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DESARROLLO Y EVALUACIÓN DE NUEVOS SISTEMAS DE ENVASES ACTIVOS DE USO ALIMENTARIO

Memoria para optar al grado de doctor

Letricia Barbosa Pereira

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Dr. ANTONIO MOREDA PIÑEIRO, DIRECTOR DEL DEPARTAMENTO DE QUÍMICA ANALÍTICA NUTRICIÓN Y BROMATOLOGÍA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

INFORMA: Que Doña Letricia Barbosa Pereira presenta el trabajo titulado "DESARROLLO Y EVALUACIÓN DE NUEVOS SISTEMAS DE ENVASES ACTIVOS DE USO ALIMENTARIO" realizado bajo la dirección del Dr. Perfecto Paseiro Losada y del Dr. José Manuel Cruz Freire, en los laboratorios de Bromatología de la Facultad de Farmacia de la Universidad de Santiago de Compostela.

Fdo. Dr. Antonio Moreda Piñeiro

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PEFECTO PASEIRO LOSADA, CATEDRÁTICO DEL ÁREA DE NUTRICIÓN Y BROMATOLOGÍA DE LA FACULTAD DE FARMACIA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA, Y JOSÉ MANUEL CRUZ FREIRE, PROFESOR TITULAR DEL ÁREA DE INGENIERÍA QUÍMICA DE LA UNIVERSIDAD DE VIGO

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Y para que así conste, se expide la presente en Santiago de Compostela en Octubre de 2013.

Fdo. Dr. Perfecto Paseiro Losada

Fdo. Dr. José Manuel Cruz Freire

Fdo. Doña Letricia Barbosa Pereira

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"No es el conocimiento, sino el acto de aprendizaje; y no la posesión, sino el acto de llegar a ella, lo que concede el mayor disfrute"

Carl Friedrich Gauss

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Abreviaturas

AAC	Antioxidant activity coefficient (coeficiente de actividad antioxidante)
ACN	Acetonitrilo
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene (Butilhidroxitolueno)
CAS	Chemical Abstracts Service
CE	Crude extract (extracto crudo)
CFU	Colony forming units (unidades formadoras de colonias)
DAD	Diode array detector (detector de díodos en línea)
DPPH	2,2'-diphenyl-1-picrylhydrazyl
DSC	Differential scanning calorimetry (calorimetría diferencial de barrido)
EC_{50}	Equivalent concentration 50 (concentración equivalente 50)
EDTA	Ethylenediaminetetraacetic acid (ácido etilendiaminotetraacético)
EFSA	European Food Safety Agency (Autoridad Europea de Seguridad Alimentaria)
ESI	Electrospray ionization

EVA	Etilenovinilacetato
EVOH	Etilenvinilalcohol
GAE	Gallic acid equivalents (equivalents de ácido gálico)
HPLC	high performance liquid chromatography (cromatografía líquida de alta resolución)
IP	Inhibition percentage (porcentaje de inibición)
LDPE	Low density polyethylene (polietileno de baja densidad)
LLDPE	Linear low densitity polyethylene (Polietileno de baja densidad lineal)
M	Concentración molar
m/m	Masa/masa
MS	Mass spectrometry (espectrometría de massas)
MAP	Modified atmosphere pakaging (envasado en atmósfera modificada)
MDA	Malondialdehyde
m/z	mass to charge ratio (relación masa/carga)
OIT	Oxidation Induction Time (tiempo de inducción de oxidación)
PA	Ascorbyl palmitate
PG	Propyl gallate (galato de propilo)
PLA	Ácido poliláctico
PE	Polietileno

PET	Politereftalato de etileno
PP	Polipropileno
PM	Peso molecular
PUFAs	Polyunsaturated fatty acids (ácidos grasos poliinsaturados)
PVC	Policloruro de vinilo
PVPP	Polyvinylpolypyrrolidone
PVPP-WS	Polyvinylpolypyrrolidone wash solution (lavados de la torta de PVPP)
ROS	Reactive oxygen species (especies reactivas al oxígeno)
SFE	Supercritical fluid extraction (extracción de fluidos supercríticos)
SPE	Solid phase extraction (extracción en fase sólida)
TBARs	Tiobarbituric acid reactive substances (sustancias reactivas al ácido tiobarbitúrico)
TBHQ	Tert-butyl hydroquinone
TG	Triglicéridos
TGA	Thermogravimetric Analysis (análisis termogravimétrica)
TOF	Time of flight (tiempo de vuelo)
UV	Ultravioleta
v/v	Volumen/volumen
w/v	Weight/volume (peso/volumen)

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Resumen

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Los compuestos fenólicos se encuentran ampliamente distribuidos en el reino vegetal, constituyen una parte esencial de la dieta humana y cada vez cobran mayor importancia debido a sus funciones bioactivas, propiciadas fundamentalmente por sus propiedades antioxidantes.

En los últimos años ha aumentado el interés por los compuestos antioxidantes de origen natural para reducir al mínimo el empleo de los aditivos sintéticos en la conservacion de los alimentos. La posible toxicidad de algunos antioxidantes sintéticos, conjuntamente con la sensibilización de los consumidores en lo que respecta a cuestiones de seguridad y toxicidad alimentaria, ha motivado un creciente interés en la identificación de antioxidantes alternativos y seguros de origen natural como la vitamina E, o extractos naturales ricos en compuestos fenólicos y/o terpénicos.

La capacidad antioxidante de una amplia variedad de vegetales, cereales, frutas, semillas y de residuos agrícolas e industriales ha sido analizada recientemente en diversos estudios, siendo los residuos agrícolas e industriales una fuente atractiva de antioxidantes naturales.

Durante el procesado y almacenamiento, tanto refrigerado como congelado, a largo plazo, ocurren reacciones de oxidación que resultan en una disminución del valor nutritivo de los alimentos y su calidad sensorial. Retardar estos procesos de oxidación es importante, no solamente para el productor del alimento, sino también para todas las personas implicadas en la cadena de producción industrial y para el consumidor final.

El uso de compuestos fenólicos y extractos obtenidos a partir de varias fuentes naturales, incluyendo residuos agro-industriales, va a tener un papel importante en el aumento de la vida útil de los alimentos. Estos compuestos, incrementan la estabilidad de los productos alimenticios a través de la prevención de la peroxidación lipídica, mediante la prevención de la descomposición de los

pigmentos naturales de los alimentos y contribuyendo a la protección y estabilización de los aromas, además, hay que tener en cuenta que poseen una considerable capacidad antimicrobiana.

Hoy en día se puede observar un incremento continuo en la demanda de la industria para aumentar la vida comercial de los productos alimenticios envasados y en la demanda de los consumidores para mejorar la seguridad, la calidad y la integridad de los alimentos. Para responder a estos cambios, a estas nuevas necesidades y desafíos, se están desarrollando nuevos sistemas de envasado conocidos como “envases activos”.

Los envases activos, a diferencia de los tradicionales, a los que se les exige que sean totalmente inertes, están diseñados para interaccionar de una forma activa y continua con su contenido, en un proceso de interacción entre envase, ambiente y alimento, que implica siempre una transferencia de masa, ya sea para ceder sustancias al contenido del envase (el alimento y su entorno) o absorber componentes desde dicho contenido y cuya finalidad es siempre la de ampliar el tiempo de conservación de los alimentos.

Las sustancias responsables de la función activa del envase pueden estar, incorporadas directamente en el material de envase, siendo por lo tanto un objetivo importante en el desarrollo de este tipo de envases el diseño de materiales funcionales que incluyan el agente activo en su estructura y que esta sustancia activa pueda actuar o ser liberada de una manera controlada.

Esta tesis se centra en el desarrollo y evaluación de nuevos sistemas de envases activos de uso alimentario que contiene compuestos de origen natural en la conservación de productos cárnicos y productos de la pesca.

Para lograr este objetivo en esta tesis se aborda el aprovechamiento de una corriente residual de la industria cervecera rica en compuestos de naturaleza

polifenólica, para la obtención de un extracto natural con capacidad antioxidante y antimicrobiana, que se utilizó para el desarrollo de nuevos envases activos de uso alimentario. Los sistemas activos desarrollados fueron utilizados en el envasado de productos cárnicos y de la pesca, y se evaluaron los beneficios sobre el producto envasado.

Los polifenoles presentes en la cerveza son extremadamente importantes en su estabilidad física. Dicha estabilidad puede ser mejorada reduciendo el contenido en polifenoles o el contenido proteico de la cerveza, ya que los polifenoles y las proteínas de alto peso molecular tienden a combinarse lentamente y formar un complejo proteína-polifenol insoluble. Estos complejos resultan de reacciones de oxidación y polimerización de los polifenoles endógenos (catequinas y proantocianidinas) y de sus interacciones con las proteínas, que se forman durante la guarda fría y que se eliminan en la etapa de filtración de la cerveza. Una de las técnicas de estabilización más empleada es la reducción del contenido en compuestos fenólicos mediante la adsorción con polivinilpolipirrolidona (PVPP). Debido a que este PVPP tiene un coste considerable, una vez que los polifenoles se adsorben en la resina de PVPP, se realiza un lavado o regeneración de la torta de PVPP con soluciones alcalinas (NaOH) en caliente, para recuperar la capacidad de adsorción de la torta de PVPP, y por lo tanto, que ésta pueda ser reutilizada en sucesivos lotes. El líquido de lavado se considera un desecho, y como tal es enviado a la estación depuradora de aguas residuales, con la consiguiente pérdida de valor como subproducto, además de ocasionar costes de depuración considerables. Esta corriente residual de PVPP se encuentra cargada fundamentalmente con compuestos polifenólicos y por lo tanto, los extractos que se pueden obtener a partir de esta corriente residual de la industria cervecera son una fuente prometedora de antioxidantes naturales con amplia aplicación.

Los capítulos 3, 4, 5 y 6 se centran en el desarrollo del método de extracción para la obtención del extracto natural a partir de los lavados de la torta de PVPP producidos en la industria cervecera. En estos capítulos se caracterizan los diferentes extractos obtenidos a escala de laboratorio, piloto e industrial y se evalúan las actividades antioxidante y antimicrobiana de cada extracto obtenido.

En el **capítulo 3** (***PATENTE Nº ES 2 347 631 B1: Procedimiento para la recuperación de sustancias antioxidantes a partir del líquido de desecho procedente del lavado de la polivinilpolipirrolidona utilizada para el tratamiento de cerveza***) de esta tesis se describe el desarrollo del método de extracción de los compuestos antioxidantes a partir de los lavados de la torta de PVPP utilizada en el proceso de clarificación de la cerveza.

El procedimiento consiste en la recuperación de sustancias antioxidantes a partir del líquido de desecho procedente del lavado de la polivinilpolipirrolidona utilizada para el tratamiento de cerveza, y que tienen posibles aplicaciones en otros ámbitos industriales, tales como la elaboración de films plásticos para el envasado de alimentos. Para ello, se lleva a cabo el lavado de la polivinilpolipirrolidona con hidróxido sódico concentrado a alta temperatura, para recuperar los compuestos polifenólicos presentes en estos licores de lavado, posteriormente, dichos licores se acidifican con ácido clorhídrico concentrado hasta pH adecuado (1-1,5) y se lleva a cabo un proceso de extracción con acetato de etilo. La extracción se realiza en un tanque agitado y luego se deja reposar para que ocurra la separación de fases por decantación. Posteriormente se separa la fase orgánica, se evapora a sequedad en un sistema rotatorio a vacío y finalmente se elimina el agua residual por liofilización.

Para la optimización del proceso, se estudiaron diferentes relaciones de fase acuosa: fase orgánica y la influencia del número de etapas de extracción, determinándose en todos los ensayos el rendimiento de extracción y la actividad

antioxidante de cada extracto obtenido. El proceso de extracción propuesto permite obtener un rendimiento de extracción entre 0,4–1,8 g de extracto/L de licor de lavado, y la actividad antioxidante de los extractos obtenidos presenta una EC_{50} entre 0,23–0,45 g/L, siendo por tanto entre 5 y 10 veces más activo que el BHT. Los compuestos antioxidantes presentes en mayor cantidad en el extracto son el ácido protocatéquico, la catequina, el ácido cafeico, el ácido p-cumárico y el ácido ferúlico que se obtienen casi en su totalidad en la primera extracción. A partir de estos estudios se comprueba que este extracto presenta un elevado potencial como agente antioxidante, se caracterizan los compuestos responsables de este poder antioxidante, se decide realizar la extracción en una única etapa y establecer como óptima la relación fase orgánica: fase acuosa de 1:2.

En el procedimiento descrito se realizó asimismo un estudio preliminar para determinar la capacidad antimicrobiana del principio activo del PVPP, para ello se ha estudiado concentraciones altas y bajas de bacterias (10^3 y 10^6 UFC/mL) con distintas concentraciones de principio activo (0,5; 1; 3 y 5%). Los microorganismos ensayados fueron: *Escherichia coli* (*E. Coli*); *Listeria monocytogenes*; *Salmonella spp*; *Escherichia coli* 0157:H; y Aerobios. Se observó que para una concentración más baja de microorganismos (10^3 UFC/mL) el principio activo desarrollado es efectivo en una concentración del 3% frente a todos los microorganismos. En el caso de Aerobios y *Salmonella* la efectividad antimicrobiana ya se hace patente a partir de una concentración del 1%.

Así pues, la invención tiene su aplicación dentro de la industria dedicada a la fabricación de cerveza, al revalorizar lo que actualmente sería un producto de desecho, y también debido al posible aprovechamiento de las capacidades antioxidante y antimicrobiana del extracto obtenido, en otro tipo de industrias (industria alimentaria, por ejemplo como aditivo, o en industrias auxiliares a la industria alimentaria, por ejemplo como materiales para envases).

En el **capítulo 4** (***Brewery waste as a potential source of phenolic compounds: optimization of the extraction process and evaluation of antioxidant and antimicrobial activities***) se amplía la información del capítulo II. En este capítulo se expone con más detalle la metodología para la determinación de la actividad antioxidante (método de DPPH), el método de HPLC-DAD desarrollado para la determinación de los compuestos antioxidantes mayoritarios en los diferentes extractos obtenidos y el método de determinación de la actividad antimicrobiana (Standard Test Method ASTM E 2149-01). El procedimiento de extracción se lleva a cabo conforme a lo descrito en el capitulo anterior, bajo las siguientes condiciones: pH=1-1.5, con agitación lenta durante 45 minutos, y tras de la separación de fases la fase orgánica se evapora a una temperatura de 40ºC. Los resultados muestran los rendimientos globales de las tres extracciones realizadas a escala de laboratorio, el rendimiento específico de cada uno de los compuestos mayoritarios obtenidos y el tratamiento estadístico de los datos obtenidos de actividad antioxidante con el método de DPPH. Se demostró que el rendimiento de la extracción para las relaciones fase acuosa: fase orgánica superiores a 1:1 (v/v) es superior al 70% en la primera extracción y que los extractos obtenidos presentan una elevada actividad antioxidante, similar al BHA y hasta 10 veces superior al BHT. El extracto natural también presenta efectividad antimicrobiana contra bacterias Gram positivas y Gram negativas seleccionadas lo que aumenta su potencial para una futura aplicación del extracto como aditivo alimentario en productos cárnicos.

El objetivo del **capítulo 5** (***Phenolic profile and antioxidant properties of a crude extract obtained from a brewery waste stream***) ha consistido en desarrollar un método cromatográfico para proceder a la identificación y cuantificación de los compuestos activos presentes en el extracto obtenido a partir de los lavados de PVPP, así como en la determinación de las propiedades antioxidantes del extracto obtenido. La extracción se procedió bajo las

condiciones óptimas definidas en los capítulos 3 y 4, pero en esta ocasión a escala piloto partiendo de 100 L de licores.

El contenido total en compuestos fenólicos presentes en el extracto obtenido fue determinado por el método de Folin-Ciocalteu. La separación, identificación y la cuantificación de los compuestos fue realizada mediante un exhaustivo análisis cromatográfico por cromatografía liquida de alta resolución en fase reversa con detector de diodos en línea (RP-HPLC-DAD). Para la confirmación positiva de la identidad de los compuestos se ha empleado la cromatografía liquida de alta resolución en fase reversa acoplado a un espectrómetro de masas con un analizador de tiempo de vuelo (RP-HPLC-ESI-TOF-MS). En el método cromatográfico desarrollado para la separación de los compuestos se utilizó una columna Kromasil C18 (250×3.2 mm, 5 µm), con una elución en gradiente con agua ultrapura acidificada con ácido acético al 0,1% y acetonitrilo, y la detección se hizo a 205, 225, 280, 325 y 372 nm de acuerdo con la clase estructural de compuestos fenólicos. La identificación se hizo comparando los tiempos de retención y los espectros UV-Vis obtenidos con los patrones comerciales bajo las mismas condiciones de análisis así como los fragmentos de masa obtenidos en el detector de masas TOF (operando en modo negativo de ionización). La monitorización y cuantificación se realizó de la siguiente forma: los ácidos hidroxibenzoicos a 225 nm, flavan-3-oles, flavanonas y flavonas a 280 nm, los derivados de la acetofenona, ácidos hidroxicinámicos y resveratrol a 325 nm, y por último los flavanoles a 372 nm, mediante el empleo del método de estándar externo.

El extracto natural obtenido está compuesto mayoritariamente por flavonoides (catequina, epicatequina, gallocatequina, epigallocatequina, quercetina) y por ácidos fenólicos (ferúlico, ρ-cumárico, cafeico y protocatéquico) que representan casi el 50% (m/m) del extracto. Las condiciones cromatográficas propuestas, garantizan una buena separación de los compuestos fenólicos

presentes en el extracto, lo que permite su aplicación en el análisis de rutina para la monitorización de los extractos cada vez que se realicen procesos de extracción a partir de los lavados de la torta de PVPP.

La actividad del extracto fue determinada por el método de DPPH y del β-caroteno. La capacidad del extracto para captar radicales libres (método de DPPH) era muy elevada (EC_{50}=0,18 g/L), superior a los antioxidantes utilizados como referencia, BHA y BHT. En cuanto a la efectividad como rompedores de la cadena oxidativa y/o inhibición enzimática (mimetizado por el método de β-caroteno) el extracto presenta un coeficiente de actividad antioxidantes de 623,8, similar al BHA (653,3) y al BHT (559,6).

En el **capítulo 6** (***Fractionation and purification of bioactive compounds obtained from a brewery waste stream***) se evalúan y se comparan dos métodos clásicos de purificación de extractos naturales con el objetivo de seleccionar el más adecuado para la purificación del extracto obtenido ahora ya a escala industrial. Los métodos utilizados en la purificación del extracto fueron la extracción en fase sólida (SPE), donde se utilizó una columna (LC-18) que se cargó con una cantidad conocida de extracto y cuya elución se realizó con mezclas de diferentes porcentajes de metanol/agua; y el método de extracción por fluidos supercríticos (SFE) donde se evaluaron diferentes condiciones de presión y de modificador (sustancia, porcentaje y flujo).

Para todas las fracciones obtenidas en los dos métodos de purificación se determinaron el rendimiento (por gravimetría), actividad antioxidante (por el método de DPPH) y la composición en compuestos fenólicos (por el método cromatográfico desarrollado en el capítulo 5).

En el proceso de purificación por extracción en fase sólida, la fracción obtenida con mayor actividad antioxidante (EC_{50}= 0,20 g/L, similar al BHA y superior al BHT) se obtuvo con la elución de 30% (v/v) de metanol. La fracción

más activa (EC_{50}= 0,23 g/L) conseguida en el proceso por fluidos supercríticos se obtuvo bajo las siguientes condiciones: temperatura de 40 ºC, presión de 140 bar, tiempo de extracción de 30 min, utilizando etanol como modificador a un flujo de 0,2 mL/min.

Los resultados obtenidos muestran que ambos procesos de purificación pueden ser utilizados para purificar el extracto natural, que mejoran las propiedades organolépticas del extracto (olor y color) y que además se obtienen fracciones con mayor actividad antioxidante que el extracto crudo. La purificación por fluidos supercríticos sería la opción a escoger, debido a que es ampliamente utilizada en la obtención y purificación de extractos de origen natural, con resultados muy positivos en lo que respecta a la eliminación de olores, residuos de solventes de extracción y eliminación de los compuestos coloreados de elevado peso molecular, mejorando las propiedades del extracto para ser utilizado de forma segura como aditivo alimentario.

Los capítulos 7 y 8 enfocan el desarrollo de envases activos utilizando el extracto natural obtenido de los licores de lavado del PVPP y la evaluación de dichos envases en la conservación de productos cárnicos.

En el **capítulo 7** (***Development of new active packaging films coated with natural antioxidants to extend the shelf life of beef***) se describe el desarrollo de films activos, incorporando extractos naturales, por la técnica de recubrimiento en films de polietileno de baja densidad (LDPE) y evaluación de la efectividad de estos films activos en la reducción de los procesos de oxidación en carne de ternera almacenada a refrigeración.

Para evaluar la efectividad del extracto natural, obtenido de los licores de lavado de la torta del PVPP, en la conservación de la carne de ternera se procedió a su evaluación in vitro por el método de DPPH conjuntamente con otros

antioxidantes utilizados como referencia, tales como BHT, propilgalato (PG) y un extracto natural de romero obtenido comercialmente. A continuación se evaluó la eficacia de estos antioxidantes cuando se dosifican a diferentes concentraciones en contacto directo con el alimento y sin contacto directo. El grado de oxidación lipídica se determinó por el método de determinación de las sustancias reactivas al ácido 2-tiobarbitúrico (TBARs). Posteriormente se desarrollaron films activos, mediante recubrimiento, con los extractos de PVPP y de romero, también a diferentes concentraciones, y se evaluó su efecto en la reducción de la oxidación lipídica de la carne de ternera por el método de TBARs (con y sin contacto directo con el alimento).

Los resultados obtenidos demostraron que el extracto natural objeto de estudio en esta tesis reduce la oxidación lipídica en la carne de ternera hasta un 70-80% respecto al ensayo control, cuando se adiciona directamente al producto (efecto similar al PG). De igual forma los films activos desarrollados con el extracto natural del PVPP pueden llegar a una inhibición en la oxidación lipídica de hasta un 80% respecto al film control durante el tiempo de almacenamiento a refrigeración.

En el **capítulo 8** (***Development of new active packaging films containing bioactive nanocomposites***) se describe el desarrollo de films activos con el extracto natural por extrusión incorporado directamente o incluido en nanoarcillas y posterior evaluación de la efectividad de estos films activos en la reducción de los procesos de oxidación en carne de ternera almacenada a refrigeración.

Previamente a la incorporación del extracto en las diferentes matrices poliméricas objeto de estudio por extrusión, se procedió a la evaluación de la estabilidad térmica del extracto natural exponiéndolo a dos temperaturas (100 y 200 ºC) durante 120 minutos. Durante la exposición a estas temperaturas se cogieron muestras a diferentes tiempos y se evaluaron la pérdida de peso (por

gravimetría), el contenido total en compuestos fenólicos (método de Folin-Denis) y la actividad antioxidante (por el método de DPPH). Los resultados demostraron una elevada estabilidad térmica del extracto frente a la temperatura por lo que se esperarían perdidas mínimas de la efectividad del extracto en el proceso de fabricación de los films activos por extrusión.

Se estudiaron dos matrices poliméricas, el polietileno de baja densidad (LDPE) y el etilenvinilacetato (EVA). A estas matrices se les incorporó extracto en una concentración de 3% (m/m) y se determinaron la actividad antimicrobiana y la migración global así como la efectividad oxidativa por el método de TBARs en carne de ternera envasada con bolsas preparadas con estos films. Los resultados demostraron que la matriz polimérica EVA era la más apropiada para la incorporación de este extracto y se prosiguió con el estudio incorporando mayores concentraciones de extracto para determinar la más adecuada para lograr una reducción de la oxidación lipídica en la carne de ternera durante su almacenamiento a refrigeración. Además, también se desarrollaron y testaron films incorporados con diferentes concentraciones de nanoarcillas activas que llevan incorporado en su interior el extracto natural.

Los resultados obtenidos muestran que los films EVA activos desarrollados con el extracto natural presentan actividad antimicrobiana y reducen la oxidación lipídica de la carne respecto al film control hasta en un 60-70%. El uso de las nanoarcillas bioactivas mejora aún mas la efectividad del envase activo reduciendo la cantidad de extracto natural necesario para obtener una reducción en la oxidación lipídica del alimento de hasta el 50% frente al film control.

Se ha observado efectividad antimicrobiana a *S. aureus* a partir de las 24 horas en los films de EVA con un 6% de principio activo (PVPP) y a partir de las 48 horas con un 6 y 9% de nanoarcilla (1,2% de PVPP).

Los films obtenidos en los capítulos 7 y 8 pueden ser utilizados en el desarrollo de nuevos envases activos de uso alimentario para ser utilizados en la conservación de productos cárnico aumentando su vida útil.

Integrado en el proyecto ENVACFISH, el **capítulo 9** (***Development of antioxidant active films containing tocopherols to extend the shelf life of fish***) enfoca el desarrollo de nuevos envases activos antioxidantes que contienen tocoferoles para el envasado de distintas especies de pescado congelado en formato de "segunda piel".

En este trabajo se evaluaron diferentes extractos naturales obtenidos comercialmente que contienen tocoferoles en dos formatos diferentes (polvo y aceite). Inicialmente se evaluaron su actividad antioxidante in vitro por el método de DPPH, su efectividad en salmón refrigerado por adición directa a la superficie del alimento (método de TBARs), así como la evaluación del extracto natural más apropiado para la incorporación en la matriz polimérica por extrusión (métodos de degradación térmica–OIT por barrido diferencial calorimétrico (DSC) y análisis termogravimétrica (TGA)).

Con los resultados obtenidos anteriormente se seleccionaron dos productos naturales y se incorporaron en la matriz de LDPE. El producto antioxidante NUTRABIOL®-T90 fue incorporado a dos concentraciones diferentes 1% (Film 2) y 5% (Film 3) que presentaron una reducción en la oxidación del salmón hasta un 30% para el Film 2 y hasta un 40% para el Film 3 respecto al film control (Film 1) durante el tiempo de almacenamiento a refrigeración. El producto TOCOBIOL®-PV contiene menor cantidad de compuestos activos que el NUTRABIOL®-T90 por lo que su incorporación en la matriz polimérica se hizo a la concentración más elevada de 5% (Film 4). El Film 4 presentó una reducción en la peroxidación lipídica alrededor de 30-35% respecto al film control.

El Film 3 (NUTRABIOL®-T90 al 5%) producido en este estudio sería el más apropiado para el desarrollo de envases activos para ser utilizados en la conservación a largo plazo del pescado congelado aumentado su tiempo de vida útil y manteniendo sus propiedades organolépticas.

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1

Introducción general

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1.1. Compuestos bioactivos antioxidantes de naturaleza polifenólica

Los compuestos fenólicos se encuentran ampliamente distribuidos en el reino vegetal, constituyen una parte esencial de la dieta humana y son de gran interés debido a sus funciones bioactivas, fundamentalmente por sus propiedades antioxidantes (Balasundram *et al*., 2006; Crozier *et al*., 2009). Los antioxidantes naturales más frecuentes están asociados con los metabolitos secundarios de plantas, tales como las vitaminas C y E (ácido ascórbico y tocoferoles), carotenoides y numerosos compuestos fitoquímicos vegetales (ácidos fenólicos, flavonoides, terpenoides, esteroles, estanoles y otras clases químicas) y tienen un papel fundamental en su morfología y fisiología (Tsao & Deng, 2004). Estos compuestos son muy importantes para el crecimiento y reproducción, ya que ofrecen protección contra patógenos, depredadores y radiación ultravioleta, además de contribuir para el color y para las cualidades sensoriales y nutritivas de las frutas y vegetales (Bravo, 1998; Naczk & Shahidi, 2006).

En las últimas décadas, estos compuestos han recibido bastante atención por parte de los consumidores y de la industria alimentaria por diversas razones. Los compuestos polifenólicos de origen natural han demostrando excelentes propiedades como aditivos alimentarios, así como su papel fundamental en la protección contra numerosas patologías debido a sus efectos vasodilatadores, anticarcinogénicos, antiinflamatorios, bactericidas, estimuladores de la respuesta inmune, antialérgicos, antivirales, efectos estrogénicos, o inhibidores de enzimas prooxidantes, como cicloxigenasa, lipooxigenasa y xantina oxidasa (Shahidi & Zhong, 2010; Scalbert & Williamson, 2000; Higdon & Frei, 2003; Kondratyuk & Pezzuto, 2004).

Muchos de los efectos beneficiosos asociados al consumo de alimentos de origen vegetal se atribuyen en gran medida a los compuestos fenólicos y están

relacionados con las propiedades químicas de éstos. Los compuestos polifenólicos son agentes reductores que junto con los agentes reductores que ya existen naturalmente en la dieta, como es el caso de la vitamina C, la vitamina E y los carotenoides, protegen los tejidos contra el estrés oxidativo (Bravo, 1998; Manach *et al*., 2005).

En este capítulo se describen brevemente la naturaleza y fuentes de origen vegetal de los principales grupos de compuestos fenólicos con actividad antioxidante, destacando especialmente los subproductos de la industria cervecera, así como el posible empleo de estos compuestos como aditivos de uso alimentarios e ingredientes en el desarrollo de alimentos funcionales con alta actividad antioxidante y beneficios potenciales para la salud humana.

1.1.1. Naturaleza de los compuestos fenólicos

Los compuestos fenólicos comprenden un amplio número de compuestos con una gran diversidad de estructuras que los hacen diferentes de otros antioxidantes. La estructura química de los polifenoles va a afectar a sus propiedades biológicas, como es el caso de la biodisponibilidad, actividad antioxidante, interacciones específicas con los receptores celulares y enzimas, entre otras. Las principales clases de polifenoles se definen de acuerdo con la naturaleza de su estructura de carbono. Estos compuestos están formados por un anillo aromático rodeado de uno o más grupos hidroxilo, y varían desde moléculas fenólicas simples hasta compuestos con elevado grado de polimerización (Bravo, 1998).

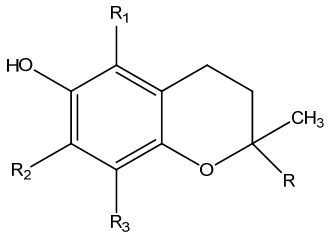
La actividad antioxidante de los compuestos fenólicos se atribuye a su facilidad para ceder átomos de hidrógeno del grupo hidroxilo aromático a un radical libre y a la posibilidad de deslocalización de cargas en el sistema de

dobles enlaces del anillo aromático (Balasundram *et al*., 2006; Tsao & Deng, 2004). Los compuestos fenólicos poseen además una estructura química ideal para captar iones metálicos (principalmente hierro y cobre) y por tanto para inhibir la formación de radicales libres a través de reacciones de Fenton (Rice-Evans *et al*., 1997).

Los compuestos fenólicos se pueden dividir en flavonoides (flavonas, flavonoles, lavanonas, flavanoles, chalconas, Isoflavonas, antocianidinas, proantocianidinas, taninos, etc.) y no flavonoides (monofenoles, ácidos fenólicos, stilbenos y lignanos) (Balasundram *et al*., 2006; Tsao & Deng, 2004; Rice-Evans *et al*., 1997). A continuación se describen los principales grupos de compuestos fenólicos de origen natural con actividad antioxidante, biosintetizados y acumulados en materiales agro-alimentarios de origen vegetal.

Tocoferoles

Los tocoferoles y tocotrienoles son monofenoles (ver Fig. 1.1) que participan en reacciones de captación de radicales libres y donación de átomos de hidrógeno. Su actividad antioxidante está ampliamente documentada y se debe principalmente al hidrógeno del grupo fenólico en el carbono en la posición C6. El α-tocoferol se considerada como el antioxidante liposoluble más eficaz en romper la cadena de radicales libres, protegiendo las membranas celulares contra los radicales peróxilo y especies de óxido de nitrógeno mutagénicas (Brigelius-Flohé & Traber, 1999; Tafazoli *et al*., 2005).

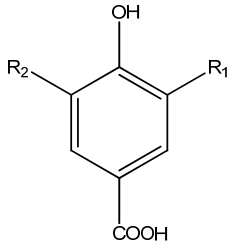


R		R_1	R_2	R_3
Tocoferol	α	CH_3	CH_3	CH_3
	β	CH_3	H	CH_3
Tocotrienol	γ	H	CH_3	CH_3
	σ	H	H	CH_3

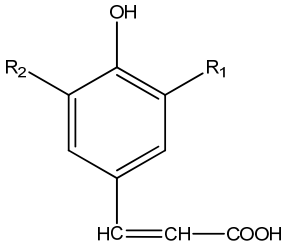
Figura 1.1. Estructura química de los diferentes tocoferoles y tocotrienoles

Ácidos fenólicos

Los ácidos fenólicos constituyen un tercio de los fenoles de la dieta y pueden encontrase de forma libre o combinados (Robbins, 2003). Se dividen en dos grandes grupos: hidroxibenzoicos y hidroxicinámicos. Los ácidos hidroxibenzoicos (estructura común C6-C1) incluyen los ácidos gálico, p-hidroxibenzoico, protocatéquico, vinílico y siríngico. Por otra parte, los acidos hidroxicinámicos son compuestos aromáticos C6-C3 con una cadena lateral en el C3, siendo los más representativos los ácidos cafeico, ferúlico, p-cumárico y sinápico (Bravo, 1998) (ver Fig. 1.2). Estos compuestos participan esencialmente en reacciones captación de radicales libres y donación de átomos de hidrógeno (Villaño, 2007).



Derivados del ácido benzoico



Deriivados del ácido cinámico

Compuesto	R_1	R_2
Ácido ρ-hidroxibenzoico	H	H
Ácido vanílico	H	OCH_3
Ácido siríngico	OCH_3	OCH_3
Ácido gálico	OH	OH

Compuesto	R_1	R_2
Ácido ρ-cumárico	H	H
Ácido ferúlico	H	OCH_3
Ácido sinápico	OCH_3	OCH_3
Ácido cafeico	OH	H

Figura 1.2. Estructura química de los diferentes ácidos fenólicos

Flavonoides

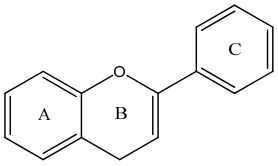
Los flavonoides son los compuestos polifenólicos más abundantes en la dieta. Son moléculas de bajo peso molecular formadas por 15 átomos de carbono dispuestos con la configuración C6-C3-C6 (dos anillos aromáticos juntos por un puente de 3 carbonos), normalmente en forma de anillo heterocíclico (Balasundram *et al*., 2006) (ver Fig. 1.3). Su actividad antioxidante, ampliamente estudiada, se debe esencialmente por su participación en la protección contra el efecto nocivo de las especies reactivas de oxigeno (ROS) con un elevado rango de efectos biológicos. Los polifenoles son compuestos con importante actividad antioxidante debido a su elevado potencial redox que les permite actuar como captadores de radicales libres, dadores de átomos de hidrógeno y secuestradores del singlete de oxígeno. Además, tienen capacidad de captar metales e inactivar

tanto fotosensibilizadores como enzimas, como pueden ser la 5-lipoxigenasa, la ciclooxigenasa, la monooxigenasa, o la xantina oxidasa (van Acker *et al*., 1996; Heim *et al*., 2002).

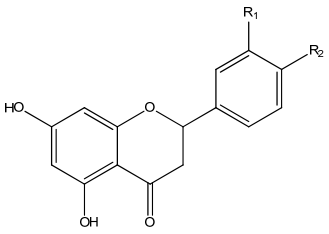
Taninos

Los taninos son compuestos de peso molecular relativamente elevado. Constituyen el tercer grupo de compuestos fenólicos más importante, y se pueden dividir en taninos hidrolizables y taninos condensados o proantocianidinas. Las proantocianidina son flavonoides poliméricos y resultan de la condensación de flavan-3-oles, principalmente (–)-epicatequina y (+)-catequina (ver Fig. 1.3). Los taninos hidrolizables son derivados del acido gálico que se esterifica en un núcleo poliólico. A su vez los grupos galoil pueden ser posteriormente esterificados y/o sufrir entrecruzamiento oxidativo para obtener taninos hidrolizables aún más complejos (Balasundram *et al*., 2006).

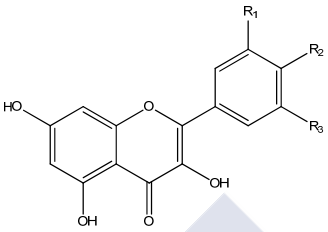
Estos compuestos pueden tener diferentes aplicaciones en los sistemas biológicos debido a su potencial como quelantes de iones metálicos, agentes precipitantes de proteínas y antioxidantes biológicos (Tian, 2012).



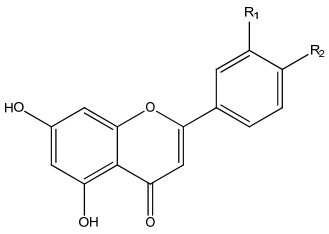
Flaván (Configuración C_6-C_3-C_6)



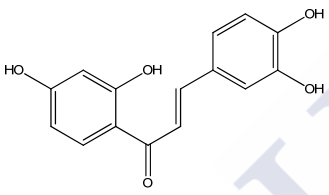
Flavanonas	R_1	R_2
Naringenina	H	OH
Hespertina	H	OCH_3



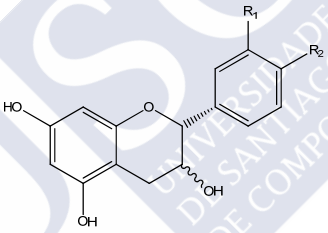
Flavonoles	R_1	R_2	R_3
Kaempferol	H	OH	H
Quercetina	OH	OH	H



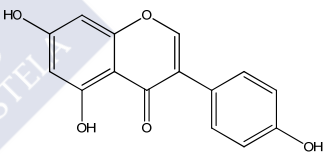
Flavonas	R_1	R_2
Apigenina	OH	H
Luteolina	OH	OH



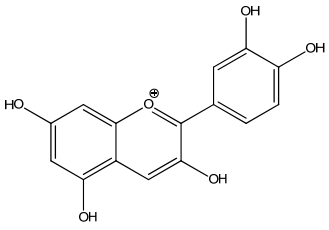
Chalconas
(Buteína)



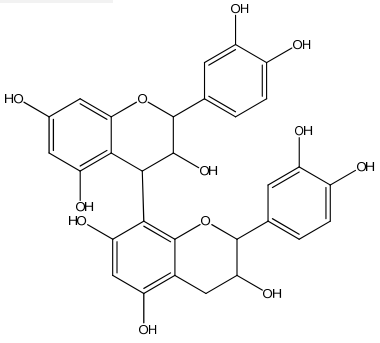
Flavanoles	R_1	R_2
Catequina (2S, 3S)	OH	OH
Epicatequina (2R, 3R)	OH	OH



Isoflavonas
(Genisteína)



Antocianidinas
(Cianidina)



Taninos
(Procianidina B1)

Figura 1.3. Estructura química de las principales clases de flavonoides

Estilbenos y lignanos

Los estibenos están presentes en la dieta en muy bajas cantidades, y el compuesto más representativo es el resveratrol, que existe en dos formas isoméricas, cis y trans, y mayoritariamente en las forma glicosilada. Los lignanos están presentes en la naturaleza en su forma libre y también son conocidos como fitoestrógenos debido a su capacidad de comportarse como agonista y antagonista del estrógeno, lo cual le confiere propiedades farmacológicas (Saleem *et al*., 2005; Scalbert *et al*., 2000). En la Fig. 1.4 se presentan un ejemplo de cada clase de compuestos.

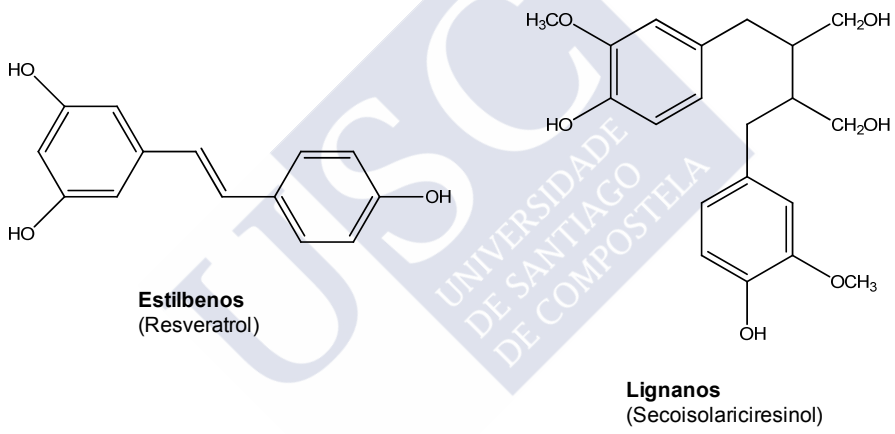


Figura 1.4. Estructura química de los estilbenos y de los lignanos

1.1.2. Fuentes naturales de compuestos fenólicos

Las frutas y los vegetales son importantes fuentes de varias vitaminas, minerales y fibras para el ser humano. Es de conocimiento general que las frutas son ricas en varios antioxidantes como el acido ascórbico, los carotenoides y los compuestos fenólicos. En diversos estudios se ha analizado la capacidad antioxidante de una amplia variedad de vegetales, cereales, frutas, semillas, y de

residuos agrícolas e industriales, siendo estos últimos una fuente atractiva de antioxidantes naturales (Balasundram *et al*., 2006; Robards, 2003; Moure *et al*, 2001; Peschel *et al*., 2006).

1.1.2.1. Alimentos ricos en polifenoles

Aunque los compuestos fenólicos estén presentes en la mayoría de los alimentos de origen vegetal, las frutas, vegetales y las bebidas son las mayores fuentes de estos compuestos en la dieta. Además, también se pueden encontrar en otras fuentes de origen vegetal tales como aceites, legumbres, cereales, especias y hierbas aromáticas, entre otros (Balasundram *et al.*, 2006). En la Tabla 1.1 se presentan algunos ejemplos encontrados en la literatura.

Tabla 1.1. Fuentes naturales de antioxidantes naturales de ingesta humana

Material	Principales compuestos	Referencia
Frutas		
Granada	Antocianidinas (cianidina, delfinidina) y flavanoles (quercetin, kaempferol)	Kim *et al*. (2002); Fischer *et al*. (2011)
Frutos rojos (cerezas, moras, etc)	Àcidos fenólicos, antocianinas, proantocianidinas, taninos hidrolizables y flavonoles (quercetina, Kaempferol), flavonas, catequinas	Seeram (2008); Robards, (2003)
Nísperos	Ácidos fenólicos (gálico y p-cumárico), flavonoides, flavanoles (catequina), carotenoides, taninos condensados	Dembitsky *et al*. (2011)
Cítricos	Flavanonas (hespertina), flavonoides, cumarinas, carotenoides y limonoides. Terpenos (presentes en los aceite esenciales), ácidos fenólicos y flavononas (naringenina)	Wilmsen *et al*. (2005); Fernandez-Lopez *et al*. (2009);
Frutos exóticos (Mango)	Catequinas, quercetina, kaempeferol, antocianidinas, acido gálico, acido elágico, galato de propilo, ácidos benzoico y protocatequico, carotenoides y vitamina C	Gonzalez-Aguilar *et al*. (2008); Vijaya Kumar Reddy *et al*. (2010); Dembitsky *et al*. (2011)
Manzana	Flavonoides, catequina, ácido clorogénico, taninos condensados	Burda *et al*. (1990); Valavanidis *et al*., (2009)
Uvas	Ácidos fenólicos, flavan-3oles (catequinas), antocianinas	Cantos *et al*., 2002; Gutiérrez *et al*., 2005
Vegetales		
Espinacas, brócoli, coles de bruselas	Flavonoides (kaempferol), acidos fenólicos (ferúlico, cafeico, sinápico)	Cartea *et al*. (2010);
Tomate	Licopeno, ácido ascórbico y α-tocopherol. Quercetina, kaempferol, naringenina y ácidos fenólicos	Frusciante *et al*. (2007); Martínez-Valverde *et al*. (2002)
Cebolla	Flavonol: quercetina y derivados de quercetina	Roldan *et al*. (2008); Gorinstein *et al*. (2008)
Ajo	Quercetina, taninos condensados y hidrolizables	Gorinstein *et al*. (2008)

Material	Principales compuestos	Referencia
Aceites vegetales		
Oliva	Tirosol y hidroxitirosol, oleuropeína, ácidos fenólicos (ferúlico, p-cumárico, cinámico, p-hidroxibenzoico, gálico, cafeico, vanílico, 3,4-dihidroxibenzoico), luteolina, apigenina Tocoferoles y tocotrienoles	Ballus *et al*. (2011)
Cereales		
Cevada, maíz, trigo negro, mijo, avena, centeno, arroz	Ácidos fenólicos (ferúlico, vanílico, cafeico, siríngico, p-cumárico), vitamina E (germen), carotenoides, lignina, lignanos (salvado)	Fardet *et al*. (2008)
Legumbres		
Guisantes amarillos y verdes, garbanzos, lentejas, frijoles, habas, y soja	Ácidos fenólicos, flavonoides y sus derivados, flavanols, flavan-3-oles, antocianidina/ antocianinas, taninos condensados, proantocianidinas, tocoferoles y vitamina C. Dentro de los flavonoides los más comunes son las isoflavonas: genisteína y daidzeína	Luthria & Pastor-Corrales (2006); Xu & Chang (2007, 2008); Amarowicz & Pegg (2008); Marathe *et al*. (2011)
Frutos secos y semillas		
Nueces, cacahuetes, pistachos, anacardos	Ácidos fenólicos, y flavonoides: catequinas, flavonoles y flavononas. Resveratrol, tocoferoles, taninos	Bartolomé *et al*. (2010); Chukwumah et al. (2012); Tomaino *et al*., (2010); Solar *et al*. (2006); Michodjehoun-Mestres *et al*. (2009)
Especias y hierbas y sus aceites esenciales		
Romero, clavo, orégano, tomillo, salvia,	Ácidos fenólicos (cafeico, ácido rosmarínico), flavonóides (apigenina), esteroles y cumarinas. Diterpenos fenólicos (timol, carvacol, cuminol, eugenol, ácido carnósico, carnosol, carvacol, rosmanol)	Viuda-Martos *et al*. (2007). Robards (2003); Zheng & Wang (2001); Bakkali *et al*. (2008)

Material	Principales compuestos	Referencia
Bebidas		
Zumos de frutas (manzana, piña, naranja, mandarina, uva, pera, albaricoque)	Ácidos fenólicos (hidroxicinámicos y hidroxibenzóicos), flavan-3-oles, flavonoles (quercetina), chalconas, flavonoides glicosilados, antocinidinas, Flavonas (apigenina, etc)	Robards (2003); Sdiri *et al*. (2012)
Té	Catequinas: (–)-epigallocatequina 3-O-gallato (EGCG), (–)-gallocatequina 3-O-gallato (GCG), (–)-epicatequina 3-O-gallato (ECG), (–)-epigallocatequina (EGC), (+)-gallocatequina (GC), y (–)-epicatequina (EC)	Bajerska *et al*. (2011)
Café	Ácido clorogénico y diterpenos (cafestol). Antioxidantes provenientes del café tostado: melanoidinas y otros productos de reacción de Maillard	Moreira *et al*. (2005); Bastos *et al*. (2007)
Mate	Ácidos fenólicos (clrogénico y cafeico)	Bastos *et al*. (2007)
Chocolate	flavan-3-oles (epicatequina), procianidinas	Schinella *et al*. (2010); Robards (2003)
Bebidas alcohólicas		
Cerveza	Ácidos fenólicos (ferúlico, cinámico, clorogénico, canilico, gálico, cafeico, o-cumárico, p-cumárico y siríngico), derivados de flavan-3-ols (cateuqina, epicatequina, procianidinas y prodelfinidina) y flavonoglicósidos	Zhao *et al*. (2010); Leitao *et al*. (2011); Whittle *et al*. (1999)
Vino	Flavonoides (quercetina, mircetina, catequina y epicatequina), acidos fenólicos (gálico, cafeico y ferulico), estilbenos (resveratrol), taninos condensados (polimeros de catequina y epicatequina) y antocianinas (cianidina, malvidina, entre otras)	Dudley *et al*. (2008); Rodrigo *et al*. (2011)

1.1.2.2. Residuos agro-industriales

Además de las frutas, vegetales y diferentes hierbas, los residuos agrícolas e industriales son fuentes atractivas de antioxidantes naturales. En los últimos años se ha enfocado con especial atención la extracción de compuestos de fuentes residuales de bajo coste de la industria agro-alimentaria. Después del procesamiento de alimentos de origen vegetal (frutas y vegetales) en la industria se forman subproductos que contienen grandes cantidades de compuestos bioactivos, incluyendo los compuestos fenólicos. Estos subproductos resultantes del procesamiento en la industria alimentaria representan una importante preocupación para el sector agro-alimentario pero también constituyen importantes fuentes de compuestos de interés que pueden ser utilizados debido a sus propiedades tecnológicas y nutricionales (Balasundram *et al*., 2006; Robards, 2003; Moure *et al*, 2001; Peschel *et al*., 2006). En la Tabla 1.2 se presentan algunos ejemplos de residuos agro-industriales que representan importantes fuentes de compuestos bioactivos.

