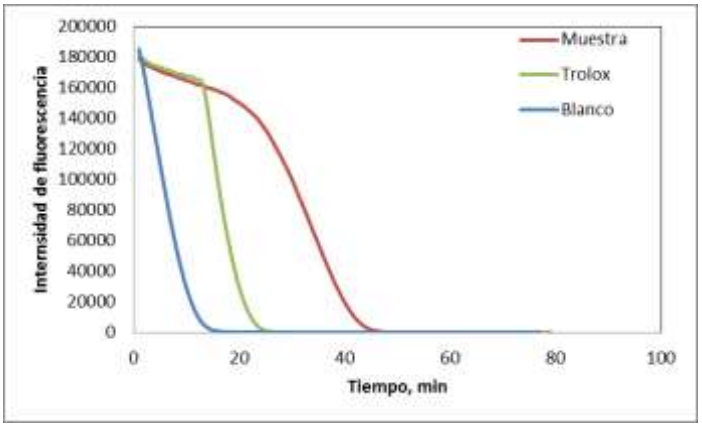



**Figura 12.** Intensidad de la fluorescencia en dos muestras y su blanco.

Normalmente se calcula el área bajo la curva a través de la ecuación obtenida de la suma de rectángulos (22):

$$AUC = \left(0{,}5 + {}^{f_5}/_{f_4} + {}^{f_6}/_{f_4} + {}^{f_7}/_{f_4} + \dots {}^{f_i}/_{f_4}\right) xCt \qquad (22)$$

Donde, $f_i$ es la intensidad de fluorescencia en el tiempo i, $f_4$ es la intensidad de fluorescencia en el tiempo inicial y *Ct* tiempo que dura el ciclo en minutos [80].

Este análisis ofrece muchas ventajas frente a los análisis DPPH y ABTS. Por ejemplo, la formación continua de radicales a esas condiciones se asemeja a la formación de radicales *in vivo*, siendo este método más parecido a las condiciones fisiológicas reales. Se puede adaptar para determinar actividad antioxidante en muestras hidrofílicas e hidrofóbicas. Actúa por transferencia del átomo de hidrógeno estableciendo una ruta definida para la actividad antioxidante de la muestra [80]. Entre las desventajas cabe destacar que es muy sensible a los cambios de temperatura y esto puede llevar a errores en la lectura. Por otra parte es

necesario el uso de un equipo con lector de fluorescencia para poder llevar a cabo la medición (no tan común como los espectofotómetros UV) [61].

Este método se ha utilizado con eficacia en zumos [82], frutas [83], vegetales [84], extractos de plantas [85], residuos [86] para determinar la actividad antioxidante.

### *1.4.4 Métodos cromatográficos*

La cromatografía líquida de alta eficacia (CLAE, HPLC con sus siglas en inglés) se ha empleado ampliamente para determinar y cuantificar la cantidad de polifenoles que poseen los alimentos [87]. Las columnas utilizadas son las de fase reversa C18, compuestas mayoritariamente por una fase sólida con un diámetro entre 2.1 y 5 mm y un tamaño de partícula de 3 a 5 µm. La denominación C18 se debe a que este tipo de columna posee una cadena de 18 carbonos en la fase sólida. La fase móvil consiste normalmente en una mezcla de una fase acuosa y una fase orgánica (metanol o acetonitrilo) y estos son acidificados mediante el ácido acético o ácido fórmico, lo cual hace que la muestra sea ionizada mejorando la detección [88]. La combinación de los solventes con diferentes columnas que se encuentran en el mercado da la posibilidad de lograr separar adecuadamente diferentes tipos de polifenoles [89]. Para la detección de los compuestos los equipos de HPLC vienen acoplados a diferentes tipos de detectores: UV, electroquímicos y fluorescentes. Sin embargo estos tienen la desventaja que no dan información sobre la estructura molecular de los compuestos. Para ello se han estado utilizando los equipos acoplados a un espectrómetro de masas (MS) que es una herramienta adecuada para establecer la estructura de los polifenoles. Existen un gran número de mejoras que se pueden aplicar al HPLC para identificar y cuantificar los componentes de una mezcla de polifenoles, además de esclarecer la estructura [89]. Se ha utilizado para determinar polifenoles en plantas [90], frutas [91], extractos [92] y residuos de agroindustrias [93,94], estableciendo la composición química y cuantificación absoluta, en el caso de que se disponga de patrón o relativa si no se dispone.

Los métodos cromatográficos también se han usado para determinar la capacidad antirradicalaria individual de cada uno de los compuestos presentes en las muestras naturales [95]. Para ello se combinan la separación aportada por la columna cromatográfica y los métodos espectrofotométricos habituales, ABTS o DPPH [96,97]. El método consiste en hacer reaccionar el radical seleccionado con los compuestos separados por la columna cromatográfica. Se calcula un tiempo de

reacción, a través de la permanencia en el bucle y a continuación se hace pasar por el detector UV a una determinada longitud de onda seleccionada. En la figura 11 se puede observar un esquema del equipo [98].

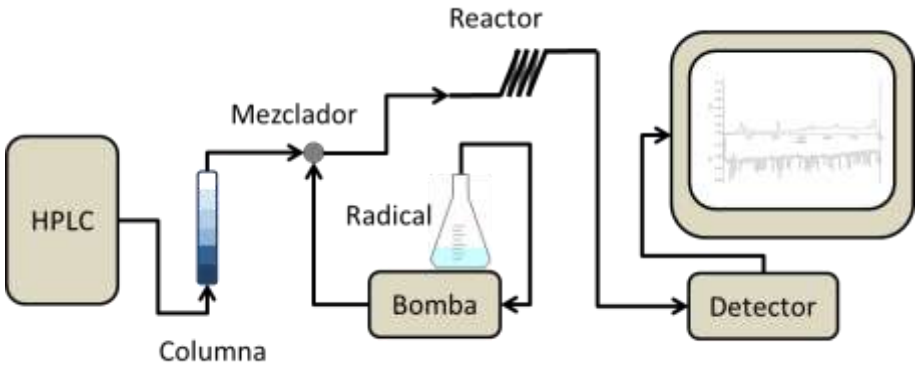



**Figura 13.** Diagrama de la determinación de capacidad antirradicalaria de una sustancia mediante el uso de un HPLC en combinación con un radical

Aplicando la inyección post-columna del radical, se han podido determinar los compuestos que aportan la capacidad antirradicalaria en extractos de borraja, entre ellos el ácido rosmarínico [64], los residuos de la pimienta (piel) [99], los isómeros del ácido clorogénico en las bebidas preparadas con café [100], en los residuos de *Calamintha grandiflora L.* [101], entre otros.

## 1.5 Estudio de la oxidación en modelos de alimentos

Para establecer si un extracto de una planta o algún alimento posee capacidad antioxidante, se aplican a matrices que simulan alimentos. Estas matrices pueden ser emulsiones, productos cárnicos y/o productos de panadería, entre otros. Entre las emulsiones tenemos, las líquidas (O/W o W/O), las emulsiones cárnicas, como salchichas, y las emulsiones altamente concentradas en acetite, como las mayonesas. Entre los productos cárnicos son estudiadas las carnes picadas procedentes de varias fuentes: Aves, ternera, cerdo, cordero, entre otros.

El seguimiento oxidativo se realiza analizando muestras de estos alimentos cada cierto tiempo. Los análisis más comúnmente utilizados son aquellos que

determinan los compuestos producidos por la oxidación primaria y secundaria de las grasas y aceites.

### *1.5.1 Valor de peróxidos (VP) por el método del ferrocianato*

El VP se usa para determinar la oxidación de un alimento a través del tiempo. Se evalúan los hidroperóxidos y peróxidos formados en las primeras fases de la oxidación lipídica. Este método se basa en la capacidad de peróxidos para oxidar los iones ferrosos a iones férricos. El tiocianato de amonio reacciona con los iones férricos, lo que proporciona un complejo coloreado que se puede medir espectrofotométricamente. Finalmente se mide la absorbancia de la solución a 500nm [102]. La calibración se hace por el método oficial.

Este método se encuentra en casi todos los análisis realizados para determinar la estabilidad oxidativa en muestras de alimentos y/o modelos, debido a su fácil uso y entendimiento. Como ejemplo podemos citar: la estabilización de aceite de girasol cuando se agrega extracto de ajo [103], efecto de diferentes polifenoles en la prevención de oxidación de aceites de pescado [104], estudio del efecto interfacial en la oxidación de las emulsiones [105], entre muchos otros.

### *1.5.2 Sustancias reactivas al ácido tiobarbitúrico (TBARS)*

Este es un método que se utiliza para determinar los productos de la oxidación secundaria de compuestos alifáticos [106]. El método refleja la formación de malondialdehído a partir de los productos de la degradación de los hidroperóxidos y peróxidos que se forman en la oxidación de los ácidos grasos poliinsaturados. Este método se basa en la determinación espectrofotométrica del complejo rosa formado después de la reacción de una molécula de malondialdehído (MDA) con dos moléculas de ácido 2-tiobarbitúrico. La longitud de onda usada para la medición de la absorbancia resultante es de 530 nm [107].

Diversos autores han utilizado este método para determinar cómo evoluciona la oxidación de un alimento a través del tiempo cuando se requiere probar el poder antioxidante de extractos naturales en alimentos que contienen grasas o cuando se quieren evaluar condiciones específicas de almacenamiento, formulación y/o preparación del alimento. Entre ellos se puede mencionar: El estudio de extractos de aguacate en hamburguesas realizadas con carne de cerdo [10], la oxidación de emulsiones tratadas con extractos de perilla [22], la evaluación de tres hierbas aromáticas como antioxidantes en emulsiones [108], estudio de la

oxidación de mortadela con diferentes niveles de nitrito de sodio [109], oxidación de carne de pollo presurizada y cocida [106], entre otros.

### *1.5.3 Compuestos volátiles*

La cromatografía de gases (GC) es sin duda el método más eficiente utilizado para la determinación cuantitativa de los hidrocarburos volátiles y proporciona datos cuantitativos muy precisos sobre las cantidades de los compuestos volátiles o volatilizados. Actualmente se usan columnas capilares cuyos rellenos, fases estacionarias poco polares y no polares que permiten una separación óptima en un tiempo corto. Las separaciones se pueden realizar isotérmicamente o en sistemas de gradiente de temperatura [110].

Los aldehídos son las sustancias volátiles más importantes producidas durante la oxidación de lípidos y se han utilizado con éxito para seguir la oxidación en una serie de los alimentos, incluidos los alimentos de origen cárnico [111]. Numerosos aldehídos se producen durante la oxidación, incluyendo octanal, nonanal, pentanal y hexanal. El hexanal es el aldehído dominante producido durante la oxidación. Los investigadores han sugerido que el hexanal es un indicador de la oxidación de lípidos de la carne de manera más eficaz que cualquier otro componente volátil. Estos componentes volátiles contribuyen al mal olor y mal sabor de los aceites y grasas oxidados y por supuesto de los alimentos que los contienen. Se determinan por CG, técnica en la que la muestra se envasa en viales que permiten un espacio entre la muestra y la tapa del vial y en la que se recogen productos volatilizados mediante fibras y que se introducen posteriormente en el circuito cromatográfico inyectan posteriormente dentro de la columna [112]. Entre los detectores mayormente usados se encuentran el detector de ionización de llama (FID) y el espectrometro de masas [113]. Si se puede contar con el detector de masas, se puede prescindir de la fibra y se inyecta directamente el gas contenido en el espacio en cabeza, permitiendo una automatización que acelera mucho la determinación.

### *1.5.4 Valor de p-anisidina*

El valor de p-anisidina es otra medida de la contenido de los productos de oxidación aldehídicos (principalmente 2-alquenales y 2,4-alkadienals) generados durante la descomposición de hidroperóxidos, y por tanto una medida de la oxidación secundaria. Se basa en la reacción de la p-metoxianilina (anisidina) y los

compuestos aldehídicos en condiciones ácidas lo cual produce sustancias de color amarillento que pueden absorber a una longitud de onda de 350 nm. El valor de p-anisidina es un indicador fiable de la rancidez oxidativa en grasas y aceites y por lo tanto un buen enfoque para medir la inhibición de la oxidación que producen los antioxidantes. Se ha encontrado una correlación significativa entre el valor de la p-anisidina y el PV. La humedad es una gran interferencia y también depende de otros factores tales como el tiempo de almacenamiento y la temperatura [112].

Entre los ejemplos en los que se ha usado, caben citar: El efecto antioxidante de microalgas en emulsiones [114], estabilidad oxidativa de aceites con extractos de sésamo [115], efectividad antioxidante de los aceites volátiles y resina de la canela (hojas y cortezas) [116], entre otros.

### *1.5.5 Tiempo de oxidación inducida (OIT)*

La calorimetría diferencial de barrido (DSC, con sus siglas en inglés) es una técnica termoanalítica que se utiliza para estudiar los cambios que se producen cuando una muestra es calentada o enfriada. Con ella se pueden detectar transiciones de fases en función de la temperatura, además de las entalpías asociadas a dicho fenómeno [117]. El instrumento está constituido por un bloque metálico con dos alvéolos cilíndricos, uno de medida y otro de referencia [118]. A continuación se esquematiza el sistema de calentamiento y medida del analizador:

El analizador se compone por un bloque cilíndrico macizo, en cuyo interior hay dos soportes o *holders*: uno de referencia y uno de la muestra a analizar. Bajo cada uno de ellos hay dos resistencias y dos sensores, ambos de platino e independientes entre sí.

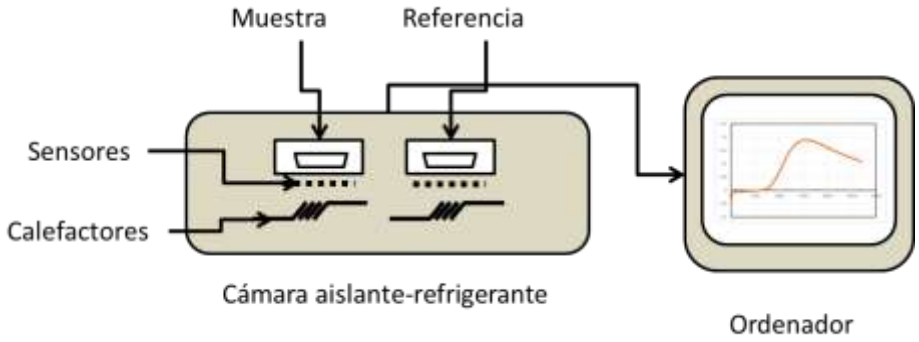



**Figura 14.** Esquema del equipo para termoanálisis.

Una de estas resistencias actúa como elemento calefactor y la otra mide la temperatura. Tanto la muestra como la referencia se someten al mismo programa de temperatura. El método consiste en suministrar calor o bien a la muestra, o bien a la referencia (según la transición sea endotérmica o exotérmica), para que la diferencia de temperatura entre ambos materiales sea nula durante la experiencia. Esto significa que si la muestra y la cápsula de referencia se calientan linealmente, las dos están a la misma temperatura. Ahora bien, en el momento en que se produce una transición de fase en la muestra a estudiar, se produce una diferencia de temperatura. Al producirse esta diferencia de temperatura, la resistencia calefactora aumenta (o disminuye, según el caso) de potencia para volver a igualar las temperaturas. De esta manera, la potencia eléctrica suministrada por la resistencia será función de la diferencia de temperatura. La programación de temperaturas y velocidades de calentamiento o enfriamiento se realiza a través de un software especializado. El equipo puede funcionar entre 100K y 950K. La regulación de la temperatura se efectúa mediante un bloque metálico que rodea los dos recintos (el de referencia y el de la muestra). A baja temperatura, la envolvente de los recintos es enfriado con un refrigerante que está circulando dentro del bloque refractario. Entre el bloque y los recintos circula un gas que favorece la transferencia térmica por convección. El estudio de las señales del DSC, llamadas termogramas, permite acceder a los parámetros térmicos de la sustancia, determinando las entalpías y las temperaturas características ($T_{onset}$ o $T_{endset}$) de los fenómenos energéticos obtenidos por las diferencias de la señal con la línea de base, que se traducen en picos de formas variadas [119–121].

Por otra parte, el DSC es una técnica que permite observar el comportamiento de una muestra cuando se somete a condiciones de temperatura y atmosfera extremas [122]. Mediante esta técnica puede someterse una muestra de aceite o grasa a una oxidación forzada, aumentando la temperatura y suministrando

oxígeno, aire o nitrógeno según sea el caso [123]. En un diagrama de tiempo *versus* energía o energía *versus* temperatura se recogen los datos obtenidos y mediante la determinación del punto de inflexión de la curva, se determina el valor correspondiente al cambio de composición de la muestra. El tiempo de oxidación inducida (OIT o IP) es aquel punto sobre el gráfico donde se observa un cambio en la pendiente de la curva registrada.

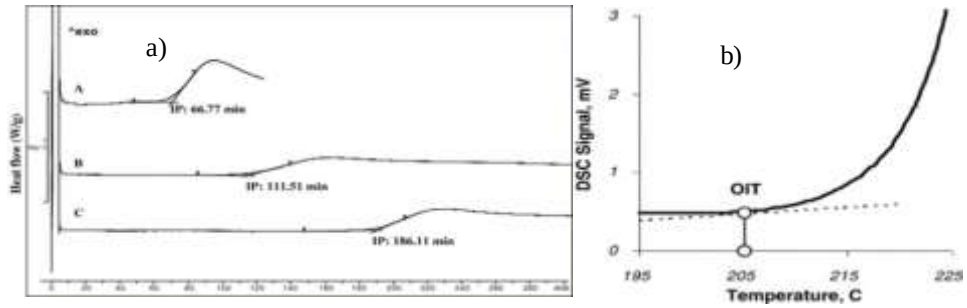



**Figura 15.** Termogramas: Isotérmico (a) [124] y no isotérmico (b) [125].

En la figura 15 se pueden observar ejemplos de los termogramas que se obtienen mediante el uso de esta técnica. Además en la figura 12 (a), se puede diferenciar los puntos de inducción para distintas composiciones (oxidación) de las muestras.

Esta técnica se ha utilizado ampliamente para determinar la estabilidad oxidativa en muestras de aceites vegetales [126,127], en emulsiones [128], en microcapsulas [122], en semillas [123], entre otros.

## 1.6 Métodos de extracción de polifenoles

Los polifenoles raramente se encuentran libres en la naturaleza. Estos compuestos se encuentran ligados generalmente a materias primas de origen vegetal y, debido a esto, para poder obtenerlos es necesario realizar extracciones. Las extracciones pueden ser convencionales o asistidas por una metodología específica, entre las que existe: Ultrasonidos, pulsos electromagnéticos, microondas, facilitado por tensioactivos, entre otras.

### *1.6.1 Extracción convencional, continua y discontinua.*

La extracción convencional es el método más usado actualmente para obtener extractos a partir de materias primas tales como frutas, vegetales y sus residuos [129]. En este método es colocado el material rico en compuestos bioactivos en contacto con solventes orgánicos, generalmente alcoholes, los cuales extraen estos compuestos. La temperatura y la agitación, en algunos casos, son fundamentales para maximizar la cantidad de compuestos extraídos del material [130]. Las extracciones convencionales pueden ser realizadas de dos maneras: por lotes y en forma continua.

Cuando la extracción se realiza por lotes, se coloca una determinada cantidad inicial de materia prima en un recipiente. Si se va a aplicar temperatura alta, éste ha de permitir el reflujo del disolvente. Normalmente se usa el soxhlet, que permite la recirculación de solvente de manera constante durante un tiempo determinado. Cada vez el solvente en contacto con el sólido es aquel que se ha evaporado y condensado, por lo cual es "fresco". En la figura 16 se observa un montaje típico de extracción usando el equipo mencionado, además de un equipo tradicional de extracción por lotes [131].

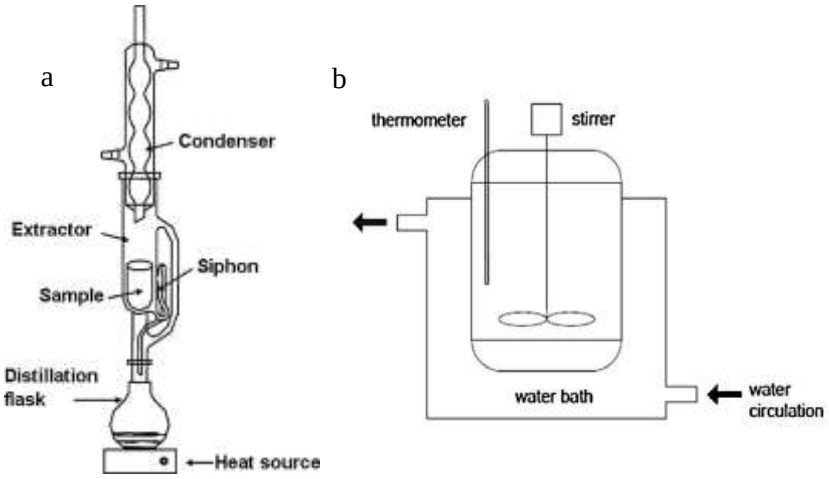



**Figura 16.** Equipo de extracción soxhlet (a)[131] y equipo de extracción convencional (b)[129].

Con este tipo de extracción se han elaborado gran cantidad de extractos, los cuales han permitido el estudio de polifenoles y su capacidad antioxidante en materiales diversos[17,132].

La extracción continua es menos utilizada que la extracción por lotes. En ella se hace pasar una corriente continua de solvente sobre una cantidad determinada de material sólido, recuperando el solvente que será analizado [133]. En la figura 17 se muestra un ejemplo de este tipo de extracción.

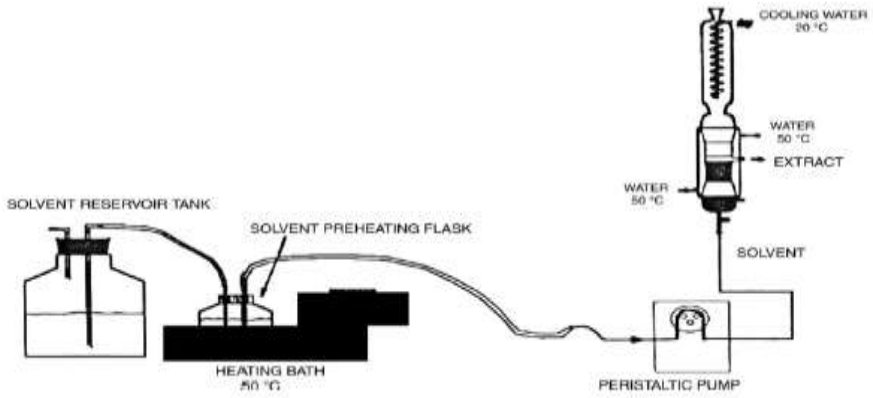



**Figura 17.** Ejemplo de extracción continua de componentes bioactivos [133]

### *1.6.2 Extracción asistida con ultrasonidos*

La extracción asistida por ultrasonidos, se lleva a cabo de la forma convencional, pero se aplican ondas ultrasónicas mediante un baño o una punta. Estas ondas ultrasónicas producen una serie de burbujas que tienen un efecto de cavitación sobre la muestra, causando micro agitación y mejorando la transferencia de masa. Por otra parte, la rotura y la creación de nuevas burbujas abre caminos a través de la célula permitiendo el paso del solvente y mejorando la extracción [129]. Esta técnica ha sido aplicada con éxito para la obtención de extractos y polifenoles a partir de semillas achiote [134], colza [135], nuez de areca [136], orégano [137], hojas de laurel [138], entre otros.

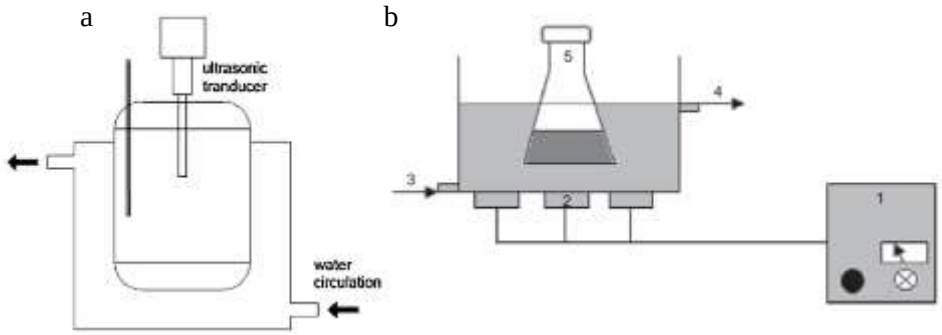



**Figura 18.** Extracción asistida por ultrasonidos: Con transductor (a) [129], en baño ultrasónico (b)[139].

### *1.6.3 Extracción usando pulsos electromagnéticos*

Diversos autores describen el uso de los pulsos electromagnéticos para mejorar los procesos de extracción de compuestos bioactivos a partir de materias primas tales como frutas y vegetales. Entre éstos se pueden citar: La extracción de polifenoles a partir de la piel de la naranja [140], extracción de colorantes a partir de la remolacha roja [141], extracción de antocianinas de patatas de color púrpura [142], extracción de betaína de la remolacha roja [143], entre otros.

El principio de esta técnica es aumentar la permeabilidad de las membranas celulares por medio de la ruptura de las mismas. Esto se logra aplicando ondas electromagnéticas a los tejidos celulares. Este aumento de la permeabilidad permite el paso del solvente a través de la membrana celular, donde son vertidos los compuestos activos; a continuación se recupera el solvente con el extracto de la planta. El pulso electromagnético se logra haciendo pasar corriente eléctrica por dos placas conductoras (electrodos) en periodos cortos de tiempo. La intensidad del pulso viene determinada por el voltaje y la distancia entre los electrodos. Intensidades menores de 0.1kV/cm se pueden considerar pulsos de baja intensidad, entre 0.1 y 1 kV/cm pulsos de mediana intensidad y mayores de 1 kV/cm pulsos de alta intensidad [144].

En la figura 19 se puede observar cómo es el esquema de aplicación de pulsos electromagnéticos a una muestra:

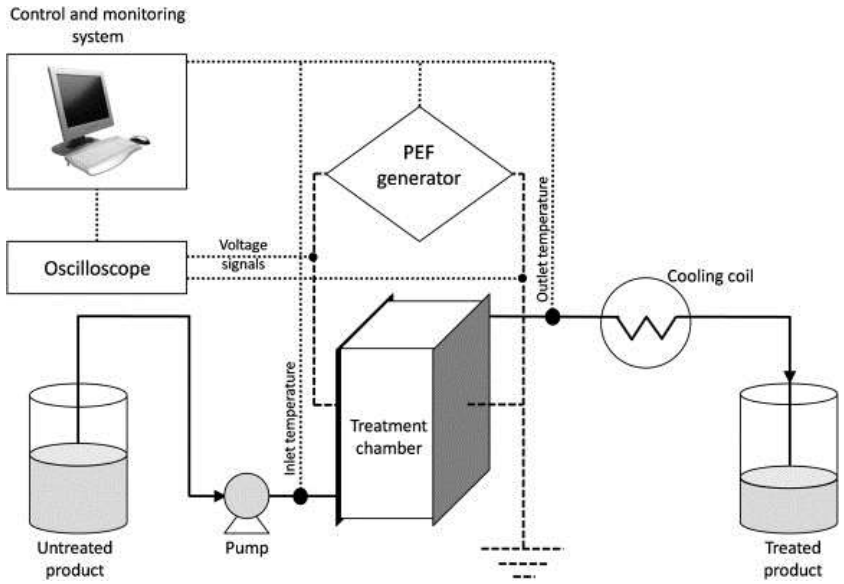



**Figura 19.** Aplicación de pulsos electromagnéticos a una muestra[145].

### *1.6.4 Extracción aplicando solventes con surfactantes.*

La adición de surfactantes se ha aplicado con éxito a la extracción de compuestos bioactivos ya que aumenta el poder de extracción de los solventes convencionales [146]. Existen dos tipos de metodologías: Usando el solvente más el surfactante, en una extracción convencional, es decir, variando la cantidad y tipo de surfactante añadido al solvente [147]; y usando el surfactante para atrapar los compuestos bioactivos y luego separarlo, por medio de la rotura del equilibrio entre el surfactante y el solvente, separándolo en dos fases [148]. El primer tipo extrae los compuestos bioactivos que quedan en el extracto junto con el surfactante. Tiene la ventaja de que el uso del extracto puede ser directo en alimentos si se usa un surfactante adecuado para ello. En la segunda metodología, también llamada extracción "de punto de nube" (CPE), tiene la ventaja de ser selectivo en la separación de compuestos ya que cada compuesto se puede unir a un surfactante específico (aniónico, catiónico o neutro) [149].

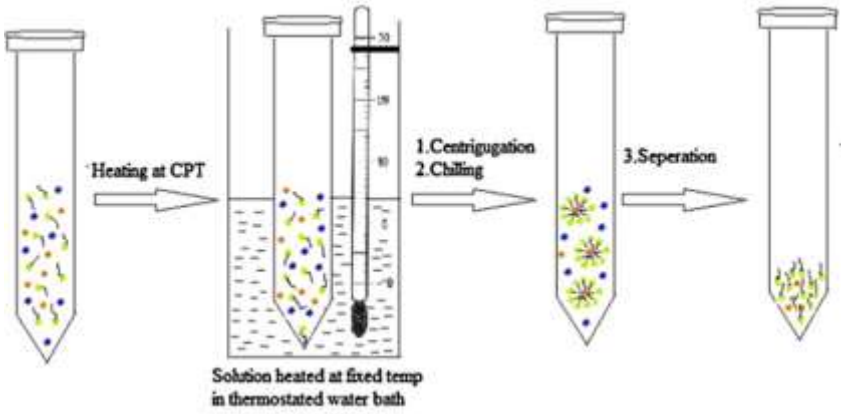



**Figura 20.** Esquema de extracción de compuestos usando la técnica CPE[150].

Como ejemplo de la extracción de polifenoles a partir de materia prima vegetal se tienen: Remoción de polifenoles y antioxidantes de zumos de frutas [147], extracción de polifenoles a partir de lodo de vino [149], obtención de polifenoles a partir del agua de prensado de la oliva [151], extracción de la teamina a partir del agua de lavado del té [152].

# 2 OBJETIVOS

El objetivo general de esta Tesis es la extracción y caracterización de compuestos bioactivos de residuos orgánicos y su uso en prevención de la oxidación en sistemas modelos de alimentos.

Dicho objetivo general se desglosa en los siguientes objetivos concretos:

- Optimizar el proceso de extracción convencional para obtener la máxima cantidad de polifenoles y capacidad antirradicalaria de los extractos, usando la metodología de superficie de respuesta.
- Analizar los extractos a través de la cantidad de polifenoles totales y su capacidad antirradicalaria, así como identificar los compuestos presentes por HPLC
- Evaluar el efecto sinérgico conjunto de compuestos antioxidantes y prooxidantes en un sistema modelo, emulsión de aceite en agua.
- Estudiar la cinética de extracción de polifenoles mediante el uso de ecuaciones matemáticas, ajustando los resultados obtenidos a una ecuación general.
- Evaluar la capacidad antioxidante de los extractos de semillas de aguacate y hojas de borraja en los lípidos de emulsiones y carne de ternera picada.
- Diseñar magdalenas con extracto de piña y analizar su capacidad antioxidante y la influencia en el sabor.

# 3 METODOLOGÍA EXPERIMENTAL Y RESULTADOS

## 3.1 Extraction of Antioxidants from Borage (Borago officinalis L.) Leaves—Optimization by Response Surface Method and Application in Oil-in-Water Emulsions

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Publicado en Antioxidants 2014, 3, 339-357; doi:10.3390/antiox3020339

**Abstract**: Borage (Borago officinalis L.) is a typical Spanish plant. During processing, 60% are leaves. The aim of this work is to model and optimize the extraction of polyphenol from borage leaves using the response surface method (RSM) and to use this extract for application in emulsions. The responses were: total polyphenol content (TPC), antioxidant capacity by ORAC, and rosmarinic acid by HPLC. The ranges of the variables temperature, ethanol content and time were 50–90 °C, 0%–30%–60% ethanol (v/v), and 10–15 min. For ethanolic extraction, optimal conditions were at 75.9 °C, 52% ethanol and 14.8 min, yielding activity of 27.05 mg GAE/g DW TPC; 115.96 mg TE/g DW in ORAC and 11.02 mg/L rosmarinic acid. For water extraction, optimal activity was achieved with extraction at 98.3 °C and 22 min, with responses of 22.3 mg GAE/g DW TPC; 81.6 mg TE/g DW in ORAC and 3.9 mg/L rosmarinic acid. The significant variables were ethanol concentration and temperature. For emulsions, the peroxide value was inhibited by 60% for 3% extract concentration; and 80% with 3% extract concentration and 0.2% of BSA. The p-anisidine value between the control and the emulsion with 3% extract was reduced to 73.6% and with BSA 86.3%, and others concentrations had similar behavior.

Keywords: RSM; rosmarinic acid; ORAC; borage leaves; extraction; emulsion; oxidation.

### *3.1.1 Introduction*

The properties of polyphenols as antioxidants have been widely recognized. They are associated with reduced risk of cancer, cardiovascular diseases, diabetes and Alzheimer's disease [153]. Most polyphenols in the human diet are supplied by plants and fruits [25]. Furthermore, antioxidants from natural sources could be used to increase the stability of food, such as the ability to prevent lipid peroxidation [132]. This damage could be catalyzed by different metals present in food (especially in meat), because the metals can participate directly or indirectly in the reaction of oxidation of lipids [154]. In addition, these metals promote the creation of reactive oxygen species (ROS) prejudicial to health [38]. Polyphenols are also used as antimicrobial agents in food preservation [27].

The worldwide demand for food has been increasing. Nowadays, fresh fruit and vegetable production is, approximately, 800,000 tons/year, without taking into account losses and waste [1].

In some studies polyphenols were found in pulp and other waste remaining from the production of fruit juices and wines [155,156]. Polyphenols can be excellent antioxidants and in some cases are better than synthetic ones [153]. New technology to treat food waste was required in order to obtain raw materials or ingredients for other processes and products [157].

Many health effects have been attributed to the borage (Borago officinalis L.) plant, such as: antispasmodic, antihypertensive, antipyretic, aphrodisiac, demulcent, and diuretic properties. It is also considered useful to treat asthma, bronchitis, cramps, diarrhea, palpitations, and kidney ailments [158]. In the food industry borage seed extracts have been used as effective antioxidants in the preparation of gelatin films from fish [159]. It was also shown to be effective in preventing oxidation in fermented dry sausages enriched with ω-3 polyunsaturated fatty acids (PUFA). As well as maintaining organoleptic properties, the borage extract was an economical and safe antioxidant source [153]. The antioxidant activity of borage meal extract was also demonstrated by Wettasinghe et al. (1999) [160] in a model meat system, where the inhibition of oxidation assessed by 2-thiobarbituric acid-reactive substances (TBARS), hexanal and total volatile formation was reported. Borage seed extracts exhibited strong metal chelating activity in an aqueous assay medium, that suggested it is a good chelating agent for food and non-food applications [154]. Bandoniene et al. (2002) [64] reported a

study that showed that borage leaf extract was an effective antioxidant in rapeseed oil. The polyphenols found in borage include rosmarinic acid, which is responsible for some of the antioxidant properties of rosemary extracts, which is also widely used by the food industry. Rosmarinic acid has a high antioxidant capacity and it is present in the majority of Lamiaceae species [64,154,161,162].

Borage leaves are a cheap raw material for the production of polyphenols, because it is a by-product of an industrial process, and in addition, the disposal of this material incurs a cost, which can be minimized by its use [153].

Response surface methodology (RSM) is a useful tool for process optimization [163], that allows the influence of independent variables on a response variable to be represented by a mathematical model that is able to reproduce the behavior of these parameters, with only a few experiments [164,165].

An experimental design commonly used in the food industry is the central composite design (CCD), which involves evaluation of the factors at various levels [166].

Several foods such as: milk, sauces and soup have an emulsion structure. This could be oil in water (O/W) or water in oil (W/O) or a combination of both. Oxidation is a principal problem of this model [167]. The oxidation of emulsions differs from oil oxidation, due to the presence of oil or water droplets and an interface between oil and water, where components partition between the phases and interact with effects on chemical reactions [168]. Furthermore, in foods, there may be synergy between antioxidants and the protein present; which may increase the antioxidant capacity and enhance the stabilization of the emulsion [169,170].

In this work, we modeled and optimized the extraction of polyphenols from borage leaves based on the total polyphenols, antiradical activity (ORAC), and the amount of rosmarinic acid. The response surface method has not been used before, but it allowed the extraction parameters to be studied for optimization of antioxidant effects in a model emulsion system.

### *3.1.2 Experimental Section*

#### *3.1.2.1 Materials*

2,2′-Azo-bis(2-amidinopropane) dihydrochloride (AAPH), was used as peroxyl radical source. Pyrogallol red (PGR), Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid), rosmarinic acid, ethanol, fluorescein, AAPH, BSA, p-anisidine (4-amino-anisole; 4-methoxy-aniline), isooctane, potassium persulfate, acetic acid (glacial) and polyoxyethylene sorbitan monolaurate (Tween-20) were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK). Folin–Ciocalteu reagent and sodium carbonate were supplied by Merck (Darmstadt, Germany). Refined sunflower oil, of a brand known to lack added antioxidants, was purchased from a local retail outlet. All compounds were of reagent grade.

#### *3.1.2.2 Borage Preparation*

The borage plant (Borago officinalis L.) was obtained in the local market, washed and the leaves were separated from other edible parts. This waste was homogenized and frozen at −80 °C for lyophilization. Then the leaves were ground into a powder by using a Moulinex mill (A5052HF, Moulinex, Lyon, France), then 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.

#### *3.1.2.3 Extraction Procedure*

Extraction was carried out in dark bottles, following the procedure described by Wijngaard et al. (2010) [155], with some slight modifications. Lyophilized sample powder (0.25 g) was blended with 15 mL of solvent of concentration specified by the CCD. It was mixed on a sample stirrer (SBS A-06 series H, Scientific Instrumentation SBS, S.L., Sabadell, Catalunya, Spain) for 1 min at 900 rpm, and then the liquid volume was increased to 25 mL with the solvent used. This mixture was placed in a bath by stirring at the required temperature and time specified by the experimental design, cooled in a refrigerator at 5 °C, centrifuged (Orto Alresa Mod. Consul, Ortoalresa, Ajalvir, Madrid, Spain) at 2500 rpm for 10 min, vacuum filtered and the loss solvent was replaced. The extract was stored at −20 °C until used for analysis.

*3.1.2.4 Total Phenolic Content (TPC)*

TPC was determined spectrophotometrically following the Folin–Ciocalteu colorimetric method [171]. 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 were mixed. The solution was allowed to react for 1 h in the dark and the absorbance was measured at 765 nm using a Fluorimetrics Fluostar Omega (BMG Labtech, Ortenberg, Germany). The total phenolic content was expressed as mg Gallic Acid Equivalents (GAE)/g dry weight.

*3.1.2.5 ORAC Assay*

Antioxidant activities of borage extracts were determined by the ORAC assay, as reported by Ninfali et al. [172]. The assay was carried out using a Fluorimetrics Fluostar Omega (Perkin–Elmer, Paris, France) equipped with a temperature-controlled incubation chamber. The incubator temperature was set at 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 and fluorescence measurements were continued for 2 h. 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)/mg of dry borage.

*3.1.2.6 HPLC*

Identification and quantification of rosmarinic acid was performed using a Waters 2695 separations module (Waters Corporation, Milford, MA, USA) system with a photodiode array detector Waters 996 (Waters Corporation, Milford, MA, USA). The column was a Kinetex C18 100A, 100 × 4.6 mm (Phenomenex, Torrence, CA, USA). Solvents used for separation were 0.1% acetic acid in water (v/v) (eluent A) and 0.1% acetic acid in methanol (v/v) (eluent B). The gradient used was: 0–12 min, linear gradient from 40% to 50% B; 12–15 min, linear gradient from 50% to 40 B. The flow rate was 0.6 mL/min, and the detection wavelength was 330 nm. The sample injection volume was 10 µL. The chromatographic peak of

rosmarinic acid was confirmed by comparing its retention time and diode array spectrum against that of a reference standard. Working standard solutions were injected into the HPLC system and peak area responses obtained. Standard graphs were prepared by plotting concentration (mg/L) versus area. Quantification was carried out from integrated peak areas of the samples using the corresponding standard graph.

#### *3.1.2.7 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, rosmarinic acid concentration). CCD uses the method of least-squares regression to fit the data to a quadratic model. The quadratic model for each response was as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \sum \beta_{ij} X_i X_j \quad (23)$$

where, Y is the predicted response; 0 is a constant; i is the linear coefficient; ii is the quadratic coefficient, ij is the interaction coefficient of variables i and j; and Xi and Xj are independent variables.