Tabla 1.2. Compuestos naturales con actividad antioxidante obtenidos a partir de diferentes ejemplos de residuos agro-industriales

Material	**Principales compuestos**	**Referencia**
Frutas y vegetales		
Uva (pepitas y piel)	Compuestos fenólicos (procianidinas, proantocianidinas, catequina, epicatequina, epigalocatequina y galato de epicatequina)	Shrikhande (2000); Torresr & Bobet (2001)
Mango, aguacate, sandía (pepitas)	Compuestos fenólicos: ácidos gálico y elágico; gallotaninos	Soong & Barlow, (2004); Schieber *et al*. (2001); Dorta *et al*. (2012)
Manzana, fresa, pera, remolacha roja	Compuestos fenólicos	Peschel *et al*. (2006)

Material	Principales compuestos	Referencia
Manzana (piel y pulpa)	Flavonoides y antocianinas. Flavonoles, flavanoles, dihidrochalconas y ácidos hidroxicinámicos (clorogenico), procianidinas	Wolfe & Liu (2003); Bhushan *et al*. (2008); Schieber *et al*. (2003)
Kiwi (piel, pulpa y residuos)	Ácidos fenólicos (cafeico, protacatéquico, ρ-hidroxibenzóico, dihidroxibenzoico, siríngico, ρ-cumárico, m-cumárico, o-cumárico ferúlico, salicilico), epicatequina, procianidina, quercetina	Sun-Waterhouse *et al*. (2009)
Cáscaras de coco seco	Acidos ferúlicos y 4-hidroxibenzoico	Dey *et al*. (2003)
Alcachofa	Ácido clorogénico, ácido cafeico y derivados	Llorach *et al*. (2002)
Alcachofa, tomate, espárragos (residuos de la industria conservera: piel, tallos)	Compuestos fenólicos	Peschel *et al*. (2006)
Brócoli, pepino, endivia, achicoria (residuos de la cosecha: tallos)	Compuestos fenólicos	Peschel *et al*. (2006)
Cereales, semillas y frutos secos		
Maíz (semilla y mazorca)	Antocianinas, ácido ferúlico, ácido 4-hidroxibenzóico, ácido vanilico, ácido ρ-cumárico, quercetina	Yang & Zhai, 2010, Torre *et al*. (2008), Parajó *et al*. (2008); Conde *et al*. (2011)
Almendra (cáscaras)	Ácido clorogénico, venílico, siríngico, quercetina	Parajó *et al*. (2008); Conde *et al*. (2011)
Castañas (erizo)	Ácidos protocatéquico, clorogénico, vanilico, siríngico, ρ-cumárico y feúlico. Flavonoides: quercetina, rutina y apigenina	Conde *et al*. (2011)
Trigo (cáscaras)	Ácido protocatéquico	Watanabe *et al*. (1997)
Centeno (salvado)	Ácido hidroxicinámico	Gallardo *et al*. (2006)

Material	Principales compuestos	Referencia
Subproductos de la producción de aceites		
Oliva (aguas residuales, hojas)	Tirosol y hidroxitirosol, oleuropeína, ácidos hidroxicinámicos (protocatéquico, cafeico, ρ-cumárico)	Obied *et al*. (2005); Garcia-Castello *et al*. (2010)
Aceite vegetal	Ácidos fenólicos y flavonoides	Matthäus (2002)
Madera y podas de olivo	Compuestos fenólicos	Parajó *et al*. (2008)
Subproductos de la producción del vino		
Bagazo de uva	Fenoles simples (ácidos gálico, protocatéquico, siríngico, ρ-cumárico, vanílico y 3,4-dihidrobenzaldehído)	Cruz *et al*. (2004); Soto *et al*. (2012)
Licores obtenidos del destilado del bagazo de uva	Ácidos protocatéquico, 4-hidroxibenzoico, clorogénico, vanílico y elágico; quercetina	Díaz-Reinoso *et al*. (2010); Conde *et al*. (2011)
Podas de sarmiento	Ácidos hidroxicinámicos (ferúlico y ρ-cumárico) y ácidos hidroxibenzóicos (gálico), vanilina y vinilguaiacol	Max *et al*. (2010)
Residuos forestales		
Eucalipto (hidrolizados de madera)	Fenoles simples (vanillina, siringaldehído, ácido vanílico, ácido protocatéquico, ácido siríngico, ácido resorcilico, ácido gálico, ácido elágico).Flavonoides (quercetina)	Cruz *et al*. (2005); Conde *et al*. (2011)
Pino (madera)	Compuestos fenólicos	Parajó *et al*. (2008)

1.1.2.3. Industria cervecera- residuos y subproductos

En la presente tesis se enfoca con especial interés la industria cervecera y su impacto en el sector agro-alimentario así como la valorización de uno de sus residuos como fuente interesante de compuestos bioactivos con valor añadido para la industria alimentaria.

Relevancia de la industria cervecera en el sector agro-alimentario

El sector de la elaboración de cerveza en España ostenta una posición destacada tanto en el contexto europeo como mundial. La producción del sector cervecero español alcanzó en 2012 los 32,2 millones de hl, siendo el cuarto productor de cerveza de la Unión Europea; y encontrándose entre los 10 principales productores del mundo (Cerveceros de España *et al*., 2013).

El sector cervecero es clave dentro del panorama agro-alimentario español. En 2011 la facturación fue de 3.000 millones de euros. La aportación a la economía nacional en términos de valor añadido supone más de 5.900 millones de euros; de hecho, el valor de la cerveza en el mercado ha resultado ser de unos 12.600 millones de euros, cifra que supone un 1,2% del PIB. Las exportaciones de cerveza española ascendieron en 2012 un 25% respecto al año anterior, hasta superar los 1,2 millones de hl comercializados en territorio nacional (Cerveceros de España *et al*., 2013)

A nivel europeo, cabe destacar, que en el año 2010 Europa fue uno de los mayores productores mundiales de cerveza (403 millones de hl) por encima de Rusia (101 millones de hl), Brasil (122 millones de hl) y Estados Unidos (207 millones de hl) y solo superado por China (466 millones de hl), que en el año 2008 fue cuando por primera vez superó a la Unión Europea, y pasó a ser el primer productor mundial de cerveza (Brink *et al*., 2011).

Aprovechamiento y valorización de los residuos de la industria cervecera

En el diagrama de la Fig. 1.5 se muestra el proceso general de producción de cerveza. Se destacan en diferentes colores las distintas operaciones agrupadas de acuerdo a las cuatro fases productivas principales: fabricación del mosto, fermentación y guarda, filtración y estabilización microbiológica/coloidal y

envasado. También se indican los diferentes residuos y subproductos que se forman durante todo el proceso de elaboración.

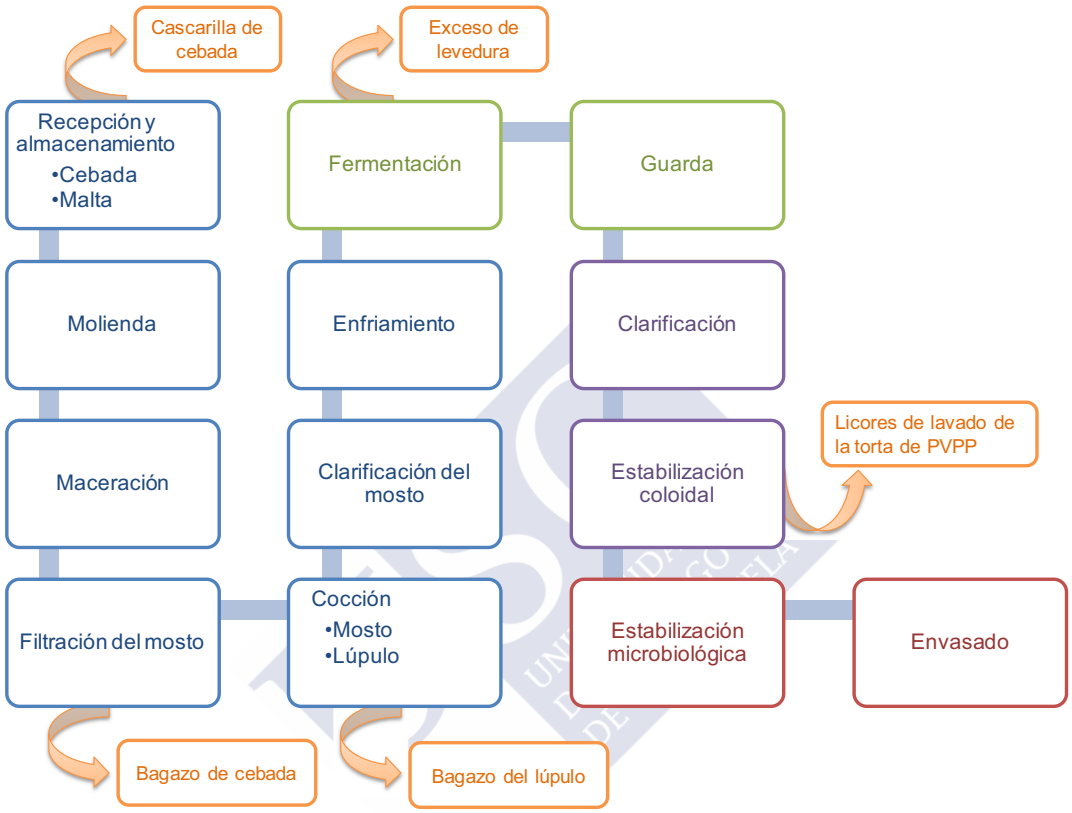


Figura 1.5. Proceso de producción de la cerveza (Canales *et al*., 2005; Mussatto, 2009)

En el proceso industrial de elaboración de la cerveza se generan inevitablemente grandes cantidades de residuos y subproductos siendo alguno de los más relevantes el bagazo de cebada, el bagazo del lúpulo y las levaduras. Desde un punto de vista medio ambiental, la eliminación de estos residuos representa una solución importante para los problemas de polución de los residuos agro-industriales y reciben especial atención por parte del sector. Varios estudios han demostrado que estos residuos y subproductos pueden tener

diferentes aplicaciones en procesos biotecnológicos debido a su compleja composición en carbono, nitrógeno y minerales pudiendo ser utilizados: en procesos fermentativos o para la producción de compuestos de valor añadido xilitol, arabitol, etanol, acido láctico, entre otros; como substrato de cultivo de microorganismos o simplemente como materiasprimas para la extracción de compuestos como azúcares, proteínas, ácidos orgánicos y antioxidantes (Mussatto, 2009; Gupta, 2010).

Además de estos subproductos, durante el proceso de elaboración de la cerveza mostrado en la Fig. 1.5, en las etapas iniciales de elaboración y malteado se generan otros residuos como la cascarilla de cebada, que puede ser utilizada como fuente de antioxidantes naturales. El grano de cebada se usa en el proceso de elaboración de la cerveza, y la cascarilla de los granos es uno de los principales subproductos, sin ningún propósito útil, y que representa hasta el 15-20% del peso seco del grano. Mediante un proceso de prehidrólisis y posterior deslignificación de la cascarilla de cebada se pueden obtener extractos naturales que contienen compuestos fenólicos y que presentan una alta actividad antioxidante (Cruz *et al*., 2007). En la Tabla 1.3 se presentan los principales subproductos que se generan en la industria cervecera y que son fuentes importantes de compuestos fenólicos con actividad antioxidante de amplia aplicación en la industria alimentaria. También se presentan las materias primas utilizadas y su contenido en compuestos fenólicos.

Tabla 1.3. Materiales, subproductos y residuos de la industria cervecera como fuente de compuestos bioactivos con actividad antioxidante estudiados y referenciados en la bibliografía hasta al momento

Material	Compuestos fenólicos	Referencias
Materias primas		
Cebada	Antocianidinas, flavonoles (quercetina, rutin), ácidos fenólicos (ferúlico, ρ-cumárico, vanílico, ρ-cumárico, protocatéquico, salicílico, sinápico, cafeico y siríngico), flavan-3-oles (catequina, epicatequina, epigallocatiquina) y proantocianidinas (oligomeros y polímeros de flavanoles: catequina y gallocatequina, proantocianidina B3 y procianidina B3). Tocoferoles y carotenoides	Goupy *et al*. (1999); Maillard *et al*. (1996); Whittle *et al*. (1999); Verardo *et al*. (2008); Holtekjølen *et al*. (2006); Dvorakova *et al*. (2008); Gupta *et al*. (2009)
Malta	Ácidos fenólicos (derivados de los ácidos cinámico y benzoico), flavan-3-oles, flavonoles (quercetina) proantocianidinas, taninos	Goupy *et al*. (1999); Maillard *et al*. (1996); Dvorakova *et al*. (2008)
Lúpulo	Terpenos, chaolconas, flavonols (kaempferol, quercetina, quercitrina y rutina), catequinas (galato de catequina y galato de epicatequina), estilbenos (resveratrol)	Zanoli & Zavatti (2008); Callemien *et al*. (2005); Magalhães *et al*. (2009)
Sub-productos		
Bagazo de cebada	Ácidos fenólicos mayoritariamente ácidos hidroxicinámicos (ácidos ferúlico, ρ-cumárico, sinápico, cafeico y siríngico). Ácidos hidroxibenzoicos (ρ-hidroxibenzoico, vanilico, siríngico y protocatéquico). Ácido clorogénico. Tocoferoles y tocotrienoles	McCarthy *et al*. (2013a); McCarthy *et al*. (2013b); Mussatto (2009); Mussatto *et al*. (2006)
Bagazo del lúpulo	Ácidos p-hidroxicumárico, gálico, ferúlico, protocatéquico y cafeico. Catequinas, flavonas y antocianidinas	Mussatto (2009)
Residuos		
Cascarilla de cebada	Vainillina, siringaldehído, ácido p-cumárico, ρ-hidroxibenzaldehído, ácido ferúlico, ácido siríngico, ácido gálico, ácido ρ-hidroxibenzoico y ácido vainíllico	Cruz *et al*. (2007)

Otro residuo que hasta al momento no había tenido gran relevancia para el sector, es el caso de los licores de lavado de la torta de polivinilpolipirrolidona (PVPP) que se obtienen en la etapa de estabilización coloidal de la cerveza (ver Fig. 1.5). Esta corriente residual puede ser una fuente importante de antioxidantes naturales, motivo por el cual fue objeto de valorización en esta tesis.

Aprovechamiento y valorización de los licores de lavado de la torta de PVPP

Los polifenoles presentes en la cerveza son extremadamente importantes en su estabilidad física. Dicha estabilidad puede ser mejorada reduciendo el contenido en polifenoles o el contenido proteico de la cerveza, ya que los polifenoles y las proteínas de alto peso molecular tienden a combinarse lentamente y formar un complejo proteína-polifenol insoluble. Estos complejos resultan de reacciones de oxidación y polimerización de los polifenoles endógenos (catequinas y proantocianidinas) y de sus interacciones con las proteínas, que se forman durante la guarda fría y que se eliminan en la filtración de la cerveza (Siebert, 1999; Callemien & Collin, 2010, Callemien & Collin, 2008). Sin embargo si quedan suficientes cantidades de proteínas y polifenoles después de la filtración puede continuar produciéndose complejos en la cerveza filtrada. La etapa de filtración (que incluye las operaciones de clarificación y estabilización coloidal) es fundamental para la obtención del nivel requerido de claridad y retrasar el enturbiamiento natural de la cerveza desde su elaboración hasta su consumo.

La estabilización coloidal implica por lo tanto, la eliminación selectiva de proteínas o taninos mediante la utilización de (Siebert, 1997; Mitchell *et al*., 2005; Mcmurrough *et al*., 1995):

- ✓ **adsorbentes** que eliminan la fracción tánica o la fracción proteica. Esta es una técnica bastante extendida que se realiza conjuntamente con la clarificación. La estabilización se consigue por aditivación en línea de alguna sustancia adsorbente que elimina bien la fracción tánica (usando PVPP, Nylon, etc.) o bien la fracción proteica (usando silicagel, bentonita, etc.), evitando de este modo que se puedan formar los complejos enturbiantes insolubles tanino-proteína;

- ✓ **enzimas proteolíticas** que reducen las moléculas de proteínas para evitar su reacción con los taninos;

- ✓ **ácido tánico** cuyo principio de acción es el contrario a los dos anteriores, ya que en lugar de eliminar o modificar los dos tipos de moléculas involucrados en el fenómeno de turbidez, se favorece la reacción entre los taninos y las proteínas, de modo que se forma un precipitado que decanta en el fondo de los tanques de guarda.

De todas estas técnicas de estabilización coloidal, una de las más empleadas es la reducción del contenido en taninos (reducción del exceso de polifenoles) mediante la adsorción con PVPP. Debido a que este PVPP tiene un coste considerable, una vez que los polifenoles se adsorben en la resina de PVPP se realiza un lavado o regeneración de la torta de PVPP con soluciones alcalinas (NaOH) en caliente, para recuperar la capacidad de adsorción de la torta de PVPP, y que esta pueda ser reutilizada en sucesivos lotes. El líquido de lavado se considera un desecho, y como tal es enviado a la estación depuradora de aguas residuales con la total pérdida de valor como subproducto, además de ocasionar costes de depuración considerables. Esta corriente residual de PVPP se encuentra cargada fundamentalmente con compuesto polifenólicos y por lo tanto, los extractos que se pueden obtener a partir de esta corriente residual de la

industria cervecera son una fuente prometedora de antioxidantes naturales con amplia aplicación.

1.1.3. Potencial y aplicación práctica de los compuestos fenólicos de origen natural

Actualmente hay un creciente interés en sustancias con propiedades antioxidantes que pueden ser utilizadas como componentes/suplementos de alimentos o como agentes específicos con propiedades farmacológicas, siendo una parte importante de la tecnología de conservación de alimentos y de cuidados de la salud. Los polifenoles pueden tener muchas aplicaciones a nivel industrial, como por ejemplo, su uso como colorantes naturales y aditivos para usos alimentarios, en la producción de cuadros, papel, en formulaciones tópicas para la dermocosmética y para la industria farmaceutica.

Los antioxidantes sintéticos de uso alimentario como el BHT, BHA y TBHQ han sido ampliamente utilizados por la industria alimentaria en la conservación de los alimentos. Sin embargo, con la crecente preocupación en lo que respecta a la seguridad de estas sustancias para la salud humana y la demanda por parte de los consumidores de productos más seguros, se viene despertando un elevado interés por los antioxidantes de origen natural como la vitamina E o extractos naturales ricos en compuestos fenólicos y/o terpénicos. La capacidad antioxidante de una amplia variedad de vegetales, cereales, frutas, semillas y de residuos agrícolas e industriales se ha analizado en diversos estudios, siendo los residuos agrícolas e industriales una fuente atractiva de antioxidantes naturales. Diversos estudios se han llevado a cabo en las últimas décadas aplicando diversos compuestos y extractos de origen natural de diversos orígenes en la conservación de diferentes alimentos (Kondratyuk & Pezzuto, 2004; Shahidi & Zhong, 2010;

Moure *et al*., 2001, Balasundram *et al*., 2006; Robards, 2003; Peschel *et al*., 2006; Schieber, 2001).

El uso de estos compuestos y extractos naturales obtenidos a partir de varias fuentes naturales, incluyendo residuos agro-industriales, va a tener un papel importante en el aumento de la vida útil de los alimentos, incrementando su estabilidad a través de la prevención de la peroxidación lipídica, de la prevención de la descomposición de los pigmentos naturales de los alimentos, y contribuir a la protección y estabilización de los aromas, además de que poseen una considerable capacidad antimicrobiana. Además de aditivos alimentarios, los extractos naturales también se pueden utilizar como nutracéuticos, suplementos alimentarios e ingredientes funcionales (Gil-Chávez *et al*., 2013).

Además de las propiedades antioxidantes, a estos compuestos se les atribuyen actividades biológicas beneficiosas para salud. Estudios epidemiológicos han demostrado que dietas ricas en frutas, vegetales y bebidas (zumos de fruta, vino, té, café, cerveza, etc.) aportan un elevado contenido en compuestos fenólicos que ofrecen protección contra varias enfermedades relacionadas con el estrés oxidativo como por ejemplo cáncer; enfermedades crónicas como son las cardiovasculares, artritis reumatoide, osteoporosis, diabetes, hipercolesterolemia, arterioesclerosis (aterogénesis) y enfermedades neurodegenerativas. Además, algunos de estos compuestos han demostrado muchos otros beneficios para la salud humana, como por ejemplo, propiedades antibacterianas, antivirales, antimutagénicas, antialérgicas, antidiarreicas, antiulcerosas, antiinflamatorias, inmunoestimulantes, inhibidora del aumento da tensión arterial, inhibidoras de la agregación plaquetária, entre otras enfermedades (Scalbert *et al*., 2000; Scalbert *et al*, 2005; Higdon & Frei, 2003; Crozier *et al*., 2009; Tajkarimi *et al*, 2010).

En la Tabla 1.4 se presentan algunos ejemplos de diversos materiales como fuentes de compuestos naturales, los compuestos más relevantes por su bioactividades así como sus posibles usos y aplicaciones en diferentes campos como pueden ser el alimentario y el farmacéutico.

Tabla 1.4. Materiales de origen natural como fuentes de compuestos bioactivos de valor añadido, sus bioactividades y potenciales usos y aplicaciones

Material	Compuestos activos	Bioactividades / posibles usos y aplicaciones	Referencia
Plantas y hierbas aromáticas			
Hojas de Ginkgo biloba	Flavonoides: Quercetina, kaempferol, y isorhamnetin (predominante)	Actividades fisiológicas en terapias contra a inflamación, enfermedades coronarias y cáncer	Gil-Chávez *et al*. (2013)
Plantago reniformes (planta)	Ácidos fenólicos (vanílico y clorogénico), flavonas (luteolin), flavonoles (quercetina), cumarinas y lignanos	Actividades antioxidante, anti-inflamatoria y citotóxica	Beara *et al*. (2012)
Orégano, estragón y tomillo	Compuestos fenólicos	Antioxidante/ compuestos funcionales para la industria alimentaria	Miron *et al*. (2010)
Albahaca y orégano	Terpenos	Anti-inflamatorio y antioxidante	Yang *et al*. (2007)
Romero, tomillo y hierba luisa (verbena)	Fenoles, proteínas, aminoácidos y azúcares	Alimentos funcionales, nutracéuticos y antioxidantes	Plaza *et al*. (2010)
Hojas de orégano	Flavonas, flavononas y flavonoles	Ingredientes de uso alimentario para adobar salchichas, carnes y ensaladas. Actividad antixiodante y beneficios para la salud	Cavero *et al*. (2006)

Material	Compuestos activos	Bioactividades / posibles usos y aplicaciones	Referencia
Hojas de romero	Antioxidantes volátiles del aceite. Fenoles totales, ácidos rosmarínico y carnósico	Alimentos funcionales. Substancias antioxidantes y anti-cancerígenas. Antioxidantes naturales para uso alimentario	Carvalho (2005); Rodríguez-Rojo *et al*. (2012)
Colza	Fenoles, tocoferoles y fosfolípidos	Prevención y tratamiento de de enfermedades crónicas: enfermedades del corazón y neurodegenerativas, edad, cáncer y artritis reumatoide	Szydłowska-Czerniak *et al*. (2010)
Saúco y 3 variedades de bagazo de uva roja	Acidos fenólicos y antocianinas	Colorantes naturales de uso alimentario y nutracéuticos	Vatai *et al*. (2008) Vatai *et al*. (2009)
Frutos			
Cascaras de cítricos	Fenoles totales	Actividad antioxidante y de captación de radicales libres. Implicación en la salud humana	Ma *et al*. (2009)
Pulpa de manzana y melocotón	Polifenoles	Promotor de la salud	Adil *et al*. (2007)
Pulpa de la manzana	Polifenoles	Efectividad contra el cáncer	Boyer & Liu (2004)
Plátano (monda) Subproducto	Fenoles totales y antocianinas	Antioxidante/ compuestos con aplicación alimentaria y con beneficios para la salud	Gonzáles-Montelongo *et al*. (2010)
Planta del pimentón rojo	Carotenoides	Agentes protectores contra el cáncer, enfermedad coronaria y enfermedad degenerativa del ojo	Rutkowska & Stolyhwo (2009)
Berenjena	Acidos fenólicos y flavonoides	Antioxidante y hepatoprotector/ ingredientes nutracéuticos	Akanitapichat *et al*. (2010)
Berenjena (piel)	Antocianinas	Antioxidante/colorante natural de uso alimentario	Todaro *et al*. (2009)

Material	Compuestos activos	Bioactividades / posibles usos y aplicaciones	Referencia
Subproductos agro-industriales			
Vaina de Parkia speciosa	Fenoles totales y flavonoides	Antioxidante/ agente nutracéutico para la protección de la salud humana	Gan & Latiff (2010)
Biomasa de salvado de arroz	Compuestos fenólicos	Efectos beneficiosos contra el cáncer y diabetes, entre otros. Aplicación en la industria alimentaria y en el ámbito de la salud y de la cosmética	Pourali *et al*. (2010)
Pepitas de la uva	Polifenoles: catequinas y proantocianidinas	Pueden ser empleadas en las industrias alimentaria, farmacéutica y cosmética Anti-carcinogénica, antiviral, anti-cancerígena y también puede actuar contra la oxidación de las lipoproteínas de baja densidad (LDL)	García-Marino *et al*. (2006); Li *et al*. (2001); Yilmaz *et al*. (2010)
Piel de la uva	Antocianinas	Aditivos de uso alimentario que aportan beneficios a la salud	He & Giusti (2010)
Piel de cebolla	Quercetina	Actividades anti-cancerígena, antiviral y anti-inflamatoria	Ko *et al*. (2011)
Germen de trigo tostado	Compuestos fenólicos y tocoferoles	Formulación farmacéutica, alimentaria y cosmética. Agentes biológicos contra insectos	Gelmez *et al*. (2009)
Semillas de cilantro	Fracciones antioxidantes	Suplementos de dieta en la industria de producción de nutracéuticos	Sahib *et al*. (2012)
Piel de cacahuetes	Compuestos fenólicos	Aplicación farmacéutica de compuestos promotores de la salud incluyendo prevención anti-cancerígena	Ballard *et al*. (2010)
Cebada y bagazo de la cebada (industria cervezera)	Acidos fenólicos y flavonoides	Aplicación farmacéutica de compuestos promotores de la salud incluyendo prevención anti-cancerígena. Aditivos de uso alimentario, aplicación de extractos en zumos de frutas, alimentos funcionales	McCarthy *et al*. (2013a); McCarthy *et al*. (2013b); Gupta *et al*. (2010)

1.2. Oxidación lipídica en alimentos

Los lípidos presentes a nivel de los sistemas biológicos y alimentarios se componen esencialmente por una mezcla de tri-, di- y monoglicéridos, ácidos grasos libres, glicolípidos, fosfolípidos, esteroles (incluido el colesterol), pigmentos y vitaminas liposolubles, siendo la mayoría de estos componentes oxidables en diferentes grados. Los triglicéridos resultan de la esterificación de una molécula de glicerol con ácidos grasos y son considerados los principales responsables para el desarrollo de la rancidez. Los triglicéridos mixtos de ácidos grasos insaturados o saturados de cadena larga que contienen un número par de átomos de carbono, son los principales componentes de las grasas naturales comestibles (Gordon, 2001).

Los lípidos desempeñan un papel importante en la calidad de determinados productos alimenticios, en particular con relación a las propiedades organolépticas que los hacen atractivos al consumidor, tales como sabor, color y textura. Por otro lado, aportan valor nutritivo a los alimentos, siendo una fuente de energía metabólica, de ácidos grasos esenciales (ácido linoleico, linolénico y araquidónico) y de vitaminas liposolubles (A, D, E y K) (Frankel, 1996; Adegoke *et al*., 1998).

La oxidación lipídica es un fenómeno espontáneo e inevitable, con una participación directa, tanto en el valor comercial de las grasas como en el de todos los productos que se fabrican a partir de ellas (alimentos, cosméticos o productos farmacéuticos). El proceso de oxidación de las grasas y aceites en los alimentos es la principal causa de los sabores y olores rancios y de los cambios en el color y en la textura de los alimentos. También es responsable de la reducción del valor nutritivo por la pérdida de vitaminas y ácidos grasos poliinsaturados, y por la disminución de la seguridad de los alimentos debido a la

formación de productos secundarios y potencialmente tóxicos y nocivos para la salud (Choe & Min, 2009; McClements & Decker, 2000)

1.2.1. Mecanismos de oxidación lipídica en alimentos

En la cadena de producción de alimentos, el estado de oxidación cambia bajo la influencia de factores biológicos, ambientales y tecnológicos. La oxidación afecta a todos los componentes de los alimentos y puede ocurrir mediante mecanismos enzimáticos o no enzimáticos, pero su impacto en la calidad de los alimentos no es uniforme.

En los alimentos, la peroxidación lipídica puede ser inducida por reacciones de radicales libres, fotooxidación, reacciones catalizadas por enzimas (lipooxigenasas), y la oxidación catalizada por metales traza dependiendo de las propiedades del producto y de las condiciones de almacenamiento o procesamiento (Frankel, 2005; .Brimberg & Kamal-Eldin, 2003).

1.2.1.1. Autooxidación

La autooxidación es el principal proceso por el cual se inicia la oxidación. Ocurre por un mecanismo radicalario entre las moléculas insaturadas de ácidos grasos y el oxígeno molecular. Se trata de un fenómeno puramente químico y bastante complejo, que implica reacciones con radicales capaces de auto-propagarse, y que dependen del tipo de acción catalítica (temperatura, iones metálicos, radicales libres, pH, luz visible y ultravioleta (UV)). Constituyendo, así, un proceso dinámico que evoluciona a lo largo del tiempo (Frankel, 2005; Brimberg & Kamal-Eldin, 2003; McClements & Decker, 2000).

Mecanismos

El mecanismo de autooxidación puede ser descrito en tres etapas distintas: Iniciación, propagación, y terminación, que se describen a continuación.

- ***Etapa de Iniciación***: Formación de radicales libres: peróxido- ($RO^{\bullet}_2$), alcoxilo ($RO^{\bullet}$) o alquilo-($R^{\bullet}$) según los esquemas de reacción [1] y [2]:

$$RH \rightarrow R^{\bullet} + H^{\bullet} \qquad [1]$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \qquad [2]$$

La formación del radical lipídico $R^{\bullet}$ es catalizado por diversos factores:

(i) Disociación térmica de hidroperóxidos;
(ii) Descomposición de hidroperóxidos catalizada por metales redox (M) de valencia variable;
(iii) Acción de la luz sobre una sustancia que actúa de sensibilizador;

propiciando la formación de otros radicales (multiplicaciones de cadena) de acuerdo con las reacciones [3] y [4]:

$$ROOH \rightarrow RO^{\bullet} + {}^{\bullet}OH \qquad [3]$$

$$2\ ROOH \rightarrow RO^{\bullet}_2 + RO^{\bullet} + H_2O \qquad [4]$$

- ***Etapa de Propagación***: los radicales lipídicos se convierten en radicales peróxido por la reacción con el oxígeno molecular según el esquema de la fórmula [5]. Los radicales lipoperóxidos inician una serie de reacciones con otras moléculas lipídicas insaturadas, resultando la formación de hidroperóxidos lipídicos y radicales libres derivados de lípidos según la reacción [6]. Sin embargo, los hidroperóxidos también pueden sufrir

descomposición y formar alcoholes, aldehídos, cetonas e hidrocarburos según el esquema de la reacción [7]. La reacción de propagación tiene lugar de forma continua siempre que haya ácidos grasos insaturados disponibles:

$$R^{\bullet} + O_2 \rightarrow RO^{\bullet}_2 \qquad [5]$$

$$RO^{\bullet}_2 + RH \rightarrow ROOH + R^{\bullet} \qquad [6]$$

$$RO^{\bullet} + RH \rightarrow ROH + R^{\bullet} \qquad [7]$$

- ***Etapa de terminación de cadena***: ocurre la reducción de los ácidos grasos insaturados, los radicales se unen entre sí, formando compuestos no radicales estables:

$$2\ R^{\bullet} \rightarrow R\text{-}R \qquad [8]$$

$$R^{\bullet} + RO^{\bullet}_2 \rightarrow ROOR \qquad [9] \quad \text{Productos estables}$$

$$2\ RO^{\bullet}_2 \rightarrow ROOR + O_2 \qquad [10]$$

Los resultados finales dependen del tipo de radicales que intervengan en la neutralización. Cuando no hay limitación de oxígeno, se forman mayores cantidades de radicales peroxilos de acuerdo con la formula [10]. En el caso de haber restricciones de oxígeno, la composición de los productos finales del tipo representado en las formulas [8] y [9] depende de varios factores, tales como: solubilidad del oxígeno en el alimento, la temperatura o la porosidad del alimento. Todas las fases del proceso de oxidación trascurren en simultáneo en una serie compleja de reacciones secuenciales y sobrepuestas.

En el transcurso de la cadena oxidativa, es posible distinguir tres etapas de evolución oxidativa: (i) desaparición de los substratos de oxidación (oxígeno,

lípido insaturado); (ii) formación de los productos de oxidación primaria; y (iii) formación de los productos de oxidación secundaria

Productos de oxidación

- ***Productos de oxidación primaria***, (peróxidos y hidroperóxidos), cuya estructura depende de la naturaleza de los ácidos grasos presentes. En los ácidos grasos poliinsaturados (PUFAs), la formación de estos compuestos está favorecida debido a la presencia de un mayor número de dobles enlaces. Los hidroperóxidos son compuesto altamente inestables que se descomponen rápidamente en radicales libres (mayoritariamente radicales alcoxilo y hidroxilo) que siguen diferentes caminos para producir una gran variedad de productos de oxidación secundarios. Los hidroperóxidos se forman y se descomponen simultáneamente y constituyen los compuestos más abundantes en condiciones de temperatura baja y moderada. Cuando estos compuestos se acumulan en niveles relativamente elevados, su descomposición es más rápida que su formación y la velocidad de oxidación global aumenta de forma exponencial llevando a un incremento significativo de compuestos de oxidación secundaria, incluyendo el inicio de polimerización, el agotamiento completo de antioxidantes de origen natural, y la detección inequívoca de rancidez que denotan el final del periodo de inducción. (Frankel, 1998; Frankel, 2005; Gordon, 2001).

- ***Productos de oxidación secundaria***, obtenidos por escisión y reordenación de los peróxidos (epóxidos, compuestos volátiles y no volátiles), cuya naturaleza y proporción dependen de diversos parámetros. La descomposición de los hidroperóxidos y un gran número de reacciones posteriores dan lugar a una mezcla compleja de productos secundarios de

oxidación que se pueden agrupar de acuerdo con el peso molecular (PM) en (Bartosz, 2013):

- ✓ ***Compuestos volátiles***, con peso molecular inferior a los triglicéridos originales, producidos por la descomposición de radicales alcoxilo. Los compuestos volátiles, provienen de la escisión hemolítica del enlace C-C en la β-posición con respecto al oxígeno del radical alcoxilo, siendo los más comunes los aldehídos, cetonas, alcoholes, alcanos, alquenos. Estos compuestos son la principal causa de las pérdidas de sabor por parte de los lípidos alimenticios que tienen impacto a concentraciones bajas;

- ✓ ***Monómeros oxidados***, o compuestos con peso molecular similar al de los TG originales, que llevan una función oxigenada en al menos uno de sus grupos acilo del acido graso. El radical alcoxilo puede participar en diferentes reacciones para producir compuestos con grupos oxigenados, principalmente, epoxi, ceto y funciones hidroxi en acidos grasos de cadena corta. Estos compuestos son los productos de oxidación secundaria más abundantes formados a temperatura ambiente o moderada y los más relevantes desde el punto de vista nutricional, ya que son fácilmente digeridos y absorbidos;

- ✓ ***Compuestos de polimerización***, (dímeros y oligómeros superiores) formados a través de la interacción de dos o más radicales de TG y por lo tanto con peso molecular más elevado que los TG no oxidados originales. Estos polímeros son los compuestos de degradación más abundantes en los aceites de fritura usados y también se forman a temperaturas ambiente y moderadas, siendo significativos al final del periodo de inducción. En los aceites ricos

en PUFAs o ácidos grasos conjugados, estos polímeros incluso pueden ser detectados antes de la aparición de la rancidez.

Principales factores implicados en la oxidación lipídica

Los fenómenos de oxidación de los lípidos dependen de mecanismos de reacción variados y extremadamente complejos, que están relacionados con el tipo de estructura lipídica y el ambiente donde se encuentran. La estabilidad oxidativa de los lípidos en las grasas y aceites, depende de varios factores (McClements & Decker, 2000; Gordon, 2001):

- **Oxígeno**, por el tipo de interfase entre los lípidos y el oxígeno (fase lipídica continúa, dispersa o en emulsión). Dado que el oxígeno es un reactivo fundamental para propagar las reacciones de autooxidación, su ausencia prevendría alteraciones oxidativas;

- La presencia de la **luz**, que actúa como catalizador de la captación de hidrógeno en la etapa de iniciación, influye en la descomposición de los hidroperóxidos que favorecen la formación de radicales libres, y acelera la oxidación;

- **Temperatura** (termooxidación), la velocidad de oxidación aumenta exponencialmente con la temperatura, y además existe una fuerte interacción entre la temperatura y el oxígeno debido a que la solubilidad del oxígeno disminuye a la medida que aumenta la temperatura;

- **Composición en ácidos grasos**, el número y la naturaleza de insaturaciones presentes (grado de insaturación) son determinantes en la velocidad del proceso de oxidación lipídica debido a la diferente reactividad de los ácidos grasos insaturados;

- La presencia de **prooxidantes**, como los iones de metales de transición (cobre y hierro) que pueden estar en el alimento en forma de impurezas traza o incluso se pueden añadir para enriquecer los alimentos. El efecto prooxidante del metal puede ocurrir con la catálisis de la composición de hidroperóxidos, y por la reacción directa con el substrato sin oxidar para producir radicales alquilo y de la activación de oxígeno molecular para producir oxígeno singlete y radicales peróxido;

- La presencia de **antioxidantes**, es de mayor importancia para la inhibición de la autooxidación en los lípidos en los alimentos. Componentes menores presentes de forma natural en los aceites, como los tocoferoles y polifenoles, ejercen un papel protector esencial contra la oxidación lipídica interrumpiendo la cadena de propagación por reacción con radicales peróxido. Otros mecanismos de protección atribuidos a los componentes menores de aditivos de aceite o alimentos son quelación de metales (polifenoles, ácido cítrico), la reducción de hidroperóxidos (ácido ascórbico) y otros;

- **Procesos tecnológicos**, procesado de los alimentos y almacenamiento.

1.2.1.2. Oxidación Enzimática

La oxidación lipídica puede producirse mediante catálisis enzimática, en particular por acción de la lipooxigenasa, por su actividad prooxidante. Esta enzima actúa a nivel de los ácidos grasos poliinsaturados (PUFAs) (ácidos linoleico y linolénico y sus esteres), catalizando la adicción de oxígeno a la cadena de hidrocarburos poliinsaturados. El resultado es la formación de peróxidos e hidroperóxidos con dobles enlaces conjugados, los cuales pueden intervenir en las diferentes reacciones de degradación, semejantes a las

observadas en los procesos de autooxidación, originando diversos productos. El proceso de catálisis enzimática transcurre con mayor especificidad, en términos de substrato y de productos finales, que el proceso de autooxidación (Choe & Min, 2009)

Un aspecto importante de la actuación de la lipooxigenasa se refiere a su capacidad para cooxidar sustratos (carotenoides, tocoferoles, clorofila, proteínas, etc.), siendo responsable de la iniciación de nuevos procesos oxidativos. Los procesos de oxidación mediante la acción de enzimas son muy frecuentes en productos cárnicos y pesqueros (Eskin & Shahidi, 2012; Min & Ahn, 2005).

1.2.1.3. Fotooxidación

El mecanismo de fotooxidación de grasas insaturadas es promovido esencialmente por la radiación UV en presencia de sensibilizadores, e implica la participación de oxígeno singlete ($^1O_2$) como agente intermediario reactivo. Los fotosensibilizadoes, tales como los pigmentos presentes en los alimentos, principalmente clorofila, hemoproteínas y riboflavina, se activan mediante la absorción de la luz. La especie excitada puede actuar como iniciador de radicales libres y transferir electrones a los lípidos para formar radicales que, a su vez, pueden reaccionar con el oxígeno (Tipo I) o interactuar con él por la transferencia de energía al oxígeno singlete no radical, una especie muy reactiva que reacciona directamente con lípidos insaturados (Tipo II). El proceso incluye reacciones con radicales, cuyo resultado es la formación de hidroperóxidos diferentes de los que se observan en ausencia de luz y de agentes sensibilizadores que por posterior degradación originan aldehídos, alcoholes e hidrocarburos (Frankel, 2005; Choe & Min, 2009).

1.2.2. Influencia de la composición y naturaleza de los alimentos en la oxidación lipídica

Además de las diferentes variables que afectan a la oxidación lipídica de los alimentos en general, algunos alimentos son especialmente susceptibles a la oxidación. Alimentos constituidos por emulsiones, pescados, productos cárnicos y alimentos procesados son ejemplos de alimentos que muestran características particulares y componentes específicos que influyen en los mecanismos de la oxidación de los lípidos.

Durante el almacenamiento refrigerado y congelado, muchos alimentos son susceptibles a la degradación por mecanismos de oxidación de lípidos. Los alimentos cárnicos y pesqueros son ejemplo de eso y serán abordados a continuación, ya que son los alimentos objeto de los estudios realizados en la presente tesis.

1.2.2.1. Productos cárnicos

Los triglicéridos, los fosfolipídos y el colesterol son los principales sustratos presentes en la carne susceptibles de sufrir oxidación lipídica. La oxidación de los fosfolípidos ocurre antes que la de los triglicéridos debido a la proximidad de los catalizadores del grupo hemo de las mitocondrias y microsomas. Durante el procesamiento y almacenamiento de productos cárnicos, la oxidación del colesterol también puede ser acelerada por radicales lipídicos formados a partir de los ácidos grasos insaturados que constituyen los triglicéridos y fosfolipidos (Gray *et al*., 1996; Min & Ahn, 2005; Osada *et al*., 2000). La estabilidad lipídica depende de la composición de ácidos grasos y del contenido de antioxidantes presentes en cada tipo de carne, así como del procesamiento o las condiciones de presentación del alimento. El oxígeno es factor clave para este tipo de

productos alimenticios. Para limitar su presencia, y así minimizar la oxidación lipídica y el crecimiento de microorganismos, se recurre al empleo de envases impermeables al oxigeno (Brandon *et al*., 2009). Sin embargo, la eliminación completa del oxígeno no es recomendable, ya que es necesario un nivel de oxígeno para mantener el contenido en oximioglobina que le confiere un color rojo brillante a la carne y que resulta muy atractivo para del consumidor.

La mioglobina y la hemoglobina son hemo-proteínas que están presentes en la carne y que tienen un papel importante en los mecanismos de la oxidación de los lípidos. Pueden actuar como aceleradores de la oxidación lipídica, y su efecto es dependiente de la concentración (a mayores concentraciones de hemoglobina y mioglobina mayores tasas de oxidación de los lípidos) (Richards & Dettmann 2003; Min & Ahn, 2005). En la carne, la oxidación de los lípidos y la oxidación de la mioglobina pueden ocurrir al mismo tiempo. La oxidación de la oximioglobina y de la mioglobia (rojo) a metamioglobina (marrón) genera productos intermediarios reactivos capaces de reforzar el proceso de oxidación lipídica y que conlleva una disminución de la calidad de la carne. Dichos compuestos actúan como catalizadores de la descomposición de los hidroperóxidos de los lípidos y son incluso más activos que los iones metálicos libres. A su vez, los lípidos oxidados también pueden contribuir a la oxidación de la mioglobina y oximioglobina por un mecanismo que todavía se desconoce. El grupo hemo puede ser liberado de la mioglobina y de la hemoglobina de las carnes durante la manipulación y el almacenamiento *postmortem*, promoviendo así también la oxidación de lípidos (Bartosz, 2013).

1.2.2.2. Productos pesqueros

Una proporción importante de los lípidos presentes en el pescado (cerca de 40%) están compuestos por los ácidos grasos poliinsaturados ω-3 (PUFAs) de

cadena larga, principalmente el ácido eicosapentaenoico (C20:5) y el ácido docosahexaenoico (C22:6), conocidos por sus propiedades beneficiosas para la salud, sobre todo en las enfermedades cardiovasculares y la aterosclerosis (Sidhu, 2003; Ruxton *et al*., 2004). Sin embargo la presencia de estos compuestos hace que los pescados y productos pesqueros sean substratos muy susceptibles a la oxidación, y los productos resultantes del proceso pueden ser potencialmente prejudiciales, anulando los efectos positivos para la salud de los ácidos grasos originales (Sidhu, 2003; Turner *et al*., 2006).

Otras consecuencias negativas que derivan del proceso de oxidación de los lípidos presentes en los productos de la pesca pueden ser cambios organolépticos inaceptables, que resultan de la formación de compuestos volátiles (propanal), alteraciones en la textura y en el color, resultantes de la interacción de los lípidos oxidados y las proteínas del pescado, y pérdidas de nutrientes esenciales y vitaminas (vitamina E) (Turner *et al*., 2006). Los compuestos volátiles que resultan de la oxidación lipídica son un problema específico de los productos pesqueros, ya que tienen valores umbrales muy inferiores a otros productos alimenticios (aceites vegetales), pudiendo ser fácilmente detectables a muy bajos niveles de oxidación (Bartosz, 2013; Ross & Smith, 2006).

La oxidación de los lípidos en el musculo de pescado puede ocurrir por procesos no enzimáticos (autooxidación y fotooxidación), o catalizada por enzimas (lipoxigenasa). De igual forma a lo que ocurre con otros alimentos, las hemo-proteínas, los metales de transición, y las lipoxigenasas son los componentes endógenos primarios involucrados en la oxidación de los lípidos del pescado. En el pescado *in vivo* existe un equilibrio entre prooxidantes (Fe-hemo presente en la hemoglobina y mioglobina, Fe no hemo, lipoxigenasas, etc) y antioxidantes (tocoferoles, astaxantina, ubiquinol, vitamina C, y enzimas como la superóxido dismutasa, etc) que están presentes en los tejidos, pero que se pierde después de la muerte cuando comienzan los procesos de hidrólisis y

especialmente los de oxidación (Adegoke *et al*., 1998; Turner *et al*., 2006; Bartosz, 2013).

1.2.3. Control de la peroxidación lipídica

La oxidación es la segunda causa más importante de deterioro de los alimentos, después de la descomposición microbiana, incluso en aquellos productos que podrían considerarse bajos en sustratos oxidables. Durante el procesado y almacenamiento a largo plazo, ocurren reacciones de oxidación y de descomposición de productos de oxidación que resultan en una disminución del valor nutritivo de los alimentos y su calidad sensorial. Retardar estos procesos de oxidación es importante no solamente para el productor del alimento, sino también para todas las personas implicadas en la cadena de producción industrial y para el consumidor final.

La oxidación puede ser inhibida por variados métodos como: evitar la presencia de oxígeno, utilizar bajas temperaturas, inactivar enzimas catalizadoras del proceso de oxidación, reducir la presión de oxígeno y el uso de envases adecuados (Sun & Holley, 2012).

Estrategias ampliamente empleadas en la industria alimentaria durante el procesado y almacenamiento para retrasar la oxidación de los lípidos son: la suplementación con vitamina E en las dietas animales, la adición de nitrito de sodio en carne curada, que además también actúa como agente antimicrobiano, manipular los alimentos a bajas temperaturas para evitar la hidrólisis de los triglicéridos y fosfolípidos por las lipasas y fofolipasas, utilizar salas de luz artificial, limitar el acceso del oxígeno a los alimentos durante el almacenamiento recurriendo al envasado a vacío o utilizando materiales de envase impermeables al oxígeno con atmosferas modificadas en su interior, y, por último, el empleo de

una combinación de antioxidantes naturales o sintéticos con sinergistas tales como el ácido ascórbico (Bartosz, 2013; Sun & Holley, 2012).

1.2.3.1. Antioxidantes de uso alimentario

Una forma de protección eficaz contra el deterioro oxidativo en los alimentos es el uso de aditivos específicos de uso alimentario que inhiban la oxidación. Estos aditivos se denominan más correctamente como inhibidores de la oxidación pero son más conocidos como antioxidantes.

Según el Reglamento (CE) Nº1333/2008 del Parlamento Europeo y del Consejo de 16 de diciembre de 2008 *sobre aditivos alimentarios, se define como "antioxidantes" las "sustancias que prolongan la vida útil de los alimentos protegiéndolos del deterioro causado por la oxidación, como el enranciamiento de las grasas y los cambios de color"* (UE, 2008).

Los antioxidantes son moléculas que presentan la capacidad de reaccionar con radicales libres, o de oxidarse con más facilidad que otras moléculas presentes, reduciendo así, o incluso eliminando, la aparición de posibles reacciones de oxidación. El antioxidante ideal deberá ser: inocuo; efectivo y activo a bajas concentraciones; soluble en el producto o sustrato oxidable; inodoro e insípido y no alterar el color del alimento; estable en un amplio rango de pH y durante el procesamiento industrial de los alimentos; compatible con los restantes constituyentes del substrato; y de fácil obtención y barato. En el uso industrial de los antioxidantes en alimentos, se debe tener en cuenta su volatilidad y estabilidad térmica, es decir, su capacidad para resistir a los tratamientos térmicos de los alimentos y seguir ejerciendo su acción protectora en el producto final (Choe & Min, 2009; Brandon *et al*., 2009).

En función del mecanismo de acción de los compuestos antioxidantes en los procesos de oxidación en alimentos, los antioxidantes se clasifican en (Choe & Min, 2009; Brandon *et al*., 2009):

- ***Sustancias que tienen capacidad de interrumpir la reacción en cadena de la formación de radicales libres***, como por ejemplo los antioxidantes de estructura fenólica (BHA; BHT; PG y tocoferoles). Estas sustancias actúan como captadores de radicales libres y/o como dadores de átomos de hidrógeno, así como secuestradores del oxígeno singlete evitando que estos reaccionen con los lípidos. Estas sustancias, al producir radicales antioxidantes relativamente estables, no propagan la cadena oxidativa. Además, compiten directamente con el sustrato lipídico RH por los radicales peroxilo;

- ***Agentes quelantes***, que ejercen una acción preventiva de la oxidación. Los compuestos de este tipo más importantes son los inactivadores de metales, como el ácido ascórbico, que desactivan los iones metálicos, promueven el inicio de la degradación de los peróxidos y retrasan la formación de aldehídos. Los agentes quelantes también pueden interferir en otros mecanismos de oxidación, inactivando los fotosensibilizadores responsables de la fotooxidacion o inactivando enzimas como las lipoxigenasas;

- ***Sustancias reductoras que tienen capacidad destructiva sobre los peróxidos*.** Se trata de compuestos capaces de inhibir la oxidación de los ácidos grasos insaturados al inducir la descomposición de los hidroperóxidos y formar compuestos estables e inactivos. Un ejemplo de estos compuestos es el α-tocoferol.

La vitamina E es un ejemplo habitual de una sustancia liposoluble que interrumpe y previene la peroxidación lipídica al convertir los radicales libres en

sustancias menos reactivas. El radical tocoferoxil es nuevamente convertido a tocoferol por antioxidantes hidrosolubles, como por ejemplo, el ascorbato y los compuestos fenólicos. La inhibición del proceso de autooxidación a partir de radicales libres por medio de los agentes antioxidantes resulta de gran importancia práctica para preservar los alimentos que contienen ácidos grasos insaturados de un posible deterioro de su calidad (Brigelius-Flohé & Traber, 1999).

Dependiendo de su origen y métodos de producción los antioxidantes se pueden clasificar como sintéticos (galato de propilo (E-310), TBHQ (E-319), BHA (E-320), BHT (E-321)), o naturales (ácido ascórbico (E-300), ácido cítrico (E-330), extractos de origen natural ricos en tocoferoles (E-306), etc).