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).

**Tabla 1.** Design variable and code.

| Extraction | Code | Temperature (°C) | Time (min) | Ethanol Concentration (%) |
|---|---|---|---|---|
| Ethanolic | | | | |
| | −1 | 60 | 10 | 30 |
| | 0 | 70 | 15 | 45 |
| | 1 | 80 | 20 | 60 |
| Aqueous | | | | |
| | −1 | 50 | 10 | |
| | 0 | 70 | 15 | |
| | 1 | 90 | 20 | |

All responses were determined in triplicate and are expressed as average ± standard deviation. The answers have a percentage deviation less than 10%.

*3.1.2.8 Oil-Water 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 BSA (0.2%), and borage extracts (3% v/v, 1% v/v, 0.3 v/v, 0.06 v/v). The emulsion was prepared by the dropwise addition of oil (sunflower oil) to the water phase, cooling in an ice bath with continuous sonication with a Vibracell sonicator (Sonics & Materials Inc., Newtown, CT, USA) for 5 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) and p-anisidine value.

*3.1.2.9 Peroxide Value (PV)*

PV was determined by the ferric thiocyanate method (Frankel, 1998) [173] (after calibrating the procedure with a series of oxidized oil samples analyzed by the AOCS Official Method Cd 8-53). Data from the PV measurements were plotted against time.

#### *3.1.2.10 p-Anisidine Value (p-AV)*

The test was performed according to the methods reported by Singh et al. (2007) [116], with some modifications. In a 10 mL volumetric flask, 0.05 g of emulsion was taken and dissolved in 25% (v/v) of isooctane at 1% of acetic acid (glacial). From this solution, 2 mL were treated with 5% (v/v) of p-anisidine reagent and kept in the dark for 10 min and absorbance was measured at 350 nm using a UV-Vis spectrophotometer (Zuzi, AUXILAB, S.L., Beriain, Navarra, Spain).

### *3.1.3 Results and Discussion*

#### *3.1.3.1 Extraction*

The extraction process was influenced by several factors including temperature, particle size, solvent, time, and solids characteristics. The polyphenols extraction was affected by increase of temperature and the solvent used [174]. Moreover, the effect of solvent-solid ratio was positive despite the solvents used, the higher solvent-solid ratio, and the higher total amount of solid obtained [130]. The total amount of polyphenols was increased by the reduction of particle size, however, with the smaller particle size results were less reproducible due to the formation of agglomerations of borage dry in samples [175]. On the other hand, the concentration used, optimal solvent and different processes promote the extraction of specific substances [157].

In our case, response values for each set of variable combinations for aqueous and ethanolic extraction were obtained (Tables 2 and 3). All responses were adjusted to a quadratic model and the values of $R^2$ were satisfactory (Table 4). Figure 21, shows the behavior of aqueous extraction, where, increasing temperature increases the amount of phenolic acid extracted, showing maximum values (Figure 21c). An increase of TPC and ORAC was observed too (Figure 21a,b). In addition, the yield of polyphenols was increased with increase in extraction time. This behavior is similar to that reported by Ballard et al. (2009) [165] in the extraction of peanut skin polyphenols. Figure 22, shows the behavior of the ethanolic extraction. It was similar to Figure 21, but with a decrease of TPC with increasing temperature (Figure 22a), and this behavior was also observed in previous studies of the effect of solvent polarity, temperature and time factors on ethanol extraction of defatted borage seed [160]. The effect may be explained by the degradation of some phenolic glycosides and flavonols at higher temperatures. The relationship between

amount of polyphenols and antioxidant values were also observed for strawberry fruit extract [166]. The TPC values for both extractions reached a similar maximum value, although the conditions were different. In this sense, the extraction of polyphenols should be linked with solvent polarity and the extraction temperature [54], and for ethanolic extraction the optimal yield of polyphenols was occurred with the process conditions 70 °C, 45% of ethanol, and 15 min; while, for aqueous extraction the optimal polyphenol yield was obtained with conditions at 98.5 °C and 15 min.

**Tabla 2.** Experimental design and responses for aqueous extraction.

| Temperature (°C) | Time (min) | TPC (mg GAE/g Dry Weight) | ORAC (mg TE/g Dry Weight) | Rosmarinic Acid (mg/L) |
|---|---|---|---|---|
| 41.72 | 15.00 | 20.75 ± 0.58 | 27.22 ± 0.33 | 1.25 ± 0.01 |
| 70.00 | 15.00 | 24.89 ± 0.90 | 101.80 ± 6.37 | 2.79 ± 0.01 |
| 70.00 | 15.00 | 24.88 ± 0.87 | 104.05 ± 2.15 | 2.79 ± 0.01 |
| 90.00 | 10.00 | 26.45 ± 0.35 | 57.67 ± 2.76 | 2.87 ± 0.36 |
| 50.00 | 20.00 | 22.42 ± 0.93 | 40.36 ± 1.16 | 1.40 ± 0.01 |
| 70.00 | 7.93 | 24.16 ± 0.79 | 89.64 ± 1.55 | 1.52 ± 0.10 |
| 70.00 | 15.00 | 25.01± 0.07 | 103.24 ± 4.27 | 2.79 ± 0.01 |
| 50.00 | 10.00 | 21.58 ± 0.12 | 83.80 ± 2.21 | 1.36 ± 0.03 |
| 70.00 | 15.00 | 25.67 ± 0.55 | 104.87 ± 5.07 | 2.80 ± 0.01 |
| 70.00 | 15.00 | 24.49 ± 0.35 | 105.37 ± 1.15 | 2.80 ± 0.01 |
| 70.00 | 22.07 | 25.49± 0.54 | 110.92 ± 3.61 | 2.80 ± 0.21 |
| 90.00 | 20.00 | 25.46 ± 0.07 | 117.05 ± 1.88 | 3.79 ± 0.12 |
| 98.28 | 15.00 | 26.72 ± 0.79 | 102.23 ± 7.59 | 3.64 ± 0.04 |

GAE: Gallic Acid Equivalent; TE: Trolox Equivalent.

The correspondence between the progressive increase of the rosmarinic acid concentration with the increase of antiradical capacity until reaching a maximum should be noted, due to the excellent antioxidant capacity of this component [161,176]. Moreover, rosmarinic acid was obtained in greater amounts by the ethanolic extraction, at 70% of ethanol solvent, where the yield was 5.6 times more than with the aqueous extraction. Other researchers have reported similar results when working with sage under the same conditions (*Salvia oficinalis*) [177]. Mhandi et al. (2007) [178] obtained an extract from borage seeds in which the amount of rosmarinic acid was similar to the maximum observed in the extractions carried out in this work. An equation that modeled the process of rosmarinic acid

extraction was developed, and it was shown that rosmarinic acid yield decreased with the increase of ethanol in the solvent. The amount of rosmarinic acid obtained in the ethanolic extraction was higher than that obtained by aqueous extraction, and this behavior was observed in other studies [162]. Variation of conditions (temperature, ratio of solid/liquid) could not give good yields of rosmarinic acid when water was used as solvent, but other phenolic compounds may be efficiently extracted with water as observed in Figure 21b, where the ORAC values with water were close to those with ethanolic extraction. The ORAC values of the extract with ethanol were only 25% more than those obtained by aqueous extraction. Other studies [64,162] relate variability in the antiradical values to the actions of the factors mentioned earlier. Extracts of orange, apple, leek, and broccoli were investigated in other studies to determine the interactions [179].

**Tabla 3.** Experimental design and responses for ethanolic extraction.

| Temperature (°C) | Ethanol Concentration (%) | Time (min) | TPC (mg GAE/g Dry Weight) | ORAC (mg TE/g Dry Weight) | Rosmarinic Acid (mg/L) |
|---|---|---|---|---|---|
| 86.82 | 45.00 | 15.00 | 23.93 ± 1.06 | 126.80 ± 2.21 | 8.82 ± 0.13 |
| 60.00 | 30.00 | 10.00 | 20.26 ± 0.71 | 108.70 ± 1.23 | 3.77 ± 0.64 |
| 80.00 | 60.00 | 20.00 | 22.58 ± 1.23 | 125.21 ± 2.77 | 14.08 ± 0.07 |
| 70.00 | 45.00 | 15.00 | 27.49 ± 1.77 | 141.77 ± 3.19 | 11.04 ± 0.46 |
| 70.00 | 45.00 | 15.00 | 27.02 ± 1.04 | 146.06 ± 3.57 | 11.13 ± 0.57 |
| 70.00 | 45.00 | 15.00 | 26.91 ± 0.92 | 144.00 ± 6.42 | 11.43 ± 0.96 |
| 70.00 | 45.00 | 15.00 | 27.13 ± 1.15 | 143.72 ± 0.35 | 11.10 ± 0.58 |
| 70.00 | 45.00 | 22.07 | 25.33 ± 1.64 | 143.79 ± 3.40 | 11.83 ± 0.53 |
| 60.00 | 60.00 | 20.00 | 23.86 ± 0.69 | 128.13 ± 2.03 | 17.20 ± 1.15 |
| 60.00 | 30.00 | 20.00 | 22.43 ± 1.07 | 109.47 ± 3.88 | 5.38 ± 0.13 |
| 53.18 | 45.00 | 15.00 | 23.38 ± 0.83 | 124.97 ± 1.29 | 11.62 ± 0.54 |
| 70.00 | 19.77 | 15.00 | 19.16 ± 0.11 | 109.26 ± 3.34 | 0.77 ± 0.02 |
| 70.00 | 45.00 | 7.93 | 24.57 ± 0.54 | 138.27 ± 1.62 | 11.68 ± 0.07 |
| 70.00 | 45.00 | 15.00 | 27.16 ± 1.21 | 143.88 ± 3.05 | 11.23 ± 0.72 |
| 80.00 | 30.00 | 20.00 | 22.27 ± 0.72 | 128.78 ± 1.81 | 5.83 ± 0.28 |
| 60.00 | 60.00 | 10.00 | 22.99 ± 0.12 | 140.22 ± 0.57 | 17.53 ± 1.30 |
| 80.00 | 60.00 | 10.00 | 23.97 ± 1.37 | 146.88 ± 4.77 | 16.64 ± 0.47 |
| 70.00 | 45.00 | 15.00 | 26.94 ± 0.87 | 143.86 ± 0.20 | 11.30 ± 0.80 |
| 70.00 | 70.23 | 15.00 | 23.50 ± 0.76 | 132.04 ± 5.82 | 20.30 ± 0.00 |
| 80.00 | 30.00 | 10.00 | 23.25 ± 0.75 | 122.09 ± 2.23 | 4.69 ± 0.78 |

GAE: Gallic Acid Equivalent; TE: Trolox Equivalent.

**Tabla 4.** Mathematical equations from response surface method (RSM) for each of the responses, with their respective value of $R^2$ and $R^2$-predicted.

| Extraction / Response | Equation | $R^2$ Value | |
|---|---|---|---|
| | | $R^2$ | $R^2$-Pred. |
| *Ethanolic* | | | |
| TPC (mg GAE/g DW) | $-132.03 + 2.37\ T + 1.18\ C + 3.13\ t - 0.013\ T^2 - 0.009\ C^2 - 0.03\ t^2 - 0.003\ T \times C - 0.014\ T \times t - 0.003\ C \times t$ | 97.8 | 84.8 |
| ORAC (mg TE/g DW) | $-544.88 + 11.18\ T + 7.75\ C + 6.97\ t - 0.068\ T^2 - 0.038\ C^2 - 0.058\ t^2 - 0.024\ T \times C - 0.009\ T \times t - 0.069\ C \times t$ | 93.5 | 51.2 |
| Rosmarinic Acid (mg/L) | $-58.94 + 0.88\ T + 1.09\ C + 0.52\ t - 0.004\ T^2 - 0.0012\ C^2 + 0.006\ t^2 - 0.004\ T \times C - 0.007\ T \times t - 0.009\ C \times t$ | 99.7 | 97.6 |
| *Aqueous* | | | |
| TPC (mg GAE/g DW) | $-132.03 + 2.371\ T + 1.18\ t - 0.002\ T^2 - 0.006\ t^2 - 0.005\ T \times t$ | 96.3 | 83.8 |
| ORAC (mg TE/g DW) | $-9.236 + 4.654\ T - 12.357\ t - 0.0538\ T^2 - 0.149\ t^2 - 0.257\ T \times t$ | 94.5 | 61.1 |
| Rosmarinic Acid (mg/L) | $-67.250 + 1.233\ T + 4.140\ t - 0.009\ T^2 - 0.146\ t^2 - 0.005\ T \times t$ | 98.5 | 89.1 |

T: Temperature (°C); C: Ethanol concentration (%); t: Time (min); Pred.: response predicted by model.

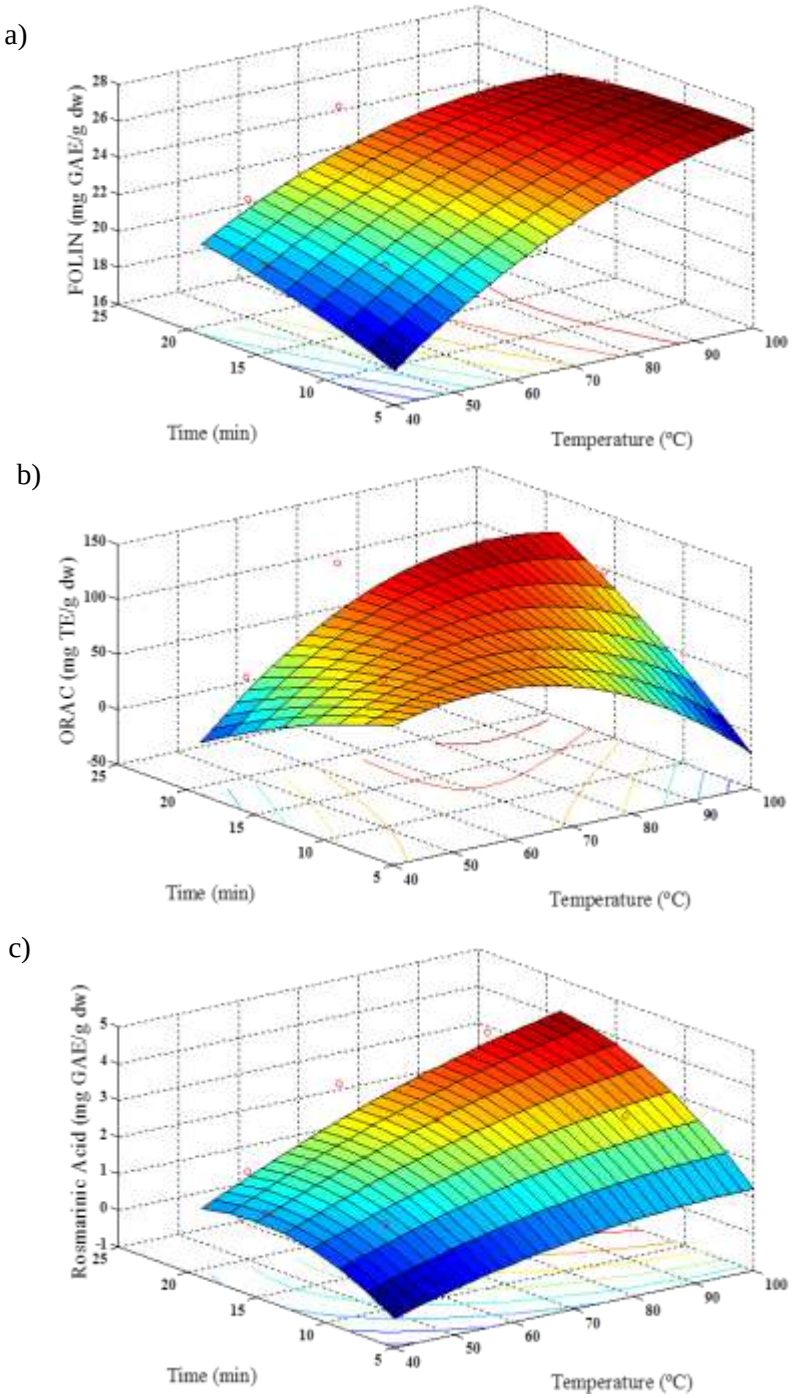


**Figura 21.** Response surface model plot, for aqueous extraction, showing the effects of time and temperature in: (a) total polyphenols contents; (b) antioxidant activity (ORAC); and (c) rosmarinic acid content.

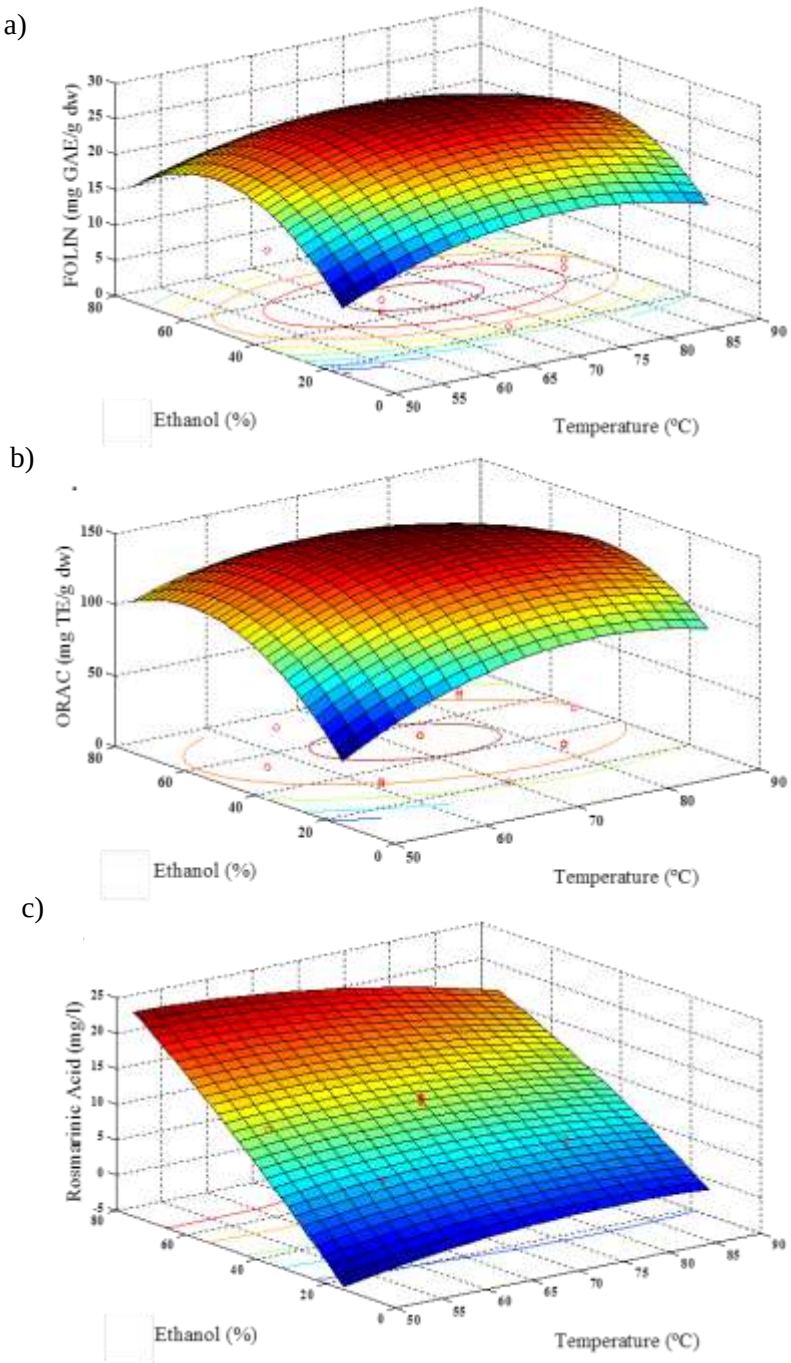


**Figura 22.** Response surface model plot, for ethanolic extraction, showing the effects of ethanol concentration and temperature on: (a) total polyphenols contents; (b) antioxidant activity (ORAC); and (c) rosmarinic acid content.

### *3.1.3.2 Response Surface Method*

The RSM has been successfully applied to identify optimal conditions for the polyphenol extraction process, since it allows a mathematical model to be developed and through it the antioxidant values are linked with the factors such as temperature, solvent type, particle size, time, as well as others. In this sense, reported investigations have related the extraction of polyphenols and their antioxidant capacity from *Castanea sativa* leaves with time, temperature and solid/liquid ratio to identify the optimal conditions [180]. Pompeu et al. (2009) [164] varied the ethanol proportion in the solvent, composition and temperature to obtain an equation that described the process adequately. The antioxidant capacity of borage extract has been studied with respect to the factors: organic solvents, temperature and time; but the yield of a specific polyphenol has not been investigated [160]. In addition, no previous studies have been performed with water as solvent and the effect of variation of extraction temperature and yield of rosmarinic acid have not been reported. The use of water as solvent has the advantages that it is environmentally safe and cheap [181].

Table 5 shows the p values for each of the coefficients of the quadratic terms obtained by using RSM. In extractions with variable time and temperature (for ORAC) and ethanol (for TPC), moreover the quadratic term (for ORAC and rosmarinic acid), had high values of p ($p > 0.05$). The high p values were attributed to fast mass transfer of phenolic compounds, which was dependent on the manner in which the sample was processed (powder) so that the quadratic term may or may not be significant [133,182]. The most significant variable was the concentration of ethanol in the ethanolic extraction, which had an influence on all responses. Similarly, for aqueous extraction the p values were high for terms including the time factor for TPC and ORAC. In addition, the constant was not significant for the model. However, for the yield of rosmarinic acid the equation coefficients were highly significant, which shows that the mathematics model may be representing the extraction adequately.

### *3.1.3.3 Validation Conditions Optimized*

Optimal conditions obtained for each of the extractions and for each type of extraction were carried out in triplicate and the responses were obtained (Table 6). For predicting responses, the model found fits well with the actual values, except in the case of the ORAC values, where it showed a deviation from the expected values

based on the value of $R^2$ predictive. Moreover, there was a difference between extractions. The ethanolic extraction gave higher values in: TPC, ORAC, and rosmarinic acid than the aqueous extraction.

**Tabla 5.** *p*-Values for each of the constants in the equation of the mathematical model.

| Extraction | Term | *p* Value | | |
|---|---|---|---|---|
| | | Response | | |
| | | TPC | ORAC | Rosmarinic Acid |
| Ethanolic | | | | |
| | Constant | 0.000 | 0.001 | 0.000 |
| | Temperature (°C) | 0.000 | 0.000 | 0.000 |
| | Ethanol (%) | 0.000 | 0.000 | 0.000 |
| | Time (min) | 0.000 | 0.092 | 0.133 |
| | Temperature (°C) × Temperature (°C) | 0.000 | 0.000 | 0.003 |
| | Ethanol (%) × Ethanol (%) | 0.000 | 0.000 | 0.022 |
| | Time (min) × Time (min) | 0.000 | 0.248 | 0.149 |
| | Temperature (°C) × Ethanol (%) | 0.044 | 0.046 | 0.001 |
| | Temperature (°C) × Time (min) | 0.003 | 0.780 | 0.031 |
| | Ethanol (%) × Time (min) | 0.236 | 0.009 | 0.000 |
| Aqueous | | | | |
| | Constant | 0.536 | 0.899 | 0.000 |
| | Temperature (°C) | 0.001 | 0.011 | 0.000 |
| | Time (min) | 0.077 | 0.045 | 0.000 |
| | Temperature (°C) × Temperature (°C) | 0.005 | 0.000 | 0.000 |
| | Time (min) × Time (min) | 0.400 | 0.299 | 0.000 |
| | Temperature (°C) × Time (min) | 0.088 | 0.001 | 0.180 |

TPC in mg GAE/g DW; ORAC in mg TE/g DW; Rosmarinic Acid in mg/L.
GAE: Gallic Acid Equivalent; TE: trolox equivalent.

### *3.1.3.4 Oil-Water Emulsions*

The progress of oxidation with time in the stored emulsion samples was measured by the PV and p-anisidine methods, which have been widely used for this purpose [183,184]. The total period of the experiment was limited to 864 h, because

at that moment the stability of the emulsion was broken since it separated into separate phases. The extract used was obtained by ethanolic extraction with the optimal conditions found earlier, since the extract has maximum values for the ORAC value and rosmarinic acid content.

**Tabla 6.** The optimal responses given by RSM for the two types of extractions.

| Extrac. | Conditions | | | Response | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Temp | EtOH | Time | TPC | | ORAC | | Rosmarinic Acid | |
| | (°C) | (%) | (min) | Pred | Actual | Pred | Actual | Pred | Actual |
| Ethanolic | 75.94 | 51.88 | 14.8 | 26.71 | 27.05 | 145.03 | 115.96 | 13.29 | 11.024 |
| Aqueous | 98.28 | - | 22.07 | 26.02 | 22.27 | 120.33 | 81.6 | 4.09 | 3.9 |

GAE: Gallic Acid Equivalent; TE: trolox equivalent; TPC in mg GAE/g DW; ORAC in mg TE/g DW; Rosmarinic Acid in mg/L.

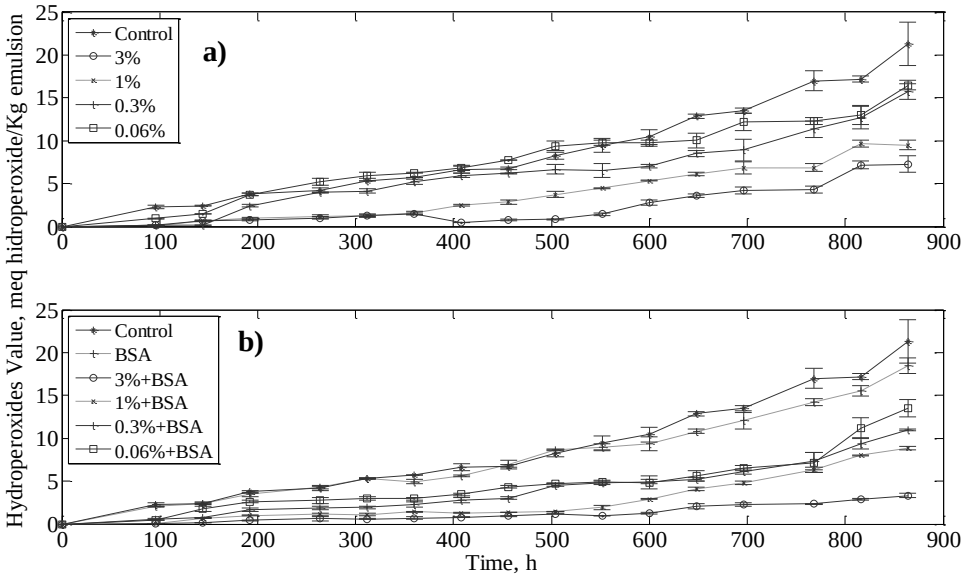


**Figura 23.** The peroxide values for the emulsions: (a) Samples with extract; (b) Samples with extract and 0.2% BSA.

Figure 23 shows the PV of the emulsion with different concentration of borage extract. In Figure 22a, a clearly increase in inhibition of lipid oxidation by increasing the extract concentration was observed and the reduction in PV suggested an effect of protection for the oil phase. This effect of borage extracts was indicated by other authors, who demonstrated the influence of the concentration of polyphenol extracts of different plants in the oxidative process in the oil [48] and they also reported the dependence of the antioxidant activity on the

type of polyphenol. Polar polyphenols were more soluble in the aqueous phase and less polar polyphenols were concentrated at the interface between oil-water [185]. In this sense, rosmarinic acid may be the cause of antioxidant activity in the aqueous phase as found by Jordán et al. (2012) [162]. The emulsions containing extract in the range of concentrations from 0.06% to 3% had an antioxidant capacity between 8% and 60% considering the control emulsion as reference. Moreover, the emulsions containing 1% and 3% of extract presented a significant difference ($p < 0.05$) in their PV with respect to the control sample.

BSA has been shown to increase antioxidant activity by synergy with phenolic antioxidants in emulsions. As in other studies, stabilization of the emulsion was observed by PV measurements [186]. The negative character of the protein and the positive charge of metal ions could explain this increase in the antioxidant capacity [167]. Furthermore, the protein contributes to physical stabilization of the emulsion and unabsorbed protein decreases lipid oxidation[50]. Figure 23b shows the PV values of the stored emulsion containing extract and BSA. It was evident that the BSA contributed to the antioxidant activity in the emulsion system, and the emulsion was significantly more stable when assessed by the PV than the sample without protein. In fact, the emulsions containing 0.2% of BSA and extract in the range 0.06% to 3% had an antioxidant capacity between 36.6% and 84% considering the control emulsion as reference. These results indicated for all concentrations employed samples containing extract and BSA were significantly more stable ($p < 0.05$) than the control emulsion with and without BSA.

Figure 24 show a comparison of PV behavior between all emulsions studied at the end of the experiment after 864 h. As was observed, the PV in the emulsion with borage extract was higher than that for the emulsion containing borage extract and BSA, and it was noted that the BSA greatly improved the antioxidant effect of the extract. The stability of the emulsion play an important role in the oxidation process, since the protein enhances droplet formation and forms a layer thereby protecting the oil droplet [187]. Moreover, it was observed that the sample containing BSA had a lower PV than the control emulsion.

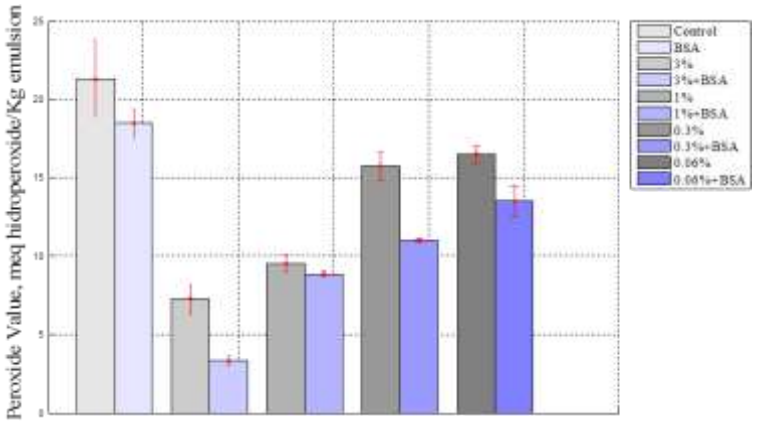


**Figura 24.** Comparison between samples containing extract and extract plus BSA at 864 h.

The secondary products of oxidation in emulsion were measured with the p-anisidine value test, and this method has been used for the determination of antioxidant capacities of different oils [116], emulsions [168] and the effects of extracts in oils [115]. The p-anisidine values (p-AV) were evaluated at three times: 408 h, 696 h, and 864 h with the same samples used for PV analysis. The determination of p-anisidine values has been used to show a synergy for the combination of both, BSA and polyphenol extract obtained from other plants like green tea [169]. Figure 25 shows that the borage extract at 3%, in combination with BSA, reduced by 86.3% the formation of secondary oxidation products in the samples, whereas the extract at 3% reduced the p-anisidine values by 73.6% at 864 h. However, the p-AV increased throughout the experiment. The presence of protein at the interface decreased the rate of lipid oxidation due to its ability to trap free radicals and bind with metals as seen earlier [105].

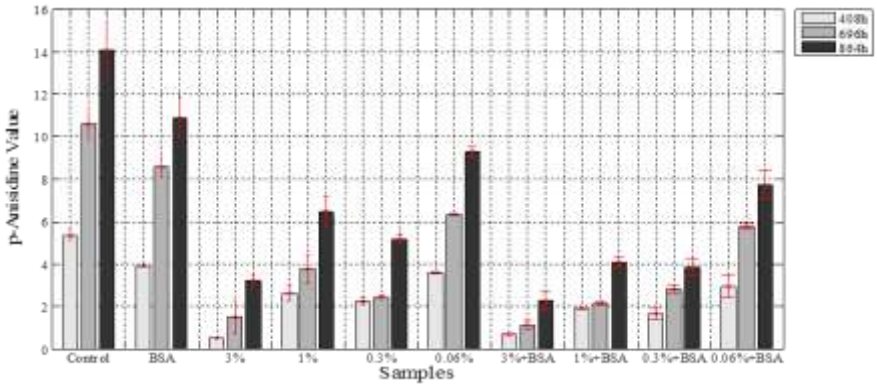


**Figura 25.** The p-anisidine values for the samples with BSA and without BSA.

In addition, studies carried out with green tea extracts and emulsion samples showed an inhibitory effect on p-AV similar to that in the current study, since the hydrophilic cathequins present in the green tea extract acted at the oil-water interface like rosmarinic acid and reduced the rate of the oxidation process [188].

### *3.1.4 Conclusions*

A model has been developed to describe the effect of several variables on extraction of polyphenols from borage leaves. The antioxidant activity (ORAC value) demonstrated optimal values for ethanolic and for aqueous extraction. The increase in antioxidant capacity can be related to the increase of the amount of rosmarinic acid; however other polyphenols may also contribute, as seen by the decrease of ORAC values with increase of extraction temperature. The decomposition of these polyphenols may explain these results. The ethanolic or aqueous extraction conditions can be chosen according to the type of phenolic acids we want to enrich in the extract, or the effect we want to obtain. The use of water for extraction of polyphenols made the extraction process more environmentally friendly, but increased the energy used. The time variable was of little significance for the model.

The use of the borage extract and the extract in combination with BSA decreased the rate of increase of PV and p-AV in an emulsion, with a synergistic effect demonstrated. This effect could be associated with the presence of rosmarinic acid in the extract and the function of BSA as a metal chelating agent at the interface.

The application of innovative technologies such as ultrasound, electromagnetic pulses, subcritical water extraction, among other, could be applied for further study in order to enhance the extraction of polyphenols [181,189,190] of the borage leaves, as well as its influence on preservation of the antioxidant properties on the extract obtained.

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

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

**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

### *3.2.1 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 [191]. 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 [15].

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 [192].

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 [25], 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 [27]. They also protect against oxidation, direct or indirect, caused by metal cations [154]. 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 [162].

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 [1,155]. Previous examples already studied 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 [193]. Also, the waste from wine and beer production includes phenolic compounds [155]. Other studies have focused on the shells of nuts, rice and wheat in which large amounts of polyphenols are found [156].

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 [10]. Among the polyphenols the (+)-catechin and (−)-epicatechin [194] and chlorogenic and protocatechuic acid, are included [9]. Previous studies on this residue have been applied to pork burgers and have been shown to be effective in preventing oxidation and microbial growth [10].

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 [195].

In order to optimize the extraction process, response surface methodology (RSM) has been used. Phenolic compounds extraction optimization from strawberries [166], apple pulp [155] and residues of chestnuts [180], are examples of this. This method establishes a multivariable mathematic model to obtain the relationship between responses and independent variables [157,164] 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.

### *3.2.2 Experimental Section*

#### *3.2.2.1 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 were acquired from Panreac Química 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.

#### *3.2.2.2 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.

#### *3.2.2.3 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 Mod. Consul, Ortoalresa, Ajalvir, 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.

#### *3.2.2.4 Total Phenolic Content (TPC)*

TPC was determined spectrophotometrically following the Folin-Ciocalteu colorimetric method [61]. 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.

**Tabla 7.** 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: Galic Acid Equivalent; TE: Trolox Equivalent; TPC: Total phenolic content; ORAC: Oxygen Radical Antioxidant Capacity; GAE: Gallic Acid Equivalents; TE: Trolox Equivalents.

*3.2.2.5 ORAC Assay*

Antioxidant activities of avocado seeds extracts were determined by the ORAC assay, as reported by Casettari *et al.* [196]. 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.

### *3.2.2.6 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 7. 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%.

### *3.2.2.7 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).

### *3.2.2.8 Peroxide Value (PV)*

PV was determined by the ferric thiocyanate method [116] (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.

### *3.2.2.9 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 Ø-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.

### *3.2.2.10 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) [197] 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 in 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.

### *3.2.3 Results and Discussion*

#### *3.2.3.1 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 [174,198–201]. 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 [166]. 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 [202,203]. 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 [163]. All these parameters are collected in Table 7 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 26 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 protocatechuic acid [9] 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 [155].

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 [174]. 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 27 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 [164].

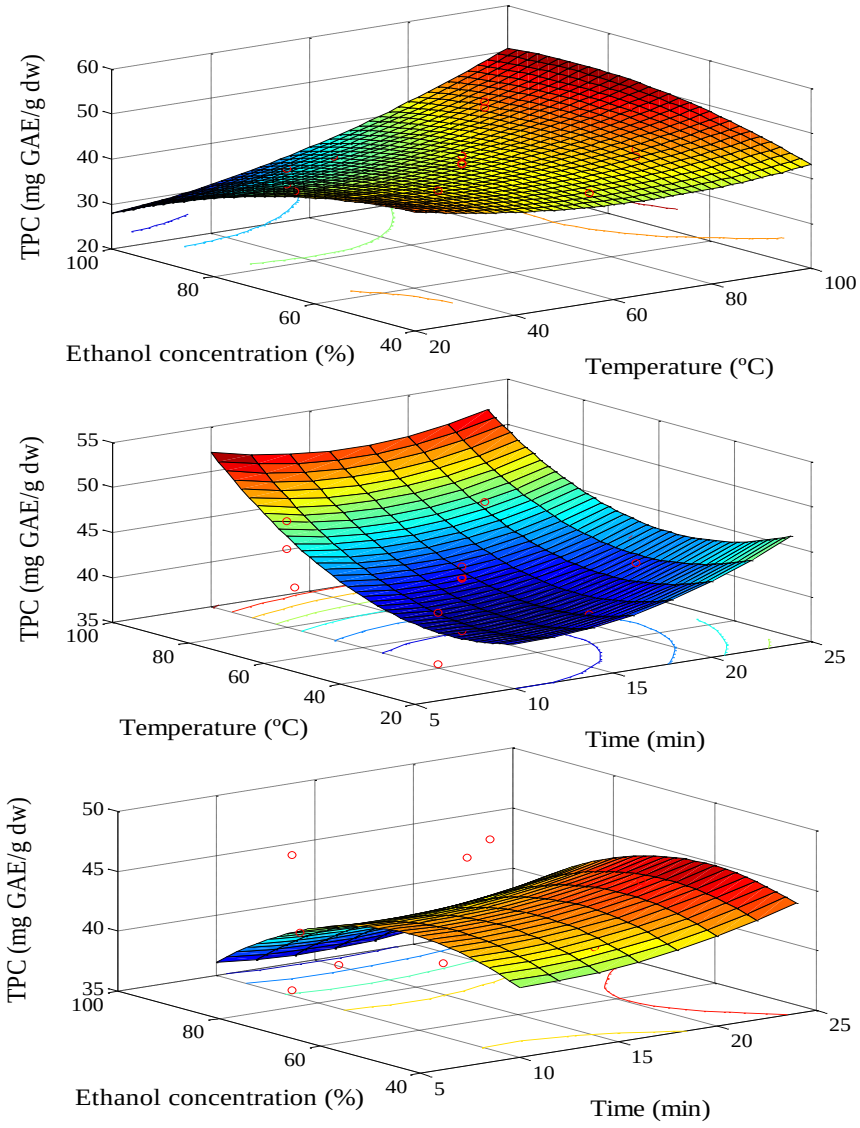


**Figura 26.** Response surface model plot: the variable is the total phenolic content (TPC) of the extract. % EtOH with temperature; temperature with time; % EtOH with time.

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 [166].

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 [163].

Table 8 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 [204].