En los últimos años ha aumentado el interés por los antioxidantes de origen natural para reducir al mínimo el empleo de los aditivos sintéticos artificiales. La posible toxicidad de algunos antioxidantes sintéticos, conjuntamente con la sensibilización de los consumidores en lo que respeta a cuestiones de seguridad y toxicidad alimentaria, ha motivado un creciente interés en la identificación de antioxidantes alternativos y seguros de origen natural (Moure *et al*., 2001; Balasundram *et al*., 2006; Robards, 2003; Peschel *et al*., 2006; Gil-Chávez *et al*., 2013). Hoy en día, se tiende cada vez más a utilizar antioxidantes naturales a partir de extractos de plantas (tocoferoles, flavonoides, polifenoles) mezclados con compuestos sinérgicos, tales como eliminadores de radicales (ácido ascórbico y ascorbatos) o quelantes de metales (ácido etilendiaminotetraacético (EDTA), fosfatos, carnosina). También, por diversas razones tecnológicas, los antioxidantes naturales están sustituyendo a los sintéticos. Así los antioxidantes naturales ofrecen ventajas respeto a su solubilidad aceite/agua, propiedades de especial interés para las emulsiones frecuentemente encontradas en alimentos. Los tejidos musculares poseen de forma intrínseca varios tipos de antioxidantes solubles en lípidos (α-tocoferol, ubiquinona), antioxidantes solubles en agua

(ascorbato), y enzimas con actividad antioxidante (superóxido dismutasa, catalasa, glutatión peroxidasa). Los antioxidantes naturales pueden ser solubles en agua o en lípidos y poseen ambas partes hidrófila y lipófila en la misma molécula (McClements & Decker, 2000; Huang *et al*., 1994). Ésta es una característica muy importante teniendo en cuenta los sistemas de alimentos que contienen lípidos, por ejemplo, productos de tipo emulsión. Otra característica importante es que pueden ser incoloros o poseer varios colores (por ejemplo, las antocianinas), además pueden tener diferentes sabores u olores, características importantes que afectan a la calidad sensorial general de los alimentos que contienen estos compuestos naturales, y por lo tanto, que les permite actuar como aditivos multifuncionales (Gil-Chávez *et al*., 2013).

1.3. Envases activos de uso alimentario

En los últimos años se han producido una serie de cambios en las preferencias de los consumidores y en la manera en que los alimentos son producidos y comercializados. Los consumidores demandan productos alimenticios cada vez más “frescos y naturales”, saludables, semiprocesados o conservados sin tratamientos térmicos y con vida útil prolongada, que resulten adecuados tanto desde un punto de vista nutricional (conservando sus propiedades nutritivas) como de sencillez en su preparación y exigen además, mayor calidad, seguridad e información de los productos a los que tienen acceso.

La industria alimentaria hace eco de esas demandas y viene realizando una serie de cambios de acuerdo a satisfacer las necesidades de los consumidores y al mismo tiempo trata de adaptarse a la globalización de los mercados. De esa manera, surge una red de distribución de los alimentos más amplia y con mayor

alcance, se incrementan los tiempos de rotación, producción, distribución y venta de los productos, (lo que a su vez reduce los costes), así como, aparece una mayor centralización en las actividades de producción e investigación (De Kruijf *et al*., 2002).

Hoy en día se puede observar un incremento continuo en la demanda de la industria para aumentar la vida comercial de los productos alimenticios envasados y en la demanda de los consumidores para mejorar la seguridad, la calidad y la integridad de los alimentos. Como consecuencia, se están poniendo cada vez más exigencias en el rendimiento del envasado de uso alimentario. Para responder a estos cambios, a estas nuevas necesidades y desafíos, se están desarrollando nuevos sistemas de envasado conocidos como “envases activos” (Danielli *et al*., 2008).

Los envases activos, a diferencia de los tradicionales, a los que se les exige que sean totalmente inertes, están diseñados para interaccionar de una forma activa y continua con su contenido, en un proceso de interacción entre envase, ambiente y alimento, que implica siempre una transferencia de masa, ya sea para ceder sustancias al contenido del envase (el alimento y su entorno) o absorber componentes desde dicho contenido y cuya finalidad es siempre la de ampliar el tiempo de conservación de los alimentos (Ahvenainen & Hume, 1997; Ahvenainen, 2003). Los materiales activos, modifican determinadas condiciones o procesos del alimento que juegan un papel determinante en la vida comercial del producto. Así pues, alteraciones provocadas por procesos químicos (oxidación, decoloración, etc.), procesos físicos (endurecimiento, deshidratación, etc.) o procesos microbiológicos (deterioro por microorganismos) pueden ser reguladas convenientemente mediante la aplicación de sistemas activos apropiados. Dependiendo de los requerimientos del producto envasado, el deterioro en la calidad puede ser reducido significativamente, y de esta manera se puede conseguir un incremento de la vida comercial (De Kruijf *et al*., 2002).

1.3.1. Diferentes tipos de envases activos de uso alimentario

Las sustancias responsables de la función activa del envase pueden estar, o bien contenidas en un recipiente separado, por ejemplo, en una bolsita de papel, o bien incorporadas directamente en el material de envase. Así pues, un objetivo importante en el desarrollo de este tipo de envases es el diseño de materiales funcionales que incluyen el agente activo en su estructura y que esta sustancia activa pueda actuar o ser liberada de una manera controlada (Coma, 2008).

Los envases activos incluyen aditivos o "potenciadores de frescura" que participan en una serie de aplicaciones del envase y, al hacerlo, mejoraran la función de la preservación del sistema de envase primario (Restuccia *et al*., 2010). A continuación se presentan algunos ejemplos con aplicación en el sistema de envasado activo para su uso en la industria alimentaria con diferentes propiedades/funciones (Brody *et al*., 2008; Vermeiren *et al*., 1999; Kerry, *et al*., 2006; Restuccia *et al*., 2010):

- **Propiedades de absorción/ eliminación**: Absorbedores de oxígeno (incluidos radicales libres del oxígeno), dióxido de carbono, etileno, olores y sabores desagradables, luz UV y absorbedores/reguladores de humedad;

- **Propiedades de liberación/emisión**: Liberadores de agentes antimicrobianos (etanol, dióxido de carbono, ácidos sórbico, benzoico, propiónico, bacteriocinas y otros), antioxidantes, aromatizantes, saborizantes, colorantes, ingredientes alimentarios, extractos y aceites esenciales de plantas con acción antimicrobiana o antioxidante;

- **Propiedades de eliminación**: Catalizadores de la eliminación de componentes de los alimentos: lactosa, colesterol;

- **Control de temperatura**: Materiales aislantes, que experimentan calentamiento espontáneo y envases de auto-enfriamiento, susceptores y modificadores de microondas, envases sensibles a la temperatura;

- **Control microbiano y de calidad**: Materiales de envasado con tratamiento en superficie para reducir la carga microbiana.

De acuerdo con Cooksey (2001) los sistemas de envases activos destinadas a mejorar la calidad y extensión vida útil de los alimentos se pueden desarrollar mediante tres procedimientos:

- **Incorporación de las substancias activas en saquitos, tiras y etiquetas** (sistemas independientes) que se incorporan o adhieren al interior del envase, pero que son parte diferenciada del mismo. Generalmente son permeables por una sola cara, orientada hacia al espacio de cabeza del envase y no al alimento para evitar el contacto y posible obstrucción o desactivación. A través de estos sistemas independientes se realiza la transferencia de masa, habitualmente gases o vapores absorbidos o liberados por el sistema, constituyendo los sistemas más ampliamente utilizados en los días de hoy (Ozdemir & Floros, 2004);

- **Incorporación directa de substancias activas en los films de envase**, incorporándolas químicamente en el propio material, en capas intermedias de los materiales multicapa, evitando así el contacto directo de la parte activa con el alimento;

- **Recubrimiento de los films con una matriz que actúa como vehículo de la sustancia activa** (segundo recubrimiento) mediante dispersión con substancias bioactivas, como pueden ser biocinas o aceites esenciales, que están en contacto directo con el alimento.

A diferencia de los sistemas independientes de saquitos, tiras y etiquetas, en el caso de los sistemas activos que llevan incorporados el aditivo en el propio film de envase benefician a la industria al simplificar el manejo y mejorar la seguridad de los consumidores ya que, el consumidor no percibe el aditivo como elemento diferenciado del envase, lo que evita el posible rechazo del consumidor y el riesgo de consumo accidental de su contenido (Suppakul, 2003; Ozdemir *et al*., 2004). Por otra parte, los materiales activos pueden estar compuestos de una o más capas o partes de diferentes tipos de materiales, tales como plásticos, papel y cartón o recubrimientos y barnices (Restuccia *et al*., 2010).

En general el agente activo (sustancias químicas, extractos o componentes obtenidos de productos naturales, aditivos antioxidantes y antimicrobianos, aromas o ingredientes alimentarios) está integrado en el propio material de envase, desde donde se libera mediante difusión al alimento (substancias no volátiles) o al espacio de cabeza del envase (substancias volátiles). Controlando los parámetros que afectan al proceso de difusión se puede conseguir una liberación controlada del agente activo (Coma, 2008).

1.3.2. Envases activos en la conservación de productos cárnicos y pesqueros

Los envases de uso alimentario protegen los productos frescos contra los efectos del deterioro, como la decoloración, mal sabor y desarrollo de mal olor, la pérdida de nutrientes, cambios de textura, patogenicidad, y otros factores cuantificables. En la carne fresca el proceso de envasado también es importante para permitir cierta actividad enzimática, para mejorar la ternura y para garantizar el color de las carnes rojas conferido por la presencia de oximioglobina. Cuando se envasan productos cárnicos y pesqueros elaborados, también es necesario

tener en cuenta factores como la deshidratación, la oxidación de lípidos, la decoloración y la pérdida de aroma (Kerry *et al*., 2006).

En el proceso de envasado de los productos cárnicos y pesqueros frescos, hay varios factores pueden influir en su vida útil, como por ejemplo el tipo de producto, la mezcla de gases, el tipo de envase y el espacio de cabeza disponible, los equipos de envasado, la temperatura de almacenamiento y los aditivos. Las tecnologías de envasado tradicionales utilizadas para la carne fresca, productos cárnicos procesados y productos de la pesca han consistido principalmente en el envasado al vacío, el envasado en atmósfera modificada (MAP) utilizando films barrera, y en el uso de envases que controlan la permeabilidad del vapor de agua y de los gases de envasado, dos factores que son los más influyentes en su deterioro (envases pasivos) (Zhou *et al*., 2010). Actualmente, la aplicación de substancias bioactivas específicas en los materiales de envasado (envasado activo) con el fin de afectar positivamente a la calidad y para extender la vida útil de los productos es una técnica muy interesante y de gran interés para este sector de la industria alimentaria.

Muchos de estos sistemas activos, se emplean en el envasado de productos cárnicos y pesqueros frescos. Entre ellos los que tiene mayor aplicación son los: absorbedores/scavengers de oxígeno; los absorbedores y emisores de dióxido de carbono; los emisores de etanol y absorbedores de olores (aplicable en el caso de pescado); los reguladores de humedad, y los envases antimicrobianos y/o antioxidantes. Todos ellos ofrecen importantes beneficios a la industria alimentaria y a los consumidores y tienen un gran mercado potencial (Vermeiren *et al*., 1999; Coma, 2008; Brody *et al*., 2008, Kerry *et al*., 2006; Gill, 2003; Sivertsvik, 2003).

1.3.2.1. Sistemas de incorporación de las substancias activas en saquitos, tiras y etiquetas

Absorbedores o eliminadores de O_2

El oxígeno es una de las principales causas de deterioro de los productos alimenticios. Elevados niveles de oxígeno en el interior del envase pueden aumentar el crecimiento bacteriano y acelerar la degradación de alimentos ricos en grasas. Aunque los alimentos sensibles al oxígeno pueden ser envasados en condiciones de atmosfera modificada o de vacío, tales técnicas no siempre facilitan la eliminación completa de oxígeno (Kerry *et al*., 2006). Sin embargo, los absorbedores de O_2 reducen y controlan de manera activa los niveles residuales de oxígeno en el interior del envase, pudiendo ser aplicados solos o en combinación con los procesos tradicionales de envasado, logrando una mejora en la extensión la vida comercial de un producto alimenticio y constituyendo una alternativa económica y eficaz a los envases tradicionales/pasivos (Ozdemir *et al*., 2004).

La utilización de los absorbedores de oxígeno aplicados a los envases de productos cárnicos y pesqueros pueden prevenir los fenómenos de oxidación tales como el enranciamiento de grasas y aceites y su consiguiente aparición de malos olores y sabores, la pérdida o cambio de los colores característicos de los alimentos, la pérdida de los pigmentos del musculo, la pérdida de nutrientes sensibles al oxígeno (vitaminas A, C, E, ácidos grasos insaturados, etc.) y el crecimiento bacteriano (Vermeiren *et al*., 1999). Estos sistemas se basan en los mecanismos de las reacciones de oxidación y utilizan diferentes tipos de sustancias que reaccionan fácilmente con el oxigeno, como son algunos compuestos orgánicos tales como ácido ascórbico, catecol, ácidos grasos insaturados (oleico, linoleico, linolénico), enzimas (glucosa oxidasa/catalasa o

alcohol oxidasa), y hierro en polvo y sales ferrosas (estos últimos de los más ampliamente utilizados y de los más efectivos), pudiendo utilizarse combinaciones de diferentes sustancias. Los eliminadores de oxígeno más comunes se pueden aplicar en el formato de saquito o tira, contenedores de la sustancia activa, que se introducen en el envase o son adheridos a la pared interior del envase (etiqueta), o a su vez, también pueden ser incorporados en el cierre o en el material de envasado a través de su disolución o dispersión en la red polimérica o a través de la inmovilización de enzimas oxidantes en su superficie (De Kruijf *et al*., 2002; Kerry *et al*., 2006).

Un caso especial dentro de los absorbedores de oxígeno son los absorbedores de radicales libres del oxígeno. Dichos absorbedores se basan en la eliminación de radicales libres a medida que estos se van formando en un proceso de oxidación en cadena que se inicia debido a la presencia de radicales libres generados a partir del oxígeno molecular. Recientemente, en algunos trabajos de investigación, se han incorporado aceites esenciales y compuestos fenólicos en los envases alimentarios con la finalidad de reaccionar con radicales libres de oxígeno y eliminarlos del espacio de cabeza, de tal manera que se ralentizaban los procesos de oxidación y se incrementaba la vida útil del alimento (Nerín *et al*., 2008; Pezo *et al*., 2008).

Absorbedores y liberadores de CO_2

La función del dióxido de carbono en el espacio de cabeza del envase es la de suprimir el crecimiento microbiano. Niveles altos de dióxido de carbono (10-80%) son deseables para alimentos como la carne y el pescado frescos con el fin de inhibir el crecimiento microbiano en la superficie y extender su vida útil. Un sistema de generación de dióxido de carbono se puede utilizar conjuntamente con absorbedores de oxígeno para mantener una atmósfera adecuada para la

conservación de determinados productos, reduciendo la tasa de respiración de los productos frescos y evitando el colapso del envase o el vacío parcial causado por eliminadores de oxígeno (Vermeiren *et al*., 1999; Suppakul *et al*., 2003). El dióxido de carbono está disponible en diversas formas, tales como sistemas basados en carbonato de hierro (II) o mezclas de hidrógenocarbonato sódico y ácido ascórbico, silica gel y oxido de calcio que han sido ya utilizados para aumentar la vida comercial de carnes frescas, incorporados en saquitos porosos o en almohadillas adsorbentes activados con la humedad o directamente incorporados en el material de envase (Kerry *et al*., 2006; Ahvenaimen, 2003; Lee, 2010).

Absorbedores o reguladores de humedad

El control de la cantidad de agua, ya sea en estado líquido o de vapor, es fundamental en algunos productos alimenticios para controlar la actividad del agua del producto así como el crecimiento bacteriano (Vermeiren *et al*., 1999). La presencia de líquidos de exudación (agua, sangre u otros fluidos) en productos cárnicos y pescados desmerece la presentación de los mismos y aumenta el riesgo de deterioro del producto. Niveles elevados de agua en el interior del envase favorecen el crecimiento de microorganismos, mientras que, excesivas pérdidas de agua pueden favorecer la oxidación de lípidos (Vermeiren et al., 1999). Además, los productos envasados con un valor alto de humedad relativa en el espacio de cabeza, son susceptibles a las fluctuaciones de temperatura durante el transporte o almacenamiento, lo cual favorece la formación de condensados y nieblas (De Kruijf *et al*., 2002). Por todo ello, es necesario el uso de controladores de humedad que pueden actuar por un proceso de absorción eliminado el exceso de agua líquida en el envase (almohadillas, hojas formadas por capas de materiales poliméricos, generalmente microporosos, conteniendo agentes higroscópicos como celulosas, propilenglicol, carbohidratos, etc) o controlando el vapor de agua en el espacio de cabeza del envase (saquitos o

etiquetas que contienen agentes deshidratantes como silica gel) (Suppakul *et al*., 2003; De Kruijf *et al*., 2002).

Absorbedores de olores y aromas

La absorción de aromas y sabores de los alimentos por parte de los materiales de envasado poliméricos, puede provocar una pérdida de la calidad e intensidad del sabor y cambios en el perfil organoléptico de los alimentos que generalmente resultan perjudiciales. Sin embargo, este efecto, se podría adaptar de una manera positiva para absorber selectivamente los olores o sabores no deseados. Dos tipos de contaminaciones que podrían ser objetivos de los envases activos/absorción de aromas son las aminas que resultan de la degradación de proteínas en el músculo de pescado y los aldehídos (hexanal y heptanal) que se forman por la descomposición de de los peróxidos resultantes de las etapas iniciales de la autooxidación de las grasas y aceites. Ambas pueden transformar una gran variedad de alimentos que contienen grasa en productos organolépticamente inaceptables (Vermeiren *et al*., 1999). Sin embargo, es muy importante que estos sistemas no sean incorrectamente utilizados enmascarando el desarrollo de malos olores producidos por los microorganismos peligrosos poniendo en riesgo a los consumidores (Brody *et al*., 2008).

1.3.2.2. Incorporación directa de substancias activas en los films de envase

Liberadores o emisores de agentes antimicrobianos/aditivos conservantes

En la literatura se pueden encontrar muchas aplicaciones sobre la incorporación de sustancias utilizadas como potenciales agentes antimicrobianos

a films y materiales plásticos para inhibir el crecimiento de microorganismos que pueden conducir al deterioro de los productos alimenticios (Kerry *et al*., 2006). Ejemplos de ellas son: el dióxido de cloro, ácidos orgánicos, respectivos ácidos anhídridos y sus sales (sórbico, benzoico, propiónico, benzoato de sodio, anhídrido benzoico), parabenos (etil p-hidroxibenzoato y metil p-hidroxibenzoato), ácidos grasos (ácido láurico, ácido palmitoleico, glicerol mono-laurato), agentes quelantes (EDTA), metales (plata, cobre, circonio, óxido de titanio), enzimas (lisozima, peroxidasa y glucosa oxidasa usada en pescado), polipéptidos (lactoferrina), polisacaridos, bacteriocinas (nisina, pediocina y natamicina), antibioticos, fungicidas, nitritos y sulfitos, y dióxido de azufre (Suppakul *et al*., 2003; kerry *et al*, 2006, Lee, 2010; Quintavalla & Vinici, 2002; Vermeiren *et al*., 1999, Zhou *et al*., 2010).

También hay varias referencias a films liberadores de biocidas como triclosan, antioxidantes como el tocoferol, compuestos fenólicos, compuestos volátiles de plantas, extractos naturales y aceites esenciales de plantas (canela, oréganos, etc), así como el empleo de polímeros bioactivos como el quitosano, tanto como antimicrobiano como vehículo de origen natural para la difusión controlada de diversos agentes activos (Moore *et al*., 2000; Vermeiren *et al*., 2002; Coma, 2008; Lee, 2010; Outarra *et al*., 2000a; Ouattara *et al*., 2000b; Cutter, 2006).

Dentro de los sistemas activos con agentes antimicrobianos también se incluyen aquellos que, en principio, no están destinados a ser liberados al contenido del envase, por ejemplo algunos sistemas (zeolitas) que contienen iones plata y que impiden el crecimiento microbiano en la interfase plástico-alimento (Quintavalla *et al*., 2002) (Danielli *et al*., 2008), enzimas inmovilizados (Vermieren *et al*., 1999) o nanopartículas de plata en celulosa (Fernandez *et al*., 2010).

Liberadores de aditivos antioxidantes

Los sistemas activos que contienen antioxidantes son utilizados para ralentizar los procesos de oxidación que afectan a la calidad de los alimentos a través de la liberación los agentes antioxidantes al alimento y/o al espacio de cabeza, donde van a ejercer su acción, generalmente como eliminadores de radicales libres. La efectividad de la utilización de los antioxidantes sintéticos, ampliamente utilizados en la industria alimentaria, como el terbutilhidroquinona (TBHQ), butil hidroxianisol (BHA) y butil hidroxitolueno (BHT) ha sido reiteradamente demostrada, así como la efectividad de antioxidantes naturales como la vitamina E (Vermieren *et al*., 1999; Moore *et al*., 2000) y los extractos naturales ricos en compuestos fenólicos y/o terpénicos (Nerín *et al*., 2006, Camo *et al*., 2008; Pereira de Abreu *et al*., 2010).

Liberadores de extractos naturales o componentes obtenidos de los mismos

Los productos naturales como aceites esenciales, extractos de plantas o componentes obtenidos de ellos, son ricos en compuestos fenólicos como flavonoides y ácidos fenólicos, que les confieren propiedades antioxidantes y antimicrobianas (Sun & Holley, 2012). La adición directa de estos aceites esenciales en productos cárnicos o pesqueros tienen un efecto inmediato en la reducción de la carga microbiana, pero puede a su vez producir alteraciones en las características sensoriales del producto (Skandamis & Nichas, 2002). Sin embargo, cuando son incorporados en los films de envase, esas alteraciones sensoriales pueden ser minimizadas y, además, los efectos positivos verse prolongados en el tiempo por un efecto de liberación controlada. La incorporación de aceites esenciales y/o sus principales componentes han sido ampliamente utilizados en la producción de films activos con propiedades antimicrobianas y

antioxidantes (Kuorwel *et al*., 2011). En la Tabla 1.5 se presentan ejemplos encontrados en la bibliografía de extractos naturales incorporados en la matriz plástica del envase aplicados a productos cárnicos y de la pesca.

Tabla 1.5. Films activos aditivados con extractos naturales y su aplicación en la conservación de productos cárnicos y de la pesca

Extracto natural	Polímero	Aplicación	Referencia
Extracto de té verde	EVOH	Antioxidante Productos de la pesca Sardinas en salmuera	Lopés-de-Dicastillo *et al*. (2012)
Proteína zeína del maíz combinada con compuestos fenólicos	LLDPE	Antioxidante Carne picada	Park *et al*. (2012)
Extracto de té verde	Quitosano	Antioxidante Carne de cerdo	Siripatrawan & Noipha (2012)
Alil-isotiocianato	PLA	Antimicrobiano	Vega-Lugo & Loong-Tak (2009)
Romero y orégano	Poliestireno/LDPE	Antioxidante Carne de cordero	Camo *et al*. (2008)
Aceites esenciales de clavo, orégano y canela (enriquecido con cinamaldehído)	PP y PE/EVOH	Antimicrobiano	López *et al*. (2007)
Romero	PP	Antioxidante Mioglobina y otros procesos oxidativos en carne fresca	Nerín *et al*. (2006)
Semillas de uva	PE multicapa LDPE coating	Antimicrobiano en carne de vaca picada	Ha *et al*. (2001)
Extracto de romero y tocoferol	PE	Antioxidante Color de la carne de ternera	Moore *et al*. (2000)

Liberadores de aromas

La incorporación de aromas en el material plástico se puede utilizar para minimizar la pérdida del olor propio del producto fresco o incluso mejorarlo o realzarlo, al abrir el envase (Gutiérrez, *et al*., 2010; López-Rubio *et al*., 2006). También puede proporcionar un medio para enmascarar malos olores provenientes de los alimentos o de su envoltura. Otras aplicaciones de los materiales de envasado liberadores de aromas incluyen la posibilidad de mejorar la calidad organoléptica del producto mediante la emisión de sabores deseables al alimento y para encapsular aromas agradables, que se liberan después de la apertura (Vermeiren *et al*., 1999).

1.3.2.3. Substancias bioactivas incorporadas como recubrimiento en la superficie de los films de envase

Una alternativa a la incorporación de substancias activas al material de envase durante la extrusión es aplicar los aditivos como un revestimiento. Esto tiene la ventaja de colocar el aditivo específico de una manera controlada sin someterlo a altas temperaturas o fuerzas de corte y además permite que el revestimiento se pueda aplicar en un paso posterior, reduciendo al mínimo la exposición del producto a la contaminación (Nussinovitch, 2009). El recubrimiento puede servir como soporte para las substancias activas permitiendo mantener altas concentraciones en la superficie del alimento. La actividad bioactiva del sistema puede deberse o bien a procesos de migración o liberación de los agentes activos, o bien por evaporación en el espacio de cabeza entre el alimento y el material de envase (Coma, 2008; Nerín *et al*., 2008; Skandamis & Nychas 2002).

Los sistemas de coating han sido ampliamente estudiados para la incorporación de agentes antimicrobianos en diferentes matrices poliméricas donde las sustancias pueden ser incorporadas en el revestimiento o bien adsorbidas a la superficie del polímero. En la Tabla 1.6 se presentan varios ejemplos de aplicaciones que se pueden encontrar en la bibliografía, utilizando diferentes materiales poliméricos y con aplicación práctica en productos cárnicos y de la pesca. También se pueden encontrar aplicaciones de extractos naturales y aceites esenciales que aplicados al envase van a tener propiedades, no solo antimicrobianas sino también antioxidantes. Los aceites esenciales tienen la ventaja de ser volátiles, siendo muy interesantes para interaccionar con/en el espacio de cabeza del envase sin entrar en contacto con el alimento. A pesar de las posibles limitaciones en lo que se refiere a la posible liberación de sabores (aromas) y de la efectividad moderada que pueden presentar por la falta de interacción directa con los alimentos, muestran mejores resultados en comparación con la adición en el propio alimento (Skandamis & Nychas, 2002).

Tabla 1.6. Films activos aditivados mediante recubrimiento con substancias activas de origen natural y su aplicación en la conservación de productos cárnicos y de la pesca

Componente activo	Polímero/ Coating	Aplicación	Proceso de liberación	Referencias
Extracto de cítricos y α-tocoferol	Bandejas de PET	Antimicrobiano Carne de pavo cocinada	Migración	Contini *et al*. (2012)
Extracto de orégano	PP	Antioxidante Carne de vacuno	Evaporación en el espacio de cabeza	Camo *et al*. (2011)
Extracto de cascarilla de cebada	LDPE	Antioxidante en pescados	Migración	Pereira de Abreu *et al*. (2010)
Nisina	PE	Antimicrobiano	Migración	Storia *et al*. (2008)
Aceites esenciales orégano y pimiento	Derivado de proteína de la leche	Antioxidante y antimicrobiano en carne	Migración	Oussalah *et al*. (2004)
Tomillo, romero, salvia (especias)	Cross-linked caseinate	Antimicrobiano en carne de ternera	Migración	Lacroix *et al*. (2004)
Nisina y α-tocoferol	Papel	Antioxidante y antimicrobiano	Migración	Lee *et al*. (2004)
Aceites esenciales asociado a atmósfera modificada	Papel impregnado en aceites esenciales	Antimicrobiano Productos cárnicos	Evaporación en el espacio de cabeza	Skandamis *et al*. (2002)
Nisina	PVC, LLDPE, nylon	Antimicrobiano Productos cárnicos Piel y muslos de pollo	Migración	Natrajan & Sheldon (2000)
Nisina	Envase plástico de vacio	Antimicrobiano en productos cárnicos	Migración	Ming *et al*. (1997)

Otro concepto con mucha demanda en la actualidad son los films comestibles bioactivos, que consisten en la incorporación de un compuesto activo en un

recubrimiento comestible por inmersión o pulverización, y su aplicación sobre los productos alimenticios.

En los films comestibles, se están utilizando diversos aditivos alimentarios tales como conservantes, antioxidantes y condimentos para mejorar la calidad y aumentar la vida útil de los alimentos. Los recubrimientos comestibles formados por polisacáridos, proteínas y lípidos, funcionan como vehículo de aditivos alimentarios, y pueden ser utilizados mediante tratamiento directo en la superficie para mejorar la calidad de los productos cárnicos y pesqueros frescos, congelados y procesados (Zhou *et al*., 2010). Pueden actuar por diversos mecanismos, siendo los más habituales los que actúan retrasando la pérdida de humedad en productos frescos o congelados, que a su vez reduce la oxidación de lípidos y la decoloración y mejora la apariencia del producto, los que disminuyen la carga microbiana y microorganismos patogénico a la superficie de productos frescos, o los que reducen la perdida de sabor y la formación de malos olores. (Kerry *et al*., 2006; Lee, 2010). Las películas comestibles y recubrimientos de superficie con sustancias bioactivas son susceptibles de ser utilizados para mejorar la conservación y agregar valor a los productos en el futuro. Algunas de las aplicaciones de recubrimiento comestible bioactivo en la conservación de productos cárnicos y pesqueros se presentan en la Tabla 1.7.

Tabla 1.7. Films comestibles activos aditivados mediante dispersión (coating) con sustancias activas de origen natural y su aplicación en la conservación de productos cárnicos y de la pesca

Componente activo	Polímero/Coating	Aplicación	Referencias
Extracto de semilla de uva y polifenoles del té	Quitosano	Antioxidante y antimicrobiano en pescado	Li *et al*. (2011)
Aceite de pescado, vitamina E	Quitosano	Antioxidante en bacalao	Duan *et al*. (2010)
Aceite de canela	Quitosano	Antioxidante y antimicrobiano en pescado	Ojagh *et al*. (2010)
Aceites esenciales de romero, clavo, tomillo, pino, etc.	Gelatina-quitosano	Antimicrobiano en bacalao	Gómez-Estaca *et al*. (2010)
Sorbitol y glicerol	Film de proteína de suero de leche	Antioxidante y propiedades sensoriales en salmón	Rodriguez-Turienzo *et al*. (2011)
Bacteriocina	Agar	Antimicrobiano en pescado (salmón)	Neetoo *et al*. (2010)
Nisina, extracto de semillas de uva, acido malico, EDTA	proteína de suero de leche	Antibacteriano en productos cárnicos	Gadang *et al*. (2008)
Aceites esenciales de romero o orégano	Gelatina con y sin quitosano	Antioxidante sardinas ahumadas refrigeradas	Gómez-Estaca *et al*. (2007)
Extractos de semilla de uva y de té verde	Film de proteína de soja	Antimicrobiano en productos cárnicos (salchichas)	Theivendran *et al*. (2006)
Aceites esenciales de orégano, romero y ajo	Film de proteína de suero de leche	Antimicrobiano	Seydim & Sarikus (2006)

1.3.3. Uso de nanomateriales en el desarrollo de nuevos envases activos

La nanotecnología en el envasado de alimentos es un área emergente y está adquiriendo cada vez mayor importancia para el sector alimentario, con resultados

prometedores y aplicaciones que ya se están desarrollando en las áreas de envasado de alimentos y de la seguridad alimentaria (Chaudhry & Castle, 2011). Los materiales de envase son la categoría de aplicaciones más importante de la nanotecnología actuales para el sector alimentario y según la EFSA, la industria de envasado de alimentos podría atraer la mayor cuota del mercado de la nanotecnología en el futuro (EFSA, 2009)

Algunas de las aplicaciones relacionadas con la nanotecnología incluyen la mejora del sabor, color, sabor, textura y consistencia de los alimentos, el aumento de la absorción y la biodisponibilidad de los alimentos o sus ingredientes (nutrientes) y el desarrollo de nuevos materiales de envasado de alimentos con mejorías en sus propiedades mecánicas, de barrera y antimicrobianas (Restuccia *et al*., 2010).

La incorporación de nanopartículas activas a matrices poliméricas puede ofrecer la doble ventaja de mejorar las propiedades del material de envase, como la resistencia mecánica, la reducción de peso, el aumento de la resistencia al calor, y la mejora de las aptitudes barrera contra el oxígeno, dióxido de carbono, radiación ultravioleta, humedad, compuestos volátiles y olores, tanto de los materiales del envase, como del propio alimento. Además imparte una funcionalidad adicional a nivel del sistema de envase activo (antimicrobiano, antioxidante), promoviendo así el alargamiento de la vida útil del alimento envasado (Bradley *et al*., 2011; Brody *et al*., 2008; Sekhon, 2010).

Los materiales de envase bioactivos necesitan ser capaces de mantener los compuestos bioactivos, ya sean éstos prebióticos, probióticos, vitaminas encapsuladas o flavonoides biodisponibles, en condiciones óptimas hasta que sean liberados al producto alimenticio (Lopez-Rubio *et al*., 2006; Brody *et al*., 2008). La encapsulación de estos compuestos bioactivos dentro del propio envase es un enfoque prometedor porque esto permitiría la liberación de los

principios activos de una manera controlable, además de conferirles protección mecánica y térmica en su interior (Sekhon, 2010).

Las nanoarcillas se encuentran como una de las opciones más interesantes para ser incorporadas en los polímeros y se encuentran entre los primeros nanocompuestos en el mercado como materiales utilizados para mejorar el envasado de alimentos (Silvestre *et al*., 2011). A pesar de que se ha informado mucho acerca de las potenciales ventajas de la utilización de la nanotecnología en la industria alimentaria, se han desarrollado todavía pocas aplicaciones prácticas para este tipo de productos. Los desarrollos más prometedores lanzados en el mercado hasta el día de hoy tienden a mejorar la calidad y la vida útil de los productos cárnicos, mediante la mejora de las propiedades mecánicas, térmicas y de barrera y la incorporación de nanocompuestos bioactivos en o sobre la película (nanocomposite/nanocoating), siendo los más comunes los de capacidad antimicrobiana (Hong & Rhim, 2008; Lim *et al*., 2010; Lopez-Rubio, 2006; Bradley *et al*., 2011).

En el nuevo concepto de recubrimiento comestible o película comestible también es posible incorporar los aditivos alimentarios y otras sustancias bioactivas para la liberación controlada, mediante el uso de nanomateriales (Sorrentino *et al*., 2007). Estos recubrimientos deben constar de ingredientes alimentarios (proteínas, polisacáridos, lípidos, etc) a los que se pueden añadir diversos agentes funcionales que incluyen antimicrobianos, agentes anti-ennegrecimiento, antioxidantes, extractos naturales enzimas, aromatizantes y colorantes (Abdollahi *et al*., 2012; Weiss *et al*., 2006). El recubrimiento comestible con compuestos activos nanoencapsulados, puede controlar de forma efectiva su liberación y proteger los productos de la humedad, el calor u otros factores, por lo que se plantea como una buena opción para mantener la calidad, especialmente para los alimentos que estén sujetos a largos períodos de almacenamiento (López-Rubio *et al*., 2006; Sorrentino *et al*., 2007).

1.3.4. Aspectos legales y seguridad alimentaria

La reglamentación relativa a los envases activos difiere a nivel mundial de país a país. Por poner un ejemplo, los eliminadores de oxígeno y los emisores de etanol han sido ampliamente aceptados en países asiáticos como Japón y Corea. Sin embargo los Estados Unidos y la Unión Europea, donde prevalecen diferentes preocupaciones sociales y donde los gobiernos ejercen una regulación más estricta, la aplicación de este tipo de envases todavía se encuentra restringida (Lee, 2010).

El concepto de material activo comprende un amplio rango de tecnologías que están a disposición de la industria alimentaria para ayudar a la resolución de problemas específicos. En Estados Unidos, Japón y Australia, muchos de estos conceptos ya están siendo aplicados, de forma exitosa, con la finalidad de incrementar la vida comercial y garantizar la calidad y la seguridad de los alimentos; mientras que en Europa, el desarrollo y la aplicación de estos sistemas todavía es limitado. Hasta finales del 2004, una de las razones que se alegaba para este retraso europeo era la restricción legislativa, que no permitía el acceso al mercado de estos sistemas innovadores.

En la actualidad, los envases activos están regulados por el Reglamento (CE) Nº 1935/2004 sobre los materiales y objetos destinados a entrar en contacto con alimentos (UE, 2004), en donde se definen y establecen requisitos generales, y por el Reglamento (CE) Nº 450/2009 que establece las normas concretas sobre materiales y objetos activos e inteligentes que deben aplicarse además de los requisitos generales (UE, 2009). De acuerdo con el reglamento, estos sistemas se denominan materiales y objetos activos en contacto con alimentos y se definen como los "*materiales y objetos destinados a ampliar el tiempo de conservación, o a mantener o mejorar el estado de los alimentos envasados, y que están diseñados para incorporar deliberadamente componentes que transmitan*

sustancias a los alimentos envasados o al entorno de éstos o que absorban sustancias de los alimentos envasados o del entorno de éstos" (UE, 2004).

Sin embargo, la realidad es que, a pesar de esta inclusión en el marco legal europeo, la industria alimentaria europea está utilizando muy tímidamente estos sistemas de envase activo. Además de las restricciones legislativas, su escasa utilización en Europa también puede ser resultado de la falta de información o conocimiento acerca de la aceptación de estos sistemas por los consumidores, del impacto económico-ambiental que puedan tener y de la eficacia de los mismos.

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2

Objetivos

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Con el aumento del consumo de alimentos cada vez menos procesados y las, cada vez mayores, exigencias de calidad por parte del consumidor, la industria alimentaria demanda envases activos que permitan conservar la calidad y extender la vida util de sus productos, disminuir el nivel de aditivos conservantes añadidos y garantizar la seguridad alimentaria de los alimentos mínimamente procesados.

En esta tesis se plantea como objetivo principal el desarrollo de nuevos envases activos, con extractos de origen natural ricos en compuestos polifenólicos, y la posterior evaluación de su aplicabilidad real como antioxidantes y antimicrobianos frente a diferentes productos alimenticios.

Por tal motivo, se propone el aprovechamiento de una corriente residual de la industria cervecera con el objeto de obtener un extracto de naturaleza polifenólica con elevada capacidad antioxidante y antimicrobiana, que será utilizado en el desarrollo de los nuevos sistemas de envases activos. Además de los extractos obtenidos de la industria cervecera, también se utilizarán otros extractos de origen natural como es el caso del extracto de romero y extractos comerciales naturales formulados con tocoferoles.

La consecución del objetivo global de la presente Tesis Doctoral pasa por el cumplimiento de los siguientes objetivos parciales:

1. Optimización del proceso de extracción para la obtención de extracto antioxidante, rico en compuestos fenólicos, a partir de los licores de lavado de la torta de PVPP.

2. Desarrollo de métodos analíticos para la identificación de los compuestos polifenólicos presentes en los extractos naturales.

3. Identificación y cuantificación de los compuestos fenólicos presentes en los extractos naturales.

4. Fraccionamiento y purificación de los diferentes compuestos fenólicos presentes en el extracto natural, para aumentar la capacidad antioxidante de los extractos naturales y eliminar posibles contaminantes que puedan afectar al alimento.

5. Verificación y evaluación de la capacidad antioxidante y antimicrobiana de los diferentes extractos naturales.

6. Desarrollo de envases activos con la incorporación de los extractos naturales en las matrices poliméricas mediante diferentes vías de inclusión: incorporándolo directamente en la matriz plástica (mediante recubrimiento o por extrusión) o previa integración en nanoaditivos.

7. Envasado de los alimentos, tanto del sector cárnico como del sector pesquero, con los nuevos envases activos desarrollados y posterior evaluación de su efectividad antioxidante y antimicrobiana bajo condiciones reales de uso.

3

PATENTE Nº ES 2 347 631 B1

Procedimiento para la recuperación de sustancias antioxidantes a partir del líquido de desecho procedente del lavado de la polivinilpolipirrolidona utilizada para el tratamiento de cerveza.

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(73) Titular/es: **SAN MIGUEL, FÁBRICAS CERVEZA Y MALTA, S.A.**
Urgel, 240
08036 Barcelona, ES

(72) Inventor/es: **Fité Luis, Benet;**
Lluís Tartera, Jaume;
Cruz Freire, José Manuel;
Paseiro Losada, Perfecto y
Barbosa Pereira, Letricia

(74) Agente: **Carpintero López Mario**

(54) Título: **Procedimiento para la recuperación de sustancias antioxidantes a partir del líquido de desecho procedente del lavado de la polivinilpolipirrolidona utilizada para el tratamiento de cerveza.**

(57) Resumen:
Procedimiento para la recuperación de sustancias antioxidantes a partir del líquido de desecho procedente del lavado de la polivinilpolipirrolidona utilizada para el tratamiento de cerveza.
El procedimiento consiste en la recuperación de sustancias antioxidantes a partir del líquido de desecho procedente del lavado de la polivinilpolipirrolidona utilizada para el tratamiento de cerveza para su aplicación en otros ámbitos industriales, tales como la fabricación de films plásticos para embalaje de alimentos. Para ello, se lleva a cabo el lavado de la polivinilpolipirrolidona con sosa (NaOH) concentrada a alta temperatura, tras lo que se procede a la extracción de las sustancias antioxidantes, las cuales son lavadas, congeladas y liofilizadas hasta garantizar la eliminación total del agua del extracto restante y su estabilización, que permita su conservación y posterior uso en otros ámbitos.

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Aviso: Se puede realizar consulta prevista por el art. 37.3.8 LP.

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DESCRIPCIÓN

Procedimiento para la recuperación de sustancias antioxidantes a partir del líquido de desecho procedente del lavado de la polivinilpolipirolidona utilizada para el tratamiento de cerveza.

Objeto de la invención

La presente memoria descriptiva se refiere a una solicitud de una patente de invención, correspondiente al proceso de extracción y secado de los principios antioxidantes del líquido de lavado de la polivinilpolipirrolidona (en adelante PVPP por motivos de simplificación), utilizada en la industria cervecera para, una vez obtenido, posibilitar su uso y valorización en otros ámbitos.

Así pues, la invención tiene su aplicación dentro de la industria dedicada a la fabricación de cerveza al valorizar lo que actualmente sería un producto de desecho y también el posible aprovechamiento de la capacidad antioxidante del extracto obtenido, en otro tipo de industrias (industria alimentaria por ejemplo como aditivo o en industrias auxiliares a la industria alimentaria, por ejemplo como materiales para envases, etc...)

Antecedentes de la invención

Dentro del ámbito de aplicación práctica de la invención, el de la elaboración de cerveza, en la misma se encuentran presentes varios tipos de polifenoles (taninos, flavonoides,...) y proteínas de alto peso molecular que tienden a combinarse lentamente y formar un turbio coloidal insoluble.

Proteína + Polifenol (Soluble) <=> Complejo Proteína-Polifenol (Turbio coloidal insoluble)

Estos complejos se forman fundamentalmente durante la guarda fría y se eliminan en la filtración de la cerveza. Sin embargo si quedan suficientes cantidades de proteínas y polifenoles después de la filtración pueden continuar produciéndose complejos en la cerveza filtrada.

El complejo proteína-polifenol presente en la cerveza filtrada se va oxidando a lo largo de la vida de la cerveza y es extremadamente sensible a la acción de la temperatura, de manera que a temperatura ambiente o superiores, puede estar en forma soluble y a medida que descienda la temperatura, inicia su aparición, por pasar a forma insoluble, así, el turbio coloidal es más o menos soluble a temperatura ambiente pero se vuelve insoluble a las temperaturas frías a las que se sirve la cerveza.

Desde la introducción de la filtración y la percepción de que una cerveza debe ser brillante, los cerveceros han adoptado una serie de técnicas para prevenir o reducir la formación de estos turbios en el envase. Estas técnicas son las denominadas de estabilización coloidal. Unas atacan y reducen la fracción de proteínas (ej. uso de enzimas proteolíticas en la guarda,..), otras la fracción de polifenoles (ej. uso de absorbentes,...) y otras consisten en que no se puedan formar los complejos insolubles.

Entre ellas cabe destacar por ser una de las técnicas de más reciente introducción en la industria cervecera y dentro de la categoría de los absorbentes, la polivinilpolipirrolidona (PVPP) que es una materia sintética que es capaz de eliminar por absorción una gran parte de los polifenoles de la cerveza.

Así, se favorece el contacto entre cerveza y este material para que se produzca la absorción de los polifenoles por parte de la PVPP hasta lo que técnicamente se considere eficiente a efectos de la estabilización coloidal de la cerveza. Una vez se ha considerado suficiente se envía a filtración la cerveza y se recupera este material o se tira a fondo perdido.

Para la recuperación de la capacidad de absorción de forma que recupere su función en los siguientes lotes, se realiza un lavado de este material (PVPP) con el fin de liberarlo de las sustancias que ha absorbido y quede a disposición para un nuevo lote.

El líquido de lavado se considera un desecho y como tal es enviado a la estación depuradora de aguas residuales con la total perdida de valor como subproducto y además ocasionando costes de depuración considerables.

Descripción de la invención

El procedimiento que la invención propone resuelve de forma plenamente satisfactoria la problemática anteriormente expuesta, permitiendo valorizar dicho líquido de lavado.

Para ello, se realiza el lavado de la polivinilpirrolidona con NaOH concentrada a alta temperatura.

Posteriormente, para recuperar los compuestos polifenólicos presentes en estos licores de lavado, se lleva a cabo un proceso de extracción con solvente orgánico con posterior evaporación con los siguientes pasos:

- Adición de ácido clorhídrico hasta conseguir un pH adecuado de entre 1 y 2,5.
- Realización de una mezcla con acetato de etilo u otro disolvente orgánico.
- Extracción con un agitador de palas.
- Reposo para conseguir la separación de fases.
- Separación de la fase orgánica.
- Evaporación de la fase orgánica a la sequedad en un sistema rotatorio a vacío.
- Eliminación del agua (Liofilización, atomización,...) del residuo obtenido para garantizar la eliminación total del agua con el fin de eliminar el agua y así estabilizar el extracto y permitir su conservación y posterior uso en otros ámbitos.
- Recogida del residuo final.

Se obtiene así un extracto caracterizado por el alto contenido en sustancias polifenólicas antioxidantes desglosadas en 15-20 compuestos fenólicos sencillos que llegan a constituir aproximadamente el 60% del extracto como el ácido protocatéquico, el ácido cafeico, el ácido ferúlico, la catequina y el ácido p-cumárico. El 40% restante serían compuestos fenólicos complejos, probablemente oligómeros de polifenoles de difícil identificación. También caracteriza el extracto obtenido por el procedimiento descrito el bajo grado de contenido en agua que es inferior al 1% , siendo su higroscopicidad muy alta.

Aunque la presente descripción se está realizando en base a una aplicación específica en el ámbito cervecero, obviamente el procedimiento es aplicable igualmente en cualquier otro ámbito industrial en el que se den unas circunstancias similares.

Descripción de los dibujos

Para complementar la descripción que se está realizando y con objeto de ayudar a una mejor comprensión de las características del invento, de acuerdo con un ejemplo preferente de realización práctica del mismo, se acompaña como parte integrante de dicha descripción, un juego de dibujos en donde con carácter ilustrativo y no limitativo, se ha representado lo siguiente:

La figura 1.- Muestra una gráfica en la que se ha representado la concentración de extracto obtenido en el proceso de extracción con acetato de etilo del licor de lavado en el que se encuentran los compuestos polifenólicos a recuperar.

La figura 2.- Muestra una gráfica similar a la de la figura anterior, en la que se han representado tres condiciones de extracción diferentes.

La figura 3.- Muestra una gráfica correspondiente al ácido protocatético obtenido en la primera extracción en función de la relación licor/acetato de etilo empleada en la primera extracción.

La figura 4.- Muestra una gráfica correspondiente al ácido cafeico obtenido en la primera extracción en función de la relación licor/acetato de etilo empleada en la primera extracción.

La figura 5.- Muestra una gráfica correspondiente al ácido ferúlico obtenido en la primera extracción en función de la relación licor/acetato de etilo empleada en la primera extracción.

La figura 6.- Muestra una gráfica correspondiente la catequina obtenida en la primera extracción en función de la relación licor/acetato de etilo empleada en la primera extracción.

La figura 7.- Muestra una gráfica correspondiente al ácido p-cumárico obtenido en la primera extracción en función de la relación licor/acetato de etilo empleada en la primera extracción.

Realización preferente de la invención

Tal y como se ha comentado con anterioridad, partiendo de la Polivinilpolipirrolidona , se procede a un lavado de la misma con una disolución de NaOH y a una temperatura alta, obteniendo líquido de desecho de la industria de la cerveza. Posteriormente, para recuperar los compuestos polifenólicos presentes en estos licores de lavado, se lleva a cabo un proceso de extracción con acetato de etilo, previa acidificación de los licores de lavado hasta pH adecuado con ácido clorhídrico concentrado.

Seguidamente se procede a la optimización de las relaciones fase acuosa:fase orgánica, así como el número de etapas del proceso extracción.

Experimentalmente se probaron varias relaciones de extracción y se comprobó el rendimiento y la actividad antioxidante obtenida en cada una de las condiciones de extracción evaluadas. El proceso de extracción propuesto permite obtener un rendimiento de extracción entre 0.4 - 1.8 g de extracto/L de licor de lavado, y la actividad antioxidante de los extractos obtenidos permite presenta una EC50 entre 0.23 - 0.45 g/L, siendo por tanto entre 5 y 10 veces más activo que el BHT.

También se detallaron la cantidad obtenida de los diferentes polifenoles para las diferentes condiciones de extracción estudiadas.

Con posterioridad se procedió a la realización de análisis tanto de los extractos como de los licores para caracterizar los polifenoles.

Los compuestos antioxidantes presentes en mayor cantidad en el extracto son el ácido protocatéquico, la catequina, el ácido caféico, el ácido p-cumárico y el ácido ferulico.

El ácido ferúlico no presenta grandes variaciones con las diferentes relaciones de licor/acetato de etilo utilizadas en la extracción.

En la primera extracción se obtienen todos estos compuestos fenólicos, pero en la segunda y tercera extracción muchos de ellos ya no están presentes.

A partir de estos estudios se comprueba que el potencial antioxidante es muy fuerte, se caracterizan los compuestos responsables de este poder antioxidante, se decide realizar la extracción en una sola fase y establecer óptima la relación fase orgánica:fase acuosa 1:2.

En el procedimiento descrito se realizó asimismo un estudio preliminar para determinar la capacidad antimicrobiana del principio activo del PVPP mediante el método de halo con el fin de ver la efectividad antimicrobiana a los microorganismos de interés propuestos por los usuarios finales para la matriz cárnica de vacuno y porcino: El método del halo es una modificación del "Antibacterial Activity Assess ment of Textile materials: Parallel Streak Method AATTCC 147-1998" Se trata de un método rápido y cualitativo que permite determinar la efectividad del compuesto a distintos microorganismos de interés.

Se ha estudiado con concentración alta y baja de bacterias (10^3 y 10^6) y con distintas concentraciones de principio activo (0,5; 1; 3 y 5%). Los microorganismos estudiados son: *Escherichia coli* (*E. Coli*); *Listeria monocytogenes; Salmonella spp; Escherichia coli 0157:H*; Aerobios.

Se observa que para una concentración más baja de microorganismos 10^3 (concentración más habitual en este tipo de situación cuando empieza la contaminación, por ejemplo, en un producto cárnico) el principio activo desarrollado es efectivo en una concentración del 3% a todos los microorganismos de interés para los usuarios finales. En el caso de *Aerobios* y *Salmonella* la efectividad antimicrobiana se hace patente a partir de una concentración del 1%, dado que se observa que no hay crecimiento de los microorganismos ensayados bajo la superficie de la muestra. Así pues, en este estudio preliminar queda demostrada la acción antimicrobiana.

La utilización del proceso descrito llevaría a valorizar un subproducto que actualmente se desecha en las industrias cerveceras con el consiguiente coste de tratamiento y depuración y dadas las condiciones de estabilidad, poder antioxidante y antimicrobiano del extracto obtenido, podría ser utilizado en otros ámbitos industriales, como por ejemplo en la fabricación de films plásticos para embalaje de alimentos u otros afines.