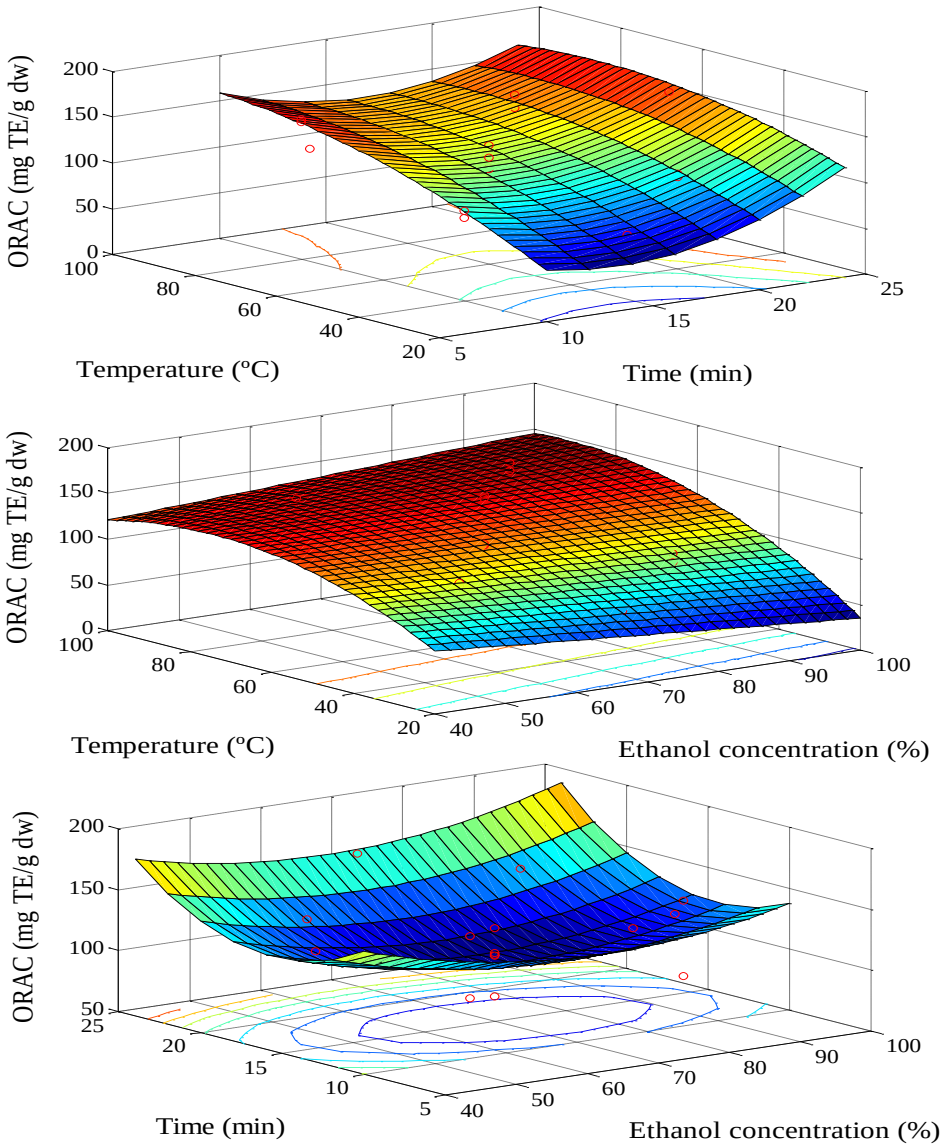


**Figura 27.** Response surface model plot: the variable is the Oxygen Radical Antioxidant Capacity (ORAC) of the extract. Temperature with time; % EtOH with temperature; % EtOH with time.

Therefore, with the exception of $T \times$ % 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 8.

Table 9 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 10 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.

**Tabla 8.** *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.

**Tabla 9.** 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.

**Tabla 10.** 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.

*3.2.3.2 Extract Optimized Effect in Oil-in-Water Emulsions (O/W)*

Figure 28 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 lyophilized 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 [105,184,187].

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 [186,205]. 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.

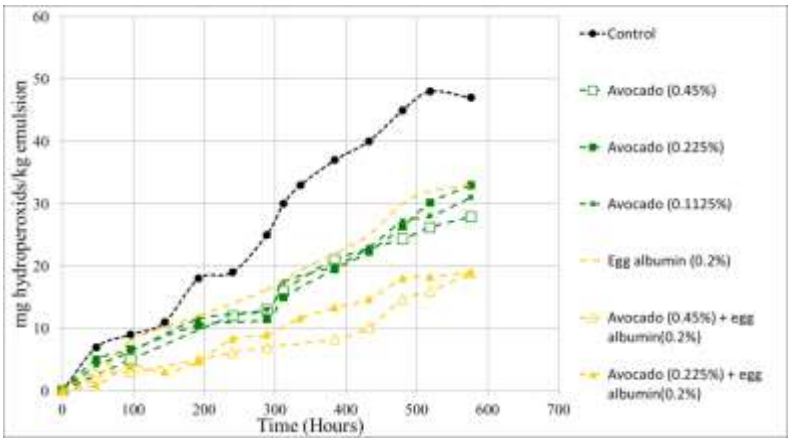


**Figura 28.** Peroxide values *vs.* time in the emulsions.

Avocado pits contain polyphenolic compounds (such as protocatechuic acid, chlorogenic acid, syringic acid and rutin), which are very strong antioxidants [9]. In 2010, Sasaki [206] 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.

### *3.2.3.3 Effect of the Extract in Burger Meat*

The TBARS method is widely used to determine the oxidation of fats and oils in foods [107,207,208]. In Figure 29, 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.* for pork burgers. That study examined protecting fat oxidation also with excellent results [209]. 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 [210].

It is not the first time avocado pits have been used in meat products. Rodríguez-Carpena *et al.* (2011) [10], 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.

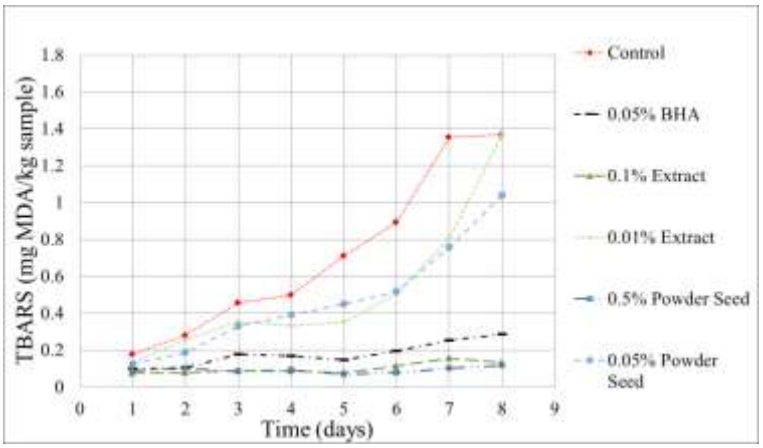


**Figura 29.** The TBARS (Thiobarbituric acid reactive substances) values for the meat emulsions.

### *3.2.4 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.

## 3.3 Improvements in the Aqueous Extraction of Polyphenols from Borage (Borago officinalis L.) Leaves. Pulsed Electric Fields (PEF) Applications.

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Publicado en Industrial Crops and Products, 2015, 65, 390-396; http://dx.doi.org/10.1016/j.indcrop.2014.11.010

**Abstract**: The aim of this work is to evaluate the effect of pulsed electric fields (PEF) (0-5 kV/cm) on the improvement of the aqueous extraction of polyphenols and antioxidant compounds from borage leaves. The extracts were assessed by using total polyphenols content (TPC) by the Folin-Ciocalteu assay and antioxidant capacity by the assay of Oxygen Radical Absorption Capacity (ORAC). Extractions along the time were conducted at different temperatures (10, 25 and 30ºC). The PEF treatments incremented the TPC and ORAC values of the extracts between 1.3 and 6.6 times for TPC and between 2.0 and 13.7 times for ORAC, compared with the control. The TPC values were plotted and fitted to the kinetic model with an adjustment of $0.96<R2<0.99$. Significant differences between the treated and control samples were found as well in this study. This procedure enhances the antioxidant capacity of the extracts and reduces extraction times.

Keywords: Borage; Pulsed Electric Fields; Antioxidant Activity; Phenolic Compounds; Aqueous Extraction.

### *3.3.1 Introduction*

Borage plant is a source of a considerable amount of polyphenols. Particularly, the highest amount can be found in the seeds (in the oil obtained by extraction) and soft tissues (in prepared extracts) [160,211]. Gallic, Chlorogenic, Rosmarinic, Syringic, p-Coumaric and Trans-cynnamic acids are the most important phenolic acids [178]. Many health benefits, such as enhancements in the cardiovascular system, respiratory system and dermatitis, are attributed to the borage [158,212]. That is the reason why it has been used as a medicinal plant by many Mediterranean communities [65].

Nowadays, due to the toxicity of synthetic antioxidants such butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ), among others, the industry has been replacing them, in some cases, with natural antioxidants. Furthermore, they are better than synthetics and have health benefits [195]. Borage extracts have been used as antioxidants and antimicrobial, even in the preparation of films of gelatin from fish, obtaining excellent results [159]. For instance, in emulsions, the peroxide value has been inhibited by 60% for a 3% extract concentration. The p-anisidine value in the emulsion with a 3% extract was reduced to 73.6% [13]. Their use in fermented dry sausages enriched with ω-3 PUFA has also shown to be effective against oxidation and not to make any difference to the organoleptic product, being an economical and safe antioxidant [211].

In some industries, borage leaves are considered by-products of the process. Therefore, it can be a cheap raw material for the production of antioxidants, which are high value-added products.

Despite producing undesirable changes in food such as the loss of their nutritional components, the thermal processes are used for the extraction and the inactivation of enzymes and microorganisms [213]. Different methods have been applied to extract the antioxidants in borage oil, leaves and edible parts: cold pressed [214] and organic solvents with heat, finding that extracted polyphenols decrease when the heat increases [160].

Application of pulsed electric fields (PEF) has been investigated as a non-thermal process to improve extraction of intracellular compounds [73,215]. This technique achieves to enhance the amount of polyphenols in tomato sauces and

grapes, as well as colour extraction plant and sugars [140,143,216,217]. In the extraction process, PEF is a treatment that consists on the application of pulses of high voltage (kV) and short duration (µs-ms) to a biological material placed between two electrodes. This voltage results in an electric field of which intensity depends on the voltage delivered and the gap between the electrodes. PEF cause a phenomenon called electroporation that consists on the increment of the cell membrane permeability to ions and macromolecules [218]. In the classic method of extraction process, the variables are temperature (30-90°C), time and solvent (usually the organic type to treat polyphenols). However, pretreating the material with PEF incorporates new variables like intensity pulse and the number of pulses used. For the extraction study in organic materials, the range of the pulse intensity used to permeabilize the cell membrane is between 1-10kV while the number of pulses are between 5-50 [219,220]. Fortunately, this can reduce or eliminate the application of heat or the use of organic solvents [220–222].

In this study, PEF have been applied in borage leaves at different temperatures and exposure times to find the differences between: content of total polyphenols and antioxidant capacity, for each one of treatments.

### *3.3.2 Material and Methods*

#### *3.3.2.1 Materials*

The 2,2-Azo-bis(2-amidinopropane) dihydrochloride (AAPH), Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid), fluorescein and sulfuric acid were purchased from Sigma–Aldrich (St. Louis, MO). Folin–Ciocalteau reactive and sodium carbonate were supplied by Merck (Darmstadt, Germany).

#### *3.3.2.2 Borage Samples.*

##### *3.3.2.2.1 Preparation of materials.*

Borage plants (Borage officinalis L.) were obtained in a local supermarket (Zaragoza, Spain). Leaves were separated from other edible parts, washed, grounded using a mill Moulinex (A5052HF), and placed in acidic water (pH:1.5) to prevent enzymatic browning. The particle size for the extraction was determined using two sieves. Only the range of particle size from 2 mm to 4 mm was studied.

*3.3.2.2.2 PEF treatment.*

PEF equipment used in this investigation was supplied by ScandiNova (Modulator PG, ScandiNova, Uppsala, Sweden). The apparatus generates square waveform pulses of a width of 3 µs with a frequency up to 300 Hz. The maximum output voltage and current were 30 kV and 200 A, respectively. The equipment consists of a direct current power supply which converts the 3-phase line voltage to a regulated DC voltage. It charges up 6 insulated gate bipolar transistor (IGBT) switching modules (high-power solid-state switches) to a primary voltage around 1000 V. An external trigger pulse gates all the modules and limits its discharge to a primary pulse signal of approximately 1000 V. Finally, a pulse transformer converts this primary 1000 V pulse to the desired high-voltage pulse.

The treatment chambers consisted of a cylindrical methacrylate tube closed with two polished stainless steel cylinders. The gap between the electrodes was 1.0 cm and the diameter of the treatment chamber for the determination of the cell disintegration index, 1.5 cm. In the extraction experiments, the gap between the electrodes and the diameter of the treatment chamber were 2.0 cm.

Actual voltage and current intensity applied were measured with a high-voltage probe (Tektronix, P6015A, Wilsonville, Oregon, USA) and a current probe (Stangenes Industries Inc. Palo Alto, California, USA) connected to an oscilloscope (Tektronix, TDS 220, Wilsonville, Oregon, USA).

PEF treatments were similar to those reported by Luengo et al. (2013) [140]. Briefly, ranging from 5 to 50 pulses of 3 µs (15 to 150 µs) were set at electric field strength ranging from 1 to 7 kV/cm. The specific energy of these treatments ranged from 0.04 to 61.1 kJ/kg. A pulse frequency of 1 Hz was used.

*3.3.2.2.3 Cell Disintegration Index (Zp).*

Cell disintegration index (Zp) was used to identify the optimal PEF treatment conditions for the pre-treatment of the borage leaves before the polyphenol extraction. This index characterizes the proportion of permeabilized cells based on the frequency dependence of conductivity of intact and permeabilized plant tissues [223]. The same treatment chamber used to apply the PEF treatments was used as measuring cell. 1.5±0.2 g of grounded borage leaves were introduced in the cell for Zp determination.

Zp analysis was carried out using impedance measurement equipment (DIL, Quakenbrück, Germany) and calculated using the equation (1):

$$Z_p = 1 - \left(\frac{K_h}{K_h'} \times \frac{(K_h' - K_l')}{(K_h - K_l)}\right); 0 \leq Z_p \leq 1 \quad (24)$$

where Kl and Kl´ are the electrical conductivities of untreated and treated material, respectively, at a low-frequency field (1 kHz) and Kh and Kh´ are the electrical conductivities of untreated and treated material, respectively, at a high-frequency field (2 MHz). The Zp varies between 0 for intact tissues and 1 for a tissue with all the cells permeabilized. The determination of Zp is based on the fact that the conductivity of intact tissues is strongly dependent of frequency because by increasing the frequency, the cell membranes become every time less resistant to the current flow. While in the low frequency range differences in conductivity between a tissue with intact cells and with rupture membrane cells are detected, in the high frequency range the intact cell membrane does not show any resistance to the current flow and no difference is detected between the conductivity of a tissue with intact cell and cells with ruptured membranes [140].

*3.3.2.3 Extraction and Analysis of Polyphenols.*

*3.3.2.3.1 Extraction procedure.*

For the extraction, 10±0.5 g of the untreated and PEF-treated borage leaves were put in a 250 ml Erlenmeyer flask that contained 100 ml of acidic water (pH:1.5). During all the extraction time, all flasks were incubated at the appropriate temperature (10, 25, 40 °C) in a water bath. Samples of 1 ml of the extraction medium were removed at different extraction times to evaluate the evolution of the total polyphenols. All experiments were performed at least in triplicate.

*3.3.2.3.2 Total Phenolic Content (TPC).*

TPC was determined by using the Folin-Ciocalteau method described by Singleton and Rossi (1965) [171]with some modifications. To 0.1 ml of borage extract, 5 ml of distilled water and 0.5 ml of Folin-Ciocalteau's reagent (2 N) were added. After 5 minutes at room temperature, 1.5 ml of a solution of sodium carbonate (20% p/v) and 2.9 ml of distilled water were added to the mixture and incubated for 90 minutes. After incubation, the absorbance was measured at 750 nm using a Unicam UV500 spectrophotometer (Unicam Limited, Cambridge, UK).

Gallic acid was used as the standard for the calibration curve and the results were expressed as mg of Gallic Acid Equivalents (GAE) per 100 g of fresh weight of borage leaves.

*3.3.2.3.3 Kinetics of polyphenols extractions by PEF treatments.*

The experimental data of TPC were fitted to the equation (2) [224], commonly used to describe the solid-liquid extraction of different intracellular compounds:

$$Y = Ymax(1-e-kt) \quad (25)$$

where Y is the TPC extracted in the solution at time t (min); Ymax is the maximum TPC obtained at equilibrium (t=∞), and k is the rate constant depending on the extraction parameters ($min^{-1}$).

*3.3.2.3.4 ORAC assay.*

Antioxidant activities of Borage extracts were determined by the ORAC assay [225]. The assay was carried out using a Fluostar Omega equipped with a temperature-controlled incubation chamber. Incubator temperature was set at 37 ºC. The extract samples were diluted 1:20 with milli-Q water. They were placed into a microplate and fluorescein (8.03 × 10-7 M) was added, with the final proportions in the well of 1/5 (sample) and 3/5 (fluorescein). An initial reading was made. After this, AAPH (0.3 M) (proportion in well 1/5) was added, and measurement was continued for 2 hours. The decrease of fluorescence over time was quantified as area under the curve (AUC, equation (3)). The samples were measured in triplicate.

$$AUC=\left(\frac{0.5+\sum_{i}^{Nc} f_i}{f_o}\right).tc \quad (26)$$

Where: fi = fluorescence units (fo, is the value of the first reading), Nc = number of cycles, tc: time of each cycle, in this case tc = 2 (minutes)

The ORAC value was calculated as follows:

A calibration curve was prepared using Trolox at different concentrations, with Trolox solution added to give a final concentration in the range between 0,5

mg Trolox/L and 14,78 mg Trolx/L, in the well. This curve was specific for the assay of the sample.

Decrease fluorescence= AUC -AUCBl

AUC = area under the curve of the sample in the well

AUCBl = area under the curve of the blank

This method includes the time and decreased fluorescence. The blank was 0.01 M phosphate buffered saline (pH 7.4). ORAC values were expressed as mg Trolox Equivalents (TE)/mg of dry borage.

*3.3.2.4 Statistical Analysis.*

The graphics and the fitted curves were made by software Matlab version 7.9.0 (The MathWorks, Inc. 2009, Massachusetts, USA). All responses were obtained in triplicate and were expressed as their average ± media deviation.

*3.3.3 Results and Discussions*

*3.3.3.1 Effect of PEF treatment in the disintegration index (Zp).*

Cell disintegration index (Zp) procedure was used to define the optimal conditions for permeabilization of borage leave cells by PEF. This index is based on the increment of the conductivity of intact cell membranes as a consequence of the electroporation caused by PEF. The relationship between the conductivity of the intact and permeabilized cells is term as Zp. Defining the process conditions to enhance polyphenol extraction from borage leaves is important in order to know the electric field strength and the treatment time, and it results in the maximum degree of permeabilization, using the least energy. The influence of the applied electric field strength (1, 3, 5 and 7 kV/cm) on the calculated values of the Zp, using equation (24) for different times of processing is presented in figure 30. It is observed that an increase in the electric field strength from 1 to 5 kV/cm resulted in an increase of the Zp. Further increments of electric field strength above 5 kV/cm did not increase the Zp. In the different electric field strengths investigated, the Zp increased rapidly at the first moments of the treatment, however, a slower increment of the cell damage was observed when the number of pulses increased. For example, at 3 kV/cm the Zp increased from 0 to 0.2 when the treatment time

increased from 0 to 50 µs and from 0.2 to 0.25 when the treatment time increased from 50 top 100 µs. Above 60 µs, continuous increase in the treatment time led to small further cell damage up until the Zp, it became practically constant indicating a saturation of the cell breakdown. Similar behavior was reported by other authors with different plant materials such as sugar beet, purple potato, orange peels or alfalfa tissues working in a range of electric fields(1-7 kV/cm), similar to those used in this investigation and treatment time in the range of microseconds (60-145 µs), obtaining Zp = 0.3 for orange peels and Zp = 1 for purple potato [142,226,227]. However, when the electric field strength applied was lower than 1 kV/cm with a treatment time in the range of microseconds, the relationships between treatment time and Zp were a convex upward that it was transformed in sigmoid curve when the saturation of the cell damage degree was observed, with Zp = 1 when 1 kV/cm is applied for 100 µs [228].

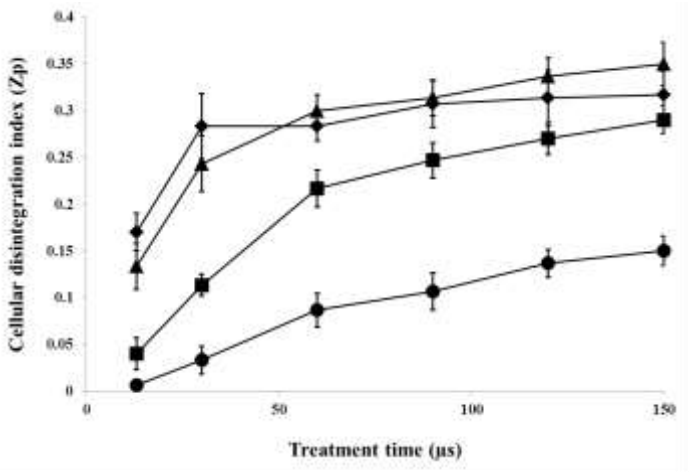


**Figura 30.** Influence of electric field strength and treatment time on the cellular disintegration index (Zp,) of borage leaves, (●) at 1 kV/cm, (□) at 3 kV/cm, (▲) at 5 kV/cm and (▼) at 7 kV/cm.

Another remarkable fact is that, independently from the applied electric field strength, the Zp value increased by the treatment time increase to approximately 60 µs (20 pulses of 3 µs). So this was the treatment time at different electric field strengths selected to investigate the effect of the application of PEF treatments on improving the extraction of polyphenols from borage leaves at different temperatures. This treatment time corresponds to a total specific energy of 1.09 and 6.18 kJ/kg at 2 and 5 kV/cm respectively.

### *3.3.3.2 Effect of PEF on the aqueous extraction of polyphenols from borage leaves at different temperatures.*

The extraction of polyphenols from plants is generally conducted after drying the plant matrix and using polar solvents such as methanol or ethanol [229]. Therefore, the process requires high energy consumption and a large amount of organic solvents. The electroporation of the plant cells by PEF with the purpose of improving extraction of polyphenols from fresh borage leaves using water as solvent is a technological innovation that may contribute to improve competitiveness and environmental protection. An additional advantage of using water as solvent for the extraction of polyphenols of green plants, such as borage leaves, is that it prevents the extraction of chlorophylls, which are only soluble in organic solvents. Extracts of green colour as consequence of the extraction of chlorophyll may result problematic for some applications of the polyphenols. Preliminary studies in our research group demonstrated that contrary to the ethanol extraction, even in the samples previously treated by PEF, chlorophylls were not extracted from fresh borage leaves when water was used as solvent.

Figure 30 shows, as an example, the evolution of polyphenol yield expressed as GAE (mg) extracted by gram of fresh borage leaves along the time for the control and for samples treated by PEF (2.5 and 5 kV/cm for 60 µs) at an extraction temperature of 25 ºC. The electroporation of the borage leaves cells by PEF of different intensity and the extraction temperature (data not shown) did not affect the shape of extraction curves. In all cases, they fit an exponential model asymptotically rising with time to a maximum value for all treatment conditions. The application of a PEF pre-treatment to the borage leaves increased the polyphenol yield and reduced the extraction time to reach a given yield. The quantity of polyphenols extracted after 1 hour of contact was very small for the control sample (see Figure 31). The polyphenol extraction yield for the PEF treated samples after 2 minutes of extraction was similar to polyphenol extraction yield for the control sample after 1 hour of extraction. Similar results were obtained by El-Belghiti et al. (2005) [230] when they investigated the kinetics of extraction of sugar from sugar beet treated by PEF, where moderate pulsed electric field treatment of intensity assured effective permeabilization of cellular membranes and significant enhancement of the solid–liquid extraction of sugar from sugar beet slices even at ambient temperature (25ºC).

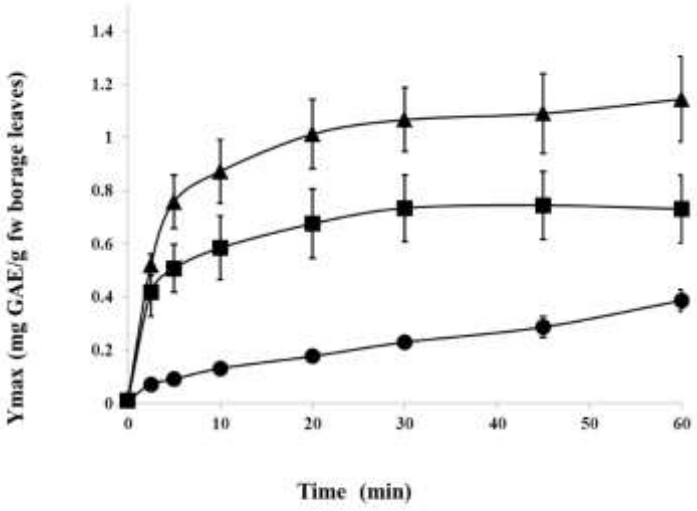


**Figura 31.** Extraction of polyphenols at 25ºC from untreated (●) and PEF treated borage leaves at 2.5 kV/cm (□) and 5 kV/cm (▲) for 60 µs along the time.

Data obtained corresponding to the extraction of polyphenols from borage leaves treated by PEF at different temperatures were fitted to the exponential model presented in equation (25). Table 11 shows the used mathematical model that is adjusted with a good fit to the experimental data ($0.87<R^2<0.95$). In the samples treated by PEF, the rate constants (k) were not affected by increasing the intensity of the electric field strength or the extraction temperature. No statistical significant differences ($p>0.05$) were found between the k-values of the extraction of polyphenols from electroporated borage leaves at 2.5 and 5.0 kV/cm. No effect of the electric field intensity on rate constants was observed either for the extraction of sugar from sugar beets, moderate pulsed electric field treatment of intensity E = 500–700 $Vcm^{-1}$ and duration $t_{PEF}$ = 0.025 s assures effective permeabilization of cellular membranes and significant enhancement of the solid–liquid extraction of sugar from sugar beet slices, even at room temperature (El-belghiti et al., 2005).

**Tabla 11.** Maximum Extraction Yield (Ymax) and Extraction Rate Obtained by Fitting the Equation 25 to the Experimental Data Corresponding to the Extraction of Polyphenols from Borage Leaves Treated by PEF at Different Electric Field Strength.

| E (kV/cm) | T (° C) | $Y_{max}$ | 95% CL | k | 95% CL | $R^2$ | RMSE |
|---|---|---|---|---|---|---|---|
| 0 | 10 | $0.153^{a}$ | 0.100-0.206 | $0.050^{abd}$ | 0.00-0.111 | 0.83 | 0.034 |
| | 25 | $0.387^{b}$ | 0.341-0.433 | $0.010^{a}$ | 0.00-0.023 | 0.95 | 0.075 |
| | 40 | $0.906^{ce}$ | 0.651-1.160 | $0.039^{b}$ | 0.012-0.066 | 0.87 | 0.167 |
| 2.5 | 10 | $0.603^{d}$ | 0.558-0.649 | $0.237^{c}$ | 0.145-0.330 | 0.88 | 0.026 |
| | 25 | $0.710^{cd}$ | 0.622-0.797 | $0.257^{cd}$ | 0.092-0.422 | 0.73 | 0.148 |
| | 40 | $0.842^{c}$ | 0.718-1.030 | $0.246^{cd}$ | 0.021-0.471 | 0.87 | 0.191 |
| 5 | 10 | $0.944^{ce}$ | 0.838-1.052 | $0.177^{cd}$ | 0.078-0.276 | 0.79 | 0.1168 |
| | 25 | $1.072^{ce}$ | 0.955-1.188 | $0.222^{cd}$ | 0.101-0.343 | 0.79 | 0.251 |
| | 40 | $1.158^{e}$ | 1.034-1.282 | $0.234^{cd}$ | 0.106-0.362 | 0.79 | 0.206 |

E: electric field strength; T: temperature; 95%CL: coefficient limit; R2: determination coefficient; RMSE: root mean square error.

Values followed by different small letter are significantly different ($p<0.05$)

Maximum extraction yield (Ymax) augmented thanks to the increment of the extraction temperature and electric field strength applied to the borage leaves before extraction. For example, in the samples treated at 2.5 kV/cm, the increment of the extraction temperature from 10 to 40ºC grow the maximum extraction yield 1.3 times and the electroporation of the borage leaves at 5 kV/cm increased the maximum extraction yield 2.4 times in comparison with the control sample. The increase of the polyphenol extraction yield with the extraction temperature and the permeabilization of the cell membranes by PEF is consistent with the mass transfer principles. The electroporation of the cell membranes that retain the intracellular content creates channels thought water and water soluble compounds can diffuse. It has been estimated that the average diffusion coefficient of a small solute in a membrane is often about a million times lower than that in the adjacent aqueous solutions [231]. Furthermore, the increment of the temperature favored extraction by both enhancing solubility and diffusivity of polyphenols [232].

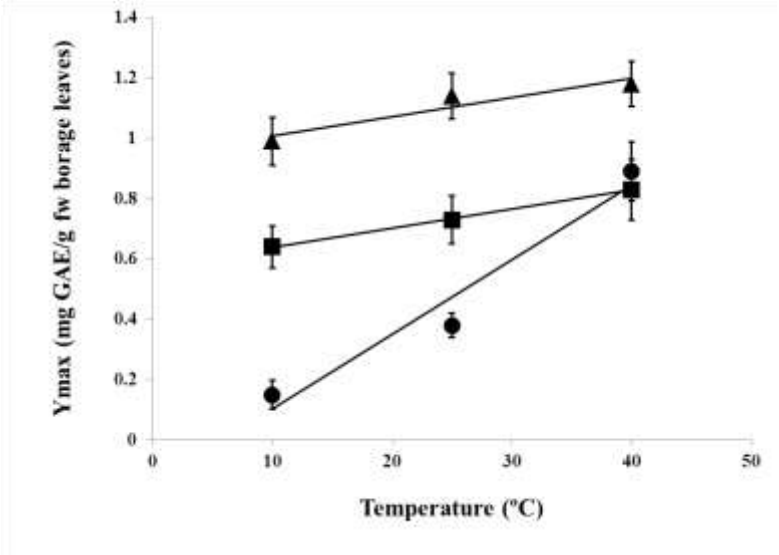


**Figura 32.** Relationship between the maximum extraction yield and the extraction temperature for the control sample (●) and borage leaves treated at 2.5 kV/cm (■) and 5 kV/cm (▲) for 60 µs.

The relationship between the maximum extraction yield and the extraction temperature for the control and samples electroporated at 2.5 and 5 kV/cm is shown in Figure 32. In the three samples, maximum extraction yield increased linearly with the extraction temperature. However, the influence of the temperature on the maximum extraction yield was higher for the control than for the electroporated samples. It is due to the greater diffusion capacity of the treated samples by pulses, without increasing the temperature the electroporation enables and eases this diffusion. Furthermore, the temperature rise also increases the solubility, especially the one of hydrophilics. The results show that it is not necessary to increase so much the temperature, fact that keeps the thermolabile compounds, for example, studies conducted to improve the extraction of anthocyanins showed that PEF and temperature treatments did not alter the degradation rate of the compound compared with the control sample ($p<0.05$) [215]. In summary, the increase of the temperature has two effects. Both the polyphenolic compounds diffusion and solubility increase. In the first effect, there is a little appreciation since the electroporation increases the diffusion [132,174,233]. The increment of the temperature from 10 to 40ºC

increased the maximum extraction yield 5.7 times for the control and 1.2 times for the samples treated at 5 kV/cm. The maximum extraction yield was higher for the sample treated at the highest electric field strength (5 kV/cm) independently from the extraction temperature. These results are no new; other authors reported that Zp is a suitable procedure to select the PEF treatment conditions that induce the highest electroporation. For instance, Angersbach, used high frequency current and voltage measurements to determine passive electrical properties, such as the polarization effect at intact membrane interfaces and field-induced electropermeability changes in the cellular materials during direct current pulses [223]. They demonstrated that ion leakage values and ruptured cells count were improved with increasing pulse number. 92.2±5.9 % of the onion cells were ruptured after 333 V/cm and 100 pulse treatment [234].

The effect of temperature on the samples electroporated was independent of the electric field strength applied (see figure 32). The straight lines, that describe the relationship between the maximum extraction yield and the extraction temperature, were parallel. Finally, according the data shown in the figure 32 the effect of electroporation on improving the polyphenol extraction was higher at lower temperatures of extraction. The treatment at 5 kV/cm increased 6.7 times the maximum extraction yield at 10ºC but only 1.4 times at 40ºC. So this effect indicates that extractions at lower temperatures could benefit from the application of PEF to increase mass transfer. It has the advantage of saving energy and obtaining specially heat sensitive intracellular compounds

#### *3.3.3.3 Antioxidant Activity of extracts obtained at different temperatures from borage leaves treated by PEF.*

The antioxidant activity of the obtained aqueous extracts was assayed by the ORAC method. This antioxidant determination method has advantages over others, because the peroxyl radical is used as a reactant with redox potential and the reaction mechanism is similar to physiological oxidants [79]. Furthermore, the assay is simple, as it measures the antioxidant activity of both lipophilic and hydrophilic compounds and it has a great biological relevance [61]. For all these reasons, the ORAC method is commonly used for the determination of the antioxidant activity of fruit and its extract obtained in aqueous or organic solvents [235–237].

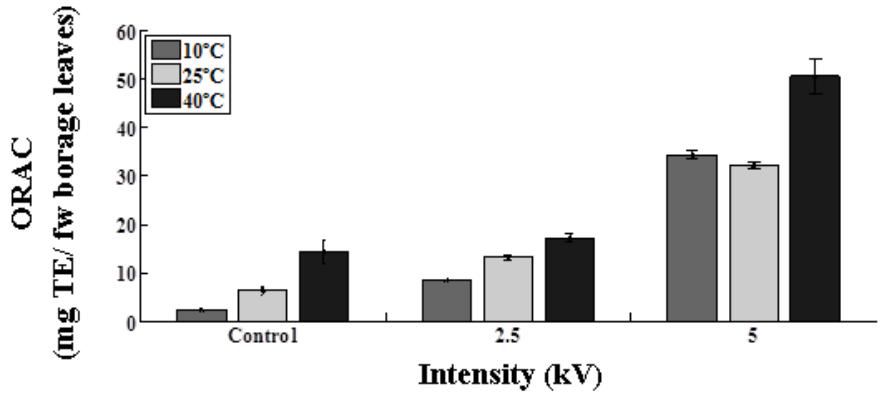


**Figura 33.** Effect of PEF treatment in the ORAC values of borage leaves extracts at 60 min.

Figure 33 shows the influence of the application of PEF treatment of different intensity on the antioxidant activity of borage leaves extracts obtained at different temperatures, after 60 minutes of extraction. Antioxidant activity of the untreated leaves is also shown by comparison. In both untreated and PEF treated samples, the antioxidant activity tended to increase with the extraction temperature. Statistical significant differences ($p>0.05$) were always found between the antioxidant activity of the extract obtained at 10ºC and 40ºC. Similar influence of the temperature on the antioxidant activity of methanol, acetone and ethanol borage extracts has been reported, where the antioxidant activity of the borage extract prepared at 25 ºC was low, but gradually increased with increasing temperature [160]. The antioxidant activity of the extracts was higher when the borage leaves were previously treated by PEF and it increased with the electric field applied. Similarly to the polyphenol extraction, no significant differences in antioxidant activity were found between the control extract and the extract obtained from the PEF treated borage leaves at 2,5 kV/cm when the extraction was performed at 40 ºC. However, when the borage leaves were treated by PEF at 5 kV/cm, the antioxidant activity of the extract increased by 3.5 times. This similar behavior between polyphenol extraction and antioxidant activity seems to indicate that polyphenols extracted from borage leaves are the main responsible for the antioxidant activity of the extracts determined by the ORAC method and that the application of a PEF treatment to the borage leaves did not impair the activity of the extracts obtained. This increase of the ORAC value as the increase of the TPC value and tendency have been reported by Alarcón et al. (2008) [85], there is a good correlation between ORAC and phenol content in herbal infusions ($r = 0.935$) and

teas (r = 0.999). Prior et al. (2005) also reported that the relationship between the TPC and the measurements by ORAC is generally good; the ORAC value increases linearly with the value of TPC. In addition, correlations between 0.93 and 0.88 have been found for the antioxidant activity and the amount of polyphenols in onions confirming that polyphenols are responsible for the antioxidant capacity [238].

Luengo et al. (2013) [140] obtained similar results evaluating the antioxidant activity of extracts of orange peels treated by PEF obtained by pressing. PEF treatments of 1, 3, 5 and 7 kV/cm increased the antioxidant activity of the extract 51%, 94%, 148% and 192%, respectively, in comparison with the not treated samples. A relation between TPC and ORAC is observed for all extractions.

### *3.3.4 Conclusion*

The PEF treatment appears an enhancement in the extraction process of polyphenols from borages leaves and its antioxidant capacities. Furthermore, it decreases the time employed in the extraction. The increase of pulse intensity shows proportionalities with the amount of extracted polyphenols. However, above 5 kV/cm, the total membrane cell is permeated and no changes were observed. Besides, the antioxidant ability of the extracts improves with the pulses intensity used for the treatment of borage leaves in the previous extractions.

Finally, having used water as a solvent adds an environmental advantage regarding with organic solvents. As well as resulting in cleaner extractions for the environment, it also complies with criteria of green chemistry concepts and industry sustainability.

## 3.4 Experimental design to monitor effects of caffeic acid, BSA and Fe (II) concentration on oxidation in a model food emulsion

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Enviado a Macrothink, en proceso de revisión

doi:10.5296/xxx.vxix.xxx URL: http://dx.doi.org/10.5296/xxx.vxix.xxx

**Abstract:** Polyphenols are the subject of intense research, which include food antioxidant. More research work is needed to clarify the mechanism involved in polyphenol activity and interactions with other components which may affect it. The aim of this study was to investigate the effects on retarding oxidative deterioration in emulsion samples containing mixtures of caffeic acid with common food components including proteins and metal ions. A simple process design based in the comparison of three components [BSA, Caffeic Acid and Fe (II)] with different concentration in model food emulsions was used to study the hypothesis that the oxidation of oil in water emulsions are function of three concentration, BSA caffeic acid and Fe (II). Effects of concentration of each component were investigated by a main component analysis. It may be observed that the concentration of BSA that maximizes the oxidation time does not depend on the remaining independent variables (concentration of caffeic acid and concentration of Fe). It may be seen that the caffeic acid has a linear influence. Whenever the caffeic acid increases, oxidation goes slower. As regards the concentration of BSA, it may be observed a non-linear behaviour that is constant for all different concentrations of Fe (II).

Keywords: experimental design: caffeic acid; antioxidants; dose-dependence; BSA; polyphenols; model food system, emulsions; Fe (II).

### *3.4.1 Introduction*

There is considerable interest in the antioxidant properties of polyphenols and their ability to reduce the risk of cancer [19,239], atherosclerosis and other cardiovascular diseases [65,240]. The capacity of polyphenols to interact with some proteins and enzymes involved in modulating signaling pathways [241,242] and their antioxidant activity, and ability to affect protein and enzyme bioavailability [169,243] are also important.

Modern consumers require high quality foods that are minimally processed and preservative free, while remaining safe. This along with tighter legislation regarding the use of preservatives has challenged the food industry. For this reason, natural antioxidant compounds, such as polyphenols, are more widely used to prevent food oxidation [193].

Caffeic acid (3,4-dihydroxycinnamic acid), is one of the most widely distributed hydroxycinnamate and phenylpropanoid metabolite in plant tissues and is present in many dietary sources including coffee beverages, blueberries, apples and cider [15].

There's a considerable amount of experimental data concerning the antioxidant activity of caffeic acid, which acts by scavenging oxygen free radicals [85,159,244] and by chelating prooxidant metal ions, like iron [245–247]. Iron is one of the most abundant transition metals in the human body, where it is essential for oxygen transport, and it reacts readily in redox reactions and is present in the aqueous phase of food emulsions [246,248,249]. Furthermore, it has a potentially deleterious effect by increasing the rate at which free radicals are generated. It is a lipid oxidation catalyst in food emulsions and its activity would be influenced by the charge on the lipid droplets [184].

Caffeic acid can interact with multi-subunit proteins and enzymes due to its capacity to establish ionic and H-bonding interactions [241,250]. Interactions of this type can cause an important modification in protein activity, or affect the antioxidant capacity of the polyphenol.

Antioxidants play an important role in retarding oxidation of low density lipoproteins (LDL) in vivo, where peroxidation of LDL is a critical initial event in atherogenesis. Hydrophilic antioxidants show greater antioxidative power than hydrophobic ones in bulk oil, while hydrophobic antioxidants show greater activity

than hydrophilic compounds in emulsions. This phenomenon is called the “Polar Paradox” [46] and is important in many foods where fat droplets are dispersed in an aqueous matrix.

Antioxidant behavior is more complex in biphasic systems such as emulsions than in bulk oil because more variables influence lipid oxidation including droplet size, emulsifiers, pH and ionic strength [184,251] among others . Some polyphenols may act as prooxidant or antioxidant agents depending on the lipid system, partly, due to differences in relative partitioning between phases [252]. Moreover, compounds with antioxidant activity may exhibit prooxidant behavior under certain conditions [193]. The activity of some polyphenols depends on concentration range, either showing reduced antioxidant activity, or even prooxidant effects at low concentrations [193,252,253]. The antioxidant behaviour of caffeic acid presents has been shown to be dose-dependent [254].

Proteins can also inhibit oxidation when they are in an aqueous medium such as the continuous phase of oil in water emulsion where they may scavenge free radicals or reduce peroxides due to their free sulfhydryl groups and ability to chelate prooxidant metals. Also protein activity depends on the concentration range, whether it is in the continuous phase or acting as an emulsifier [255–258].