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REIVINDICACIONES

1. Procedimiento para la recuperación de sustancias antioxidantes a partir del líquido de desecho procedente del lavado de la polivinilpolipirrolidona utilizada para el tratamiento de cerveza **caracterizado** por que en el mismo se lleva a cabo el lavado de la polivinilpolipirrolidona con una disolución de sosa del 0,5% al 4% y a una temperatura entre 60 y 100ºC, tras lo que se procede a la extracción con solvente orgánico o por otra tecnología de extracción de las sustancias antioxidantes.

2. Procedimiento según la reivindicación anterior **caracterizado** por que en el proceso de extracción con solvente orgánico con posterior evaporación, se llevan a cabo los siguientes pasos:

- Adición de ácido clorhídrico hasta conseguir un pH de entre 1 y 2,5,
- Realización de una mezcla con acetato de etilo con una relación de fase acuosa:fase orgánica del orden de 1:0,25 a 1:20,
- Extracción con un agitador de palas a temperatura ambiente y con una remoción suave,
- Reposo para conseguir la separación de fases,
- Recogida de la fase orgánica,
- Evaporación de la fase orgánica a la sequedad en un sistema rotatorio a vacío, sin aumentar la temperatura por encima de los 60ºC.

3. Procedimiento según la reivindicación anterior **caracterizado** por que el solvente orgánico utilizado en el proceso de extracción es preferentemente acetato de etilo.

4. Procedimiento según cualquiera de las reivindicaciones anteriores **caracterizado** por que el residuo obtenido es lavado, congelado y liofilizado hasta garantizar la eliminación total del agua del extracto restante y su estabilización, que permita su conservación y posterior uso en otros ámbitos.

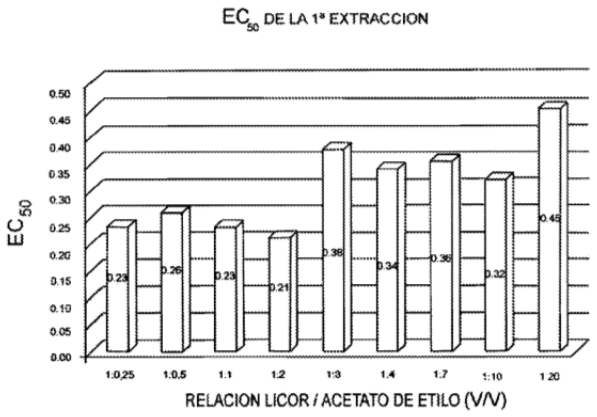
EC50 DE LA 1ª EXTRACCION
EC50
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.23
0.26
0.23
0.21
0.38
0.34
0.36
0.32
0.45
1:0,25
1:0,5
1:1
1:2
1:3
1:4
1:7
1:10
1:20
RELACION LICOR / ACETATO DE ETILO (V/V)

FIG. 1

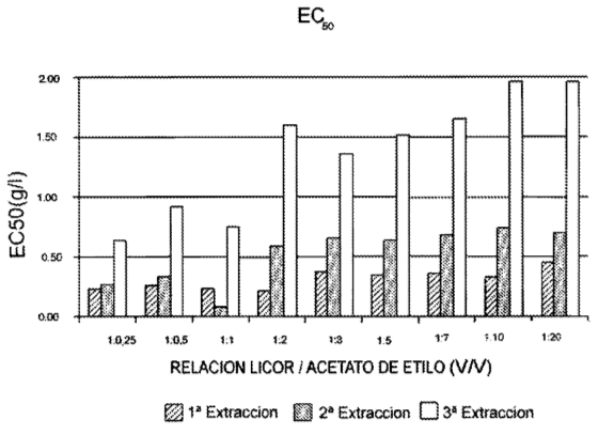
EC50
EC50(g/l)
2.00
1.50
1.00
0.50
0.00
1:0,25
1:0,5
1:1
1:2
1:3
1:5
1:7
1:10
1:20
RELACION LICOR / ACETATO DE ETILO (V/V)
1ª Extraccion
2ª Extraccion
3ª Extraccion

FIG. 2

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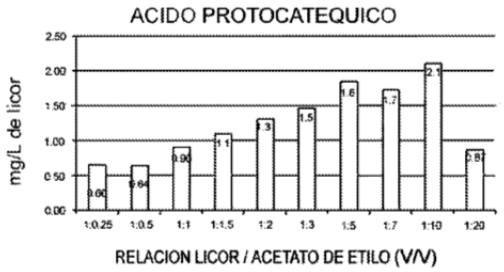

FIG. 3

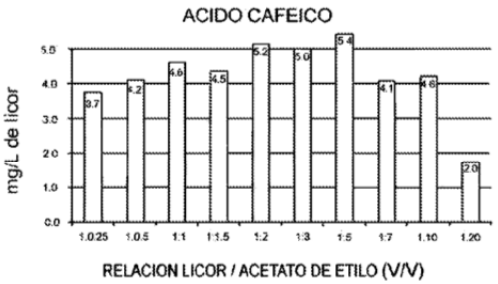

FIG. 4

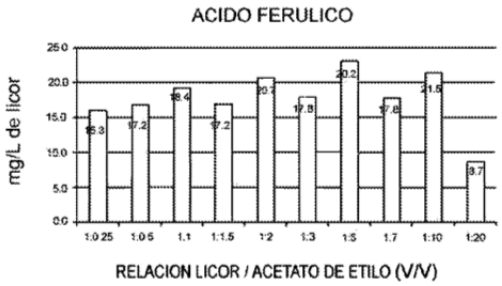

FIG. 5

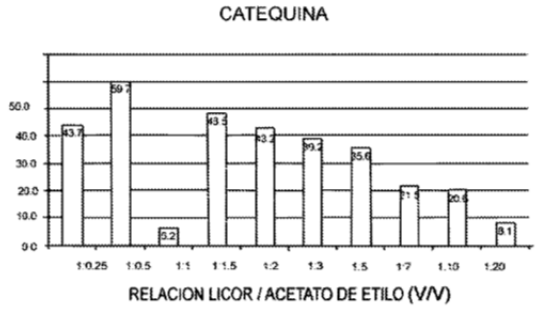
CATEQUINA
50.0
40.0
30.0
20.0
10.0
0.0
43.7
59.2
6.2
43.5
40.2
39.2
35.6
20.6
9.1
1:0.25
1:0.5
1:1
1:1.5
1:2
1:3
1:5
1:7
1:10
1:20
RELACION LICOR / ACETATO DE ETILO (V/V)

FIG. 6

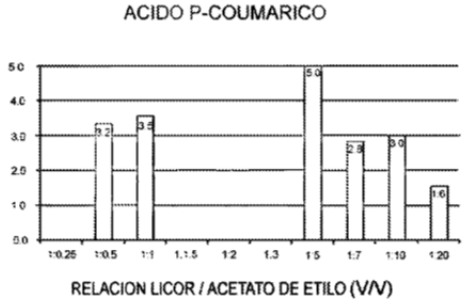
ACIDO P-COUMARICO
5.0
4.0
3.0
2.0
1.0
0.0
3.2
3.5
5.0
2.8
3.0
1.6
1:0.25
1:0.5
1:1
1:1.5
1:2
1:3
1:5
1:7
1:10
1:20
RELACION LICOR / ACETATO DE ETILO (V/V)

FIG.7

4

Brewery waste as a potential source of phenolic compounds: optimization of the extraction process and evaluation of antioxidant and antimicrobial activities

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Abstract

This paper reports the development of an extraction process that allows selective recovery of polyphenols from a brewery waste stream. The antioxidant activity of this extract (EC_{50} = 0.23–0.3 g/L) was very high, similar to that of butylated hydroxyanisole (BHA) and higher than that of other synthetic antioxidants such as butylated hydroxytoluene (BHT). Crude extracts exhibited a high level of antimicrobial activity against Gram-positive and Gram-negative bacteria, when applied at concentrations of 1% and 3% (w/v). The antioxidant and antimicrobial activities can probably be attributed to the major phenolic compounds, such as protocatechuic, caffeic, p-coumaric and ferulic acids and catechin. With this procedure, the waste polyphenols could be used as cheap source of natural compounds, with potential applications in the food and health sectors. Substitution of synthetic additives with safe and effective natural additives may be of further benefit to the food industry.

Keywords: Brewery waste stream, polyvinylpolypyrrolidone (PVPP), phenolic compounds, natural antioxidants, antimicrobial activity.

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4.1. Introduction

Beer is one of the most commonly consumed alcoholic beverages. It is an extremely complex mixture of bioactive substances, including minerals, carbohydrates, amino acids, vitamins and phenolic compounds, of high nutritional value (Duarte *et al*., 2002; Siebert, 1999). Polyphenols are considered as the main natural antioxidants in raw brewing materials and beer (Whittle, Eldridge, & Bartley, 1999). The phenolic compounds present in beer, derived from barley malt (around ≈70%) and from hops (around ≈30%), are responsible for the overall antioxidant activity of the beverage (Callemien & Collin, 2010). Malt and barley extracts have been characterised by their high content of flavon-3-ols, as well as flavonols and phenolic acids, which confer the extracts with a high antioxidant activity (Goupy, Hugues, Boivin, & Josèphe Amiot, 1999; Maillard, Soum, Boivin, & Berset, 1996). Some compounds in hops also contribute to the biological stability of beer, and it has been assumed that hops protect beer from bacterial contamination. The antimicrobial activity is due to a mixture of α-acids and β-acids, essential oils, and polyphenolic compounds (Cleemput *et al*., 2009; Vaughan, O'Sullivan, & van Sinderen, 2005).

Polyphenols have many industrial applications as natural additives with antimicrobial and/or antioxidant properties, e.g. in the cosmetic and food industries. In addition, phenolic compounds have positive health benefits in the prevention of human diseases associated with oxidative stress (Scalbert, Manach, Morand, Remesy, & Jimenez, 2005).

Nowadays, there is an increasing interest in phytochemicals as new sources of natural antioxidants and antimicrobial agents (Robards, 2003). The use of synthetic antioxidants in the food industry, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tert-butyl

hydroquinone (TBHQ), and ascorbyl palmitate (PA), is severely restricted by legislation concerning both the application and permitted concentrations (Regulation (EC), 2008). Moreover, there is some safety concerns related to the residual toxicity of chemical preservatives (EFSA, 2012). Therefore, the recent worldwide tendency to avoid or at least decrease the use of synthetic additives has created a need for alternative cheap, renewable, natural and possibly safer sources of natural compounds with antioxidant and antimicrobial activities to stabilise foods against oxidative rancidity and microbial spoilage. Extraction of such compounds from waste materials, e.g. agro-industrial waste generated by the food processing industry, has been considered in some studies (Cruz, Moldes, Bustos, Torrado, & Domínguez, 2007; Moure *et al*., 2001).

Different studies have shown that polyphenols are extremely important for the physical stability of beer during storage. Thus, beer may become turbid due to the oxidation and polymerisation of endogenous polyphenols and their interaction with proteins (Siebert, 1999). Catechin and proanthocyanidins (dimers and trimers of catechin, epicatechin and gallocatechin) have displayed haze-forming activity with peptides in model systems, and there is evidence that proanthocyanidin dimers of catechin and/or epicatechin are the main polyphenols involved in haze formation in beer (Callemien & Collin, 2008; Callemien & Collin, 2010).

Two basic procedures are used to prevent haze formation and thus extend the shelf life of beer: reduction of the concentration of haze active proteins, and/or reduction of the concentration of haze active polyphenols. Reducing the polyphenol content, by adsorption with polyvinylpolypyrrolidone (PVPP), is an efficient way of stabilising beer due to its selectivity for phenolic compounds related with its structure. PVPP selectively absorb phenolic compounds by formation of hydrogen bonds between the hydroxyphenolic group and the amide of PVPP (PVPP mimics the structure of a protein) (Mitchell, Hong, May, Wright, & Bamforth, 2005; Siebert & Lynn, 1997). Treatment of beer with PVPP always

produces a decrease in total polyphenols, total flavanols, simple phenolic acids, flavonol glycosides, catechins, proanthocyanidins, and complexes of polyphenols and proteins (Mcmurrough, Madigan, & Smyth, 1995). After use, the PVPP resin is regenerated in the brewery, in a washing process that generates large quantities of a waste stream. This waste stream, which contains large amounts of polyphenols, is a promising alternative and economical source of natural antioxidants and antimicrobials with several applications in the food industry.

The aim of this study was to obtain a crude extract that could be used as a source of antioxidants for use in the food industry. For this purpose, an extraction process was developed to enable selective recovery of polyphenols from the waste stream generated during regeneration of PVPP. The yield of crude extracts containing phenolics was optimised by testing different ratios of waste to solvent in a three-step extraction procedure, and the major phenolic compounds were identified and quantified by high performance liquid chromatography (HPLC). In addition, the radical-scavenging activities of the different extracts and the antimicrobial effects of the crude extract were tested against Gram-positive and Gram-negative bacteria.

4.2. Materials and methods

4.2.1. Chemicals

Ethyl acetate (GR for analysis), methanol (GC≥99.9%), hydrochloric acid (fuming 37%), glacial acetic acid and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). Ultrapure water was prepared in a Milli-Q filter system (Millipore, Bedford, MA, USA). 2,2′-diphenyl-1-picrylhydrazyl (DPPH) was supplied by Fluka Chemie AG (Buchs, Switzerland). Protocatechuic acid, caffeic

acid, (+)- catechin hydrate, ferulic acid, p-coumaric acid, 2, 6-Di-tert-buthyl-4-methylphenol (BHT) and 2(3)-tert-butyl-4-hydroxyanisole (BHA) were provided by Sigma-Aldrich (Steinhein, Germany).

4.2.2. Starting material

The PVPP sludge (containing polyphenolic compounds), which remains after clarifying beer, was washed with a solution of NaOH (2% (w/v)) at room temperature. The brewery waste stream (i.e. the PVPP washing solution [PVPP-WS] generated during desorption of the polyphenols from the PVPP resin) was supplied by a brewery (San Miguel, Fábricas Cerveza y Malta, S.A., Spain).

4.2.3. Extraction of the antioxidant compounds from the PVPP washing solution

The extracts were obtained according to the process shown in Fig. 4.1. The PVPP-WS (100 mL), of initial pH 13.5, was acidified with HCl to pH≈1.5. The phenolic compounds were extracted with ethyl acetate solvent. The samples were extracted three times, and different ratios (v/v) of PVPP-WS: ethyl acetate were tested (1:0.25; 1:0.5; 1:1; 1:1.5; 1:2; 1:3; 1:5; 1:10). The extraction was carried out with constant stirring for 45 minutes at room temperature. The samples were protected from the light at each step of the extraction procedure. The organic and aqueous phases were separated and each organic phase was collected separately and evaporated to dryness under vacuum at 40 ºC in a rotary evaporator. The extraction yield of each phase was determined gravimetrically. Finally, each extract was redissolved in methanol, to determine the antioxidant activity, and in a solution of methanol and water (60:40), for HPLC-DAD analysis.

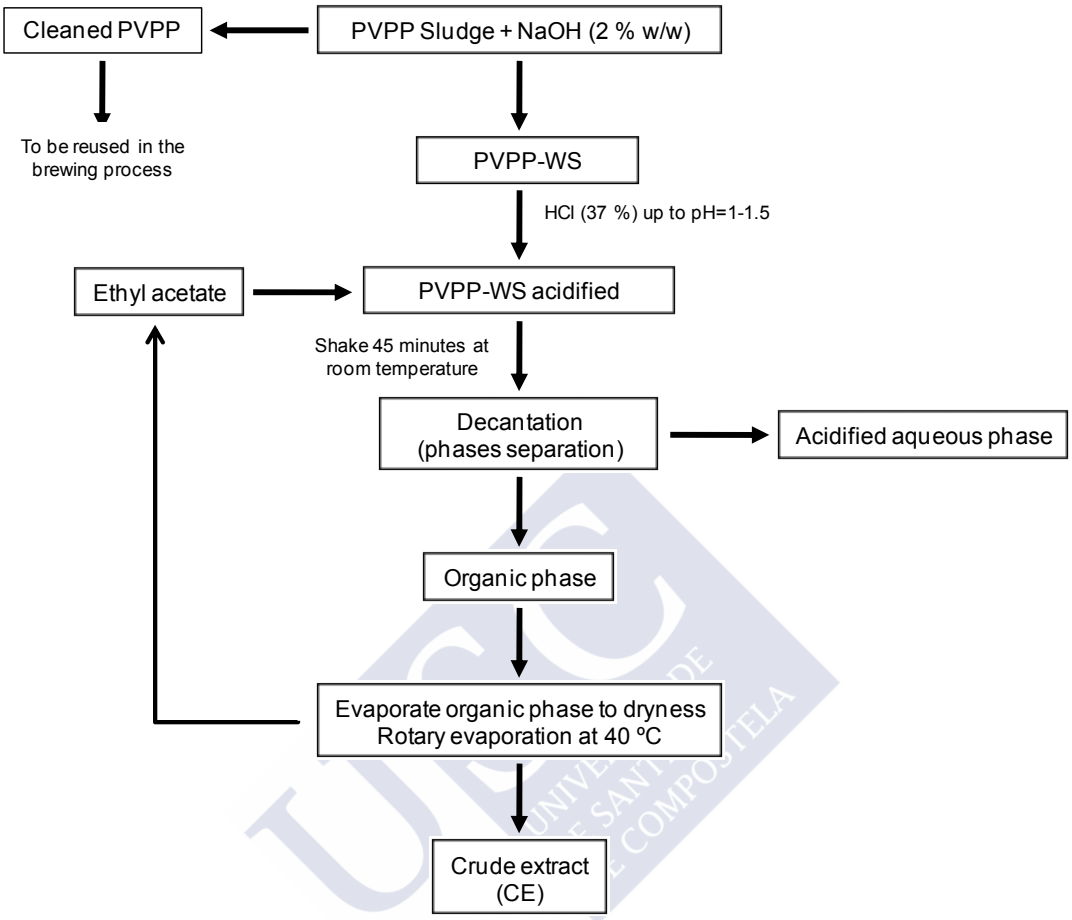

Figure 4.1. Extraction procedure used to obtain the crude extracts containing phenolic compounds

4.2.4. HPLC analysis of phenolic compounds

Chromatographic analysis was carried out with an HPLC system (model 1100 HP, Hewlett–Packard, Waldbronn, Germany) controlled by HP ChemStation® chromatographic software. Polyphenol separation was performed, with a Kromasil C18 column (250 × 4.6 mm, 5 µm particle size) (Teknokroma, Barcelona, Spain), at 38 °C. A sample volume of 50 µL was injected into the column and eluted with a

constant flow rate of 1.0 mL/min. Gradient elution was applied, with Milli-Q water (0.1% acetic acid) (A) and acetonitrile (B) as solvents, as follows: 90% A and 10% B for 5 min; 5–35 min, linear gradient to 50% B and held for 8 min; 43–45 min, linear gradient from 50% to 10% B. Continuous scanning was performed by DAD, in the range 190–700 nm, and the chromatograms were acquired at 280 and 325 nm.

Phenolic compounds were identified by comparison of the retention times and UV spectra with those obtained with pure standard injected under the same chromatographic conditions. Monitoring and quantification were carried out at 280 nm, for protocatechuic acid and catechin, and at 325 nm, for caffeic, p-coumaric and ferulic acids. The compounds were quantified by the external standard method with six-point calibration curves generated by linear regression (correlation coefficients >0.999 for all phenolic compounds).

4.2.5. Determination of antioxidant activity with the DPPH radical scavenging method

Antioxidant activity determination of the different extracts was performed by the 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method described by von Gadow, Joubert, and Hansmann (1997), with slight modifications.

The solutions of each extract and of two synthetic antioxidants commonly used in the food industry, BHA and BHT, were prepared in methanol. Fifty microliters of the different methanolic solutions of the antioxidant extracts and reference antioxidants were added to 2 mL of methanolic DPPH solution (3.6×10^{-5} mol/L). The resulting mixture was homogenised and held in the dark for 16 min at room temperature. The decrease in DPPH absorbance was measured at 515 nm. All determinations were performed in triplicate. The inhibition percentage

(IP) of the DPPH radical by antioxidant compounds was calculated according to the following equation: IP = $[(A_0–A_{16})/A_0] \times 100$, where A_0 is the absorbance of the control at initial time, and A_{16} is the absorbance of the antioxidant at 16 min.

The antioxidant activity of the extracts was calculated as the equivalent EC_{50} concentration, which indicates the specific antioxidant activity of the antioxidant or antioxidant extract expressed as g/L. Calculation of the EC_{50}, which is the concentration of antioxidant required to inhibit 50% of the initial DPPH radical, enables comparison of the antioxidant activity of all samples under the same experimental conditions.

4.2.6. Determination of antimicrobial activity

The antimicrobial activity was assessed by the Standard Test method ASTM E 2149-01 for determining the Antimicrobial Activity of Immobilised Antimicrobial Agents Under Dynamic Contact conditions (E2149-01, 2002).

The microorganisms used to test the antimicrobial efficiency were *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Salmonella spp* ATCC 13311, *Listeria monocytogenes* ATCC 13932; *Escherichia coli serotype* O157:H7 (non-toxigenic) ATCC 700728 and Total Aerobic Mesophilic flora isolated from minced meat.

These microorganisms were used to prepare cell suspensions of 10^6 and 10^3 CFU/ml of total aerobic flora and other bacteria, respectively, in buffered peptone water. The cell suspensions were placed in contact with different concentrations of crude extracts, ranging from 1% to 3% (w/v), to test the antimicrobial activity. The suspensions (each microorganism + crude extract) were then incubated at 37 °C for 24 and 72 h. Ten-fold serial dilutions of each suspension were then made and spread onto different agar plates, which were incubated at optimal growth

conditions of time/temperature for quantification of the number of viable cells by colony forming units per ml (CFU/ml). The bacterial population measures are the means from duplicate experiments performed per species, and the data obtained were used to calculate the microbial death rate.

The antimicrobial activity of the crude extract was calculated according to the following equations: Microbial Death Rate (mean log10 density) = B−A, where, A is the $\log_{10}$ density of bacteria for the flask containing the crude extract and bacteria after the specified contact time, and B is the log10 density of bacteria for the flask used to determine "A" before addition of the crude extract.

4.2.7. Statistical analysis

Data were analysed by analysis of variance (ANOVA) and significant differences were assessed by the Tukey's multiple range test at $p < 0.01$ using the IBM SPSS Statistics 20.0.0 statistical software package. Data are presented as the mean ± standard deviation.

4.3. Results and discussion

4.3.1. Selection of extraction parameters

The extraction procedure was optimised for the brewery waste stream (PVPP-WS) containing polyphenols. The following experimental variables were evaluated with the aim of improving the efficiency of the extraction procedure: pH, PVPP-WS: solvent ratio, extraction steps and temperature. The extraction procedure was carried out under non-oxidative conditions (Alonso-Salces *et al*., 2001).

The most commonly used and most effective solvents for extracting polyphenols from vegetables, fruits and agro-industrial wastes are polar solvents, such as ethanol, methanol and acetone. In this study, ethyl acetate was selected because the waste is aqueous and because apolar solvents are among the most commonly used solvents for removing polyphenols from water (Cruz, Domínguez, Domínguez & Parajó, 1999 and Moure *et al*., 2001). Furthermore, ethyl acetate yields more phenolic compounds as it selectively extracts low and high molecular weight polyphenols (Fernández de Simon, Cadahía, Conde, & García-Vallejo, 1996).

The effect of pH is also important in the extraction process, and some studies have reported maximum solubility of polyphenols at pH 3–4 in the organic phase. Increased antioxidant activity of aqueous fractions after treatment under acidic conditions has been reported and is probably due to altered phenol composition (Moure *et al*., 2001; Tabart *et al*., 2007). A study by Kalt, McDonald, and Donner (2000) with blueberry extracts showed that the extracts obtained at pH 1 exhibited a significantly higher antioxidant capacity than those obtained at pH 4 and 7. Therefore, in order to obtain extracts with higher antioxidant activity, the pH used in this study was ≈1–1.5.

The extraction time and temperature conditions were optimised simultaneously. The extraction yield was lower at temperatures above 40 °C, possibly because of degradation of polyphenolic compounds caused by hydrolysis, internal redox reactions and polymerisation (Alonso-Salces *et al*., 2001). With the purpose of obtaining extracts with the highest antioxidant activity as possible, without loss of active compounds susceptible to temperature, the extraction procedure was carried out at room temperature. A long extraction time might imply a time-consuming and uneconomical procedure without significant increases in the amount of phenols extracted (Larrauri, Sánchez-Moreno, & Saura-Calixto, 1998). After testing different extraction times in previous assays (data not shown), an extraction time of 45 min under non-oxidative conditions was chosen to ensure a good yield and obtain extracts with the highest possible scavenging activity. The extraction procedure requires a controlled stirring speed to prevent formation of emulsions. Such emulsions make phase separation difficult and consequently reduce the extraction yield.

4.3.2. Extraction yield of crude extracts

With the purpose of obtaining a crude extract containing the maximum yield of polyphenols under defined experimental conditions, an extraction procedure involving three steps was carried out with PVPP-WS and ethyl acetate at ratios (v/v) between 1:0.25 and 1:10. The efficiency of the three-step extraction procedure for improving the removal of phenolic compounds was evaluated. The results obtained, expressed in g of extract/L PVPP-WS, are shown in Fig. 4.2.

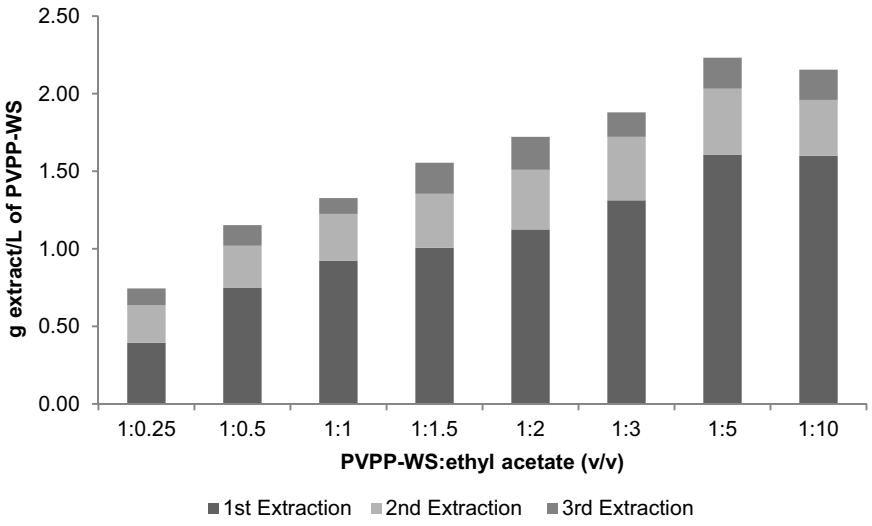

Figure 4.2. Recovery yield from three-step extractions with different ratios (v/v) of PVPP-WS: ethyl acetate

The extraction yield increased as the volume of ethyl acetate in the solution was increased. This was observed for almost all fractions obtained, which were collected separately, at each step of the procedure. Nevertheless, the difference was less discernible in the third step. The extraction yield ranged between 0.4 and 2.2 g of extract/L of PVPP-WS. At a PVPP-WS: solvent ratio of 1:10, the extraction yield obtained was the same as at a ratio of 1:5, indicating that the extraction was complete at the latter ratio. The best yields, ranged between 70% and 75% (w/w), were obtained in the first extraction, except at the lower ratios tested (1:0.25–1:1). The most effective ratios (v/v) of PVPP-WS: ethyl acetate were 1:2, 1:3, 1:5 and 1:10, and the yields of extract were between 1.01 and 1.61 g/L. The second and third extraction yields were lower than the first. The second extraction yield was almost the same for the different ratios used (approximately 0.35–0.40 g/L), except for cases in which lower ratios were used (1:0.25–1:1). In the latter, the extraction yields were slightly higher because the first extraction was less effective due to a lower volume of ethyl acetate used. This could be due to saturation of the solvent.

In the third-step of the extraction, the yields were even lower than the second (with extracts around 0.20 g/L), and therefore the third extraction was no longer economically justified. For PVPP-WS:ethyl acetate ratios (v/v) higher than 1:1, the yields in the first extraction were more than 70% of the extracts obtained from the entire procedure, and consequently the inclusion of the second and the third steps is not economically justifiable.

4.3.3. Phenolic compounds present in crude extracts

The main phenolic compounds present in the highest quantities in the crude extracts obtained in the different extraction steps were identified and quantified by HPLC-DAD. The results, expressed as mg of phenolic compound/ L of PVPP-WS, are shown in Fig. 4.3. As expected, the main phenolic compounds present in the crude extracts are simple phenolic compounds, mainly phenolic acids and the flavan-3-ol catechin, which are the main causes of beer haze (Callemien and Collin, 2008; Callemien and Collin, 2010). Catechin and ferulic acid are the main compounds present in the crude extract (Fig. 4.3a and b), followed by caffeic acid (Fig. 4.3c), p-coumaric acid (Fig. 4.3d) and protocatechuic acid (Fig. 4.3e). These results are consistent with those of other studies that reported cinnamic acids in beer at the same ratios, with ferulic acid being the most abundant, followed by caffeic and p-coumaric acids (Piazzon, Forte, & Nardini, 2010). Furthermore, the concentrations of phenolic acids present in the PVPP-WS crude extracts are higher than in other extracts obtained from other by-products obtained in the brewery industry, such as malt extracts, barleys and spent grains (Goupy *et al*., 1999, McCarthy, O'Callaghan, Piggott, FitzGerald, & O'Brien, 2013a; McCarthy *et al*., 2013b).

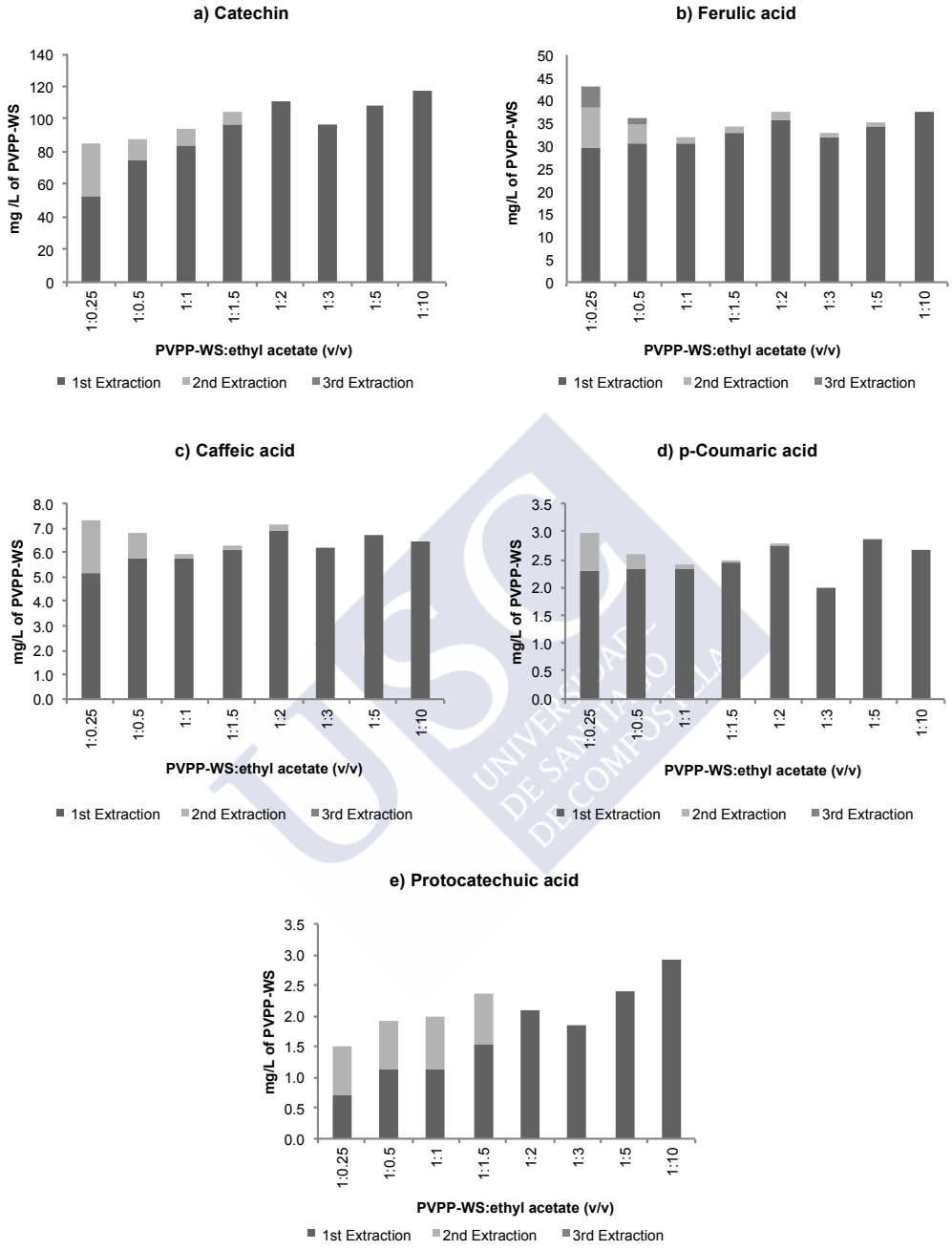

Figure 4.3. Content of the main phenolic compounds present in each crude extract obtained from the PVPP-WS at each solvent ratio tested: a) catechin, b) ferulic acid, c) caffeic acid, d) p-coumaric acid and e) protocatechuic acid

At the lowest ratios used (1:0.25–1:1.5), the second step was required to extract all compounds. Almost all of the compounds were completely extracted in the first step of the procedure. At a PVPP-WS: solvent ratio of 1:1, most of the compounds were completely extracted in a single step. Nonetheless, at a ratio of 1:2, greater amounts of each compound were extracted, i.e. this ratio is sufficient to yield the main phenolic compounds obtained in the residual stream. Although protocatechuic acid appeared to be the compound most difficult to yield, it was completely extracted at a PVPP-WS: solvent ratio of 1:2. Ferulic acid is the only compound for which a third extraction step is required at lower ratio (1:0.25 and 1:0.5). The active compounds present in the extracts obtained in the second and third extraction steps may be other types of compounds different from simple phenolics.

4.3.4. Free radical scavenging activity of crude extracts

The DPPH free radical-scavenging activities of each crude extract obtained from each step of the three-stage extraction, in comparison with reference antioxidants commonly used in the food industry (BHA and BHT), are shown in Table 4.1.

The antioxidant activity of the test samples is highest at lower EC_{50} values. The antioxidant activities of the reference synthetic antioxidants, BHA and BHT, are consistent with previously reported values (Cruz *et al*., 2007). The results indicate that all the extracts obtained in the three extractions and with the different solvent ratios tested, exhibited considerably higher DPPH free radical-scavenging activities than BHT. The free radical-scavenging activity of the extracts obtained in the first step of the extraction procedure was higher than in the extracts yielded in the second step, which in turn was higher than in the third step for all ratios tested. This may be due to the fact that major phenolic compounds with high antioxidant

activity were extracted in the first step (Fig. 4.3). Ferulic acid and catechin, which are present in highest amounts in the crude extracts, are possibly the main cause of the high antioxidant activity, since phenolic acids are known antioxidants that act as free radical acceptors (Piazzon *et al*., 2010).

Table 4.1. Antioxidant activity of each fraction obtained with different ratios of PVPP-WS: ethyl acetate

	EC_{50} (g/L)		
PVPP-WS: ethyl acetate ratio (v/v)	**1st Extract**	**2nd Extract**	**3rd Extract**
1:0.25	0.234^{aA}±0.005	0.269^{bB}±0.003	0.637^{bC}±0.007
1:0.5	0.260^{bA}±0.003	0.326^{cB}±0.002	0.897^{cC}±0.008
1:1	0.234^{aA}±0.004	0.450^{dB}±0.007	0.746^{dC}±0.009
1:1.5	0.546^{cA}±0.009	0.869^{eB}±0.008	1.73^{eC}±0.008
1:2	0.213^{dA}±0.003	0.585^{fB}±0.003	1.60^{fC}±0.007
1:3	0.378^{eA}±0.004	0.654^{gB}±0.008	1.36^{gC}±0.004
1:5	0.341^{fA}±0.001	0.642^{gB}±0.006	1.52^{iC}±0.003
1:10	0.322^{gA}±0.007	0.742^{iB}±0.010	1.96^{jC}±0.008
BHA		0.241^{a}±0.006	
BHT		2.62^{h}±0.002	

∗Mean values (n = 3) followed by different lower case letters superindexes within the same extraction step and capital letters superindexes within the same row denote statistically significant differences at $p < 0.01$.

The crude extracts obtained in the first step of the extraction with the first three ratios applied (1:1.25, 1:0.5 and 1:1), demonstrated high antioxidant activities of around 0.23–0.26 g/L, comparable to the high activity exhibited by

BHA. Therefore, the first extraction was insufficient because the second and third fractions were still fairly active, with EC_{50} values lower than 0.50 g/L. These results, combined with the extraction yield obtained (see Fig. 4.2) and the HPLC-DAD results (see Fig. 4.3), confirm that, at the lowest ratios, the extraction process was not complete and a large number of active compounds were not extracted in the first step because the volume of ethyl acetate was not sufficient and further extraction steps would be necessary. However, the simple phenolics identified were completely extracted in the second step, so that in the third step, other active compounds were extracted. This also applies to the second and third extractions, for ratios higher than 1:1, and phenolic compounds identified by HPLC-DAD were completely extracted in the first step.

An increase in the volume of ethyl acetate had a positive effect on the extraction efficiency, and a similar trend was observed for all antioxidant activities expressed in EC_{50}, which may be related to the presence of high contents of simple phenolic compounds identified in the present study. This may be attributed to ethyl acetate, the polarity of which is suitable for extracting polyphenols. Nevertheless, in the first step of the extraction procedure, in which higher proportions of ethyl acetate were used (1:3 and 1:5), the extracts exhibited lower antioxidant activity, with values of 0.378 g/L for ratios of 1:3 and 0.341 g/L for the largest volume of ethyl acetate (1:5). Therefore, although yields were higher with the larger volumes of ethyl acetate used, no other simple phenolic compounds were identified (see Fig. 4.3). This confirms that polyphenolic compounds that display a high degree of antioxidant activity are the first to pass to the organic phase. Therefore, the lowest extraction ratios are those associated with the lowest EC_{50}, and the extracts obtained after the third step have the highest EC_{50} in which the antioxidant activity is due to other less active compounds. The increase in EC_{50} is due to the fact that other higher molecular weight compounds of lower antioxidant capacity are extracted with the most active polyphenolic compounds, which are usually simple polyphenols.

At the lowest PVPP-WS: solvent ratios used in the study (1:0.25 and 1:0.5), complete extraction of polyphenolic compounds was not achieved in the first step. This was confirmed by the results obtained with the DPPH method, in which both extracts exhibited a high degree of antioxidant activity. The DPPH radical scavenging capacity of crude extracts obtained under PVPP-WS: solvent ratio 1:2 displayed the highest specific activity (EC_{50} = 0.213 g/L) similar to that of BHA and 12 times higher than that of BHT. Furthermore, DPPH radical scavenging capacity of PVPP-WS crude extracts are higher than other extracts obtained from other by-products obtained in the brewery industry, such as barleys, spent grains, malt extracts, and barley husk (Cruz *et al*., 2007, Goupy *et al*., 1999; McCarthy *et al*., 2013b).

4.3.5. Antimicrobial activity of crude extracts

The antimicrobial activity of the crude extract was evaluated at two concentrations (1% and 3% (w/v)). Selection of the concentrations used was based on the results of a previous qualitative assay in which different concentrations were tested with different microorganisms. The microorganisms were selected by taking into account the most important microorganisms that affect meat and meat products, considering future use of the crude extract as a possible food additive and the food products in which it could be used.

The antimicrobial activities of crude extracts, against Gram-positive bacteria (*S. aureus*, *L. monocytogenes*) and Gram-negative bacteria (*Salmonella spp*, *E. coli* and *E. coli* 0157:H7), are shown in Fig. 4.4a and b. The log 10 values of colony-forming units per milliliter log/ml are plotted to illustrate the bacterial reduction by the mean log 10 CFU per milliliter over time. A decrease of one logarithmic unit (log 10) is equivalent to a 90% reduction in the bacterial population.

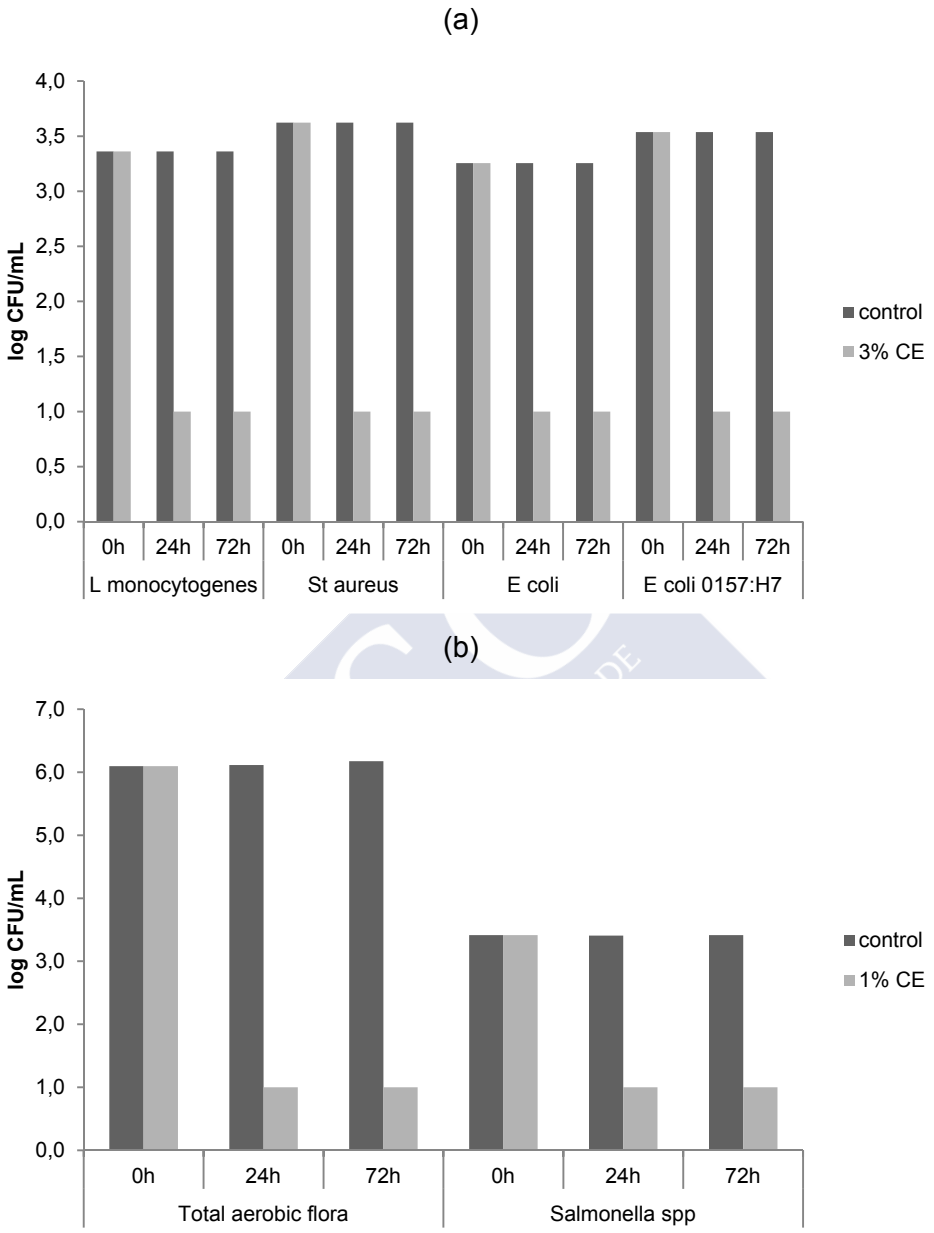

Figure 4.4. Log10 of the number of viable cells per milliliter (log CFU/ml) of microorganisms (a) in media supplemented with crude extract (CE) applied at 3% and (b) in media supplemented with crude extract (CE), applied at 1%

The 3% (w/v) crude extract reduced the density of *L. monocytogenes*, *S. aureus*, *E. coli* and *E. coli* 0157:H7 by more than 2 × log 10 after 24 and 72 h of exposure relative to the control (Fig. 4.4a).

The 1% (w/v) crude extract exhibited a high degree of inhibition against total aerobic flora and a 5 log reduction in growth was achieved after 24 h of exposure (Fig. 4.4b). Salmonella spp showed more than 2 × log 10 reduction after contact with the 1% (w/v) crude extract for 24 and 72 h.

In addition to the contribution of phenolic compounds to the high antioxidant activity of the extract (as mentioned above), these compounds may also be responsible for the antimicrobial properties. According to the findings of Puupponen-Pimiä *et al*. (2001), ferulic and caffeic acids may be the main compounds responsible for the antimicrobial effect of the crude extract. However other compounds such as flavonoids could be also responsible for the antimicrobial activity (Cushnie & Lamb, 2005). Flavonoids, such as catechin, which are present in PVPP-WS extracts, have showed antimicrobial activity (Rauha *et al*., 2000; Yilmaz, 2006).

The inhibitory effect of the crude extract was noteworthy. The growth and final population of the all microorganism tested were lower than in the control. Some studies have shown clear evidence of the effect of phenolic compounds against Gram-positive bacteria. However, the effect against Gram-negative bacteria was not as evident (Shelef, Naglik, & Bogen, 1980). In the present study, the crude extract obtained from the brewery waste stream appears to be equally effective against both groups of bacteria. These findings are consistent with those reported by Tajkarimi, Ibrahim, and Cliver (2010) that *E. coli* was sensitive to natural extracts obtained from herbs and spices.

4.4. Conclusions

In summary, crude extracts of phenolic compounds with high antioxidant activity can be obtained from the brewery waste stream produced during regeneration of the PVPP resin used to clarify beer. These extracts, which comprise a waste product from the brewing industry, may be used as cheap source of natural compounds with dual functions (as antioxidant and antimicrobial agents) of vast application.

This conventional extraction procedure was optimised to obtain a quantitative extraction. The comparative study of extraction yields and antioxidant activities of the extracts obtained, with different ratios of ethyl acetate and sequential extraction steps, from the PVPP washing solution, showed that a single-step extraction at a PVPP-WS:ethyl acetate ratio of 1:2 (v/v) was the most effective, with a yield in the first extraction step of more than 1 g of extract/L of PVPP-WS.

The crude extract obtained from this by-product, under the conditions selected, exhibited a high antioxidant activity, comparable to that of the synthetic antioxidant BHA. Therefore, the product may be a good source of natural antioxidants that could be used to replace the synthetic antioxidants used in the food industry. Furthermore, the extract exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria. The numbers of bacteria were reduced by more than 99%.

In summary, because of its high antioxidant and antimicrobial activity, the crude extract is potentially useful as a source of food additives aimed at increasing the shelf life of food.

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5

Phenolic profile and antioxidant properties of a crude extract obtained from a brewery waste stream

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Abstract

The main aims of this study were to determine the phenolic profile of a crude extract obtained (at pilot scale) from a brewery waste stream and to evaluate the antioxidant activity of the extract. The total phenolic content was determined by the Folin–Ciocalteu assay, which revealed that 50% of the extract comprised phenolic compounds. The polyphenols, identified and quantified by RP-HPLC–DAD and HPLC-ESI–TOF-MS, were mainly flavonoids (catechin, epicatechin, gallocatechin, epigallocatechin, quercetin) and phenolic acids (ferulic acid, p-coumaric acid, caffeic acid, protocatechuic acid). The crude extract displayed a high DPPH radical scavenging activity (0.18 g/L), similar to that of BHA (0.248 g/L) and higher than that of BHT (2.54 g/L). All three tested products displayed a similar ability to decrease oxidative bleaching of β-carotene (antioxidant activity coefficient of 623.8 for the crude extract, 653.3 for BHA, 559.6 for BHT). This type of brewery waste stream may be a promising source of natural antioxidants to replace the synthetic antioxidants currently used in the food industry.

Keywords: Natural extract, Polyphenols, HPLC–UV-DAD**,** HPLC-ESI–TOF-MS**,** Antioxidant activity.

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5.1. Introduction

Phenolic compounds are widely distributed in foods of plant origin such as fruits, vegetables and beverages (fruit juice, wine, tea and beer), which are the major sources of these compounds in the human diet (Robards, 2003; Scalbert & Williamson, 2000).

Polyphenols are a large structurally diverse group of natural antioxidants and have been extensively studied in relation to their chemistry, sources, antioxidant activity and health effects (Higdon & Frei, 2003; Shahidi & Zhong, 2010). In recent years, numerous studies have associated the consumption of foods rich in polyphenols with the prevention of cardiovascular diseases, certain types of cancer and other diseases related to oxidative damage (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005).

Natural antioxidants can be isolated as pure compounds from different materials, and used for food preservation, as supplements/functional food, for medicinal purposes, and in cosmetics (Schieber, Stintzing, & Carle, 2001). A large field of research on natural antioxidants has focused on phenolic compounds, in particular flavonoids and hydroxycinnamic acids, with the aim of using these compounds to replace synthetic substances that are widely used as antioxidants in foods, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertbutylhydroquinone (TBHQ), in response to consumer preference and some health concerns (Balasundram, Sundram, & Samman, 2006; EFSA, 2011, 2012; Regulation, 1333/2008, 2008).

Numerous agro-industrial by-products have been studied as potential sources of natural antioxidants (mainly phenolic compounds) and some of these products have been found to have activities comparable to synthetic antioxidants (Moure *et al*., 2001; Peschel *et al*., 2006; Schieber *et al*., 2001).

Polyphenols are usually minor compounds in the brewing process. About 70% of phenolic compounds in beer are derived from barley malt and the remaining 30% are derived from hops (Callemien, Jerkovic, Rozenberg, & Collin, 2005; Goupy, Hugues, Boivin, & Josèphe Amiot, 1999). Nevertheless, numerous studies have shown that polyphenols are extremely important to brewers because they influence several stages of the brewing process and the overall stability of beer, such as haze formation, color, taste, filtration, foam stability, and redox state (Callemien & Collin, 2010; Mikyska, Hrabak, Haskova, & Srogl, 2002). The proanthocyanidins and catechins of barley are known to be involved in the formation of beer haze because of their affinity for proteins.

There is evidence that the main polyphenols involved in haze formation in beer are the proanthocyanidin dimers of catechin and/or epicatechin (Mcmurrough, Madigan, & Smyth, 1995; Siebert, 1999; Siebert, Carrasco, & Lynn, 1996). The polyphenol content can be reduced by stabilizing and clarifying the beer with a polymeric adsorbent resin, polyvinylpolypyrrolidone (PVPP), which prevents haze formation and extends the shelf life of the product (Mcmurrough *et al*., 1995; Mitchell, Hong, May, Wright, & Bamforth, 2005). However, non-haze active polyphenols, which are considered to be important antioxidants with several applications in the food industry, are also removed during such treatment (Whittle, Eldridge, & Bartley, 1999). As PVPP is an expensive product, it is regenerated in breweries in a process that involves cleaning the used resin with NaOH solution to remove the polyphenols that had been adsorbed. This process generates a cleaned PVPP resin, which can be reused, and a PVPP washing solution (waste stream), which contains the polyphenols. This waste stream is therefore a potentially cheap source of natural antioxidants with a vast application.