Experimental studies have shown synergistic effects between caffeic acid, polyphenols and other compounds in different substrates. Some studies show that combinations of components have higher antioxidant activity than the original products but other studies report equal or lower antioxidant activity of mixtures [104,259,260]. Previous studies of our research group have shown that the combination of caffeic acid with bovine serum albumin (BSA) has better antioxidant properties in an emulsion than both the protein and the polyphenol separately.

Iron is an active catalyst for lipid oxidation of foods. Iron is present in plant foods either as a residue from processing equipment or from plant enzymes. In the case of animal products, haem pigments, including myoglobin and hemoglobin, may act as the source of catalytic iron species. Lipid oxidation in biological systems is often characterized by a lag-phase, during which little if any lipid oxidation products are detected. However, radicals are continuously generated during this period but are quenched by various types of substrates. Iron species are well

established as responsible for initiation of oxidative processes. The present study investigates the prooxidant activity of ferrous ions, Fe (II), in emulsions.

In this context, the aims of this study are: i) to characterize and compare the antioxidant activity of caffeic acid in the presence of Fe (II) ions and bovine serum albumin (BSA), and ii) to show how concentrations of the different components influence the oxidation rate.

### *3.4.2 Materials and Methods*

#### *3.4.2.1 Chemicals*

Caffeic acid, bovine serum albumin (BSA), ferric chloride and Tween 20 were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK). Refined sunflower oil of a brand known to lack added antioxidants was purchased from a local retail outlet.

#### *3.4.2.2 Removal of tocopherols from sunflower oil*

Tocopherols were removed from sunflower oil by column chromatography using alumina as described by Yoshida [158].

#### *3.4.2.3 Preparation of emulsions*

Oil-in-water emulsions (20.2 g) were prepared, as previously described (Almajano et al., 2007b), by dissolving Tween-20 (1%) in water containing caffeic acid (0-2.5 mM) and BSA (0-1 % in weight). The oil was added dropwise to the aqueous sample cooled in an ice-bath while sonicating for 5 min. in total.

#### *3.4.2.4 Storage and sampling of emulsions*

All emulsions were stored in triplicate in 50 mL glass beakers in the dark (inside an oven) 30 ºC. Two aliquots of each (0.005 - 0.1 g depending on the extent of oxidation) were removed periodically for peroxide value (PV).

#### *3.4.2.5 Analytical methods*

PV was determined by the ferric thiocyanate method [261] after calibrating the procedure with a series of oxidised oil samples analysed by the AOCS Official Method Cd 8-53.

### *3.4.2.6 Statistical analysis*

Data from the PV measurements were plotted against time. The times to reach 40 meq/kg (PV) were determined for each stored sample. PV induction times were analyzed by one-way analysis of variance (ANOVA) to determine the pooled standard deviation. The mean values within each test were compared by a two-sample t-test by using the pooled standard deviation to determine significant differences.

### *3.4.3 Results and Discussions*

Figure 34 shows the PV of the emulsion with different concentration of Fe (II), BSA and caffeic acid. In figure, a clearly increase in inhibition of lipid oxidation by increasing the concentration was observed and the reduction in PV suggested an effect of protection for the oil phase. Polar polyphenols were more soluble in the aqueous phase and less polar polyphenols were concentrated at the interface between oil-water [185]. In this sense, caffeic acid may be the cause of antioxidant activity in the aqueous phase as found by others authors [262].

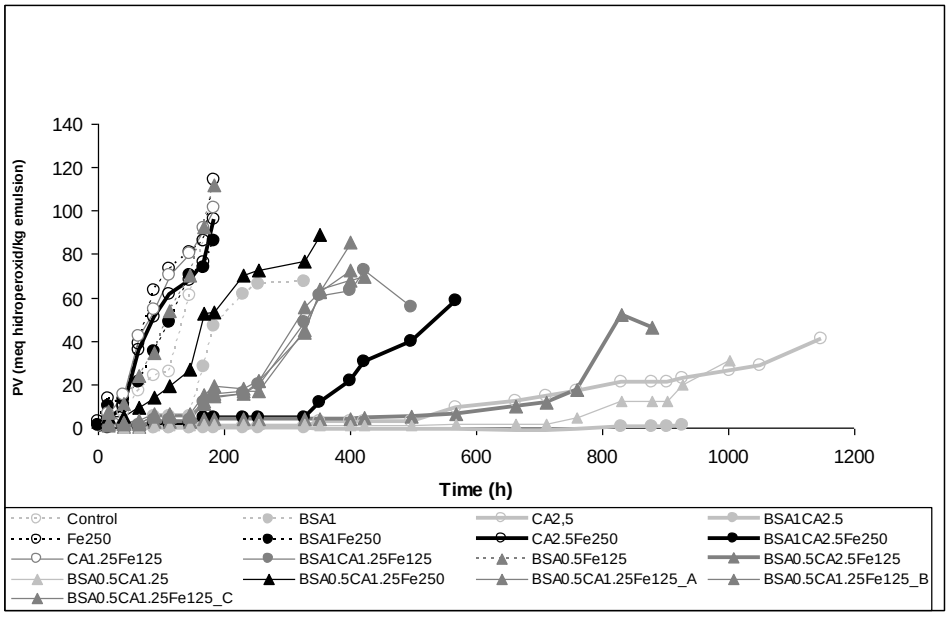



**Figura 34.** Peroxide value change with time of samples containing caffeic acid [0-2.5 mM], BSA [0-1%] and Fe(II) [0-250 µM] during storage at 30ºC

The design was done following a fractional factorial points, central points and star points. Table 12 shows the concentrations of additives and the time for the emulsion to reach PV=40 meq/kg.

**Tabla 12.** Times (h) for oil-in-water emulsions stored at 30ºC to reach PV = 40 meq/kg emulsion. (Range of times for triplicate samples)

| BSA (%) | Caffeic Acid (mM) | Fe (II) (µM) | Time (h) |
|---|---|---|---|
| 0 | 0 | 0 | 111.6 $^{(a,b,c)}$ ± 11.8 |
| 1 | 0 | 0 | 177.9 $^{(c)}$ ± 2.9 |
| 0 | 2.5 | 0 | 1196.5 $^{(g)}$ ± 50 |
| 1 | 2.5 | 0 | > 1200 ** |
| 0 | 0 | 250 | 67.4 $^{(a)}$ ± 8.6 |
| 1 | 0 | 250 | 97.9 $^{(a,b)}$ ± 5.1 |
| 0 | 2.5 | 250 | 74.5 $^{(a)}$ ± 9.4 |
| 1 | 2.5 | 250 | 464.9 $^{(e)}$ ± 21.2 |
| 0 | 1.25 | 125 | 67.8 $^{(a)}$ ± 12.9 |
| 1 | 1.25 | 125 | 307.9 $^{(d)}$ ± 21.3 |
| 0.5 | 0 | 125 | 96.3 $^{(a,b)}$ ± 2.8 |
| 0.5 | 2.5 | 125 | 789.8 $^{(f)}$ ± 21.5 |
| 0.5 | 1.25 | 0 | > 1100 ** |
| 0.5 | 1.25 | 250 | 156.6 $^{(b,c)}$ ± 3.3 |
| 0.5 | 1.25 | 125 | 314.3 $^{(d)}$ ± 4.0 |
| 0.5 | 1.25 | 125 | 310.6 $^{(d)}$ ± 8.3 |
| 0.5 | 1.25 | 125 | 297.1 $^{(d)}$ ± 7.5 |

(Values with the same superscript letter are not significantly different, $p<0.05$)

The range was chosen according to the concentrations that are commonly present in food emulsions (Table 13). The maximum concentration of 2.5 mM caffeic acid was 5 times more than the reported concentration with highest antioxidant power in an emulsion [173]. Based on previous studies [205], 250 µM Fe (II) also represents 5 times more than the normal concentration present in foods

that contain Fe (II) ion [247]. Oxidative stability for the composition at the central point of the chosen range was repeated 3 times each in triplicate, and was found to be highly reproducible (Table 12).

**Tabla 13.** Factor and levels of experiment

| Factors | Min | Max |
|---|---|---|
| BSA Concentration (*A*, %) | 0 | 1 |
| Caffeic acid concentration (*B*, mM) | 0 | 2.5 |
| Fe(II) concentration (*C*,μM) | 0 | 250 |

Inspection of the data for oxidative stability, before analysing the data mathematically, showed several interesting points. The samples were classified into 5-6 groups:

Samples which started to oxidise from the very first moment. Here we could find 6 mixtures, two of them with caffeic acid, with Fe (II) and without BSA protein. The behaviour of the rest was predictable since they corresponded to the control and the Fe (II) samples.

The second group contained only two mixtures. In this case, samples also oxidised rapidly, but the induction period was longer. These samples corresponded to the maximum concentration of BSA protein and the sample with intermediate concentration of BSA and caffeic acid and the maximum concentration of Fe (II).

The third group included the three experiments which represent the central point, and were very reproducible. The group also includes mixtures with intermediate concentrations of Fe (II) and caffeic acid, and the highest BSA concentration.

The fourth group only contains one mixture; with the highest concentration of caffeic acid and Fe(II) without BSA.

The final group includes samples that show an induction period higher than 600 hours, which can also be considered as divided in two. But since this division is not clear, we consider them together. This group includes mixtures which have the maximum concentration of caffeic acid (with and without BSA); the intermediate point without Fe(II) and the maximum concentration of caffeic acid; and the intermediate concentrations for the other components.

This classification summarises the results from the times to reach PV = 40 meq hydroperoxide/kg emulsion, shown in Table 1. The samples containing caffeic acid and BSA, without Fe(II), did not reach this peroxide value throughout the experiment (1200 hours) because the emulsion separated before oxidation had occurred.

In order to provide predictive power for mixtures that had not been studied, mathematical analysis was applied.

The data shown in Table 14 were processed by a main component analysis. In the first stage, the three main factors (A, B, C), the three second order interactions (AB, AC, BC), and the third order interaction (ABC) were considered. The objective was to establish which main and crossed factors were statistically significant, and what their impact on oxidation would be. Considering a 95% confidence interval, the main factors with statistical significance are: the BSA concentration (A), caffeic acid concentration (B) and the Fe (II) concentration (C), with a p-value lower than 5%. Moreover, the crossed factor B·C (caffeic acid concentration · Fe (II) concentration) was considered significant with a p-value lower than 5%. This model has a R2 adjustment of 98%.

**Tabla 14.** Design of experiments and experimental data for A: BSA [0-1%], B: caffeic acid [0-2.5 mM] and C: Fe(II) [0-250 μM]

| *Design* | *A* | *B* | *C* | *Tm1* | *Tm2* | *Tm3* |
|---|---|---|---|---|---|---|
| Fractional Factorial design $2^3$ | -1 | -1 | -1 | 107.35 | 102.50 | 124.94 |
| | 1 | -1 | -1 | 176.97 | 181.30 | 175.57 |
| | -1 | 1 | -1 | 1277.79 | 1033.99 | 1345.34 |
| | 1 | 1 | -1 | 2000.0 | 2000.0 | 2000.0 |
| | -1 | -1 | 1 | 57.65 | 70.39 | 74.18 |
| | 1 | -1 | 1 | 94.04 | 103.76 | 95.96 |
| | -1 | 1 | 1 | 69.80 | 68.32 | 85.37 |
| | 1 | 1 | 1 | 487.58 | 491.41 | 415.69 |
| Central Points | 0 | 0 | 0 | 318.64 | 310.54 | 313.70 |
| | 0 | 0 | 0 | 328.14 | 304.12 | 299.60 |
| | 0 | 0 | 0 | 304.09 | 281.23 | 306.10 |
| Star Points | 0 | 1.25 | 125 | 60.7 | 59.88 | 82.69 |
| | 1 | 1.25 | 125 | 286.91 | 307.40 | 329.54 |
| | 0.5 | 0 | 125 | 97.24 | 93.14 | 98.52 |
| | 0.5 | 2.5 | 125 | 788.76 | 811.81 | 768.80 |
| | 0.5 | 1.25 | 0 | 1700.0 | 1700.0 | 1700.0 |
| | 0.5 | 1.25 | 250 | 155.24 | 154.14 | 160.43 |

In the second stage the curvature was studied, and that is why a central point repeated 9 times (3 triplicates) was added. Results may be seen in Table 15. The curvature is significant and the fractional factorial design applies to linear models; therefore, new data should be included in order to determine the curvature degree and the variables that have this behaviour.

**Tabla 15.** Curvature

| Predictor | Coef | StDev | T | *p* |
|---|---|---|---|---|
| *Lac of fit* | -219.31 | 81.67 | 2.69 | 0.013 |

In the third stage new data were added, points centred on faces, with the purpose of establishing with which variables the curvature is associated and the curvature degree of each variable involved in the experiment. A factorial analysis was carried out considering just those main, crossed and quadratic factors which are significant with a p-value lower than 5%. The analysis of main factors, their interactions and quadratic terms is shown in Table 16. It may be observed that A (BSA concentration) and C (Fe(II) concentration) are the significant quadratic terms. However, B (caffeic acid concentration) showed a linear behaviour.

**Tabla 16.** Regression analysis terms

| Predictor | Coef | StDev | T | *p* |
|---|---|---|---|---|
| Constant | 396.02 | 46.40 | 8.54 | 0.000 |
| *A* | 150.84 | 35.61 | 4.24 | 0.000 |
| *B* | 399.71 | 35.61 | 11.23 | 0.000 |
| *C* | -434.73 | 35.61 | -12.21 | 0.000 |
| *B·C* | -319.43 | 39.81 | -8.02 | 0.000 |
| $A^2$ | -294.16 | 64.70 | -4.55 | 0.000 |
| $C^2$ | 446.29 | 64.70 | 6.90 | 0.000 |

This analysis results in the following regression equation for the reduced model:

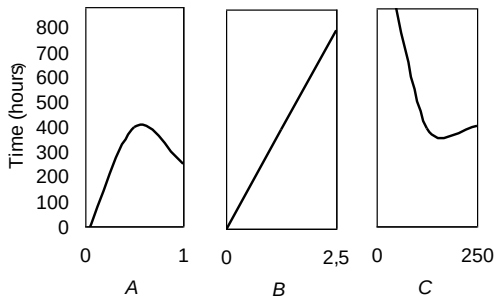
Tm=396.02+150.84A*+399.71B*-434.74C*-319.43B*C*-294.16A2*+446.29C2* (26)

Where * means that the variables are defined within a range from -1 to 1.

Table 6 shows the results of an ANOVA and the $R^2$ adjustment coefficient. It may be observed that this new regression made with fewer factors causes a 90% reduction in the adjustment coefficient. Then the new adjustment coefficient corrected for the reduced model remains at 79%. The ANOVA shows a satisfactory

behaviour, despite the elimination of the second order crossed factors A·B (BSA concentration · caffeic acid concentration) and A·C (BSA concentration · Fe(II) concentration) the third order interaction A·B·C (BSA concentration · caffeic acid concentration · Fe (II) concentration) and the quadratic factor B2 (caffeic acid concentration 2).

Figures 35-37 show the trends in the model behaviour. Figure 35 shows the changes in the three main factors. The B factor (caffeic acid concentration) has a linear behaviour, as the concentration increases, oxidation decreases proportionally. However, the A and C factors have a non-linear behaviour characterized by local maxima and minima. Increase in the BSA concentration at concentrations less than 0.5% reduces the rate of oxidation but, above 0.5% increase in BSA concentration increases the rate of oxidation. An increase in the Fe (II) concentration accelerates oxidation when the concentration is lower than 125 μM, but for higher




concentrations increase in Fe (II) reduces the rate of oxidation.

**Figura 35.** Plots of the main effects on stability of changes in A: BSA [0-1%], B: caffeic acid [0-2.5 mM] and C: Fe(II) [0-250 μM]

Figure 36 shows the interaction term B·C (caffeic acid concentration · Fe (II) concentration). The concentration of caffeic acid is a determining factor. If caffeic acid concentration is high, increase in B.C reduces oxidation. However, in this model, if both concentrations, Fe (II) and caffeic acid, are small, increase in B·C may reduce or promote oxidation, depending on Fe (II) concentration. On the other hand, Fe (II) works always as an oxidizing agent, if the concentration is high. Authors report similar results [22].

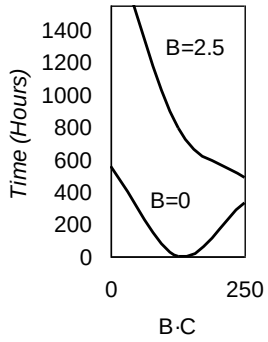



**Figura 36.** Plot of interaction effects on stability between B: caffeic acid [0-2.5 mM] and C: Fe(II) [0-250 µM]

Figure 37 shows the response surface. It may be seen that the caffeic acid has a linear influence. Whenever the caffeic acid increases, oxidation goes slower, this behavior has been observed in other study with extract rich in rosmarinic acid (Borage extract) (Segovia et al., 2014). As regards the BSA concentration, again it may be observed a non-linear behaviour that is constant for all different concentrations of Fe (II)..

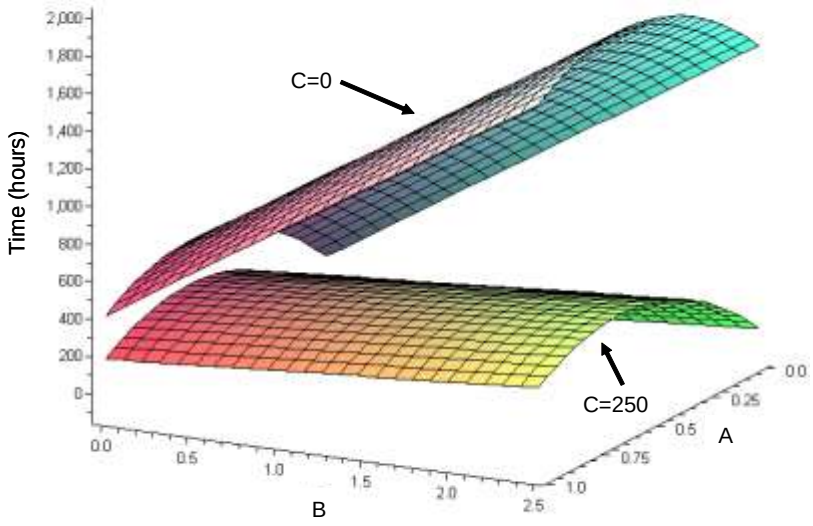



**Figura 37.** Surface response for effects on stability of A: BSA [0-1%], B: caffeic acid [0-2.5 mM] and C: Fe(II) [0-250 µM]

Differentiating (1) with respect to A (BSA concentration) and setting the derivative equal to zero, the value of the BSA concentration that maximizes the oxidation time is obtained.

$$\delta Tm/\delta A^* = 0 \rightarrow A=0.63\% \quad (27)$$

It may be observed that the concentration of BSA that maximizes the oxidation time does not depend on the remaining independent variables (caffeic acid concentration and Fe (II) concentration).

Differentiating (1) with respect to C (Fe(II) concentration), the following equation is obtained:

$$\delta Tm/\delta C^* = 0 \rightarrow C=141.3+36.01B \quad (28)$$

Then the Fe(II) value associated with the local optimum point depends on the caffeic acid concentration, and it is a linear function, so if the caffeic acid concentration increases, the Fe(II) concentration that defines the optimum point increases too. If the concentration of Fe (II) is within [141.3 - 231.3µM] then the equation (3) is the restriction that allows the caffeic acid concentration required to maximize the oxidation time to be determined. Under other conditions, for example if [Fe(II)]=50 µM, the local optimum point is found for the highest caffeic acid concentration in this study, which was 2.5 mM.

### *3.4.4 Conclusion*

The analysis of the effects of the main components has allowed a response surface to be constructed in which the oxidation time (Tm) is a function of BSA, caffeic acid and Fe (II) concentration.

The BSA concentration that maximizes the oxidation time (0.63 %) is independent of the other variables (caffeic acid and Fe (II) concentration). The oxidation has a linear behaviour with respect to the caffeic acid concentration, in the studied range (0-2.5 mM).That is, increasing the caffeic acid concentration reduces the oxidation rate and we achieve higher induction periods. There is a

correlation between the effects of Fe (II) and caffeic acid concentrations on oxidation velocity. This relation is given by the equation:

[Fe (II)] that maximizes the oxidation time = 141.3 + 36.0 [caffeic acid]

## 3.5 Pineapple waste extract for preventing oxidation in model food systems

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Enviado a Journal of Food Science

**Abstract:** Pineapple (*Ananas Comosus*) is consumed in the form of chunks (canned), cubes, fruit salad and also in juices, concentrates and jams. In these processes, the waste generated represents a high percentage of the total fruit. Some studies have shown that residues of certain fruits, such as pineapple, have the same antioxidant activity than the fruit pulp. So although these residues are discarded, they could be used as an alternative source of polyphenols, as natural antioxidants. This study is focused in the antioxidant activity of wastes obtained in the production of pineapple and their application. The polyphenols scavenging activity was determined by the Oxygen Radical Antioxidant Capacity (ORAC). The antioxidant potential is measured in emulsions (o/w) and in muffins, where the primary oxidation (by peroxide value, PV) and the secondary oxidation (by Thiobarbituric Acid Reactive Substances, TBARs) were analyzed. In addition the muffins were analyzed by means of a triangular sensory test. PV method showed a reduction 59% in emulsions and 91% in the muffins. The reduction in TBARs values for emulsions were 27 % and for muffins were 51%. The triangular sensory test reflected that the samples with the extract are not distinguished (with $\alpha$=0.05).

Keywords: Pineapple, waste, extract, antioxidant, muffins, emulsion.

### *3.5.1 Introduction*

It is known that polyphenols can prevent illness (heart disorders, inflammatory processes [18], cancer [39], hypertension [263], infections[264]) due to some of its properties. These polyphenols are consumed in our diet through fruits and vegetables [51].

Pineapple (*Ananas comosus*) is an American fruit which belongs to bromeliaceae family. It is the third tropical fruit most important worldwide and also one of the most produced [265]. In her composition has vitamin C, carbohydrates (sugar), proteins, water, fiber, minerals (calcium, phosphor and iron) and bioactive components such as polyphenols [7,266].

It is proved that the skin, pulp and juice of pineapples have antioxidant activity due to its polyphenols. For example, pineapple juice is used to help throat pain and dizziness [266] and in Thailand it is used to treat dysuria [265] Flavonoids and phenolic acids are the main polyphenols in pineapples [7]. Myricetin is a flavonoid which is linked with antioxidant activity in food and also takes part in cellular reactions controlling the reproduction of tumor cells [267].

Caffeic acid, p-coumaric acid and ferulic acid are phenolic acids present in pineapples which have antioxidant, anticancer and antimicrobial activity[268].

Pineapple industry produces lot of waste (skin, seeds, center) which is difficult and expensive to treat. Moreover it damages the environment [2,7,195]. However, these wastes from pineapples can be reused as raw material to obtain polyphenols with antioxidant activity [2,7].

It is known that other wastes in industry are used in this way (as colourants, fibers, with antimicrobial effect) with satisfactory results [1].

Plant extracts and plant wastes are often used in reducing the oxidation velocity in fats and oils. Rosemary extracts [108], borago waste extracts [13], avocado seeds extracts [11], pear extracts [22] and gentian lutea extracts [48], between others, have showed high effectivity to delay the oxidation in emulsions and meat. In fact, model food system are the more adequate model used to study effectively antioxidant action of natural extracts [48,185]. For example, emulsions are models used to simulate and check the antioxidant behavior in the prevention

lipid oxidation [253,269]. These emulsions can be high or low concentration in oil. It depends on the food analyzed [262,270].

For the other hand, it is also relevant that bakery products (bread, cakes, muffins and cookies) are also used as models. They are valuables in order to study the influence of different factors in fat and oils oxidation [262,271–273]. For example, in products designed for children, such as cookies, it is often reduced the addition of synthetic antioxidant. This action avoids that undesired compounds can be formed [273,274].

Lipid oxidation study in food has been relevant and for this reason the mathematical model of reactions behavior has been modelled. It is used to foresee the food oxidative stability more exactly. In this sense, soya bean ferment extract [48], cookies [273] and pork sausages [107] have been used as oxidative stability models.

The aim of this project is to study waste extracts of pineapples and its radical scavenging power and apply these extracts in two food models: emulsion and muffins, to evaluate the effectivity in delaying the lipid oxidation.

### *3.5.2 Material and Methods*

#### *3.5.2.1 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) was used as standard. Both and rosmarinic acid, ethanol, fluorescein, isooctane, potassium persulfate, acetic acid (glacial) and polyoxyethylene sorbitan monolaurate (Tween-20) were purchased from Sigma-Aldrich Company Ltd. (Madrid, Spain). Folin–Ciocalteu reagent and sodium carbonate were supplied by Merck (Darmstadt, Germany). All compounds were of reagent grade. Refined sunflower oil, of a brand known to lack added antioxidants, was purchased from a local retail outlet.

#### *3.5.2.2 Pineapple preparation*

The pineapple (*Ananas comosus*) was obtained in the local market; the peel was separated from other edible parts. This waste was homogenized and ground by

using a Moulinex mill (A5052HF, Moulinex, Lyon, France). Finally, the waste was stored in a refrigerator at 4ºC until use (24-48 h).

The homogenate was centrifuged (Orto Alresa, Madrid, Spain) at 2500 rpm for 10 min and the supernatant was collected and stored at -20ºC. This sample was called No-treatment sample (NTS).

#### *3.5.2.3 Total Phenolic Content (TPC)*

TPC was determined spectrophotometrically following the Folin–Ciocalteu colorimetric method [275]. Sample diluted 1:4 with milli-Q water was stirred in triplicate. The final concentration in each of the 96 wells plate was: 7.7% v/v sample, 4% v/v Folin-Ciocalteu's reagent, 12 % saturated sodium carbonate solution and 84,3% of milli-Q water. The mixture was allowed to react in the dark for 1 h and the absorbance was measured at 765 nm using a Fluorimetrics Fluostar Omega (BMG Labtech, Ortenberg, Germany). The total phenolic content was expressed as mg Gallic Acid Equivalents (GAE)/g fresh weight.

#### *3.5.2.4 HPLC and on line HPLC–ABTS+ radical scavenging activity analysis*

The method for identification of peak with antioxidant activity was described by Aini et al., (2014)[275]. The instrument was a Waters 2695 separations module (Waters Corporation, Milford, MA, USA) system with a photodiode array detector Waters 996 (Waters Corporation, Milford, MA, USA). The column, a Kinetex C18 100A, (100×4.6 mm). Solvents used for separation were 0.1% acetic acid in water (v/v) (eluent A) and 0.1% acetic acid in acetonitrile (eluent B). The gradient was, from 100 to 50% A in 25 min, to 100% A in 5 min. The flow rate was 0.7 ml / min. Detection wavelength were 280, 320 and 734 nm. The sample injection volume was 10 µl. The chromatographic peaks of gallic acid and ferulic acid were confirmed by comparing their retention times and diode array spectra with that of their reference standards. The pump for ABTS injection was a Merk-Hitachi HPLC gradient pump (Model L-6200) with a 0.2 ml/min flow, an ABTS concentration was of 0.03% (w/v).

#### *3.5.2.5 ORAC Assay*

Antioxidant activities of extracts were determined by the ORAC assay, as reported by Segovia et al. (2014) [13]. The assay was carried out using a Fluorimetrics Fluostar Omega (BMG Labtech, Ortenberg, Germany) equipped with

a temperature-controlled incubation chamber. The incubator temperature was set at 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. This method includes the time and decrease of fluorescence. The area under the curve (AUC) was calculated. A calibration curve was made in each plate 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)/ml of extract.

*3.5.2.6 Oil-Water Emulsions*

Oil-in-water emulsions were prepared as reported by Skowyra et al (2014) [22], with slight modifications. Samples were made (in triplicate). 4g of NTS were added in the emulsion (final weight 20,2 g). 2 concentrations of BHA were used as positive control, to compare.

The emulsions were homogenized by Ultra-Turrax (Ika®-Werke, Staufen, Germany). All emulsions were stored in 60 mL glass beakers in the dark at 35 °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) and TBARS value. All the samples were made in triplicate

*3.5.2.7 Peroxide Value (PV)*

PV was determined by the ferric thiocyanate method (Frankel, 1999) [276] (after calibrating the procedure with a series of oxidized oil samples analyzed by the AOCS Official Method Cd 8-53). Data from the PV measurements were plotted against time.

*3.5.2.8 Emulsion oxidation mathematical modeling*

The equation (29) described by Wardhani et al (2013) [48], which is a method to adjust the data in a known mathematical equation, is used.

$$L = \frac{L_m}{1+exp\left(2+\frac{4v_m}{L_m}(\lambda - t)\right)} \quad (29)$$

Were, L is the measurement if lipid oxidation, Lm the maximum value of oxidation, λ is the stagnation phase, νm is the maximum velocity constant of the reaction and t is the time.

#### *3.5.2.9 Muffins model food*

Walker et al (2014) [277] method has been used to elaborate muffins with little changes. The mass composition was: 32g of beaten egg, 32g of sugar, 25g of flour, 15.6ml of oil (without their natural antioxidants), 2g of bakery leavening and 4g of water (or each of the extracts; the water was in the control)

The mixture was put in the oven (model HPL 840, Teka, Zaragoza, Spain) at 180ºC for 20 min. When the muffins were cooked, they were introduced in an incubator at 25 ºC. Some samples were taken periodically and analyzed.

#### *3.5.2.10 Muffins Oil extraction*

5g of sample are taken and introduced in 15ml tubes with 4ml of hexane that is added to extract the oil. The sample is homogenizes with an Ultra-Turrax (Ika®-Werke, Staufen, Germany), centrifuged (Orto Alresa, Madrid, Spain). The floating waste is collected and the hexane is evaporated by a sample-concentration (Techne, FSC400D. Beacon Road, Stone, Staffordshire, ST15 OSA, UK) using nitrogen gas. The final sample of oil is kept at -20 ºC until the analysis.

#### *3.5.2.11 Thiobarbituric Acid Reactive Substances (TBARS)*

Secondary oxidation was determined by the concentration of thiobarbituric acid-reactive substances (TBARS) using the method described by Segovia et al. (2014) [11] with some modifications. A sample (0.01 g for the emulsions or 0.5 grams for the muffins) was dispensed in tubes, in the dark, and 1 mL of EDTA to prevent the oxidation, was added. The samples were homogenized for 5 min with an Ultra-Turrax (Ika®-Werke, Staufen, Germany) with 5 mL of TBARS reactive (Trichloroacetic acid, 9.2%; Hydrochloric acid, 2%; Thiobarbituric acid, 0.22%, w/w final). During homogenization, the tubes were placed in an ice bath to minimize the development of oxidative reactions. The samples were filtered and the solution 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.

#### *3.5.2.12 Sensory evaluation*

Rotondi et al (2004) [278] was the method used to sensory evaluation. This method is discriminatory (triangular test). The differences between muffins with or without extract are looking for. NTS, in the same composition as in the muffins recipe when doing oxidation stability test, was used as the extract. Three muffins were arranged together, two identic and one different. The different one was asked to be detected by the panelist. Muffins with extracts were called A, and muffins without extracts were called B. Plates with AAB and BBA combinations were prepared. 30 panelists were asked (15 men and 15 women) to answer. The samples were repeated randomly. Significance level chose was 5%. One question about the preference was included in the test.

#### *3.5.2.13 Statistical analysis*

The adequacy of the equation was determined by evaluating the coefficient of determination ($R^2$). Statistical significance of the model was determined at the 5% probability level ($\alpha = 0.05$). The graphics and the 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%.

### *3.5.3 Results and Discussion*

#### *3.5.3.1 Antioxidant capacity in extracts*

NTS sample was analyzed to evaluate the polyphenols quantity and oxidant capacity by ORAC analysis. The polyphenols were 68,13mg of GAE/g of fresh sample and antioxidant capacity was 24,27 µM TE/ml of extract. These values are inside the estimated range reported in another linked study about pineapples [279].

In order to characterize the possible polyphenols found in greater amounts in the sample and to know their antioxidant capacity, it has made a study of the sample with the use of HPLC and HPLC adding online the ABTS radical. This method can be used for a rapid assessment of radical scavenging properties of separated compounds in chromatographic column, which are present in complex

mixtures: such as plant extracts [101]. The figure 37 shows the result obtained and Table 17 are possible polyphenols found in the NTS extract.

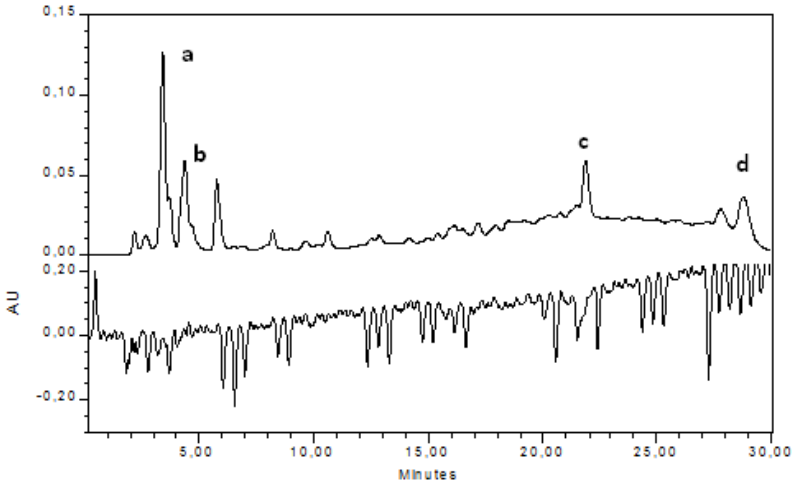


**Figura 38.** HPLC and HPLC-ABTS+ on line analysis from pineapple extract.

**Tabla 17.** Polyphenols identify in the chromatogram.

| Peak | Rt (min) | λ (nm) | Posible compound |
|---|---|---|---|
| a | 3.45 | 215.7, 278.0 | Gallic acid (std) |
| b | 4.43 | 253.3, 370.2 | Ellagic acid |
| c | 21.82 | 216.8, 323.1 | Ferulic acid (std) |
| d | 27.78 | 219.2, 325.5 | Caffeic acid |

Std marked polyphenols were compared with standards

Radical ABTS + has some advantages over of DPPH radical. First, because the former is water soluble and therefore more sensitive to substances having radical scavenging activity in aqueous extracts [76]. Second, in 5 min the reaction is completed. The figure 38 shows that the extract has a high antioxidant activity throughout the chromatogram. That's mean that even compounds are not detected or the signal is very small these provide radical scavenging activity. It could be

because is due to others compounds (not polyphenols) that are not detected in this conditions. Moreover, it is important to remark that all the identified peaks have radical scavenging activity and is known, from other studies, that gallic acid, ellagic acid, ferulic acid and caffeic acid have antioxidant capacity and all can be found in pineapple and their residues [279,280].

### *3.5.3.2 Effects on emulsions*

To examine the effect of pineapple samples in emulsion, PV method was done to check primary oxidation and TBARS to check secondary oxidation products. The study lasted 35 days. In figure 39 it can be seen the time evolution of lipid oxidation by PV in emulsions. BHA in 2 concentrations is used as positive control (BHA 1, 0.000125% w/w and BHA 2, 0.002% w/w).

As is logical and expected, the first sample that reaches this value is the control. There are not significate differences between the BHA1 and the extract. This shows that the extract retards oxidation in the early stages, ie, in the initial formation of hydroperoxides. Obviously there are many examples where it is demonstrated that the natural extracts delaying lipid oxidation. For instance, Skowyra et al (2014) demonstrated the antioxidant properties of artemisia annua extracts in model food emulsion [77].

At the endpoint of the experiment, after 35 days of oxidation at 35 ° C in the incubator with constant stirring, if set to control 100%, the value achieved by each of the samples with respect to this total is 1.55, 59.9, 93.25% for BHA 1, NTS y BHA 2, respectively. Other waste food products, as the borage by Segovia et al (2014), reported similar results within the oil in water emulsions [13].

Pineapple extracts have a high amount of polyphenols which are directly related to the antioxidant capacity. Among them it would highlight the family of hidrocinamics acids (ferulic acid, p-coumaric acid, caffeic acid) and the hidrobenzoics acids family (salicílic acid, p-hydroxybenzoic) and myrcetin. All these polyphenols which are in the extracts are linked with antioxidant capacity [281].

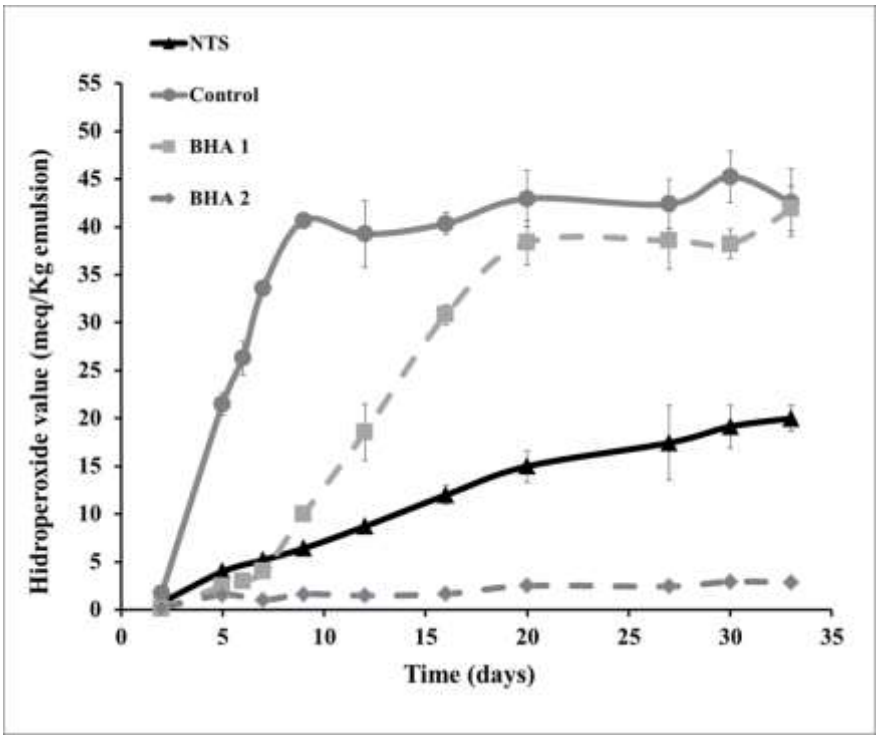


**Figura 39.** The PV evolution's vs time

All results have been modeled using equation (29). The results are showed in Table 18. The equation (29) has been used to model a wide range of biological and chemical processes of sigmoidal tendency. Wardhani et al. (2013) [48], applied it for oxidation food models when soya extract is added. In that study, it was shown that the equation can be well adjusted for blend peroxide values and TBARS values, with an $R^2$ between 0.10-0.99 for the conjugated dienes and the TBARs value. The low $R^2$ values were found in the sample using the high concentration of synthetic antioxidant. In this study, the behavior is similar [48]. The $R^2$ is higher for the natural antioxidant, the extract and the lower concentration of BHA (between 0.96 and 0.93) and is lower (0.68) in the most concentrated BHA sample. It looks that cannot be applied due to the emulsion was not oxidized.

If we consider the velocity reaction constant of the samples, it is observed that they follow this order: Control>BHA1>NTS>BHA2. This is a logical oxidation order where this value decreases when pineapple extracts are added. Moreover, for BHA 2 sample, oxidation doesn't increases in the time studied.

**Tabla 18.** Coeficients adjusted with equation (1).

| Sample | Parameters | | | |
|---|---|---|---|---|
| | $L_m$ | $v_m$ | λ | $R^2$ |
| Control | 42,02 ± 1,75[a] | 8,12 ± 0,14 | 2,57 ± 1,28 | 0,97 |
| NTS | 20,05 ± 3,80[bc] | 0,94 ± 0,10 | 3,44 ± 4,56 | 0,93 |
| BHA1 | 36,95 ± 3,62[b] | 4,75 ± 0,28 | 8,41 ± 2,62 | 0,95 |
| BHA2 | 3,12 ± 2,64[c] | 0,07 ± 0,10 | -11,20 ± -21,86 | 0,68 |

The averages that do not share a letter are significantly different (α =0,05)

TBARS values are used to show how is the substances evolution's produced in the secondary oxidation. In figure 3, the values obtained in this study are shown. In the last day (35) if 100% is assumed for the control, the different samples achieve 45.92, 13.47 y 97.37% for NTS, BHA1 y BHA2 extracts respectively. The pineapple waste extract is between both concentration of BHA.