Several analytical methods have been proposed for the qualitative and quantitative evaluation of polyphenols in different food samples (Tsao & Deng, 2004). Analysis of the diverse phenolic compounds isolated from beer and beer

products is rather complicated. Reverse-phase high-performance liquid chromatography coupled with photodiode array detection (RP-HPLC–DAD) is the technique most commonly used to separate and quantify antioxidants such as the phenolic compounds in beer, hops and barley malt (Callemien *et al*., 2005; García, Grande, & Gándara, 2004; Leitao *et al*., 2011; Verardo, Bonoli, Marconi, & Caboni, 2008). HPLC coupled with mass detection is also widely used to identify unknown polyphenolic compounds and proanthocyanidins (Robards, 2003; Verardo *et al*., 2008). Whittle *et al*. (1999) have investigated proanthocyanidins (dimers and trimers of catechin, epicatechin and gallocatechin) in barley, by HPLC-ESI–MS. Callemien and Collin (2008) have also used RP-HPLC-ESI(−)–MS/MS to identify proanthocyanidin isomers in beer extracts. More recently, high performance liquid chromatography/ time-of-flight mass spectrometry (HPLC/TOF-MS) has also been used to separate and characterized phenolic compounds in different natural extracts (Iswaldiy *et al*., 2011; Linares *et al*., 2011).

Several studies have shown that extracts obtained from the brewing industry contain phenolic acids, mainly ferulic acid and p-coumaric acid, and that these extracts display some antioxidant activity (Cruz, Moldes, Bustos, Torrado, & Dominguez, 2007; Leitao *et al*., 2011; Piazzon, Forte, & Nardini, 2010). Malt and barley extracts are characterized by high contents of flavan-3-ols and also flavonols and phenolic acids, which confer the extracts with high antiradicalar activity (Dvorakova *et al*., 2008; Maillard, Soum, Boivin, & Berset, 1996; Verardo *et al*., 2008).

The main aims of this study were to scale-up the extraction process developed at laboratory scale, determine the phenolic profile of the crude extract obtained from the brewery waste stream and to evaluate the antioxidant activity of the extract with a view to its potential use in the food industry. The total phenolic content of the crude extract was determined by the Folin–Ciocalteu test, and the phenolic compounds present in the extract were identified and quantified by

chromatographic techniques (HPLC–DAD and HPLC-ESI–TOF). The antioxidant activity of the extract was evaluated by the DPPH test and the β-carotene bleaching assay.

5.2. Materials and methods

5.2.1. Reagents, solvents and standard phenolic compounds

Ethyl acetate (GR for analysis), methanol (GC≥99.9%), hydrochloric acid (fuming 37%), glacial acetic acid and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). Ultrapure water was prepared in a Milli-Q filter system (Millipore, Bedford, MA, USA). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and gallic acid were supplied by Fluka Chemie AG (Buchs, Switzerland). 2, 6-Di-tert-buthyl-4- methylphenol (BHT), 2(3)-tert-butyl-4-hydroxyanisole (BHA) and Folin–Ciocalteu reagent were provided by Sigma-Aldrich (Steinhein, Germany).

Polyphenol standards were supplied by different companies, as follows: 3-hydroxy-3-methylglutaric acid, protocatechuic acid, gentisic acid, caffeic acid, (−)-epicatechin, sinapic acid, 3,5-dimethoxy-4-hydroxyacetophenone, resveratrol, (±)-naringenin, epigallocatechin, (+)-catechin hydrate, vanillic acid, ferulic acid,m-coumaric acid, quercetin, kaempferol, gallocatechin, p-coumaric acid and apigenin by Sigma-Aldrich (Steinhein, Germany); 4'-hydroxyacetophenone, gallic acid, ellagic acid, chlorogenic acid, syringic acid, isoquercetin, salicylic acid and formononetin by Fluka Chemie AG (Buchs, Switzerland); and homovanillic acid, 4-hydroxybenzoic acid and 4'-hydroxy-3'-methoxyacetophenone by Alfa Aesar (Karlsruhe, Germany).

5.2.2. Starting material

The brewery waste stream (i.e. the PVPP washing solution [PVPP-WS] generated during desorption of the polyphenols from the PVPP resin) was kindly supplied by the Mahou-San Miguel Group (Spain).

5.2.3. Pilot scale extraction of the polyphenols from the PVPP washing solution (PVPP-WS)

An aliquot of the PVPP-WS (100 L) was acidified with HCl to pH= 1.5. The sample was extracted, with 200 L of ethyl acetate, by stirring for 30 min at room temperature. Organic and aqueous phases were separated by decantation, and the organic phase was collected and evaporated to dryness under vacuum at 40 °C. Residual water was removed from the extract by lyophilization and the recovery yield was determined. Finally, the crude extract was reconstituted in methanol for determination of the total phenolic content and antioxidant activity. For the chromatographic analyses, the extract was reconstituted in an appropriate amount of 60:40 methanol/water (v/v).

5.2.4. Determination of total phenolic content (TPC)

The content of total phenolic compounds in the crude extract was determined according to the Folin–Ciocalteu colorimetric method described by Singleton, Orthofer, and Lamuela-Raventos (1999), with some modifications.

An aliquot (100 µL) of sample solution was mixed with 3.75 mL of ultrapure water in a test tube, and 0.5 mL of Folin–Ciocalteau reagent (diluted 1:1 (v/v) with ultrapure water) was added. After 2 min, 0.5 mL of 20% sodium carbonate anhydrous solution was added to the mixture. The solution was allowed to stand

for 1 h at room temperature, and the absorbance of the resulting blue complex was measured at 765 nm in a dual-beam spectrophotometer (Uvikon XL, Bio-Tek Instruments, Milan, Italy). All determinations were performed in triplicate. Quantification was carried out on the basis of the standard curve of commercial gallic acid, and the concentration of total phenolic compounds was expressed as mg of gallic acid equivalents (GAE)/g of dry extract.

5.2.5. Reversed phase high performance liquid chromatography analysis with UV-diode array detection (RP-HPLC–UV-DAD)

Chromatographic analysis was performed with a HPLC system model 1200 HP (Hewlett-Packard, Waldbronn, Germany) controlled by HP Chemstation® chromatographic software. The system was equipped with a vacuum degasser, a quaternary pump, an autosampler, a column thermostat system, and a diode array detector (DAD).

The polyphenols were separated on a reverse phase Kromasil C18 column (250×3.2 mm internal diameter, 5 µm particle size) (Phenomenex, Barcelona, Spain) thermostated at 38 °C. A gradient elution method was applied. The following solvents constituted the mobile phase: milli-Q water 0.1% acetic acid (solvent A) and 100% ACN (solvent B). The elution conditions were as follows: 0–5 min, 90% A and 10% B; 5–35 min, linear gradient from 10% to 50% B; 35–43 min, 50% B isocratic; and 43–45 linear gradient from 50% to 10% B. Finally, the column was washed and reconditioned. The mobile phase flow rate was 0.5 mL/min during the entire analysis and the sample injection volume was 20 µL. Scanning was performed continuously at wavelengths between 190 and 700 nm, and the chromatograms were acquired at 205, 225, 280, 325 and 372 nm.

5.2.6. High performance liquid chromatography/time-of-flight mass spectrometry (HPLC-ESI-TOF-MS)

Chromatographic analyses were performed in a second Agilent 1100 Series HPLC system (Hewlett-Packard HP, Waldbronn, Germany) consisting of a binary pump, a degasser and an autosampler. The same column, under the same conditions and gradient elution program described above were used to confirm identification of the phenolic compounds. The HPLC system was coupled to an orthogonal-accelerated TOF mass spectrometer (Bruker micrOTOF from Bruker Daltronics, Bremen, Germany), which was equipped with an electrospray ionization (ESI) source operating in the negative ion mode with spectra acquired over a mass range from m/z 50 to 1000. The optimum values of the ESI-MS parameters were as follows: capillary voltage, +4500 V; drying gas temperature, 200 °C; drying gas flow, 9.0 L/min; and nebulizing gas pressure, 2.5 bar. The mass spectrometer was controlled by MicrotofControl® software (Bruker), and the accurate mass data for the molecular ions were processed by DataAnalysis 3.3® software (Bruker Daltonics).

5.2.7. Identification and quantification of polyphenolic compounds

The individual phenolic compounds for which standards were available were identified by comparing the retention times and UV–vis spectra (absorbance maximum (λmax)) with those obtained by injecting standards under the same HPLC conditions and with MS fragmentation data provided by TOF (MS).

Stock solutions of each standard compound were prepared in a 60:40 (v/v) methanol: water mixture. The solutions were injected individually into the HPLC column and eluted under the analytical conditions and gradient elution method described above determining their chromatographic retention times and collecting

UV spectra by the DAD detector and MS fragmentation data from the ESI-TOF-MS detector. A UV spectral library of standard compounds was established in the laboratory and the extracted compounds were identified by comparison of typical mass fragmentation patterns from the samples and standards.

Individual phenolic compounds were quantified by the external standard method. A six-point regression curve of the UV absorption data was constructed after analysis of commercial standards at the wavelength of maximum absorbance of each analyte, and the phenolics were expressed as mg/g dry extract. Monitoring and quantification were carried out as follows: at 225 nm for phenolic acids; at 280 nm for flavan-3-ols, flavanones and flavones; at 325 nm for acetophenone derivates, hydroxycinnamic acids and resveratrol; and at 372 nm for flavonols. External linear calibration curves were determined under the same conditions, and correlation coefficients of >0.999 were obtained for all of the compounds. All stock standard solutions were stored at 4 °C in darkness.

5.2.8. Determination of antioxidant activity

The antioxidant activity was measured in two different ways. The methods used to evaluate the antioxidant activity were the 2,2- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method and the β-carotene bleaching method. In both assays, two synthetic compounds with antioxidant properties, BHA and BHT, which are commonly used in food industry, were also tested as controls. All solutions were prepared in methanol immediately before use and were protected from the light.

5.2.8.1. DPPH• scavenging assay

The antioxidant activity of the crude extract was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging method, which determines the hydrogen-donating antioxidant ability, according to the method described by von Gadow, Joubert, and Hansmann (1997), with slight modifications.

An aliquot of crude extract solution (50 µL) was added to 2 mL of the DPPH methanolic solution (3.6×10^{-5} M). The mixture was shaken vigorously on a vortex mixer (MS2 Mini Vortex Shaker IKA®) and left to stand in the dark at room temperature. The decrease in DPPH absorbance was measured at 515 nm after 16 min in a dual-beam spectrophotometer (Uvikon XL, Bio-Tek Instruments, Milan, Italy). All determinations were performed in triplicate. The inhibition percentage (IP) was calculated according to the following equation:

$$\mathrm{IP} = \frac{\mathrm{A_0} - \mathrm{A_{16}}}{\mathrm{A_0}} \times 100$$

where A_0 is the absorbance of the control at initial time; A_{16} is the absorbance of the antioxidant at 16 min.

The antioxidant activity of the crude extract was calculated as the EC_{50} concentration, which indicates the specific antioxidant activity of the antioxidant or antioxidant extract expressed as g/L. The EC_{50} is the concentration of antioxidant required to inhibit 50% of the initial DPPH radical and allows comparison of the antioxidant activity of all samples under the same experimental conditions. EC_{50} values were obtained from the linear regression curve data obtained from different concentrations (ranging 0.1 to 3.5 g/L) of each antioxidant compound or extract used against the inhibition percentage of DPPH free radical (IP).

5.2.8.2. Beta-carotene bleaching assay

The ability of the crude extract to decrease the oxidative bleaching of β-carotene in a β-carotene/linoleic acid emulsion was carried out according to the spectrophotometric method described by Miller (1971).

Crystalline β-carotene (2.0 mg) was dissolved in 10 mL of chloroform. One mL of this solution was then transferred to a round-bottom flask containing 20 mg of purified linoleic acid and 200 mg of Tween 40. The chloroform was removed by rotary evaporation at 40 °C, and 50 mL of oxygenated distilled water was added to the flask under vigorous stirring to form a stable emulsion. Methanolic antioxidant solution (0.2 mL) was added immediately to an aliquot (5 mL) of this emulsion, and the zero-time absorbance was recorded at 470 nm in a double beam spectrophotometer (Uvikon Spectrophotometer 922, Kontron Instruments, Italy), against a control containing methanol instead of antioxidant solution. Samples and controls were placed on a block heater at 50 °C and the antioxidant activity was evaluated comparatively, according to the ability of the sample to prevent oxidation of the emulsion β-carotene/linoleic acid, monitored by the loss of color at 470 nm (until complete discoloration of the carotene, in≈3 h). The experiment was conducted twice with replicates for all treatments.

The antioxidant activity was measured by the antioxidant activity coefficient (AAC), calculated according to the following equation:

$$\mathrm{AAC} = \frac{\mathrm{A_{E120}} - \mathrm{A_{C120}}}{\mathrm{A_{C0}} - \mathrm{A_{C120}}} \times 1000$$

where A_{E120} is the extract absorbance at 120 min, A_{C120} is the control absorbance at 120 min, and A_{C0} is the absorbance at initial time (zero-time).

5.2.9. Statistical analysis

Antioxidant activity data were analyzed by analysis of variance using SPSS statistical software, version 19.0 (SPSS Inc., USA) package and significant differences were assessed by Tukey's multiple range test ($p<0.05$). Data are presented as the mean ± standard deviation.

5.3. Results and discussion

5.3.1. Extraction yield of the pilot scale extraction

The extraction yield was 148.7 g of crude extract from 100 L of the waste stream (see Table 5.1). In industrial brewing, approximately one liter of this waste stream (PVPP-WS) is generated for every 138 L of beer produced. Therefore, taking into account the amounts of beer produced by the top beer producing countries in 2010: Europe (403 million hectoliters), China (466 million hectoliters) and United States (207 million hectoliters), up to 434, 502 and 223 t of the crude extract could be obtained in these countries, respectively (Brink *et al*., 2011).

Table 5.1. Extraction yield, total phenolic content, radical scavenging activity (DPPH method) and β-carotene bleaching of the crude extract obtained from the brewery waste stream (PVPP-WS) and of synthetic antioxidants (BHA, BHT) usually used in food industry

	Extraction yield	Total phenolic content (TPC)	Antioxidant activity*	
Antioxidant compound	**g of crude extract/100 L of PVPP-WS**	**mg GAE/g dry extract**	**DPPH radical scavenging EC_{50} (g/L)**	**β-carotene bleaching AAC**
Crude extract	148.7	449±6.91	0.186±0.001[a]	623.8±11.31[a]
BHA	-	-	0.248±0.004[b]	653.3±15.31[b]
BHT	-	-	2.54±0.011[c]	559.6±3.542[c]

5.3.2. Determination of the total content of phenolic compounds

It is well known that phenolic compounds contribute directly to the antioxidant activity of natural extracts, and therefore it is important to evaluate the total phenolic content in the crude extract obtained from the PVPP-WS. The results obtained, expressed as gallic acid equivalents, are shown in Table 5.1. Expressed as absolute values, the extract contains 449 mg of phenolic compounds/g of crude extract, so that around 50% of the compounds present in the crude extract are phenolic compounds. Several researchers have determined the total phenolic content of different materials used in the brewing process. One study, which characterized different types of beer, showed that total polyphenol contents ranged from 366 to 875 (GAE) mg/L of beer (Piazzon *et al*., 2010). Another study that determined the total phenolic content of the aqueous/methanolic extracts and aqueous/acetone extracts of different barley varieties and the corresponding malts, reported TPC values ranging between 358 and 867 mgGAE/kgdw formalt, and values ranging between 211 and 606 mg GAE/kgdw for barley (Dvorakova *et al*., 2008). Another study reported a total polyphenol content of around 23 mg

GAE/gdw in hops (Kähkönen *et al*., 1999). The high content of phenolic compounds in the crude extract obtained in the present study may be due to the nature of the waste stream and to the process used to obtain the extract, after recovery of the excess phenolic compounds that interact with proteins to form haze in beer.

5.3.3. Identification and quantification of phenolic compounds

It is important to separate, identify and quantify individual phenolic compounds in order to characterize the crude extract and reveal the phenolic profile. For this purpose, an exhaustive chromatographic analysis was performed by HPLC–DAD, and identification of the phenolic compounds in the crude extract was positively confirmed by HPLCESI–TOF.

5.3.3.1. Identification of phenolic compounds by HPLC-ESI–TOF

Polyphenolic compounds were successfully separated and identified by RP-HPLC–DAD, and the identity of the different compounds was confirmed by analysis of their molecular ions (MS) and the main fragments obtained by HPLC-ESI–MS (TOF).

A single gradient was developed for several phenolic compounds as a rapid and efficient means of characterizing the crude extract and for quality control. After reviewing the bibliography on the main polyphenols present in brewing materials (hops, barley malt and beer), three standard mixtures of 30 commercially available phenolic compounds were selected as being representative of these brewing materials and were used to optimize the chromatographic method. As a final test, the crude extract was injected to evaluate the performance of the chromatographic separation.

HPLC analysis revealed that the crude extract is a complex mixture of phenolics that are difficult to resolve. Several phenolic compounds, which are representative of diverse structural types, were identified. The chromatographic separation of the polyphenols present in the crude extract is shown in Fig. 5.1, which represents a chromatogram obtained at 280 nm by HPLC–DAD.

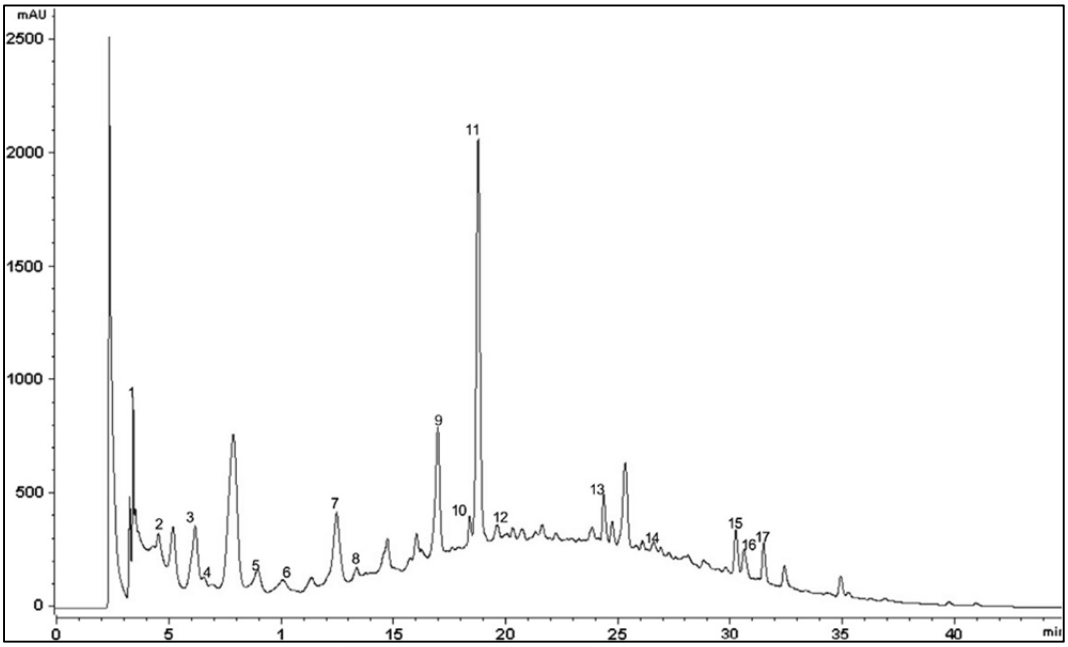

Figure 5.1. HPLC chromatogram of the crude extract monitored at 280 nm

The HPLC-ESI–TOF method was used to identify the phenolic compounds in the crude extract. The phenolic compounds were identified on the basis of elemental composition data determined from accurate mass measurements in negative ionization mode and comparison with the available standards. The phenolic compounds identified in the crude extract are summarized in Table 5.2, which includes the retention time (t_r), maximum absorption wavelengths (λmax), structural class, molecular formula, and fragment ion masses of each compound. The peak name in the table corresponds to the peak labels in Fig. 5.1.

Seventeen phenolic compounds were identified in the crude extract obtained from the waste stream. As expected, these correspond to compounds known to be the main cause of beer instability, such as flavan-3-ols, flavonols and phenolic acids (Mcmurrough *et al*., 1995; Siebert, 1999; Siebert *et al*., 1996).

The phenolic acids identified in the crude extract were divided into two groups: benzoic acid derivates and cinnamic acids. The first group was composed by gallic acid (peak 1), protocatechuic acid (peak 3) and 4-hydroxybenzoic acid (peak 6) detected at UV λ_{max} 225 nm. The cinnamic acids identified at λmax 325 nm were caffeic acid (peak 7), p-coumaric acid (peak 9) and ferulic acid (peak 11). These results are supported by the HPLC profile, retention time and the MS spectrum obtained with the standard compounds.

Peaks 2, 4, 5 and 8,with fragments m/z 245.08, 137.03, characteristic of flavan-3-ol compounds, were detected at UV λ_{max} 280 nm. Peak 5 and peak 8 correspond to catechin and epicatechin, respectively, with the same $[M–H]^-$ at m/z 289.07 and presented product ions at m/z 245.08 and 137.03. Peak 2 and peak 4 had $[M–H]^-$ at m/z 305.07, which displayed fragments at m/z 289.07, 245.08, 137.03, characteristic of gallocatechin and epigallocatechin, respectively. In addition to the characteristic fragments common to all flavan-3-ols, these compounds displayed a fragment corresponding to catechin (m/z 289.07).

The following flavonols were detected at λmax 372 nm: quercetin (peak 14), isoquercetin (peak 10), and kaempferol (peak 17). The crude extract also contains naringenin (peak 16), which belongs to the class of flavanones detected at λmax 280 nm. Peak 12, identified as acetosyringone, was detected at λmax 280 nm, the characteristic wavelength for acetophenone derivates. A compound that belongs to the stilbenoids was detected at UV λmax 325 nm and identified as resveratrol (peak 13). Finally, a flavone was also detected at two characteristic λmax, 280 and 325 nm, and identified as apigenin (peak 15).

Table 5.2. Identification of the phenolic compounds in the crude extract, from their spectral characteristics and negative ions, by RP-HPLC-DAD and HPLC-ESI-TOF-MS

Peak	Name	t_r (min)	Molecular Formula	$[M-H]^-$ m/z	Main fragments m/z	λ_{max} (nm)	Structural class
1	Gallic acid	3.59	$C_7H_6O_5$	169.02	125.03	225	Benzoic acids derivates
2	Gallocatechin	4.54	$C_{15}H_{14}O_7$	305.07	289.07, 261.08, 137.03	280	Flavan-3-ols
3	Protocatechuic acid	6.11	$C_7H_6O_4$	153.02	119.03	225	Benzoic acids derivates
4	Epigallocatechin	6.91	$C_{15}H_{14}O_7$	305.27	289.07, 245.08, 137.03	280	Flavan-3-ols
5	Catechin	9.36	$C_{15}H_{14}O_6$	289.07	245.08, 137.03	280	Flavan-3-ols
6	4-hydroxybenzoic acid	10.12	$C_7H_6O_3$	173.03	137.03	225	Benzoic acids derivates
7	Caffeic acid	12.72	$C_9H_8O_4$	179.04	135.04	325	Cinnamic acids
8	Epicatechin	13.62	$C_{15}H_{14}O_6$	289.07	245.08, 137.02	280	Flavan-3-ols
9	p-Coumaric acid	17.13	$C_9H_8O_3$	163.04	119.05	325	Cinnamic acids
10	Isoquercetin	18.41	$C_{21}H_{20}O_{12}$	463.09	301.04	372	Flavonols
11	Ferulic acid	18.89	$C_{10}H_{10}O_4$	193.05	178.03, 134.04	325	Cinnamic acids
12	Acetosyringone	19.78	$C_{10}H_{12}O_4$	195.03	135.04	280	Acetophenone derivates
13	Resveratrol	24.40	$C_{14}H_{12}O_3$	227.07	-	325	Stilbenoids
14	Quercetin	26.71	$C_{15}H_{10}O_7$	301.04	271.03, 179.00, 151.01	372	Flavonols
15	Apigenin	29.90	$C_{15}H_{10}O_5$	269.05	255.07, 151.01	280, 325	Flavones
16	Naringenin	30.19	$C_{15}H_{12}O_5$	271.06	151.01, 119.06	280	Flavanones
17	Kaempferol	30.63	$C_{15}H_{10}O_6$	285.04	257.05, 151	372	Flavonols

5.3.3.2. Quantification of the individual phenolic compounds by HPLC–DAD

The content of each polyphenol present in the lyophilized extract obtained by the extraction performed in the pilot plant, identified and quantified by HPLC–DAD (Fig. 5.1) and positively identified by HPLC-ESI–TOF in negative ionization mode (Table 5.2), is shown in Table 5.3.

Table 5.3. Total and individual phenolic compounds (mg/g) in the crude extract

Phenolic compound	mg/g	Phenolic compound	mg/g	Phenolic compound	mg/g
Benzoic acid derivates		***Cinnamic acids***		***Flavonols***	
Gallic acid	9.377	Caffeic acid	13.70	Isoquercetin	15.89
Protocatechuic acid	16.69	p-Coumaric acid	11.41	Quercetin	15.13
4-hydroxybenzoic acid	3.145	Ferulic acid	39.52	Kaempferol	14.76
Total	*29.21*	*Total*	*64.63*	*Total*	*45.78*
Flavan-3-ols		***Flavanones***		***Acetophenone derivates***	
Gallocatechin	162.2	Naringenin	5.065	Acetosyringone	14.57
Epigallocatechin	50.59				
Catechin	41.30	***Flavones***		***Stilbenoids***	
Epicatechin	17.18	Apigenin	7.066	Resveratrol	6.473
Total	*271.3*				
Total phenolic compounds present in the crude extract, 444.1 mg/g					

Almost all of the compounds were identified and quantified in the present study, although a small number of compounds remained unidentified. The sum of phenolic compounds identified in the crude extract was 444.1 mg/g of crude

extract. This is consistent with the total phenolic content determined by the Folin–Ciocalteu method (around 500 mg/g of crude extract).

Major phenolic compounds present in the crude extract include flavan-3-ols, phenolic acids (benzoic acid derivates and cinnamic acids) and flavonols. The major compounds were flavan-3-ols, such as gallocatechin, epigallocatechin and catechin (162.2 mg/g, 50.59 mg/g and 41.30 mg/g respectively), and also epicatechin, although at lower concentrations. Flavan-3-ols correspond to around 50% of the total polyphenols present in the crude extract. These results were expected because of the origin of the waste stream and concentration of the compounds removed from the beer. Ferulic acid was present at higher concentrations (39.52 mg/g), followed by caffeic acid and p-coumaric acid (13.70 and 11.41 mg/g of crude extract, respectively). These results are consistentwith some studies that reported phenolic acids in beers at the same ratios, with ferulic acid being the most abundant, followed by caffeic and p-coumaric acids (Piazzon *et al*., 2010). The flavonols present in the crude extract – isoquercetin, quercetin and kaempferol – were equally distributed in the crude extract at a concentration of around 15 mg/g of crude extract for each compound. Acetosyringone was also present at the concentration of 14.57 mg/g of crude extract. Other classes of compounds, such as apigenin, naringenin and resveratrol, were detected at concentrations of 7.066, 5.065 and 6.473 mg/g of crude extract respectively.

The content of phenolic compounds in crude extracts depends on the plant species, cultivation technique and, in the case of food products, on the technological procedures used to process the raw material. Environmental factors such as maturity, seasonal conditions and production area also contribute to the variability in the phenolic compounds present in natural extracts (Leitao *et al*., 2011; Robards, 2003). This type of waste stream is generated during production of different types of beer, and the phenolic content of each type of beer may vary significantly on the basis of the different types of beer that are obtained from

different varieties of barley and malt (Goupy *et al*., 1999; Piazzon *et al*., 2010). Therefore, each crude extract obtained by this extraction process must be characterized.

5.3.4. Antioxidant activity

Free radical scavenging is generally recognized as the accepted mechanism whereby antioxidants inhibit lipid oxidation (Brand-Williams, Cuvelier, & Berset, 1995; Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007). In this study, an indirect method was used to determine the free radical scavenging capacity of the crude extract. A direct competitive method based on the competitive β-carotene bleaching during the autoxidation of linoleic acid in aqueous emulsion, which often occurs in food systems, was also used to obtain information about the efficacy of the crude extract to prevent lipid oxidation of food (Roginsky & Lissi, 2005).

5.3.4.1. Free radical scavenging capacity of crude extract — DPPH assay

Scavenging of free radicals and quenching of peroxide radicals terminating the peroxidation chain reaction are antioxidant properties of great significance (Brand-Williams *et al*., 1995).

The data on the ability of DPPH radical scavenging of the crude extract and the synthetic antioxidants BHA and BHT, commonly used in food industry, are shown in Table 5.1.

The antioxidant activities of the reference synthetic antioxidants, BHA and BHT, are consistent with previously reported values (Cruz *et al*., 2007). The crude extract, obtained from the PVPP-WS under the operational conditions of

extraction, displayed the highest specific activity (EC_{50}=0.186 g/L), similar to that of BHA and higher than that of BHT (a low EC_{50} value indicates high antioxidant activity).

The high radical scavenging activity displayed by the crude extract may be attributed to the high content in phenolic compounds, as such activity is known to be greatly influenced by the phenolic composition of the sample (Maillard *et al*., 1996). Flavonoids and particularly phenolic acids display strong antioxidant properties (Leitao *et al*., 2011). The antioxidant activity of different brewing materials has been related to phenolic acids such as gallic acid, caffeic acid, p-coumaric acid and ferulic acid. Hydroxycinnamic and hydroxybenzoic acids are known antioxidants that act as free radical acceptors and chain breakers (Piazzon *et al*., 2010). Catechin has been described as the flavan-3-ol responsible for the high free radical scavenging activity of barley and malt. Other studies have shown that flavonoids, such as the flavan-3-ols gallocatechin and epigallocatechin, are responsible for the high antioxidant activity of brewing materials (Dvorakova *et al*., 2008; Whittle *et al*., 1999). Large amounts of the most active phenolic compounds were present in the crude extract obtained from the waste stream (Table 5.3), and these are probably responsible for the high antioxidant activity demonstrated by the natural extract.

5.3.4.2. Antioxidant activity in β-carotene-linoleic acid–β-carotene bleaching assay

The β-carotene-linoleic acid bleaching assay mimics the ability of compounds in biological systems to reduce carotene consumption by breaking chain propagation reaction or/and enzymatic inhibition. In this assay, the activity of linoleic acid-derived alkylperoxyl radicals causes the chromophore degradation of β-carotene throughout the duration of the experiment. The progress of oxidation

reaction is tracked by spectrophotometry as β-carotene bleaching, and the results depend on the inherent activity of the antioxidant to scavenge these reactive oxygen species.

The results of the β-carotene bleaching assay are shown in Table 5.1. The crude extract was capable of protecting β-carotene from degradation caused by alkylperoxyl radicals during the reaction time. The AAC value (623.8) is very similar to the values obtained for the synthetic antioxidants used in the food industry (BHA and BHT). The crude extract was very effective in protecting β-carotene from oxidation and displayed similar effectiveness to BHA and greater activity than BHT. The results indicate that the crude extract may be capable of preventing the occurrence of damaging free radical chain reactions in susceptible biological and non-biological systems.

5.4. Conclusions

A crude extract was obtained (at pilot scale) from a brewery waste stream, with an extraction yield of 0.15% (w/v). The total phenolic content of the extract was around 50%, as determined by the Folin–Ciocalteu assay and confirmed by RP-HPLC–DAD and HPLC-ESI–MS TOF analyses. The proposed gradient elution method enabled successful chromatographic separation of the phenolic compounds present in the crude extract. This method may be applied for the routine screening of crude extracts obtained from brewing waste and for the identification and quantification of polyphenols. Flavonoids and phenolic acids, which were the main compounds present in the crude extract, were probably the main responsible of the high antioxidant activity, which was similar to that of the synthetic antioxidants used in the food industry (BHA and BHT). This fact

encourages the prospect of their commercialization as powerful natural antioxidants to be used as food additives in order to increase the shelf life of food by preventing lipid peroxidation and protecting from oxidative spoilage during storage time.

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6

Fractionation and purification of bioactive compounds obtained from a brewery waste stream

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Abstract

The brewery industry generates waste that could be used to yield a natural extract containing bioactive phenolic compounds. We compared two methods of purifying the crude extract—solid-phase extraction (SPE) and supercritical fluid extraction (SFE)—with the aim of improving the quality of the final extract for potential use as safe food additive, functional food ingredient, or nutraceutical. The predominant fractions yielded by SPE were the most active, and the fraction eluted with 30% (v/v) of methanol displayed the highest antioxidant activity (0.20 g L^{-1}), similar to that of BHA. The most active fraction yielded by SFE (EC_{50} of 0.23 g L^{-1}) was obtained under the following conditions: temperature 40 ºC, pressure 140 bar, extraction time 30 minutes, ethanol (6%) as a modifier, and modifier flow 0.2 $mLmin^{-1}$. Finally, we found that SFE is the most suitable procedure for purifying the crude extracts and improves the organoleptic characteristics of the product: the final extract was odourless, did not contain solvent residues, and was not strongly coloured. Therefore, natural extracts obtained from the residual stream and purified by SFE can be used as natural antioxidants with potential applications in the food, cosmetic, and pharmaceutical industries.

Keywords: Residual stream, SPE, SFE, bioactive compounds, natural extract, antioxidant activity.

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6.1. Introduction

Bioactive phenolic compounds are widely distributed in nature and are the most abundant antioxidants in the diet, being common components of fruits, vegetables, and beverages (Scalbert, & Williamson, 2000; Martins *et al*., 2011). Numerous studies have associated the consumption of foods rich in bioactive compounds, such as phenolic compounds, with the prevention of cardiovascular diseases, certain types of cancer, and other diseases related to aging (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005). The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity. These bioactive compounds may be a major determinant of the antioxidant potentials of foods, and they may therefore be a natural source of antioxidants (Balasundram, Sundram, & Samman, 2006). Antioxidants are widely used in food products to prevent or delay the oxidation of fats and oils (EU, 2008). The recent worldwide trend to avoid or at least reduce the use of synthetic additives, such as BHT and BHA, has created the need to identify natural (and possibly safer) alternative sources of food antioxidants (EFSA, 2011; EFSA, 2012). In recent years, there has been a growing interest in the use of natural antioxidants in the food industry, not only for application as preservatives but also because of their benefits to human health (Shahidi, & Zhong, 2010; Gil-Chávez *et al*., 2013).

Beer production is an extensively studied biotechnological process that generates various by-products. The most common byproducts are generated from the main raw materials used to make beer, that is, barley malt, hop, and yeast. These by-products can be used in biotechnological processes, such as fermentative processes for the production of value added compounds (e.g., xylitol, ethanol) as substrates for culturing microorganisms and as raw material for extraction of compounds such as antioxidants (Mussatto, 2009).

Beer contains a large variety of phenolic compounds which are derived from the biotechnological fermentation of barley malt (70%) and hop (30%) and which are responsible for the overall antioxidant activity of the beverage (Goupy, Hugues, Boivin, & Amiot, 1999; Callemien, & Collin, 2007). Numerous studies have shown that polyphenols are extremely important for the physical stability (a fundamental quality parameter) of beer (Callemien *et al*., 2007). During storage of beer, colloidal haze forms as a result of the complexes that polyphenols form with proteins and polypeptides (Siebert, 1999). The negative impact of malt and hop polyphenols on haze stability can be minimized by using polyvinylpolypyrrolidone (PVPP) resin to stabilize beer and consequently extend its shelf life. Stabilization with PVPP removes a substantial portion of the haze active and nonhaze active polyphenols from beer, and these polyphenols can subsequently be recovered from the PVPP by an alkaline treatment (Mitchell, Hong, May, Wright, & Bamforth, 2005). Therefore, a natural extract containing bioactive phenolic compounds with high antioxidant activity can be obtained from the alkaline residual stream generated after cleaning the PVPP in the brewery industry, by extraction in a solvent such as ethyl acetate (Barbosa-Pereira, Angulo, Paseiro-Losada, & Cruz, 2013).

The composition of the extract will depend on the solvent used and also on the quality of the original material, its composition, genetic factors, environmental conditions, storage conditions, and any prior treatment. In order to obtain a high quality extract with antioxidant activity that is suitable for use in the food, cosmetic, and pharmaceutical industries, the extract must be purified to remove all inert and undesirable components, so as to improve the antioxidant activity of the extract and minimize any odour, taste, and colour (Peschel *et al*., 2006).

A purification process that removes fractions with limited antioxidant activity enables a good level of antioxidant activity to be obtained from relatively small amounts of the original natural extract. Moreover, it is also important to obtain pure

extracts to ensure the identity and safety of antioxidant compounds to be used as food additives (Diaz-Reinoso, Moure, Dominguez, & Parajó, 2006).

In the present study, we evaluated two methods of purifying the crude extract—solid-phase extraction (SPE) and supercritical fluid extraction (SFE). SPE has been widely used for clean-up and purification of extracts as well as preconcentration of juices, wines, and beer. Phenolic compounds are readily fractionated by several formats of SPE in different materials of natural origin; elution with methanol on reverse phase columns is the most popular method of separating these compounds (Robards, 2003; García, Grande, & Gándara, 2004; Onofrejová *et al*., 2010).

Extraction and recovery of valuable compounds are the most common uses for SFE, which operate at low temperatures, in the absence of oxygen, and typically use CO_2 as extraction solvent (SC-CO_2). These features make SFE an ideal technique for extracting bioactive compounds (Herrero, Cifuentes, & Ibañez, 2006). The most obvious advantages of SFE are that it is clean and environmentally friendly. Direct SC-CO_2 extraction is not recommended for by-products obtained on a large scale and that contain small amounts of bioactive compounds (Diaz-Reinoso *et al*., 2006). However, SFE has been used to purify crude extracts yielded by organic solvents, to improve their purity and their biological properties without thermal or chemical degradation. As CO_2 is a non-toxic, inexpensive, noninflammable, volatile solvent, it can be used in a variety of different conditions (Babovic *et al*., 2010; Vicente *et al*., 2013). The extraction efficiency of SC-CO_2 can be optimized by changing the density of CO_2 (varying pressure and temperature), the modifier (e.g., organic solvent), modifier percentage, or time, among other parameters. Due to the apolar nature of CO_2, the use of modifiers (e.g., ethanol) can significantly improve the recovery of the phenolic compounds due to the polarity of these compounds (Diaz-Reinoso *et al*., 2006).

The aims of the present study were (i) to evaluate the efficiency of the SPE and SFE techniques to purify natural antioxidants obtained from brewery waste and (ii) to determine the recovery yield and the radical-scavenging activity of the fractions obtained. Chemical analysis of the fractions by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to a diode array detector (DAD) was carried out to identify and quantify the polyphenols responsible for the antioxidant activity.

6.2. Materials and methods

6.2.1. Reagents, solvents and standard phenolics

Ethyl acetate (GR for analysis), methanol (≥ 99.9%), absolute ethanol, hydrochloric acid (37%), glacial acetic acid, and acetonitrile (ACN, HPLC grade) were obtained from Merck (Darmstadt, Germany). Ultrapure water was prepared using a Milli-Q filter system (Millipore, Bedford, MA, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH, ≥ 85%) and gallic acid (≥ 98%) were supplied by Fluka Chemie AG (Buchs, Switzerland). 2,6-Di-tert-buthyl-4-methylphenol (BHT, 99.0%) and 2(3)- tert-butyl-4-hydroxyanisole (BHA, 98%) were provided by Sigma-Aldrich (Steinhein, Germany). Supercritical carbon dioxide, CO_2 SCF (purity: 99.998%), was supplied by Air Liquide (Spain).

Polyphenol standards were supplied as follows: protocatechuic acid (≥ 97.0%), caffeic acid (≥ 98.0%), (−)-epicatechin (≥ 90%), acetosyringone (97%), resveratrol (≥ 99%), (±)- naringenin (95%), epigallocatechin (≥90%), (+)-catechin hydrate (98%), ferulic acid (99%), quercetin (≥ 98%), kaempferol (≥97.0%), gallocatechin (≥ 98%), p-coumaric acid (≥ 98.0%), and apigenin (≥ 97%) by Sigma-Aldrich (Steinhein, Germany); gallic acid (≥ 98.0%), syringic acid (≥ 97%),

isoquercetin, and salicylic acid (≥ 99.0%) by Fluka Chemie AG (Buchs, Switzerland); and homovanillic acid (98%), 4-hydroxybenzoic acid (99%), and acetovanillone (98%) by Alfa Aesar (Karlsruhe, Germany).

6.2.2. Sampling

In beer production, a clarification step is essential to improve beer stability. As a result of this process, a PVPP sludge is obtained in the brewing industry. The PVPP sludge loaded with polyphenolic compounds was washed with a NaOH solution (2% (w/w)) at room temperature. After the NaOH-PVPP was filtered, a cleaned PVPP resin and a PVPP washing solution (PVPP-WS) containing phenolic compounds were obtained (see Fig. 6.1). The residual stream generated after the PVPP cleaning process was kindly supplied by Mahou-San Miguel, Spain.

6.2.3. Industrial Plant Scale Extraction of the Antioxidants from PVPP Sludge

The PVPP-WS (1000 L) was acidified to pH 1.5 with HCl (37%), and polyphenolic compounds were extracted with ethyl acetate (2000 L) by stirring for 30 minutes at room temperature. The organic and aqueous phases were separated by decantation, and the organic phase was collected and evaporated to dryness at 40 ºC. The residual water was removed from the extract by lyophilisation before the recovery yield was determined gravimetrically, and the dry extract was used in fractionation experiments (see Fig. 6.1).

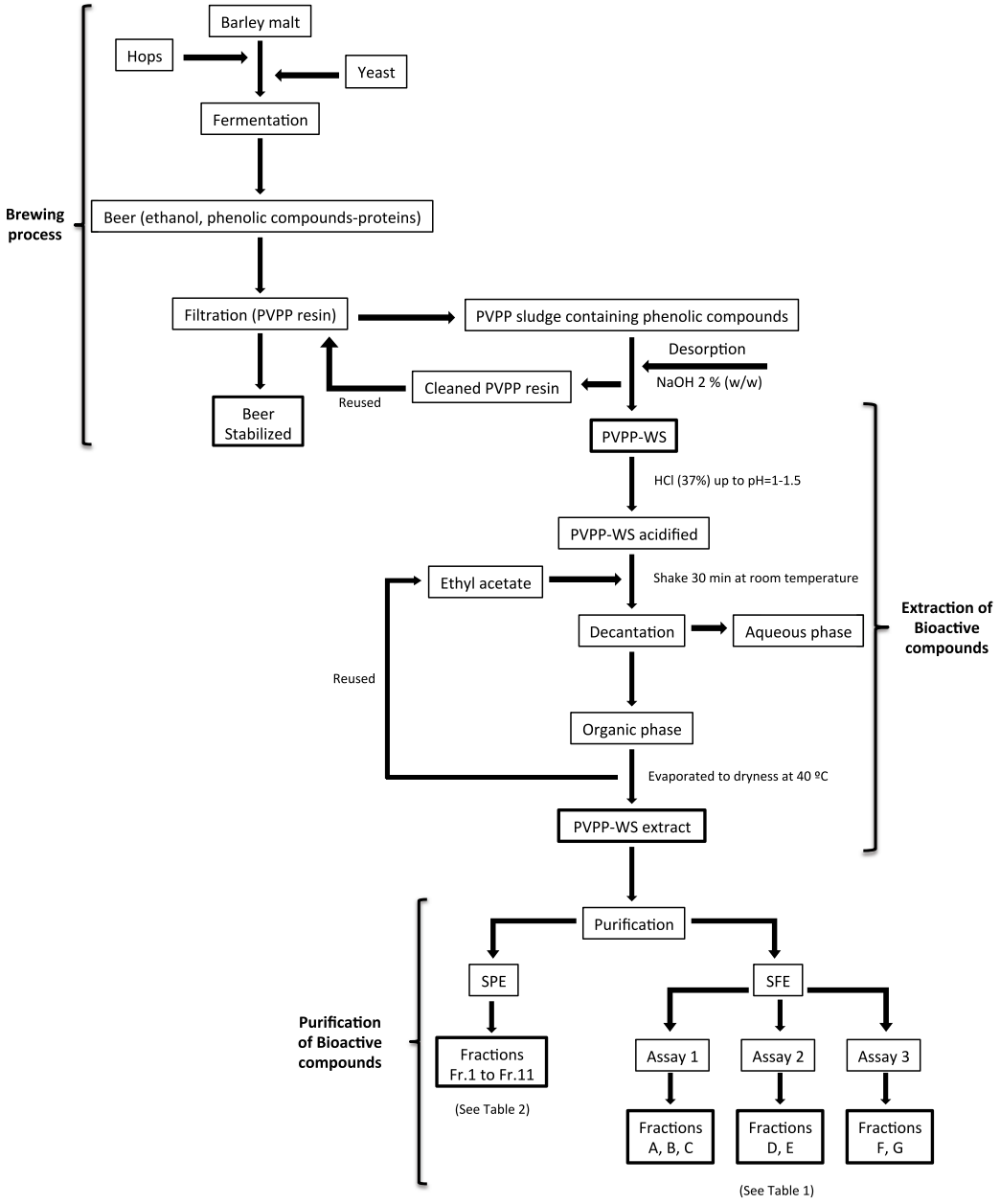

Figure 6.1. Schematic representation of the brewing process, extraction, and purification of the PVPP-WS extract containing bioactive compounds

6.2.4. Fractionation and Purification of PVPP Crude Extract

6.2.4.1. Solid-Phase Extraction (SPE)

SPE was performed with super clean cartridges (LC-18 20 mL, from Supelco, Germany) and 5 g of reversed-phase sorbent (modified silica with octadecyl groups). The crude extract (50 mg) was dissolved in 10 mL of water and loaded on the cartridge. The natural extract was eluted with different percentages of methanol (v/v): 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and finally 100% of methanol, so that eleven separate fractions were obtained at the end of the process. All fractions were evaporated to dryness, under vacuum at 40◦C, in a rotary evaporator, and finally redissolved in methanol for further analysis. The recovery yield of each fraction was determined gravimetrically, and the antioxidant activity of each fraction was measured by the DPPH radical-scavenging test. The phenolic compounds responsible for the antioxidant activity were determined by HPLC-DAD.

6.2.4.2. Supercritical Fluid Extraction

The crude extract was fractionated using a supercritical fluid SCF R100 system (Thar Technologies, Inc.) equipped with a 5mL SFE cell (Thar Technologies, Inc.). Different extraction conditions were tested in three different assays. In each assay, 1 g of sample was submitted to the fractionation procedure. The assay conditions are shown in Table 6.1.

Assay 1—Pressure Mode (Pressure Range)

The experiment was run at 40 ºC, and CO_2 was automatically fed into the system by the CO_2 pump to maintain a constant pressure. For fractionated

separation of the different compounds present in the crude extract, pressure of between 100 and 300 bar (100, 120, 140, 160, 200, 250, and 300bar) was applied. The extraction time was 30 minutes at each pressure tested. Experiments were carried out with and without modifier. Ethanol was used as a modifier, and two different flow rates (0.1 mL min^{-1} and 0.2 mL min^{-1}) were tested.

Assay 2—Flow Mode (Pressure Range)

The trial was run at 40 ºC, and CO_2 was fed into the system at a flow rate of 3 g min^{-1}. The pressure range and extraction time at each pressure tested were the same as described earlier. The modifier (ethanol) was applied under different conditions: 3% and 6% of modifier at flow rate of 0.1 mL min^{-1} and 0.2 mL min^{-1}, respectively.

Assay 3—Flow Mode (Percentage of Modifier Range)

The experiment was run at 40 ºC, and CO_2 was fed into the system at a flow rate of 3 g min^{-1}. In this test, pressure was maintained constant at 140 bar. Two modifiers, ethanol and methanol, were tested within a range of 0 and 3% (0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%). Single extracts obtained under the three different test conditions were evaporated to dryness under nitrogen steam. The recovery yield of each fraction was determined gravimetrically. Fractions were characterized by HPLC-DAD, and the antioxidant activity of each was determined by the free radical method DPPH.

Table 6.1. SFE operational conditions tested

Assay	T (ºC)	Time (min)	CO_2 flow (g min^{-1})	Pressure (bar)	Modifier	Modifier (%)	Modifier flow (mL min^{-1})	Fractions obtained
1	40	30	- (*)	100, 120, 140, 160, 200, 250, 300	None	None	None	A_{100}, A_{120}, A_{140}, A_{160}, A_{200}, A_{250}, A_{300}
					Ethanol	- (*)	0.1	B_{100}, B_{120}, B_{140}, B_{160}, B_{200}, B_{250}, B_{300}
						- (*)	0.2	C_{100}, C_{120}, C_{140}, C_{160}, C_{200}, C_{250}, C_{300}
2	40	30	3	100, 120, 140, 160, 200, 250, 300	Ethanol	3	0.1	D_{100}, D_{120}, D_{140}, D_{160}, D_{200}, D_{250}, D_{300}
						6	0.2	E_{100}, E_{120}, E_{140}, E_{160}, E_{200}, E_{250}, E_{300}
3	40	30	3	140	Ethanol	0, 0.5, 1, 1.5, 2, 2.5, 3	0.0, 0.02, 0.03, 0.05, 0.06, 0.08, 0.1	F_0, $F_{0.5}$, F_1, $F_{1.5}$, F_2, $F_{2.5}$, F_3
					Methanol			G_0, $G_{0.5}$, G_1, $G_{1.5}$, G_2, $G_{2.5}$, G_3

* In pressure mode, neither CO_2 flow nor modifier flow were controlled.

6.2.5. Separation and Quantification of Bioactive Phenolic Compounds (RP-HPLC-DAD)

Chromatographic analysis was performed on an HPLC system model 1200 HP (Hewlett-Packard, Waldbronn, Germany), equipped with a diode array detector (DAD) and controlled by HP Chemstation chromatographic software.

Chromatographic separation of polyphenols was carried out on a reverse phase Kromasil C18 column (250 × 3.2mm internal diameter, 5 μm particle size) (Phenomenex, Barcelona, Spain). The solvents constituting the mobile phase were milli-Q water 0.1% acetic acid (solvent A) and 100% ACN (solvent B). The gradient program was as follows: 0–5 min, 90% A and 10% B; 5–35 min, linear gradient until reaching 50% B at 35 min; 35–43 min, 50% B isocratic; 43– 45 linear gradient from 50% to 10% B; and finally, the column was washed and reconditioned. The mobile phase flow rate was 0.5 mLmin−1 during the entire analytical run, the column temperature was set at 38 ºC, and the sample injection volume was 20 μL. A scan in the range of 190 to 700 nm was continuously performed by DAD.