The tendency is the same as the previous one, the primary oxidation. The pineapple waste extract avoids the creation of substances in the secondary oxidation in 45,92%. This result shows that although having a high concentration of first oxidation compounds, the polyphenols in extracts are capable of avoiding the formation of secondary compounds. This fact is due to the easiness of polyphenols to give a hydrogen atom [253].

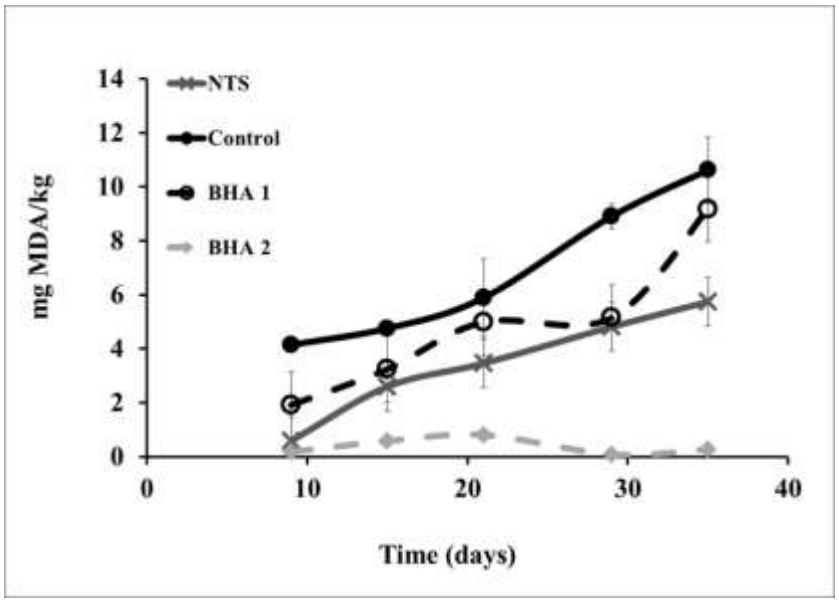


**Figura 40.** Values in emulsions and its evolution in time

*3.5.3.3 The effect on muffins*

To study muffins with natural extracts, the pineapple waste extract was added in the muffins. The oxidation evolution was analyzed during 35 days. Figure 40 and 41 shown the primary and secondary oxidation results in the last day. Assuming the PV in the control 100% the value for the muffins containing the pineapple extract is only 9.74% of the control sample (Figure 40). For TBARS values the muffins with the extract achieve the 48.76% of the control (Figure 41). That fact shows that the extract works as an antioxidant in the oils used in muffins and also that they don't change its properties in the cooking process. In similar conditions, Aleman et al (2010) [282], studied the efficacy of tocopherol extract, citric acid and ascorbil palmitate when they are added at bakery products. They show that these products had antioxidant activity in primary and secondary oxidation. The secondary oxidation with the formation of different odorous products in oils and fats are not desirable because they change sensorial properties of them[10]. The use if pineapple extract can modify this situation delaying its apparition.

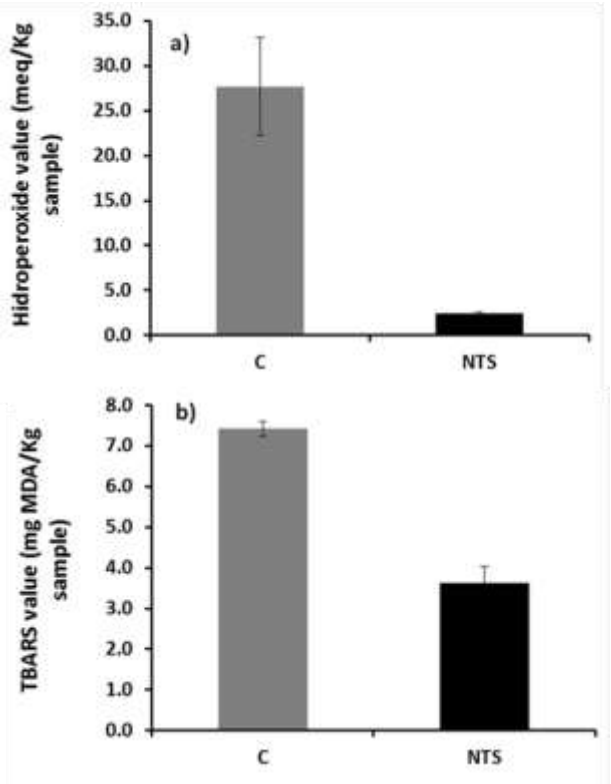


**Figura 41.** PV (a) and TBARS values (b) for muffins samples in 35 days of treatment

*3.5.3.4 Sensory test results*

To know if there are differences between muffins with or without extract, it is rune a sensory discriminative test (triangular test). Rotondi et al. (2004) [278], have used this test to know if panelists could differentiate between olive oil sample with different maturation degree. The result was that they could not distinguish between the samples and also conclude that the sensory profile reported was the same.

It has been made both combination, BAA and ABB, randomized in order to minimize the bias in the results. For BAA combination, a total of 11 panelists detected the difference and in ABB a total of 15 panelists detected the different muffins. Considering the table for 95% of significance level and a total of 30 panelists, 15 people should notice the difference. This fact shows that it was easier to detect the muffin with pineapple among two standards than otherwise. In the analysis of 60 samples (BAA plus ABB), it can be said that, with a 5% level of confidence, the muffins could not be distinguished.

Answering the question about which muffins were more appealing, the half of the people answering A and the other half B. And also the presentation way had influence. That result reflects that it is more likely to prefer the combination with only one muffin with pineapple among the three samples and that fact could be influenced by the moment of deciding which combination they prefer.

#### *3.5.3.5 Conclusions*

This study reveals that it is possible to use pineapple wastes of industry in order to produce extracts which contains polyphenols with antioxidant capacity. This extracts will allow us to protect food to lipid oxidation in similar emulsions and bakery products such as muffins.

Moreover, the used mathematic model shows that the process of oxidation in the food models studied follow a known biologic process and by this way it is easier to facilitate the prediction of their behavior in time evolution.

## 3.6 Avocado seeds: a comparative study of the antioxidant capacity (oxidation oil, OIT, EPR and other radical scavenging methods)

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Enviado a Journal of Agricultural and Food Science

**Abstract:** Increasingly, consumers want products containing little or no synthetic compounds. The bones of avocado, a residue of the food industry can be used to obtain extracts with high antioxidant power.

Two types of extractions were performed: with pure methanol and ethanol-water (50%). Radical scavenging methods have values between 1310 and 263 µmols TE/g of mass dissolved for ORAC and ABTS respectively.

Furthermore the individual contribution of each of the compounds present in the extract was analyzed. The sum of all them signifies 86% of the total.

Finally, the power to delay oil oxidation in a moderate oxidation follow by PV and TBARs and forced oxidation, by OIT and measured by DSC were analyzed. The concentration of 0.75% extract of avocado seed means a delay in the oxidation close to 80% as measured by OIT.

This implies which this residue may have a use as a natural antioxidant, and give added value to organic waste.

Keywords: avocado seed, EPR, radical scavenging methods, OIT-DSC, emulsion

### *3.6.1 Introduction*

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 [25], thanks to their antiradical power. They are very useful in food products, not only for the commented previously, but also because they prevent lipid peroxidation due to the attack of free radicals [27]. In addition, they protect against oxidation, direct or indirect, caused by metal cations [154]. These cations stimulate the creation of reactive oxygen species (ROS), which are harmful to the health. Besides, it should be noted that polyphenols have been used as preservatives, protecting against microorganisms [162].

The process of food 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. It is interesting to obtain harmless substances with high antioxidant power, thus, what was a waste becomes a "high value-added" product [1,155]. Previous examples already studied [4,222,283] 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 [193]. Also, the waste from wine and beer production includes phenolic compounds [155]. Other studies have focused on the shells of nuts, rice and wheat in which large amounts of polyphenols are found [156].

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 [10]. Among the polyphenols the (+)-catechin and (−)-epicatechin [194] and chlorogenic and protocatechuic acid, are included [156]. Previous studies on this residue have been applied to pork burgers and have been shown to be effective in preventing oxidation and microbial growth [10].

It has also been shown that the extract of seeds of avocado (SAE) is useful in preventing oxidation of model food systems such as emulsions of sunflower oil

in water (10% oil) and in real foods such as minced beef. In both cases it has been found that can slow the oxidation over 60% [11].

The objective of this work is manifold. On the one hand compare different traditional methods of radical scavenging, Including the EPR methodology with the real-methoxy radical. Moreover analyze the contribution of each of the components present in the residue. Finally compare different techniques forced oxidation of sunflower oil including the OIT

### *3.6.2 Materials and methods*

#### *3.6.2.1 Chemicals*

Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid), ethanol, fluorescein, AAPH, BHA 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). Iron(II) sulfate (FeSO4), DMPO, H2O2, MeOH, Trichloroacetic acid and hydrochloric acid were acquired from Panreac Química S.L.U. (Barcelona, Spain). All compounds were of reagent grade.

#### *3.6.2.2 Materials*

Refined sunflower was purchased from a local retail outlet. Sunflower was passed through alumina as described by Skowyra et al. (2014) [77] in order to remove the tocopherols. The avocado (Persea americana) was obtained in the local market; the seeds were separated from other edible parts. 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. It was homogenized and frozen at −80 °C for lyophilization. Finally, the powder was stored in a dark bottle in a desiccator until use.

#### *3.6.2.3 Extraction Procedure.*

Extraction was carried out in dark bottles. Lyophilized powder (0.25 g) was blended with 25 mL of solvent (50% methanol-water). This mixture was placed under stirring in a refrigerator at 4 °C overnight, centrifuged (Orto Alresa, Madrid, Spain) at 2500 rpm for 10 min and the supernatant was separated as extract. The ethanol was eliminated by rotavaporation and the extract was freeze-dried and

stored until used for analysis. To obtain the sample for EPR pure methanol was used.

*3.6.2.4 Total Phenolic Content (TPC).*

TPC was determined spectrophotometrically following the Folin-Ciocalteu colorimetric method [13,61]. A sample diluted 1:4 with milli-Q water was stirred in triplicate. The final concentration in each one of the 96 wells plate 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.

*3.6.2.5 Scavenging activity was determined by 3 different methods*

ORAC [11]; FRAP [22] and TEAC [284] The results were expressed as µmol Trolox equivalents (TE)/ g of dry weight or µmol ferulic acid equivalents/ g of dry weight.

*3.6.2.6 Determination of Methoxy Radical Scavenging Activity by EPR.*

The method was reported by Azman et al. (2014) [285]. The extract was prepared in deoxygenated MeOH. A spin-trapping reaction mixture consisted of 100 µL of DMPO (35 mM), 50 µL of H2O2 (10 mM), 50 µL of avocado methanol extract (0−8.13 g/L) 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 FeSO4 (2 mM). The final solutions (250 µL) were passed to a narrow quartz tube (inside diameter = 2 mm) and introduced into the cavity of the EPR spectrometer. The spectrum was recorded 12 min after the addition of the FeSO4 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, 9.876 GHz; microwave power, 30.27 mW; center field, 3522.7 G; sweep width, 100 G; receiver gain, 5.02 × 104; modulation frequency, 100 kHz; modulation amplitude, 1.86 G; time constant, 40.96 ms; conversion time, 203.0 ms.

Each measurement was carried twice. The first derivative of the absorption signal was integrated in duplicate, resulting directly proportional to the concentration of the remaining radical adducts values, when the competitive reactions of the methoxy radical with DMPO and the antioxidant were completed. Then, these values were compared with those obtained with ferulic acid, used as standard.

*3.6.2.7 HPLC-DAD.*

Identification and quantification was performed using a Waters 2695 separations module (Meadows Instrumentation, Inc) system with a photodiode array detector Waters 996 (Meadows Instrumentation, Inc). A Kinetex C18 100A, (100×4.6 mm) was used as column. Mobile phase was 0.1% acetic acid in water (v/v) (eluent A) and 0.1% acetic acid in acetonitrile (eluent B). The gradient used was: 0–2 min, isocratic gradient from 0% B; 2-40 min, linear gradient from 0%-15% B and 40-50 min linear gradient from 15%-0% B. The flow rate was 0.8 ml / min. Detection wavelength were 280 and 330 nm. The sample injection volume was 10 µl. The chromatographic peaks were confirmed by comparing their retention times and diode array spectra against of their reference standards and the chlorogenic acid was confirmed by MS HPLC-MS. 100 to 500 ppm working standard solutions were injected into the HPLC to obtain the calibration curve plotting concentration (mg/L) versus area. Quantification was carried out from integrated peak areas of the samples using the corresponding standard graph.

*3.6.2.8 HPLC-ABTS/on-line.*

For the analysis of the radical scavenging activity of each of the compounds by ABTS radical, a pump Merk-Hitachi HPLC gradient pump (Model L-6200) was coupled, with a 0.2 ml/min flow with ABTS concentration of 0.03% (w /v). It was allowing a perfect mixture, without any alteration, because there was a 3 m tube before to arrive to the detector.

The reading wavelength was 734 nm. The calibration curve to quantify the results was made with Gallic acid.

*3.6.2.9 Peroxide Value (PV).*

PV was determined by the ferric thiocyanate method [116](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.

*3.6.2.10 TBARS Assay.*

Oil secondary oxidation was determined by the concentration of thiobarbituric acid-reactive substances (TBARS) using the method described by Gallego et al. (2013) [108] with slightly modifications. An amount of each sample was taken and the TBARs reagent (15 % trichloroacetic acid, 0.375 % thiobarbituric acid and hydrochloric acid 2.1 %) was added in the ratio 1:5. Immediately the samples were added in an ultrasonic bath (Prolabo brand equipment) and immersed in a water bath pre-heated to 95 ºC. Samples were centrifuged and the absorbance of the supernatant was measured at λ= 531 nm. The results are expressed as mg MDA/kg of oil.

*3.6.2.11 Oxidation Induction Time analysis (OIT-DSC ).*

Differential Scanning Calorimetry (DSC) experiments were performed with a DSC 820 from Mettler Toledo (Schwerzenbach, Switzerland) under isothermal conditions (100 ºC) and with an air flow of 10 mL / min. The samples (5.00±0.25 mg) were weighed into 40 µL aluminium DSC open crucible in order to allow the oil to be in contact with the oxygen stream. An empty crucible was used as reference.

*3.6.3 Results and Discussion*

*3.6.3.1 TPC and Radical Scavenging Activity*

There are various authors working with different extracts form avocado seeds.

**Tabla 19.** Different radical scavenging values found in the Bibliography

| Study | Sample | Extractions condition | Antioxidant Activity | | | | References |
|---|---|---|---|---|---|---|---|
| | | | TPC (mg GAE/ g DW) | ORAC (µmol TE/ g DW) | ABTS (µmol TE/ g DW) | FRAP (µmol TE/ g DW) | |
| 1 | Hass | Acetone 70%/Water 29,.%/ Acetic Acid 0.3% v/v, RT | 122.85 ± 3.81 * | 1021.00 ± 28.57 * | — | — | Wang et al. (2010) |
| 2 | — | Methanol 75%/ Water 25% v/v | 292.00±9.81 | — | 173.3 | — | Pahua-Ramos et al. (2012) |
| 3 | — | Ethanol 50%/ Water 50% v/v, 70ºC | 88.2±2.2 | — | 725±39.4 | 1484±15.7 | Soong and Barlow (2004) |
| 4 | Hass | Methanol 70%/ Water 30% v/v | 35.11±9.88 | — | 1784.85 ±604.44 * | — | Rodríguez-Carpena et al. (2011) |
| 5 | — | Ethanol 56%/ Water 44%v/v, 63ºC | 45.01 | 622.64 | — | — | Segovia et al. (2014) |
| 6 | Hass | Methanol 80% / Water 20 % v/v, 60ºC | 9.51 ± 0.161 | 210.00 ± 14.00 | 94.00 ± 0.7 | — | Kosińska et al. (2012) |

*: Results obtained from data provided in articles. RT: Room temperature.

Table 19 shows the conditions of extraction and the results obtained. The conversion to the same units to be able to compare has been done. There is a large scatter in the results and it is difficult to justify. On one hand, the highest value obtained is in the extraction performed with 75% methanol by Pahua Ramos (292 mg GAE / g Dry weight) [9], while the lower value is obtained in the extraction with 80% methanol at 60ºC, 30 times lower (9,51mg GAE / g Dry weight) obtained by Kosinska [286]. In the present study the TPC are 30.98 ± 0.68 mg GAE/g DW with the Ethanol 50% extraction and 4ºC overnight. It is similar to the amount described by Rodríguez-Carpena et al. (35.11 ± 9.88 mg GAE/g DW) with methanol 70% [10].

Only three authors report results for the ORAC and the dispersion is similar. Results range from above 1000 µmol TE/g DW [287]up to those values close to 200 [286]µmol TE/g DW, that is 5-fold less. The value obtained in the present study is the highest, more than 1300 µmol TE/g DW. The explanation is that the TPC could do the determination of some compound without radical scavenging activity, because is a not selective method and it depends on of the other variables. Neither the relationship between the two values (ORAC / TPC) has similarity. It may be 8, 13, 22 or 42.

Using the ABTS as radical, the dispersion is not less. The higher value (1784 µmol TE/g DW [210] is greater almost 20 times that one of the inferiors (94 µmol TE/g DW,[24]). The value obtained in this study is in the lower range (263 µmol TE/g DW ).

With all these data, the extraction conditions, the sample origin and the methodology affect so much to the results could be said.

In the present study is the first time that all popular radical scavenging methods are presented, establishing a comparison between them, besides working with two different extractions. For the other hand, a novel method is incorporated, the reaction to methoxy radical, determined by EPR. The Table 20 shows the results with different units. The EPR analyzes a competitive reaction to DMPO [285,288,289] which acts also scavenging the radicals generated "in situ".

**Tabla 20.** Radical scavenging values obtained with pure methanol and ethanol/water

| Method | Extractions Condition | |
|---|---|---|
| | Methanol, 4ºC, 24h | Ethanol/Water (50:50), 4ºC, 24h |
| TPC (mg GAE/ g DW) | 25.35±0.77 | 30.98±0.68 |
| ORAC (µmol TE/ g DW) | 1240±70 (0.59±0.03 ‡) | 1310±40 |
| ABTS (µmol TE/ g DW) | 123.74±2.46 (0.015±0.00 ‡) | 263.58±17.85 |
| FRAP (µmol TE/ g DW) | 316.60±6.87 (0.19±0.00 ‡) | 438.89±7.32 |
| EPR (g FAE/ g DW) | 0.53±0.07 | |

‡: g FAE/ g DW

Table 20 shows the radical scavenging values obtained with pure methanol and ethanol/water (50%). There is not a value for the EPR with ethanol/water because there is an interference with the water in the determination. The standard used is the ferulic acid for a similar reason, to avoid interferences and to facilitate the solubility in the adequate concentration.

For the EPR determination the value for the SAE is 0.53±0.07 FA eq/g DW. It is less that other values obtained previously for Azman et al in the White tea (1.33±0.3 FA eq/g WT) [285] and similar to the other plants as perilla, caesalpinia (results not published yet).

In extracts of plant products are many individual compounds contributing to the overall antioxidant activity. While we would highlight the interactions between them and the synergistic effect, it is also important to consider the individual

contribution to the antioxidant activity exerted by the compounds separately. To do this, after the separation by HPLC with a gradient polarity (as I is contained in materials and methods) the injection of the ABTS radical, generated "in situ" was done. So, the negative peak corresponds to the radical scavenging activity. The higher negative area corresponds to the higher antiradical activity.

In direct chromatogram, prior to injection of ABTS, they have been identified catechin, epicatechin 3-O-caffeoylquinic acid (chlorogenic acid isomer) at concentrations of 20.10 mg / L extract, 27.89 mg / L extract and 51.59 mg / L extract, respectively. In addition, there are 3 peaks belonging to the family of flavonoids. Kosinska, and Rodriguez-Carpena also found these compounds in amounts of 57.5 µg /g DW and 282.7 mg / 100 g DW respectively [210,286]. Figure 41 shows HPLC performed. The chlorogenic acid is the polyphenol in high quantity. This acid is found in many natural plant extracts and has been amply demonstrated its influence on the antioxidant capacity and capture hydroxyl radicals [290] because contains a catechol group which makes it especially effective for capturing free radicals [291,292] .

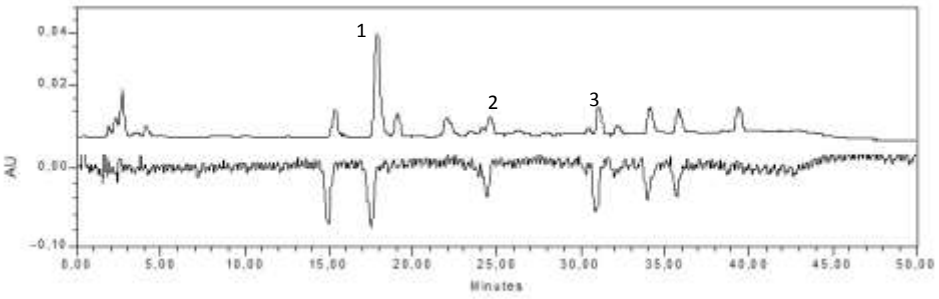


**Figura 42.** HPLC chromatogram of the extract of SAE. Chlorogenic acid (1), Catechin (2), Epicatechin (3).

Figure 42 also includes the "negative" peaks, which are in the chromatogram having antiradical activity. This method has already been described in earlier publications and is effective, fast and sensitive analysis for individual components [96,293,294]. Table 21 lists the values quantified with gallic acid.

**Tabla 21.** SAE composition and antioxidant capacity of their compounds.

| Name | RT | Area HPLC | mg/L | AREA HPLC-ABTS | % | Antioxidant capacity (mg GAE/L) | % of antioxidant activity in the total extract |
|---|---|---|---|---|---|---|---|
| **Procyanidin 1** | 15,36 | 199682 | — | 1956638 | 21,76% | 53,12 | 16,3% |
| **Chlorogenic Acid** | 17,88 | 1011205 | 51,59 | 1901135 | 21,14% | 51,86 | 16,0% |
| **Catechin** | 24,59 | 157538 | 20,10 | 977869 | 10,88% | 30,84 | 9,5% |
| **Epicatechin** | 31,07 | 233557 | 27,89 | 1574801 | 17,51% | 44,43 | 13,7% |
| **Procyanidin 2** | 32,21 | 80373 | — | 265837 | 2,96% | 14,63 | 4,5% |
| **Procyanidin 3** | 34,12 | 216547 | — | 1169588 | 13,01% | 35,21 | 10,8% |
| **Procyanidin 4** | 35,82 | 192750 | — | 960683 | 10,68% | 30,45 | 9,4% |
| **Catechin 1** | 39,40 | 205953 | — | 184943 | 2,06% | 12,79 | 3,9% |

The first flavonoid (RT 15.36 min) with the chlorogenic acid and the epicatechin, are those that provide the largest percentage of radical scavenging activity. Below are catechin and other flavonoids. The sum of the percentages of the individual peaks provides over 84% of the radical scavenging activity with ABTS radical. The difference to 100 can be due to the synergistic effect between the different compounds or also other compounds not presents in the separation by this HPLC method. In any case, the percentage of individual peaks is very high and justifies the major part of the antiradical activity. All of them are known antioxidants present in coffee, tea and other plant extracts [295–298]. And they have been used successfully in preventing lipid oxidation in food [49,85,285].

### *3.6.3.2 Protective effect of SAE in fatty acid mixture from sunflower oil.*

To evaluate the antioxidant activity in a model system (sunflower oil stripped of its natural antioxidants) different percentages of SAE were added. The control sample was prepared without anything (no antioxidant) and the positive controls were prepared with BHT, synthetic antioxidant (BHT). These samples were asked two types of analysis. The first was forced oxidation at moderate temperature (23 days at 35 ° C). The evolution of primary oxidation was performed by peroxide value and secondary oxidation by thiobarbituric acid reactive compounds (TBAR's). The second method is a very forced oxidation, which determines the OIT by DSC at 100ºC and 10 L/min of air.

The evolution of PV results over time in moderate oxidation (35 ºC) is set out in Figure 43. In addition, the Table 22 lists the induction time of each and the slope or rate of oxidation at the time of starting. Vaidya shown similar results

working with walnut oil and grape seed oil [299]. Three different concentrations (0.25 %, 0.50 % and 0.75 %) of SAE have been studied. The effect in the antioxidant activity is proportional to the concentration. And, in this range of concentrations, has not reached a concentration that can have pro-oxidant effect.

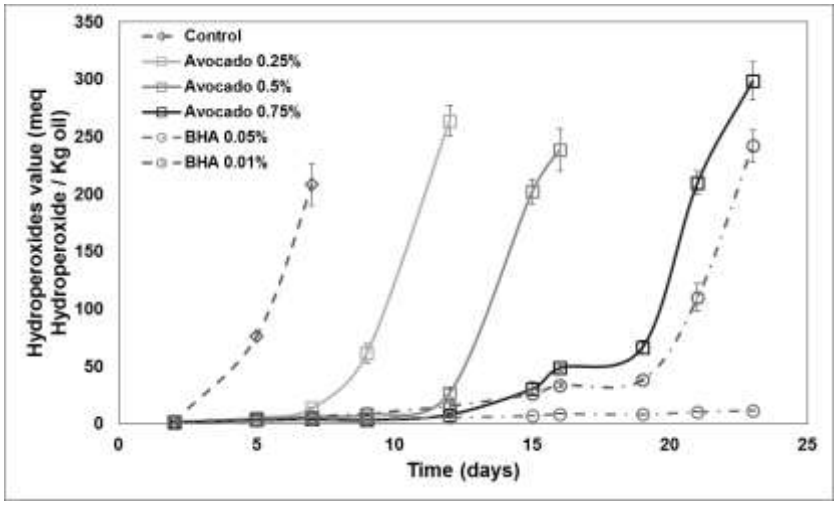


**Figura 43.** Changes in the peroxide value in sunflower oil fatty acid mixture at 35ºC in

For the concentration of 0.75% of SAE the maximum PV are reached at t = 500 h, which compared with the same value for the control (t = 100 h) implies a reduction of oxidation, with increased shelf life longer than 5 times. The results are similar to those obtained with 0.1% BHA. The lower concentration of SAE values (0.25%) also has a protective effect, although lower (2 times delayed with respect to the control sample). Abdelazim worked with sesame extract and found similar delay of the oxidation. Sesame cake extract at concentration of 200 ppm has stabilization efficiency comparable to commonly-employed synthetic antioxidants BHT and BHA at their legal limit, but has lower efficiency than that of the synthetic antioxidant TBHQ [115]. And also it has been successfully worked with pure compounds as chlorogenic acid and caffeic acid in the presence of mixtures triacylglycerols and they found similar delay in the samples oxidation, where at 2.8×10-4M both acids show equal effective- ness and strength. At concentrations above 10×10-4M caffeic acid appears as a much more effective and stronger inhibitor [295].

**Tabla 22.** Parametres for the different methods to calculate the antioxidant activity

| Sample | IT (days)‡ | OIT (min)† | $VP_{10}$ * (meq Hydroperoxide/ Kg Oil) | VP Slope (meq Hydroperoxide/ Kg Oil x days) | $TBARS_{15}$§ (mg MDA/ Kg Oil) | TBARS Slope (mg MDA/ Kg Oil x days) |
|---|---|---|---|---|---|---|
| **Control** | 5,00 | 28,51 | — | 66,08 | 42.09±1.92$^{a}$ | 3,64 |
| **Avocado 0.25%** | 8,31 | 41,55 | 263.79±13.28$^{a}$ | 67,52 | 11.47±1.60$^{b}$ | 1,27 |
| **Avocado 0.5%** | 11,65 | 43,22 | 26.74±3.50$^{b}$ | 54,21 | 6.69±1.04$^{b}$ | 0,86 |
| **Avocado 0.75%** | 18,85 | 52,75 | 7.73±0.98$^{c}$ | 58,02 | 1.79±0.38$^{c}$ | 0,19 |
| **BHA 0.01%** | 19,17 | 47,54 | 15.37±2.28$^{d}$ | 51,00 | 2.76±0.08$^{d}$ | 0,26 |
| **BHA 0.05%** | — | 127,79 | 5.23±0.19$^{e}$ | — | 0.99±0.00$^{e}$ | 0,06 |

‡: Data from VP graphics. †: Data from DSC graphics. *: Hydroperoxide value at 10 days of experiment.
§: TBARS value at 15 days of experiment.
Means within each column with different superscripts are significantly (P < 0.05) different.

The control has a negligible induction time (Table 22). All other samples have an induction time possible to calculate. This fact is a distinguishing feature with the previous studies [48]. This demonstrates the high antioxidant activity of SAE in the concentrations used.

Figure 44 shows the evolution of TBARs over time. The first values (at the beginning of the oxidation, before the hydroperoxides have been formed) are negligible, but the increase starts from the 8th day which coincides with a significant increase in the compounds obtained by the primary oxidation. The sample with 0.25% of SAE has a delay of 43% over the control one while the sample with 0.75% the percentage of delay is 77%.

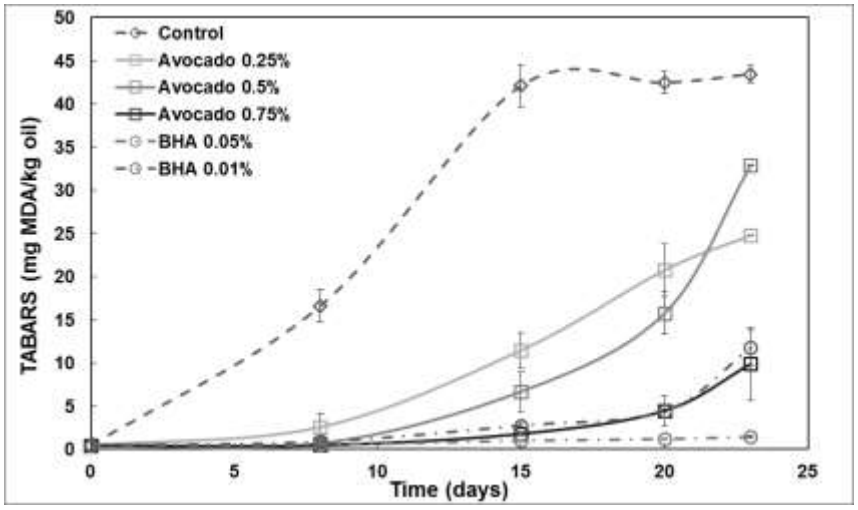


**Figura 44.** Changes in the TBARS value in sunflower fatty acid mixture at 35ºC in dark

The second method allows to obtain comparable results, but in hours, versus days of the first. It is to perform a forced oxidation with oxygen in the conditions studied and measured by DSC. They are shown in Figure 45 and Table 22. It is not the first time this method is used to calculate the oxidation. For example, as was used in Cocoa Butter [300] and also in other oils such as soybean, cottonseed, canola, and sunflower [301] with good results to determine the time it takes to oxidize a fat and consequently the protective effect that make the extract or antioxidant added. The considerable reduction of time (less than 6 hours per sample) allows to have a quick and reliable method applied in the food industry, to assess the protective effect of antioxidants or, where applicable, potential synergies that may decrease the final amount of a particular synthetic antioxidant.

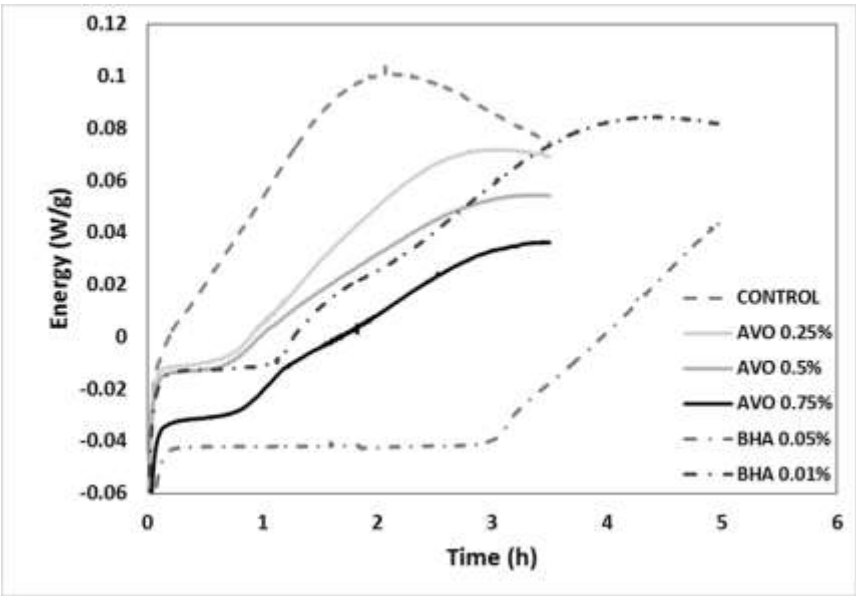


**Figura 45.** Isothermal analysis to determinate OIT value for Sunflower fatty acid mixture.

The control displays an immediate oxidation in the conditions employed, where the OIT is very difficult to see. No significant differences were found between the two lower concentrations of SAE used. Both have an OIT somewhat higher 41-43 minutes, representing more than 45% protection against the forced oxidation if compared with control. Nevertheless, the higher concentration (0.75%) the OIT has a value close to 53 min, which is above the value achieved with the

lower concentration of BHA (0.01%) and an increase of 85% in the stability of the fatty acid mixture analyzed (Table 22). As in other studies where they have applied natural extracts to prevent oxidation of fish oils [302] sunflower oil high in oleic acid and castor oil natural extracts prevent oxidation [303], the SAE possesses antioxidant activity due to the influence of the components found in the extract.

### *3.6.4 Conclusion.*

The SAE is effective as a natural antioxidant. The main antiradical activity is due to polyphenols catechin, epicatechin, 3-O-caffeoylquinic acid (chlorogenic acid isomer), plus 3 compounds of the flavonoid family. Its individual activity has been demonstrated by HPLC post column injection of ABTS, for different radical scavenging methods, including the EPR and also in the protection of sunflower oil oxidation devoid of its natural antioxidants. The degree of oxidation was followed by traditional methods (PV, TBARS) and also has determined the OIT. Both are correlated, which states that could be determined only by the OIT, saving material and time.

## 3.7 Study of solid-liquid extraction of total polyphenols from avocado (Persea americana) pit

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Enviado a Industrial Crops and Products

**Abstract**: Increasingly, polyphenols are the focus of many food and cosmetic industries due to their high antioxidant capacity. Avocado seed is a by-product that contains a large amount of extractable polyphenols. This fact makes it a promising candidate for the cheap and sustainable extraction of phenolic compounds. The aim of this work is to evaluate the effect of ultrasound power (0-104 W) and temperature (20-60 ºC) on the extraction of total polyphenols from avocado seed using water as a green solvent. Higher polyophenol content and antioxidant capacity were obtained when rising temperature and ultrasound power. Different mathematical models (Peleg's, empirical, film theory and Fick's law) were also used to find the one that best fit the extraction kinetics. Models based on film theory and Fick's law were able to predict the ultrasound-assisted batch and continuous extractions, respectively, with accuracy. Using a model based on Fick's law, diffusion coefficients of polyphenols in both fast and slow stages were calculated for the extractions. In addition, a linear relation between total polyphenolic content and antioxidant capacity was proposed.

Keywords: Avocado; Polyphenols; Extraction; Modelization; Diffusivity; Ultrasound

### *3.7.1 Introduction*

The food industry is generating many organic by-products and it is well known that the food wastes such as seeds, hulls, wood, bark, roots and leaves are potential sources of antioxidants compounds [192,194,304]. In this work, avocado seed is proposed to be a potential candidate for polyphenol extraction. There are several studies about avocado and its high antioxidant capacity, as well as its large amount of extractable polyphenols, from quantifying its total polyphenolic yield until how it prevents food matrixes or emulsions oil-water from oxidation [10,194,264,305,306].

In the other hand, the intake of polyphenols as natural substances through the diet, such as food products enriched with them, is a fact whose popularity has greatly increased in recent years. It is widely accepted that high intakes of fruit and vegetables prevent people from some diseases due to the presence of various antioxidants [267].

Pharmaceutical and food industry have been candidates to use natural antioxidants, particularly polyphenols, since they have numerous reported benefits [195]. Furthermore, the use of synthetic antioxidants, like BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene), are restricted because concern is expressed about the possible negative effects on human health [307].

In addition, it is necessary to point out that processes, such as solid-liquid extraction, can be modelized and simulated thanks to the numerical methods and computational advances. It is important to modelize the extraction of polyphenols and estimate their effective diffusivity due to their potential uses in industry. A model will be necessary in order to analyze and design an extraction industrial process [130,156,308]. Unfortunately, most of studies about these extraction processes are scarce and their point is not from engineering [156].

The solid-liquid extraction of polyphenols is a multi-phase and unsteady-state transfer mass operation, where the concentration of the solute inside the solid varies continuously. Experimental studies about kinetics extraction are required to estimate effective diffusivity. The liquid phase concentration as a function of time is used to adjust the experimental data to fit theoretical models, which are under some hypothesis and specific kinetics parameters [309].

It is true that there is a large number of mathematical models that can be applied to extraction kinetics. These models are generally based on modifications of Fick's law, such as the film theory, which has been used to modelize the extraction of bioactive compounds from plants. In this sense, it is noteworthy that empirical equations like Peleg's model can provide a proper fitting to extraction processes.

Several experimental studies about estimating effective diffusivities and mass transfer coefficients of polyphenols (or specific phenolic compounds) from a food by-product have been carried out for these years. Diffusivities and models are estimated by expressions from Fick's second law of diffusion [132,233,296,310,311]. In these works, many variables such as temperature, ultrasound power and extraction time in batch extractions are studied [11,13,129,310–312].

The aim of this work was to modelize the solid-liquid ultrasound-assisted extraction in batch and continuous of total polyphenols from avocado seed, as well as estimate the effective diffusivity of polyphenols and its influence with temperature and ultrasound power. Antioxidant activity was also studied to find its relation with total polyphenol yield.

### *3.7.2 Materials and methods*

#### *3.7.2.1 Sample preparation*

Avocado (Persea gratissima) seeds from domestic consumption were used. The seeds were manually separated from avocado. The fresh seeds were ground by using a Moulinex mill (A5052HF, Moulinex, Lyon, France). Afterwards, the particle size was normalized using both number 8 and 10 sieves. The particle radius was calculated as the average of these sieves pores. Finally, the ground seeds were stored in a dark bottle under refrigeration at 4ºC until use.

#### *3.7.2.2 Ultrasound-assisted batch extraction (UABE)*

Batch extraction was carried out following the procedure described by Segovia et al. (2014) [13] with some slight modifications. About 8 ± 0.1 g (measured precisely) of avocado seeds were extracted in batch with 500 mL of water. The extractions were carried out in sealed flasks. These experiments were performed in triplicate at different temperatures (20, 40 and 60 ºC). At specific intervals of time, 1000 µL of extraction were sampled and put in the fridge (away

from light) at 4 ºC. After two hours, the samples were analysed to determinate total phenolic content and antioxidant capacity.

Ultrasound assisted extraction was performed in an ultrasonic bath (Type T 710 DH, 580VA, 40 KhZ, PROLABO, Germany) using the procedure mentioned above. The ultrasonic output ranged from 0 to 80% (100% equals 130 W).

#### *3.7.2.3 Ultrasound-assisted Continuous extraction (UACE)*

The experimental extraction setup was similar to what Pinelo et al. (2006) [133] reported. The column extractor was 0.75 cm of radius and 10 cm of height. The extractor was placed in the ultrasonic bath described above and the same experimental conditions were applied. Extraction was accomplished by continuous pumping fresh water (4.17 mL/min) through the column. The solvent was pumped upward from the bottom. The outlet extract was sampled at specific intervals of time to record the polyphenol concentration and antioxidant capacity. At the end of the process, all these extracts were collected and stored as the final one.

#### *3.7.2.4 Determination of total polyphenolics content (TPC)*

Total phenol content of the extract was determined using the Folin–Ciocalteu reagent method with a slight modification [11]. Samples were taken from the prepared extracts. The sample was placed in a plate by triplicate, adding 4% (v/v) of the Folin-Ciocalteu's reagent, 12% (v/v) sodium carbonate anhydrous solution (20%) and finally 80 µl of milli-Q water. Allowed to react for 1 hour in the dark room, the absorbance was measured at 765 nm using a Fluorimetrics Fluostar Omega (Perkin–Elmer, Paris, France). The total phenolic content was expressed as mg Gallic Acid Equivalents (GAE)/l, also, mg GAE/fresh matter in final extract.

#### *3.7.2.5 Determination of total antioxidant capacity (ORAC assay)*

Antioxidant activities of avocado extracts were determined by the ORAC assay, as reported by Segovia et al. 2014 [13]. The assay was carried out using a Fluorimetrics Fluostar Omega (Perkin–Elmer, Paris, France) equipped with a temperature-controlled incubation chamber. The incubator temperature was set at 37ºC. 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 hours. 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 mmol Trolox Equivalents (TE)/mg of fresh matter in final extract.

*3.7.2.6 Calculation of the diffusion coefficients*

A model based on Fick's law was used in order to find the diffusion coefficients for each stage of the extraction:

$$\frac{C_\infty - C}{C_\infty} = \frac{6}{\pi^2}\left[f_1 exp\left(-\frac{\pi^2 D_1 t}{r^2}\right) + f_2 exp\left(-\frac{\pi^2 D_2 t}{r^2}\right)\right] \quad (30)$$

where $f1$ and $f2$ are the fractions of the solute, which are extracted with diffusion coefficients D1 and D2, respectively. C∞ is concentration in equilibrium, C is concentration in time t and r2 the particle radius.

The next conditions were established to apply the above equation [129]:

(1) Symmetrical and porous sample particles. The geometry of solid particles is assumed to be spherical with radius of r or thin plate with half thickness of L.

(2) The solid particle is assumed to be of a pseudo- homogeneous medium. The concentration of the active compounds in the solid particle depends on time and radius, r or thickness, x.

(3) Uniform distribution of active compounds in the sample matrix.

(4) Homogeneous mixing between solvent and plant sample particles. The concentration of the solute in the solvent only depends on time.

(5) The mass transfer of active compounds from the solid is a diffusion phenomenon in which the diffusion coefficient is independent of time.

(6) Diffusion of the solute and other compounds are in parallel and no interaction between them.

(7) External mass transfer resistance is negligible. The concentration of the solute in the solvent at the interior of the solid particle is equal to the concentration of the solute in the bulk solvent.

According to the method followed by Hojnik et al. (2008) [312], in later stages of the extraction, only the second term on the right-hand side of Eq. (30) remains significant. The parameter $D_2$ is obtained from the slope and the parameter $f_2$ from the intercept of the curve where ln[C∞/(C∞-C)] is plotted as function of time t. In earlier stages of the extraction, the second exponential term is close to unity and with the addition of $f_2$ from the previous calculation, $D_1$ and $f_1$ can be determined.

### *3.7.2.7 Mathematical modelling of UABE*

The equations of Table 23 have been applied in order to establish a model of curves obtained experimentally. The choice of the best model was based on the analysis of the highest correlation coefficient ($R^2$), lowest sum of squares due to error (SSE) and root mean squared error (RMSE) of experimental data to the equations.

**Tabla 23.** Mathematical models used in the kinetic study of UABE

| Model | Equation |
|---|---|
| Empirical | $C = C_w[1 - exp(-k_w t)] + C_d[1 - exp(-k_d t)]$ |
| Peleg's | $C = C_o + \frac{t}{k_1 + k_2 t}$ |
| Rate Law | $C = \frac{t}{\frac{1}{h} + \frac{t}{C_\infty}}$ |
| Film Theory | $\frac{C}{C_\infty} = 1 - (1 - b)e^{-kt}$ |

### *3.7.2.8 Mathematical modelling of UACE*

For the continuous extraction, Eq. (30) from Fick's law was applied to find and compare the diffusion coefficients as Petrović et al. (2012) [313] reported.

*3.7.2.9 Statistical methods*

The analysis of variance (ANOVA) was done. A significance level of 5% was considered to determine significant differences between two samples, where every sample was taken by triplicate.

Matlab R2013a (The Mathworks Inc., Natick, MA, USA) was used to make the analysis.

*3.7.3 Results and discussion*

*3.7.3.1 UABE modelling*

In order to evaluate the use of ultrasound in the extraction, the change in amount of extracted polyphenols over time was determined. Fig. 45 shows the effect of ultrasound on the extraction of polyphenols from avocado seed at 60 ºC. At 20 and 40 ºC, a similar trend was followed (data not shown). Independent variables such as temperature, power and time were chosen based upon our previous studies (Segovia et al. (2014) [11]). Therefore, maximum temperature was set at 60 ºC, preventing polyphenols from degradation. The range of ultrasound power (0–100 W) was selected according to reported works that studied the extraction of active compounds from black chokeberry[314], Epimedium [315] and sweet tea tree [316], among others.

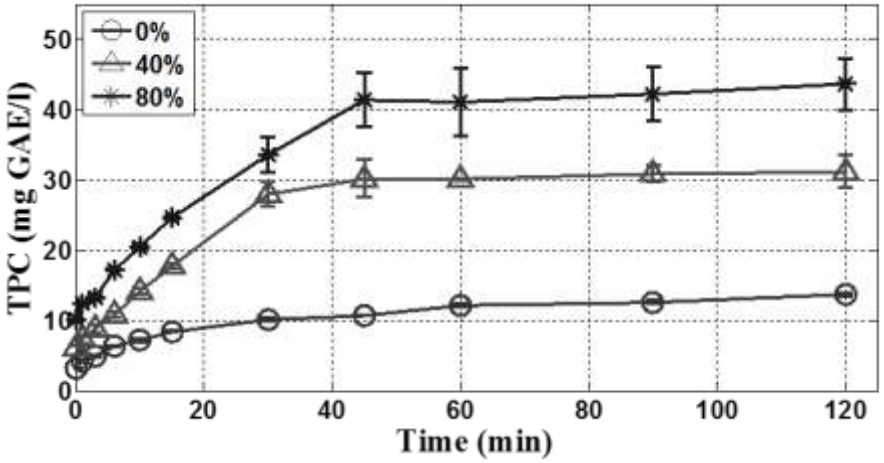


**Figura 46.** The influence of ultrasound power on UABE kinetics of total polyphenols from avocado seed at 60 ºC.

As seen in Fig. 46, the extraction of polyphenols was enhanced when ultrasound power was raised, as expected. Similar results were reported on other organic samples[317,318] . While the equilibrium was reached in less than one hour thanks to the ultrasound extraction, classical extraction methods using solvents at moderated temperatures needed longer time [132,233]. This fact is due to ultrasounds break the cells improving the diffusion of solvents into the tissue and consequently, the extraction. Furthermore, ultrasounds enhances the solubility of the compounds in the extracting solvent [319]. In some extractions, like those at 40 ºC and 0-40 % power, the equilibrium was reached later than one hour. As shown Fig. 47, at 20 and 60 ºC, there were significant differences between the extractions with and without ultrasounds. However, at 40 ºC, extractions at 0 and 40% ultrasound power did not show any significant difference between them.

The following equation was used to measure the ultrasound effect on the extraction [31]:

$$Ultrasound\ effect\ (\%) = \frac{A-B}{B} x100 \quad (31)$$

where A is the extracted amount of polyphenols using ultrasounds and B is the extracted amount without using them.

At 60 ºC, extractions at 40 and 80% ultrasound power showed a 125% and 208% ultrasound effect, respectively. Therefore, ultrasound power played an important role in enhancing the extraction yield. The rising of extracted polyphenols thanks to ultrasound application was also reported for rape seeds, an 88% ultrasound effect,[135] as well as black chokeberries, from 25 to 85% effect [314]. It is noteworthy that avocado seeds have the highest reported values of ultrasound effect so far.

For the purpose of describing the polyphenol extraction by mathematical models, 4 different equations were applied: two empirical (Peleg and empirical models), one from modified Fick's law (film theory) and the last one called Rate Law. In this kind of extractions, two phases are included: (i) penetration of solvent into the cells of the plant and solubilisation of the polyphenolic compounds (fast); and (ii) diffusion of the solubilised compounds from the inner part of the plant into the solution (slow) [129].

**Tabla 24.** Error analysis for fitting experimental data to different models in UABE of polyphenols from avocado seed

| Model | Conditions | | $R^2$ | SSE | RMSE |
|---|---|---|---|---|---|
| | | | | | |
| Peleg´s | 20 | 0 | 0.9 | 1.5 | 0.4 |
| | | 40 | 0.87 | 2.51 | 0.53 |
| | | 80 | 0.85 | 8.99 | 0.99 |
| | 40 | 0 | 0.81 | 2.31 | 0.51 |
| | | 40 | 0.88 | 1.82 | 0.45 |
| | | 80 | 0.93 | 10.7 | 1.09 |
| | 60 | 0 | 0.84 | 20.96 | 1.53 |
| | | 40 | 0.92 | 85.36 | 3.08 |
| | | 80 | 0.88 | 211.8 | 4.85 |
| Empirical | 20 | 0 | 0.93 | 1.2 | 0.41 |
| | | 40 | 0.9 | 1.96 | 0.53 |
| | | 80 | 0.88 | 7.51 | 1.04 |
| | 40 | 0 | 0.95 | 0.68 | 0.31 |
| | | 40 | 0.96 | 0.65 | 0.3 |
| | | 80 | 0.96 | 6.42 | 0.96 |
| | 60 | 0 | 0.91 | 4.59 | 1.29 |
| | | 40 | 0.95 | 51.1 | 2.7 |
| | | 80 | 0.93 | 116.5 | 4.08 |
| Rate Law | 20 | 0 | 0.9 | 1.5 | 0.41 |
| | | 40 | 0.87 | 2.51 | 0.53 |
| | | 80 | 0.85 | 8.99 | 0.99 |
| | 40 | 0 | 0.81 | 2.31 | 0.5 |
| | | 40 | 0.88 | 1.83 | 0.45 |
| | | 80 | 0.94 | 10.7 | 1.09 |
| | 60 | 0 | 0.84 | 20.96 | 1.53 |
| | | 40 | 0.92 | 85.36 | 3.08 |
| | | 80 | 0.88 | 211.8 | 4.85 |
| Film Theory | 20 | 0 | 0.96 | 0.05 | 0.07 |
| | | 40 | 0.98 | 0.01 | 0.04 |
| | | 80 | 0.94 | 0.06 | 0.08 |
| | 40 | 0 | 0.96 | 0.03 | 0.06 |
| | | 40 | 0.99 | 0.01 | 0.03 |
| | | 80 | 0.93 | 0.05 | 0.07 |
| | 60 | 0 | 0.98 | 0.02 | 0.04 |
| | | 40 | 0.99 | 0.01 | 0.04 |
| | | 80 | 0.99 | 0.01 | 0.03 |

The results of $R^2$, SSE and RMSE for the different models are presented in Table 24. Peleg, Empirical and Rate Law models yielded high $R^2$ values, which ranged from 0.81 to 0.96, and high SSE and RMSE, not indicating a good relation to the experimental data [320]. The film theory was the model that best fitted to the experimental data as shown in Table 24. This model is based on Fick's law and assumes that extraction occurs due to the washing and diffusion processes [129]. In literature, film theory has been described to model the solid-liquid extraction of bioactive compounds from plants, having a $R^2$ higher than 0.94 [321].

**Tabla 25.** Parameter values entertained by the mathematical model labeled as film theory for UABE of polyphenols from avocado seed.

| Temperature, ºC | Power, % | | | | | |
|---|---|---|---|---|---|---|
| | 0 | | 40 | | 80 | |
| | b×10 | -k×10$^{-2}$ | b×10 | -k×10$^{-2}$ | b×10 | -k×10$^{-2}$ |
| 20 | 1.39 | 3.80$^{b}$ | 3.1 | 1.18$^{a}$ | 2.16 | 4.12$^{a}$ |
| 40 | 2.20 | 2.05$^{b}$ | 1.83 | 2.01$^{b}$ | 2.62 | 5.39$^{a}$ |
| 60 | 2.93 | 3.06$^{b}$ | 1.68 | 5.00$^{a}$ | 2.25 | 4.11$^{a}$ |

b constant is dimensionless and k is min$^{-1}$.

Different letters in superscript indicate significant differences in mean values.

According to film theory model, Table 25 shows that the coefficients in first stage (fast, b) are higher than those in second stage (slow, k). This trend was also observed in the extraction of oil from sunflower seeds [322].

### *3.7.3.2 UACE modelling*

The evolution of polyphenol concentration as a function of extraction time is shown in Fig. 47. Extractions at 20 and 40 ºC are not reported since they present a similar trend. It is possible to distinguish two phases in the process: (i) the TPC of extracts decreases concomitantly with the increase in extraction time; (ii) a stabilization of TPC was reached when the extraction time was prolonged (beyond 50 min in this case). At 20 ºC, there were only significant differences between extractions at 0 and 80% ultrasound power. At 40 ºC, 0 and 40% ultrasound powers did not show significant differences in polyphenolic yield content. Finally, at 60 ºC, the ultrasound power did not have any significant influence on TPC extracted in

continuous. Therefore, the rise of temperature decreased the influence of ultrasound power on continuous extractions.

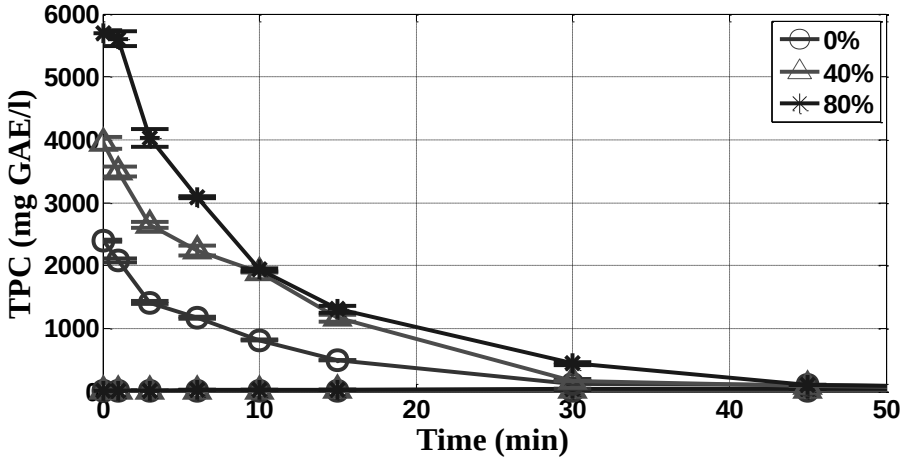


**Figura 47.** The influence of ultrasound power on UACE kinetics of total polyphenols from avocado seed at 60 ºC.

Using the Eq. (31), at 20 ºC, ultrasound effects of 280 and 260% were found in extractions at 80 and 40% ultrasound power, respectively. At 40 ºC, a significant ultrasound effect of 270% was only observed when 80% ultrasound power was applied. Finally, at 60 ºC, ultrasound effects of 150 and 105% were found in extractions at 80 and 40% ultrasound power, respectively. All these results clearly demonstrate that ultrasound is a competitive and effective extraction technology. Similar results were also reported in the extraction of polyphenols from grape marc [139] and black seed [323], among others.

**Tabla 26.** Parameter values entertained by the mathematical model labeled as film theory for UACE of polyphenols from avocado seed.

| Model | Conditions | | $R^2$ | SSE | RMSE |
|---|---|---|---|---|---|
| | Temperature, ºC | Power, % | | | |
| Fick Law | 20 | 0 | 0.99 | 0.01 | 0.03 |
| | | 40 | 0.99 | 0.01 | 0.03 |
| | | 80 | 0.97 | 0.05 | 0.07 |
| | 40 | 0 | 0.94 | 0.06 | 0.08 |
| | | 40 | 0.86 | 0.22 | 0.15 |
| | | 80 | 0.97 | 0.04 | 0.06 |
| | 60 | 0 | 0.99 | 0.02 | 0.04 |
| | | 40 | 0.99 | 0.02 | 0.04 |
| | | 80 | 0.99 | 0.01 | 0.03 |

**Tabla 27.** Error analysis for fitting experimental data to Fick's law model in UACE of polyphenols from avocado seed

| Temperature, °C | Power, % | | | | | |
|---|---|---|---|---|---|---|
| | 0 | | 40 | | 80 | |
| | $f_1$ | $f_2$ | $f_1$ | $f_2$ | $f_1$ | $f_2$ |
| 20 | 1.56 | $0.22^{b}$ | 1.48 | $0.08^{ab}$ | 1.52 | $0.25^{a}$ |
| 40 | 1.15 | $0.42^{b}$ | 1.47 | $0.48^{b}$ | 1.29 | $0.26^{a}$ |
| 60 | 1.47 | $0.14^{a}$ | 1.52 | $0.06^{a}$ | 1.65 | $0.04^{a}$ |

Different letters in superscript indicate significant differences in mean values.

In attempts to predict the evolution of UACE, a mathematical model based on Fick's law Eq. (30) was used. Thanks to this, diffusion coefficients were determined (Table 26), as well as the fractions of solute that are extracted in each stage. Fitting experimental data to this model corroborates that the extraction process can be also divided into both fast and slow stages. As shown in Table 27, values of $R^2$ were in the range from 0.86 to 0.99, suggesting a good fit to the model. Moreover, values of SSE and RMSE are close to 0, confirming a satisfactory quality of data prediction.

### *3.7.3.3 Diffusion coefficients in UABE and UACE*

Fick's law has been commonly employed to determine diffusion coefficients by several authors who study the extraction of bioactive compounds [139,324]. In this study, the used diameter (1.09 mm) was calculated as the average of both number 8 (2.36 mm) and 10 (2.00 mm) sieve pores. Table 28 shows the calculated coefficients using Eq. (30). The values of these coefficients change depending on the independent variables and damage of cell membranes caused by sonication. In some cases, the temperature plays an important role in the extraction stages [312].

**Tabla 28.** Diffusion coefficients of phenolic compounds for UABE and UACE from avocado seed

| Temperature, °C | Power, % | | | | | |
|---|---|---|---|---|---|---|
| | 0 | | 40 | | 80 | |
| | $D_1$ | $D_2$ | $D_1$ | $D_2$ | $D_1$ | $D_2$ |
| UABE | | | | | | |
| 20 | 0.58 | 0.63 | 0.31 | 1.86 | 1.62 | 0.2 |
| 40 | - | - | - | - | 0.36 | 1.76 |
| 60 | 4.10 | 0.30 | 0.47 | 3.90 | 0.23 | 3.93 |
| UACE | | | | | | |
| 20 | 0.88 | 0.009 | 1.45 | 0.028 | 1.17 | 0.058 |
| 40 | 1.12 | 0.036 | 1.27 | 0.029 | 1.43 | 0.062 |
| 60 | 1.78 | 0.058 | 1.15 | 0.045 | 1.43 | 0.042 |

In batch extractions, diffusion coefficients do not present a linear relation with the temperature or ultrasound power. Furthermore, it is commonly reported that a single stage is present in this kind of extraction [156].

However, under no sonication, continuous extractions are similar to classical ones: the rise of temperature and ultrasound power resulted in the increase of diffusion coefficient of polyphenols. At 40% power, the coefficients decreased at fast stages and rose at slow ones when increasing temperature. Firstly, the washing of external layers of cell is prevailing and then the diffusion starts inside the cell. The opposite happened at 80% power, when fast stages rose decreasing the coefficients at slow stages. This fact was due to a greater damage in cell walls [312].

### *3.7.3.4 Antioxidant capacity*

Antioxidant capacity of extracts was determined by using ORAC assay. This assay is widely used to found antioxidant capacity of extracts from plants and food [325]. While all collected extracts were analyzed as only one in UACE, the last collected extract was analyzed in UABE. As shown in Fig. 48, the rising of ultrasound power can result in the increase of total antioxidant capacity, since permeability of cell membranes is enhanced under sonication. Furthermore, the

antioxidant capacity is related to extracted polyphenol content [11,325]. In this work, a linear relation between ORAC and TPC was found, yielding $R^2$ values that range from 0.78 to 0.99 as shown in Table 29. This correlation was also reported in other works [326,327][51,52]. Soong & Barlow (2004) [194] found a linear relation but $R^2$ coefficient ranged from 0.828 to 0.966.

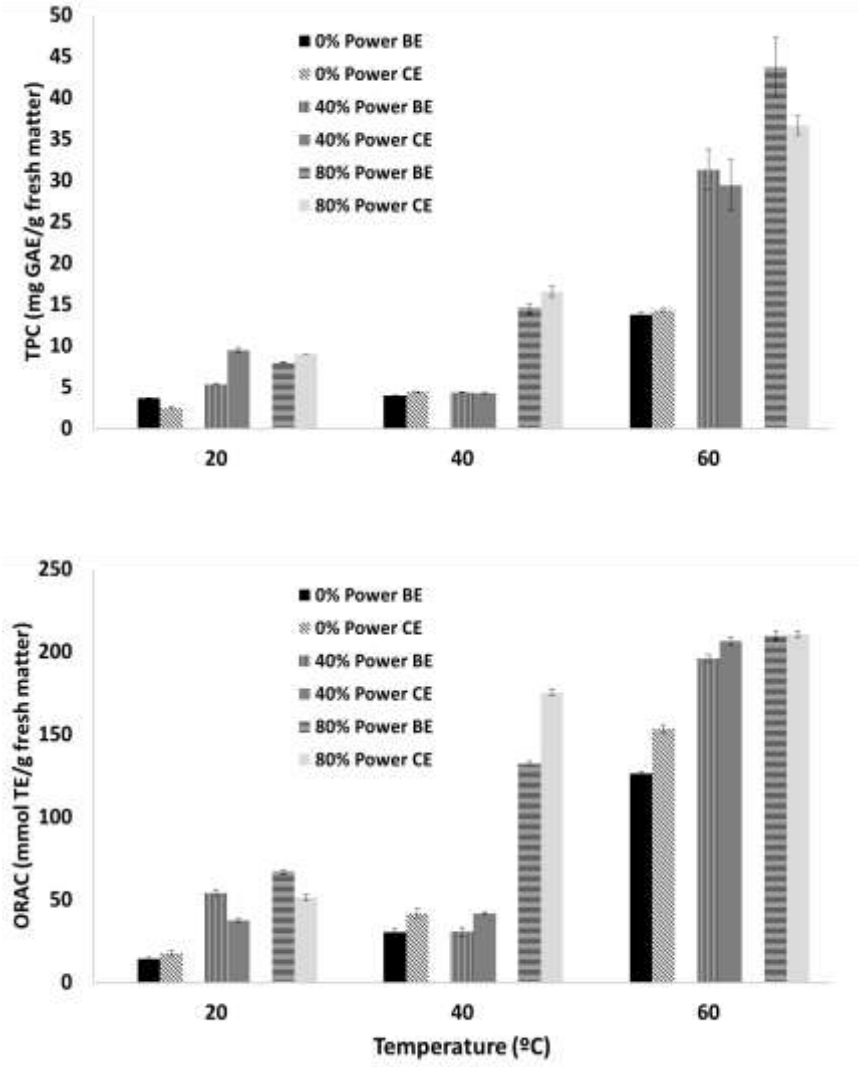


**Figura 48.** The effect of temperature and ultrasound power on total polyphenol content (a) and antioxidant capacity (b) of extracts in batch and continuous (BE: batch extractions; CE: continuous extractions).

While there was a progression in total polyphenol content and antioxidant capacity at 20 and 60 ºC, there was none at 40 ºC and 40% ultrasound power. Under

these conditions, the extraction yield from avocado seed was not enhanced, indicating that ultrasound power and temperature did not have any effect on mass transfer [328].

**Tabla 29.** Proposed correlations between TPC and ORAC in UABE and UACE (0-80% power) of polyphenols from avocado seed.

| Condition | Equations | $R^2$ |
|---|---|---|
| UABE | | |
| 20ºC | $TPC = 0.072 \times ORAC + 2.42$ | 0.86 |
| 40ºC | $TPC = 0.102 \times ORAC + 1.02$ | 0.99 |
| 60ºC | $TPC = 0.323 \times ORAC - 27.81$ | 0.93 |
| UACE | | |
| 20ºC | $TPC = 0.203 \times ORAC - 0.23$ | 0.78 |
| 40ºC | $TPC = 0.091 \times ORAC + 0.51$ | 0.99 |
| 60ºC | $TPC = 0.346 \times ORAC - 38.90$ | 0.93 |

*3.7.4 Conclusions*

The experimental results showed that temperature and ultrasound power had a significant influence on the extraction of polyphenols from avocado seed. It is noteworthy that higher polyophenol content and antioxidant capacity were obtained when rising temperature and ultrasound power. A linear relation between total polyphenol content and antioxidant capacity was found as well. Furthermore, water, which is considered as a green solvent, was used for extracting the polyphenols.

Thanks to the use of mathematical models, a better understanding of the extraction process divided into two stages (one fast and one slow) was provided. Models based on film theory and Fick's law were able to fit the ultrasound-assited batch and continuous extractions, respectively, with accuracy. Using Fick's law model, diffusion coefficients of polyphenols cold be determined in both stages. UACE reached the equilibrium a 17% faster than UABE, saving energy and reducing processing costs.

Overall, ultrasound-assisted extractions proved to be more effective than classical ones. In addition, modelization of solid-liquid extraction would make

possible the potential use of avocado seeds as a sustainable and cheap source of polyphenols in industry.

# 4 DISCUSION GLOBAL DE RESULTADOS

## 4.1 Extracción de polifenoles.

Los resultados de la extracción de polifenoles de los residuos de la borraja de forma convencional se muestran en las figuras 21 y 22. En el comportamiento de la extracción acuosa (figura 21), se observa la influencia de la temperatura (60-80ºC) y el tiempo (10-20 min). Se alcanzan los máximos de extracción de polifenoles y ácido rosmarínico cuando estas variables son altas. El aumento representa hasta un 29% en TPC y un 191% en ácido rosmarínico. Pinelo et al (2005) [130] publicaron un comportamiento similar al extraer polifenoles a partir de orujo de uva. La temperatura es una variable que posee mucha influencia en la extracción de compuestos bioactivos a partir de materia prima orgánica. La difusión de los compuestos se encuentra directamente relacionada con la temperatura y es por ello que un aumento o disminución de la temperatura afecta el proceso de extracción [132,329,330]. Por otra parte, la figura 22 recoge los resultados de la extracción usando una mezcla etanol-agua (30-60%). El efecto del etanol es notable, el tiempo y la temperatura para alcanzar el máximo de extracción es menor.Ssin embargo la extracción de ácido rosmarínico aumenta en un 70% con respecto a la extracción con agua como solvente. El efecto del solvente también es observado en otros trabajos realizados con borraja (semilla desgrasada) donde el solvente tuvo un efecto en el índice de la actividad antioxidante del 33% [160] ya que la polaridad del solvente tiene marcada influencia en la extracción de polifenoles [324]. También se observa la relación que existe entre el ácido rosmarínico con la capacidad antioxidante de los extractos ya que este polifenol posee gran capacidad antirradicalaria [252]. Otros extractos, elaborados con té de salvia, poseen entre 12.2-296 mg/L de ácido rosmarínico. Dicho té de salvia es conocido por su uso medicinal y su capacidad antioxidante [331].

En los extractos elaborados a partir de semilla de aguacate usando como solvente etanol-agua (40-80%), también se observa la influencia de las variables Temperatura y porcentaje de etanol en la extracción. En el caso del etanol, la concentración de polifenoles es inversamente proporcional a la de etanol; disminuye hasta un 21%. Este hecho puede ser debido a la respuesta de los componentes bioactivos a la polaridad del solvente [324]. Sin embargo, con respecto a la temperatura (60-80ºC), la cantidad de polifenoles totales es directamente proporcional al aumento de la Temperatura. Se incrementa en un 15% en el intervalo de tiempo estudiado (5-45 min) pero las diferencias no son estadísticamente significativas. Uno de los polifenoles responsables de la acción

antioxidante de este tipo de extractos es el ácido clorogénico [9,332]. Diversos autores han demostrado la presencia de este ácido fenólico en extractos elaborados de la misma manera [286,333].

## 4.2 Optimización en la extracción convencional de polifenoles.

Se ha aplicado la metodología de superficie de respuesta lo que ha permitido optimizar el proceso de extracción convencional para los residuos de la borraja y las semillas de aguacate. No es la primera vez que se usa la RSM en la optimización. Experiencias previas, aplicadas con éxito son en piel de patata [334], con residuos de vino [71], con residuos de frutas y verduras [335].

En la optimización del proceso de extracción a partir de residuos de borraja, se obtuvieron ecuaciones que parametrizan la cantidad de polifenoles totales (TPC), la capacidad antirradicalaria medida por el métdo ORAC y la cantidad de ácido rosmarínico cuantificado por HPLC. El ajuste, $R^2$, para la extracción acuosa estuvo entre 94.5 y 96.3; y para la extracción con el solvente etanol-agua de 97.8 y 99.7. Esto representa un muy buen ajuste para las ecuaciones obtenidas, además de que se puede afirmar que representan adecuadamente el proceso de extracción [155]. Los valores de las variables que permiten obtener el punto optimizado fueron: 75.94ºC, 51.88%, 14.8 min y 98.28ºC, 22.07 min, para la extracción con solvente etanol-agua y extracción acuosa respectivamente. La diferencia entre la cantidad de polifenoles obtenida por la ecuación y la real fue de un 1.25% (solvente etanol-agua) y 16.8% (solvente agua).

Para el proceso de extracción de las semillas de aguacate se obtuvieron dos ecuaciones; una para la TPC y otra para el ORAC. El ajuste, $R^2$, para estas ecuaciones se encontró entre el 94.7 y 96.7. Sin embargo, debido a que el ajuste para predecir de la ecuación para optimizar la TPC era bajo (57.0), se construyó una nueva ecuación sin tomar en consideración los factores con menor significancia ($p>0.05$) y sus interacciones. Esto mejoró el ajuste de la ecuación y disminuyó la diferencia entre los valores de TPC des 14.97% a 3.13%. Este tipo de ajuste se realiza con el fin de que la ecuación represente lo más fielmente la extracción [166]. El punto optimizado para obtener la máxima cantidad de polifenoles fue de 63ºC, 56% de etanol y 23 min. Para la mayor cantidad de ORAC el punto se encontró en 93.6ºC, 44.7% de etanol y 7 min.

## 4.3 Aplicación de tecnologías novedosas para mejorar la extracción de polifenoles.

Para mejorar los procesos de extracción se han aplicado metodologías asistidas que permiten aumentar la cantidad de compuestos extraídos y/o disminuir la cantidad de tiempo empleado para su extracción. En el presente trabajo, la extracción asistida por ultrasonido y el uso de pulsos electromagnéticos se han usado para mejorar el proceso de extracción convencional.

Para la borraja, se aplicaron tres tratamientos con pulsos electromagnéticos a 0 kV/cm, 2.5 kV/cm, 5 kV/cm; el solvente utilizado fue el agua; la extracción se llevó a cabo en un tiempo de 60 min y se realizó a 10, 25 y 40ºC. A una temperatura de 10ºC la diferencia entre el control y la muestra tratada con 5kV/cm incrementó en un 520% y para 2.5 kV/cm el incremento fue de 290%. Esta diferencia todavía aumenta más si se eleva la temperatura de extracción. Estos resultados concuerdan con los publicados por otros investigadores donde para la extracción de polifenoles a partir de piel de naranja, el incremento va desde un 20% hasta un 159% [140]. Todo lo expuesto refleja el concepto teórico del efecto de los pulsos electromagnéticos, que es aumentar la permeabilidad de la pared celular permitiendo el vertido del contenido celular dentro del solvente [232].

En la extracción de polifenoles a partir de la semilla de aguacate se ha aplicado la extracción asistida por ultrasonido, de forma convencional y continua. El solvente usado fue el agua, la potencia del equipo fue establecida en 0, 40 y 80%; y la temperatura entre 20 y 60ºC. El uso de ultrasonido ha producido un incremento en la extracción de polifenoles de un 280%, 270% y 150% a una temperatura de 60ºC y a una potencia del 80%. Este comportamiento también se ha observado en otros extractos elaborados con orujo de uva [336] y colza [135]. La razón principal de esta mejora en la extracción, es debida a que la aplicación de ultrasonidos aumenta la solubilidad de los componentes bioactivos presentes en la muestra. Además la formación y rotura de burbujas alrededor de la membrana celular permite el paso de compuestos a través de ella y por tanto, la transferencia de masa [129].

## 4.4 Aplicación de los extractos a modelos de alimentos

Los extractos obtenidos, tanto de borraja como de aguacate y piña, se han utilizado en alimentos modelo, para evitar la oxidación de las grasas y/o aceites de carne de ternera picada, emulsiones y magdalenas.

En las emulsiones, todos los extractos mostraron su eficacia para retardar la oxidación lipídica tanto la oxidación primaria como la secundaria. El extracto de borraja usado en concentraciones que van desde 0.06% hasta un 3% redujo la formación de productos de la oxidación primaria en un 8% hasta un 60% respectivamente. Además, al ser agregada una proteína tal como el BSA en un 0.2% la reducción ha sido mayor, desde un 36.6% hasta un 84% respectivamente. Esto se debe a que la proteína ordena mejora la estructura de las micelas en la emulsión, creando una superficie alrededor de estas que interacciona con los polifenoles y evita el contacto con las sustancias que pueden oxidar el aceite [167,168,173]. El valor de p-anisidina también se vio afectado; por ejemplo, una concentración del extracto del 3% (m/m) redujo la formación de sustancias secundarias de la oxidación en un 73.6% y si se combina con un 0.2% (m/m) de BSA llega hasta un 86.3%.

Con el extracto de aguacate se ha trabajado a dos concentraciones: 0.45% y 0.255%. Además se ha analizado la influencia de la proteína de huevo en una concentración del 0.2% (m//m). Se ha reducido el tiempo de formación de hidroperóxidos en un 260 y 220% respectivamente. Esta inhibición en la formación de estos productos ha sido observada en otros estudios cuando se usan extractos de genciana a una concentración de 0.5% y BSA al 0.1% [284]. El extracto de aguacate también se utilizó directamente en aceite de girasol desprovisto de sus antioxidantes naturales, para evitar la oxidación. Con una concentración del 0.75%, se ha observado un aumento en la estabilidad frente a la oxidación y tiene un efecto protector que radica en la capacidad de formar una interfase entre el aire y el aceite. También influye el poder quelante del extracto disuelto en el aceite [47]. Por otra parte, el extracto aplicado a carne picada redujo la formación de los productos de oxidación secundaria en un 90%, lo cual refleja su efectividad para proteger de la oxidación en diferentes modelos de alimentos.

Con el fin de estudiar mejor la interacción polifenol-BSA-Fe, se diseñó un estudio que incorporaba un solo polifenol (ácido cafeico) con una proteína (BSA) y Fe (II). Se ha determinado la influencia de estos tres factores en la oxidación de

emulsiones. Un incremento en la concentración del ácido caféico ha aumentado la estabilidad oxidativa de la emulsión, la concentración ideal del BSA se encuentra en un 0.5% y el Fe (II) a bajas concentraciones puede tener minimizado su efecto con la presencia del ácido cafeico y la BSA. Sin embargo, a altas concentraciones, se produce una oxidación que no puede ser minimizada. Este tipo de sistema es muy complejo y con muchos factores a valorar [46]. El ácido cafeico es un compuesto polar y esto favorece la protección de las emulsiones desde la fase acuosa [337] y además tiene una actividad quelante ante el Fe (II) [338].

Por último, se ha usado un extracto de residuos de piña para evitar la oxidación en magdalenas elaboradas con aceite de girasol. Éste ha evitado la formación de productos de oxidación primaria en un 91.26% y de oxidación secundaria en un 51.94%. En el extracto se identificaron 4 ácidos fenólicos: gálico, elágico, ferúlico y cafeico; todos con una gran actividad antioxidante [260]. Además la cantidad de polifenoles totales fue de 68.13 mg de GAE/g de muestra fresca y la capacidad antioxidante fue de 24.27 µM TE/ml. Todos estos valores se encuentran dentro del rango publicado por otro estudio en el que se determinan estos valores para la piña tratada y no tratada [279]. El estudio se completó con la obtención de ecuaciones que modelizan la oxidación y permiten ajustar los valores de PV (ecuación (29)). Ecuaciones similares han sido útiles para modelizar procesos biológicos y químicos de tendencia sigmoidal. Wardhani et al. (2013) [48], aplicaron la ecuación (29) al estudio de la oxidación en modelos de alimentos cuando se añade un extracto de soja fermentada. En ese estudio, encontraron que se obtiene un buen ajuste para los valores de dienos conjugados y para valores de TBARS, con un $R^2$ entre 0.99-0.10. Los valores inferiores de $R^2$ se encuentran en muestras en las que se ha aplicado el antioxidante sintético. En nuestro caso, el comportamiento fue similar con una $R^2$ que va desde 0.96 hasta 0.68. También el valor inferior corresponde a la muestra de BHA más concentrada, que no se oxida en el tiempo que dura el estudio.

## 4.5 Cinética de extracción de polifenoles.

El estudio sobre la cinética de la extracción de polifenoles fue realizado para las semillas de aguacate, en condiciones de extracción convencional y aplicando extracción asistida por ultrasonido, además, por lotes y de forma continua.

Con el fin de establecer un modelo matemático que describiera la extracción de polifenoles por lotes en las muestras, se aplicaron cuatro ecuaciones, dos de ellas tipo empíricas (Peleg model, empirical model), una de la ley de Fick modificada (Film Theory) y por último la ecuación Rate Law. En este tipo de extracciones es posible visualizar dos etapas; la de extracción rápida y la de extracción lenta. En la etapa de extracción rápida el solvente penetra dentro de la matriz de la planta facilitando la disolución de los compuestos dentro del solvente. En la zona de difusión los compuestos se dispersan desde dentro de la célula de la planta hasta el solvente; este paso es el más lento [320]. Los resultados de los ajustes se pueden ver en la tabla 2.

Las ecuaciones de Peleg, Empirical y Rate Law poseen valores de $R^2$ similares que se encuentran entre 0.81 y 0.96, considerablemente altos. Sin embargo, al estudiar los valores de SSE y RMSE, se observan valores altos, lo que indica que las ecuaciones no representan el comportamiento de la extracción adecuadamente [321]. Por otra parte, la ecuación que mejor representa la extracción con valores altos de $R^2$ y valores bajos de SSE y RMSE, es la ecuación basada en la Film Theory, que es una modificación de la obtenida por la Ley de Fick, en donde se pueden apreciar dos etapas de extracción, etapa de lavado y etapa de difusión [129]. La ecuación de Film Theory se ha utilizado para modelizar procesos de extracción en plantas, con una correlación $R^2$ mayor a 0.94 [321]. En la tabla 24 se muestran los coeficientes de la ecuación de Film Theory.

Los coeficientes de la etapa de extracción rápida son altos con respecto a los coeficientes en la etapa de extracción lenta comportamiento similar al observado en la extracción de aceite de semillas de girasol [322].

Para establecer una ecuación que permita modelizar el comportamiento de la extracción en continuo, se ha utilizado la ecuación de la ley de Fick, en la que se han determinado los coeficientes y las fracciones correspondientes a cada etapa de la extracción. Cuando se realizaron los cálculos se pudo notar que la extracción consta, al igual que la extracción por lotes, de dos etapas: la etapa rápida y etapa lenta de extracción. Los valores de $R^2$ se encuentran entre 0.86 y 0.99, lo cual indica una buena adecuación al modelo. Además la cercanía de los valores de SSE y RMSE al valor cero afirma que la ecuación representa bien este tipo de extracción.

# 5 CONCLUSIONES

Las principales conclusiones de la presente Tesis Doctoral son:

1. Del residuo de borraja se pueden extraer antioxidantes. A través de la extracción convencional con solventes etanólicos (etanol de 30 al 60% de concentración v/v) se obtiene una cantidad de polifenoles totales de 27.05 mg equivalentes de ácido gálico/ g de materia seca y una capacidad antirradicalaria (medida por el análisis capacidad de absorción de radicales de oxígeno, ORAC) de 115.96 mg trolox equivalente/g de materia seca. Además, se identifica el ácido rosmarínico como uno de los principales polifenoles presentes en el extracto, con una concentración de 11.024 mg/l de extracto. Dicho compuesto es uno de los principales responsables de la capacidad antirradicalaria.

Con agua como disolvente, la cantidad de polifenoles totales obtenidos es de 22.27 mg equivalentes de ácido gálico/ g de materia seca, la capacidad antirradicalaria (medida por ORAC) es de 81.6 mg trolox equivalente/ g de materia seca y contiene 3.9 mg/l de extracto de ácido rosmarínico. Si se usan pulsos electromagnéticos se observa hasta un incremento del 2.4 veces la cantidad obtenida con agua con una intensidad de pulsos 5 kV/cm.

2. La semilla de aguacate contiene la cantidad suficiente de antioxidantes como para justificar el proceso de extracción a nivel industrial. Con las extracciones convencionales se obtiene un rendimiento de 45.01 mg GAE/ g de materia seca en la cantidad de polifenoles y de 154.3 µM trolox equivalente/ g de materia seca, en la capacidad antirradicalaria medida por ORAC. El uso de la extracción asistida por ultrasonido incrementa la extracción de los polifenoles hasta un 200%, si se aplica una potencia de 104 vatios y una temperatura de extracción de 60ºC. En este extracto, es posible identificar familias de procianidinas, catequina y epicatequina, además de un isómero del ácido clorogénico, compuestos con gran efecto antioxidante.