Individual phenolic compounds were identified by comparing their retention time and their UV spectrum with those obtained by injecting standards in the same HPLC conditions. Phenolic acids were monitored and quantified at 225 nm, flavan-3 ols, flavanones, flavones, and acetophenone derivates at 280 nm, hydroxycinnamic acids and resveratrol at 325 nm, and flavonols at 372 nm.

6.2.6. Antioxidant Activity, DPPH Assay

The antioxidant activity of phenolics in the crude extract and its fractions, obtained during the purification processes, were determined by the DPPH (2,2-

diphenyl-1-picrylhydrazyl) radical scavenging method described by von Gadow, Joubert, and Hansmann (1997) with slight modifications.

Standard solutions of the different antioxidant fractions and of two synthetic compounds with antioxidant properties, BHA and BHT, which are commonly used in the food industry, were prepared in methanol. An aliquot of antioxidant (50 μL) was added to 2 mL of DPPH radical methanolic solution (3.6×10^{-5} M), shaken vigorously on a vortex shaker (MS2 MiniVortex Shaker IKA), and left to stand in the dark at room temperature. The absorbance was measured at 515 nm, after 16minat room temperature, in a dual-beam spectrophotometer (Uvikon XL, Bio-Tek Instruments, Milan, Italy). All determinations were performed in triplicate. The decrease in absorbance was converted to inhibition percentage of the DPPH (IP), according to the following equation:

$$\mathrm{IP} = \frac{\mathrm{A}_0 - \mathrm{A}_{16}}{\mathrm{A}_0} \times 100$$

where A_0 is the absorbance of the control at initial time; A_{16} is the absorbance of the sample after 16 minutes.

The concentration of antioxidant compound or fraction required to achieve 50% inhibition of the radical DPPH (equivalent concentration = EC_{50}) was determined from the linear regression curve obtained by plotting the different concentrations of antioxidant compound or fraction used (within the range 0.1 to 3.5 g L^{-1}) against the inhibition percentage of the DPPH (IP).

6.3. Results and discussion

In this study, a new by-product (PVPP-WS) was considered as a natural source of antioxidant-rich bioactive compounds with several potential applications.

The extraction procedure used to obtain the crude extract has already been tested at laboratory scale and pilot plant scale in previous studies. With the overall aim of enabling the brewing industry to implement this extraction process in industrial plants, the present study investigated the scaling up of the extraction process and the purification of the bioactive phenolic compound extracted. The extraction yield was approximately 0.1%. In the brewery industry, around one litre of this waste stream (PVPP-WS) can be generated from every 138 L of beer produced. Approximately 403 million hectolitres of beer were produced in Europe in 2010, which means that up to 400 tons of this crude extract could be obtained in Europe every year (Brink *et al*., 2011).

The crude extract must be processed (by purification and fractionation) as its brown colour would hinder its use as a food additive. In addition, more information about the composition of the crude extract in bioactive phenolic compounds could be obtained from different fractions to determine the correlation between the antioxidant activity and the phenolic compounds or group of phenolic compounds present in the fractions.

6.3.1. Solid-Phase Extraction Results

Solid-phase extraction is generally used for sample clean-up, fractionation, purification and/or preconcentration of natural extracts. In this study, eleven differently coloured fractions containing phenolic compounds were obtained (see Fig. 6.1).

6.3.1.1. Extraction Yield and Antioxidant Activity

The recovery yield and the radical scavenging activity were determined for each fraction obtained at each solvent ratio applied to the cartridge. The results are shown in Table 6.2, along with the colour of each fraction. The antioxidant activity of the synthetic antioxidants commonly used in the food industry was also evaluated to compare the potential of the natural compounds as food additives. The crude extract was also tested to evaluate whether the fractionation process yielded fractions that were more or less active than the crude extract.

The most active fractions and the best yields obtained in the SPE process corresponded to the first seven fractions eluted with a solvent mixture from 0%–60% of methanol. This showed that the natural extract and the polyphenolic compounds are water soluble but the addition of methanol yielded the most active fractions. Therefore, the fraction obtained with 30% (v/v) of methanol exhibited the highest antioxidant activity. All the fractions that display a notable level of antioxidant activity (fractions 1–7) were coloured, particularly fractions Fr. 4, Fr. 5, and Fr. 6, which also displayed the highest degree of antioxidant activity against the free radical DPPH. This is consistent with the results described by Woffendem, Ames, and Chandra (2001) in a study evaluating the relationship between antioxidant activity and colour of crystal malt extracts. Fractions 8, 9, 10, and 11 were colourless, and the antioxidant activity was lower than that of the other extracts.

The EC_{50} values of Fr. 3, Fr. 4, Fr. 5, and Fr. 6 were similar to that of the synthetic antioxidant BHA used in food industry. Except for the last 4 fractions yielded, all the fractions obtained from the crude extract showed a higher DPPH radical scavenging capacity than the antioxidant BHT, also commonly used in food industry. In this study, the antioxidant capacity of the crude extract (EC_{50} =0.32 g L^{-1}) was also calculated: (a) to compare the capacity of this extract and the

fractions obtained; and (b) to evaluate whether the antioxidant activity increased as a result of the purification process. Purification of the crude extract yielded fractions with higher antioxidant activity than the crude extract.

Table 6.2. Recovery yield, radical scavenging activity (DPPH) and colouration of each fraction (Fr.) obtained by SPE with different % of methanol

Sample	% of methanol	Recovery yield (% w/w)[a]	DPPH (EC_{50})[b]	Colour
Fr. 1	0	10.4	0.44	Wheat
Fr. 2	10	18.1	0.30	Burnt orange
Fr. 3	20	16.2	0.27	Brown
Fr. 4	30	19.4	0.20	Maroon
Fr. 5	40	18.3	0.26	Dark brown
Fr. 6	50	8.19	0.23	Dark brown
Fr. 7	60	6.71	0.89	Ochre
Fr. 8	70	1.59	7.02	Colourless
Fr. 9	80	0.324	6.64	Colourless
Fr.10	90	0.615	8.25	Colourless
Fr. 11	100	0.194	14.3	Colourless
Crude extract	-	-	0.32	Dark brown
BHA	-	-	0.24	-
BHT	-	-	2.67	-

[a] Values expressed as % of dry crude extract

[b] Values expressed as g L^{-1} of extract (fraction)

6.3.1.2. HPLC-DAD-UV Analysis of the Antioxidant Fractions

The results of the identification and quantification of major polyphenolic compounds present in each fraction obtained in the fractionation process (SPE) by HPLC-DAD-UV are shown in Table 6.3.

Table 6.3. Phenolic compounds present in the different fractions, obtained with a LC-18 column, identified and quantified by HPLC-DAD

	Fraction											Crude extract
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**-**
Antioxidant compound	**mg g^{-1} of fraction**											**mg g^{-1}**
Gallic acid	193	-	-	-	-	-	-	-	-	-	-	20.1
Gallocatechin	178	641	-	-	-	-	-	-	-	-	-	132
Protocatechuic acid	-	72.9	9.51	-	-	-	-	-	-	-	-	15.5
Epigallocatechin		86.2	96.4	-	-	-	-	-	-	-	-	30.6
Catechin	-	114	75.5	-	-	-	-	-	-	-	-	29.9
4-Hydroxybenzoic acid	-	6.51	7.49	-	-	-	-	-	-	-	-	2.54
Caffeic acid	-	13.7	66.0	-	-	-	-	-	-	-	-	14.1
Epicatechin	-	-	132	-	-	-	-	-	-	-	-	21.4
p-coumaric acid	-	-	73.9	1.83	-	-	-	-	-	-	-	11.4
Isoquercetin	-	-	166	-	-	-	-	-	-	-	-	28.3
Ferulic acid	-	-	29.6	138	-	-	-	-	-	-	-	33.7
Acetosyringone	-	-	35.2	50.9	-	-	-	-	-	-	-	14.6
Resveratrol	-	-	-	-	-	51.6	14.5	-	-	-	-	5.35
Quercetin	-	-	-	-	31.7	117	-	-	-	-	-	14.5
Apigenin	-	-	-	-	-	58.8	59.7	-	-	-	-	8.10
Kaempferol	-	-	-	-	-	25.3	23.4	120	-	-	-	6.06
Naringenin	-	-	-	-	-	38.7	171	11.8	-	-	-	14.6
Total (mg g^{-1})	371	934	692	191	31.7	292	269	132	-	-	-	403

The different ratios of solvents yielded different fractions. These fractions displayed different levels of antioxidant activity because the polyphenolic content varies considerably with solubility.

The first fractions, which exhibited the highest level of antioxidant activity (see Table 6.2), contained the highest amounts of polyphenolic compounds (Table 6.3). Fractions 1 and 2 contain large amounts of gallocatechin, which largely accounts for the high radical scavenging activity exhibited by these two fractions. Compounds with flavonoid structure such as catechin generally display a higher level of antioxidant activity than nonflavonoid compounds (Zhao, Chen, Lu, & Zhao, 2010).

Fraction 4 displayed the highest level of antioxidant activity, mainly due to the high content of ferulic acid, a phenolic compound. However, Fraction 3, which displayed a similar level of antioxidant activity to Fraction 4, also contains large amounts of antioxidant compounds such as epigallocatechin, caffeic acid, p-coumaric acid, and isoquercetin (Goupy *et al*., 1999). Hydroxycinnamic and hydroxybenzoic acids are known antioxidants that act as free radical acceptors and chain breakers (Sroka, & Cisowski, 2003). Caffeic, ferulic, and p-coumaric acids have been widely studied and reported to be major contributors to the antioxidant activity of beer (Zhao *et al*., 2010; Leitao *et al*., 2011). In the present study, the natural extract was obtained after the treatment of the beer, and, as expected, this crude extract was loaded with these bioactive compounds. Fractions 2 and 3, which contain these compounds, were the most active free radical scavengers. These findings may be attributed to a combined, synergistic, and/or additive action. This type of action has previously been observed to result in an increased antioxidant potential (Moure *et al*., 2001).

The recovery yields and the phenolic contents of fractions 6 and 7were lower than those of the other fractions. However, both of these fractions displayed some

antioxidant activity, mainly due to the flavonols. Fractions 6 and 7 contain the flavonols quercetin and kaempferol at similar concentrations. Fractions 8, 9, 10, and 11 were extracted using high contents of methanol, and no phenolic compounds were detected in the fractions.

6.3.2. Supercritical Fluid Extraction

SC-CO_2 has been used successfully to purify crude extracts by concentrating the bioactive compounds (e.g., antioxidants) in the extract and also by removing contaminants. In the present study, several fractions were obtained from the SFE fractionation assay under different test conditions (see Fig. 6.1).

6.3.2.1. SFE Fractions (Extraction Yield, Colour, and Antioxidant Activity of Each Fraction)

The extraction yield of the different antioxidant fractions obtained under the different operational conditions (see Table 6.1) and the colour of each fraction are shown in Table 6.4. The extraction yield ranged from 0.12% (fractionation test A), for the extraction without cosolvent (see Table 6.4a), to 41.5% (fractionation test E) for the extractions in which ethanol was used as a modifier (see Table 6.4b). The results showed that the use of a modifier is mandatory essential for successful fractionation of the crude extract. This is consistent with data reported by various authors who obtained high extraction yields from diverse natural matrices by using modifiers (Diaz-Reinoso *et al*., 2006).

Results showed that the increase in the percentage of modifier increases the amount of extract (see Table 6.4b). The volume of the extract collected at each pressure is more constant in the flow mode (see Table 6.4b, tests D and E) than in pressure mode (see Table 6.4a, tests B and C). Moreover, when higher amounts

of the modifier are used, less pressure is required, as observed in tests C (flow rate of 0.2 mL min^{-1} twice that of B) and E (6% of modifier twice that of D), in which almost all compounds were yielded at a pressure of 120 bar. These results indicated that the optimal conditions for the extraction process of the crude extract are those used in test E, which yielded most phenolic compounds and fractions with the highest antioxidant activities (see Table 6.5) similar to BHA and higher than that of BHT (see Table 6.2). Moreover, under these conditions, the fractions were paler, which is an advantage as the strong brown colour of the crude extract may hinder its use as a food additive. However, the fractions obtained in high amounts were always darker, as, for example, E_{120} (see Table 6.4b).

The darkest fractions, which contained more phenolic compounds per weight of extract fraction (see Tables 6.4a, 6.4b and 6.4c), displayed a high radical scavenging capacity (see Table 6.5). Colourless fractions, which did not appear to contain phenolic compounds, did not display any scavenging activity of the radical DPPH. This is further indication that fractions colour depends on the content of active compounds, which are also responsible for the antioxidant activity of the fractions.

In the present study, methanol was also evaluated as a modifier in test G (see Table 6.4b). The results showed that ethanol is more suitable for the extraction process because the phenolic compounds of interest were obtained with a lower percentage of ethanol (1.5%), while 2% methanol was required to obtain compounds such as ferulic acid and gallocatechin (data not shown), the main compounds present in the crude extract. Selection of a suitable modifier and/or adjustment of the modifier percentage is essential for successful fractional separation of the antioxidant compounds present in the crude extract.

Table 6.4. (a) SFE yield determined gravimetrically (w/w) and colour of fractions A, B, and C. (b) SFE yield determined gravimetrically (w/w) and colour of fractions D and E. (c) SFE yield determined gravimetrically (w/w) and colour of fractions F and G

(a)

	A		B		C	
P (bar)	Yield (%)	Colour	Yield (%)	Colour	Yield (%)	Colour
100	0.02	Colourless	0.24	Wheat	0.02	Colourless
120	0.02	Colourless	7.71	Dark brown	23.04	Dark brown
140	0.02	Colourless	4.27	Burnt orange	6.39	Brown
160	0.00	Colourless	2.77	Burnt orange	0.06	Wheat
200	0.02	Colourless	12.7	Maroon	0.01	Colourless
250	0.02	Colourless	8.09	Dark brown	0.07	Ochre
300	0.02	Colourless	3.29	Maroon	0.03	Colourless
Total	0.12%		39.1%		29.6%	

(b)

	D		E	
P (bar)	Yield (%)	Colour	Yield (%)	Colour
100	0.89	Brown	3.27	Burnt orange
120	0.73	Brown	19.83	Dark brown
140	0.76	Brown orange	5.67	Burnt orange
160	1.42	Burnt orange	3.65	Burnt orange
200	1.13	Burnt orange	3.84	Burnt orange
250	0.79	Burnt orange	2.56	Burnt orange
300	0.98	Burnt orange	2.64	Burnt orange
Total	6.7%		41.5%	

(c)

	F		G	
% Modifier	Yield (%)	Colour	Yield (%)	Colour
0	0.04	Orange	0.13	Wheat
0.5	0.07	Coral	0.08	Wheat
1.0	0.33	Wheat	0.31	Wheat
1.5	0.58	Wheat	0.28	Wheat
2	0.44	Wheat	0.58	Wheat
2.5	0.61	Wheat	0.41	Wheat
3	1.08	Wheat	0.59	Wheat
Total	3.15%		2.38%	

6.3.2.2. HPLC-DAD-UV Analyses of the Antioxidant Fractions

The fractions obtained by SFE were analysed by HPLC-DAD-UV to the characterization of bioactive compounds in the crude extract.

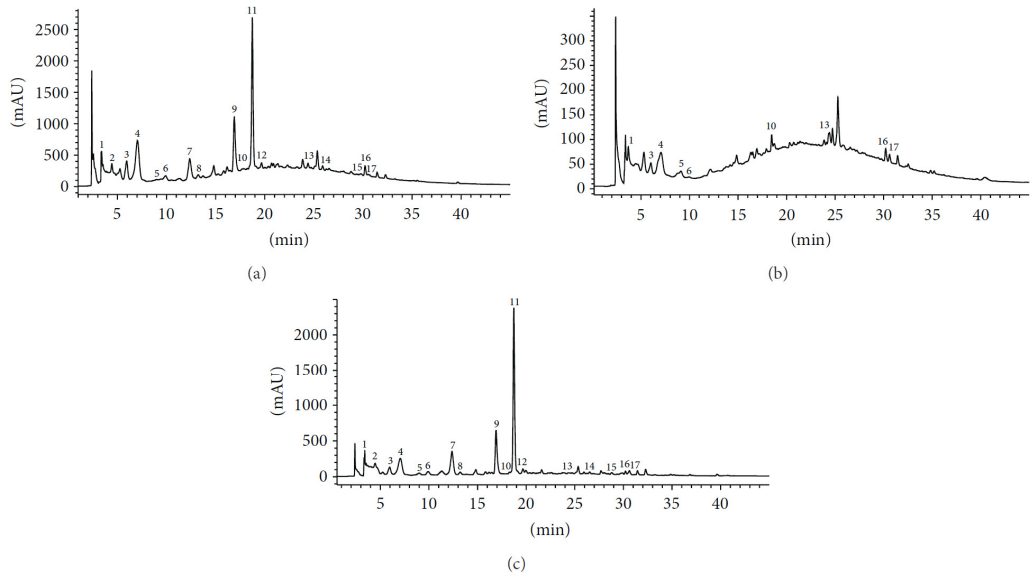

Figure 6.2. Chromatograms acquired at 280 nm by HPLC-DAD-UV. (a) Chromatogram of the crude extract without fractionation process. (b) Chromatogram of the residue of the crude extract that remains in the extraction cell after the fractionation process (E_{Cell}). (c) Chromatogram of the fraction obtained under optimal conditions (E_{140})

Fig. 6.2 shows, as a representative example, the chromatogram of the crude extract before fractionation by SFE (Fig. 6.2a) and the chromatograms obtained after the fractionation procedure under optimal conditions, the extraction cell (Fig. 6.2b) and the fraction E_{140} (Fig. 6.2c). Chromatogram C (Fig. 6.2c) shows that SFE fractionation improves the purity of the phenolic compounds (clearly observed from the base line of the chromatogram) relative to the crude extract (Fig. 6.2a). It is

also evident that the impurities in the crude extract remain in the extraction cell, as verified by the peak obtained in chromatogram B. However, phenolic compounds and probably other compounds not identified in the present study remain active in the extraction cell (E_{Cell}), which confers antioxidant activity to the residue in the extraction cell (see Table 6.5).

Table 6.5. Antioxidant activity of SFE extracts determined by DPPH. Results are expressed as EC_{50} ($g\ L^{-1}$)

	Fraction								
	Pressure Mode			**Flow mode**				**Flow mode**	
P (bar)	**A**	**B**	**C**	P (bar)	**D**	**E**	% modification	**F**	**G**
Cell	-	-	0.23	Cell	0.20	0.22	Cell	0.26	0.20
100	n.d.	2.65	37.73	100	0.33	0.74	0	21.99	0.64
120	n.d.	0.36	0.41	120	0.51	0.36	0.5	1.57	0.68
140	n.d.	0.27	0.25	140	0.62	0.23	1.0	0.59	3.52
160	n.d.	0.29	0.28	160	0.46	0.21	1.5	0.97	1.42
200	n.d.	0.34	1.00	200	0.74	0.22	2	0.88	0.80
250	n.d.	0.21	0.20	250	0.34	0.23	2.5	0.64	0.67
300	n.d.	0.10	3.12	300	0.42	0.34	3	0.54	0.42

The phenolic compounds present in the fractions obtained under optimal conditions in this study (fractionation E) are shown in Table 6.6. The number of phenolic compounds shown in the table corresponds to the numbers in the chromatograms in Figures (6.2a, 6.2b, and 6.2c). The phenolic compounds were identified and quantified in this study for all the fractions obtained under the different assay conditions (data not shown). The qualitative composition of phenolic compounds in the factions yielded by SFE is the same in almost every fraction obtained under the different test conditions assayed (see Table 6.6). The

main bioactive phenolic compounds identified in the fractions were as follows: ferulic acid, p-coumaric acid, caffeic acid, protocatechuic acid, catechin, gallic acid, gallocatechina, and epigallocatechin. These results are consistent with those obtained with the SPE procedure (see Section 6.3.1.2).

Table 6.6. Phenolic profile of each fraction obtained under SFE conditions of test E: Temperature 40 ºC, extraction time 30 minutes, modifier ethanol (6%), modifier flow 0.2 ml min^{-1}. Phenolic compounds are expressed as mg g^{-1} of fraction

Peak no.	Phenolic compound	SFE fractions (mg g^{-1})							
		E_{Cell}	E_{100}	E_{120}	E_{140}	E_{160}	E_{200}	E_{250}	E_{300}
1	Gallic acid	1.04	0.24	1.05	1.68	2.61	3.02	3.44	3.07
2	Gallocatechin	171	10.7	14.8	33.9	41.0	33.3	29.8	20.9
3	Protocatechuic acid	-	1.99	4.12	9.19	12.8	14.9	16.7	16.0
4	Epigallocatechin	1.43	10.7	10.2	-	-	-	-	-
5	Catechin	6.10	3.40	3.36	6.88	11.8	11.7	15.7	16.7
6	4-Hydroxybeinzoic acid	-	3.58	1.96	4.78	4.22	2.72	2.36	1.55
7	Caffeic acid	-	7.29	7.38	17.1	19.65	14.8	13.3	9.52
8	Epicatechin	-	-	-	-	0.23	0.14	0.17	0.40
9	p-coumaric acid	-	7.97	5.89	13.5	13.9	9.66	8.45	6.26
10	Isoquercetin	2.51	-	-	-	-	3.71	3.75	1.43
11	Ferulic acid	-	35.7	27.6	69.0	70.1	47.0	38.9	22.0
12	Acetosyringone	-	2.92	0.83	3.62	2.07	1.18	3.10	2.55
13	Resveratrol	-	-	0.54	1.68	0.37	0.46	0.60	0.80
14	Quercetin	0.57	0.65	0.98	1.91	2.61	2.83	3.13	2.96
15	Apigenin	-	-	0.04	0.66	0.78	0.72	0.79	0.93
16	Kaempferol	1.48	0.94	1.65	3.17	4.42	3.18	6.24	6.64
17	Naringenin	0.39	0.40	0.78	1.69	2.45	2.75	3.26	3.52
Total (mg g^{-1})		11.0	88.9	82.5	172.7	192.8	155.9	152.3	117.0

The phenolic compounds were not successfully separated by the SFE fractionation procedure as in the SPE fractionation (LC-18 column). However, the purity of the fractions yielded by the SFE was greater than that of the fractions yielded by SPE. Moreover, large amounts of pure fractions containing the main bioactive phenolic compounds were obtained by SFE, which makes this technique the most promising for purification of the crude extract.

Regarding the antioxidant activity, the fractions with the highest contents of polyphenolic compounds are those with the highest antioxidant activity. However, the antioxidant activities of the different fractions did not differ significantly because the composition of bioactive compounds was also very similar.

6.4. Conclusions

Industrial extraction of a natural extract containing bioactive compounds was successful and could be implemented in the brewing industry to recover a residue with added value. The dried ethyl acetate crude extract was purified by two alternative procedures to improve its quality (antioxidant activity and organoleptic properties) for potential use as a food additive.

Both purification procedures yielded fractions with better organoleptic properties (odour and colour) and with higher antioxidant activity than the crude extract. The fractions that display strong antioxidant activity may be suitable for use as food additives (to increase the shelf life of food by preventing lipid peroxidation and protecting from oxidative spoilage during storage). Moreover, these bioactive compounds may be a good source of compounds with several applications in the food industry, as food ingredients and nutraceuticals, in the cosmetics, and in pharmaceutical industries.

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7

Development of new active packaging films coated with natural antioxidants to extend the shelf life of beef

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Abstract

The aim of the present study was to develop active packaging films containing natural antioxidants and to evaluate their capacity to enhance the oxidative stability of beef during refrigeration. The antioxidant activity of a natural extract obtained from a brewery residual waste (PVPP-WS) was evaluated and compared with that of a commercial rosemary extract and two synthetic antioxidants (BHT and propyl gallate). Different concentrations of each antioxidant were also added directly to beef samples, resulting in a reduction in lipid oxidation of up to 70-80% relative to the control. Active antioxidant films coated with PVPP-WS extract reduced lipid oxidation by up to 80%, relative to the control, during cold storage. The use of active packaging films containing natural extracts could extend the shelf life of meat products and should therefore be of great interest in the food industry.

Keywords: Lipid oxidation, beef, natural antioxidants, coating, active packaging.

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7.1. Introduction

Lipid peroxidation is a major cause of deterioration of meat quality during processing, distribution and refrigeration, thereby reducing shelf stability and acceptability. Lipid oxidation can produce changes in meat quality parameters, such as organoleptic properties and nutritional value, and it leads to the generation and accumulation of compounds that may pose risks to human health (Gray, Gomaa, & Buckley, 1996).

The shelf life of meat can be extended by using antioxidants, antimicrobials and packaging materials (Zhou, Xu, & Liu, 2010).

Synthetic antioxidants have long been used in the food industry to prevent or minimize lipid oxidation in food products. Because of the growing concern about the potential health hazards associated with synthetic antioxidants commonly used in the food industry (e.g. BHT, BHA and PG) and the increased consumer demand for natural products, there is a growing interest in the use of naturally occurring antioxidants for use in food processing (Shahidi, & Zhong, 2010). The antioxidant properties of natural extracts are mainly due to the presence of phenolic compounds, and therefore the antioxidant effect of such extracts is similar to that of synthetic phenolic antioxidants.

Residual waste substances obtained from agro-industrial by-products offer a practical and economic source of potent antioxidants that could replace synthetic preservatives (Balasundram, Sundram, & Samman, 2006).

Polyvinylpolypyrrolidone washing solution (PVPP-WS) extract is a natural extract obtained from a brewery waste stream. The high antioxidant activity of the extract has been demonstrated in different in vitro experiments carried out by the authors of the present study (Barbosa-Pereira, Angulo, Paseiro-Losada, & Cruz,

2013). During storage of beer, colloidal haze can develop as a result of the formation of complexes between polypeptides and polyphenols (Siebert, 1999). The negative impact of polyphenols on haze stability is minimized by using polyvinylpolypyrrolidone resin (PVPP) to stabilize beer and extend its shelf life. PVPP stabilization removes a substantial part of the polyphenols in beer (both haze and non-haze active polyphenols), which can be recovered from the PVPP by an alkaline treatment (Mitchell, Hong, May, Wright, & Bamforth 2005).

This natural extract, which contains phenolic compounds with high antioxidant activity, can be obtained from the alkaline residual stream generated in brewery industry after cleaning the PVPP. The antioxidant activity of PVPP-WS extract is related to the high concentrations of phenolic compounds, such as flavonols (catechin, gallocatechin and epigallocatechin) and hydroxycinnamic and hydroxybenzoic acids (gallic acid, caffeic acid, p-coumaric acid and ferulic acid), which act as free radical acceptors and chain breakers and are therefore responsible for the high free radical scavenging activity of the extract (Barbosa-Pereira, Angulo, Paseiro-Losada, & Cruz, 2013; Barbosa-Pereira, Pocheville, Angulo, Paseiro-Losada, & Cruz, 2013).

Natural extracts from many herbs and spices have been studied and used to extend the shelf life of foods (Balasundram *et al*., 2006; Fernández-Lopez, Zhi, Aleson-Carbonell, Pérez-Alvarez, & Kuri, 2005; Sánchez-Escalante, Djenane, Torrescano, Beltrán, Roncales, 2003). Rosemary extract has been shown to possess strong antioxidant activity because of the high contents of phenolic diterpenes (e.g. carnosic acid, carnosol and rosmanol) and phenolic acids (e.g. rosmarinic acid), which act as oxidative chain breakers via electron donation (Zheng, & Wang, 2001).

Rosemary extracts have been widely used in the food industry, and many authors have reported their effectiveness in reducing lipid oxidation in meat

products. Nevertheless, most studies reported in the relevant literature involve the direct addition of rosemary to the packaged food, and relatively few deal with the inclusion of rosemary in the packaging material (Sánchez-Escalante *et al*., 2003; McBride, Hogan, & Kerry, 2007; Nerín, *et al*., 2006).

Active packaging is currently one of the most dynamic technologies used to preserve the quality of food via the release of active agents from the packaging film. Release of the active agents can be controlled over an extend period of time to maintain or extend the quality and shelf-life of products, without the need for direct addition of any substances to foodstuff (Zhou *et al*., 2010; Lee, 2010).

Some studies have evaluated how antioxidants (such as BHT, BHA, alpha-tocopherol and natural extracts) incorporated in packaging film migrate out of the film and retard lipid oxidation in the stored foodstuffs (Moore, Han, Acton, Ogale, Barmore, &Dawson, 2000; Barbosa-Pereira *et al*., 2013). The incorporation of rosemary extract in active packaging film has been described by Nerín et al. (2006), with promising results in relation to extending the shelf life of beef.

Another concept in active packaging is the addition of bioactive substances by a coating process. The development of coatings with antimicrobial capacity has been studied in relation to preservation of meat products (Zhou *et al*., 2010; Lee, 2010; Kerry, O'Grady, & Hogan, 2006; Bonilla, Atarés, Vargas, & Chiralt, 2012). Antimicrobial and antioxidant additives can also be incorporated by coating them onto food packaging materials to control spoilage by oxidation and to preserve food quality (Vermeiren, Dvelieghere, van Beest, & de Kruijf, Debevere, 1999; Lee, An, Lee, Park, & Lee, 2003). However, studies reporting antioxidant effects of active films coated with natural extracts are scare.

The aim of this study was to evaluate and compare the antioxidant effect of two natural extracts (PVPP-WS and rosemary extract) and two synthetic antioxidants (BHT and PG) on the oxidative stability of beef during cold storage.

The natural extracts were used to coat active films with antioxidant properties. Finally, the effectiveness of the new antioxidant active packaging films in delaying lipid oxidation of beef during cold storage was evaluated in samples in which the packaging was with and without direct contact with the food sample.

7.2. Materials and methods

7.2.1. Chemicals

Butylated hydroxytoluene (BHT) (99.0%, CAS No. [128-37-0]), sodium azide (99.0%, CAS No. [26628-22-8]), 2-thiobarbituric acid (TBA) (≥98%, CAS No. [504-17-6]) and trichloroacetic acid (TCA) (puriss. p.a. 99.5%, CAS No. [76-03-9]) were purchased from Sigma-Aldrich (Steinheim, Germany). Propyl gallate (PG) (98%, CAS No. [121-79-9]), 1,1,3,3-tetraethoxypropane (TEP) (purum ≥95% (GC), CAS No. [122-31-6]) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (TECHN ≥85%, CAS No. [1898-66-4]) were supplied by Fluka Chemie AG (Buchs, Switzerland). Methanol (GC≥99.9%, CAS No. [67-56-1]), orthophosphoric acid (85% GR for analysis, CAS No. [7664-38-2]) and ethanol (absolute for analysis, CAS No. [64-17-5]) were provided by Merck (Darmstadt, Germany).

7.2.2. Natural extracts

PVPP-WS extract: this extract, which contains natural antioxidants, was obtained from a residual stream generated during the PVPP cleaning process in the brewing industry. The PVPP washing solution (PVPP-WS) was acidified to pH 1.5 with HCl (37%), and polyphenolic compounds were extracted with ethyl acetate by stirring for 30 minutes at room temperature. Organic and aqueous

phases were separated by decantation and the organic phase was collected and evaporated to dryness at 40 ºC. Residual water was removed from the extract by lyophilisation to yield the dry crude extract (Barbosa-Pereira, Angulo, Paseiro-Losada, & Cruz, 2013). The content of phenolic compounds is shown in Table 7.1a.

Rosemary extract: commercial rosemary extract (MS-198-08 GIN 601331 Rosemary Extract P) was supplied in powder form by INGRENAT (Ingredientes naturales SL., Murcia, Spain). The composition of the rosemary extract is shown in Table 7.1b.

Table 7.1. (a) Total and individual phenolic compounds (mg g^{-1}) in the PVPP-WS extract. (b) MS-198-08 GIN 601331 Phenolic composition of the rosemary extract

(a)

Phenolic compound (mg g^{-1})					
Benzoic acid derivates	***38.1***	***Cinnamic acids***	***59.2***	***Flavonols***	***48.9***
Gallic acid	20.1	Caffeic acid	14.1	Isoquercetin	28.3
Protocatechuic acid	15.5	p-coumaric acid	11.4	Quercetin	14.5
4-hydroxybenzoic acid	2.54	Ferulic acid	33.7	Kaempferol	6.06
Flavan-3-ols	***214***	***Flavanones***	***14.6***	***Acetophenone derivates***	***14.6***
Gallocatechin	132	Naringenin		Acetosyringone	
Epigallocatechin	30.6				
Catechin	29.9	***Flavones***	***8.10***	***Stilbenoids***	***5.35***
Epicatechin	21.4	Apigenin		Resveratrol	

Total phenolic compounds present in the PVPP-WS extract, 403 **mg g^{-1}**

(b)

Compound (mg g^{-1})	
Phenolic diterpenes	***101.32***
Carnosic acid	87.18
Carnosol	14.14
Excipient	
Maltodextrin	898.68

7.2.3. Instruments

The concentration of 2-thiobarbituric acid reactive substances (TBARs) and the absorbance in the DPPH method were determined in a dual-beam spectrophotometer (Uvikon XL, Bio-Tek Instruments, Milan, Italy).

Secondary oxidation compounds were extracted from beef by use of a T 25 ULTRA-TURRAX digital homogenizer and an MS2 Mini Vortex Shaker (both from IKA).

7.2.4. Antioxidant activity of natural extracts – DPPH method

The antioxidant activity of the natural extracts was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method described by von Gadow, Joubert, and Hansmann (1997), with slight modifications.

Standard solutions of the different antioxidants and of two synthetic compounds with antioxidant properties usually used in the food industry, PG and BHT, were prepared in methanol. An aliquot of antioxidant (50 µL) was added to 2

mL of DPPH radical methanolic solution (3.6×10^{-5} M), and the solution was shaken vigorously on a vortex shaker and left to stand in the dark at room temperature, for 16 min at room temperature. The absorbance of the solution was then determined at 515 nm in a spectrophotometer. All determinations were performed in triplicate. The decrease in absorbance was converted to DPPH inhibition percentage (IP), according to the following equation:

$$\mathrm{IP} = \frac{\mathrm{A}_0 - \mathrm{A}_{16}}{\mathrm{A}_0} \times 100$$

where

A_0 is the absorbance of the control at initial time;

A_{16} is the absorbance of the sample after incubation for 16 minutes.

The concentration of antioxidant compound or natural extract required to achieve 50% inhibition of the radical DPPH (Equivalent concentration=EC_{50}) was determined from the linear regression curve (i.e. the different concentrations of antioxidant [within the range 0.1 to 5 g L^{-1}] plotted against the percentage of free radical scavenging activity [IP%]).

7.2.5. Processing of active packaging samples by coating

Several formulations of the antioxidant compounds plus a low density polyethylene (LDPE) matrix were prepared. Active films were prepared with different antioxidant contents and percentages of compounds to study the antioxidant effectiveness of the polymer formulations in the beef. All coatings were applied with a rod of 40 microns (TR40) at a speed of 90 mm/sec. The final

grammage is similar to that used in the packaging industry (3.2 g m^{-2}). The formulations are shown in Table 7.2.

Table 7.2. Formulations of the LDPE coatings (% (w/w))

Formulations	Natural extracts	
	Rosemary	**PVPP-WS**
Film Control	-	-
Film R 3%	3%	-
Film R 10%	10%	-
Film R 20%	20%	-
Film PVPP-WS 3%	-	3%
Film PVPP-WS 10%	-	10%
Film PVPP-WS 20%	-	20%

7.2.6. Evaluation of antioxidant activity in food

7.2.6.1. Beef samples

Freshly cut beef, of thickness 1 cm, was purchased in a local retail store. The meat was divided into small pieces of 50 ± 1 g and of surface area 8.5 × 8.5 cm. A small amount of sodium azide (approx. 1mg) was applied to the surface of beef samples to prevent microbial spoilage during storage.

7.2.6.2. Evaluation of antioxidant effect of direct addition of the antioxidant to beef

In this preliminary test, different concentrations of natural extracts (PVPP-WS and rosemary) and synthetic antioxidants (BHT and PG) were added to different pieces of beef. Two different concentrations of each antioxidant were used (0.05% and 3%, w/w). Addition of an intermediate concentration of the PVPP-WS extract (0.5%) to the surface of beef was also tested. The effect of the different antioxidants in the headspace of the container enclosing the beef sample was also tested, at the highest concentrations tested for direct contact with sample (3% for the synthetic antioxidants and rosemary extract and 0.5 and 3% for PVPP-WS extract). Samples without antioxidant compounds were used as controls.

The beef samples to which the different antioxidant compounds were added were stored at 4 ºC for 17 days. Samples were collected after 3, 7, 10, 14 and 17 days for TBARs determination.

7.2.6.3. Effectiveness of active packaging films on lipid stability of beef

Beef samples were wrapped in films coating with the natural extracts (PVPP-WS and rosemary extracts), which covered the entire meat surface. Three concentrations of extracts were used to coat the films (3, 10 and 20%, w/w). The effect of the natural extracts was also evaluated in the headspace of the container enclosing the beef sample, using the highest concentration tested for direct contact (20%). Two control tests were performed in this assay: the control film without the addition of natural extracts and the control of meat samples not covered with the film. The meat samples were placed in a glass dish and stored at 4 °C for 16 days. Lipid oxidation of samples was evaluated periodically (0, 3, 6, 9, 13 and 16 days).

7.2.6.4. Lipid oxidation analysis – TBARs

Lipid oxidation of beef was determined by analysis of 2-thiobarbituric acid reactive substances (TBARs), according to a slightly modified version of the extraction procedure described by Witte, Krause, and Bailey (1970).

Five grams of beef, to which BHT was added (0.2 mg mL^{-1}), were extracted with 45.5 mL of extraction solution containing 10% trichloroacetic acid (TCA) in 0.02 M orthophosphoric acid to a final volume of 50.0 mL. Samples were mixed in an Ultra-Turrax homogenizer for 30 seconds and then filtered through a nylon membrane filter (0.45 µm). Five mL of extracted solution and 5 mL of TBA solution (0.02 M) were added to a test tube, which was then sealed with a screw cap, homogenized on a vortex shaker and then incubated in an oven at 100 °C for 40 minutes. Finally, the samples were cooled at room temperature, and the absorbance was measured by spectrophotometer at 530 nm against a blank containing 5 mL of TCA solution and 5 mL of TBA reagent.

Malondialdehyde (MDA) is one of the largest relatively stable end products generated during secondary lipid oxidation. The concentration of MDA was calculated from the calibration curve obtained using 1,1,3,3-tetraethoxypropane (TEP), a precursor of MDA, at concentrations between 0 and 5 mg L^{-1}. Results were expressed as milligrams of malondialdehyde per kilogram of sample (mg MDA kg^{-1} of beef).

7.2.7. Statistical analysis

Data were analysed by analysis of variance (ANOVA) and significant differences were assessed by the Duncan's multiple range test at $P < 0.05$ using the IBM SPSS Statistics 20.0.0 statistical software package. Data are presented as the mean ± standard deviation.

7.3. Results and discussion

Initial screening was carried out to determine the potential of a natural extract obtained from the brewery waste stream as a natural antioxidant. This was done by use of an indirect method that determines the in vitro antioxidant ability of the antioxidant compounds and natural extracts as free radical scavengers of the radical DPPH. Thereafter, a direct method (TBARs) was used to evaluate the effect of the antioxidants to prevent the oxidative degradation of lipids with the consequent production of compounds such as conjugated hydroperoxides and aldehydes. A preliminary test was performed, prior to formulation of the active films, to evaluate the effectiveness of the natural extracts by adding different concentrations of each directly to the surface of the beef samples. A second test was carried out to evaluate how effective the active films were preventing lipid oxidation in beef samples packed with and without direct contact with active films.

7.3.1. Antioxidant properties of natural antioxidants

The capacity of natural extracts and synthetic antioxidants to scavenge free radicals was determined using the radical DPPH. Synthetic antioxidants commonly used in the food industry, BHT and PG, were used as reference compounds for comparative purposes. The commercial rosemary extract was used as a reference natural extract, since it has been extensively studied and its antioxidant effectiveness demonstrated by several authors (Erkan, Ayranci, & Ayranci, 2008; Romano, Abadi, Repetto, Vojnov, & Moreno, 2009). The results of the comparisons are shown in Table 7.3. The EC_{50} value is reciprocally related to the antioxidant activity of the tested sample.

Table 7.3. Free radical scavenging activity – DPPH

Antioxidant	**EC_{50} (g L^{-1})***
BHT	2.64±0.025[a]
PG	0.046±0.002[b]
Rosemary extract	2.10±0.013[c]
PVPP-WS extract	0.346 ±0.007[d]

* Values are means of three determinations. Different letters mean statistically significant differences at $P < 0.05$.

Natural extracts exhibited higher radical scavenging activity than the synthetic antioxidant BHT. The best results were obtained with PG (EC_{50} = 0.046 g L^{-1}), which is consistent with data obtained in other studies that have demonstrated the high antioxidant activity of PG (Martinez-Tome, Jimenez, Ruggieri, Frega, Strabbioli, & Murcia, 2001). The natural extract obtained from the brewery waste stream (PVPP-WS extract) exhibited a high level of antioxidant activity (EC_{50} = 0.346g L^{-1}), which was seven times higher than that of BHT and six times higher than that of the rosemary extract. The EC_{50} value was lower for the rosemary extract (2.10 g L^{-1}) than for BHT, which is consistent with previously reported data (Romano *et al*., 2009; Almela, Sánchez-Muñoz, Fernández-López, Roca, & Rabe, 2006). However it should also be noted that the commercial rosemary extract used in this study is a commercial extract that contains excipients and therefore is not a pure extract (see Table 7.1b).

7.3.2. Antioxidant effectiveness of natural products added directly to the surface of beef

The antioxidant effect of the different antioxidants was determined in beef samples by the TBARs method. The data obtained for the reference antioxidants (synthetic antioxidants and rosemary extract) applied at 2 different concentrations (low: 0.05% and high: 3%) and the PVPP-WS extract applied at 3 concentrations (low: 0.05%, medium: 0.5% and high: 3%) to the surface of beef samples, and also tested in the headspace of the containers, are shown in Table 7.4. Lipid oxidation of beef samples increased during the storage time at 4ºC in different degrees and the control test (without addition of antioxidant) yielded high TBARs values. All the antioxidants applied in this assay reduced lipid oxidation in beef relative to that in the control test. At the lowest concentration tested in the present study (0.05% (w/w) (L)), the highest effect was obtained with the PG, in which lipid oxidation was inhibited during the entire storage time (see Fig. 7.1a). The other antioxidants reduced the lipid oxidation relative to the control test in the follow order: PVPP-WS extract > rosemary extract > BHT, to a lower degree than PG. The PVPP-WS and rosemary extracts displayed a similar effect against lipid oxidation with a reduction around 40-50% with respect to the control test during the storage time. Similar results were obtained at the highest (3% (w/w) (H)) and lowest 0.05% (L) concentrations tested (see Fig. 7.1b). PG was the most active compound and had a slightly greater effect than that observed when applied at 0.05% (w/w) (L), with complete inhibition of lipid degradation. The reductions in the effects of BHT and rosemary extract were similar to those obtained when applied at the lower concentration. The results are consistent with data obtained by other authors in beef products, i.e. lower TBARs values obtained with the rosemary extract than with BHT at the same concentration (Bozkurt, & Belibağlı, 2008; McBride *et al*., 2007, Trindade, Mancini-Filho, & Villavicencio, 2010). However,

differences were greater with a natural extract of PVPP-WS, with an almost complete reduction in lipid oxidation, similar to PG.

We also evaluated the effect of the antioxidants in the scavenging of free radicals present in the headspace of the containers enclosing the meat samples, in tests with the highest concentration of antioxidant (3% (w/w) (H-VP)). The system inside the container was prepared so that the packaging film was not in direct contact with the meat sample. The results obtained are shown in Fig. 7.1c. No significant effect on lipid oxidation was observed in the beef samples. Rosemary has been reported to be a promising antioxidant extract with positive effects as a scavenger of free radicals in vapour phase in model systems (Nerín, Tovar, & Salafranca, 2008). It is possible that the concentration tested in the present study was not high enough in the vapour phase to have a positive effect in reducing lipid oxidation.

The effect of the PVPP-WS extract on the lipid oxidation of beef was tested at different concentrations with direct contact with the food sample (Fig. 7.1d.). The addition of the natural extract containing phenolic compounds enhanced the oxidative stability of beef when compared with the control test. The results show that higher concentrations of polyphenols had greater antioxidative effects. Samples in which the lowest concentration of antioxidant extracts (0.05%) was used are those in which lipid oxidation was least reduced, although decreases of around 40% were obtained. Lipid oxidation was inhibited in samples in which highest concentrations of antioxidant extract (0.5% and 3%) were used at the intermediate concentration (0.5%). The TBARs values were approximately 70% lower than in control samples, and at the high concentration (3%), the TBARs values were approximately 80% lower than in the control sample (all tested during cold storage).

Table 7.4. TBARs values obtained in beef samples spiked with natural and synthetic antioxidants, stored at 4 ºC during 17 days*

		TBARs (mg MDA kg^{-1} of beef)					
		Storage time (days)					
Antioxidant	**Concentration % (w/w)**	**0**	**3**	**7**	**10**	**14**	**17**
Control	0	0.280^{A}±0.021	5.37^{abB}±0.761	9.82^{aC}±0.085	12.4^{abCD}±0.182	13.9^{aD}±1.52	13.5^{abD}±0.421
BHT (L)	0.05	0.280^{A}±0.021	3.25^{deB}±0.240	6.23^{bC}±0.324	8.31^{deD}±0.635	10.2^{bcE}±0.487	12.5^{abcF}±0.062
BHT (H)	3	0.280^{A}±0.021	3.41^{deB}±0.053	8.57^{cC}±0.327	7.97^{deC}±0.859	11.4^{bD}±0.500	12.5^{abcD}±0.1.77
BHT (H-HS)	3 HS	0.280^{A}±0.021	4.75^{bB}±0.939	8.72^{cC}±0.461	11.1^{acD}±0.417	15.5^{deE}±0.684	12.6^{acD}±1.04
PG (L)	0.05	0.280^{A}±0.021	0.483^{gB}±0.113	0.560^{dB}±0.087	0.207^{gA}±0.011	1.33^{gD}±0.064	0.914^{dC}±0.004
PG (H)	3	0.280^{AB}±0.021	0.506^{gBC}±0.020	0.695^{dC}±0.090	0.190^{gA}±0.009	1.06^{gD}±0.239	0.716^{dC}±0.114
PG (H-HS)	3 HS	0.280^{A}±0.021	6.30^{cB}±0.324	9.29^{eC}±0.383	9.61^{cdC}±0.332	14.4^{aD}±1.03	15.8^{fE}±0.385
Rosemary extract (L)	0.05	0.280^{A}±0.021	3.62^{dB}±0.069	6.28^{bB}±0.025	7.03^{efC}±2.02	9.08^{cD}±0.539	9.36^{gD}±1.00
Rosemary extract (H)	3	0.280^{A}±0.021	2.73^{efB}±0.100	4.96^{fC}±0.444	6.54^{efC}±0.1.48	9.50^{bcD}±0.451	6.84^{hC}±0.311
Rosemary extract (H-HS)	3 HS	0.280^{A}±0.021	5.94^{acB}±0.131	11.0^{gC}±0.012	13.5^{bD}±0.415	18.3^{fE}±0.041	14.1^{bD}±0.364
PVPP-WS extract (L)	0.05	0.280^{A}±0.021	2.88^{defB}±0.018	5.36^{fC}±0.026	6.51^{efC}±0.007	8.84^{cD}±1.36	8.40^{gE}±0.065
PVPP-WS extract (M)	0.5	0.280^{A}±0.021	2.37^{fBC}±0.072	2.28^{hBD}±0.082	2.01^{gD} ±0.101	3.49^{hE}±0.075	2.59^{eC}±0.248
PVPP-WS extract (H)	3	0.280^{A}±0.021	2.41^{fB}±0.205	1.64^{lC}±0.145	1.36^{gC}±0.117	2.47^{ghB}±0.142	2.05^{deD}±0.042
PVPP-WS extract (M-HS)	0.5 HS	0.280^{A}±0.021	5.32^{abB}±0.076	10.2^{aC}±0.299	12.7^{abD}±0.338	16.8^{efE}±0.716	15.8^{fE}±0.897
PVPP-WS extract (H-HS)	3 HS	0.280^{A}±0.021	5.22^{abB}±0.281	10.1^{aC}±0.026	10.3^{cdCD}±1.25	15.7^{deE}±0.566	11.4^{cD}±0.258

* Mean values (n=3) followed by different lower case letters superindexes within the same time and capital letters superindexes within the same row denote statistically significant differences at $p < 0.05$.