3. El residuo de la piña muestra una cantidad de polifenoles de 68.13 mg GAE/ g de materia fresca, además de una capacidad antirradicalaria de 24.27 µM trolox equivalente/ml de extracto. Los polifenoles identificados en este extracto son ácidos fenólicos: Gálico, elágico, ferúlico y cafeico.

4. En el estudio de las extracciones convencionales de los extractos de borraja y aguacate se obtuvieron las optimizaciones por medio del cálculo de rendimientos mediante RSM (response surface method). En el caso de la borraja, el

punto óptimo de extracción fue de 75.9 ºC, 52% etanol y 14.8 min, que representa simultáneamente el punto en el que se encuentra la mayor cantidad de polifenoles, de capacidad antioxidante y de contenido de ácido rosmarínico en el extracto. En el extracto de semillas de aguacate, el punto máximo de cantidad de polifenoles y capacidad antioxidante obtenido en la optimización se encuentra en 63ºC, 56% de concentración de etanol y 23 min de extracción.

5. Se ha aplicado la ecuación usada comúnmente en la descripción de los procesos de extracción de componentes intracelulares, tanto a la extracción convencional de borraja como a la realizada con pulsos electromagnéticos. El ajuste ($R^2$) está comprendido entre 0.73 y 0.95. Se observa que no existen diferencias significativas entre las constantes, k, obtenidas para el modelo, independientemente de la temperatura y la intensidad del pulso aplicado de 2.5 y 5 kV/cm.

6. Se han aplicado las ecuaciones: Empirica, Peleg's, ley de velocidad y la teoría del film en la extracción de polifenoles de semillas de aguacates. Tanto para la extracción convencional como para la extracción asistida por ultrasonidos el ajuste de las ecuaciones es bueno; el $R^2$ varía entre 0.81 y 0.99. La ecuación que mejor se ajusta es la correspondiente a la de la teoría de film, con el error cuadrático medio (RMSE) entre 0.03 y 0.07. Además se observa un mejor ajuste cuando se aplica la extracción asistida por ultrasonido. Los coeficientes de difusión muestran una marcada influencia en el comportamiento de la extracción si se aplica la extracción asistida por ultrasonidos.

7. El ácido cafeico tiene una actividad antioxidante remarcable en el seno de las emulsiones de aceite en agua. Presenta efecto sinérgico con la presencia de la proteína BSA y puede llegar a proteger de la oxidación incluso en presencia de un agente prooxidante con el catión Fe (II). Con una concentración de 2.5 mM de ácido cafeico se retrasa 9 veces la producción de un total de 40 miliequivalentes de hidroperóxidos/kg emulsión respecto a la emulsión control. Al agregar Fe (II) se observa una aceleración en la oxidación de la emulsión.

8. Los extractos de borraja presentan buenas propiedades antioxidantes en modelos de alimentos tales como emulsiones O/W. Muestran una reducción de formación de hidroperóxidos de un 60% si la concentración final del extracto en el seno de la emulsión es del 3%. Además se reduce el valor de p-anisidina en un 73.6%. El uso de BSA en la emulsiones presenta un efecto sinérgico y aumenta el efecto protector.

9. El extracto de aguacate usado en una concentración del 0.45% en emulsiones ha impedido la formación de productos de oxidación primaria en un 90%. El extracto usado directamente en aceite de girasol, ha reportado una reducción en la formación de compuestos hidroperóxidos. Además se observa en los diagramas térmicos que la velocidad de oxidación disminuye con respecto al control. El valor del tiempo inducido de oxidación (OIT) es desde 53 min para una concentración del extracto de 0.75% y de 42 min para el 0.25%. El valor del control es de 29 min.

10. El uso del extracto de aguacate en modelos cárnicos demuestra su efectividad. Una concentración del 0.1% (m/m) de extracto liofilizado inhibe los productos de oxidación secundaria en un 90% con respecto al control.

11. El extracto de piña en magdalenas reduce la formación de compuestos de la oxidación lipídica primaria en un 60% y los productos de oxidación secundaria en un 46 % con respecto al control. Se ha obtenido la ecuación que representa la evolución cinética de la oxidación con un ajuste de $R^2$ de 0.97. Las velocidades de reacción varían con la concentración del extracto en la magdalena; la velocidad más alta corresponde al control, mientras que la más baja, más ralentizada, corresponde a la que tiene mayor concentración del extracto. El análisis sensorial, con pruebas discriminativas triangulares demuestra que no se pueden distinguir las muestras que incorporan el extracto de aquellas que no lo tienen.

Como conclusión global, se ha demostrado que el uso de subproductos de la agroindustria es muy útil para la elaboración de extractos naturales que incorporan antioxidantes y es una materia prima importante que pasa de ser un residuo por el que se ha de pagar a ser un producto que permite obtener compuestos de alto valor añadido. Ralentizan la oxidación de las grasas en diversos modelos de alimentos. Los subproductos de la industria de la producción de borraja (hojas), aguacate (semillas) y piña (epicarpio) contienen gran cantidad de polifenoles y muestran una remarcable capacidad antioxidante. Además, estos desperdicios pueden ser material para la producción de polifenoles tales como el ácido rosmarínico y clorogénico empleando las tecnologías adecuadas para su refinación.

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# 7 ANEXOS

## 7.1 Anexo 1. Lista de publicaciones y Congresos fruto de esta Tesis

### PUBLICACIONES

(1) Segovia, F.; Luengo, E.; Corral-Pérez; J., Raso, J.; Almajano, MP.. Improvements in the aqueous extraction of polyphenols from borage (Borago officinalis L.) leaves by pulsed electric fields: Pulsed electric fields (PEF) applications. Ind. Crops & Products 2014, doi:10.1016/j.indcrop.2014.11.010

(2) Segovia, F.; Sánchez, S.; Iradi, M.; Azman, N.; Almajano, MP. Avocado Seeds: Extraction Optimization and Possible Use as Antioxidant in Food. Antioxidants 2014, 3, 439–454.

(3) Segovia, F.; Lupo, B.; Peiró, S.; Gordon, M.; Almajano, MP. Extraction of Antioxidants from Borage (Borago Officinalis L.) Leaves—Optimization by Response Surface Method and Application in Oil-in-Water Emulsions. Antioxidants 2014, 3, 339–357.

(4) Segovia, F.; Almajano, M.P. Onion Consumption and Health. Nova Science Publisher 2012, 91-120. Book chapter.

(5) Francisco Segovia Gómez, Hernan González, and María Pilar Almajano. Experimental design to monitor effects of caffeic acid, BSA and Fe(II) concentration on oxidation in a model food emulsion. Enviado a Macrothink.

(6) Francisco Segovia Gómez and María Pilar Almajano. Pineapple waste extract for preventing oxidation in model food systems. Enviado a Journal of Food Science.

(7) Francisco Segovia Gómez and María Pilar Almajano. Avocado seeds: a comparative study of the antioxidant capacity (oxidation oil, OIT, EPR and other radical scavenging methods). Enviado a Journal of Food Science and Technology

(8) Francisco Segovia Gómez, Juan José Corral; María Pilar Almajano. Study of solid-liquid extraction of total polyphenols from avocado (Persea americana) pit. Enviado a Industrial Crops and Products

Otras publicaciones:

Skowyra, M.; Gallego, M.; Segovia, F.; Almajano, MP. Antioxidant Properties of Artemisia Annua Extracts in Model Food Emulsions. Antioxidants 2014, 3, 116–128.

Skowyra, M.; Falguera, V.; Azman, N.; Segovia, F.; Almajano, MP. The Effect of Perilla Frutescens Extract on the Oxidative Stability of Model Food Emulsions. Antioxidants 2014, 3, 38–54.

Azman, N.; Segovia, F.; Martínez-Farré, X.; Gil, E.; Almajano, MP. Screening of Antioxidant Activity of Gentian Lutea Root and Its Application in Oil-in-Water Emulsions. Antioxidants 2014, 3, 455–471.

Gallego, M. G.; Gordon, M. H.; Segovia, F. J.; Skowyra, M.; Almajano, MP. Antioxidant Properties of Three Aromatic Herbs (Rosemary, Thyme and Lavender) in Oil-in-Water Emulsions. J. Am. Oil Chem. Soc. 2013, 90, 1559–1568.

## COMUNICACIONES ORALES Y POSTER EN CONGRESOS

**VII Congreso Español de Ingeniería de Alimentos (CESIA 2012). Spain 2012.**

"Optimización de la extracción de compuestos con actividad antioxidante en residuos de borraja". Poster.

**International Porous and Powder Materials. Symposium and Exhibition. Turquia 2013.**

"Polyphenol quantification of pineapple through fermentation process and the use in muffins". Poster.

"Modeling polyphenol extraction of avocado pit through the mass transfer coefficient". Poster.

**13th Mediterranean Congress Of Chemical Engineering. Spain 2014.**

"Oxidative Stability "Natural" Milk Mayonnaise Evaluation With Differential Scanning Calorimeter (Dsc)". Comunicación Oral.

"Continuous Ultrasound Extraction Of Borage Polyphenols: Determination Of Total Polyphenols And Effective Diffusivity". Poster.

## 7.2 Anexo 2. Otras publicaciones

*Antioxidants* **2014**, *3*, 38-54; doi:10.3390/antiox3010038

OPEN ACCESS

*antioxidants*

ISSN 2076-3921

www.mdpi.com/journal/antioxidants

*Article*

# The Effect of *Perilla frutescens* Extract on the Oxidative Stability of Model Food Emulsions

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*Received: 5 December 2013; in revised form: 9 January 2014 / Accepted: 16 January 2014 / Published: 22 January 2014*

**Abstract:** The polyphenolic profile of leaves and stalks of *Perilla frutescens*, was assessed as a source of natural antioxidants. The amount of caffeic and rosmarinic acids, determined by high-performance liquid chromatography (HPLC), were 0.51 mg/g dry weight (DW) and 2.29 mg/g DW, respectively. 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 65.03 mg Trolox equivalents (TE)/g DW, 179.60 mg TE/g DW and 44.46 mg TE/g DW, respectively. *P. frutescens* extracts also showed good antioxidant properties in 10% sunflower oil-in-water emulsions during storage at 32 °C. Perilla extract at 320 ppm was as effective as butylated hydroxyanisole (BHA) at 20 ppm in slowing down the formation of hydroperoxides as measured by peroxide value, thiobarbituric acid reactive substances and hexanal content. The results of this study indicate that extract of *P. frutescens* may be suitable for use in the food matrix to help achieve potential health benefits.

**Keywords:** perilla; polyphenols; antioxidants; oil-in-water emulsions; lipid oxidation

## 1. Introduction

In most foodstuffs, lipid oxidation is a severe problem that causes rancid odors and flavors, modifies texture and color, and decreases shelf life [1]. These changes degrade functional and nutritional compounds of food, damage essential fatty acids, and produce oxidized polymers, which could raise safety concerns. Especially, this process is favored in oil-in-water emulsions due to the large contact surface between the oxidizable lipid hydroperoxides in emulsion droplets and water-soluble prooxidants resulting in the propagation of oxidation reactions [2]. To avoid this problem, synthetic antioxidants are commonly used, such as butylated hydroxytoluene and butylated hydroxyanisole [3]. However, in recent years, consumers have become increasingly concerned about the impact of food and food ingredients on their own health and this attitude has caused several changes throughout the food industry [4]. As part of these changes, food companies have been forced to seek for natural-origin counterparts for a number of ingredients that fulfill technological functions [5].

Plant extracts rich in phenolic compounds may be a good alternative to synthetic antioxidants to prevent lipid oxidation. Plants produce phenolic compounds to deal with reactive oxygen species and free radicals produced during photosynthesis. Inside the plant structure, lipid peroxidation has been known to associate with tissue injuries and disease conditions. Plant phenolic compounds can act as protective factors delaying the onset of lipid oxidation and decomposition of hydroperoxides in living tissues [6]. Therefore, such compounds are expected to play a similar role inside food matrices, provided that a suitable extraction and conservation method is found and optimized. In the process of testing new plant extracts as antioxidants, two major difficulties arise. On the one hand, there are several factors in the extract-obtaining process that must be analyzed, the most important of which are the method and the solvent used. These factors will determine the antioxidant content and activity of the resulting mixtures [7]. On the other hand, very often the results obtained when studying the isolated products using the *in vitro* tests correlate poorly with their ability to avoid oxidative impairment of foods [8,9]. This lack of equivalence is the consequence of complex interactions among the components of the food matrix, which may limit or enhance the activity of the tested extract. Therefore, functionality tests must be performed in model foods that consider the most important conditions of the actual food in which they are to be applied. In this way, oil-in-water emulsions have become a standardized model food to test the protective effect of antioxidant products against lipid oxidation [10]. Phenolic extracts of certain plant materials have been shown to neutralize free radicals in model systems. Widely used culinary herbs of the *Lamiaceae* family, such as rosemary, thyme, marjoram, and oregano, have gained the interest of many research groups [11–14].

*Perilla frutescens* (*Lamiaceae* family) is a traditional Chinese medicinal plant that is commonly used for a variety of diseases such as depression, inflammation, bacterial and fungal infections, allergy, intoxication, some intestinal disorders, and even tumors [15,16]. In Asian countries, such as Japan, Korea, and China, its leaves are commonly added to sushi, garnishes, and soups, and young raw leaves are often used to wrap cooked food. Its health-promoting effects have been mainly attributed to its content of phenolic acids (e.g., rosmarinic acid), flavonoids, and triterpenoids [17,18]. These components provide the extracts of this plant with proved antioxidant, anti-inflammatory, antibiotic, and antipyretic properties. Moreover, *Perilla frutescens* seed oil (known as PFSO) has also been shown to be a rich source of unsaturated fatty acids, especially omega-3 linolenic acid [19]. Although

not yet reported in the literature, perilla 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 purple perilla (*Perilla frutescens*) 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 as the primary oxidation products and thiobarbituric acid reactive substances and hexanal content as the secondary products. In addition, the content of the main phenolic compounds in perilla cultivated in Spain has been quantified.

## 2. Experimental Section

### *2.1. Raw Material*

Purple perilla was grown in a greenhouse (Balaguer, Spain). Stalks and leaves of perilla were collected, dried, and ground to a homogenous powder in collaboration with the company, Pâmies Horticoles. The powder was stored in darkness, at room temperature until extraction. Refined sunflower oil was purchased in a local market.

### *2.2. Reagents*

6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), gallic acid, rosmarinic acid, caffeic acid, phosphate buffered saline (PBS), 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS), 2,2,-azobis-(2-methylpropionamide) dihydrochloride (AAPH), fluorescein ($C_{20}H_10Na_2O_5$), and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were purchased from Sigma–Aldrich Company Ltd. (Gillingham, UK). Folin-Ciocalteu reagent, absolute ethanol, aluminum oxide, ferric chloride ($FeCl_3$), ammonium thiocyanate ($NH_4SCN$), anhydrous sodium carbonate, and Tween 20 were of analytical grade, from Panreac (Barcelona, Spain).

### *2.3. Extraction*

Air-dried and finely ground perilla was weighed (4 g) and extracted with 60 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.

### *2.4. Total Phenol and Flavonoid Content*

Total polyphenol content (TPC) of extracts was determined by colorimetric spectrophotometry following the Folin-Ciocalteu method [20], slightly modified and adapted for microplates. Samples were taken from the extract solutions, diluted 1:30 (v:v) and Folin-Ciocalteu reagent (4% by volume), 20% sodium carbonate solution (30.8% by volume), and Milli-Q water were added. Samples were

well mixed and left in the dark for 1 h. 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 perilla extracts was measured according to the method of Jia *et al.* [21]. Each sample (500 µL) was mixed with 5% $NaNO_2$ (75 µL), and then left to stand for 5 min at room temperature. The mixture was sequentially mixed with 150 µL 10% $AlCl_3$, 500 µL 1 M NaOH and 275 µL distilled water. 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 (CE/g DW).

### *2.5. Antioxidant Capacity Determination*

#### 2.5.1. ABTS Assay

The first method used was the 2,2′-azino-bis-3-ethylbenzo-thiazoline-6-sulphonic acid (ABTS) discoloration assay [22]. The assay is based on the ability of an antioxidative compound to quench the $ABTS^{\cdot+}$ radical relative to that of a reference antioxidant such as Trolox. A stock solution of ABTS radical cation was prepared by mixing ABTS solution with a potassium persulfate solution at 7 mM and 2.45 mM final concentration, respectively. The mixture was maintained in the dark at room temperature for 16 h before use. The working $ABTS^{\cdot+}$ solution was produced by dilution of the stock solution in 10 mM PBS (pH 7.4) incubated at 30 °C to achieve an absorbance value of 0.7 (±0.02) at 734 nm. An aliquot of 20 µL of diluted extract was added to $ABTS^{\cdot+}$ radical working solution (180 µL). For the blank and standard curve, 20 µL of PBS or Trolox solution were used, respectively. Absorbance was measured by means of a UV-vis spectrophotometer (Fluostar Omega, Perkin-Elmer, Paris, France) at 734 nm and percent inhibition was calculated as per Skowyra *et al.* [5]. The radical-scavenging capacity of extracts was quantified as mg of Trolox equivalent per gram of dry weight (TE/g DW).

#### 2.5.2. The Oxygen Radical Absorbance Capacity (ORAC) Assay

The oxygen radical absorbance capacity (ORAC) method was adapted from Ou *et al.* [23]. The assay was performed with an automated microplate reader and 96-well plates. Diluted extract (40 µL) was transferred by pipette into each well and then 120 µL of 1.34 µM fluorescein working solution in phosphate buffer (13.3 mM) at 37 °C were added to each sample. The plate was placed in a spectrophotometer (Fluostar Omega, Perkin-Elmer, Paris, France) and incubated at 37 °C. The initial fluorescence was recorded at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. 2,2′-Azobis(2-metylpropionamide) dihydrochloride (AAPH, 40 µL, 30 mM) was then added to each sample well and the fluorescence was measured immediately and every 2 min thereafter for 120 min. For the calibration curve, solutions of Trolox were prepared in the range of 8–58 µM. The ORAC value for each extract was calculated using a regression equation relating Trolox concentration and the net area under the fluorescence decay curve. Results are expressed as mg of Trolox equivalents per gram of dry weight (TE/g DW).

2.5.3. FRAP Assay

The FRAP assay was performed as described by Benzie & Strain [24] with some modifications. The FRAP reagent was prepared with acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in HCl, 40 mM) and $FeCl_3$ (20 mM). The proportions were 10:1:1 (v:v:v), respectively. A suitable dilution of the extract was added to the FRAP reagent (1:30, v:v) and incubated at 37 °C. The assay was performed by means of an automated microplate reader (Fluostar Omega, Perkin-Elmer, Paris, France) with 96-well plates. The absorbance at 593 nm at time zero and after 4 min was recorded. The analysis was performed in triplicate and values were determined from a calibration curve of Trolox (ranging from 2.5 to 33 µM). The results are expressed as mg of Trolox equivalent per gram of dry weight (mg TE/g DW).

*2.6. Determination of Cinnamic Acid Derivatives by High-Performance Liquid Chromatography (HPLC)*

HPLC analyses of the perilla extracts were carried out using an Acquity UPLC System (Waters, Milford, MA, USA) with photodiode array (PDA) detector. Perilla extracts (10 µL) were injected onto an analytical $C_{18}$ column (Symmetry, 5 µm, 3.9 × 150 mm, Waters) at 25 °C. The mobile phase was composed of 0.5% formic acid (v/v) in acetonitrile (eluent A) and 0.5% formic acid (v/v) in water (eluent B). The gradient program was as follows: 10% A (20 min), 35% A (4 min), 10% A (6 min). Total run time was 30 min. The absorbance at 330 nm was measured to detect the cinnamic acid derivatives (caffeic acid and rosmarinic acid). The standard was identified by its retention time and its concentration was calculated by comparing the peak area of samples with that of the standard. Standard solutions with concentrations ranging from 10 to 100 ppm were then prepared by diluting the stock standard solution with water. The perilla extracts were filtered through a 0.45 µm filter for HPLC analysis.

*2.7. Oil-in-Water Emulsion System*

2.7.1. Removal of Tocopherols from Sunflower Oil

Tocopherols were removed from sunflower oil by column chromatography using activated alumina, as described by Yoshida *et al.* [25]. The oil was stored at −80 °C prior to emulsion preparation (up to 2 days). The fatty acid composition of the filtered sunflower oil is shown in Table 1. The fatty acid composition was determined using a method based on that of Conde *et al.* [26]. The sunflower oil used contained linoleic acid (52.38%) and oleic acid (34.51%) as the main unsaturated fatty acids.

2.7.2. 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) during 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 perilla extract was redissolved in ethanol 50% (v/v) and added directly to the emulsion and homogenized, obtaining final concentrations of 80 and 320 ppm (C1 and

C2, respectively). For the negative control, no extract was added, and the positive controls were prepared with Trolox (40 ppm) and BHA (20 ppm) dissolved in ethanol.

All emulsions were stored in triplicate in 60 mL amber bottles in the dark, with constant elliptical movement and allowed to oxidize at 32 ± 1 °C for 30 days.

**Table 1.** Fatty acid composition of sunflower oil.

| Fatty acid name | Numerical symbol | Amount (%) |
|---|---|---|
| **Saturated** | | **12.79** |
| Palmitic acid | C16:0 | 6.99 ± 0.08 |
| Stearic acid | C18:0 | 4.16 ± 0.04 |
| Arachidic acid | C20:0 | 0.33 ± 0.01 |
| Behenic acid | C22:0 | 0.96 ± 0.02 |
| Lignoceric acid | C24:0 | 0.35 ± 0.02 |
| **Unsaturated** | | **87.21** |
| Oleic acid | C18:1 (*n*-9) | 34.51 ± 0.11 |
| Eicosenoic acid | C20:1 (*n*-9) | 0.32 ± 0.03 |
| Linolenic acid | C18:2 (*n*-6) | 52.38 ± 0.23 |

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

Peroxide value (PV) was measured periodically (every 2 or 3 days, the time of storage) using aliquots of 0.05–0.1 g of each sample and determined by the ferric thiocyanate method [27], after calibrating the procedure with a series of oxidized oil samples analyzed by the AOCS Official Method Cd 8-53 [28].

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

2.7.4. Measurement of Secondary Oxidation by TBARs and Hexanal Methods

The thiobarbituric acid reactive substances (TBARs) assay was performed as described by Maqsood and Benjakul [29], with some modifications. One milliliter 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) for 5 min and 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.

Hexanal was measured according to Waraho *et al.* [2], with some modifications, using a TRACE gas chromatography equipped with mass spectrometry MS DSQI (ThermoFisher Scientific, Waltham, MA, USA) and TRIPLUS auto-injector. 1 mL of emulsion was weighted into a head space vial and equilibrated at 60 °C for 30 min. Aliquots (1 mL) of the head space were injected onto a DB-624 column (60 m × 0.32 mm × 1.8 µm). Initially, the oven temperature was set to 60 °C, maintained at

this value for 2 min, then raised up to 220 °C at 8 °C/min and maintained for 5 min. The injection port was operated in the split mode. The carrier gas was helium at flow rate of 1.8 mL/min. A flame ionization detector was used at a temperature of 260 °C. Hexanal concentrations were determined from peak areas using a standard curve prepared with hexanal standard solutions.

### *2.8. Statistical Analysis*

TPC, TFC, $ABTS^{\cdot}$, ORAC, and FRAP measurements were performed in triplicate on triplicate samples. PV, TBARs, and hexanal 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 and Flavonoid Content of Extract*

The total polyphenols and flavonoids in the ethanolic extracts of *P. frutescens* leaves and stalks are shown in Table 2. The perilla extract contained 22.67 ± 0.52 mg gallic acid equivalent (GAE)/g DW and 2.90 ± 0.07 mg catechin equivalent (CE)/g DW. Hong and Kim [17] reported a similar content in perilla leaves (12.15 mg GAE/g DW and 7.23 mg rutin equivalent (RE)/g DW, respectively) using 70% ethanol in refluxed extraction for 24 h. Similarly, Kee *et al.* [30] found a value of 27.10 mg GAE/g DW after aqueous extraction. However, studies involving methanol extraction of perilla leaves have reported highest values than those obtained in the present study, in the range of 0.7–1.1 g GAE/100 g fresh weight (FW), using a mixture of water-methanol-formic acid (15:80:5) [31]. Lin *et al.* [15] also studied the extraction of phenolics and flavonoids from perilla leaves and stalks separately and the methanolic extract of stalk had higher polyphenol and flavonoids content than that of leaves (137.40 mg GAE/L and 205.75 mg RE/L, respectively). Consequently, the extraction method and the solvent used play a key role in the extraction of polyphenols and flavonoids from plant material. Likewise, Hong *et al.* [32] studied phenolic-enriched fraction from *P. frutescens* and the highest total phenolic and flavonoid level was detected in the ethyl acetate fraction from the water extract (373.92 mg GAE/g fraction and 86.63 mg RE/g fraction, respectively). Whatever the considered extraction method or solvent, perilla was shown to have polyphenol level like typical culinary herbs from the same family (*Lamiaceae*), such as basil [33], spearmint [30], marjoram, and salvia [34].

**Table 2.** Polyphenol and flavonoid content and antioxidant activity of perilla extract.

| Method | Amount detected [a] |
|---|---|
| Total phenol content (mg GAE/g DW) | 22.67 ± 0.52 |
| Total flavonoid content (mg CE/g DW) | 2.90 ± 0.07 |
| ABTS (mg TE/g DW) | 65.03 ± 2.98 |
| ORAC (mg TE/g DW) | 179.60 ± 6.02 |
| FRAP (mg TE/g DW) | 44.46 ± 1.55 |

[a] Values are mean ± standard deviation ($n = 3$).

*3.2.* In Vitro *Antioxidant Activity of Extract*

Antioxidant activity of the extract from *P. frutescens* was assessed by three different methods: $ABTS^{+}$, ORAC, and FRAP (Table 2). The use of several methods provides more comprehensive information about the antioxidant properties of the original product [35]. An $ABTS^{+}$ value of 65.03 ± 2.98 mg TE/g DW, an ORAC value of 179.69 mg TE/g DW, and a FRAP value of 44.46 mg TE/g DW were measured in the ethanolic leaf and stalk extract. Similarly, Muller-Waldeck [31] found the $ABTS^{+}$ value of 0.8–1.3 g TE/100 g fresh weight (FW) in perilla leaves using 80% methanol with 5% formic acid. Lin *et al.* [15] reported that a scavenging abilities of the methanolic extracts of stalk and leaf from *P. frutescens* at 1.5–25 μg/mL on DPPH radicals were in the range of 18.7%–91.0% and 6.7%–63.1%, respectively. Meng *et al.* [36] also found an activity of 114–167 μmol TE/100 mL of aqueous perilla extract as assessed via DPPH assay. Hong *et al.* [32] described antiradical activity (DPPH) and reducing power of the phenolic-enriched fractions from *P. frutescens*, finding strong reducing power and effective radical scavenging activity (89.48%–90.74%) of the ethyl acetate fractions of methanolic extracts. They also reported the antioxidant activity (in β-carotene/linoleic acid system) of the phenolic-enriched fractions from perilla leaves, finding that at 500 μg/mL chloroform fractions of 70% ethanol extract presented remarkable antioxidant abilities in the linoleic acid emulsion system. Tawaha *et al.* [34] assessed the antioxidant activity of selected plant species such as rosemary, marjoram and salvia by $ABTS^{+}$ method, obtaining in all cases lower values (of the order of μmol TE/g DW) than those found for perilla in this study. In addition, Hossain *et al.* [33] reported that the ORAC values for basil and parsley were 17.57 and 13.25 g TE/100 g DW, respectively. This value is much lower than those found in the current study.

*3.3. Quantitative Analysis of Cinnamic Acid Derivatives*

Lately, phenolic compounds, such as rosmarinic acid or caffeic acid have aroused increasing interest due to their antioxidant activity, which improves the stability of lipid-containing foods [26,37] and their possible beneficial effects on human health [38]. The concentrations of rosmarinic acid (2.29 ± 0.09 mg/g DW) and caffeic acid (0.51 ± 0.02 mg/g DW) were compared with those reported in the literature (Table 3).

**Table 3.** Content of rosmarinic acid and caffeic acid in the perilla extracts (mg/g DW).

| Rosmarinic acid | Caffeic acid | Solvent | Place of cultivation | Reference |
|---|---|---|---|---|
| 39.5 | ND | Water:acetone:hydrochloric (20:80:1) | Japan | Natsume *et al.* [39] |
| 3.4–10 | 0.05–1.2 | Water with 0.01 M $H_2SO_4$ | China and Japan | Meng *et al.* [36] |
| 0.21–3.76 | ND | 70% EtOH | Geochang, Korea | Hong and Kim [17] |
| 51.37–155.50 | ND | MeOH with ethyl acetate fraction | Geochang, Korea | Hong *et al.* [32] |
| 29.28–54.76 | 1.09–3.86 | MeOH with 1% TFA | Yeongnam, Korea | Kang and Lee [40] |
| 26.84 | 1.32 | Water at 100 °C | Miryang, Korea | Yang *et al.* [38] |
| 2.29 | 0.51 | 50% EtOH | Spain | This paper |

It is well known that the different extraction conditions lead to different amount of polyphenols in plant extracts. Although many studies have reported the content of cinnamic acid derivatives in *P. frutescens*, researches on the content of the main phenolic compounds in perilla cultivated in Europe has not been measured yet.

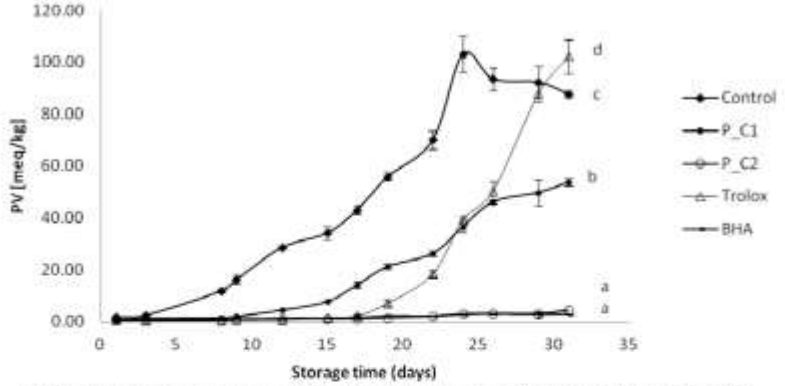
The utilization of perilla opens interesting possibilities for the development of functional foods not only in Asian countries, but also in Europe. Moreover, the content of cinnamic acid derivatives in perilla extract was higher or similar to those reported by Lee [41] in the dried plants (*Lamiaceae*), such as basil, marjoram, oregano, rosemary, or thyme, which are common culinary herbs.

### *3.4. Antioxidant Activity of Extracts in Model Emulsion System*

In addition to their effects on human health upon direct ingestion, one of the main applications of antioxidant extracts is their use in the food industry to avoid or delay oxidation of perishable systems [42]. In this study, this technological ability has been assessed in oil-in-water emulsions as a model food used to test the 50% ethanol extract of perilla. The oxidation was periodically followed by measurement of peroxide value (as indicator of primary oxidation products) and TBARs value and hexanal content (as indicator of secondary oxidation products) during storage at 32 ± 1 °C for 30 days. In addition the change in pH was monitored, as 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 six days. The next samples to reach this level of deterioration were P_C1 perilla at 80 ppm (after 15 days) and Trolox at 40 ppm (after 19 days). Other samples: P_C2 and BHA were stable until the end of the experiment (after 30 days, PV was <10 meq/kg). Perilla extract at 320 ppm (P_C2) was as effective as BHA at 20 ppm in preventing the oxidation in emulsions during storage. The mean peroxide value recorded for control emulsions after 24 days, was more than twice the value obtained for emulsions containing the perilla extract at 80 ppm and Trolox at 40 ppm (control: 103.17 meq/kg; perilla C1: 36.55 meq/kg; Trolox: 39.42 meq/kg). Kiokias *et al.* [43] 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. Although the time needed to reach these values was remarkably shorter in this study, the concentration of antioxidant extracts was much lower as well (80 and 320 ppm). Kiokias and Varzakas [44] reported that a 10% cottonseed oil-in-water emulsion with quercetin at 1.5 mmol/kgr took 2.7 days at 60 °C to reach a PV of 67.07 meq/kg. In addition, Maisuthisakul *et al.* [45] reported that a 10% oil-in-water emulsion with 100 mg/kg teaw (*Cratoxylum formosum* Dyer) extract took 4.55 days at 60 °C to reach a PV of 50 meq/kg. In this work, the emulsion containing 80 ppm of perilla extract exceeded this value at the 30th day at 32 °C. Ramful *et al.* [9] 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.* [46] reported that tea extracts added to sunflower oil-in-water emulsion were very effective in their 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. In the same study the rosemary extract (0.03%) in the early stages of storage under these conditions had moderate antioxidant activity up to PV of 20 meq/kg.

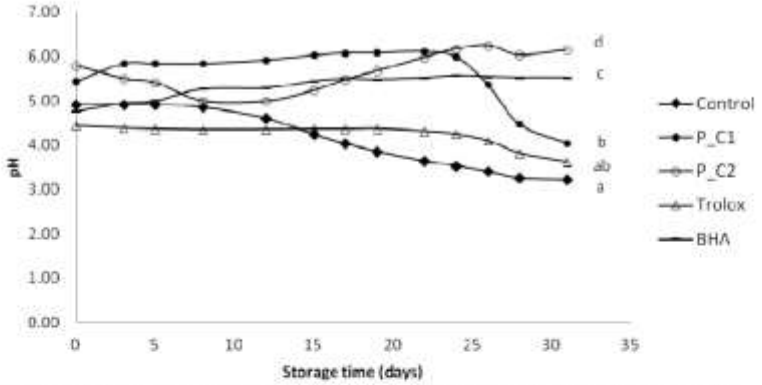
**Figure 1.** Evolution of primary oxidation (peroxide value) in model food system (O/W emulsion 10% of oil) with different concentration of perilla ethanolic extracts (C1: 80 ppm and C2: 320 ppm).




[a–d] Values with different letters at 30th day of storage are significantly different ($p < 0.05$).

Hydroperoxides are the main primary products of lipid oxidation, but they are highly unstable and easily break down into secondary compounds, resulting in the appearance of aldehydes, ketones, epoxides, or organic acids, which may lead to changes in the pH [46]. In addition, since it is known that many antioxidant molecules are less effective when the pH is low [47], this parameter was also measured as a potential indicator of oil-in-water emulsions oxidation. From an initial average value of 5.12, the samples without any antioxidant addition and with Trolox tended to stabilize their pH at 3.22 and 3.62, respectively after 30 days (Figure 2). In the P_C1, P_C2 and BHA samples the pH slowly increased during storage, but in P_C1 pH it decreased rapidly after 24 days, reaching the value of 4.05. Observing this relationship confirmed that the pH fell as PV increased. Gallego *et al.* [12] reported that following the order of primary oxidation, the pH showed a decline (from 6 to 3) proportional to the rate of oxidation in oil-in-water emulsion with 100 ppm of different extracts from rosemary, thyme, and lavender. Sorensen *et al.* [48] also reported that lipid oxidation increased when pH was decreased from 6 to 3 in a 10% oil-in-water emulsion. Moreover, Zhou *et al.* [49] found that the pH appeared to play a significant role in controlling the net antioxidant and pro-oxidant capacity of polyphenols in lipid model systems. The addition of (−)-epigallocatechin-3-gallate (EGCG) at 5–100 μM to food emulsions was observed to exhibit pro-oxidant activity in low pH (pH 2–4). On the other hand at higher pH values studied (pH 5–7), lower levels of primary and secondary oxidation products were detected in samples with 25–500 μM EGCG.

**Figure 2.** Evolution of pH in model food system (O/W emulsion 10% of oil) with different concentration of perilla ethanolic extracts (C1: 80 ppm and C2: 320 ppm).

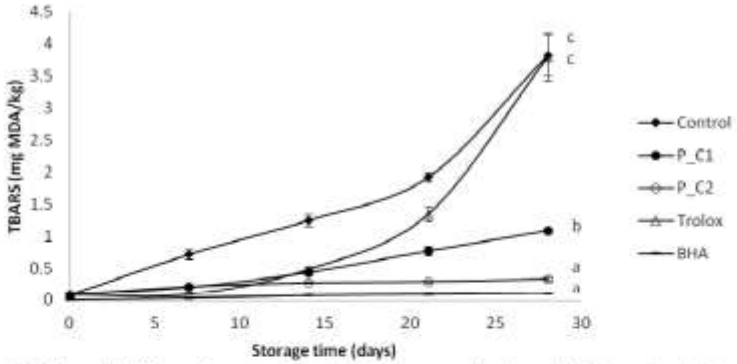



[a–d] Values with different letters at 30th day of storage are significantly different ($p < 0.05$).

In the secondary oxidation stage, volatile compounds (e.g., alcohols and aldehydes) are formed by the decomposition of lipid hydroperoxides. In particular, volatile aldehydes have great importance as an indicator of oxidation due to their considerable contribution to the aroma and flavor deterioration of the final product [50]. Secondary oxidation products in the emulsions were monitored by measurement of the TBARs (Figure 3) and the hexanal content (Figure 4). After four weeks, TBARs values of emulsions containing perilla extract and BHA were lower than that of the control (3.82 mg MDA/kg) and the Trolox-containing sample (3.57 mg MDA/kg). BHA was the most effective antioxidant followed by perilla extract P_C2 and P_C1. Garcia-Iñiguez *et al*. [13] 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. In addition, Poyato *et al*. [14] reported that in olive oil-in-water emulsions after 48 h of storage at 65 °C, TBARs value was stable and low, with no differences between lyophilized aqueous extract of lemon balm (477 ppm) and BHA (200 ppm). Dimakou and Oreopoulou [51] found that polar (paprika, marigold, bixin) and hydrophobic (β-carotene, lycopene) carotenoids exerted antioxidant effect measured by TBARs test during thermal accelerated autoxidation (60 °C) of sunflower oil-in-water emulsions, stabilized by Tween 20.

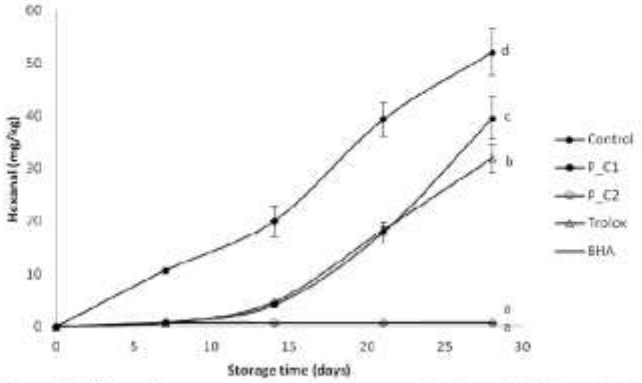
Similar to TBARs, the results of hexanal content after four weeks of storage showed that BHA and perilla extract (P_C2: 320 ppm) were the most effective antioxidant, followed by Trolox and P_C1. The protective effect of *P. frutescens* should be attributed to its content of the well-recognized antioxidant, like hydroxycinnamic acids and possibly other polyphenols. In the literature, other authors have described the antioxidant effect of caffeic acid and rosmarinic acid in model food emulsions. Caffeic acid (5 mmol/kg emulsion) showed good antioxidant properties in both 30% sunflower oil-in-water and 20% water-in-sunflower oil emulsions (pH 5.4) during storage at 50 °C [50]. In the model corn oil-in-water emulsions rosmarinic acid (50 μM) inhibited hexanal formation during storage at 55 °C for 24 days [1].

**Figure 3.** Evolution of secondary oxidation (TBARs) in model food system (O/W emulsion 10% of oil) with different concentration of perilla ethanolic extracts (C1: 80 ppm and C2: 320 ppm).




a–c Values with different letters at 30th day of storage are significantly different ($p < 0.05$).

**Figure 4.** Evolution of secondary oxidation (hexanal content) in model food system (O/W emulsion 10% of oil) with different concentration of perilla ethanolic extracts (C1: 80 ppm and C2: 320 ppm).




a–d Values with different letters at 30th day of storage are significantly different ($p < 0.05$).

In the present study positive correlations between TBARs and hexanal ($R^2$ = 0.9106) levels and also between PV and both TBARs and hexanal ($R^2$ = 0.9446 and $R^2$ = 0.9431, respectively) levels in oil-in-water emulsions were found.