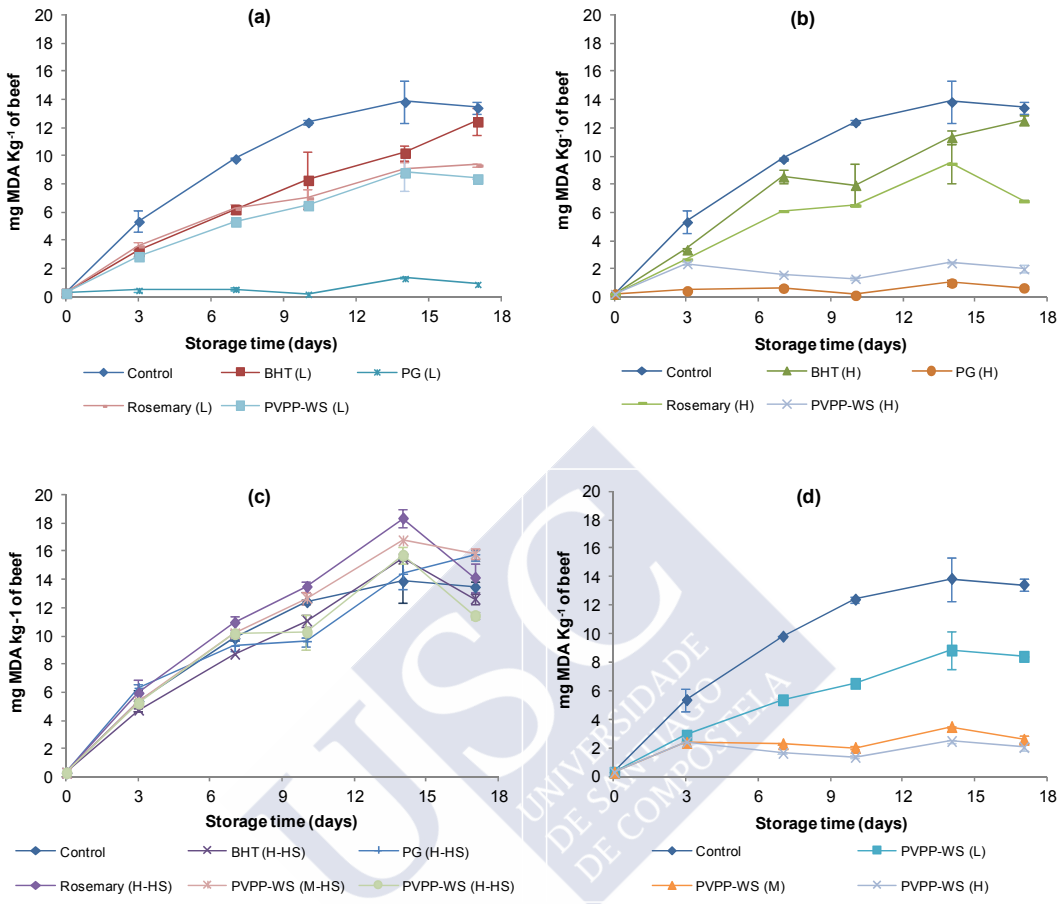

Figure 7.1. Effect of natural and synthetic antioxidants, added at different concentrations, on the lipid stability of beef stored at 4 ºC during 17 days: (a) low concentration (L: 0.05%) and (b) high concentration (H: 3%). (c) Effect of antioxidants in the headspace of the container. (d) Effect of different concentrations of PVPP-WS extract (L: 0.05%, M: 0.5% and H: 3%) in direct contact with sample and in the headspace of the container

7.3.3. Evaluation of antioxidant effect of the active films coated with natural extracts

When incorporated into the polymer, antioxidant compounds will not be directly available (as they were in the previous assay, in which the extracts were

added directly to the surface of the beef samples). In this test, films coated with 3, 10 and 20% of the natural extracts ($w_{extract}/w_{polymer}$) were evaluated. The effect of these films in the headspace of the container was again tested with films to which the highest concentration of extracts was added (20%). Because the main aim of this work was to develop active films containing natural antioxidants, to replace synthetic antioxidants commonly used as food additives, the antioxidant activity of the natural extracts (rosemary and PWPP-WS extracts) was tested. The antioxidant effect of active films was determined, using the TBARs method, as the capacity to inhibit lipid oxidation in beef samples during the storage. The results, expressed as mg MDA Kg^{-1} of sample, are shown in Table 7.5. Two control tests were performed, a test without film to evaluate the oxidation of beef (control without film), and a test of LDPE film without antioxidant extract added -to evaluate the effect of the film on the surface of the meat sample (control film). LDPE films spiked with rosemary extract at different concentrations (3, 10 and 20% (w/w) yielded films R 3%, R 10% and R 20% respectively (see Table 7.2). Film R 20% HS is the same as film R 20%, except that it is not in direct contact with the samples, exerting its effect in the headspace of the container. The same nomenclature (see Table 7.2) was used with the films incorporating the PVPP-WS extract at the same concentrations (PVPP-WS 3%, 10%, 20% and 20% VP). All the active films tested exhibited an effect that protects the beef from oxidation (see Fig. 7.2). The rosemary extract-coated active films (Fig. 7.2a) in contact with meat samples yielded a reduction of approximately 50% in the TBARs values. The results obtained with the films coated with the PVPP-WS extract (see Fig. 7.2b) showed that higher concentrations of this natural extract exert higher antioxidative effects. The PVPP-WS extract was more effect in reducing lipid oxidation than the rosemary extract. This effect was more evident at the highest concentration applied (20%), which delayed the onset of oxidation until the ninth day, after which slight oxidation was observed, but which was much lower than that observed in the other samples under study, with a reduction of 80% respect to the control test

without film. The active films in direct contact with the meat samples are shown in Fig. 7.2c. The greatest effect was obtained with the PVPP-WS 20% film. However, all the active films substantially reduced lipid oxidation in beef. The control film in contact with the surface reduces the lipid oxidation because less sample surface is in contact with the oxygen present in the vapour phase of the container (Fig. 7.2c) when compared with the control without film. This may explain the effect of the active films in the headspace of the container (Fig. 7.2d). The natural extracts, particularly rosemary, had some effect as scavengers of oxygen and free radicals responsible for the oxidation process. This is consistent with previous findings (Nerín *et al*., 2008). The PVPP-WS extract was also effective, although to a lesser extent. However, inhibition of oxidation was less than that obtained with a simple polymer in close contact with the sample without active compounds. It appears that close contact between the active film and the meat sample is required for a large reduction in lipid oxidation in meat samples.

The active films developed in the present study have proven to have an inhibitory effect on the lipid oxidation process comparable to that observed after direct addition of the antioxidants to the meat sample (see section 7.3.2). The results obtained with the PVPP-WS 20% film (produced with the PVPP-WS extract) showed the effectiveness of the natural extract obtained in the brewery industry and confirm the possible use of the extract as a food antioxidant, as recognised for rosemary extract (Bozkurt *et al*., 2008; Trindade *et al*., 2010; Fernandez-Lopez *et al*., 2005). The results obtained with the food samples are consistent with those obtained with the DPPH method (see section 7.3.1), which confirms that these natural extracts display a radical scavenging capacity and that this may be one of the mechanisms of action whereby the extracts reduce lipid oxidation in meat samples.

Table 7.5. TBARs values obtained in beef samples packed with different active films and with both controls, stored at 4 ºC during 16 days

		TBARs (mg MDA kg^{-1} of beef)					
		Storage time (days)					
Film name	Film description	0	3	6	9	13	16
Without film	Control without film	0.576^{A}±0.077	6.01^{aB}±0.602	8.13^{aC}±0.352	10.7^{aD}±0.589	13.0^{aE}±1.11	12.1^{aF}±0.366
Control	Control film	0.576^{A}±0.077	3.50^{cB}±0.054	5.85^{bBC}±0.389	6.64^{bCD}±0.61	7.96^{bD}±0.41	9.25^{bD}±1.25
R 3%	Rosemary 3%	0.576^{A}±0.077	1.94^{dB}±0.152	2.44^{cC}±0.273	4.21^{cD}±0.271	5.51^{cE}±0.400	5.97^{cF}±0.761
R 10%	Rosemary 10%	0.576^{A}±0.077	0.941^{deA}±0.456	1.72^{cdB}±0.111	3.98^{cC}±1.38	4.78^{cD}±1.41	5.61^{cE}±0.351
R 20%	Rosemary 20%	0.576^{A}±0.077	1.05^{deA}±0.041	1.10^{cdA}±1.02	3.62^{cB}±0.602	4.92^{cC}±1.49	6.70^{cD}±0.238
R 20% VP	Rosemary 20% HS	0.576^{A}±0.077	4.36^{bcB}±0.844	7.03^{abC}±1.58	7.60^{bC}±0.127	11.0^{dD}±1.10	12.3^{aD}±2.20
PVPP-WS 3%	PVPP-WS 3%	0.576^{A}±0.077	1.45^{deAB}±0.022	2.20^{cB}±0.220	4.15^{cC}±0.831	6.04^{cD}±0.688	6.43^{cD}±0.691
PVPP-WS 10%	PVPP-WS 10%	0.576^{A}±0.077	0.635^{eA}±0.024	1.42^{cdA}±0.511	4.09^{cB}±0.177	4.85^{cB}±0.580	5.35^{cB}±0.953
PVPP-WS 20%	PVPP-WS 20%	0.576^{A}±0.077	0.590^{eA}±0.017	0.630^{dA}±0.024	0.747^{dA}±0.294	2.48^{eB}±0.634	2.83^{dB}±0.550
PVPP-WS 20% VP	PVPP-WS 20% HS	0.576^{A}±0.077	4.94^{abB}±0.310	7.87^{aC}±0.479	11.0^{aD}±0.020	11.0^{dD}±0.758	11.3^{abD}±0.895

* Mean values (n=3) followed by different lower case letters superindexes within the same time and capital letters superindexes within the same row denote statistically significant differences at $P < 0.05$.

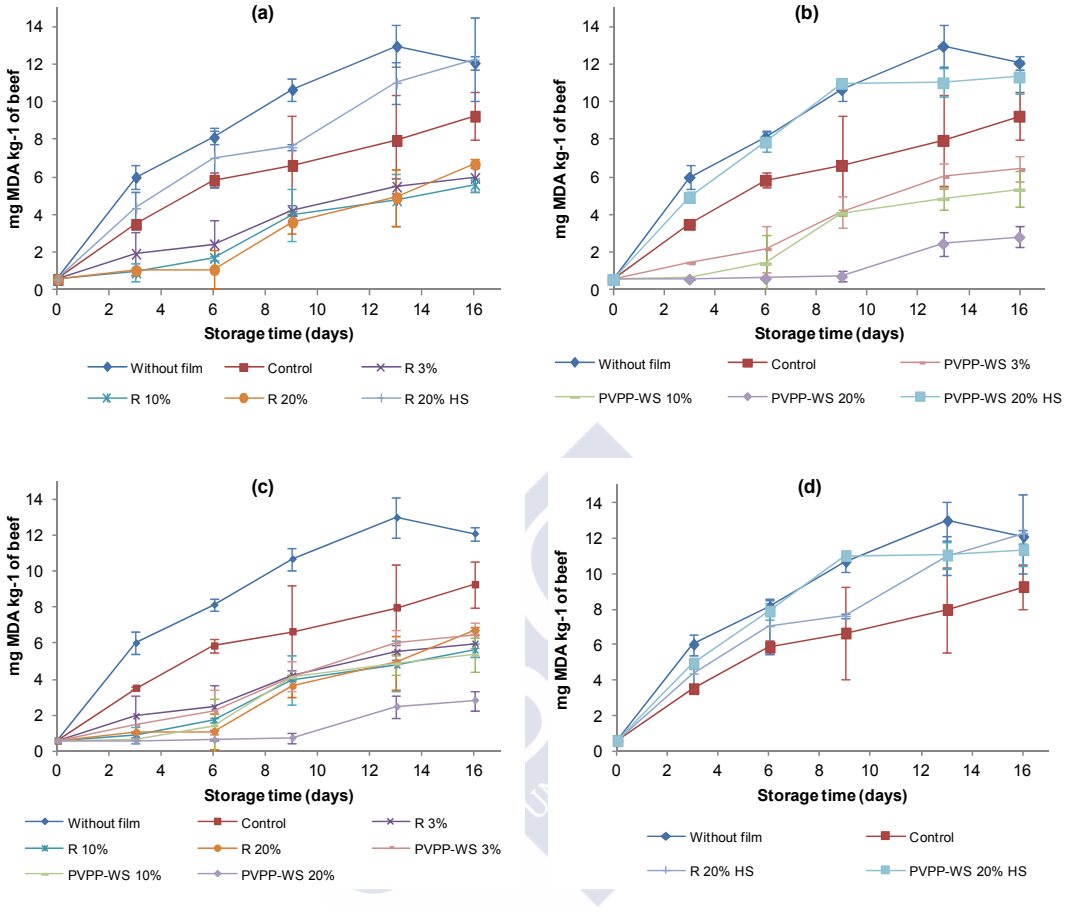

Figure 7.2. Lipid oxidation in beef packed with active films coated with different concentrations of natural extracts: (a) rosemary extract, (b) PVPP-WS extract, (c) in direct contact with food, and (d) in the headspace of the container (i.e. contact via the vapour phase), during cold storage

7.4. Conclusions

The natural extracts tested displayed a higher degree of radical scavenging activity than BHT, a synthetic antioxidant commonly used in food industry. Natural extracts proved to be effective antioxidants and reduced lipid oxidation when added directly to the surface of refrigerated beef samples. Therefore, it may be possible to use these extracts to replace synthetic antioxidants. PVPP-WS extract displays a similar antioxidant effect to PG. Active films containing natural extracts were successfully produced, and an assay was performed to evaluate the effect in extending the shelf life of beef during cold storage. The use of active films coated with natural antioxidants enhanced oxidative stability of beef relative to that obtained with the film control. The greatest differences were observed in samples with which films containing the highest concentration of extract were used. The best results were obtained with the natural extract obtained from the brewery residual stream (PVPP-WS), which reduced lipid oxidation by up to 80%. These active films appear promising for the development of an active antioxidant packaging for use with meat products in the food industry.

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8

Development of new active packaging films containing bioactive nanocomposites

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Abstract

The aim of this study was to develop and evaluate the effectiveness of active packaging films produced by extrusion with a natural extract obtained from a brewery waste product. The thermal stability of the natural extract, which contains phenolic compounds, was first established. The extract was then incorporated into ethylene vinyl acetate (EVA) and low density polyethylene (LDPE) films. Migration, antimicrobial activity and lipid oxidation tests showed that EVA film was the most suitable for incorporating the natural extract and protected beef samples from oxidation better than the control film. Finally, EVA film was spiked with different concentrations of the natural extract (3% and 6% (w/w)) or functionalized nanoclays prepared using the natural extract (3%, 6% and 9% (w/w)). The films spiked with the highest concentrations of extract or functionalized nanoclays provided the best results and reduced the oxidation of beef samples by around 50% or more, relative to control films. The active films developed in the present study show promise for use in the food industry.

Keywords: Active packaging films, bioactive compounds, antimicrobial activity, antioxidant activity, functionalized nanoclays.

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8.1. Introduction

Spoilage of fresh beef is mainly characterized by microbial activity and by the oxidation of lipids and pigments (Sun, Holley, 2012). Lipid oxidation, which is one of the main causes of deterioration of meat quality during refrigerated storage, reduces the stability and acceptability of food and is often a decisive factor in determining the shelf life of food (Gray, Gomaa, & Buckley, 1996). Reducing the oxidation process is an important challenge to food producers and, indeed, everyone involved in the entire food chain *from the farm to the fork*.

The shelf life of fresh meat can be extended by using antioxidants, antimicrobials and appropriate packaging materials (Zhou, Xu, & Liu, 2010). One of the aims of food packaging is to protect the product from harmful environmental factors, by acting as an inert barrier (Vermeiren, Devlieghere, van Beest, de Kruijf, & Debe- vere, 1999). Active packaging is currently one of the most dynamic technologies used to preserve the quality, safety and sensory properties of food. The active packaging interacts positively with product and environment to improve/preserve the quality of food longer than conventional packaging (Kerry, O'Grady, & Hogan, 2006). Some types of active packaging allow the controlled release of bioactive substances (antimicrobials or antioxidants that have previously been added to the package), thus avoiding the direct addition of the active agents to the food product (Lee, 2010). Most studies in this field have concerned active packaging developed with antimicrobial agents, O2 scavengers and moisture-control systems. Antimicrobial agents can be directly incorporated into packaging films and have been extensively studied for use with meat products (Coma, 2008; Zhou, Xu, & Liu, 2010).

Many research studies in recent years have focused on developing active packaging containing natural antioxidants. Thus, some authors have incorporated antioxidants such as BHT, BHA, alpha-tocopherol and natural extracts into packaging films and assessed how these migrate and retard the oxidative process of foods during storage (Moore, Han, Acton, Ogale, Barmore, & Dawson, 2000; Barbosa-Pereira *et al*., 2013c). Moore *et al*. (2003) concluded that the incorporation of active substances into packaging may be more effective than the direct use of additives on the meat surface.

Growing concern about the potential health hazards caused by synthetic additives has led to renewed interest in the use of naturally occurring antioxidants/antimicrobials (Shahidi, & Zhong, 2010).

Active packaging systems have been developed by using natural extracts such as rosemary, oregano and green tea to increase the oxidative stability of different meat products and thus to extend their shelf life (Nerín *et al*., 2006; Camo, Lorés, Djenane, Beltrán, & Roncalés, 2011, Siripatrawan, & Noipha, 2012).

Waste products from fruit and vegetable processing provide a practical and economic source of potent antioxidants/antimicrobials that could replace synthetic preservatives (Balasundram, Sundram, & Samman, 2006, Moure, A., Cruz, J. M., Franco, D., Domínguez, J.M., Sineiro, J., Dominguez, H., *et al*., 2001).

In the present study, a natural extract obtained from a brewery waste stream (PVPP-WS extract) was used to develop active packaging film. This natural extract has a high content of phenolic compounds such as flavonols (catechin, gallocatechin and epigallocatechin) and hydroxycinnamic and hydroxybenzoic acids (gallic acid, caffeic acid, p-coumaric acid and ferulic acid). These compounds confer the extract with a high level of free radical scavenging activity as they act as free radical acceptors and chain breakers (Barbosa-Pereira, Angulo, Paseiro-Losada, & Cruz, 2013a, Barbosa-Pereira, Pocheville, Angulo,

Paseiro-Losada, & Cruz, 2013b). We have also confirmed the antimicrobial activity of the PVPP-WS extract against both Gram-positive and Gram-negative bacteria (Barbosa-Pereira *et al*., 2013b). The antimicrobial activity of different natural extracts and essential oils that contain phenolic compounds has been demonstrated in several studies. (Burt, 2004, Cushnie, Lamb, 2005; Moreno, Scheyer, Romano, Vojnov, 2006, Tajkarimi, Ibrahim, & Cliver, 2010, Holley, & Patel, 2005, Puupponen-Pimiä *et al*., 2001, Rauha *et al*., 2000).

Active packaging films have been produced with natural extracts with both antimicrobial and antioxidant properties (Siripatrawan, & Noipha, 2012, Catalayud, López-de-Dicastillo, López-Carballo, Vélez, Muñoz, & Gavara, 2013).

Recent studies have focused on the use of nanocomposites in developing new types of active packaging (Silvestre, Duraccio, & Cimmino, 2011; Bradley, Castle, & Chaudhry, 2011). Abdollahi *et al*. developed an active bionanocomposite film with a synergic effect between nanoclays and natural extracts with antioxidant and antimicrobial activities (Abdollahi, Rezaei, & Farzi, 2012). In the present study, functionalized nanoclays were prepared using the natural extract obtained from a brewery waste stream and incorporated into a polymer film to develop an active packaging film.

The main aims of this study were to develop an active packaging film with both antioxidant and antimicrobial properties and to evaluate the effect of these films on the stability of beef during refrigeration. For this purpose, the suitability of the natural extract for incorporation into a polymeric matrix by extrusion was assessed. The suitability of the polymeric matrix was also evaluated, as well as the optimal concentration of the natural extract and the overall migration and antimicrobial activity of the active films. Finally, the antioxidant effect of active packaging films, spiked with natural extract and with the functionalized nanoclays, was evaluated on refrigerated samples of beef packed in the films.

8.2. Materials and methods

8.2.1. Chemicals

Butylated hydroxytoluene (BHT) (99.0%); 2(3)-tert-butyl-4-hydroxyanisole (BHA) (98%); sodium azide (99.0%); 2-thiobarbituric acid (TBA) (≥98%) and trichloroacetic acid (TCA) (puriss. p.a. 99.5%) were purchased from Sigma-Aldrich (Steinheim, Germany). 1,1,3,3-tetraethoxypropane (TEP) (purum ≥95% (GC)), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Denis reagent (purum) and gallic acid were supplied by Fluka Chemie AG (Buchs, Switzerland). Anhydrous sodium carbonate (99.9%), orthophosphoric acid (85% GR for analysis), methanol (GC≥99.9%), absolute isooctane and absolute ethanol were provided by Merck (Darmstadt, Germany). Ultrapure water was prepared in a Milli-Q filter system (Millipore, Bedford, MA, USA).

8.2.2. The natural extract

PVPP-WS extract: the extract containing natural antioxidants was obtained from a residual stream generated during a brewery cleaning process. The waste polyvinylpolypyrrolidone washing solution (PVPP-WS) was acidified to pH 1.5 with HCl (37%), and polyphenolic compounds were extracted with ethyl acetate by stirring for 30 minutes at room temperature. Organic and aqueous phases were separated by decantation and the organic phase was collected and evaporated to dryness at 40 ºC. Residual water was removed from the extract by lyophilisation to obtain a dry crude extract. The PVPP-WS extract was obtained on an industrial scale and characterized in previous studies (Barbosa-Pereira *et al*., 2013a; Barbosa-Pereira *et al*., 2013d).

8.2.3. Evaluation of the suitability of the natural extract for incorporation into food packaging films

8.2.3.1. Thermal stability

Experiments were carried out under an oxidizing atmosphere in an oven at two different temperatures, 100 and 200 ºC, for 120 minutes, to determine the thermal stability of the natural extract containing antioxidant compounds. Samples of the natural extract (approx. 0.20 g) were collected at different times (5, 10, 15, 30, 60 and 120 min) and cooled at room temperature in a desiccator before gravimetric determination of the non-volatile fraction. The thermooxidative stability of the natural extract samples was assessed by measuring the phenolic content and antioxidant activity.

8.2.3.2. Total phenolic content – Folin-Denis

The total phenolic content of the natural extract samples was determined spectrophotometrically, by the Folin-Denis method, according to Waterman and Mole (1994).

An aliquot (100 µL) of sample solution was mixed with 8.4 mL of ultrapure water in a test tube, and 0.5 mL of Folin-Denis reagent was added. After 1 min, 1 mL Na_2CO_3 solution (10%) was added and the solution was allowed to stand for 30 min at room temperature. Absorbance of the resulting blue complex was measured at 760 nm in a dual-beam spectrophotometer (Uvikon XL, Bio-Tek Instruments, Milan, Italy). All determinations were made in triplicate. The total phenolic content of the samples was determined by comparison against the standard curve of commercial gallic acid, and expressed as gallic acid equivalents (GAE).

8.2.3.3. Radical scavenging activity – DPPH

The antioxidant activity of the natural extract was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method described by von Gadow, Joubert, and Hansmann (1997), with slight modifications.

Standard solutions of the natural extract samples were collected at different times and prepared in methanol. Two synthetic compounds with antioxidant properties and commonly used in the food industry, BHA and BHT, were used as controls. An aliquot of each antioxidant (50 µL) was added to 2 mL of DPPH radical methanolic solution (3.6×10^{-5} M), shaken vigorously in a vortex and left to stand in the dark at room temperature. The absorbance was measured at 515 nm in a spectrophotometer, after 16 min at room temperature. All determinations were made in triplicate. The decrease in absorbance was converted to percentage of DPPH inhibition (PI), according to the following equation:

$$\mathrm{IP} = \frac{\mathrm{A}_0 - \mathrm{A}_{16}}{\mathrm{A}_0} \times 100$$

where,

A_0 is the absorbance of the control at initial time;

A_{16} is the absorbance of the sample at 16 minutes.

The concentration of antioxidant compound or natural extract required to yield 50% inhibition of the radical DPPH (equivalent concentration=EC_{50}) was determined from the linear regression curve data obtained from different concentrations of antioxidant (within the range 0.1 to 5 g L^{-1}) against the inhibition percentage of DPPH free radical (IP %).

8.2.4. Nanocomposites

Functionalized montmorillonites were prepared by combining untreated nanoclays provided by Nanobiomatters S.L. (Paterna, Spain) and 20% of PVPP-WS extract (MMT + 20% PVPP-WS). The natural extract incorporated in the functionalized nanoclays represented 20% (w/w) of the weight of the nanomaterial, so that the content of PVPP-WS extract used to produce the films minimized consumption of natural extract.

8.2.5. Polymer formulations

Several formulations of the PVPP-WS natural extract with different polymeric matrices were prepared by extrusion. Active films with different contents (w/w) of natural extract were processed, to study the antioxidant effectiveness of the polymer formulations with meat samples.

The matrices used were low density polyethylene (LDPE), ALCUDIA 2008F, provided by Repsol YPF; and ethylene vinyl acetate copolymer (EVA) 25% (w/w) vinyl acetate, ELVAX®3182, provided by Dupont.

The nanocomposites (functionalized nanoclays) were also incorporated into EVA films. The formulations are shown in Table 8.1.

The polymers with active extract were processed in a Microcompounder (Haake Minilab) with a co-rotating twin-screw extruder. The mixtures were processed at a speed of 150 rpm at 150 ºC for 6 minutes. To obtain the extruded films, the Microcompounder premix material was pelletized and then fed into a Polylab rheometer at a mass temperature of 160 ºC. The thickness of the extruded films was approximately 100 µm.

Table 8.1. Film formulations

Formulation	Polymer	PVPP-WS	PVPP-WS nanoclay[a]
$LDPE_C$	LDPE	-	-
$LDPE_{A1}$	LDPE	3%	-
EVA_C	EVA	-	-
EVA_{A1}	EVA	3%	-
EVA_{A2}	EVA	6%	-
EVA $Nano_C$	EVA	-	-
EVA $Nano_{A1}$	EVA	-	3%
EVA $Nano_{A2}$	EVA	-	6%
EVA $Nano_{A3}$	EVA	-	9%

[a]The films were formulated with functionalized nanoclays prepared with 20% of PVPP-WS extract, so that the amount of natural extract used to produce these films is lower than in the films to which the extract was directly added.

8.2.6. Overall migration test

In a first screening test to evaluate the most suitable polymer for developing the active packaging, the overall migration of LDPE and EVA films (control and spiked at 3% in natural extract) were tested to determine the total amount of non-volatile substances that could migrate into foodstuffs from active films, according to CEN standard EN 1186-14:2003. The test was performed at 40 °C for 10 days in 95% (v/v) ethanol, and at 20 °C for 2 days in isooctane to simulate room temperature storage (more stricter storage conditions than in reality). Exposure was by total immersion (both sides in contact), although in practice only one side of the film will be in contact with food. Samples of film were cut to produce specimens of 1 dm^2 and immersed in a glass tube with 100 mL of food simulants.

The final polymer formulations spiked at highest concentrations were tested with three food simulants: distilled water, 95% ethanol (v/v) and isooctane (alternative simulants to D2), in contact with one side of the material (CEN standard EN 1186-5:2002, CEN standard EN 1186-14:2003). As the most suitable simulants for meat are water and oil, and considering that beef is stored under refrigeration, the distilled water and 95% ethanol (v/v) samples were kept in a controlled chamber at 20 ºC for 10 days in, while isooctane samples in were tested at 20 °C for 1 day.

At the end of the incubation period, the films were removed and the simulants were evaporated to dryness. The residue was weighed with an analytical balance (Mettler AE260) (± 0.1 mg precision) to determine the overall migration value in mg dm^{-2} of polymer. Three determinations were made with each sample, and the final migration value was calculated as the average of the three determinations.

8.2.7. Evaluation of antimicrobial activity of the films

The antimicrobial activity of the active films was assessed by the ASTM E 2149-01 standard test method for determining the Antimicrobial Activity of Immobilized Antimicrobial Agents Under Dynamic Contact conditions (E2149-01, 2002). The test was carried out to evaluate (quantitatively) the antimicrobial effectiveness of the active films spiked with natural extract against selected spoilage microorganisms. Initially, LDPE and EVA films spiked with PVPP-WS extract (active additive) were analysed against Staphylococcus aureus CECT 239, Escherichia coli ATCC 8739, Salmonella spp CECT 443, Listeria monocytogenes CECT 935; Escherichia coli serotype O157:H7 (non-toxigenic) CECT 4972, and Total Aerobic Mesophilic flora isolated from minced meat. LDPE and EVA films without additives were tested as controls. The EVA films (control and spiked with 3% and 6% PVPP-WS extract) were then tested against microorganisms that were

most susceptible to the natural extract (Staphylococcus aureus CECT 239 and Escherichia coli ATCC 8739). EVA NANO films (control and spiked with 6% and 9% functionalized nanoclays) were also evaluated against Staphylococcus aureus CECT 239.

8.2.8. Evaluation of antioxidant effectiveness of the films with food samples

8.2.8.1. Beef samples

Freshly cut beef, of thickness 1 cm, was purchased in a local retail store. The meat was divided in small pieces of 6±1 g and a small amount of sodium azide (approx. 1mg) was added to the surface of the meat, to prevent microbial spoilage during storage.

8.2.8.2. Evaluation of antioxidant effectiveness of the active packaging films

Samples of meat were packaged in individual bags prepared with all film formulations shown in Table 8.1. In the first test, the following were evaluated: control LDPE and LDPE spiked with 3% of PVPP-WS extract ($LDPE_{C}$, $LDPE_{A1}$, respectively); control EVA and EVA spiked with 3 and 6% of PVPP-WS extract (EVA_{C}, EVA_{A1} and EVA_{A2}, respectively); and EVA film spiked with 3% of the functionalized nanoclays (EVA $NANO_{A1}$). The packaged beef samples were stored at 4 ºC for 14 days. Analysis for 2-thiobarbituric acid reactive substances (TBARs test) was carried out after 3, 6, 9 and 14 days of storage. In a second assay, EVA films spiked with 6% and 9% of the functionalized nanoclays (EVA $NANO_{A2}$ and EVA $NANO_{A3}$, respectively) were tested against the control film (EVA $NANO_{C}$). Beef samples were removed after 3, 6, 9 and 13 days for TBARs determination.

8.2.8.3. Lipid oxidation analysis – TBARs

Lipid oxidation of beef meat was determined by the TBARs test, according to the extraction procedure described by Witte, Krause, and Bailey (1970), with slight modifications.

Five grams of beef, to which BHT was added (0.2 mg mL^{-1}), were extracted with 45.5 mL of extraction solution containing 10% trichloroacetic acid (TCA) in 0.02 M orthophosphoric acid to a final volume of 50.0 mL. The samples were mixed in an Ultra-Turrax homogenizer 30 seconds and then filtered through 0.45 µm nylon membrane filters. Five mL of extracted solution and 5 mL of TBA solution (0.02 M) were placed in a test tube with a screw cap, homogenized by vortex, and then incubated in an oven at 100 °C for 40 minutes. Finally, the samples were cooled at room temperature, and the absorbance was measured by spectrophotometer at 530 nm against a blank containing 5 mL of TCA solution and 5 mL of TBA reagent.

The concentration of malondialdehyde (MDA) was calculated from the calibration curve obtained using 1,1,3,3-tetraethoxypropane (TEP) (a precursor of MDA), as standard, in a concentration range between 0 and 5 mg L^{-1}. Results were expressed as milligrams of malondialdehyde per kilogram of sample (MDA mg kg^{-1} of beef).

8.2.9. Statistical analysis

Data were analysed by analysis of variance (ANOVA), and significant differences were assessed by the Duncan's multiple range test (at $p < 0.05$), by using the IBM SPSS Statistics 20.0.0 statistical software package. Data are presented as means ± standard deviations.

8.3. Results and discussion

8.3.1. Thermal stability and antioxidant activity of natural extract

The thermal stability of the natural extract was tested to assess whether the antioxidant compounds underwent thermal degradation at the temperature at which the polymers (LDPE and EVA) were processed (Fig. 8.1.). Moreover, the stability to high temperatures was also confirmed by determining the capacity of the natural extract to scavenge free radicals by DPPH method of each collected sample. The results obtained for each sample, expressed as EC_{50}, are shown in Table 8.2.

Fig. 8.1 shows the total phenolic contents and the weight loss of the natural extract exposed to different temperatures for various times. Before the exposure to high temperatures, phenolic compounds constituted 61.1% of the natural extract. The percentage weight loss in the natural extract was around 8.9% (w/w) after exposure to a temperature of 100 ºC for 120 min, and the percentage of phenolic compounds in the natural extract was 5.1% lower than in the initial extract (see Fig. 8.1a). However, there was no loss of antioxidant activity by the natural extract. The EC_{50} value of each sample of natural extract obtained at different times during the assay indicated that the antioxidant activity of the natural extract remained constant (see Table 8.2). The weight reduction after exposure to a temperature of 200 ºC for 120 min was 23.1% (w/w); the total phenolic content was around 60% in all samples (see Fig. 8.1b) and the antioxidant activity increased (see Table 8.2). These results can be attributed to the presence of compounds with low or even no antioxidant activity in the volatile fraction of the extract, so that there is a relative increase in the concentration of the active compounds resulting in more active extracts.

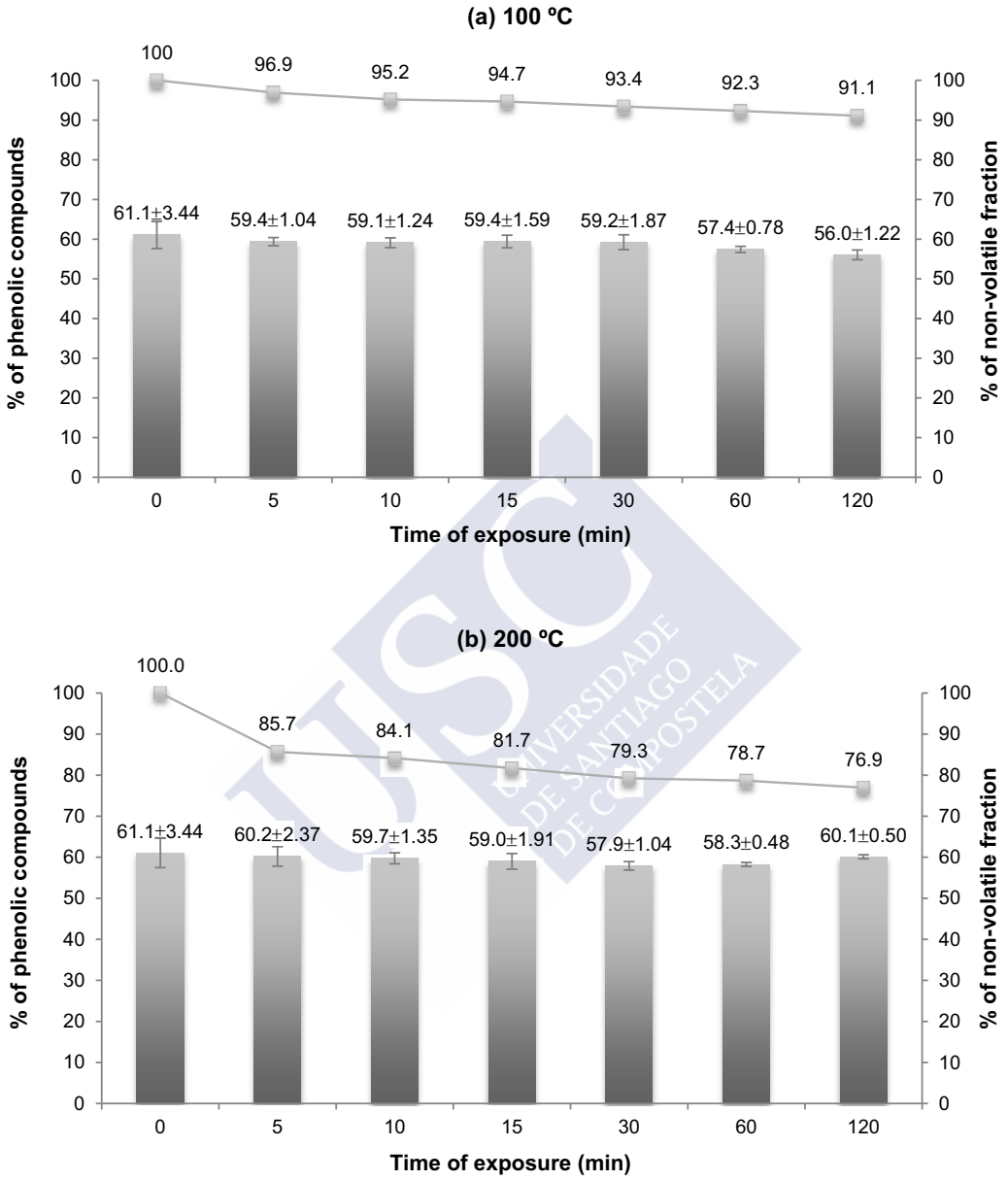

Figure 8.1. Thermal stability of the natural extract exposed at temperatures of 100 ºC (a) and 200 ºC (b) for 120 minutes. Evaluation of the total phenolic contents determined by Folin-Denis method (left axis) (represented by bars) and the percentage of non-volatile fraction determined gravimetrically (right) throughout the exposure time (represented by straight lines)

Table 8.2. Antioxidant activity, determined by the DPPH method, of the natural extract exposed to different temperatures. Results are expressed as EC_{50} (g L^{-1})

Time (min)	EC_{50} (g L^{-1})*	
	100 ºC	200 ºC
0	0.316^{a}±0.006	0.355^{a}±0.018
5	0.344^{a}±0.018	0.254^{a}±0.022
10	0.316^{a}±0.070	0.335^{a}±0.017
15	0.304^{a}±0.016	0.299^{a}±0.004
30	0.292^{a}±0.017	0.245^{a}±0.010
60	0.371^{a}±0.049	0.215^{a}±0.006
120	0.307^{a}±0.011	0.199^{a}±0.008
BHA	0.259^{a}±0.056	
BHT	2.497^{b}±0.192	

*Values are means of three determinations. Different letters indicate significant differences ($p < 0.05$).

The results obtained in the present study are consistent with previous findings. Cruz *et al*. (2007) showed that temperature has only a slight influence on the phenolic content and antioxidant activity of natural extracts obtained from industrial wastes, which showed a greater ability than synthetic antioxidants (BHT and BHA) to retain antioxidant activity after heating. These authors also suggested that the compounds resulting from the thermal degradation may be more active than those in the initial extracts (Cruz, Conde, Domínguez, & Parajó, 2007). Taking into account that LDPE and EVA extrusion is carried out at 150 ºC for 6 min, the natural extract can be blended with the polymer (at 160 ºC) with only a slight loss of active compounds and no loss of the antioxidant capacity.

8.3.2. Overall migration

After testing the thermal stability, the PVPP-WS extract was used to produce different film formulations (see Table 8.1.) by extrusion in LDPE and EVA polymers. These films were used in the overall migration assay.

Overall migration tests with food simulants were carried out to determine the total amount of non-volatile substances that might migrate into foodstuff from active films. The overall migration levels from LDPE and EVA films (control films and films spiked at 3% with PVPP-WS extract) in 95% ethanol (v/v) and isooctane are shown in Table 8.3a. The first screening test was performed at room temperature (i.e. unrealistic storage conditions) to evaluate how the natural extract is released from polymers spiked with the PVPP-WS extract. The EVA polymeric matrix proved most suitable for the active packaging because it released higher concentrations of the active compounds (i.e. the overall migration of the active films spiked with PVPP-WS extract was higher than from the control and $LDPE_{A1}$ films). This is important because the films must release the active compounds to the food for the positive benefits to be obtained.

In the second part of the study, overall migration tests were carried out with food simulants (meat products) to determine the total amount of non-volatile substances that migrate into foodstuff by single side contact under refrigeration (i.e. realistic storage conditions). In this assay, the EVA films samples spiked with the highest concentrations of PVPP-WS extract (EVA_{A2}) and functionalized nanoclays (EVA $NANO_{A3}$) were evaluated (Table 8.3b).

Table 8.3a. Overall migration data in 95% ethanol (v/v) and isooctane for LDPE and EVA films (total immersion)

Packaging sample	Packaging material	Natural extract (% PVPP-WS)	Food simulant	Time (days)	Temperature (º C)	Migration (mg dm^{-2})*
$LDPE_C$	LDPE	0	95% ethanol	10	40	0.8^{a}±0.3
$LDPE_{A1}$	LDPE	3	95% ethanol	10	40	1.9^{a}±0.1
$LDPE_C$	LDPE	0	Isooctane	2	20	4.6^{a}±0.4
$LDPE_{A1}$	LDPE	3	Isooctane	2	20	3.0^{a}±0.5
EVA_C	EVA	0	95% ethanol	10	40	4.5^{a}±0.2
EVA_{A1}	EVA	3	95% ethanol	10	40	12.6^{b}±0.7
EVA_C	EVA	0	Isooctane	2	20	22.1^{c}±0.7
EVA_{A1}	EVA	3	Isooctane	2	20	25.5^{c}±0.4

* Values are means of three determinations. Different letters indicate significant differences ($p < 0.05$).

Table 8.3b. Overall migration from EVA active films in water, 95% ethanol (v/v) and isooctane (single face contact)

Packaging sample	Packaging material	Natural extract (%PVPP-WS)	PVPP-WS Nanoclays (%)	Food simulant	Time (days)	Temperature (º C)	Migration (mg dm^{-2})*
EVA_C	EVA	-	-	Distilled Water	10	20	0.10^{a}±0.10
EVA_{A2}	EVA	6	-	Distilled Water	10	20	0.87^{a}±0.51
EVA $NANO_{A3}$	EVA	-	9	Distilled Water	10	20	0.07^{a}±0.06
EVA_C	EVA	-	-	95% ethanol	10	20	2.27^{ab}±1.02
EVA_{A2}	EVA	6	-	95% ethanol	10	20	5.30^{c}±0.62
EVA $NANO_{A3}$	EVA	-	9	95% ethanol	10	20	4.23^{bc}±0.38
EVA_C	EVA	-	-	Isooctane	1	20	4.20^{bc}±0.35
EVA_{A2}	EVA	6	-	Isooctane	1	20	5.07^{c}±1.01
EVA $NANO_{A3}$	EVA	-	9	Isooctane	1	20	4.07^{bc}±0.61

* Values are means of three determinations. Different letters indicate significant differences ($p < 0.05$).

The EVA active films tested comply with the limits established by current legislation with respect to the overall migration values, which are all lower than 10 mg dm^2 (Commission Regulation (EU) No. 10/2011). The overall migration was higher when the active films were in contact with 95% ethanol and isooctane (fat simulants) than when in contact with distilled water. When in contact with 95% ethanol, migration from EVA active films containing i) natural extract at the highest concentration tested (EVA_{A2}) and ii) functionalized nanoclays (EVA $NANO_{A3}$) was twice as high as from the control film (EVA_{C}). The differences were not as evident when the films were in contact with isooctane. The overall migration from EVA $NANO_{A3}$ was lower because the total concentration in PVPP-WS extract is lower and probably also because the natural extract that when being functionalized in the nanoclay has more difficulty to migrate.

8.3.3. Antimicrobial activity of the active films

In the present study, the antimicrobial activity of the different active films spiked with PVPP-WS natural extract against selected spoilage microorganisms was evaluated and compared with that of control films. The LDPE and EVA films (controls and active films spiked with 3% natural extract: $LDPE_C$, $LDPE_{A1}$, EVA_{C} and EVA_{A1}) were tested against different microorganisms (see section 8.2.7). The LDPE active films did not display antimicrobial activity (data not shown), and the EVA active film (EVA_{A1}) was the only film with antimicrobial activity (see Table 8.4.). EVA again proved to be the most effective polymer in the active packaging. EVA films (control and spiked with 3% and 6% PVPP-WS extract) were tested against microorganisms that are most susceptible to the natural extract (Staphylococcus aureus CECT 239 and Escherichia coli ATCC 8739) (Table 8.4).

Table 8.4. Antimicrobial activity of the different active films, spiked with PVPP-WS extract or functionalized nanoclays, against *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* CECT 239, during storage for 6 days

Film sample	Population (log CFU ml^{-1})*				Cell death (%)	Cell death (%)	Cell death (%)
	0 h	24 h	48 h	6 days	24 h	48 h	6 days
EVA_C							
E. coli	$4.56^{A\dagger}$±0.00	$4.54^{A\dagger}$±0.00	$4.52^{A\ddagger}$±0.00	$4.50^{A¥}$±0.01	-	-	-
S. aureus	$4.41^{a\dagger}$±0.00	$4.38^{b\dagger}$±0.05	$4.41^{a\dagger}$±0.02	$4.42^{a\dagger}$±0.01	-	-	-
EVA_{A1}							
E. coli	$4.56^{A\dagger}$±0.00	$4.52^{A\ddagger}$±0.02	$4.55^{A\dagger}$±0.01	$4.57^{A\dagger}$±0.01	-	-	-
S. aureus	$4.41^{a\dagger}$±0.00	$4.28^{c\ddagger}$±0.02	$3.79^{b¥}$±0.02	$1.69^{c◊}$±0.05		66	99
EVA_{A2}							
E. coli	$4.56^{A\dagger}$±0.00	$4.51^{A\ddagger}$±0.01	-	<1	-	-	99.94
S. aureus	$4.41^{a\dagger}$±0.00	$2.30^{f\ddagger}$±0.02	$1.30^{f¥}$±0.03	$1.30^{d¥}$±0.05	99.16	99.85	99.85
EVA $NANO_C$							
S. aureus	$4.41^{a\dagger}$±0.00	$4.52^{a\ddagger}$±0.01	$3.45^{c¥}$±0.01	$3.15^{b◊}$±0.01	-	-	-
EVA $NANO_{A1}$							
S. aureus	$4.41^{a\dagger}$±0.00	$4.14^{d\ddagger}$±0.00	$3.37^{d¥}$±0.04	<1	-	90.96	99.94
EVA $NANO_{A2}$							
S. aureus	$4.41^{a\dagger}$±0.00	$3.78^{e\ddagger}$±0.02	$2.39^{e¥}$±0.01	$1.30^{d◊}$±0.00	53.49	98.1	99.84
EVA $NANO_{A3}$							
S. aureus	$4.41^{a\dagger}$±0.00	$3.78^{e\ddagger}$±0.01	$1.30^{f¥}$±0.00	$1.30^{d¥}$±0.00	53.49	98.84	99.84

* Mean values (Log CFU mL^{-1}) ± standard deviation. Within each column, different capital superscript letters, for *E. coli*, and, lower case superscript letters for *S. aureus*, indicate significant differences ($p < 0.05$). Within each row, the different symbols (†, ‡, ¥ and ◊) indicate significant differences ($p < 0.05$).

The EVA film spiked with the lower concentration of extract (EVA_{A1}) was active against *S. auerus* after 48 h of contact. EVA_{A2} was effective against both microorganisms tested during long incubation times for E. coli and throughout the entire incubation time for *S. aureus*. All EVA NANO active films showed antimicrobial activity against S. aureus during incubation, except for EVA $NANO_{A1}$ at 24 h. The best results were obtained with the EVA $NANO_{A2}$ and EVA $NANO_{A3}$ films. Inclusion of the functionalized nanoclays improved the effectiveness of the active packaging film, even though they contained lower amounts of natural extract.

Several authors have reported that phenolic compounds contribute to the antimicrobial activity. The PVPP-WS natural extract used to develop the active films contains large amounts of such bioactive compounds. According to the findings of Puupponen-Pimiä *et al* (2001), ferulic and caffeic acids are probably the main compounds responsible for the antimicrobial effect of the PVPP-WS natural extract used to develop the active films tested in this study. However, other compounds such as flavonoids present in the PVPP-WS extract may also be responsible for the antimicrobial activity. Compounds such as catechins, apigenin, naringenin, quercetin and kaempferol, all of which are present in PVPP-WS extract, have displayed antimicrobial activity against *S.aureus* and *E. coli* (Rauha *et al*., 2000, Mori, Nishino, Enoki, & Tawata, 1987, Yilmaz, 2006).

8.3.4. Antioxidant activity of the active films in food

According to the results of the overall migration tests and antimicrobial activity, EVA films appear to be the most suitable type of film for incorporating the natural extract and provided the best results. To confirm this, the antioxidant activity was also tested in beef samples (Table 8.5a).

Table 8.5. (a) TBARs values (mg MDA kg^{-1} of sample)* obtained in beef samples packed with different active films spiked with PVPP-WS extract or with the lowest concentration of functionalized nanoclays and stored at 4 ºC for 14 days. (b) TBARs values (mg MDA kg^{-1} of sample)* obtained in beef samples packed with different active films spiked with higher concentrations of functionalized nanoclays and stored at 4 ºC for 13 days

(a)

Film sample	Storage time (days)				
	0	3	6	9	14
LDPE C	0.399^{A}±0.046	6.61^{aB}±0.149	8.06^{aC}±0.838	15.6^{aD}±1.06	18.3^{aE}±0.640
LDPE A1	0.399^{A}±0.046	4.22^{dB}±0.464	8.98^{aC}±1.20	15.2^{bD}±0.072	14.9^{bcD}±0.110
EVA C	0.399^{A}±0.046	5.02^{cB}±0.149	8.09^{aC}±1.26	14.3^{cD}±0.905	16.6^{abE}±1.01
EVA A1	0.399^{A}±0.046	2.63^{fB}±0.507	3.86^{bB}±0.089	8.46^{dC}±0.876	12.9^{cD}±1.02
EVA A2	0.399^{A}±0.046	3.28^{eB}±1.02	3.28^{bB}±0.820	5.86^{eC}±0.038	5.43^{dC}±0.704
EVA NANO A1	0.399^{A}±0.046	5.79^{bB}±0.785	8.85^{aC}±0.570	11.8^{fD}±0.564	13.1^{cE}±0.625

(b)

Film sample	Storage time (days)				
	0	3	6	9	13
EVA NANO C	0.274^{A}±0.025	0.550^{aA}±0.271	3.69^{aB}±0.821	6.92^{aC}±0.253	9.17^{aD}±0.534
EVA NANO A2	0.274^{A}±0.025	0.544^{aA}±0.000	1.55^{bB}±0.456	4.69^{bC}±1.03	8.36^{bD}±0.873
EVA NANO A3	0.274^{A}±0.025	0.673^{aA}±0.375	1.34^{bB}±0.035	1.87^{cC}±0.115	7.06^{cD}±0.121

* Different lower case superscript letters (within the same time) and capital superscript letters (within the same row) denote statistically significant differences in the mean values (n=3) at $P < 0.05$.

The TBARs values did not differ between the active film spiked with antioxidant extract ($LDPE_{A1}$) and the control LDPE film ($LDPE_{C}$). The $LDPE_{A1}$ film did not display any antioxidant activity relative to the control film once food

packaged with $LDPE_{A1}$ had undergone the same degree of oxidation as meat packaged with the control film (see Fig. 8.2a).