Different extracts of *P. frutescens* have demonstrated free-radical scavenging [17,31,36,40] and antioxidant action in rats [38,52]. These properties of perilla have been associated with cinnamic acid derivatives and other polyphenolic compounds. Caffeic acid (CA) and rosmarinic acid (RA) are the major compounds in *P. frutescens* that showed the hepatoprotective effect against t-BHP-induced

oxidative liver damage. *In vitro* and *in vivo* treatments with perilla leave extracts and with combined CA and RA gave almost the same efficacy of liver protection against oxidative stress. *In vivo* treatment of combined CA and RA resulted in a more than proportional increase in antioxidant enzymes and reduced levels of indicators of hepatic toxicity, compared to CA only treatment suggesting that the stronger hepatoprotective effect of perilla is brought about by the combination of CA and RA. In an *in vivo* study, samples pretreated with 1000 mg/kg of body weight (BW) of perilla extract showed no signs of toxicity. After the conversion of the effective dosage in rats into a dose based on the surface area of humans they obtained the perilla extract equivalent in humans of 162 mg/kg BW, which equates to 9.7 g of perilla extract o 205 g of perilla fresh leaves [38].

The concentration of the perilla extract was selected so as not to exceed the non-toxic pharmacological doses. Therefore, the addition of 320 mg/kg of perilla extract to food emulsions would serve a twofold purpose: (i) to support the pharmacological doses of these extracts by using those food emulsion system as vectors; and (ii) to improve the fat stability and, hence, the nutritional properties of those emulsion systems.

## 4. Conclusions

This study showed that the extract of *Perilla frutescens* is a potential source of natural antioxidant to be used as a lipid oxidation inhibitor in food industry. In addition, food emulsions appear to be useful vectors in supplying the daily dosage of *P. frutescens* extract in consumers, which may positively affect their health. Further research into the enrichment of food products with bioactive substances extracted from *P. frutescens* should be conducted because there is still not sufficient knowledge about their activity during food processing, nor about their interactions with other food components.

## Acknowledgments

The authors would like to thank Pàmies Horticoles for kindly supplying us the plants. The authors also thank Margarita Dâvila, Esther Miralles, Pilar Teixidor and Lourdes Berdié for technical support.

## Conflicts of Interest

The authors declare no conflict of interest.

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

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*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 Pànies Horticoles. 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, $ABTS^{\bullet}$, 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 |

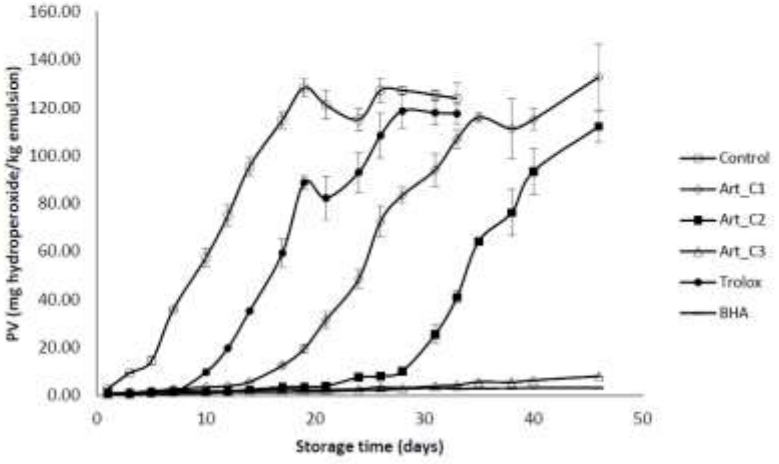
### *3.2. Antioxidant Activity of Extracts in Model Emulsion System*

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.

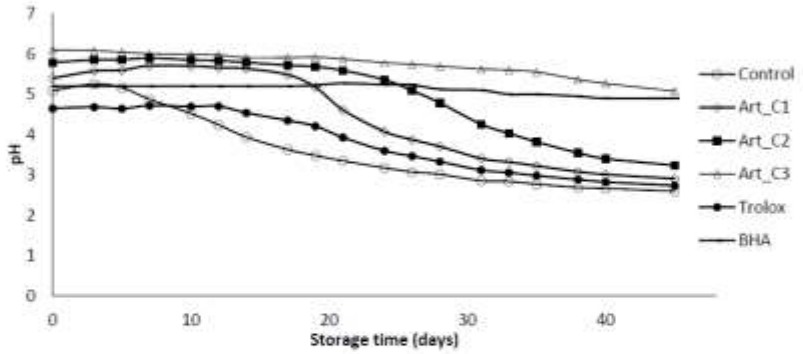
**Figure 1.** Evaluation of primary oxidation (peroxide value) in a model food system (O/W emulsion 10% of oil) with different concentrations of *A. annua* (C1: 0.20 g/L; C2: 0.65 g/L and C3: 2 g/L).




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).



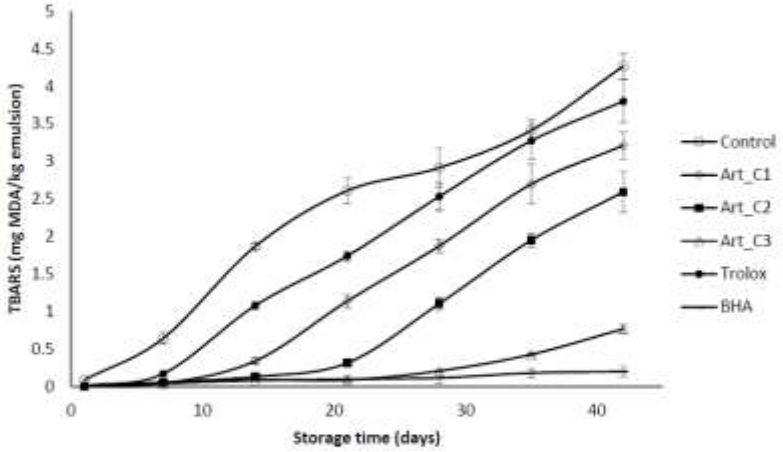

Secondary oxidation products in the emulsions were monitored by measurement of the TBARs (Figure 3). After 6 weeks, TBARs values in emulsions containing *A. annua* extracts and BHA were lower than that those in the control (4.27 mg MDA/kg) and the Trolox-containing sample (3.80 mg MDA/kg). BHA was the 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 Horticoles for kindly supplying us the plants.

## Conflicts of Interest

The authors declare no conflict of interest.

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*Antioxidants* **2014**, *3*, 455-471; doi:10.3390/antiox3020455

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ISSN 2076-3921

www.mdpi.com/journal/antioxidants

*Article*

# Screening of Antioxidant Activity of *Gentian Lutea* Root and Its Application in Oil-in-Water Emulsions

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*Received: 25 January 2014; in revised form: 10 April 2014 / Accepted: 12 June 2014 / Published: 19 June 2014*

**Abstract:** *Gentiana Lutea* root (*G. Lutea*) is a medicinal herb, traditionally used as a bitter tonic in gastrointestinal ailments for improving the digestive system. The active principles of *G. Lutea* were found to be secoiridoid bitter compounds as well as many other active compounds causing the pharmacological effects. No study to date has yet determined the potential of *G. Lutea* antioxidant activity on lipid oxidation. Thus, the aim of this study was to evaluate the effects of an extract of *G. Lutea* on lipid oxidation during storage of an emulsion. *G. Lutea* extracts showed excellent antioxidant activity measured by DPPH scavenging assay and Trolox equivalent antioxidant capacity (TEAC) assays. An amount of 0.5% w/w *G. Lutea* lyophilise was able to inhibit lipid oxidation throughout storage ($p < 0.05$). A mixture of *G. Lutea* with 0.1% (w/w) BSA showed a good synergic effect and better antioxidant activity in the emulsion. Quantitative results of HPLC showed that *G. Lutea* contained secoiridoid-glycosides (gentiopiocroside and sweroside) and post column analysis displayed radical scavenging activity of *G. Lutea* extract towards the ABTS radical. The results from this study highlight the potential of *G. Lutea* as a food ingredient in the design of healthier food commodities.

*2.4. Determination of Free Radical Scavenging Activity Assays*

2.4.1. TEAC Assay

The antioxidant capacities of *G. Lutea* were measured by using a modified TEAC assay, which was performed as described by Miller *et al.* [20]. The TEAC assay was based on the reduction of the $ABTS^{\bullet+}$ radical cation by the antioxidants present in the samples. $ABTS^{\bullet+}$ radical cation (7 mM, final concentration) was dissolved before adding potassium sulphate (2.45 mM, final concentration) and allowing the mixture to stand in the dark up to 16 h. Phosphate Buffer Solution (PBS, 10 mM) with the $ABTS^{\bullet+}$ radical cation was incubated at room temperature for 30 min before used. Then, the mixture of the $ABTS^{\bullet+}$ radical cation was adjusted to an absorbance of 0.73 ± 0.2 nm, using a microplate reader (Fluostar Omega, BMG Labtech, Ortenberg, Germany). The TEAC values for the different concentrations of each compound were interpolated from the trolox calibration curve and expressed as milligrams of trolox equivalent per gram of dry weight sample (mg TE/g DW sample).

2.4.2. DPPH Assay

The effect of the extracts on the scavenging of DPPH radical was determined according to the method adapted from Madhujith *et al.* [21] with slight modifications. The sample was diluted 1:20 (v/v) and DPPH radical in methanol (5.07 mM) was made for the study. Then, the sample (10% v/v) and DPPH solution (90% v/v) were added to the well of the microplate. The absorbance was measured at 517 nm over every 15 min for 75 min. The results were expressed as mg TE/g DW sample.

2.4.3. Superoxide Activity Xanthine/Xanthine Oxidase (X/XO)

The method was based on the developed method of Valentao *et al.* [22] and modified for application in microplates by Lopez *et al.* [23]. All test samples were dissolved in a 50 mM phosphate buffer to simulate the environment in which the reaction occurs in the body. The sample was mixed with 145 µM of a solution of xanthine, 50 µM of a solution of NBT and incubated in 37 °C. The sample extract was diluted from 1:10 to 1:100 (v/v) for the study. Finally, 0.29 U/mL of enzyme xanthine oxidase solution was added and the absorbance was recorded at 560 nm every 2 min. The value of $IC_{50}$ was calculated to determine the inhibition rate of *G. Lutea* in the reaction.

*2.5. Determination of Antioxidant Activity in o/w Emulsion*

2.5.1. Removal of Tocopherols from Sunflower Oil

Alumina was placed in an oven at 200 °C for 24 h, and then removed and allowed to cool in a desiccator until it reached room temperature. Sunflower oil triacylglycerol was passed twice through the alumina in a column to remove the tocopherols as described by Yoshida *et al.* [24]. Finally, the filtered oil was stored at −80 °C until use.

## 2. Experimental Section

### *2.1. Plant Material*

Commercially dried *G. Lutea* was kindly supplied by Manatial de la Salut (Barcelona, Spain), a registered herbal company. Reagents used were: thiobarbituric acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, methanol, hydrogen chloride, aluminium oxide, ferrous chloride, anhydrous sodium carbonate, ethanol 96%, Phosphate Buffer Solution (PBS) and ammonium thiocyanate from Panreac (Barcelona, Spain). Gallic acid, 2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2–carboxylic acid (Trolox), NBT (nitrobluetetrazolium), Bovine Serum Albumin (BSA), Xanthine and Xanthine-oxidase from Sigma-Aldrich (Gillingham, UK).

### *2.2. Extraction of G. Lutea*

Dried roots of *G. Lutea* were finely ground using a standard kitchen food processor. Ground *G. Lutea* (5 g) was extracted in two ways; (1) with 50:50 (v/v) methanol:water and (2) with water, always in the ratio 1:10 (w/v). The extraction was performed at 4 ± 1 °C for 24 h, in the dark with constant stirring. The extract solutions of *G. Lutea* were recovered by filtration using Whatman Filter paper, 0.45 µm. 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 methanol was removed under vacuum using a rotary evaporator (BUCHI RE111, Postfachi, Switzerland) and kept frozen at −80 °C for 24 h. All extracts were dried in a freeze dryer (Unicryo MC2L −60 °C, Martinsried, Germany) under vacuum conditions at −60 °C for 3 days to remove moisture. Finally, *G. Lutea* lyophilize (freeze dried) were weighed to determine the concentration recovered (g/L) and the extraction yield (%) as Zhang *et al*. [18]. Samples were then weighed and kept protected from light in a desiccator until use.

### *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 Santas *et al*. [19]. The sample was diluted 1:25 (v/v) in order to be in the range of absorbance. The final concentration (v/v) for the mixture was; sample 7.7%, Folin reagent 4% and saturated sodium carbonate solution 30.8%. The mixture was finally diluted with Mili Q water, shaken and incubated in the dark for 1 h. Absorbance at 765 nm was measured using a microplate reader (Fluostar Omega, BMG Labtech, Ortenberg, Germany) against water as a blank. Gallic acid was used to prepare a standard calibration, and the results were expressed as mg of Gallic acid equivalents/g dry weight (mg GAE/g DW).

2.5.2. Preparation of Emulsion

Oil in water emulsion was prepared by dissolving Tween-20 (1%, final concentration) in Milli Q water and adding oil (10%, final concentration). To form an emulsion, the oil was added drop wise to the solution of Tween-20 and water, which was kept cold, and sonication process was continued for 5 min. All samples were redissolved in ethanol-50% (v/v) to obtain the final concentration in the emulsion. The final samples were prepared either (i) control (no addition); (ii) 0.35% (w/w) Trolox (positive control); (iii) 0.1% (w/w) BSA; (iv) 0.5% (w/w) lyophilise *G. Lutea*; (v) 0.5% (w/w) lyophilise *G. Lutea* mixed with 0.1% (w/w) BSA; (vi) 0.2% (w/w) lyophilise *G. Lutea* and (vii) 0.2% (w/w) lyophilise *G. Lutea* mixed with 0.1% (w/w) BSA. The emulsion for each sample was prepared in quadruplicate, obtaining a total of 28 samples and stored in the dark and allowed to oxidize at 37 °C. The pH of the samples was measured four times for each sample (pH meter GLP21, Crison Instruments, Barcelona, Spain) as a parameter to investigate its correlation with PV.

2.5.3. Determination of Peroxide Value (PV)

The primary oxidation products were measured using peroxide value (PV) according to the thiocyanate method of the Association of Official Analytical Chemists (AOAC) 8195 [25]. Ferrous chloride solution was prepared in hydrochloric acid (1 M) with the addition of iron chloride (II) (2 mM, final concentration). Ammonium thiocyanate solution was prepared in water (2 mM, final concentration). The assay was performed with a drop of emulsion in the range from 0.007 to 0.01 g, diluted with ethanol. From this solution the required amount of sample, varying according to the degree of oxidation, was taken in a cuvette and ethanol (96%) was added. Ferrous chloride and ammonium thiocyanate solutions were added, each in a proportion of 1.875% (v/v), final concentration. The absorbance was measured spectrophotometrically at $\lambda$ = 500 nm. The results are expressed as meq hydroperoxides/kg of emulsion.

2.5.4. Determination of Secondary Oxidation by Thiobarbituric Acid Reactive Substances (TBARS)

The TBARS method was adapted from Gallego *et al.* [26]. The TBARS reagent was prepared (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid and hydrochloric acid 2.1% v/v). One mL of each emulsion was taken and the TBARS reagent was added in the ratio 1:5 (v/v). Immediately the samples were added to an ultrasonic bath (5 min) and after immersing in a water bath preheated to 95 °C (20 min) the samples were centrifuged and the absorbance of the supernatant was measured at $\lambda$= 531 nm. The results are expressed as mg malondialdehyde (MDA)/kg of emulsion.

*2.6. Statistical Analysis*

Statistical analysis was performed using a one-way analysis of variance ANOVA using Minitab 16 software program (Minitab, Inc., Paris, France). When a statistically significant difference was found, Tukey's tests were performed and the statistical significance was set at $p < 0.05$. The results were presented as mean values ($n \geq 3$).

*2.7. HPLC and Post-Column HPLC-ABTS$^{\bullet+}$ Radical Scavenging Method*

The method for identification of peaks with antioxidant activity was that used by Koleva *et al.* [27] with some modifications. The instrument was a Waters 2695 separations module (Meadows Instrumentation Inc., Bristol, USA) system with a photodiode array detector Waters 996 (Meadows Instrumentation Inc., Bristol, USA). The column used was a Kinetex C18 100A, (100 × 4.6 mm, Phenomenex, Torrence, CA, USA). Solvents used for separation were 0.1% acetic acid in water (v/v) (eluent A) and 0.1% acetic acid in methanol (v/v) (eluent B). The gradient used was isocratic, 75% A. The flow rate was 0.6 mL/min. Detection wavelength was 230 nm (to see the peaks) and 734 nm (to see the ABTS radical). The sample injection volume was 10 µL. The chromatographic peaks of gentiopicroside and sweroside were confirmed by comparing their retention times and diode array spectra with that of their reference standards. The pump for ABTS post-column injection was a Merk-Hitachi HPLC gradient pump (Model L-6200, Hitachi High Technologies America Inc., Schaumburg, Illinois, IL, USA) with a 0.2 mL/min flow; ABTS concentration was of 0.03% (w/v).

## 3. Results and Discussion

*3.1. Analysis of Total Polyphenols and Free Radical Activity Assays*

On average, from 5 g of dried *G. Lutea* extracted with aqueous methanol 50:50 (v/v) and water alone, it was possible to recover 1.5 ± 0.05 g and 1.0 ± 0.04 g of lyophilised, respectively. The concentration recovered was proportional to the extraction yield shown in Table 1. Previous studies reported that gentiopicroside compound, an active compound that signifies the main bitter principle in *G. Lutea* was still preserved at almost 83.5% after drying [28].

**Table 1.** Extraction yield, total phenolic content (TPC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Trolox equivalent capacity assay (TEAC) and enzymatic activity of *G. Lutea*.

| Activity *G. Lutea* | Extraction Solvent | |
|---|---|---|
| | $H_2O$ | 50:50 MeOH:$H_2O$ |
| Extraction yield (%) | 20.00 ± 0.9 | 29.10 ± 0.3 |
| Total phenolic content (g GAE/g DW) | 3.79 ± 1.7 | 12.03 ± 1.8 |
| DPPH (µmol of TE/g DW) | 12.34 ± 1.5 | 15.89 ± 0.5 |
| TEAC (µmol of TE/g DW) | 33.28 ± 1.5 | 48.90 ± 1.8 |
| Superoxide activity (mg/mL) | 30.00 ± 2.8 | 23.21 ± 2.8 |

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

The concentration of total polyphenols and the value of antioxidant activity assays were determined and the results are shown in Table 1. The extract of *G. Lutea* in methanol-50% showed higher phenolic content and antioxidant activity than the water extract. The total phenolic content of *G. Lutea* extracts allowed the estimation of all phenolic acids, flavonoids, anthocyanins, nonflavonoids and many classes

of polyphenol compounds present in the samples. On the other hand, Nastasijevic *et al*. determined the total polyphenol content of *G. Lutea*in in water extract as being slightly higher compared to different concentrations of aqueous ethanol and methanol extracts [10].

Water extracts of *G. Lutea* showed the lowest activity in free DPPH$^{\bullet}$ scavenging activity compared to methanol-50% extract, similar to previous research from Kintzios *et al*. [14]. It is not the first time that the antioxidant activity of *G. Lutea* by DPPH method has been carried out. However, variation in results may be due to the plant age, solvent, method and system used throughout the experiment [10,14,29].

For the TEAC assay, the finding was consistent with the DPPH method where the aqueous methanol extract showed higher activity than the water extract. TEAC assay indicated the extract potency used as a source of antioxidants based on the ability of the antioxidant compound to scavenge the long-life radical cation ABTS$^{\bullet+}$. In the results shown in Table 1 it can be appreciated that the methanol-50% extract has a higher capacity to scavenge ABTS$^{\bullet+}$ radicals and consequently shows a higher antioxidant activity than DPPH assay. To the best of our knowledge, this is the first report of the antioxidant activity of extracts from *G. Lutea* roots assessed using the TEAC methods.

Some of the previous reports showed that the antioxidant activity of plant extracts correlates with the phenolic content [30] and the yield of phenolic components from herbs is higher with methanol-50% rather than with water as extract. The mixtures of alcohol and water have been more efficient in extracting compounds and give a better yield than the corresponding mono-component solvent system. Xanthones such as isogentisin and gentisin and its derivatives are one of main sources of phenolic compounds in *G. Lutea* and are expected to be more soluble in aqueous alcohol. A study showed good correlation between phenolic content and antioxidant activity [31] whereas another found no correlation [32].

In the present work, an effective antioxidant activity in *G. Lutea* was found. Methanol-50% extract exhibited $O_2^{\bullet-}$ scavenging activity, measured using the X/XO system (Table 1), with an $IC_{50}$ at 23.21 ± 2.8 mg/mL. Water extract of *G. Lutea* showed lower scavenging activity than methanol aqueous extract, with $IC_{50}$ = 30.00 ± 2.8 mg/mL. These results are consistent with Kusar *et al*. [29], who demonstrated the effect of superoxide activity of *G. Lutea* leaf and root in methanol extracts, with $IC_{50}$ inhibition value of 11.1 mg/mL and 8.2 mg/mL, respectively. Kusar and co-workers' findings were accomplished by X/XO reaction mixture with DEPMO-OOH scavenger that transformed the reaction to a stable radical measured by electro spin resonance (ESR). Valentao *et al*. [22] observed the phenolic acids (*p*-coumaric acid, ferulic acid, sinapic acid and kaempferol) exhibited superoxide scavenger activity and an inhibitory effect on XO. Considering the results obtained from TPC assay, it may be anticipated that *G. Lutea* extract has antioxidant activity achieved by the scavenging of superoxide radical and XO inhibition.

### *3.2. Antioxidant Effect in Stored o/w Emulsion*

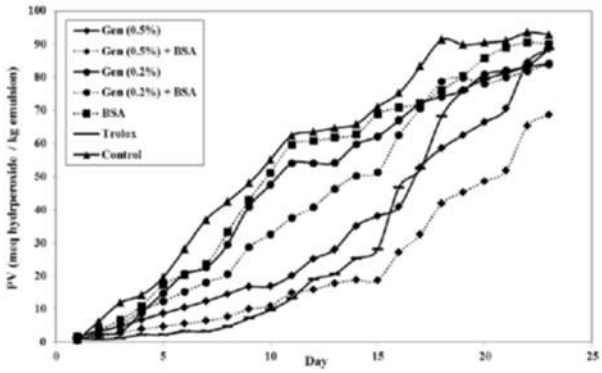
Many strategies on laboratory scale have been developed to improve the stability of shelf life in food models including adding a minimum amount of natural plants to delay the oxidation rate. The effect of *G. Lutea* on inhibiting lipid oxidation in oil-in-water (o/w) emulsion as a food model has not been described. In this study it also has been carried out to determine the synergic effect of *G. Lutea*

with BSA in o/w emulsions. The oxidation in o/w emulsions was measured in two stages of oxidation; primary oxidation product (Peroxide Value) and secondary oxidation products (TBARS). In addition the change in pH was monitored, since pH tends to fall during oxidation.

3.2.1. Evolution of Peroxide Value (PV)

Figure 1 shows the evolution of PV *vs.* time. The control (without extract added) showed the highest oxidation throughout the storage time followed by the emulsion with only BSA (0.1%). The sample containing Trolox (0.35%, positive control) and the samples containing extracts, were not oxidized during the first 10 days. They show significant difference from the control ($p < 0.05$). The time required for the emulsions to reach a peroxide value of 10 meq hydroperoxides/kg of emulsion was determined as a standard to measure the stability of emulsion. The limits of fat product (animal, plant and anhydrous) margarine and fat preparation were set <10 meq hydroperoxides/kg as a guarantee of the product quality [33]. When the peroxide value of the sample is measured as greater than 15 meq hydroperoxide/kg, the sample is considered rancid, which may alter the color, taste and nutritional quality due to the deterioration of the lipid. The control was the first sample to reach 10 meq hydroperoxides/kg of emulsion which occurred rapidly in two days. The emulsion with BSA exhibited a similar deterioration rate to the control, revealing that BSA, in this concentration of 0.1%, does not provide any antioxidant effect in the emulsions. Positive control samples (Trolox) showed good antioxidant effect over 11 days and begin to oxidize rapidly after 15 days, reaching 88 meq hydroperoxides/kg emulsion on the final days of the experiment.

**Figure 1.** Change of peroxide value over time stored at 37 °C (each value is expressed as mean ($n = 3$)).




Adding 0.2% *G. Lutea* to the emulsion, with or without adding BSA, did not result in any relevant effect towards oxidation in the first stage (PV <10 meq hydroperoxides/kgin time <3 days). There is a significant difference between 0.2% antioxidant sample with BSA and in its absence ($p < 0.05$). The sample with 0.5% *G. Lutea* showed antioxidant activity towards lipid degradation first at 15 days and gradually oxidized after 15 days ($p < 0.05$). Finally, the sample with *G. Lutea* 0.5% and BSA 0.1%

displayed the lowest PV, with significant differences with the other samples throughout storage time ($p < 0.05$); it took almost 10 days to reach above 10 meq hydroperoxides/kg of emulsion.

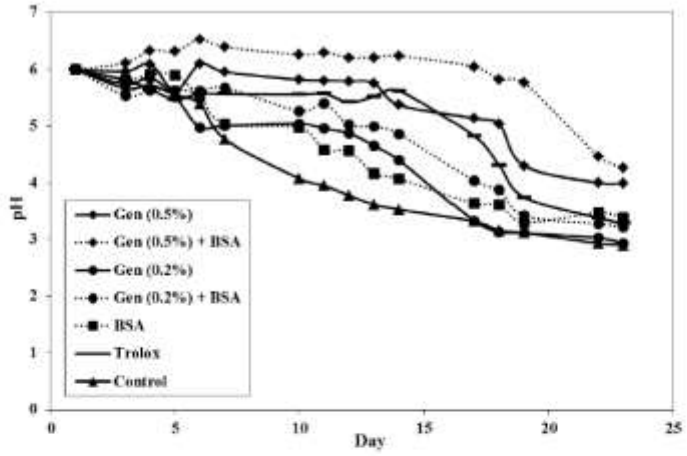
Almajano *et al.* [34] reported that some antioxidant compounds such as EGCG and caffeic acid mixed with BSA cause a marked increase of the antioxidant activity in an emulsion. Since BSA is known to be surface active [35], the increase of antioxidant activity in emulsions containing a mixture of antioxidant and BSA could be due to BSA binding with the antioxidant and transporting it to the oil water interface, where it is highly effective in reducing the rate of oxidation. The authors also stated that the antioxidant molecule had bound to the BSA protein, proved by TEAC assay, and showed a progressive increase in the radical scavenging $ABTS^{\bullet+}$ with the storage time over several days [34]. The mixture of 0.1% BSA and 0.5% *G. Lutea* in emulsion exhibit the lowest oxidation rate compared to all samples shown in this experiment. These results showed for the first time the important effect of *G. Lutea* extract on lipid oxidation with synergic effect to BSA tested in an o/w emulsion. This concentration of 0.5%, demonstrated the best antioxidant effect throughout the storage period.

3.2.2. Evolution of pH over Time

Since decomposition of hydroperoxide measured in PV assay is acidic, the pH change in the sample is considered inversely proportional to the PV. Thus, the pH measured is a parameter of which its correlation with PV can be investigated. Antioxidant activity in food models is less effective under low pH conditions. However, some antioxidant compounds such as carnosic acid and carnosol (found in rosemary) have been reported to have high antioxidant activity at lower pH, which is at pH 4–5 [36]. Figure 2 shows the changes of pH value on emulsions over 23 days storage. Overall, the decrease in pH value throughout storage was of a similar order with increased primary oxidation measured in the PV assay. These results agreed with the studies done by Frankel *et al.* [16]. They found that the lipid oxidation in emulsion is slower at higher pH, and decreased when oxidation is accelerated. All samples started nearly at neutral pH and 0.5% *G. Lutea* with 0.1% BSA showed the highest value throughout storage. Similar to PV, the pH of the sample with 0.2% *G. Lutea* with or without BSA showed higher pH than control but the value was not significant throughout storage ($p > 0.05$). The pH of 0.5% *G. Lutea* with BSA and 0.5% *G. Lutea* alone, showed significant differences compared to all samples during storage period ($p < 0.05$). The behavior of pH of the sample with 0.5% *G. Lutea* with BSA was stable until 19 days before it started to decrease.

Skowyra *et al.* [37] demonstrated that the pH and PV have the best correlation with $R^2 = 0.9648$. Our results are in agreement with them and it can be described that the antioxidant activity in o/w emulsion which is stable at pH 6 showed an inverse relationship at a lower PV value. Some authors also reported a similar agreement of pH change which was inversely proportional to the lipid oxidation [26,38,39]. Meanwhile, Mancuso *et al.* [40,41] suggested the initial oxidation of emulsion depended on pH, by varying the effect of emulsifier. The authors observed a higher oxidation rate occurring at pH 7 rather than pH 3 o/w emulsion. Results may be due to the iron solubility increasing at low pH and allowing iron to be partitioned into the continuous phase, whereas insoluble iron at high pH may precipitate onto the emulsion droplet surface resulting in an increase in the lipid oxidation.

**Figure 2.** Change of pH over time, stored at 37 °C (each value is expressed as mean ($n = 3$)).



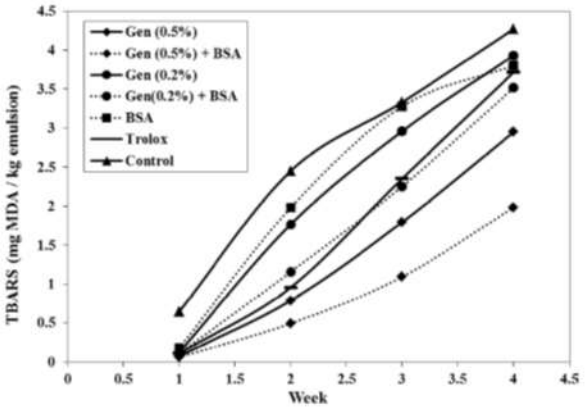

3.2.3. Evolution of Thiobarbituric Acid Reactive Substances (TBARS)

One of the compounds produced from secondary oxidation in lipids is MDA (malondialdehyde) which can be measured by the TBARS method. The secondary lipid oxidation is responsible for the alteration of flavor, rancid odor and the undesirable taste in foods [42]. Secondary oxidation products were monitored by TBARS assay and are shown in Figure 3. Similar to PV, the control had the most rapid increase in TBARS followed by the BSA sample. TBARS values for samples treated with 0.5% gentian powder, with and without BSA, experienced below 1.2 mg MDA/kg sample over the first 21 days and showed prominently lower than positive control up to 4 weeks ($p < 0.05$). The sample with 0.2% of *G. Lutea* alone does not display significant delay in lipid oxidation ($p > 0.05$), meanwhile 0.2% *G. Lutea* with BSA and positive control showed significant different during 20 days ($p \leq 0.05$). From the TBARS results exhibited, the synergic effect between both the concentration of *G. Lutea* and BSA in the emulsion during the storage time is demonstrated and the samples with both (gentian and BSA) show the lowest oxidation rate compared to all samples.

After 23 days, all samples are oxidized with above 1.2 mg malondialdehyde/kg sample even though *G. Lutea* with the BSA mixture showed minimum rise and had the best antioxidant effect in the emulsion. This behavior is not new. It has been previously reported that artificial antioxidants such as Trolox, epigallocatechingallate (EGCG), caffeic acid are more stable in emulsions during storage in the presence of BSA than in its absence [34].

*G. Lutea* has compounds which of family of iridoids, scoiridoids and xanthones [3]. It could be developed as antioxidant agent and radical scavengers and may contribute to the decrease of lipid oxidation [43]. The water soluble antioxidant molecular structures differ in the number of phenolic hydroxyl groups, their location and the carboxylic acid group [34]. Thus, the range of structure presented may allow any possible interaction with the BSA to be detected.

**Figure 3.** Change of Thiobarbituric Acid Reactive Substances (TBARS) over time, stored at 37 °C (each value is expressed as mean ($n$ = 3)).




Many findings determined such amount of natural plants work as antioxidant efficiently; depended on variation of system tested and plant preparation. However, the more important is to identify the minimum concentration required to reduce lipid oxidation significantly throughout storage time. Adding minimum amount of natural plant not only can delay lipid oxidation, but also to avoid some changes on sensorial quality and flavoured. Analysis on our data showed for *G. Lutea* required as minimum 0.5% (w/w) would be beneficial for reducing the velocity of lipid oxidation significantly in both primary and secondary oxidation in emulsion system ($p < 0.05$). Adding also 0.1% (w/w) of BSA gave better effect of the antioxidant activity towards emulsion, compare to sample with *G. Lutea* alone. The study confirmed the potential of edible *G. Lutea* to prevent oxidation of emulsion.

### *3.3. HPLC Analysis of G. Lutea and the Total Antioxidant Activity Based on Post-Column On-Line Coupling ABTS*$^{\bullet+}$

The HPLC analysis is targeted to identifysecoiridoid-glycoside, the bitter constituent occurred in the extract shown in (a) and (b). Results in Table 2 presentsecoiridoid glycoside; gentiopicroside, swerosideand amarogentin were the important compounds in the *G. Lutea*. The highest content of secoiridoidin *G. Lutea* extract in methanol-50% weregentiopicroside (1805 ± 62 mg/L extract) and the amount of sweroside found was 72 ± 4 mg/L extract. It was not possible to identifyamarogentin in the extract, meanwhile only low traces of amarogentinwere found in various commercial *G. Lutea* (less than 0.09%) [3]. Carnat *et al.* [28] also reported that amarogentin can only be found in some fresh root of *G. Lutea*. There are comprehensive studies measuring the active compound in *G. Lutea* using bioassay-guided fractionation such as HPLC [3], CapilaryElectrophoris [44] and Thin Layer Chromatography [45]. From the literature study, there is constituent of secoiridoidthat has not been identified in this study (swertiamarin)are believed to be existed in the extract [2]. This is most likely due to the objective of the method was not optimize the quantification of the traces but to measure the antioxidant activity of each compound in the *G. Lutea* extract.

**Table 2.** Amount of secoiridoid-glycoside quantified by HPLC.

| Sample | Concentration (mg/L) |
|---|---|
| Gentiopocroside | 1805 ± 62 |
| Sweroside | 72 ± 4 |
| Amarogentin | n.d |

n.d = not detected.

Aberham *et al.* [3] analyzed the active compound of 12 commercial samples of *G. Lutea* root. They found that swertiamarin was shown to have consistent occurrence between 0.21% and 0.45% and gentiopicroside was the most dominant compound in the sample up to 9.53%. Meanwhile, Ando *et al.* [46] reported that gentiopicroside was not detected from the fresh roots of 3-year-old *G. Lutea*. In contrast, Hayashi *et al.* [47] described that one year root contains high amounts of gentiopicroside and amarogentin and decreases over 5 years.

The Gentianaceae family is well known for its intensive bitter root used as a tonic for the digestive system with many pharmacological benefits. There are other plants such as *Swertiachirayita* [48] and *Lonicera japonica* [49] that also possess similar compounds of secoiridoid-glycosides (swertiamarin and sweroside).

Investigation of the main individual compounds in *G. Lutea* root was previously developed and optimized by Aberham *et al.* [2,3]. Furthermore, a substantial number of studies have demonstrated the effect of the secoiridoid group on the scavenging function to generate free radicals [7,13,48]. Wei *et al.* [13] reported that five secoiridoids, including gentiopicroside, sweroside, swertiamarin and sweroside, did not show any scavenging ability towards free radicals by *in vitro* DPPH assay. However, taking into account their report, it was desirable to explore more individual extracted compounds by isolating the compounds followed by a biochemical assay, such as ABTS radical, to measure their activity. Online post-column methods are very dependable because they combine systems for investigating different features of the sample simultaneously. Our initial observation of using *in vitro* ABTS assay showed that gentiopicroside and sweroside displayed no scavenging activity towards ABTS radicals (data not shown) while the activity of these compounds is similar towards DPPH radicals. However, our finding showed an activity of amarogentin analyzed by ABTS *in-vitro* assay (644.5 ± 17.5 mg eq TE/L sample). However, the amarogentin was not identified in the extract, as discussed above. This is in contrast with Phoboo *et al.* [48] who observed no scavenging activity of amarogentin towards DPPH radical.

The HPLC separated analytes reacted with ABTS radical post column (see Figure 4) and the reduction was detected as a negative peak at 734 nm. In Figure 5, the chromatographic analysis showed gentiopicroside (a) and sweroside (b) detected in *G. Lutea* extract and unknown compounds, (c) and (d), detected as negative peaks using the ABTS radical assay, which indicated that these components had free radical scavenging activity. The result of antioxidant response peaks (negative peak) of the *G. Lutea* compounds, expressed as mg galic acid equivalent (GAE)/L extract, indicates their relative contribution to the antioxidant activity of the extract with concentration taken into account. Analysis of *G. Lutea* extract of total antioxidant activity had been reported several times mainly measured by DPPH *in vitro* assay [10,14]. Even though we were unable to identify the

compound relevant to the scavenging activity in the post-column ABTS assay, the results showed that the antiradical capacity of *G. Lutea* is not related to gentiopicroside and sweroside. There are many other compounds that maybe related to the scavenging activity in the extract such as xanthone-glycosides.

**Figure 4.** Scheme of HPLC-ABTS for screening of antioxidant compounds in *G. Lutea* root extract.

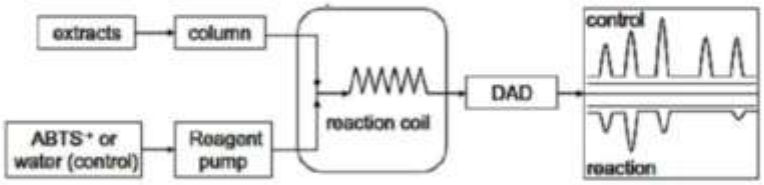
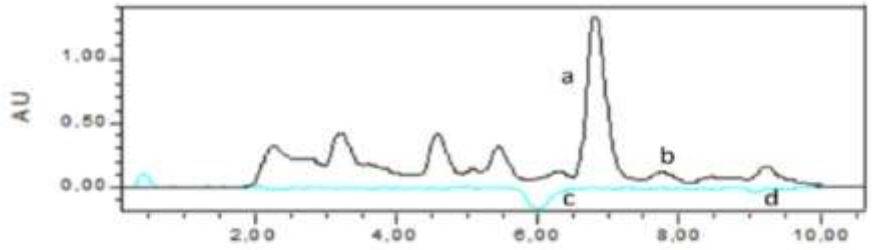



**Figure 5.** Chromatogram of *G. Lutea* root extract obtained direct and from the post-column HPLC-ABTS$^{\bullet+}$ radical scavenging method. (**a**) Gentiopicroside; (**b**) Sweroside; (**c**) antiradical activity by unknown compound; 31.33 ± 1.16 mg GAE/L and (**d**) antiradical activity by unknown compound; 8.30 ± 0.12 mg GAE/L.




Nevertheless, our finding showed that the 0.5% *G. Lutea* extracts is able to delay the process of oxidation and give better storage stability as emulsion. However, this activity is not due to the bitter compounds (gentiopicroside amarogentin and sweroside) presented in our extract. Studies (from the revised literature) proved that xanthone compounds such as gentioside, gentisin and isogentisin found in *G. Lutea* have antiradical activity even though their constituents possess remarkable activity in pharmacological study

## 4. Conclusions

*G. Lutea* has valuable pharmacological properties due to the bitter properties of its secoiridoid-glycosides; their extraction using methanol-water mixture was better than water alone. *G. Lutea* extract showed excellent antioxidant activity in aqueous methanol measured by DPPH scavenging activity assay and Trolox equivalent capacity assay (TEAC) methods (15.89 and 48.90 µmol of TE/g DW, respectively). *G. Lutea* lyophilize can be applied as antioxidants in oil-in-water emulsions. 0.2% (w/w) of lyophilize. *G. Lutea* does not inhibit lipid oxidation significantly.

An amount of 0.5% (w/w) *G. Lutea* lyophilise exhibited antioxidant activity towards primary and secondary oxidation in an o/w emulsion. Adding 0.1% (w/w) BSA with *G. Lutea* in an emulsion showed a synergic effect and better activity in delaying lipid oxidation.

Gentiopicroside and sweroside found in HPLC analysis do not show any antiradical capacity in *G. Lutea* aqueous methanol extract. However, total antiradical capacities shown in post-column measurements presented activities of 31.33 ± 1.16 mg GAE/L and 8.30 ± 0.12 mg GAE/L towards the ABTS free radical. This study confirmed that *G. Lutea* roots as a source of edible natural antioxidants have potential to be used by the food industry.

**Acknowledgments**

The authors acknowledge the special fund from The Technical University of Catalonia, University Malaysia Pahang and Malaysia Government for the financial support of this research.

**Author Contributions**

Nurul Aini Mohd Azman and Francisco Segovia did the experimental section and the first writing; Xavier Martínez-Farré and Emilio Gil helped in the writing and correction of English; Maria Pilar Almajano has been the supervisor and manager of all the process.

**Conflicts of Interest**

The authors declare no conflict of interest.

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