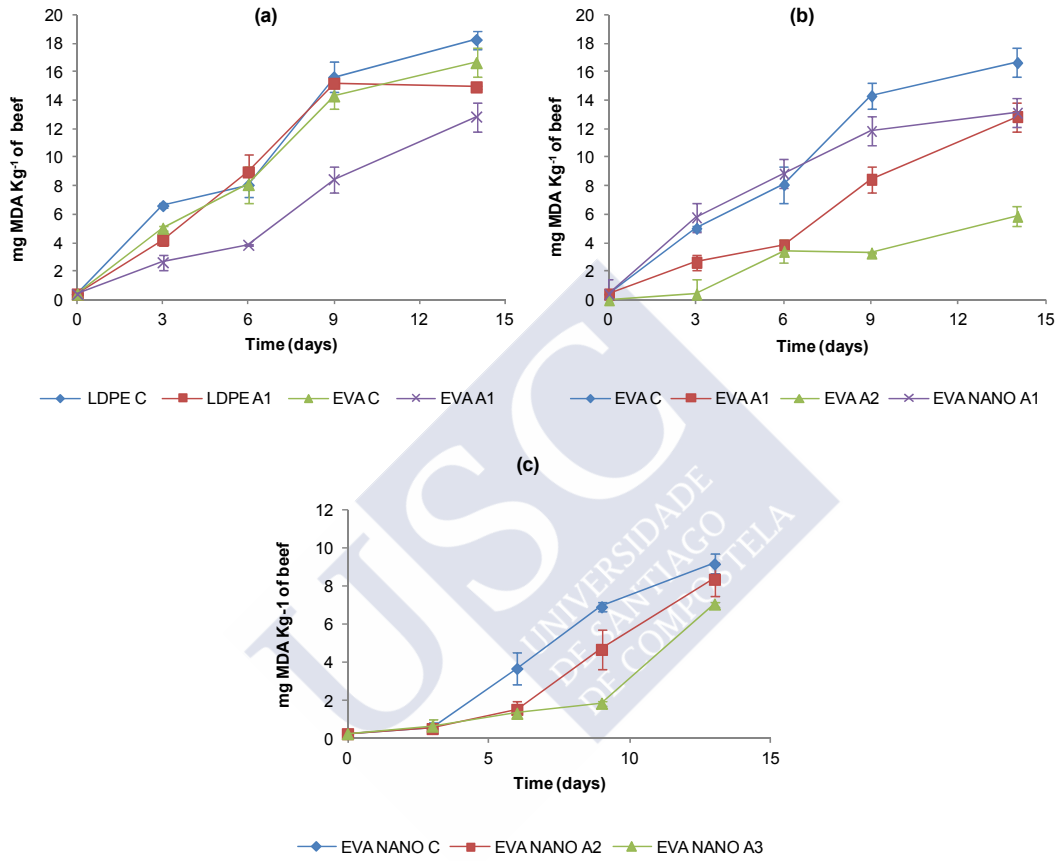

Figure 8.2. Lipid oxidation of beef packed with active films determined by measuring the TBARs-reacting substances during storage at 4 ºC. Evaluation of antioxidant effectiveness of the following: (a) LDPE and EVA films controls and LDPE and EVA films spiked with the same concentration of natural extract; (b) EVA film control, EVA films spiked with different concentrations of natural extract and EVA films spiked with the lowest concentration of functionalized nanoclays; (c) EVA film control and EVA films spiked with different concentrations of functionalized nanoclays

These results are consistent with those of the antimicrobial and overall migration tests, since the active compounds appear to be trapped in the polymeric matrix and no effect against meat spoilage was observed. The active EVA film (EVA_{A1}) containing natural antioxidants provided better protection against lipid oxidation than the control film. In the first nine days of storage, the oxidation inhibition was around 40-50% for the EVA_{A1} film and decreased thereafter. The results obtained confirmed that the most suitable polymeric matrix for formulating the active film containing the natural extract was EVA. A higher concentration of natural extract was also tested (EVA_{A2} spiked with 6% PVPP-WS extract). At the same time EVA films spiked with 3% functionalized nanoclays (EVA $NANO_{A1}$) were also tested for their effectiveness in inhibiting oxidation of beef lipids (Table 8.5a). The active films containing natural antioxidant were more successful than the control film (EVA_C) in reducing oxidation of the beef samples (see Fig. 8.2b). Higher concentrations of polyphenols enhanced the antioxidant effects of the films. The greatest differences were observed in samples packaged with the films with the highest concentration of extract (EVA_{A2}), and the TBARs value decreased by around 60-70% during storage. The film containing 3% nanocomposites (EVA $NANO_{A1}$) had the least inhibitory effect relative to the control, with reductions in TBARs values of around 20% after ninth day of storage. Since the amount of natural extract used in the nanocomposites was lower than when spiked directly in the polymeric matrix (because the functionalized nanoclays include only 20% of natural extract), another two more active films were prepared with 6% and 9% of functionalized nanoclays (EVA $NANO_{A2}$ and EVA $NANO_{A3}$ respectively). The results regarding the effectiveness of these active films in reducing lipid oxidation of beef are shown in Table 8.5b. The higher concentration of natural extract (EVA $NANO_{A3}$) increased the antioxidant effect relative to the control film (see Fig. 8.2c). After the sixth day of storage, the reduction in lipid oxidation of beef was around 60% with both active films. After 9 days, the inhibition was lower for EVA $NANO_{A2}$ (around 30%) and but higher for EVA $NANO_{A3}$ (73%). The EVA $NANO_{A3}$ film,

which contains a higher concentration of natural extract, exhibited a high antioxidant activity and the effect lasted longer during storage. However, it is important to note that the EVA $NANO_{A3}$ films only contained 1.8% of PVPP-WS extract (20% of PVPP-WS extract contained in 9% functionalized nanoclays).

8.4. Conclusions

The natural extract obtained from a residual waste exhibited a high degree of thermal stability, conserving the antioxidant properties that make it suitable for the development of active films by extrusion. Antioxidant active films were successfully developed by extrusion. The EVA films spiked with the natural extract protected the food sample (beef) better than the control film, by retarding lipid oxidation and antimicrobial growth. The use of functionalized nanoclays (prepared with the natural extract) improves the effectiveness of the active packaging with and minimizes the amount of natural extract required.

The active films produced in the study could be used to develop active packaging for use in the food industry to extend the shelf life of meat products. Furthermore, the additive used is a natural product obtained from an agro-industrial residual source and is therefore likely to be more acceptable to consumers than similar products based on synthetic antioxidants.

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9

Development of antioxidant active films containing tocopherols to extend the shelf life of fish

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Abstract

With the purpose of develop active films for fish, five natural products with antioxidant properties containing tocopherols were selected and their antioxidant activity in vitro was tested using the DPPH method. In addition these antioxidants were also tested directly on the salmon muscle using the TBARs method. Besides, thermal degradation and differential scanning calorimetry tests allow us to select the products for incorporation into the polymer matrix. Then, two natural products have been selected and incorporated in low density polyethylene films. Film 2 and Film 3, which contain product C at 1 and 5% respectively, presented a reduction in lipid oxidation up to 30% for Film 2 and to 40% for Film 3. Film 4, which contains 5% of product D, reduced lipid oxidation during storage around 30–35%. The results showed that Film 3 was the most effective to salmon conservation at long-term storage. These films could be used as future active packaging by the food industry for the salmon conservation to extend its shelf life.

Keywords: Lipid oxidation; Natural antioxidants; Tocopherols; Active films; LDPE; Salmon.

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9.1. Introduction

Seafood has recently received more attention from consumers as important source of nutritional components (unsaturated fatty acids, minerals and vitamins) that have positive benefits in nutrition and human health. The nutritional significance of fish and fish oils intake is due to their high content of essential long-chain omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which protect against heart disease, cancer, hypertension, rheumatoid arthritis, depression, diabetes, among other health benefits (Richards & Hultin, 2002; Sidhu, 2003). The high content of PUFAs in fish muscle, as well the presence of relevant prooxidant compounds, promote the development of enzymatic and non-enzymatic rancidity leading to organoleptic, physical and nutritional quality losses in food (Erickson, 1997; Frankel, 1993; McClements & Decker, 2000).

Lipid oxidation is the main cause of spoilage of fish during its processing and storage, being a key factor in the shelf life of food (Frankel, 1993, 1998a, 1998b). As a consequence of this deterioration process, hydroperoxides are formed (primary oxidation) which are unstable and decompose relatively quickly into aldehydes, ketones, alcohols, acids, esthers or hydrocarbons (secondary oxidation). These secondary oxidative products are responsible for changes in color, texture, odor and flavor in food (Erickson, 1997; McClements & Decker, 2000).

The use of antioxidants is an effective way to prevent or minimize lipid oxidation in food products, delaying the formation of toxic oxidation products maintaining the nutritional quality and prolonging their shelf life (Huang, Ou, & Prior, 2005). Antioxidants act by inhibiting the oxidation of lipids and other molecules avoiding the damaging effects of oxidation in animal tissues. The major

mechanisms of action in protecting the food from the oxidation process include the free radical scavenging and chain breaking by inhibiting the initiation or propagation phases of free radical chain reactions of lipid oxidation, metal chelation and singlet oxygen quenching (Aidos, Masbernat-Martinez, Luten, Boom, & van der Padt, 2002; Huang *et al*., 2005).

Antioxidants may be present naturally as food components as in the case of tocopherols or added during food processing (Choe & Min, 2009). In many situations, food processing is responsible for the loss of these endogenous compounds activity and required the addition of synthetic antioxidants such as 2 (3)-tert-butyl-4-methoxyphenol (BHA), butylated hydroxytoluene (BHT) and propyl gallate, which are widely used in the food industry. In recent years, several studies have shown evidence of some toxicity by synthetic antioxidants such BHT and BHA that may possess carcinogenic effects at high levels (EFSA, 2011 and EFSA, 2012). For this reason, and for human health benefits, the use of natural antioxidants such as tocopherols, ascorbic acid or natural extracts with high levels of polyphenols are increasingly used as antioxidants in food. Besides, these natural compounds are being effective in the prevention and/or treatment of diseases caused by free radicals (Frankel, 1996; Shahidi & Zhong, 2010).

Tocopherols are antioxidants extensively used as food additives by being effective, fat soluble and inhibitors of oxidation reactions because their efficient chain-breaking capacity, demonstrating effectiveness against lipid peroxidation and other oxidative situations caused by the presence of free radicals (Brigelius-Flohé & Traber, 1999; Ohkatsu, Kajiyama, & Arai, 2001). To prevent and/or delay the food spoilage, antioxidants are directly added as food additives or could be added indirectly through the packaging material, using what is known as active packaging.

Active packaging represents a new packaging concept with great interest in food industry. This system allows the release of the active components into the food and has shown great potential in the preservation of the food quality being an alternative to traditional packaging. Antioxidants such as BHT, BHA and α-tocopherol have been incorporated in plastic materials in order to protect the polymer matrix from degradation during processing. The α-tocopherol has been used in different situations to stabilize plastic materials such as low density polyethylene (LDPE) and polypropylene (PP) proving to be a good preservative of these materials (Wessling, Nielsen, & Giacin, 2000).

Nevertheless, the possibility of antioxidant compounds migrating from the plastic material being in contact with food has been observed. A study performed by Wessling, Nielsen, Leufvén, and Jägerstad (1999) suggests that LDPE spiked with α-tocopherol can be used as active packaging, for foods with high fat content, releasing the antioxidant and extending the shelf life of the product.

The aim of this study is to evaluate and compare the antioxidant effectiveness of different natural antioxidant products obtained commercially which contains tocopherols, on the stability of salmon during refrigerated storage. Besides, the most suitable antioxidant for their incorporation into LDPE is selected. With the results obtained, the products to be used in the development of active packaging with antioxidant properties were selected and studies were conducted for assessment of their effectiveness in the delay of lipid oxidation in the salmon muscle.

9.2. Materials and methods

9.2.1. Chemicals

2(3)-tert-butyl-4-hydroxyanisole (BHA) (98%, CAS No. [25013-16-5]); butylated hydroxytoluene (BHT) (99.0%, CAS No. [128-37-0]); sodium azide (99.0%, CAS No. [26628-22-8]); 2-thiobarbituric acid (TBA) (≥98%, CAS No. [504-17-6]) and trichloroacetic acid (TCA) (puriss. p.a. 99.5%, CAS No. [76-03-9]) were purchased from Sigma–Aldrich (Steinheim, Germany). 1,1,3,3-tetraethoxypropane (TEP) (purum ≥95% (GC), CAS No. [122-31-6]) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (TECHN ≥85%, CAS No. [1898-66-4]) were supplied by Fluka Chemie AG (Buchs, Switzerland). Methanol (GC ≥ 99.9%, CAS No. [67-56-1]); orthophosphoric acid (85% GR for analysis, CAS No. [7664-38-2]) and ethanol (absolute for analysis, CAS No. [64-17-5]) were provided by Merck (Darmstadt, Germany). Irganox® 1076 (CAS No. [2082-79-3]) and Irgafos® 168 (CAS No. [31570-04-4]) were kindly supplied by Ciba–Geigy, Switzerland.

9.2.2. Commercial products containing natural antioxidants

The natural products with antioxidant activity used, TOCOBIOL®, TOCOBIOL® GL, NUTRABIOL®-T90, TOCOBIOL® PV, NUTRABIOL®-T50 PV, were supplied by Biotecnologías Aplicadas, S.L., BTSA, (Madrid, Spain). The presentation of the products TOCOBIOL®, TOCOBIOL® GL, NUTRABIOL®-T90 was in oil form and products TOCOBIOL® PV, NUTRABIOL®-T50 PV were in powder form. Table 9.1 shows the composition of the different natural antioxidants tested. This information was provided by the company.

Table 9.1. Antioxidant products composition

Oils with antioxidant properties						Powders with antioxidant properties			
A – TOCOBIOL®		**B – TOCOBIOL® GL**		**C – NUTRABIOL®-T90**		**D – TOCOBIOL®-PV**		**E – NUTRABIOL®-T50 PV**	
Tocopherols	18.6%	Tocobiol:	25.0%	Tocopherols	90.2%	Tocobiol	65.0%	Tocopherols	50.3%
Sterols	9.70%	Tocopherols	18.6%	Alpha-tocopherol	15.5%	Tocopherols	18.6%	Alpha-tocopherol	12.5%
Squalene	4.30%	Sterols	9.70%	Gamma and Beta-tocopherol	63.3% 21.1%	Sterols	9.70%	Gamma and Beta-tocopherol	59.6% 27.9%
Monoglycerides	19.8%	Squalene	4.30%	Delta-tocopherol	9.80%	Squalene	4.30%	Delta-tocopherol	9.70%
Vegetable oil		Monoglycerides	19.8%	Vegetable oil		Monoglycerides	19.8%	Vegetable oil	
		Rest: vegetable oil	25.0%			Rest: vegetable oil		Excipient:	40.0%
		Propyl gallate	35.0%			Excipient:		Silica gel	
		Excipients:	15.0%			Silica gel	35.0%		
		Lecithin							
		Propylene glycol							

9.2.3. Materials

9.2.3.1. Fish sample

Salmon (Salmo salar) was selected to be used in the antioxidant effectiveness assays. The salmon was supplied to the laboratory, in frozen slices with a thickness between 1 and 1.5 cm, by the company Elaborados FREIREMAR SA.

For the antioxidant effectiveness assessment, salmon was defrosted at room temperature and its skin and bones removed from the flesh. To prevent microbial spoilage during storage, a small quantity of sodium azide (approx. 1 mg) was added on the surface of salmon.

9.2.3.2. Processing of polymer formulations

Several formulations of the antioxidant compounds with LDPE matrix were prepared. The recipes of formulations are shown in Table 9.2. The matrix used was a low density polyethylene (LDPE), provided by Repsol YPF (MFI; 20 g min^{-1}; density; 919 kg m^{-3}). Only the antioxidants compounds with a suitable thermal stability were mixed with the polymer.

Blends with LDPE and antioxidants were prepared in an internal mixer Brabender W50EHT at 150 ºC. Besides the natural antioxidants, the commercial antioxidants IRGAFOS® 168 (secondary antioxidant) and IRGANOX® 1076 (primary antioxidant) were also added in the usual proportions in industry in order to prevent the oxidation of polymer matrix.

In order to study the antioxidant effectiveness of the polymer formulations in the fish, active films with different antioxidant contents were processed. These films were also spiked with the commercial antioxidants IRGAFOS® 168 and IRGANOX® 1076 always at the same concentration of 0.2 and 0.4% respectively. For the preparation of the extruded films, a Brabender DSE-20 twin-screw extruder with a flat die was used (the barrel and die temperature were 140 and 150 °C respectively and the screw speed 45 rpm). The melted polymer formulations were cooled on a chilled roll and stretched in the machine direction. The chilled roll was kept at 80 °C with a rotation speed of 1.2 rpm. The thickness of the extruded films was between 80 and 150 μm. Then, bags with dimensions of 6 cm × 6 cm were thermosealed from the extruded films.

Table 9.2. LDPE formulations

	Natural antioxidants			Commercial antioxidants	
Formulations	C	D	E	IRGAFOS 168	IRGANOX 1076
LDPE 1	5%				
LDPE 2	1%				
LDPE 3	0.5%				
LDPE 4		5%			
LDPE 5		1%			
LDPE 6		0.5%			
LDPE 7			5%		
LDPE 8			1%		
LDPE 9			0.5%		
LDPE 10				0.2%	0.4%
LDPE 11	5%			0.2%	0.4%

9.2.4. Instruments

The concentration of 2-thiobarbituric acid reactive substances (TBARs) and the absorbance in the DPPH method were determined on a dual-beam spectrophotometer Uvikon XL, Bio-Tek Instruments, Milan, Italy.

On the extraction process of secondary oxidation compounds from fish muscle, a homogenizer T 25 digital ULTRA-TURRAX® and a shaker MS2 Mini Vortex Shaker IKA® were used.

Thermal stability of the antioxidant compounds was evaluated using a TGA-7 thermo balance (Perkin–Elmer).

The thermooxidative stability of the polymeric formulations (antioxidant product and polymer matrix) was assessed by Differential Scanning Calorimetry tests measuring its oxidation induction time (OIT). A Perkin–Elmer series 7 DSC isothermally was used to OIT measurements.

9.2.5. Methods

9.2.5.1. Determination of antioxidant effectiveness

The methods employed in the evaluation of antioxidant activity were the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method and the determination of thiobarbituric acid reactive substances (TBARs) method.

9.2.5.1.1. Free radical scavenging activity, DPPH assay

The determination of antioxidant activity of different natural products was carried out by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method, which determines the hydrogen-donating antioxidant ability, according to the method described by von Gadow, Joubert, and Hansmann (1997).

Standard solutions of the different antioxidant products were prepared in methanol as well as two synthetic compounds with antioxidant properties usually used in the food industry, BHA and BHT. The purpose to use these synthetic compounds was to compare the activity of natural products with those that are highly active. All solutions were prepared immediately before used and the concentration was within 0.1–3.5 g L^{-1} range. A methanolic solution of DPPH radical 3.6 × 10^{-5} M was prepared daily and protected from the light. 50 µL of different methanolic solutions of antioxidant product was added to 2 mL of DPPH solution and shaken. Absorbance was determined at 515 nm in a spectrophotometer after 16 min. All determinations were performed in triplicate. The absorbance decrease was converted to percentage of DPPH inhibition (PI) according to the following equation:

$$\mathrm{IP} = \frac{\mathrm{A}_0 - \mathrm{A}_{16}}{\mathrm{A}_0} \times 100$$

where,

A_0 is the absorbance of the control at initial time;

A_{16} is the absorbance of the sample at 16 min.

In order to obtain data of antioxidant activity comparable for all samples, the concentration of product required to achieve a 50% inhibition of the radical DPPH

was determined. This Equivalent Concentration 50 (EC_{50}) indicates the specific antioxidant activity of the product expressed as g L^{-1}. EC_{50} values were obtained from the linear regression curve data obtained from different concentrations of each compound used against the percentage of free radical scavenging activity (PI%). The lower the EC_{50} value, the higher is the antioxidant activity of the tested sample.

9.2.5.1.2. Determination of thiobarbituric acid reactive substances – TBARs

Malondialdehyde (MDA) is one of the largest relatively stable end products generated from secondary lipid oxidation of polyunsaturated fatty acids (PUFA). This compound reacts with 2-thiobarbituric acid forming a pink-colored compound which was determined spectrophotometrically by measuring the absorbance at 530 nm (St. Angelo, 1996).

TBARs were determined using the extraction procedure described by Witte, Krause, and Bailey (1970) with slight modifications. Five grams of fish muscle, which was added BHT (0.2 mg mL^{-1}), were extracted with 45.5 mL of extraction solution containing 10% trichloroacetic acid (TCA) in 0.02 M orthophosphoric acid to a final volume of 50.0 mL. Samples were mixed in Ultra-Turrax 1 min and then filtered through 0.45 µm nylon membrane filter. In a test tube with screw cap, 5 mL of extracted solution and 5 mL of TBA solution (0.02 M) were homogenized by vortex and afterward incubated in an oven at 100 °C for 40 min. Finally, samples were cooled at room temperature, and the absorbance was measured by spectrophotometer at 530 nm against a blank containing 5 mL of distilled water and 5 mL of TBA reagent.

MDA concentration was calculated using the calibration curve obtained using 1,1,3,3-tetraetoxipropano (TEP), a precursor of MDA, in a concentration range

between 0 and 5 mg L^{-1}. Results were expressed as milligrams of malondialdehyde per kilogram of sample (MDA mg kg^{-1} of salmon).

9.2.5.2. Evaluation of antioxidant effectiveness of the different natural products in direct contact with the salmon

Different concentrations of natural products were added to different pieces of salmon with a surface area of 3.1 × 4 cm^2 and weighing 15 ± 1 g. Two different concentrations of each natural product (A, B, C, D and E) were used. Antioxidant products solutions were prepared in ethanol. The concentrations applied resulted in 30 mg dm^{-2} (A1, B1, C1, D1 and E1) and 125 mg dm^{-2} (A2, B2, C2, D2 and E2) on the salmon surface. Different pieces of salmon muscle additivated with the different compounds were stored at 4 °C. Trial period was performed for 10 days and samples were collected for TBARs determination at 3, 7 and 10 days.

9.2.5.3. Evaluation of antioxidant suitability to incorporation in film

In order to know the thermal stability of the antioxidant compounds, dynamic experiments were conducted under oxidant atmosphere in a thermo balance. The antioxidant samples were tested from 50 °C to 70 °C at heating rate of 10 °C min^{-1}.

The thermooxidative stability of the polymeric formulations (antioxidant product and polymer matrix) was assessed measuring its oxidation induction time (OIT). The sample temperature was stabilized for 2 min at 200 °C under inert atmosphere, which was subsequently switched to oxygen atmosphere to start the test. Analyses were carried out according to EN 728. The OIT was measured as the onset point at which the DSC thermogram suffers a suddenly drop respect to the instrument baseline. The maximum time in the experiments was 240 min.

9.2.5.4. Evaluation of antioxidant effectiveness of the films additivated with commercial products

To perform this study, different pieces of fish (±20 g) were packed in individual LDPE bags containing different concentrations of selected antioxidant compounds. A LPDE film without natural antioxidants incorporated was used as control film.

Packed salmon was stored at 4 °C for 21 days. Analyses of TBARs were performed at 4, 7, 11, 14, 18 and 21 days.

9.3. Results and discussion

Several authors make distinction between antioxidant capacity and reactivity of the antioxidant compounds. The antioxidant capacity provides information about the duration of the antioxidant action, i.e. the ability to delay oxidative degradation, while the reactivity is characterized by the beginning of antioxidant process by a known concentration of an antioxidant or a mixture of antioxidants, determined by the reactivity of the/these antioxidant/s to free radicals in the corresponding reaction (Roginsky & Lissi, 2005). For this reason, in this work, two analytical methods were used. An indirect method to determine the antioxidant products ability to free radical scavenging (DPPH) and a direct method which evaluates lipid oxidation in food where antioxidants were added (TBARs).

In this study, three different assays were conducted. The first one was to evaluate the effectiveness of several commercial natural antioxidants, formulated with tocopherols, by adding different concentrations directly on the surface of the fish muscle. The second one was to study the different thermal properties of

antioxidants in order to select the most suitable to process by extrusion. Finally, films additivated with the selected antioxidant products which showed better results in the previous mentioned test, were evaluated.

9.3.1. Antioxidant properties of commercial products

The capacity to scavenge free radical is an antioxidant property of great significance as well as the quenching of the peroxide radicals terminating the peroxidation chain reaction (Brand-Williams, Cuvelier, & Berset, 1995). The data obtained for the ability of DPPH radical scavenging by natural antioxidant products (Table 9.1) was compared with reference antioxidants BHA and BHT, usually used in food industry. Results of the antioxidant activity determined by DPPH method are shown in Table 9.3.

Table 9.3. Free radical scavenging activity

Antioxidant	EC_{50} (g L^{-1})
A	1.49 ± 0.075
B	0.435 ± 0.050
C	0.401 ± 0.062
D	3.25 ± 0.095
E	0.704 ± 0.047
BHT	2.53 ± 0.085
BHA	0.273 ± 0.025

The EC_{50} values obtained for the synthetic antioxidants used as reference, BHA and BHT, are in agreement with values found in the literature (Cruz, Moldes, Bustos, Torrado, & Dominguez, 2007).

The results obtained indicate that all antioxidant extracts tested presented antioxidant activity and the natural product with highest radical scavenging activity was product C followed by B, E, A and D which had the lowest antioxidant activity among the products studied.

Except for D, all the products tested demonstrate higher antioxidant activity compared to BHT, synthetic antioxidant commonly used in food industry.

Antioxidants products B and C have displayed higher antioxidant activity presenting EC_{50} values of 0.435 and 0.401 g L^{-1} respectively, comparable to the reference synthetic antioxidant BHA which presented an EC_{50} of 0.273 g L^{-1}.

Comparing the three TOCOBIOL® products, the most active was B followed by A and finally D. The highest antioxidant capacity presented by product B may be due to the fact that in its composition in addiction to 25% of TOCOBIOL® product also includes 25% of the synthetic antioxidant propyl gallate, used in the food industry due to its high antioxidant activity (Martinez-Tome *et al*., 2001), and therefore showed greater antioxidant activity than the product A (EC_{50} of 1.49 g L^{-1}). Product D was less effective in radical scavenging because it is composed by only 65% of TOCOBIOL® product which is the responsible for its antioxidant activity.

Comparing both NUTRABIOL® products, C product presents the highest radical scavenging capacity to free radicals because is the one which contains highest percentage of tocopherols.

Of the two products tested in powder form, antioxidant E showed highest antioxidant capacity with an EC_{50} value of 0.704 g L^{-1} because of its high percentage of tocopherols (50.3%). The same was observed for the oils, the product with highest proportion of tocopherols, product C (90.2%), was the most

effective, i.e. presented the highest antioxidant activity with an EC_{50} value of 0.401 g L^{-1}.

9.3.2. Antioxidant effectiveness of commercial products directly on salmon

To evaluate the effectiveness of these compounds directly on food, salmon was selected, and lipid oxidation was evaluated during storage at 4 °C.

The study was divided into two parts and samples were analyzed at different days. In the first part of this study the antioxidant products in the oil form were tested and in the second one antioxidant products in powder form. In each assay there was a control test prepared as samples but without the addition of antioxidant products.

9.3.2.1. Evaluation of antioxidant effectiveness of the oil products

The antioxidant effectiveness of the different products tested in the salmon muscle was determined by the TBARs method. The data obtained with the 3 oils applied at 2 different concentrations used on the salmon muscle are shown in Table 9.4a.

Lipid oxidation of salmon muscle samples increased during the storage in different degrees being the control test (without addition of antioxidant), which showed high TBARs values.

All antioxidants applied in this assay reduced lipid oxidation in salmon compared to the control test. At the lowest concentration of the study (30 μg dm^{-2}), product B showed better effectiveness, reducing notably lipid oxidation compared to control assay. For the C antioxidant, also at higher concentration of antioxidant

employed in the salmon muscle (125 μg dm^{-2}) lower antioxidant effect was observed. After 10 days the antioxidant effect presented by the two concentrations of product C is approximately the same, with TBARs values around 2.4 mg MDA kg^{-1} sample, which corresponds to a reduction in lipid oxidation around 40% respect to control assay. Analyzing the data obtained, it can be seen that the oil, which presented better antioxidant effect in salmon is product B, and for the two concentrations evaluated, the higher is the concentration used higher the antioxidant effectiveness. B1 and B2 concentrations reduced lipid oxidation about 70% being B2 sample slightly more effective. Antioxidants A and B applied at the highest concentration, i.e. 125 mg dm^{-2} (A2 and B2), remain oxidation values below 1 mg kg^{-1} MDA of sample which is equivalent to a deterioration reduction in food around 75% compared to control assay. For compounds A and B it can be seen that the higher the concentration used, the greater reduction of oxidation in fish was observed, while in the product C this effect was not observed. This could be due to that C was the compound with highest concentration of tocopherols (see Table 9.1) and presented the highest EC_{50} in the DPPH method (EC_{50} = 0.401 g L^{-1}), and subsequently, when applied to the food at the same concentrations that were used with other products, it could have a prooxidant effect caused by this high amount of tocopherols.

Numerous authors have described this prooxidative effect of α-tocopherols after applying this product at high concentrations (Brigelius-Flohé & Traber, 1999; Frankel, 1996; Huang, Frankel, & German, 1994).

At very high concentrations, the prooxidant effect of α-tocopherol occurs during the induction period of primary oxidation where hydroperoxides are formed in large amounts. This happens because at high concentrations, α-tocopherol loses its activity to inhibit the formation of these hydroperoxides demonstrating a low effectiveness in the primary oxidation inhibition. However this effect was not observed in the secondary oxidation where a significantly delaying of secondary

products formation is verified (Huang *et al*., 1994; Tafazoli, Wright, & O'Brien, 2005; Tong, Sasaki, McClements, & Decker, 2000). Thus, the results obtained using the C antioxidant product could be due to a possible loss of activity of the compound as result of the large amount of tocopherols present in high concentrations because only a small amount of α-tocopherols consumed in the oxidation reaction and a large part remains available for further reactions that occur simultaneously and leads to an oxidation enhancement (Decker, Warner, Richards, & Shahidi, 2005; Fuster, Lampi, Hopia, & Kamal-Eldin, 1998).

9.3.2.2. Effectiveness of products in powder form

TBARs values obtained from salmon additivated with two commercial products in powder form with antioxidant properties, D and E, at two different concentrations are displayed in Table 9.4b.

Analyzing the data obtained in this assay and, taking into account that the control was not much oxidized, which makes difficult the comparative, improvements were observed in the conservation of fish additivated with antioxidant products at the concentrations 1 and 2 (30 and 125 mg dm^{-2} respectively), except for the D2. Antioxidant effectiveness of product E in fish muscle increased with the increasing of the concentration employed. After 3 days of storage, the antioxidant effectiveness was higher than 50% compared to control test.

On the seventh day of trial, the degree of inhibition remained for the D1, E1 and E2 but in less percentage (52, 22 and 40% respectively). Powder E showed an EC_{50} of 0.70 g L^{-1} in the DPPH method indicating the strong antioxidant/radical scavenger activity as well that the product had antioxidant activity in vitro. This antioxidant effect was confirmed when applied directly into the salmon muscle and

consequently could be considered as a possible antioxidant product to be used in fish conservation.

Antioxidant D displayed the highest EC_{50} of the five compounds tested being the product that showed lower antioxidant activity in DPPH assay. However, added at concentration of 30 mg dm^{-2} (D1) on the sample of salmon demonstrated antioxidant effect in the 7 days of storage, while at higher concentration (D2) a possible prooxidant effect was observed because lipid oxidation did not decrease. Compound D seems to be an example of antioxidant that although it did not demonstrate great antioxidant activity by the indirect method (EC_{50} = 3.25 g L^{-1}), it was effective against lipid peroxidation of food (Table 9.4b). Therefore, methods to determine free radical scavenging such as DPPH method are very useful to make a screening of the antioxidants, however to verify their effectiveness in food, methods to evaluate the food deterioration must be used.

The antioxidant effectiveness in food depends on a variety of factors such as polarity, solubility and metals quenching activity as well as the interaction with other food components (Decker *et al*., 2005; Roginsky & Lissi, 2005). Antioxidant D presents a very similar behavior to that observed with the antioxidant oil C in terms of a possible pro-oxidant effect or inhibition of antioxidant activity when applied at high concentrations.

Table 9.4. (a) TBARs data obtained from salmon muscle in contact with different concentrations (30 and 125 mg dm^{-2}, for samples 1 and 2 respectively) of antioxidant oils (described in Table 9.1) during storage at 4 °C. (b). TBARs values obtained from salmon muscle in contact with different concentrations (30 and 125 mg dm^{-2}, for samples 1 and 2 respectively) of antioxidant powders (described in Table 9.1) during storage time at 4 °C

(a)

	TBARs (mg malondialdehyde kg^{-1} salmon muscle)						
Time (days)	Control	A1	B1	C1	A2	B2	C2
0	0.31±0.12	0.31±0.12	0.31±0.12	0.31±0.12	0.31±0.12	0.31±0.12	0.31±0.12
3	2.2±0.21	0.83±0.41	1.2±0.12	2.2±0.41	0.61±0.05	0.49±0.09	2.4±0.38
7	3.9±0.17	2.6±0.04	0.57±0.08	2.0±0.29	0.68±0.09	0.63±0.10	2.9±0.16
10	3.9±0.17	3.5±0.14	1.0±0.21	2.4±0.05	1.1±0.25	0.79±0.12	2.3±0.01

(b)

	TBARs (mg malondialdehyde kg^{-1} salmon muscle)				
Time (days)	Control	D1	E1	D2	E2
0	0.21±0.19	0.21±0.19	0.21±0.19	0.21±0.19	0.21±0.19
3	2.4±0.90	0.66±0.27	0.96±0.32	0.68±0.43	0.52±0.01
7	2.5±0.43	1.2±0.56	2.0±0.11	5.2±0.48	1.5±0.64

9.3.3. Thermal stability and antioxidant effectiveness of compounds

The thermal stability of the five antioxidants was evaluated and the most suitable ones, that is, which do not suffer thermal degradation at the LDPE processing temperature, were chosen for blending with polymer matrix.

Fig. 9.1 displays the thermograms obtained from the TGA experiments. The A and B compounds have a lower thermal stability than others. The degradation process of A and B antioxidants start from 100 °C, whereas in the other ones (C, D and E), it begins at 250 °C (see onset in each curve) approximately. Keeping in mind that LDPE processing temperature is around 150 °C (indicated by the arrow in the graph), the antioxidants A and B were ruled out to blend with the polymer.

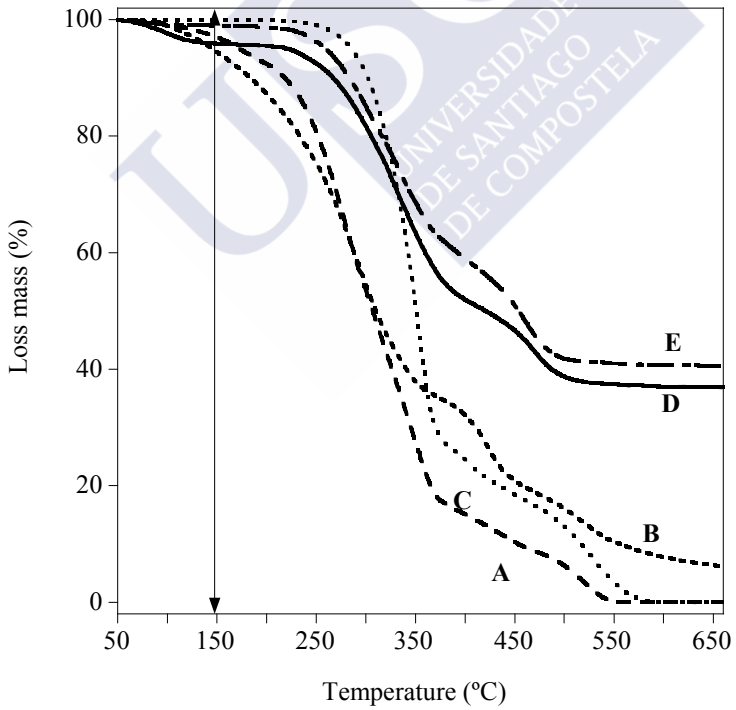

Figure 9.1. TGA thermograms of antioxidant compounds described in Table 9.1

Then, the antioxidant activity of the different LDPE/antioxidant blends was evaluated measuring the OIT by DSC. The oxidation induction time increases with the increase of antioxidant amount. This effect is observed with three antioxidants, C (Blends LDPE 1–3), D (Blends LDPE 4–6) and E (Blends LDPE 7–9) (Fig. 9.2). Comparing same formulations, the antioxidant activity is quite similar in blends with antioxidant C and E (the maximum time assayed) but it is much lower with D compound.

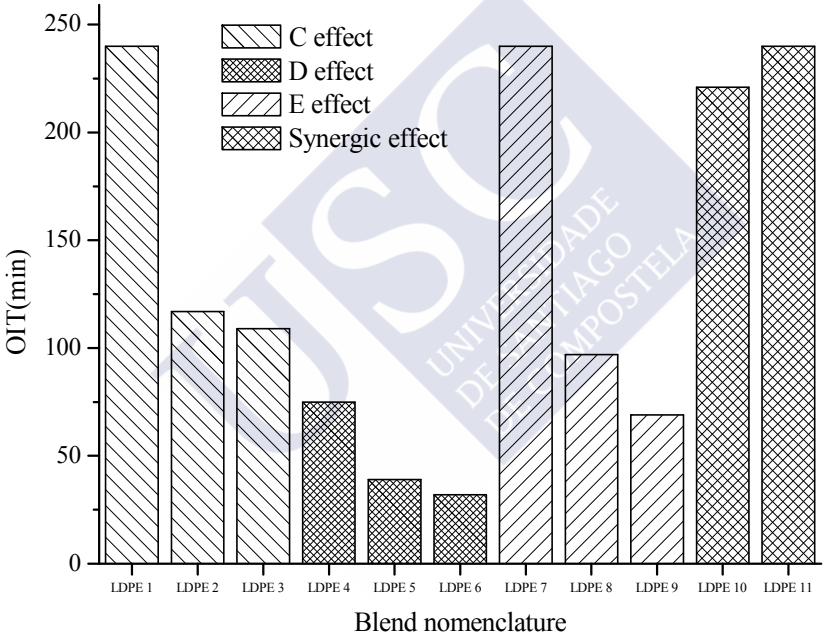

Figure 9.2. OIT of LDPE blends described in Table 9.2

On the other hand, these results reveal a behavior of studied antioxidants as long-term antioxidant. Therefore, OIT values increase with increasing natural antioxidant levels, characteristic behavior of phenolic compounds. However,

phosphate stabilizers alone do not increase the OIT of polyethylene in the temperature range of 180–210 °C (Phease, Billingham, & Bigger, 2000).

To prevent the matrix oxidation during the extrusion and the premature consumption of the natural antioxidants, the two commercial antioxidants (IRGANOX® 1076 and IRGAFOS® 168) were added to the polymer blends. The OIT data of samples LDPE 10 and LDPE 11 (the maximum time assayed) shown an available level of antioxidant in the film to be released to fish.

Based on the results obtained, the natural antioxidants that should be selected to incorporate in the films to be tested in the salmon packaging assay would be C and E. Once these two products are constituted by the same active principle Nutrabiol® but in different proportions, and for the purpose of testing two different products with different compositions in antioxidant active compounds, the products selected to incorporate to LPDE films were C and D. Thus, two different formulations in tocopherols can be evaluated as well as their effectiveness against lipid peroxidation getting more information about the possible application of these antioxidant products as ingredients of active films.

In summary, the C and D antioxidants together with the commercial antioxidants (Irgafos® 178 and Irganox® 1076 at 0.2 and 0.4% respectively) were chosen to prepare the LDPE active films. The active films formulations selected were: Film 2 and 3 incorporated with natural product C at 1% and 5% respectively; Film 4 with natural product D at 5%. Film 1 with only commercial antioxidants was used as control.

9.3.4. Evaluation of antioxidant effectiveness of the active films

When incorporated into the plastic matrix, compounds with antioxidant activity will not be available as in previous assays, which were added directly into fish

muscle. In addition, during films processing, that occurs at high temperatures and aggressive conditions, the active compounds could be reduced and its antioxidant capacity be affected. Therefore, based on the results of direct adding tests, commercial products which showed evidences of great effectiveness in inhibition of lipid oxidation in salmon were selected. Both of the products exhibited better results at lower concentrations and a possible pro-oxidant effect when applied at the highest concentration demonstrating their high activity.

Compound C showed a high antioxidant activity when applied at low concentration, a high antioxidant activity by DPPH method and in its composition contains a high percentage of tocopherols. Therefore it was incorporated into the plastic matrix at two different concentrations (1% and 5%). Compound D was incorporated into the plastic matrix only at the highest concentration of 5% because showed less antioxidant activity by DPPH method (EC_{50} = 3.25 g L^{-1}). The antioxidant effect of films additivated with selected products was determined by the capacity to inhibit lipid oxidation in salmon muscle during storage at 4 ºC by TBARs method.

Fig. 9.3 shows the TBARs values, expressed as mg MDA Kg^{-1} of sample, obtained in the salmon muscle packed with the three films additivated with the natural products containing tocopherols compared to control film (Film 1). Results showed that lipid oxidation increased in all samples during storage but at different rates. Control test presented a maximum of oxidation of approximately 5.8 mg MDA kg^{-1}, while the samples packed with active films presented TBARs values ranging within 3–4 mg MDA kg^{-1}.

Salmon packed with films spiked with antioxidant product C in the two concentrations of active ingredient applied at 1 and 5% (Film 2 and Film 3 respectively), experimented an important lipid oxidation decrease, and the greatest antioxidant effect was verified at higher concentration tested.

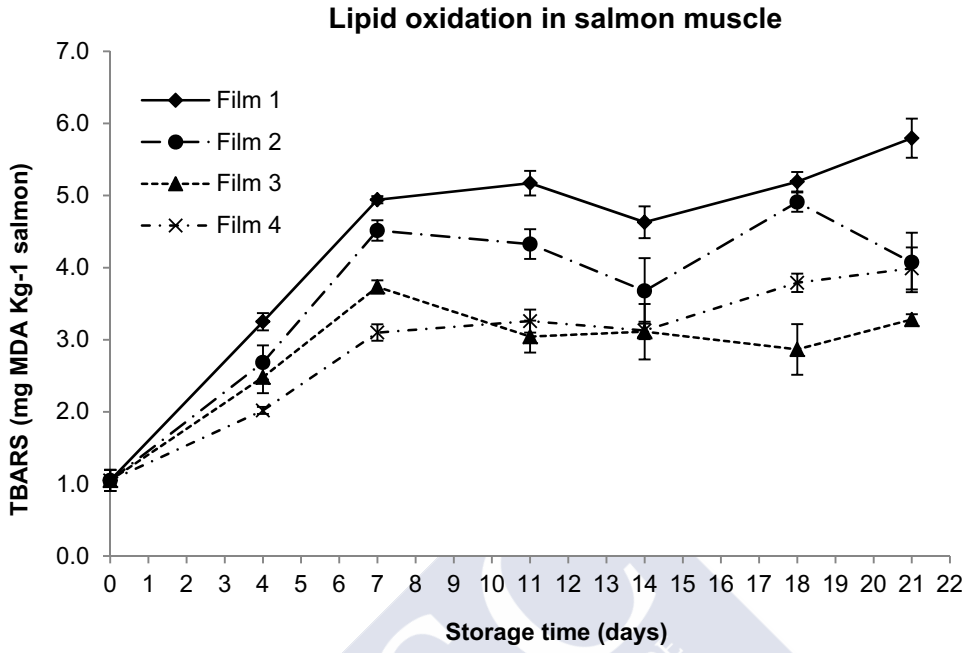

Figure 9.3. Lipid oxidation (TBARs) in salmon packed with antioxidant films stored at 4 °C during 21 days (Film 1: control; Film 2: 1% antioxidant C; Film 3: 5% antioxidant C; Film 4: 5% antioxidant D)

At the first days of assay, no main differences between the two concentrations tested was observed, however, after the 7th day, differences were more remarkable. At 7th day, lipid oxidation was reduced around 25% for Film 3, while for Film 2 this decrease was less than 10% compared to control (Film 1). After day 11 of assay, Film 3 showed an oxidation decrease to around 40%. In the same period less reduction, around 15%, was observed for the Film 2, which at the end of the assay was decreased by 30%. TBARs values of fish packed with Film 4, which contains 5% of antioxidant product D also showed a significant reduction in lipid oxidation of salmon throughout entire assay compared to control, increasing food stability. During the first 7 days of study, a reduction around 40% was

observed to Film 4, and after that, oxidation decreasing remained constant around 30–35% until the end of the assay.

Comparing the curves of the films spiked with the two products applied in this study at the same concentration (5%), it can be pointed out that both films showed important antioxidant effectiveness and there were no main differences between the two compounds. There was a small difference over the first days of analysis where Film 4 (which contains TOCOBIOL® PV) was more effective than Film 3, while at the end of the assay Film 3 is the most effective film. This may be due to the high amounts of tocopherols presents in Film 3 (product C).

Active films used had shown an inhibitory effect on the lipid oxidation process comparable to the effect observed with direct addition of antioxidants to the fish muscle. Results obtained with Films 2 and 3 confirm the effectiveness of compound C as well as the higher concentration incorporated into the films greater the effectiveness of them. Results also confirmed the high antioxidant effect of compound D, which although did not showed high antioxidant activity in the free radical scavenging method (DPPH), it showed great effectiveness against salmon lipid oxidation.

9.4. Conclusions

The results of antioxidant activity obtained by free radical scavenging DPPH method confirm that the natural products employed in this trial have important antioxidant effectiveness.

The order of free radical scavenging activity of the natural products tested was: NUTRABIOL®-T90 > TOCOBIOL® GL > NUTRABIOL®-T50 PV > TOCOBIOL® > TOCOBIOL® PV. Then, it can be said that considering free radical

scavenging capacity, NUTRABIOL® products were the most active in both forms of powder or oil. These compounds constituted mainly by natural tocopherols could replace synthetic antioxidants in food industry.

The addition of these compounds extensively inhibited lipid oxidation of salmon muscle during the storage time, up to 70%. These results indicate that these compounds could be used as food additives in order to prevent lipid oxidation during storage time.

After the incorporation of antioxidant products into plastic matrices, positive effects on reduction of lipid oxidation, up to 40%, in fish were still observed, being able to be used for extending salmon shelf life.

These results indicate that these active films containing tocopherols could be used to develop a promising active packaging to be applied in food preservation by food industry.

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Conclusiones generales

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Seguidamente se puntualizan las principales conclusiones extraídas de los trabajos que forman parte de esta Tesis Doctoral.

Relativo a la obtención del extracto natural a partir de los licores de lavado de la torta de PVPP, generados en la industria cervecera, y su posterior caracterización:

Primera:

Se ha desarrollado y optimizado en una patente, un proceso de extracción de los compuestos polifenólicos presentes en los licores de lavado de la torta de PVPP. La invención tiene su aplicación dentro de la industria cervecera, al revalorizar lo que actualmente sería un producto de desecho y que constituye una fuente de bajo coste de compuestos naturales con capacidad antioxidante y antimicrobiana y que pueden tener elevado potencial de aplicación en la industria alimentaria como aditivos. Este proceso se ha optimizado a nivel laboratorio, en planta piloto y después se implementó a escala industrial.

Segunda:

Se ha desarrollado un método cromatográfico para la separación, identificación y cuantificación de los compuestos fenólicos, presentes en el extracto natural, por RP-HPLC-DAD y HPLC-ESI-TOF-MS. Las condiciones cromatográficas propuestas, garantizan una buena separación de los compuestos fenólicos presentes en el extracto, lo que permite su aplicación en el análisis de rutina para la monitorización de los extractos cada vez que se realicen procesos de extracción a partir de los lavados de la torta de PVPP.

El extracto natural está compuesto mayoritariamente por flavonoides (catequina, epicatequina, gallocatequina, epigallocatequina y quercetina) y por

ácidos fenólicos (ferúlico, ρ-cumárico, cafeico y protocatéquico) que representan casi el 50% (m/m) del extracto crudo. Aunque la composición y/o cantidades de los compuestos fenólicos presentes en el extracto puedan cambiar ligeramente de acuerdo con el lote de los licores de lavado, los extractos obtenidos presentan los mismos compuestos mayoritarios y una elevada capacidad antioxidante.

Tercera:

La actividad antioxidante del extracto natural obtenido a partir de esta corriente residual de la industria cervecera, ha sido demostrada mediante diferentes métodos y comparada con antioxidantes sintéticos ampliamente utilizados en la industria alimentaria. El extracto natural tiene una actividad antioxidante similar a la del BHA y muy superior a la del BHT. El extracto natural también presenta efectividad antimicrobiana contra bacterias Gram positivas (*S. aureus*, *L. monocytogenes*) y Gram negativas (*Salmonella spp*, *E. coli* y *E. coli* 0157:H7), que fueron seleccionadas teniendo en cuenta la futura aplicación del extracto como aditivo alimentario en productos cárnicos.

Por lo tanto, los extractos que se pueden obtener a partir de esta corriente residual de la industria cervecera son una prometedora fuente de compuestos naturales susceptible de ser utilizados con agente antioxidantes y conservantes en alimentos.

Cuarta:

Los dos procesos desarrollados para la purificación del extracto natural, tanto el de extracción en fase sólida como el de extracción con fluidos supercríticos, han permitido obtener fracciones menos coloreadas, más puras y con un actividad antioxidante considerablemente superior a la del extracto crudo, lo que contribuye a mejor las propiedades del extracto para su utilización como aditivo alimentario.

Relativo al desarrollo de envases activos de uso alimentario para el envasado de productos cárnicos refrigerados

Quinta:

El extracto natural ha mostrado elevada efectividad en la reducción de la oxidación lipídica de la carne de ternera almacenada a refrigeración, cuando está en contacto directo con el alimento. Los resultados obtenidos han demostrado que el extracto posee una efectividad similar al propilgalato, y es más efectivo que antioxidantes sintéticos como el BHT y que otros antioxidantes comerciales naturales, como el extracto de romero.

También se ha comprobado que el extracto natural apenas ejerce su efecto antioxidante sobre el producto cuando se encuentra en el espacio de cabeza del envase, es decir, en ausencia de contacto directo con el alimento.

Sexta:

Se han desarrollado films activos recubiertos con el extracto antioxidante obtenido a partir de los licores de lavado de la torta de PVPP y films recubiertos con extracto de romero. Se ha comprobado que a mayor concentración de extracto en la superficie del film, mayor es su efectividad en la reducción de los niveles de oxidación. Se ha demostrado que el extracto de PVPP posee una excelente actividad antioxidante cuando está en contacto directo con el alimento. Los films que contienen mayor concentración de extracto incorporado han logrado una reducción del grado de oxidación del alimento hasta un 80% respecto al film control, frente al 50% de reducción obtenido con el extracto de romero.

Séptima:

El extracto obtenido a partir de los licores de lavado de torta de PVPP ha demostrado una elevada estabilidad cuando se somete a elevadas temperaturas, manteniendo sus propiedades antioxidantes con pérdidas mínimas en su contenido en compuestos fenólicos. Esto hace que este extracto resista a las temperaturas de procesado de los films por extrusión sin que pierda su efectividad.

Se han desarrollado films activos con el extracto natural incorporado mediante extrusión en dos matrices poliméricas (LDPE y EVA), con el fin de mejorar la conservación del producto cárnico, fresco y almacenado a refrigeración, por contacto directo. Los films activos preparados por extrusión con LPDE, no presentan una elevada efectividad antioxidante, ya que no hay una buena accesibilidad de los compuestos antioxidantes al alimento, pero lo film elaborado con EVA, si que han desarrollado un importante efecto antioxidante sobre el alimento, reduciendo la oxidación del alimento hasta un 70% respecto al control.

Octava:

El uso de nanoarcillas bioactivas preparadas con el extracto natural, ha aumentado la eficacia de los films desarrollados, ya que requieren el empleo de menores cantidades de extracto activo. Los films de EVA que contienen el nanoaditivo, han conseguido reducir un 50% la oxidación del producto cárnico, durante el tiempo de almacenamiento a refrigeración, con una concentración de extracto en el film del 1,2%, frente al 6% necesario para conseguir un efecto similar cuando el extracto se incorpora directamente a la matriz polimérica sin nanoarcilla.

Relativo al desarrollo de envases activos de uso alimentario para el envasado de pescado congelado

Novena:

Se ha comprobado que la adición directa de productos comerciales naturales, que contienes tocoferoles, ha reducido hasta un 70% el grado de oxidación de productos de la pesca almacenado a congelación. Con estos antioxidantes comerciales naturales se han desarrollado por extrusión films activos que han permitido reducir la oxidación lipídica hasta un 40% respecto a los envases control, con el consiguiente incremento en la vida útil del alimento envasado. Siendo por tanto estos films de gran aplicación para el envasado de distintas especies de pescado congelado en formato de “segunda piel”.